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Biology of memory

Edited by G. Ádám

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Akadémiai Kiadó, Budapest

BIOLOGY OF MEMORY

Proceedings of the Symposium held at the Biological Research Institute in Tihany, 1 to 4 September 1969

Edited by
G. ÁDÁM

The lectures delivered at this scientific meeting were centred around the chemical and electrical changes in the cell in the course of learning as well as the structural background of memory functions. Much attention has been devoted to the controversial problem of memory transfer. The volume contains the full material of the conference including both lectures and discussions.

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J. SZENTÁGOTHAI

Vol. 10

AKADÉMIAI KIADÓ, BUDAPEST 1971

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at the Biological Research Institute in Tihany,
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EDITED BY

G. ÁDÁM M. D., D. Sc. (biol.)
Associate Member of the Hungarian Academy of Sciences
Professor of Physiology
Eötvös Loránd University, Budapest

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Technical editor:

ERZSÉBET M. SZÁSZ, Ph. D.

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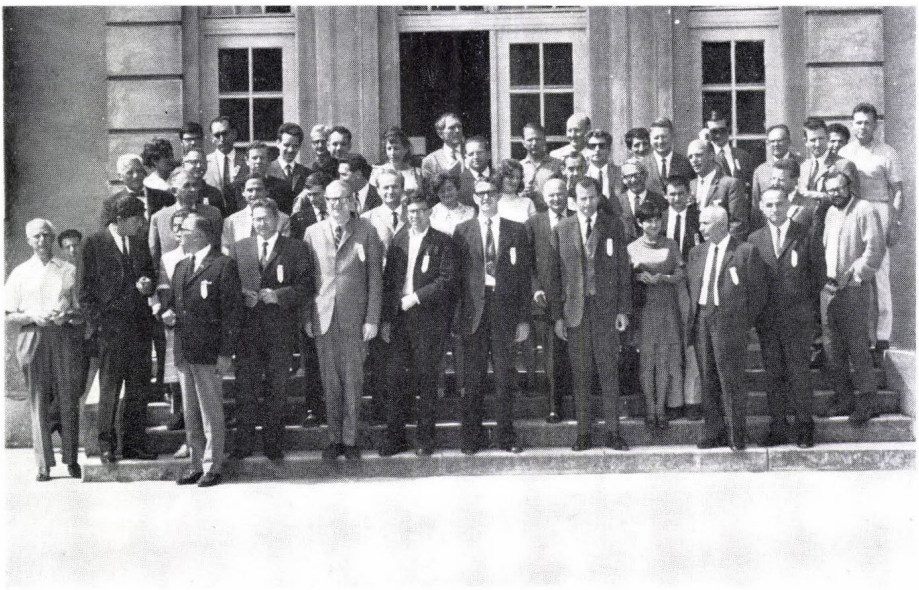

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LIST OF PARTICIPANTS

G. Ádám, Eötvös Loránd University, Budapest, Hungary
K. Biró, Eötvös Loránd University, Budapest, Hungary
B. Bohus, University Medical School, Pécs, Hungary
W. L. Byrne, University of Tennessee, Memphis, U.S.A.
W. C. Corning, University of Waterloo, Ontario, Canada
V. Csányi, University Medical School, Budapest, Hungary
J. Daliers, UCB Dipha, Brussels, Belgium
G. F. Domagk, University of Göttingen, Göttingen, G.F.R.
W. Domschke, University of Göttingen, Göttingen, G.F.R.
L. Dömötör, Institute of Neurosurgery, Budapest, Hungary
J. A. Dyal, University of Waterloo, Ontario, Canada
W. B. Essman, City University of New York, Flushing, U.S.A.
J. Faiszt, Eötvös Loránd University, Budapest, Hungary
O. Fehér, József Attila University, Szeged, Hungary
J. Feldman, University Medical School, Edinburgh, Great Britain
S. Freed, New York Medical College, New York, U.S.A.
R. Galambos, University of California, La Jolla, U.S.A.
R. M. Gaze, University Medical School, Edinburgh, Great Britain
M. Gerbner, Psychological Institute of the Hungarian Academy of Sciences, Budapest, Hungary
P. Greguss, Railway Scientific Research Institute, Budapest, Hungary
L. Hiripi, Biological Research Institute, Tihany, Hungary
G. Horn, University of Cambridge, Cambridge, Great Britain
H. Hydén, University of Göteborg, Göteborg, Sweden
R. Yu. Ilyutchenok, Institute of Physiology, U.S.S.R. Academy of Sciences, Novosibirsk, U.S.S.R.
J. Izsák, Eötvös Loránd University, Budapest, Hungary
F. Katona, Institute of Neurosurgery, Budapest, Hungary
K. Kelemen, University Medical School, Budapest, Hungary
I. Kiss, Biological Research Institute, Tihany, Hungary
F. Klingberg, Karl Marx University, Leipzig, G.D.R.
B. Knoll, University Medical School, Budapest, Hungary
J. Knoll, University Medical School, Budapest, Hungary
T. Kukorelli, Eötvös Loránd University, Budapest, Hungary
E. Kuzmann, Eötvös Loránd University, Budapest, Hungary
E. Lábos, Biological Research Institute, Tihany, Hungary

I. Láng, Biological Section, Hungarian Academy of Sciences Budapest, Hungary
K. Lehotzky, Institute of Work Hygiene, Budapest, Hungary
K. Lissák, University Medical School, Pécs, Hungary
I. Madarász, University Medical School, Szeged, Hungary
J. V. McConnell, University of Michigan, Ann Arbor, U.S.A.
I. Mészáros, Eötvös Loránd University, Budapest, Hungary
Lj. Mihailović, University of Belgrade, Yugoslavia
E. Mikolás-Szász, Eötvös Loránd University, Budapest, Hungary
S. Narikashvili, Institute of Physiology, Georgian Academy of Sciences, Tbilisi, U.S.S.R.
F. Obál, University Medical School, Szeged, Hungary
T. N. Oniani, Institute of Physiology, Georgian Academy of Sciences, Tbilisi, U.S.S.R.
A. Orosz, Eötvös Loránd University, Budapest, Hungary
I. Pelyhe, Eötvös Loránd University, Budapest, Hungary
M. Pollack, City University of New York, Flushing, N.Y. U.S.A.
J. Pusztai, Eötvös Loránd University, Budapest, Hungary
H. Røigaard-Petersen, University of Aarhus, Aarhus, Denmark
F. Rosenblatt, Cornell University, Ithaca, New York U.S.A.
J. Salánki, Biological Research Institute, Tihany, Hungary
K. S. Rózsa, Biological Research Institute, Tihany, Hungary
A. Sarkadi, Institute of Neurosurgery, Budapest, Hungary
F. Sebestyén, University Medical School, Pécs, Hungary
G. Székely, University Medical School, Pécs, Hungary
J. I. Székely, University Medical School, Budapest, Hungary
J. Szentágothai, University Medical School, Budapest, Hungary
K. Takácsi-Nagy, Akadémiai Kiadó, Publishing House of the Hungarian Academy of Sciences, Budapest, Hungary
I. Tomka, Institute of Neurosurgery, Budapest, Hungary
G. Ungar, Baylor College of Medicine, Houston, U.S.A.
I. Varanka, Biological Research Institute, Tihany, Hungary
O. L. Wolthuis, Medical Biological Laboratory, Rijswijk, Holland
R. Zech, University of Göttingen, Göttingen, G.F.R.
I. Zs. Nagy, Biological Research Institute, Tihany, Hungary

PREFACE

This international Symposium on the biology of memory was held in the Biological Research Institute at Tihany, Lake Balaton in September 1969. The meeting covered nearly all biological aspects of recent research on elementary learning and memory storage. This complex approach proved to be fruitful: the lectures, discussions, as well as the the chairmen's concluding remarks reflected the main problems of this most exciting, but rather controversial field of interdisciplinary research.

The volume contains the thirty papers read at the Symposium, arranged in a different sequence than they had been presented during the four-day meeting. These changes made by the Editor were necessary in order to follow the logical succession of the different topics, rather than the chronology of the lectures, which was inevitably influenced by the itineraries of some of the participants from abroad. Consequently the sequence of the concluding remarks had to be altered, too. The division of the book into six parts according to the different topics is tentative because of the multiple overlappings. The discussions of the lectures had to be digested, since the presentation of a complete record would have been difficult.

The Symposium was organized by the Department of Comparative Physiology of Eötvös Loránd University, Budapest, each member of which — engaged in electrophysiological and biochemical research of memory mechanism — has been an enthusiastic manager of the conference. The staff of the host Institute deserves gratitude as well for the excellent help in organization. Thanks are due to Dr. Erzsébet M. Szász for the perfect technical preparation of the book, and to Mrs. L. Keviczky for the accurate clerical assistance.

I wish to express my acknowledgement to the Publishing House of the Hungarian Academy of Sciences for the beautiful presentation of the volume.

Budapest, March 1970

G. Ádám

OPENING ADDRESS

by

G. ÁDÁM

On behalf of the Organizing Committee I would like to express my warmest greetings and welcome to all participants of our Symposium, especially to those colleagues who have come from abroad. We are delighted to have you here in Hungary, in Tihany, for our first conference on learning and memory.

The biological aspects of memory storage are hotly debated in these days; it is also—unfortunately—a "fashionable" problem, and that is why it seems to me very important at least to speak a common language, to have a mutual understanding concerning the main physiological, biochemical and pharmacological questions of this highly complicated brain mechanism.

We think that this Symposium is an appropriate occasion to start such a mutual understanding. Distinguished workers from Eastern and Western Europe and from America have come here to the picturesque Lake Balaton, to this fine Institute to reduce to the same denominator some of the main questions of information storage.

A few weeks ago at the International Congress of Psychology in London several symposia on learning and memory took place. I must confess that an atmosphere of scepticism and pessimism has been characteristic of these meetings. One of our distinguished colleagues even alluded to his "swan-song" in this controversial field. I cannot agree with such a pessimistic view. Let us hope that our Symposium will not notify the decline, but the start of a real understanding!

WELCOMING ADDRESS

by

J. Salánki

During the past few years it has become traditional that a the end of the summer this Institute provides an opportunity for some national or international scientific meeting, and we are all truthfully very pleased. We are especially glad when the programmes of these meetings cover fields satisfying not only the interest of those who are directly working in it but, being of general importance, attract attention widely among biologists and non-biologists. It may be stated without exaggeration that the problems of learning and memory belong to this category of recent biological investigations. On the other hand, it is unquestionable that our knowledge is rather restricted in this field, and that the various trends of research are rather controversial. Therefore, a Symposium like this, where scientists from different laboratories exchange ideas and discuss the latest experiments and results, might prove to be very fruitful.

In this Institute we do not work directly on the biology of memory. Nevertheless, our Department of Experimental Zoology, investigating the physiology, functional morphology and chemistry of the nervous system in lower animals is carrying out research on those structures and mechanisms which also serve as basic elements for learning and memory. Research on the neurobiology of invertebrates has called attention in many cases to the fact that in the approach to some basic problems lower animals may be of excellent service either because of analogy or just as a result of divergency. Since the phylogenetical development of function is probably valid for all neuronal processes, I am convinced—as shown also by the programme of this Symposium—that the comparative approach is of great importance also in the forthcoming discoveries in problems of memory. For this very reason we are particularly glad to welcome here this Symposium.

Section I

MORPHOLOGICAL BACKGROUND OF MEMORY PROCESSES

MEMORY FUNCTIONS AND THE STRUCTURAL ORGANIZATION OF THE BRAIN

by

J. SZENTÁGOTHAI

Department of Anatomy, University Medical School,
Budapest, Hungary

(1) GENERAL CONSIDERATIONS

Neural organization is essentially a device for the recording and processing of information, including its storage and use for the control of various physiological functions. This task is accomplished, in multicellular organisms of the animal kingdom, by a set of special cells—the neurones (and certain auxiliary elements: the glial cells)—in which the above general properties of living matter have developed to a remarkably high degree. In living organisms a device for handling of information could be based, theoretically, on two principles: (*i*) a purely chemical information system with specific chemical encoding and signals but no specificity of the connecting channels,* or (*ii*) a multichannel "connectivity" system based on specific connexions, in which the information is encoded and transmitted in some very general and propagated change of the physiological state of the individual channels. As is well known, neither of these principles is encountered in the animal kingdom in its pure or even in a predominant form. In the neural organization both principles are applied in a very large variety of combinations. The most general—although by no means unique—combination is that information is conducted within the limits of the cell either by propagated impulses for longer distances or perhaps by electrotonic changes for shorter ones, whereas transmission from one cell to another is effected by chemical signals.

It is quite obvious that even in nervous systems based on the combination of the two, one mechanism might become so dominant that it completely overrides the significance of the other. One might conceive quite easily a nervous system having at its disposal only a single kind of neurotransmitter and two possible kinds of subsynaptic receptors, one producing excitation on receipt of the mediator and the other producing inhibition. Such a nervous system would, obviously, require a very high degree of connexion specificity, and it would be also quite uneconomical from the point of view of "specific integration capacities", i.e. the amount and variety of possible functional "decisions" calculated for the number of structural elements.

Conversely, one might conceive a nervous system in which larger distances between various parts of the nervous system (or of the body) would be bridged by nerve fibres with the capacity to conduct impulses very rapidly,

* The endocrine system is based on this principle.

but with rather poor connectivity at both ends of the fibres. The terminals of these fibres could be conceived to discharge very specific neurotransmitters into regional common tissue spaces, from which then the numerous receptors of the local nerve cells could pick up the combination of chemical information they are able to interpret and to use.*

Although many examples could be cited from various parts of the nervous system in which either of the two mechanisms becomes dominant, an overall dominance of one principle against the other does not occur. The two basic principles of construction and function are interwoven in the most intricate way and in most cases both aspects can be recognized depending on the viewpoint of our consideration. It might be rewarding to carry through such speculations to their ultimate consequences, with special consideration of the "specific integrative capacity" of the nervous tissue in the above-mentioned sense. Calculations could be made, for example, on how many bits of information could be processed by a simple neurone network of the relay nucleus type if it were built on the classical structural principle of the neurone theory with axon terminals arranged side-by-side on the surface of the dendrites or of the somata, and using as transmitter a single kind of neurohumour or two neurohumours: one excitatory and another inhibitory. And this might be compared with the information processing capacities of another network with all other parameters equal, but having complex synaptic arrangements (glomerular synapses etc.) in which the transmission of impulses would be envisaged to occur not primarily on the principle of connectivity but on that of the specificity of a variety of mediators. Although we do not have anything but vague ideas on how such a synaptic system might work, it is quite obvious that the integrative capacities of the second neurone network would be larger by order of magnitude. Hence a nervous system built on this principle ought to be more economical than one built predominantly on the principle of specific connectivity.

Taking into account these considerations, however speculative for the time being, it would be sheer naivity to try to explain anything but the most elementary nervous mechanisms on the basis of one of the two fundamental functional aspects. Already at the rather basic level of information processing—under which I mean here nothing more than the spatio-temporal transforms that happen to any pattern of excitation while being transmitted from one level of the CNS to another**—we would be at a loss if we were to explain everything on the basis of connectivity, i.e. to solve all problems arising by means of simple switching. The difficulties, evidently,

* Anatomical study of the CNS on the electron microscope level has revealed a considerable number of complex synaptic arrangements (glomeruli etc.) that are surrounded, and probably separated from the environment, by glial elements. It would be completely possible that the glomeruli (Szentágothai, 1965*a*, 1970*a*) and synaptic cartridges (Szentágothai, 1969, 1970*b*) would be such "regional tissue spaces" into which various kinds of neurotransmitters might be released by the several axon terminals present. The detailed connectivity in such assemblies might be unimportant.

** Most conveniently one might think of the transforms that occur in the afferent pathways from receptor fields to cortex.

increase if we begin to consider higher levels of neural functions, such as storage and retrieval of information. Even if we were convinced that information is stored for long terms in the nervous system in changes of macromolecules, neither distribution to the ultimate elements that can be stored, nor their retrieval would ever be explained without proper understanding of the connectivity or circuitry aspects. Thinking in general terms of information handling—both in living organisms and in technical devices—it is not the storage in some molecular conformation change that is the main problem, but how the message that is to be stored is broken down into its elements, how these are distributed to their sites of storage, and how the message can be retrieved by assembling the elements appropriately and in reasonable time (Elsasser, 1958).

We do know very little as yet of the logic of breaking down any message by the CNS into storable elements, although we have now some knowledge how the main sensory pathways can assemble elementary information picked up by the receptors, into messages that are meaningful to the animal. We are now labelling certain nerve cells, at various levels of the sensory systems, as "novelty detectors", "sameness detectors", "size- and movement detectors", "direction detectors" etc. All of these are complex properties that could not be perceived by any single kind of receptor. The basic condition for such processing is an extremely complex and specific convergence and evidence is accumulating quickly about the very high specificity of individual neurons, at all levels of the CNS, with respect to precisely determined specific convergences from very different sources. We know of neurones having not only multi-modal convergence within a given sensory region (for example skin) but convergence upon individual neurones from entirely different sense organs. It is the question whether such cells, or groups of cells connected to such multi-sensory cells, could retain complex information in the form of some *Gestalt*. Or, conversely, whether information reaching such cells is again broken down into elements and stored as such, distributed to hundreds or even millions of cells. At this point psychology will be an essential tool in understanding the logic of the brain in breaking down (and reassembling) *Gestalt* type messages into storable bits of information.

But irrespective of whether messages are stored in the CNS as *Gestalt* type complexes in specific cells of very highly selective convergence patterns, or whether this *Gestalt* is broken down again into elements further distributed, both solutions would need an unbelievable degree of specific connectivity. In addition, this connectivity cannot be of a one-way character, it must be reciprocal. Indeed, over all the CNS there is everywhere an extremely rich, multiplex and reciprocal connectivity. In this respect the usual textbook diagrams of pathways and neurone chains are highly misleading by not showing reciprocity and multiplicity of connections to which attention was called first by Lorente de Nó (1933). If this character of connectivity is considered, the rudimentary capacities of lower centres to retain traces of earlier functional events for certain duration of time become highly significant. We will no longer think that this could be explained primarily by assuming that it ought to be due to changes at specific synaptic sites.

Instead it will be realized that it is the assembly of neurons rather, with a multitude of possible changes (to be discussed in the second part of this paper), that could be the material basis of the retained trace. Extrapolating this to higher levels, it is quite obvious to assume that the capacity for retaining traces of functional events will be somehow related to the richness and multiplicity of internal connexions. If this speculation were correct, we would have to assume that centres with low and stereotyped internal connectivity would be less able to retain traces (memory) and form new functional connections (learning) than those having a rich, multiple, and reciprocal connectivity. The cerebellum, for example, with its remarkably low internal (associative) connectivity and stereotyped structure, ought to be considered in this line of reasoning as a "limited-purpose computer" for the solution of actual problems and of little if any significance in long term retaining of traces. The cerebral cortex, conversely, with its spectacular wealth, multiplicity and high specificity of connexions both inside restricted areas (intracortical) and between neighbouring and distant areas (associative and commissural), and of connexions relayed over subcortical nuclei, would be the ideal place for both memory and learning; as we all know it indeed is.

Considering the "lightning" speed with which messages can be stored away and even more with which they may be retrieved, one has also to speculate on how quickly information from any given site of the CNS might reach any number of other sites. This would mean in anatomical terms: how many synapses, on the average, would be needed in order to reach from a given neurone any other possible neurone of the CNS? Although we have no direct information at hand in answering this question, the number is remarkably low if one tries to think it over from what we know about CNS connectivity in general. It would be a fair guess to assume that the number ought to be somewhere around ten—as an order of magnitude. Restricting this speculation to the cerebrum, the number might even be reduced by a factor of two. This means that within ten synapses any point of the CNS would be connected—potentially—with any other. Consequently, the nervous system could be regarded, with minor restrictions, as something like a continuous medium to which, for example, the principle of holography (Julesz and Pennington, 1965; Pribram, 1966; Longuet-Higgins, 1968; Westlake, 1967; Gabor, 1968; Greguss, 1968) could be certainly applied, at least theoretically. This is by no means a suggestion that memory is, in fact, based on a principle analogous to that of holography. I am implying only that the anatomical structure of the nervous system would certainly not rule out—or one might even say that it would not seriously contradict—the assumption that memory functions could be based on principles analogous to holography.

(2) REPERCUSSIONS OF FUNCTION UPON STRUCTURE

After these general considerations it is obviously expected from the anatomist to tell about the possible changes that may occur in neural structures as the consequence of functional load or deprivation. Such changes have been

observed since long and many neurobiologists have felt that memory and learning functions might be related in some way or other to the phenomena of growth (Hebb, 1949) and its reverse (i.e. to building up or breaking down of living matter). As with all other tissues, also in the neural tissues use or functional load increase the mass and structural order of matter, whereas disuse leads to its decrease and loss. That functional efficiency again is related to mass and order of structure is common knowledge. These relations have been shown to be operational in the nervous system in quite a number of structural parameters.

(*a*) *Overall volume of nervous tissue.* It has been known for a long time that so-called transneuronal atrophies manifest themselves most clearly by a reduction of grey matter volume. However, as discussed in more detail elsewhere (Szentágothai and Hámori, 1969), transneuronal atrophies are probably produced by the loss of other trophic relations and by that of function. They cannot, therefore be considered as pure functional atrophies. An atrophy that is more likely to be purely functional occurs in the spinal grey matter after removal in young animals (dogs, 14 days of age) of the long bones (femur, tibia and fibula) of the lower limbs (Szentágothai and Rajkovits, 1955). The reduction in volume of the ventral horn is 9·5 per cent as compared with the opposite side (which may be considered as functionally overloaded) at the age of 3–5 months. The reduction in tissue volume (4–8 per cent) is less clear in the dorsal horn, but this is understandable when considering the functional changes that are likely to occur in a limb deprived of its skeleton (subsection *b*). A similar atrophy is experienced in the lateral geniculate body if young animals are deprived of patterned vision (Wiesel and Hubel, 1963). As cell bodies constitute only a minor fraction of the total volume of grey matter it is most likely that the major factor in the overall volume reduction is caused by the dendritic tree. A "withering" of the dendrites or somewhat less elaborate and profuse branching at their peripheries might cause quite considerable changes in tissue volume. It is also known from the study of later stages in neural tissue development that elaboration and a certain "shaping" of the dendritic tree to its final adult pattern is one of the latest steps in the maturing of the tissue. This is by no means a simple process running in one direction by growing out more branches to larger distances. On the contrary, many examples could be cited especially in neurons having dendritic trees of very elaborate and specific shape and branching patterns* that maturing means withdrawal of dendritic branches that have grown too far and do not fit into the final branching pattern. This process strikingly resembles the pruning of trees. This aspect shall be discussed in some more detail in subsection (*d*).

(*b*) *Fibre diameter* is a structural parameter of nervous tissue that is relatively easy to measure and to compare. A considerable amount of evidence has been accumulated on nerve caliber spectra under various cir-

* Nerve cells that have been labelled by Ramon-Moliner and Nauta (1966) as "isodendritic".

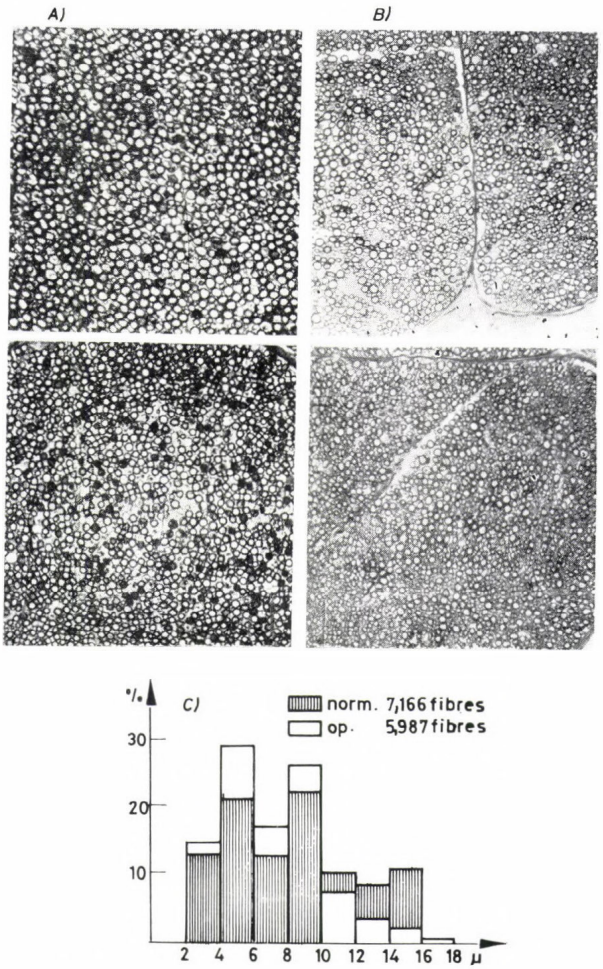

Fig. 1. *A*. Transverse section of ventral root (L_7) of a 4-month-old dog on intact functionally overloaded (above), and (below) same from the operated side, where the long bones (femur, tibia and fibula) have been removed at 14 days of age. *B*. Transverse section of dorsal root (L_7) from normal side (above) and operated side (below) from the same dog. *C*. Numbers of myelinated fibres and caliber-spectra from dorsal roots (L_7) of same dog, showing a significant loss in myelinated fibres and disappearance of the third peak corresponding to the large caliber (16–18 μ) afferents on the operated side (Szentágothai and Rajkovits, 1955).

cumstances of regeneration and transneuronal atrophy; however, it is not clear, whether the observed changes can be directly related to functional deprivation or overload. Hypertrophy of nerve fibres that supply functionally overloaded muscle has been shown by MacEdds (1950) and an obvious difference can be seen between both the ventral (Fig. 1*A*) and the dorsal (Fig. 1*B*) roots supplying a limb deprived of the long bones and the contralateral functionally overloaded limb (Szentágothai and Rajkovits, 1955). At inspection the difference seems more apparent in the ventral root, however, when counting the myelinated fibres, a reduction in number by 16·5 per cent is obtained for the dorsal root, as against a 7 per cent reduction only in the ventral root. The caliber spectrum (Fig. 1*C*) shows a complete disappearance of the third peak corresponding to the large 14–16 μ diameter fibres. This might indicate that the large caliber muscle afferents suffer specifically from the loss of the skeleton. As these fibres do not have significant synaptic connections in the dorsal horn (Szentágothai, 1967), the larger reduction in grey matter volume of the ventral horn (mentioned in subsection *a*) would have a simple explanation. The great loss in myelinated fibres can be explained only by assuming that a number of small caliber fibres that are normally myelinated have not developed a myelin sheath.

Another experimental model studied by Szentágothai and Rajkovits (1955) was the optic nerve of animals whose eyelid had been sutured shortly after birth. The caliber spectra of the two optic nerves of the same animal, with one eye sutured and the other left open, showed the same difference as the dorsal roots in the previously described model with removal of the long bones of the limb.

(*c*) *Synapse size* has been envisaged by several neurobiologists as a possible means by which functional load or deprivation might have a direct repercussion of effectivity of synaptic transmission. Few attempts have, so far, been made at determining the size changes that might be expected to occur under extreme circumstances. Changes were detectable, although not sufficiently significant, after removal of the long bones of the lower limb. The changes in synapse size were quite obvious in the base of the dorsal horn and the intermediate zone of the spinal grey matter after excision in early youth of the peripheric nerves and prohibiting their regeneration (Szentágothai and Rajkovits, 1955). As shown in a stereodiagram and histograms of the optic profiles of terminal knobs (Fig. 2), the difference in synaptic size is quite convincing. Trophic changes cannot be ruled out under such circumstances, however, as the change in the other structural parameters (grey matter volume, fibre diameter) was significant and of the same direction in the experiments both with removal of the long bones and with excision of the peripheric nerves, it stands to reason that reduction of synapse size occurs also under circumstances of purely functional deprivation and/or overload.

An attempt was made at recording, in a more indirect way, changes in the optic pathway on the level of the electron microscope (EM) after suturing the eyelids at an early age. As it would be extremely difficult to get reliable direct information on the size of the optic nerve terminals of the geniculate body in EM pictures, the ratio of synaptic surface profile length

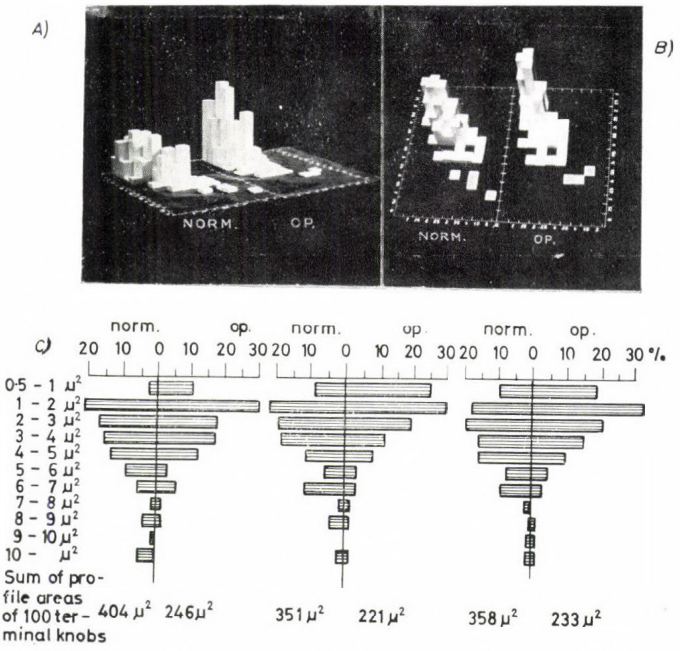

Fig. 2. *A* and *B*. Size of synaptic knobs at the base of the dorsal horn and in the intermediate zone of L_6 on the normal side of a 5-month-old dog (left) and on the operated side (right) after excision of all peripheric nerves from hindlimb at 7 days of age. The photographs show two views of stereohistograms in which the diameters of synaptic knobs are indicated in size classes of 1, 1·5, 2, 2·5, . . . μ, the longer diameters growing from back to front and the shorter ones from left to right. Height of the white prisms corresponds to per cent occurrence of terminal knobs that belong to the various size classes. *C*. Same in simplified surface area histograms in three different animals (Szentágothai and Rajkovits, 1955).

versus overall length of optic terminal profile surface was determined (Szentágothai and Hámori, 1969). In the characteristic synaptic glomeruli of the LGB advantage was taken of the fact that optic terminals can easily be recognized in the EM picture and can be separated from axon terminals of other origin. As seen in Table 1, the ratio is clearly lower in animals with sutured eyelids as compared with their normal litter-mate controls. This does not show, of course, that there is any difference in absolute size of the optic terminals. But it does indicate a dependence upon normal vision of the size of synaptic contact surface, which is obviously more important than the size of the terminal.

(*d*) *Cell migration and process formation* has been suspected since long ago to be in some connection with specific and neural functions mainly on

TABLE 1

Contact profile length of optic terminals in the cat LGB expressed as percentage of total optic profile circumference

Type of contact	Controls		Eyelids sutured after birth	
Contact with dendritic profile	34·6 ± 3·2	73·7	26·2 ± 1·8	53·7
Contact with monoptic axons	39·1 ± 2·9		27·5 ± 1·4	
Contact with glial elements	26·4 ± 5·1		46 ± 0·7	
Synaptic thickenings of optic terminals	12·4		8·9	

the basis of phylogenetic observations and reasoning. It appears as though nerve cells would tend—in the long phylogenetic scale—to migrate toward their chief sources of stimulation or to direct their dendrites in these directions (Ariens-Kappers, 1921). However naive the early concepts on "neurobiotaxis" might seem to us today, the basic facts are real enough, and they have received some unexpected support from the field of experimental neurology. Shofer et al. (1964) have shown with a most impressive example that after removal in the cerebellum of the superficial parallel fibres by X-ray irradiation* the dendrites of the Purkinje cells turn down in a weeping-willow fashion towards the undamaged deeper parallel fibres. Movements of Golgi 2nd type interneurons during the maturation of the primary optic cortex can be arrested by depriving animals of their vision (Valverde, 1968). A similar arrest of the movement of external granule cells in the cerebellar cortex towards the depth has been shown by Hámori (1969) to occur in the crossed cerebellar-atrophy after lesion of the cerebral cortex. As discussed at some length recently (Szentágothai and Hámori, 1969), such observations furnish no evidence at all to cells or dendrites being attracted directly by the presence of axon terminals or by neural stimuli. The mechanism works, obviously in an indirect way, probably over a complex intermediate chain of causal links, but the end result is still the same.

(*e*) *Changes in the internal structure of neurones* have not been, so far, sufficiently studied under extreme circumstances of functional load and deprivation. There are quite clear morphological signs of shifting the protein synthesis machinery to higher or lower gear according to functional load, but this will be discussed from a more general viewpoint in Section 3. It would be logical to assume that the relatively crude changes observed on the light microscope level (subsections *a* and *b* ought to have their equivalents in the ultrastructure of the processes. Very little, unfortunately, has been known about such changes. It appears though that in hypothalamic nuclei, which can be driven functionally by experimental interference with endocrine mechanisms, the dendritic microtubular systems become hypertrophic in nerve cells that otherwise show clear signs of increased

* Their cells are in most laboratory mammals in an undifferentiated neuroblastic stage at birth and can be destroyed easily by X-irradiation, while the cells of the deeper parallel fibres have differentiated earlier and are much less sensitive.

protein synthesis (Szentágothai, 1965*b*). This accidental observation has not yet been studied in detail, so that it should be treated with due reserve.

(*f*) *Changes in the complexity of synaptic articulation surfaces* begin now to emerge as a field of major importance. Various kinds of structural arrangements have been brought into focus by electron microscopy that exhibit clear evidence of the repercussion of specific function on nerve tissue structure. Dendritic spines have been shown on the light microscope level to depend very strongly on intact distant connections (Globus and Scheibel, 1967; Valverde, 1968) and even on orderly function (Valverde, 1967). Spine-like processes of LGB relay neuron dendrites, which in normal dogs by the age of two months project into deep invaginations of the optic afferents, have been shown not to develop at all if the eyelids are sutured shortly after birth (Szentágothai, 1968; Szentágothai and Hámori, 1969). Micro-spines or spinules are found in many synaptic regions (Szentágothai, 1965*a*; Eccles et al., 1967, Chapter VII) and even ordinary dendritic spines may show secondary outgrowths — so-called secondary spines that appear to establish secondary synapses in addition to the usual single synaptic contact of the spine (Hámori and Szentágothai, 1964). The most puzzling in these spinules and secondary spines is their variability both in size and in number. They may be abundant in one animal and may be rare or lacking in another in the same type of synapse. The only logical explanation is the assumption that such additional complexities of the synaptic contacts are induced by some unknown factor in the functional history of the animals in which they occur.

(3) FUNCTIONALLY INDUCED STRUCTURE CHANGES AND PROTEIN SYNTHESIS

It remains now to find a common biological frame for the structural changes induced by functional load or deprivation and to relate them to the mechanisms of growth. There is no doubt that most, if not all, of the changes described in Section 2 can be related in some way or other to protein synthesis. It has long been known that functional load enhances the synthesis of proteins and the building of various other plasma constituents in a wide variety of tissues. The most conspicuous signs of increased protein synthesis, such as increase in the nucleolar size and nuclear size, plasma basophilia have been widely used by histologists even before more exact modes of assays became available for assessing increased or decreased specific-functional activity. The relations between specific function and protein synthesis have been analyzed since the early studies of Hydén (1943) and Hamberger and Hydén (1949) in many ways and from several aspects. It has been shown quite recently (Wegener, 1970) that spatial patterns of physiological stimulation can be matched with similar patterns of increased protein synthesis. It has become clear by now that the basic cellular mechanisms underlying developmental process formation, growth and maturation of processes (chiefly of axons and their terminals), process regeneration, and functionally induced structural changes are essentially similar.

It would be, however, an extreme simplification to consider the neurone with respect to protein synthesis, or more generally to the building up of tissue matter—simply as another kind of cell reacting to functional load in the general way, with an increased synthesis of its materials. From the observations reported in Section 2, subsection *b* it becomes obvious that functional load increases the formation of myelin. As myelin is synthetized and formed by Schwann cells in the periphery and by oligodendroglia in the centre, the assumption that specific function has repercussions not only upon the nerve elements themselves but also upon the surrounding glial cells seems logical. Quite clear ultrastructural changes can be seen in glial processes in hypothalamic nuclei that are functionally driven (Szentágothai et al., 1968).* Attention has been called to the importance of metabolic interrelations between glia and neurones by Hydén (1964), and important biophysical and physiological aspects of these interrelations have been unravelled by Kuffler (1967). Interesting speculations about the possible significance of myelin in the storage of informations have been made by MacKay (1954).

The problem of protein synthesis in neurones is further complicated by the selective specificity in the site of the structural changes. From the accumulated evidence it appears that functionally induced structural changes are not simply a shift of the synthetic machinery to higher gear, but building up of matter and structure at very specific sites. Strangely, the major structural change is more often recorded at the postsynaptic site. Dendritic growth, dendritic protrusions, spines and spinules are all elements showing the highest degree of structural plasticity (Szentágothai and Hámori, 1969). Whether this can be related to the local availability of protein synthetic machinery (ribosomes, occasionally of Golgi systems, specific groupings of mitochondria and of various kinds of endoplasmic membrane systems, etc.) is unknown. Uptake of amino acids starts undoubtedly in the nerve cell bodies (Droz and Leblond, 1963) and an early movement of the incorporated material towards dendrites can be readily observed, but this does not preclude very specific mechanisms of synthesis in the distal parts of dendrites. Axon terminals lacking in ribosomes might be envisaged as depending more on supply of materials through the axons and on local uptake. For local uptake of materials from the intercellular space axons have the ultrastructural machinery in the form of very active pinocytosis, and uptake from local source of specific neurohumours into axon terminals has been unequivocally shown (Wolfe et al., 1962). In spite of that, accumulation of great numbers of mitochondria in axon terminals might indicate rather specific metabolic activities in presynaptic sites. Certain differences in histochemical staining properties of the mitochondria of the cell body or proximal dendrite and of the synaptic axon terminals in the same neurone (Hajós and Kerpel-Fronius, 1969) might also point to specific differences between the metabolic machinery in different parts of the neurone.

* The hypothalamus has the advantage that some of its nuclei can be specifically driven by interfering with various endocrine functions.

(4) GROWTH VERSUS PROTEIN HYPOTHESIS OF MEMORY

The concept of memory traces—the elusive engramme—being encoded in the ribonuclear self-reproducing machinery of the nerve cell has the obvious advantage of giving an intelligible molecular explanation of both the long term stability and specificity of the encoded information. The growth concept, on the other hand, has the merit of giving at least a faint idea of how anything that is encoded in some molecular change of the neurone might be retrieved as a change in the pattern of electrogenic events. It is, of course, important to realize from the material reviewed in Section 2 that the structural change induced by any specific functional event cannot be imagined as some quantitative change in one or the other of the structural parameters of the neurone. On the contrary, every stored element ought to be enviasged as a whole pattern of minute molecular changes in: (*i*) the central self-reproducing (nuclear and cytoplasmic) machinery, (*ii*) the various kinds of material conveying machineries of neuron processes (neurotubuli, neurofilaments),* (*iii*) the ultrastructural and macromolecular organization of the myelin sheaths, (*iv*) the local metabolic and synthetizing machinery (ribosomal, mitochondrial, endoplasmic membrane, etc.) of the immediate postsynaptic elements and postsynaptic portions of dendrites and axons, (*v*) various structures and geometric parameters of the postsynaptic structures (spines, spinules, dendritic protrusions and crests),** and (*vi*) in the protein-lipid (and possibly other macromolecular) structures of the synaptic membranes. It can be imagined what amount of specific information might be encoded in such a complex pattern of possible changes. And what is more, many of these changes are readily available for the "readout" by having an immediate repercussion upon the principal physiological parameters (thresholds, conduction, amplitude, recovery, repetition rates, quantity and time parameters of mediator release, etc.) of the neurone.

If looked upon in this way, there is no contradiction or antagonism whatever between the two concepts. They are complementary to each other and in many respects only two different aspects of the same thing. It is also in this way of reasoning that the apparent contradictions between the connectivity and chemical specificity aspects, from which we started out at the beginning (Section 1), are being resolved, at least in perspective.

The embryonic development of the nervous system, according to all information available, requires a refined and highly selective system of presumably biochemical specificities, which secures that various kinds of neurones establish connexions in strictly predetermined fashion. Nothing, virtually, is known about how axon terminals recognize the specific neurones with which they have to establish synaptic contacts. It is probable though that macromolecules attached to the outer surface of the membranes will decide whether an axon terminal arriving at a certain site at a certain stage

* It is impossible to enter here even marginally into a discussion of the fascinating problem of the mechanisms that ensure material movements of flows at various speeds in neurone processes (for ref. see recent review of this problem by F. O. Schmitt, 1968).

** I have discussed the various kinds of postsynaptic structure differentiations of the dendrites recently elsewhere (Szentágothai, 1970*b*).

of development establishes a synapse with the first neurone it encounters, or whether it has to grow on for a few tens of microns in order to find another more appropriate neuron. The surface properties responsible for establishment (or conversely avoiding establishment) of a contact are not distributed equally on the surface of the neurones, as can be deduced from the fact that synapses between the neurons are not established randomly in any part of the postsynaptic neurone, but on a strictly determined part of the receptive surface and with determined geometry. The mechanisms for the specific selection of appropriate connections are not inherent to the neuroblasts from the very beginning of their existence, but are gradually emerging in an epigenetic fashion from various inductions that they receive according to their position. There is thus ample evidence from the field of descriptive, and particularly from experimental, neuroembryology for not only a refined system of specificities (probably macromolecular in nature) but even for one that emerges in a crude and general form and that becomes more and more refined with development and maturation. It is thus by no means inconceivable that memory and learning are but further steps of neural specification in the postnatal development.

These considerations and analogies are certainly entirely inadequate to serve as models for a realistic approach to the problems of memory and learning. They might, however, help to set the stage for, or give something resembling, a preliminary framework into which our speculations and future efforts have to be fitted.

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FUNCTIONAL CONTROL OF NERVE FIBRE CONNECTIONS

by

R. M. GAZE[a] and M. J. KEATING[a, b]

Neurobiology Research Unit, Physiology Department, Edinburgh University, Great Britain

Most physiological studies of memory mechanisms involve experimentally produced alterations in neural activity followed by a search for any long-term structural or functional changes that may be induced by the experimental procedure. An alternative methodological approach would be to study a nervous system in which gross observable changes in structure and function are taking place and to attempt to elucidate the mechanisms producing these changes. The developing nervous system provides just such a situation. Since synaptic changes feature prominently in most current physiological theories of memory, investigations of the factors involved in the formation of synaptic connections are highly relevant. Of particular interest would be the demonstration that specific patterns of neural connectivity could be produced by specific changes in the functional activity of the developing nervous system, since any such functionally induced changes in connectivity could yield useful information for the further study of memory mechanisms. We propose to discuss the functional control of fibre connections in relation to one strictly circumscribed area of neural activity—the developing anuran visual system.

As a result of the work of Sperry and his associates (Sperry, 1943, 1944, 1945, 1951; Sperry and Miner, 1949; Attardi and Sperry, 1963; Sperry and Arora, 1965) it is now widely accepted that the ontogenesis of certain neural connections reflects the existence of refined chemospecificity mechanisms which match up appropriate sets of neurones with one another or with the non-neural periphery. A considerable amount of the experimental work which led up to the hypothesis of neuronal specificity has been done on the amphibian visual system and in this system it appears that (*i*) the ganglion cells of the retina become differentiated one from another during embryogenesis by the development of individual, presumably biochemical, differences. This differentiation of the retina occurs separately and at different times across the three spatial axes of the retina (Székely, 1954; Eakin, 1947; Jacobson, 1967). (*ii*) The tectum undergoes a comparable and matching chemospecification during development, such that matching fibres from the retina eventually terminate at the appropriate tectal locus by a process akin to 'sniffing' the specificities. (*iii*) There exists also, probably, a form of pathway specification such that growing fibres, under

[a] Present address: National Institute for Medical Research, Mill Hill, London N.W. 7.
[b] Barry Stevens Memorial Fellow, Mental Health Research Fund.

favourable circumstances, will choose the correct alternative when presented with a Y-junction spatial choice (Attardi and Sperry, 1963). The hypothesis of neuronal specificity has received considerable support over the past ten years from electrophysiological experiments on nerve regeneration and development (Gaze, 1959, 1960, 1967; Gaze and Jacobson, 1963; Gaze et al., 1963, 1965; Jacobson and Gaze, 1965).

The spatial specification of the retina manifests itself early during the development of the eye, while the eye anlage is still in a prefunctional (previsual) state. Thus retinal specification of this nature is independent of visual function. Operations performed on the embryonic eye after the retina has become specified and before retinotectal connections and visual function appear, result in an abnormality of visually controlled behaviour which closely corresponds to the type of abnormality produced by the operation in the spatial relationship between the eye and the brain. Thus an eye rotated 180° results in the eventual development of visuomotor behaviour which is also back-to-front and upside-down. Such maladaptive behaviour persits indefinitely with little or no sign of improvement as a result of visual function.

These experiments on the amphibian visual system gave rise to the hypothesis of neuronal specificity; and the hypothesis can account very nicely (though only, so far, at a superficial level) for the phenomena from which it was derived. But the part of the visual system that was studied in these experiments was the direct contralateral retinotectal projection, which in these animals covers the entire visual field. At the time of Sperry's early experiments this contralateral projection was the only visual projection recognized. There exists also, however, an *ipsilateral* visual projection in the frog (Zagorul'ko, 1957; Gaze, 1958; Gaze and Jacobson, 1962) and recent evidence (Gaze and Jacobson, 1963; Keating and Gaze, in preparation) suggests that the pathways concerned in the ipsilateral projection involve passage of impulses first to the contralateral tectum and then back, via an intertectal linkage, to the ipsilateral tectum. This projection is thus composed of two stages (at least): a first stage, which is part of the direct contralateral retinotectal projection, studied in the classical experiments of Sperry and others; and a second stage, which involves connection, probably via the post-optic commissures (Keating and Gaze, in preparation), between appropriate parts of the two tecta.

One of the observations made by Gaze and Jacobson (1962) in their electrophysiological investigation of the ipsilateral projection in the frog, was that, within the binocular field, one point in visual space projected via *both* eyes to one point on the left tectum and to another (not necessarily symmetrical) point on the right tectum. Only those points lying in the midsagittal plane of the animal would project to symmetrical positions on the two tecta.

In the direct contralateral retinotectal projection, nasal field (temporal retina) projects to rostral tectum and temporal field (nasal retina) to caudal tectum; and since the nasal extremity of the field for the right eye lies to the left of the animal while the nasal extremity of the field for the left eye lies to the right of the animal, any object extended in the horizontal direc-

tion in the binocular visual field must be represented extended in one direction rostrocaudally on the left tectum and in the opposite direction on the right tectum (Fig. 1).

The observations (Gaze and Jacobson, 1962) that one point in the binocular field projects via *both* eyes to one point on the left tectum and to another point on the right tectum, thus indicates that, through one eye, the ipsilateral projection of the nasotemporal field axis will have the reverse orientation to the contralateral projection of this axis. And since the second stage of the ipsilateral projection involves an intertectal linkage, this linkage must connect rostral contralateral tectum with more caudal ipsilateral tectum, and more caudal contralateral tectum with rostral ipsilateral tectum (Fig. 1).

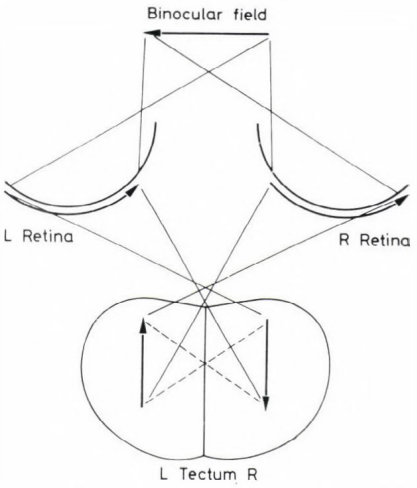

Fig. 1. Diagram representing the projection, rostrocaudally on the two tecta, of the nasotemporal binocular field axis for each eye. For both the right and left eye the nasal aspect of the field projects rostrally on the tectum, with more temporal field represented more caudally. The contralateral tectal representation of the field arrow thus points in a different direction on each tectum. Since one point in binocular visual space projects via both eyes to one tectal point on one side and to a different tectal point on the other, the intertectal connections effectively existing are indicated, in outline, by the dashed lines. The representation of the intertectal connections indicates merely the overall result of the connections, not the pathway.

We may now represent the contralateral and ipsilateral projections of the nasotemporal field axis in the form of a linear diagram (Fig. 2*a*). The intertectal connections that form the second stage of the ipsilateral visual projection are precise and retinotopically organized. How might they be set up during development? If we argue that they are formed by a mechanism of neuronal specificity akin to that which is operative in the formation of the contralateral retinotectal projection, then, as is the case with this latter projection, rotation of the eye after the time of intertectal specification should result in inversion of the ipsilateral projection. This is a necessary requirement of hypothesis; since with prespecified intertectal connections, inversion of the first stage of the ipsilateral projection must result in inversion of the second stage also (Fig. 2*b*).

The experimental findings in this situation are different. If the eye of a *Xenopus* is rotated during larval life, after retinal specification has occurred, its contralateral field projection is, of course, rotated; but its ipsilateral field projection is *normal* (Fig. 2*c*). And moreover, the contralateral projection from the normal eye in this situation is, naturally, normal, whereas the ipsilateral field projection from the *normal* eye is *rotated*, and to the same extent as the rotation of the operated eye (Gaze et al., 1970). These

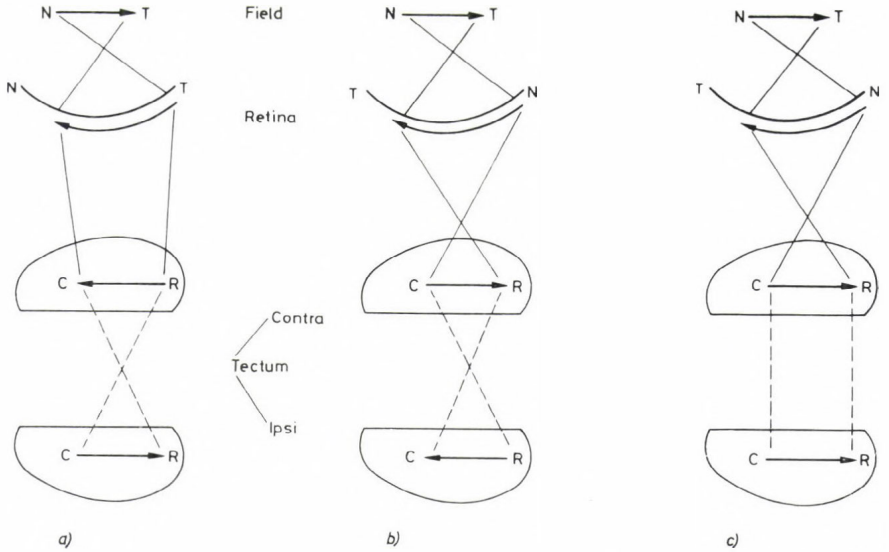

Fig. 2. (*a*) Linear representation of the contralateral and ipsilateral projections of the field of the right eye; (*b*) Linear approximation to what might be expected in the case of an eye rotated by 180° if the intertectal connections were determined by a specificity mechanism akin to that determining the contralateral retinotectal projection. In this case both contralateral and ipsilateral projections of the field are rotated; (*c*) Actual field projections found in animals with one eye rotated by 180°. The contralateral projection from the rotated eye is rotated while the ipsilateral projection is not. N, nasal; T, temporal; C, caudal; R, rostral.

findings exclude a simple prefunctional specificity system as the mechanism responsible for the elaboration of the retinotopically organized ipsilateral projection in this frog.

The situation in the case of an animal with a rotated eye may be represented as in Fig. 3. Both projections to one tectum are rotated (and congruent); that is, the contralateral projection from the rotated eye and the ipsilateral projection from the normal eye both go to one and the same tectum and are both rotated to the same extent; and both projections to the other tectum, that is, the contralateral projection from the normal eye and the ipsilateral projection from the rotated eye, are normal (Fig. 3). This means that the pattern of intertectal connections seen in an animal with a rotated eye is different from that seen in a normal animal (Fig. 2*c*). Keating (1968) has proposed that the intertectal connections that make up the second stage of the ipsilateral projection depend on a *functional* interaction between the inputs from the two eyes.

The abnormality in the ipsilateral projection from the normal eye reflects accurately the abnormality in the contralateral projection from the operated eye to the same tectum; and similarly, the normality of the ipsilateral projection from the operated eye to the other tectum reflects the normality

of the contralateral projection from the normal eye to that tectum. It seems thus that the ipsilateral projection from one eye is dependent on the input to the same tectum from the contralateral eye. The ipsilateral projection appears to require an interaction, at tectal level, between the two eyes. And since it is one point in visual space that projects via both eyes to one point on the tectum, no matter what the relative positions of the retinae, it is the *field* that is of importance rather than the geography of the retinal point stimulated; the interaction that occurs appears to be a functional interaction.

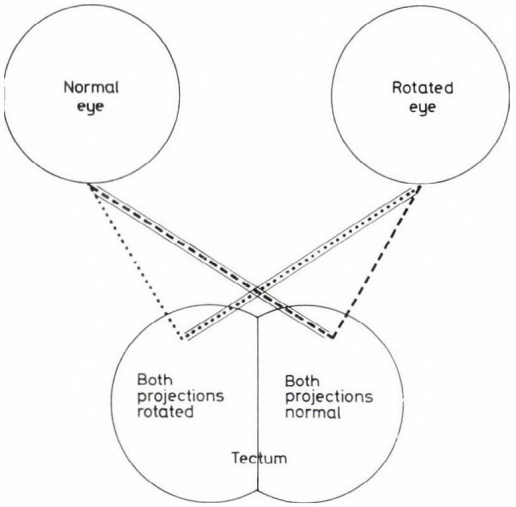

Fig. 3. Summary of the projections formed following rotation of a larval amphibian eye. Projections to the left tectum are shown by dots, those to the right tectum by dashes. In each case, the contralateral projections are shown within tramlines. The ipsilateral connections merely show the effective functional link, not the pathway.

The hypothesis is that, at some stage of development, the impulses from one eye cross to their appropriate position (determined by the mechanisms of prefunctional specificity) on the contralateral tectum and thence recross to that point on the ipsilateral tectum which is simultaneously receiving a similar spatiotemporal pattern of excitation via the other eye from the same point in the binocular field. Points on the two tecta receiving similar excitation patterns become specifically linked together (Keating, 1968; Gaze et al., 1970).

The hypothesis of functional interaction can account for the development of the normal ipsilateral projection, for that obtained after rotation of a larval eye and for that found in animals with 'compound eyes' (Gaze et al., 1963, 1965). In agreement with the hypothesis is the fact that, during larval life, when the eyes (in *Xenopus*) look out laterally, there is no ipsilateral projection. The first ipsilateral responses are found at about stage 62 (Nieuwkoop and Faber, 1956), during metamorphosis, when the eyes have started to move round to their adult position. Furthermore, the ipsilateral projection found in animals which have had one eye anlage removed early in development, is abnormal in that it is diffuse; in this case there has been no possibility of functional interaction between the two visual inputs since only one is present.

At the present time the developmental stages over which the interaction mechanism may work are being investigated. Preliminary results would indicate that this time may cover a period during and just after metamorphosis.

The significance, if any, of this developmental mechanism for the study of learning or memory is, of course, unknown. It may be relevant, however, that somewhat comparable mechanisms have been proposed concerning the mode of formation of conditioned reflexes (Grastyán, 1967; Hebb, 1949). And whereas the importance of functional interaction in the preservation of the normal binocularity of visual cortical cells in cats has been demonstrated by Hubel and Wiesel (Hubel and Wiesel, 1963, 1965; Wiesel and Hubel, 1963, 1965), the observations described here and treated in detail by Gaze et al. (1970) appear to demonstrate the formation of a new, precise, spatial arrangement of fibres as a result of binocular interaction between the two eyes, wich could involve visual function.

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Note added in proof. Since this paper was submitted we have investigated the pattern of intertectal linkages in *Xenopus* reared in the dark (*J. Physiol.* in press). In these animals, an approximately normal ipsilateral visual projection was found; the significance of these, and other relavant results, will be discussed in following papers.

DISCUSSION

F. Rosenblatt: Is there a time-lag between the appearance of contralateral and ipsilateral connections in the rotated eye experiments?

R. M. Gaze: In the experiments that I have been describing, the eye-rotations were performed early in larval life and the recordings made some time after metamorphosis, when both projections had appeared; so these experiments do not allow us to answer that question. However, as I said in my talk, the ipsilateral projection in Xenopus only appears at or shortly after metamorphosis, whereas the contralateral projection appears early in larval life. In this sense, certainly, the contralateral projection appears in normal development before the ipsilateral one.

F. Rosenblatt: Do you have any evidence that would distinguish between the alternatives of a single fibre termination hunting for a point of contralateral activity, or a large network of potential fibre pathways, from which the appropriate one is selected?

R. M. Gaze: We have indirect evidence only on this point. Firstly, the development of an ipsilateral projection in animals with only one eye, even though this projection is abnormal, would suggest that various intertectal connections may exist even in the absence of interaction. Secondly, in the

early stages of regeneration of the frog optic nerve (Gaze, R. M. and Jacobson, M., 1963, *Proc. Roy. Soc.* **B 157**, 420) we found that fibres from a small localised region of the retina could transmit activity to a wide region of the tectum; this suggests extensive branching of the fibres at this time, which is not normally found.

G. HORN: How specific is the restoration of the contralateral connections after rotating the eye? Is the columnar organisation restored?

R. M. GAZE: The contralateral projection may be restored in a highly specific fashion after eye rotation and nerve section. The result may be just as if the eye had been rotated without nerve section. The columnar organisation may also be restored.

G. HORN: Within a column, is the horizontal organisation restored?

R. M. GAZE: Yes. As was first shown by Maturana et al. (Maturana, H. R., Lettvin, J. Y., McCulloch, W. S. and Pitts, W. H., 1959, *Science 130*, 1709), the depth organisation may be restored after nerve regeneration. However, we have found (Gaze, R. M. and Keating, M. J. 1968, *J. Physiol. 200*, 128–129) that the *relative* depths of various response types is less after regeneration. This presumably reflects the diminished thickness of the superficial layers of the tectum after nerve section and regeneration. The regenerating fibres appear to grow down into the tectum until they meet up with their appropriate sites of termination, rather than to grow down for a predetermined distance and there terminate.

O. L. WOLTHUIS: If you were to project light into two different areas of the left and right eye, would you get false connections in the tectum? According to your hypothesis this would occur.

R. M. GAZE: This should occur under certain circumstances and we are currently trying to do comparable experiments. We would like to be able to deviate the optic axis of one eye by optical means and see whether this upsets the ipsilateral projection. So far the technical difficulties are proving formidable. We have so far one case of a 'naturally produced' optical defect (two lenses; Gaze, Keating, Székely and Beazley, 1970) which appeared to produce the expected result. We are currently also rearing animals in the dark to observe the effect on the ipsilateral projection.

S. FREED: To what extent does the animal behave as if the remembered events before the eye was rotated?

R. M. GAZE: We have not made behavioural observations, since these can tell us nothing useful about the actual connections formed. But the earlier work of Stone and Sperry (reviewed by Sperry, 1951, in *Handbook of Experimental Psychology*. p. 236, Ed. S. S. Stevens, Wiley, New York) indicates that these animals are completely misled by eye rotation and that they show no sign of learning or adaptation to their altered visual environment for periods of years.

FRONTAL LOBE INJURY AND MEMORY FUNCTIONS

by

M. GERBNER

Psychological Institute of the Hungarian Academy of Sciences, Budapest, Hungary

Learned reactions are markedly altered by injuries of the prefrontal area and the excitable motor areas of the frontal lobe. Hyper-reactivity, perseveration, deterioration of discriminative performance, discrimination reversal and delayed reaction as well as de-inhibition of differential conditioned reflexes are frequently observed in lesions of the prefrontal area (Jacobsen, 1935, 1936; Allen, 1939, 1941, 1943, 1949; Konorski et al., 1952; Lawicka and Konorski, 1959, 1961; Konorski and Lawicka, 1964; Brutkowski et al., 1956; Brutkowski, 1959, 1964; Brutkowski and Dabrowska, 1963; Pribram, 1961; Pribram et al., 1964; Gross, 1963; Gross and Weiskrantz, 1964). Well-defined sensory disorders were, however, not found, and the injuries of the prefrontal cortex did not influence the execution of motor acts (Rosvold and Mishkin, 1961; Battig et al., 1962; Brutkowski, 1964, 1965; Luria, 1966*a*, *b*). The syndrome observed after a prefrontal lesion has often been considered to involve an impairment of act-inhibition (Kalischer, 1911; Stanley and Jayness, 1949), or a loss of inhibition in competing response tendencies (Brush et al., 1961; Battig et al., 1962; Brutkowski, 1964, 1965; Mishkin, 1964; Shumilina, 1966). This hypothesis is presently in the best agreement with the observed experimental facts. This explanation of the reaction changes observed after prefrontal injuries involves an alteration of the elementary memory functions, i.e. memory traces and their elaboration.

Concerning the loss of motor responses, frequently observed after injuries of the excitable motor region of the frontal lobe, two contradictory explanations were forwarded a long time ago. According to the first one this loss would be due to a defect of merely the executive mechanisms not affecting the memory functions (Ferrier, 1890; Leyton and Sherrington, 1917; Bucy, 1934, 1949; Walshe, 1947; Penfield, 1954; Pribram et al., 1955/56). The second one claimed the presence of memory disfunctions and did not believe in the existence of disorders in the motor mechanism (Hitzig, 1874; Franz, 1907). Thus an analysis of the two fundamental solutions possible seems to be unavoidable for the investigator of the problems which relate the frontal lobe to memory functions.

METHODS

Dogs standing on a platform in a sound-proof chamber were used. The right foreleg of the animal was placed passively on the feeder and food was offered. After several repetitions the animal became capable to carry out this limb movement actively. When this occurred, a 300 cps generator sound was introduced, and only the reactions which were elicited by this stimulus were reinforced. After several associations the animal could carry out the limb movement in response to the stimulus, i.e. conditioned instrumental motor reflex developed. As soon as the response became firmly established a 700 cps sound was presented without reward. In due time, the initially generalized motor response failed to operate in the latter case as an indication of the development of a conditioned instrumental differentiation. Concurrently, any response given to the sound of a buzzer was reinforced by food except when the buzzer sound was preceded by the ringing of a bell 5 sec earlier (called conditioned inhibitor). Proceeding in parallel with the conditioned instrumental differentiation this procedure developed a conditioned inhibition. The criterion to perform the operative lesion was either a response level at which the animal responded correctly to the reinforced stimulus in 95 per cent of the trials, and uncorrect responses to the non-reinforced stimulus did not exceed 5–10 per cent, or when the number of training sessions attained 200. The respective frontal lobe areas were aseptically injured by subpial suction under intravenous potentiated anaesthesia. After a recovery phase of 5 to 7 days we investigated the effects of the lesions on the performance of learned reactions. The dogs were sacrificed after the post-operative experiments. Their brains were removed and fixed in formol solution. The area of the lesion was identified macroscopically by using ferric chloride and ferrous cyanide to stain the grey matter.

THE EFFECTS OF THE VARIOUS LESIONS

The most significant effects were found in association with the following localization of lesions:

(1) In the frontal gyrus, i.e. the dorsal prefrontal area.
(2) In the lateral part of the anterior sigmoid gyrus, i.e. the lateral premotor area.
(3) In both the frontal and the anterior sigmoid gyri.
(4) In the motor area lying between the cruciate and coronal sulci (Fig. 1).

The prefrontal lesions did not affect the reinforced conditioned reflexes. On the other hand, erroneous, i.e. non-reinforced responses, as well as intersignal reactions were more frequent after the injury in all animals. After the injury of the lateral premotor area four of the animals lost their learned responses completely, and the fifth one partially. Thus the lesion of the prefrontal area interfered with the inhibition of non-reinforced reactions (in agreement with the observation of the authors mentioned), while premotor lesions resulted in a deterioration of reinforced responses (Fig. 2, left side).

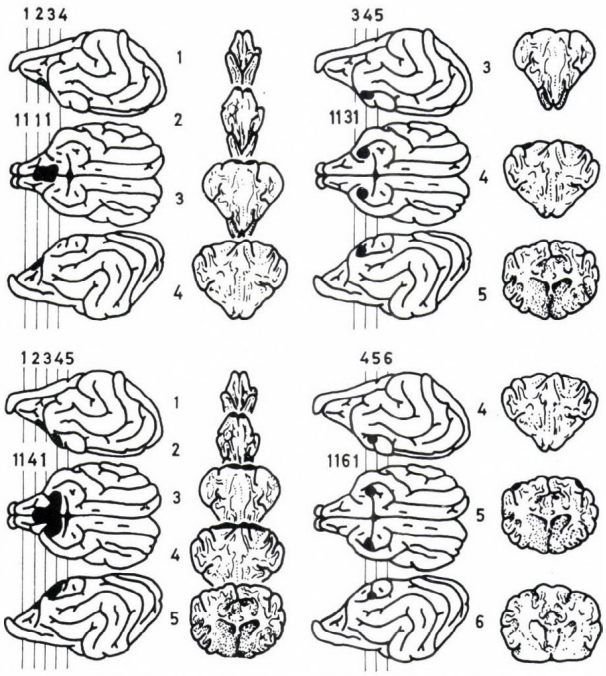

Fig. 1. The typical location of lesions.

The conditioned response was completely lost in the four animals in which both the prefrontal and the premotor areas were injured. The reinforced response remained intact only in the case of the fifth dog whose premotor lesion was the least severe. In the latter case the errors committed by responding to non-reinforced stimuli increased in the manner observed after prefrontal lesions. Since solitary prefrontal lesions involved a more frequent manifestation of erroneous non-reinforced reactions, this result indicates that the presence of the premotor area is a necessary condition to obtain such errors in case the prefrontal area had been injured (Fig. 2, middle part).

The reflex-loss following the premotor lesion might be attributed to a defect in the motor mechanism. Accordingly, we determined and injured the motor region whose threshold-current stimulation had previously evoked an optimal contraction of the contralateral leg. All animals showed motor disorders; they raised their forelegs higher than before the lesion, and crossed them. Sometimes the leg was bent backward from the ankle. However, the conditioned response was lost in only one dog. Thus the loss of conditioned reactions does not derive from an impairment of the motor mechanism (Fig. 2, right side).

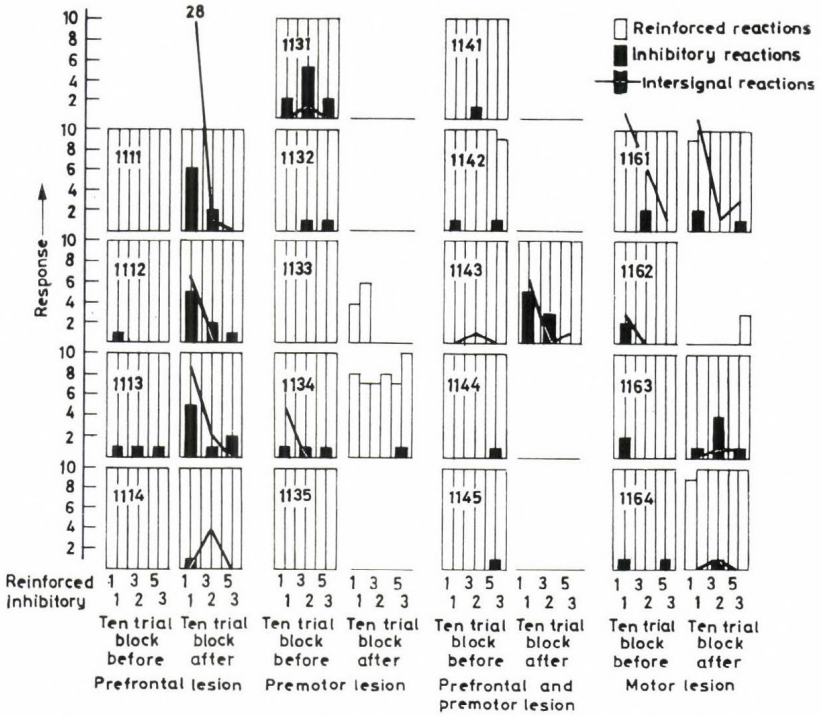

Fig. 2. The effect of the lesions performed in the respective areas of the frontal lobe. 10 trials are one unit on the abscissa. Ordinate, number of responses. Correct responses to the reinforced stimulus are indicated by white bars, erroneous ones given to the non-reinforced stimulus by black bars. The lines show the number of intersignal responses. Numbers represent individual animals before and after the lesion.

IMPAIRED INHIBITION OF RESPONSES AFTER PREFRONTAL INJURY

After a prefrontal injury the non-reinforced differential stimulus and the conditioned inhibitor elicited fewer erroneous responses than the partially reinforced component of the conditioned inhibitory complex. We found also a more elevated rate of intersignal responses. It has to be noted here that at the very beginning of the training trials such responses were reinforced (Fig. 3).

Thus the effect of partial reinforcement was manifested also when the inhibition of the response was impaired by the prefrontal lesion. In agreement with a previous similar observation of Brutkowski et al. (1956), the extent of the impairment of the inhibition depended also on the occasions of the reinforced employment of the same stimulus. Consequently, the impaired inhibition of responses after prefrontal injury is not simply the

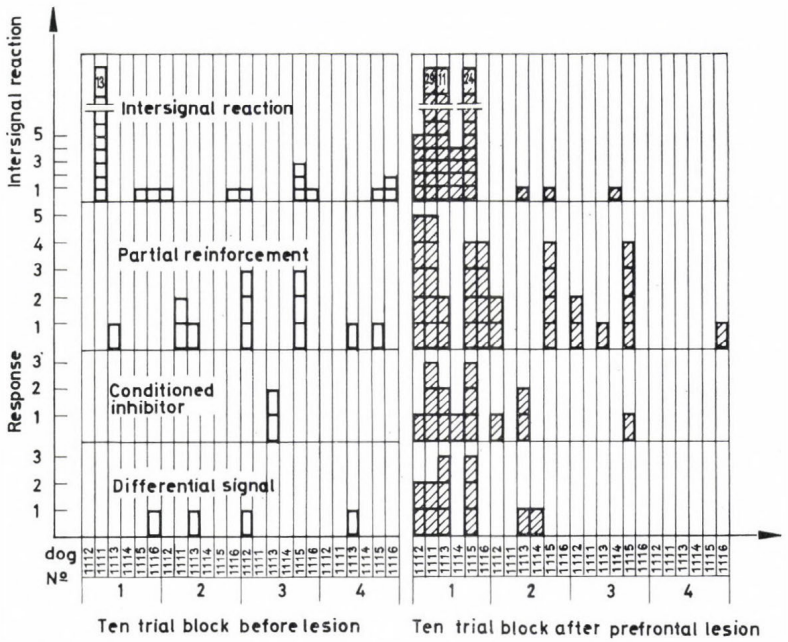

Fig. 3. The impairment of response inhibition after prefrontal injury. Ten non-reinforced trials are one unit on the abscissa. 40 trials each before and after the injury performed in six animals; numbers represent individual animals. The erroneous responses given to the various non-reinforced stimuli are indicated on the ordinate. White and striated bars represent erroneous responses before and after the prefrontal lesion, respectively.

result of a defect restricted to the inhibitory memory functions. In case it was employed, the memory function of a previous partial reinforcement may also become operative.

THE LOSS OF RESPONSES AFTER PREMOTOR INJURY

The conditioned reactions lost after the injury of the lateral premotor area became again elicitable in four of the animals after 60–120 trials. Concurrently, their erroneous responses given to the non-reinforced stimulus increased for a time to a higher level than before the lesion. One half of the animals also showed an increase in the erroneous intersignal reactions (Fig. 4).

In four other animals the premotor lesion caused a lasting loss of conditioned responses. The conditioned leg reaction was re-taught when the animals failed to react in any of the 300 trials. Re-learning was fast, in two of the animals even faster than the first learning. However, the non-

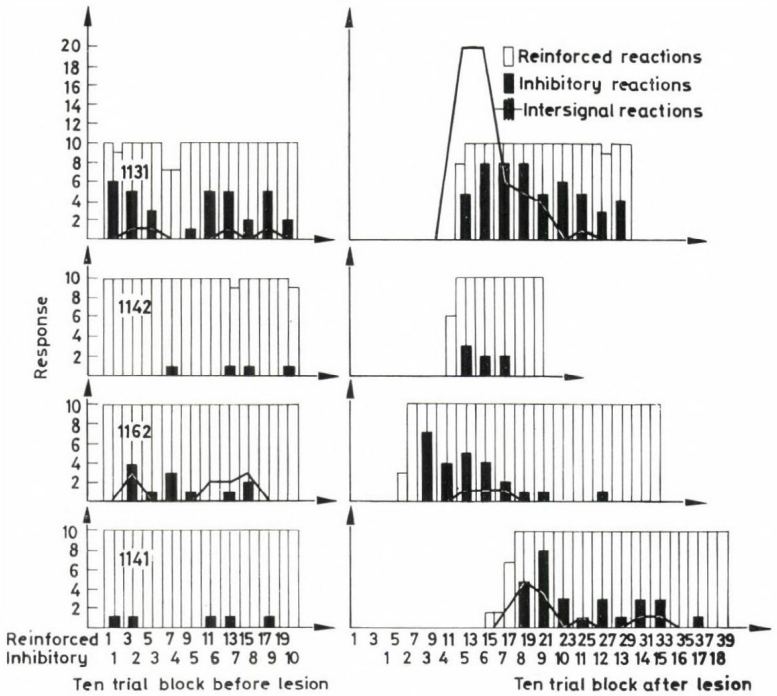

Fig. 4. Spontaneous recovery of responses after premotor injury. For legends see Fig. 2.

reinforced stimulus evoked fewer errors than before, in contrast to the animals with spontaneous reflex recovery. The inhibition once acquired was not damaged in connection with the lasting loss and re-learning of conditioned responses following premotor lesions (Fig. 5).

The temporary increase of erroneous responses observed in spontaneous recovery seems to suggest the action of a compensatory mechanism. Such over-compensation occurs also in other natural and artificial regulating systems based on an antagonistic mechanism. Concerning the origin of this compensation only tentative answers may be given. In the lasting loss of reactions after a premotor injury no effective compensation could be found. Accordingly, over-compensation did not occur during re-learning.

A HYPOTHESIS CONCERNING THE STRUCTURE OF FRONTAL LOBE MEMORY FUNCTIONS

The prefrontal lesion was shown to interfere with the inhibition of non-reinforced responses. On the other hand, the premotor injury impaired the performance of reinforced reactions. The defects in the two fundamental manifestations of elementary learning, namely the response to reinforced

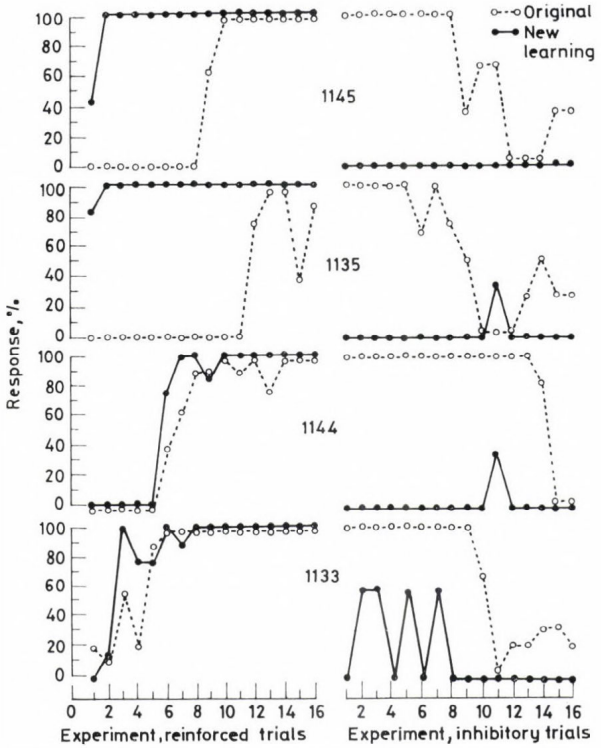

Fig. 5. A comparison of original and re-learning following the loss of responses by premotor injury. Abscissa, sessions; ordinate, the number of responses expressed as a percentage of administered stimuli. Numbers denote individual animals. Reinforced trials are shown on the left side of the figure, and non-reinforced ones on the right side.

stimuli, and its inhibition in case of other stimuli, depend on the injured region of the frontal lobe.

However, the number of errors committed after prefrontal injury is not only related to the defect in the inhibition evoked by the non-reinforced application of the stimulus, but also to the number of the administered reinforcements. Furthermore, the erroneous reactions observed after prefrontal injury seem to be mediated by premotor mechanisms. Again, the increased number of erroneous responses which were observed during the spontaneous return of reflexes after premotor injury may be attributed to an antagonistic compensatory mechanism.

All these changes in the elementary acquired responses that follow the injury of the investigated regions of the frontal lobe might derive from a defect in the system storing and elaborating memory traces. In the suggested corresponding system for memory functions (*i*) the prefrontal struc-

ture would take an active part in the inhibition of responses which were associated with non-reinforced information, (*ii*) premotor activity would cooperate in such learned responses that derived from reinforced information, (*iii*) the two regions would act in combination, i.e. the relationship of the two directions of this memory function would be reflected by a quantitative probability of the response, and the premotor mechanism would mediate the effect of prefrontal injury, and (*iv*) the intervention of a compensatory mechanism might be postulated. The present experiments indicate that the prefrontal and premotor areas of the frontal cortex are part of a system of response regulation which exerts its influence in conjunction with other structures of elementary learning and retention. By utilizing its stored information this system governs the responses which are linked to actual stimuli.

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MEMORY AND BRAIN STEM FUNCTION

by

F. KATONA

Institute of Neurosurgery, Budapest, Hungary

> "This is a gift that I have simple, simple; a foolish extravagant spirit, full of forms, figures, shapes, objects, ideas, apprehensions, motions, revolutions: these are begot in the ventricle of memory, nourished in the womb of pia mater, and delivered upon the mellowing of occasion."
>
> (Shakespeare: Love's Labour's Lost)

> "There are those who dispose the faculties according to the ventricles, like Michael Scot, Thomas Aquinas, Albertus Magnus etc. locate the memory here."
>
> (William Harvey: Anatomy)

In the 16th century it was just as commonplace as it is today that memory is located in the brain. Literature, philosophy, theology and medical science all agreed upon this question, and following the trend of the Alexandrian school of medicine they placed the faculty of human memory in the posterior ventricle of the brain.

Our scientific approach more refined and cleared by errors made during the past, knows at least where not to search for memory traces, and avoids at present the ventricular system, which was favoured for 2000 years.

The present trend in research of memory and memory transfer seems to avoid any question of localized organization in the central nervous system. Upon the traits of Bethe, Lashley etc. the whole brain, the complete volume of the central nervous system is taken out and is given homogenized into another animal to control possible results of transferred behaviour. On the other hand, neurophysiology looks for a more definite site in the brain, the temporal lobe and its correlate system.

While up-to-date methods of neurophysiology make use of old theories of the localizationist and equipotentialist clinical medicine seems to be at a loss in this question?

This meeting has proved that even those who apply the classic psychobiological methodology in research on learning, memory, and memory transfer and work with whole brain in rats, rely upon clinical evidence. Thus several remarks have been made on the well-known observations of Penfield et al. regarding temporal lobe function and human memory. Localization and antilocalization is partly confused due to difficulties in transfer technique in large numbers of animals, where ablation methods combined with learning and transfer would decrease the statistical figures produced by the easier non-localization method. However, one would sug-

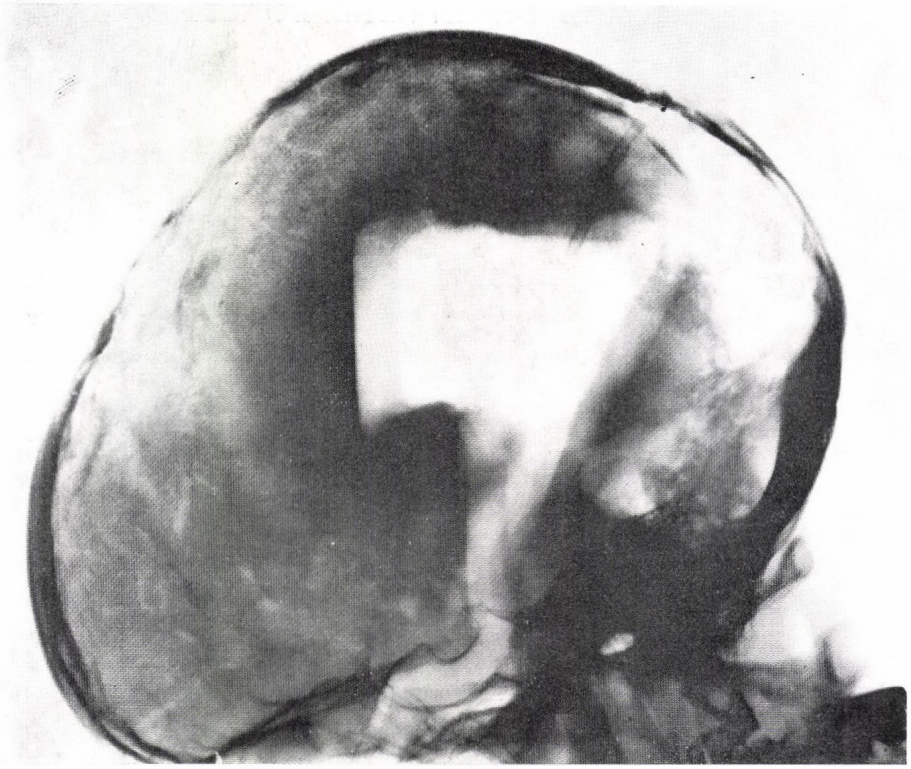

Fig. 1.

gest this trend as a method of choice, at least from the clinician's point of view.

In clinical practice where memory problems emerge usually we deal with some damage of the brain. It should be pointed out that Penfield's observations were made only on epileptic patients, which fact has not been mentioned here.

If we look for serious generalized damage in the brain affecting large areas in order to satisfy the school of Lashley, we come to the disease of infantile hydrocephalus. In some cases the enlarged ventricular system, the pressure of the cerebrospinal fluid on the brain leaves but a thin layer of 1–3 cm of the cortex. A considerable part of the brain is ablated, and most damage is usually done to the frontal and temporal lobes, whereas subcortical structures are better preserved.

It is clear that behaviour of these infants is regulated by subcortical mechanisms, and elementary reflexes develop and function according to inborn patterns.

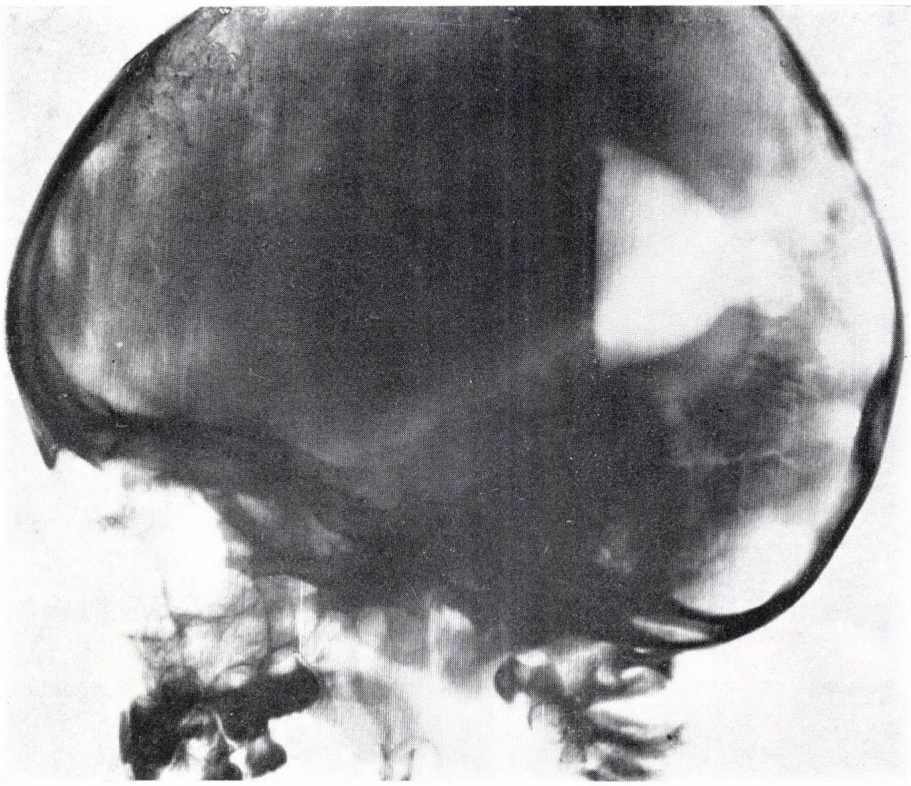

Fig. 2.

However, in some cases where functional ability is preserved, certain elements of learning and storage can definitely be observed. Such an element is time and feeding. No doubt that the relation between feeding and time is a well-known primitive memory pattern in normal infants. This connection of feeding and time is maintained in hydrocephalic infants in spite of considerable loss in brain volume.

Though the overall volume of the brain decreases considerably in such cases, no part of the hemispheres is entirely lost in the sense of ablation. The outer layer of the lobes, e.g. are preserved (Figs 1 and 2).

In such cases elementary orientation and learning is present. Time reaction to feeding develops to the hour, and shifting of this timing is stabilized again and again in the infants as long as conscience is maintained. The tolerance of basic mental function to extreme volume losses is one of the reasons why hydrocephalic infants can be successfully treated by the method of ventriculoatrial shunt. Diminishing the volume of the CSF in the ventric-

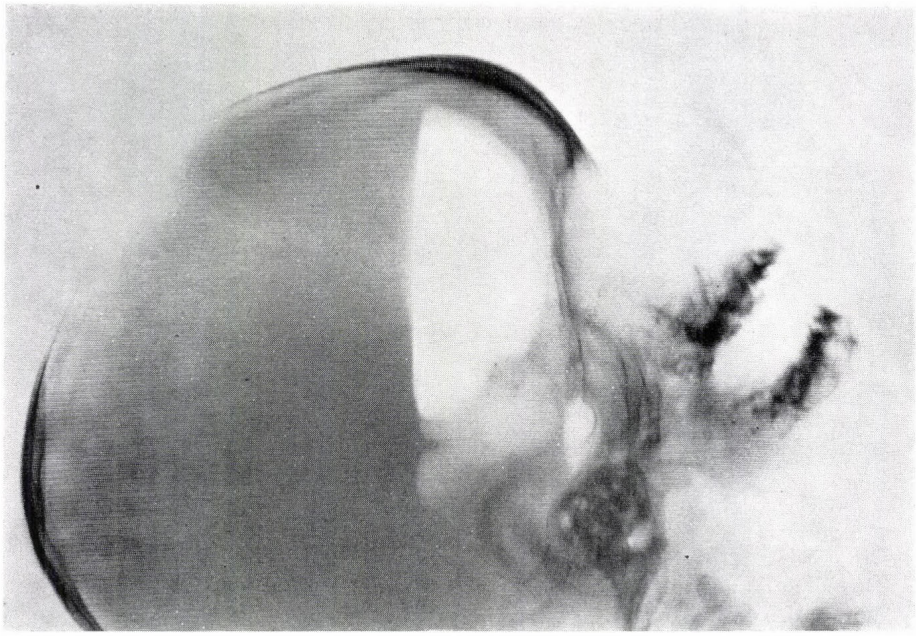

Fig. 3.

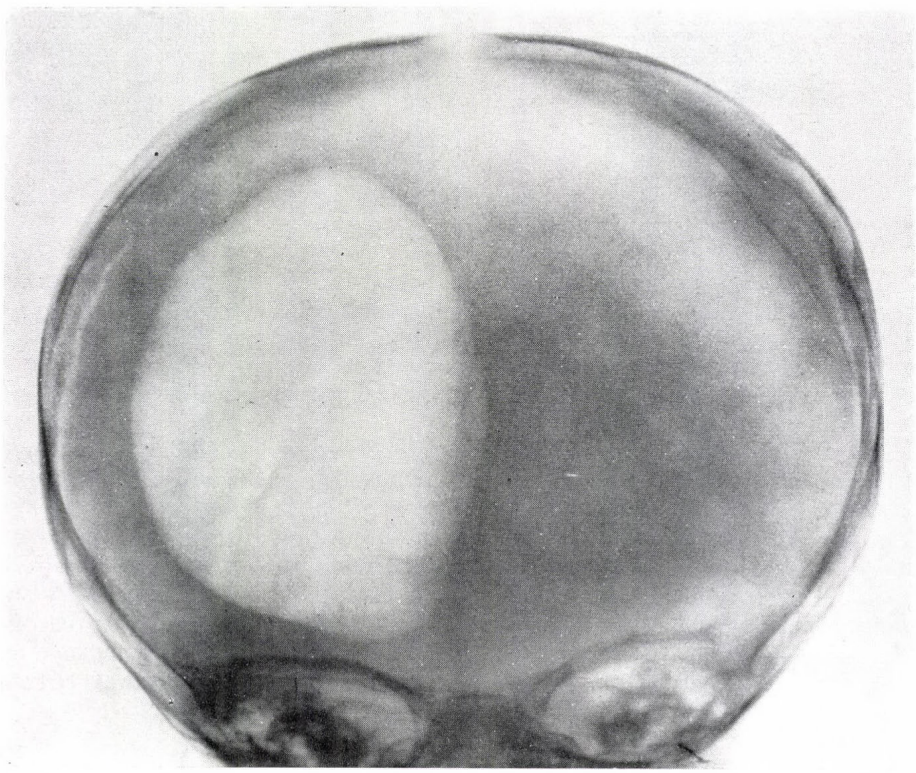

Fig. 4.

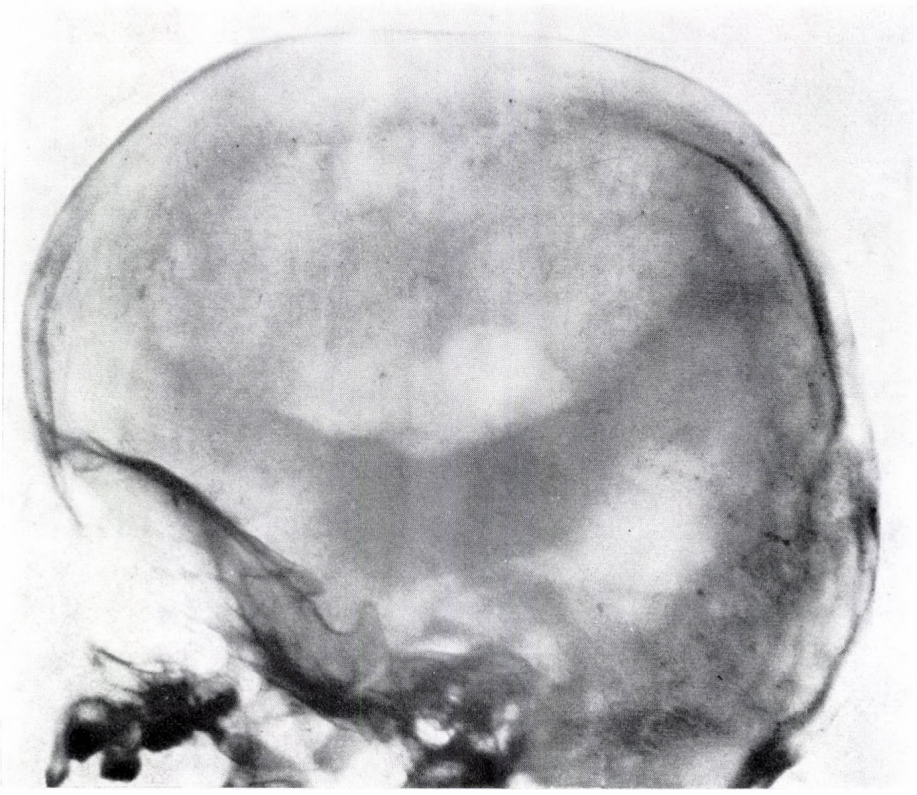

Fig. 5.

ular system decreases the pressure acting on the brain and a successive expansion increases the total volume of the hemispheres. Mental development takes place as a result of elementary learning and storage maintained even during expansion of the fluid and shrinkage of the brain.

These cases are basically different from the ablative methods applied by Lashley and followers in order to study intelligence performances. Nevertheless nature sometimes produces spontaneous ablations as a result of congenital malformation. In such cases sometimes complete lobes, e.g. the temporal or frontal lobes do not develop at all or only in a rudimental form. It is well known that other lobes of the brain may compensate the loss and they are discovered only incidentally. Sometimes there are no mental defects in these cases even in the absence of complete lobes of the brain (Figs 4 to 8).

However, if both lobes are completely involved bilaterally, severe disorders of intelligence, learning, and storage develop. In cases of serious unilateral damage, sometimes considerable learning capacity is preserved and

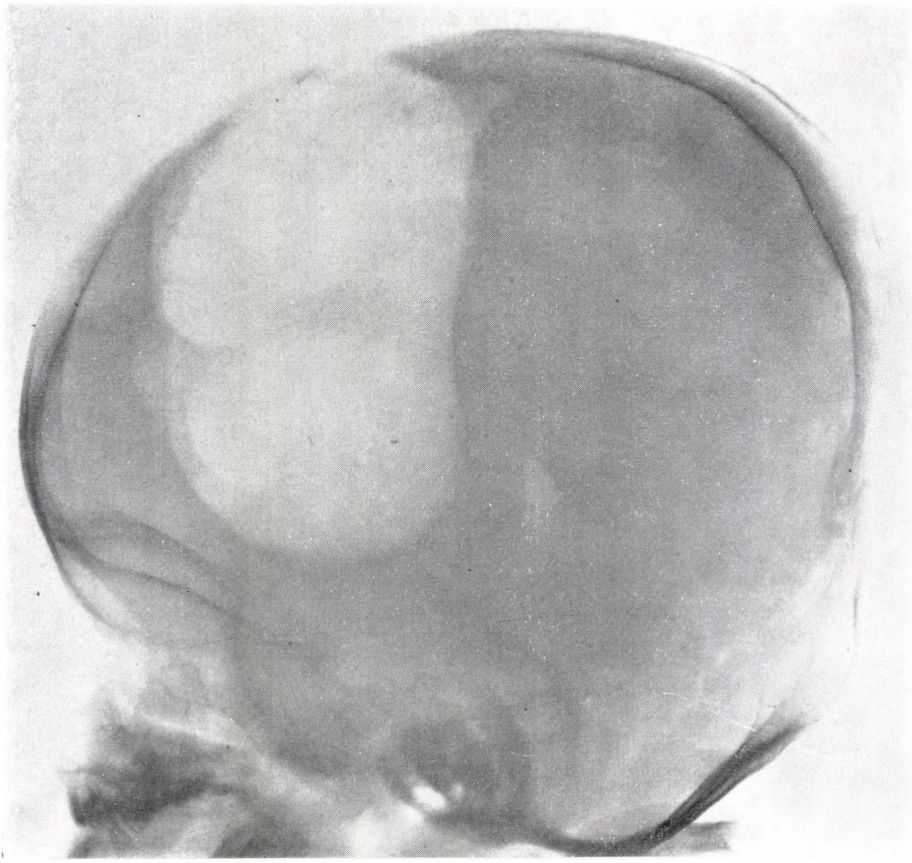

Fig. 6.

not only elementary orientation develops, but a thorough contact is built up with the surrounding world.

It is well known in clinical practice that hemispherectomy of the non-dominant part does not alter grossly intelligence performances.

How loss of brain volume interferes with memory and storage is a problem of clinical medicine. Hydrocephalus developing in small children does not alter mental faculties if the pressure builds up slowly and the brain can yield owing to the expanding skull. In a typical case, for example, a gradually developing hydrocephalus owing to a tumour blocking the ventricular system dilated the ventricles. Nevertheless the 4-year-old boy was in an excellent mental state. His memory was completely intact, he was able to recite long poems (Figs 9, 10).

However, if lesions of the brain stem are suspected or proved there is always a chance to face severe disturbances of memory even if conscience

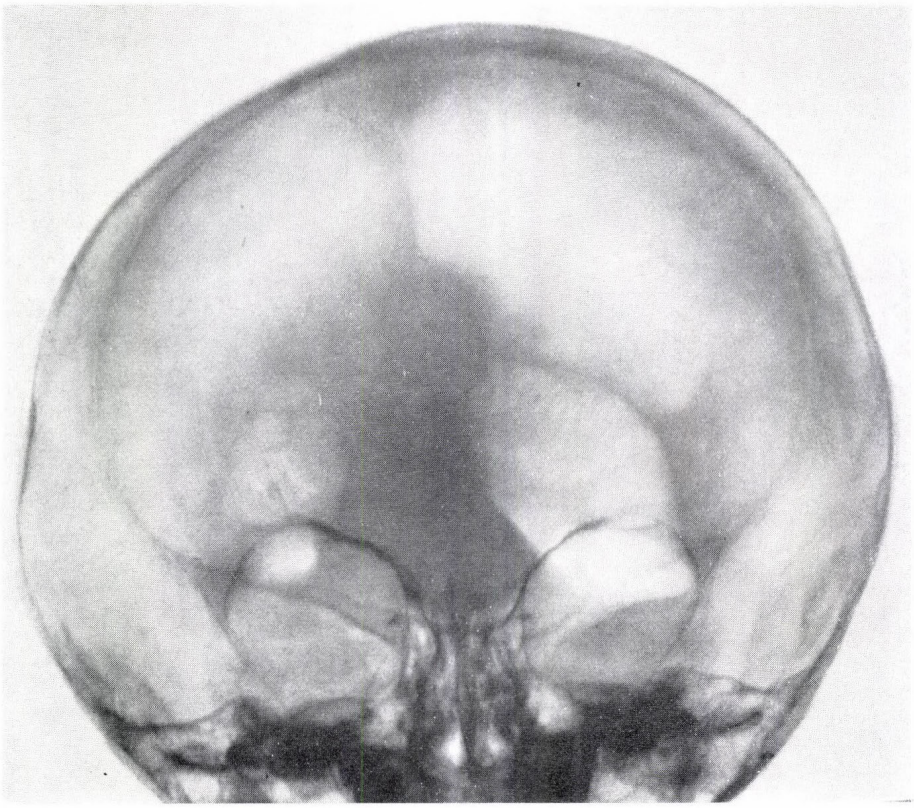

Fig. 7.

is not disturbed and mutism does not develop. In such patients sometimes astonishing memory performances are experienced. Long poems are recited, if the trigger is pulled by telling the first sentence. However, after finishing the poem, it will not be recited again upon telling the title or the story in it, only upon reciting the first sentence. There is no possibility to learn anything new.

In more serious cases even self-feeding is difficult, the patient has to learn elementary processes by long strenuous repetitions, while conscience is maintained and the EEG shows signs of deep lesion.

Thus clinical medicine has to preserve the conception of definitively localized systems in the brain organizing the function of memory storage and reproduction. There is a long way to reach an elementary understanding about the organization of elementary memory. The analysis of postnatal human development and thorough clinico-physiological study of each clinical case is the method of choice clinical medicine is able to offer in this respect.

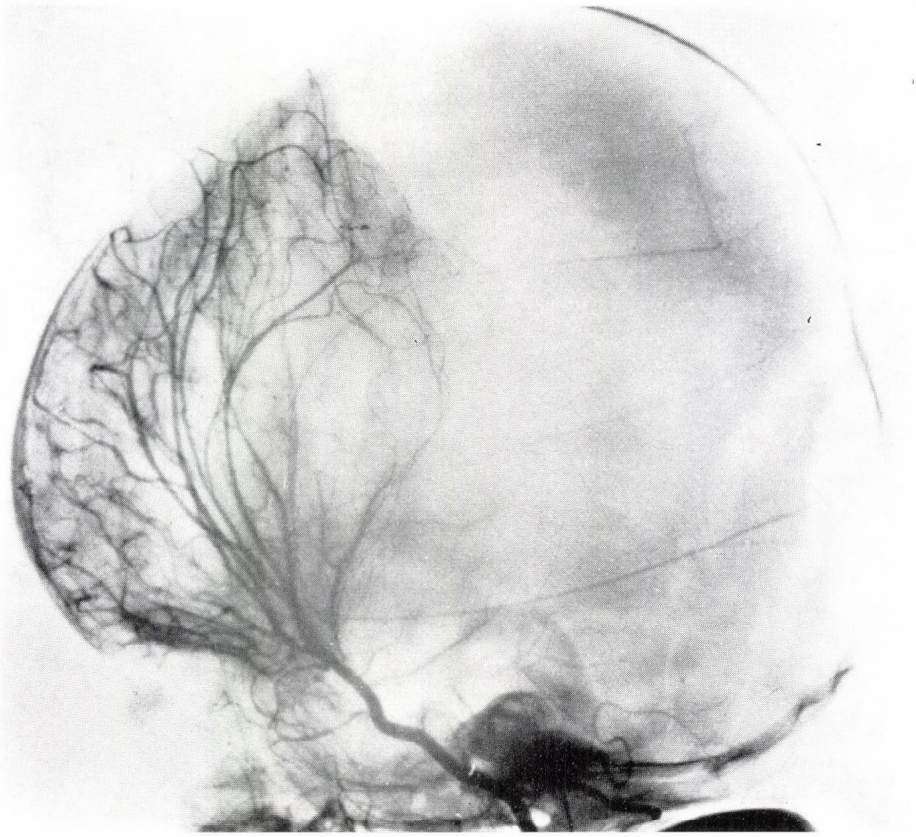

Fig. 8.

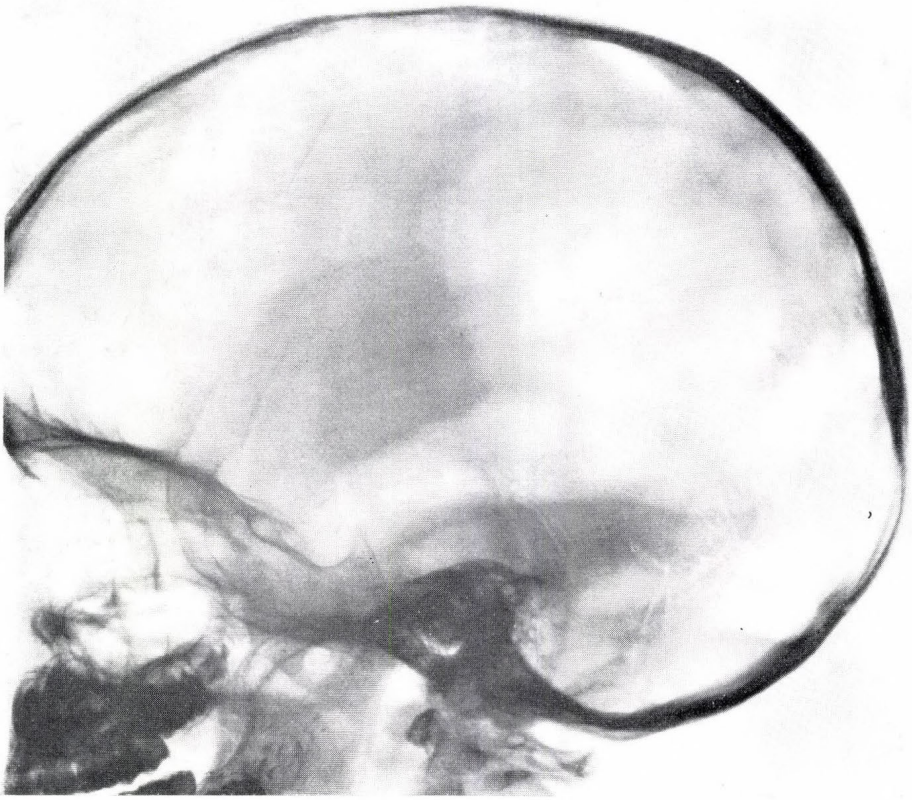

Fig. 9.

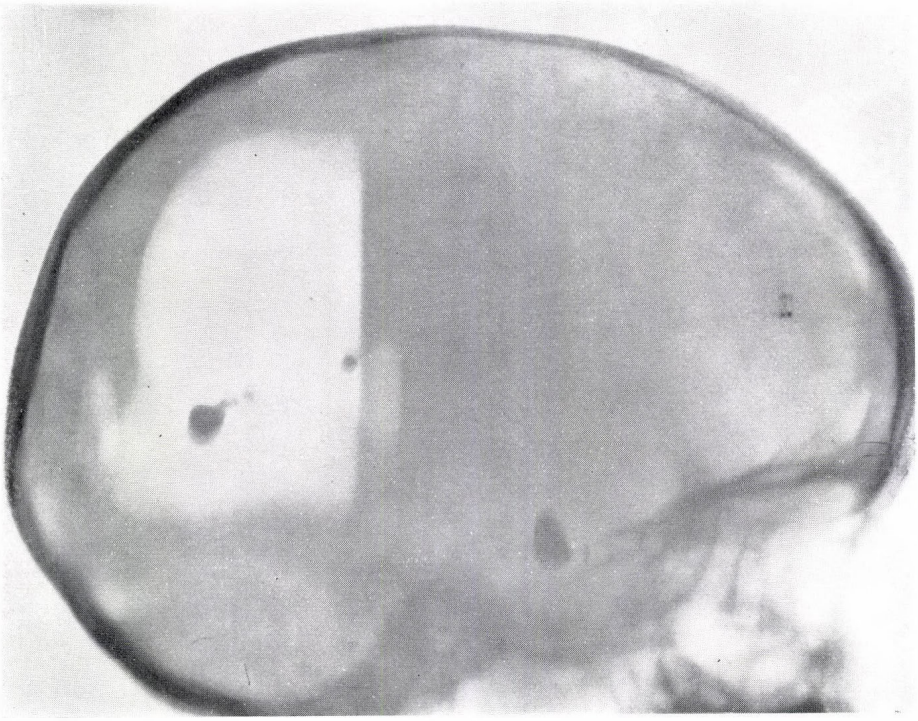

Fig. 10.

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CONCLUDING REMARKS

by the Chairman

S. NARIKASHVILI

To-day we heard a series of most interesting lectures. I think it is unnecessary to comment on each of them, I would rather raise some general problems related to the mechanism and structural background of memory processes.

We all know about the extreme importance of the investigation of these plastic phenomena. The research in this direction is important not only from the point of view of neurophysiology and behavioural sciences, but also from that of neurokybernetics and technical sciences. These very different, nevertheless related disciplines must make a common effort to elucidate such problems as the imprinting, storage, consolidation and recall of memory contents, the process of forgetting, etc. Apparently the first step to approach these most complicated problems must be the clarification of morphological, biochemical and physiological changes related to information processing and learning. We must resolve the dilemma: should we concentrate on one main problem, the problem of the mechanism of learning, or rather investigate the whole series of unresolved questions related to the fate of information in the central nervous system. Of course it would be preferable to go step by step, but our knowledge regarding elementary processes is very scarce. Therefore, maybe it is better in the present situation to study all these problems from many aspects and directions at the same time, without—however—pretending to elucidate the basic mechanisms of the origin of these processes. The investigations concerning mechanisms of memory are at present in a permanent change, in a period which we can call "blind searching".

Taking into consideration the situation outlined above, the experiments with sensory deprivation and artificial environmental enrichment mentioned in Professor Szentágothai's lecture seemed to be the most important. We know from the literature a great number of papers dealing with morphological, physiological and biochemical changes in the state of sensory deprivation and in an enriched environment, namely in the early stages of individual development. It seems to me that this neuro-ontogenetical direction of investigations could be very fruitful in understanding the specific changes and processes underlying learning and memory.

I would like to call your attention to another problem, to that of reverberating impulses circulating on closed neuronal circuits. This process investigated by our team may underlie the phenomenon of short-term memory. Unfortunately we do not know exactly the real nature of such

circular events; with the exception of the interesting data of Verzeano and Negishi there is no evidence on the way of propagation of such impulses. Starting from the morphological description of Lorente de Nó we simply presume the existence of such reverberating phenomena. If we wish to clarify the basic events of the first stage, the labile period of memory functions, we cannot avoid the collection of direct evidence on self-reinforcing reverberating neuronal circuits. I invite you to such common efforts. Thank you for your attention.

Section II

MECHANISMS OF MACROMOLECULAR STORAGE

DO SPECIFIC BIOCHEMICAL CORRELATES TO LEARNING PROCESSES EXIST IN BRAIN CELLS

by

H. Hydén and P. W. Lange

Institute of Neurobiology, Faculty of Medicine, University of Göteborg, Göteborg, Sweden

The present paper will deal mainly with the biochemical changes observed in neurones during three different learning experiments. First, however, some experiments showing differences in the protein composition of nerve and glia cells will be described. Of the learning experiments, the first is a case of instrumental learning in rats, in which changes in the synthesis of three acidic neuronal proteins and in the RNA base composition of neurons occurred; the arguments that these changes are specifically related to the training and that they are an expression of increased gene activation will be presented. In the next study, the protein changes observed in brain cells during simple sensory conditioning in rats will be described, and it will be argued that these are due to an increased level of attention rather than to learning, *per se*. Finally, some RNA data on neurons from monkeys performing a visual discrimination test will be reported.

NEURONAL AND GLIAL PROTEINS

Four years ago, Moore and collaborators (Moore, 1965; Moore and McGregor, 1965) described a brain-specific protein, called S100, because it is soluble in saturated ammonium sulfate. It is an acidic protein, has a molecular weight of around 20,000, constitutes 0·1 % per cent of the brain proteins and moves close to the anodal front in electrophoresis. It develops after 12 days postnatally in the rat and is present only in nervous tissue. Thirty per 100 moles of its amino acids are acidic. It contains 30 per cent glutamic acid and no tryptophane. S100 can be further separated into at least 3 fractions, of which 2 have a high turnover and react immunologically with antiserum against S100 (McEwen and Hydén 1966). The S100 protein is not linked to carbohydrates (Fig. 1).

Hydén and McEwen (1966) have shown by antiserum precipitation reactions supported by the Coons (1957) technique that S100 is mainly a glial protein which in nerve cells is found only in the nuclei. Recently, Benda et al. (1968) confirmed its presence in glia and showed its 10-fold growth in a clonal strain of glial tumors. Perez and Moore (1968) have also presented evidence that S100 is mainly a glial protein. Moore and Perez (1966, 1968) have described another brain-specific protein which seems to

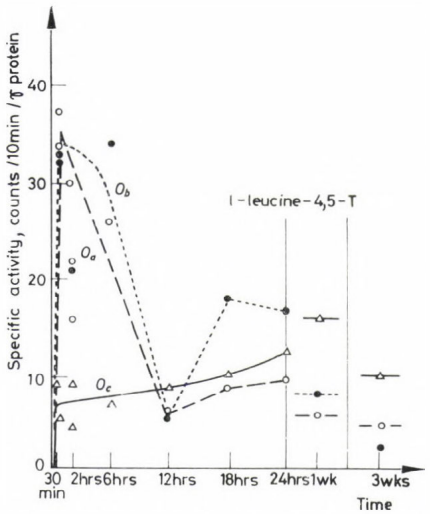

Fig. 1. Specific radioactivity of bands O_a, O_b, and O_c separated on 11·2 per cent polyacrylamide gels as a function of time between isotope injection and sacrifice. Radioactivity was determined after combustion of slices of the polyacrylamide gels by liquid scintillation counting. Isotope: 1-leucine-4, 5 T.

be localized exclusively to the nerve cells and which has been named the 14-3-2 protein.

There is evidence for the existence of still other brain-specific soluble proteins. MacPherson and Liakopolou (1965) have described one in the β-globulin range, Kosinski and Grabar (1967) have described five soluble proteins, and Warecka and Bauer (1967*a*, *b*) recently described an α-glycoprotein rich in neuraminic acid, which develops three months after birth in man and is probably derived from glia. Bennett and Edelman (1968) have purified and characterized still another acidic brain-specific protein.

AN IMMUNOLOGICAL STUDY OF DEITERS' NUCLEUS

We have examined the properties of antibodies prepared against neurones and glia (Mihailović and Hydén, 1969) obtained from Deiters' nucleus in the continuing attempt to identify brain-specific proteins in them. The antigens in brain cells presumably number in the order of hundreds: Huneeus-Cox (1964), as well as Huneeus-Cox et al. (1966) for instance, successfully prepared antisera against eleven antigens in preparations of squid axoplasm that did not include the external membranes. In our study antigen consisted of glia material dissected from the Deiters' nucleus of the rabbit by the free-hand technique previously described (Hydén, 1959). The dissection was carried out at 4 °C, with careful removal of capillaries and nerve cell bodies and processes; in this way, 3·2 mg of Deiters' nucleus glia was collected from 40 rabbits. The other antigen consisted of 1·3 g of whole Deiters' nucleus, containing both neurons and glia, dissected from 100 rabbits.

Each of these antigens was homogenized and mixed with both complete and incomplete Freund's adjuvant. A group of six rhesus monkeys weighing 3–3·5 kg were injected intramuscularly with 0·6 ml of one or the other emulsion once a week for 4 weeks. None ever showed neurological symptoms, or signs of tuberculosis. The animals were bled after one week. On day 44 each monkey received a booster injection of 0·2 ml of its antigen emulsion precipitated with $Al_2(SO_4)_3$, and was bled one week later. These sera were tested on Ouchterlony plates against extracts of glia and of Deiters' nucleus, and their precipitation activities against sucrose-Triton X-100 extracts of both glia and of Deiters' nucleus material were also evaluated. In addition,

the micromethod for double diffusion in one dimension in glass capillaries previously described (Hydén and McEwen, 1966) was used as an assay system, the Coons (1957) multiple layer indirect method for immunofluorescence applied to cryostat sections through the Deiters' nucleus, with evaluation of the specific fluorescence appearing in the nerve and glia cells was also used. Some samples of the antisera were absorbed in two or three steps with sucrose-Triton X-100 homogenates of glia and of rabbit spleen, while others were twice absorbed with rabbit spleen and then absorbed with glia.

Tables 1 to 4 summarize some results of these studies. Both the anti-Deiters' nucleus and the antiglia sera formed well-defined precipitates with $\mu g/\mu l$ amounts of their respective antigens (Table 1). Table 2 shows that the antiglia serum formed precipitates with the glia but not with nerve cells obtained from Deiters' nucleus, and that no precipitates formed when normal rabbit serum was used against these antigens.

TABLE 1

A) Gel precipitation reactions (+) between anti nucleus Deiters' antiserum (1 : 512) and a homogenate of nucleus Deiters

Antigen $\mu g/\mu l$	Reaction	Antigen $\mu g/\mu l$	Reaction	Antigen $\mu g/\mu l$	Reaction	Antigen $\mu g/\mu l$	Reaction
8·20	—	0·80	+	0·30	+	0·05	—
4·10	—	0·50	+	0·20	+	0·02	—
2·10	—	0·40	+	0·10	+	0·01	—
1·00	—						

B) Gel precipitation reactions (+) between anti Deiters' glia antiserum (1 : 512) and an antigen homogenate of Deiters' glia

Antigen $\mu g/\mu l$	Reaction	Antigen $\mu g/\mu l$	Reaction
0·67	+	0·08	—
0·60	+	0·04	—
0·16	+	0·02	—

Table 3 shows the results of an antigen dilution study: homogenates of isolated nerve cells and of the same volumes of glial cells were tested against the antiglia antiserum in the dilution 1 : 512. Even when 300 isolated nerve cells were used no precipitation was obtained, but glial homogenates gave well-defined precipitates.

Precipitates were obtained when the anti Deiters' glia antiserum was tested against glia dissected from other parts of the brain, e.g. from the hypoglossal nucleus and from the spinal cord and cerebral cortex, but none

TABLE 2

Gel precipitation reactions (+) between anti Deiters' glia antiserum and 0·9 μg of protein extracted from nerve and glia cells dissected from Deiters' nucleus. Normal serum controls negative in each case

Antiserum dilution	Protein from	
	Nerve cell	Glia cell
1 : 64	—	+
1 : 128	—	+
1 : 256	—	+
1 : 512	—	+
1 : 1024	—	—
1 : 2048	—	—

TABLE 3

Precipitation reaction between anti Deiters' glia antiserum (1 : 512) and homogenates of Deiters' nerve cells and corresponding volumes of glia. Neuronal protein estimate based on 12,000 $\mu\mu$g of protein per cell. Glial protein per unit volume estimated at 50 per cent neuronal

Deiters' neurones		Precip.	Deiters' glia (same volume as nerve cells)	
Number of nerve cells	Calcul. protein 10^{-6} g		Calcul. protein 10^{-5} g	Precip.
300	3·6	—	1·8	+ 2 ppt
150	1·8	—	0·9	+ 2 ppt
70	0·9	—	0·45	+ 2 ppt
60	0·72	—	0·36	+ 1 ppt
30	0·36	—	0·18	+ 1 ppt
15	0·18	—	0·09	—
6	0·09	—	0·045	—
3	0·045	—	0·022	—

appeared against homogenates of motor neurones, pyramidal nerve cells of the hippocampus and granular cells from the cerebellum, all containing from 3·5 to 0·1 μg of protein per microlitre.

Antiserum against the whole Deiters' nucleus gave two precipitation lines with both glia and nerve cells as antigens. However, when this antiserum was absorbed with glia or with spleen, only the nerve cell homogenates gave precipitates (Table 4).

The results with the fluorescence technique matched those obtained with the immunodiffusion technique as summarized in the Tables. Experiments were carried out according to the multiple layer method of Coons (1957, 1958). Five μ thick cryostat sections through the lateral vestibular nucleus were first dried (sometimes left overnight in the refrigerator at +4 °C)

TABLE 4

Number of precipitation lines after absorption of anti Deiters' nucleus antiserum. Antigen: homogenates from 120 isolated nerve cells and corresponding amount of glia containing 1·6 μg used in each case. All dilutions tested (1 : 2, 1 : 4, 1 : 8, 1 : 16) gave the same result

	Protein from	
	Nerve cells	Glia cells
Unabsorbed	2	2
Absorbed with glia	1	0
Absorbed with spleen	1	0

and subsequently fixed in cold acetone for 30 sec. After being washed for 5 min in the buffered saline, the sections were covered with the antiserum to be investigated for 30 min. After thorough washing (3×5 min) in a cold pH 7·1 phosphate buffered saline, a goat–antimonkey globulin–gamma-globulin conjugated with fluorescein isothiocyanate (Difco product) was applied to the sections for 30 min and the excess removed by repeated washing (again 3×5 min) in the buffer. Control sections heated with normal monkey serum and with conjugated gammaglobulin only were regularly used with each experimental series. The sections were finally mounted in a small drop of buffered glycerol (9 parts of glycerol, 1 part of buffered saline) under a coverslip and immediately observed in a Zeiss fluorescence microscope. After the photographs were taken using the high speed Ektachrome film with exposures varying from 1 to 5 sec, the sections were restained with Ehrlich's hematoxylin–eosin. The fields previously photographed were identified under the light microscope, and rephotographed in black and white, thus enabling comparison of conventional microscopical appearances of the structural details with fluorescent pictures.

Antiserum to whole Deiters' nucleus when absorbed with glia, or with spleen, or with both, gave no fluorescence in glial cells, but did so in nerve cells; this fluorescence was localized to the outer rim of the cell body and to the dendritic processes, which could be traced through the section by their brilliant fluorescence, suggesting that the antigens were localized in the plasma membranes. The reaction was positive furthermore in the nerve cell nucleus, but not at the site of the nucleolus.

From these observations the following conclusions can be made. Neurones and glia differ with respect to antigen composition. This is an interesting finding from the point of view that both types of cells develop from the same type of ectodermal stem cell. The question is then whether the antigens are specific for the type of cell in which they occur. Judged by the absorption experiments, the neuronal antigens seem to be specific for that type of cell. It should be noted that the neurone-specific antigens were concentrated to the processes, to the outermost part of the cell and to the nucleus, and especially to the nuclear membrane.

It seemed on the other hand clear that the antigens in the glia were not glia-specific. They were localized all over in the cell body, but not in the nucleus. If the immunological organ specificity is considered, it seems to be due to the presence of antigens in the neurones.

On the other hand, glial cells possess protein which is confined only to the nerve tissue and which they share with neurones, namely the acidic S100 protein. The presence of this antigen cannot be demonstrated by the method used in this study to prepare immune sera (Levin and Moore, 1965).

ALTERED PROTEIN SYNTHESIS DURING TRAINING

EXPERIMENT 1: HANDEDNESS TRANSFER

(A) Incorporation of ^{3}H-leucine into the acidic protein fractions 4 and 5

If a narrow glass tube is arranged a few centimetres from the floor, filled in its lower third with protein pills, 4 mm diameter and slightly tilted downward at its lower end, rats will reach down into the tube to retrieve the pills, one by one. They generally use either the left or right hand as they perform this task, and they can be induced to transfer this handedness (Hydén and Egyházi, 1964). When tested in free-choice reachings, all the rats in the present study showed clear preference for the left or right hand in 23 out of 25 reaches. A wall was then placed parallel to the glass tube so as to prevent use of the preferred paw; the rats began to retrieve the food pills with the non-preferred paw. When given two training periods of 25 minutes per day in this situation, their performance, measured as the number of successful reaches per 25 minutes, increased linearly up to day 8. Performance curves were obtained on all rats used in our experiments and were similar to that shown in Fig. 2, which demonstrates the performance curve of a separate set of 12 rats during 16 days. Once learned, this new behaviour is retained for a long time. Since no stress (surgical, mechanical or shock) is applied to induce the new behaviour, this procedure has distinct advantages over other behavioural experiments used in rats.

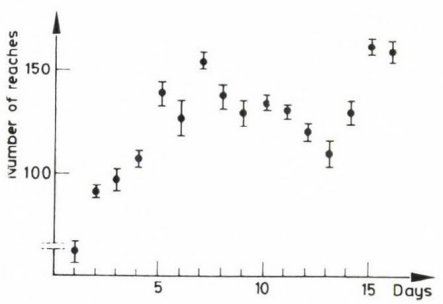

Fig. 2. Performance at reversal of handedness as the average number of successful reaches for 10 rats trained 2×25 minutes per day during 16 days.

To trace protein synthesis during this learning, the rats under fluothane anaesthesia received 60 μC of ^{3}H-leucine in 60 μl intraventricularly in both hemispheres half an hour before their final training period. Hippocampal nerve cell samples were then taken for analysis 15 minutes after the last training period. Nerve cells of the hippocampus were selected because (*i*) several clinical and behavioural studies have shown the importance of this structure for the formation of long-term memory

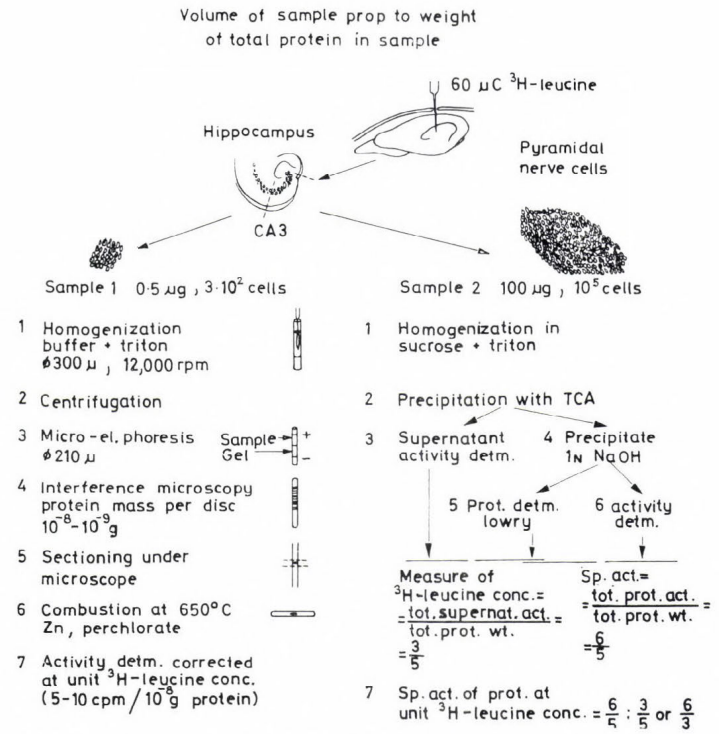

Fig. 3. Outline of the microdisc electrophoresis procedure for separation of 10^{-7} to 10^{-9} g of protein and evaluation of incorporation of radioactive amino acid into the individual fractions. Volume of sample is proportional to weight of total protein in sample.

(see e.g. Meissner, 1966; Ojemann, 1966; Penfield, 1952; Buresova et al., 1962; Adey et al., 1964); (*ii*) its bilateral destruction results in severe defects in learning and formation of memory (Meissner, 1966; Ojemann, 1966); (*iii*) during attentative learning, impedance changes occur in the hippocampus (Adey et al., 1964); and (*iv*) no memory is formed if protein synthesis in the hippocampus is inhibited by 90 per cent (Barondes and Cohen, 1967; Flexner and Flexner, 1968).

The micromethod used for protein analysis was as follows. About 300 pyramidal cells from the CA3 region of the hippocampus, separately dissected out freehand on a cooling table, were analysed for protein by a technique already described (Hydén and Lange, 1968*a*).* An outline of this

* One may ask why it seems necessary to struggle with such minute amounts of material and with the dissection of such small areas within the brain. As an answer, we would like to advocate the view that altered synthesis, if any, is more likely to be found in a uniform cell population from an area that is clearly involved functionally. In a mixed cell population from a whole brain such changes easily disappear in the background noise.

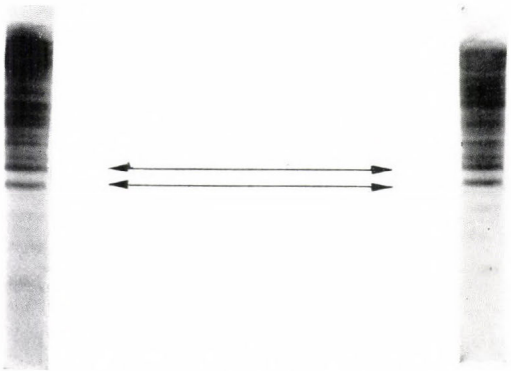

Fig. 4. Protein of pyramidal nerve cells of the hippocampus, CA3 region, separated on 400 μ diameter polyacrylamide gels, and stained with amido black. Fractions 4 and 5 from the anodal front are indicated by arrows.

procedure is given in Fig. 3. The left side of the scheme gives the various steps leading to the value of the specific activities per amount of protein in each protein microfraction.

Since these specific activity values vary, because of variation in the local concentration of ^{3}H-leucine, the correction procedure shown on the right side of Fig. 3 was applied in order to allow a comparison of values from identical parts of both hemispheres or from different animals. This was accomplished in a separate experiment, where the relation between the uncorrected specific activities and the concentration of the free ^{3}H-leucine in the hippocampal nerve cells was determined and found to be linear. Dividing the specific activity values obtained by the values of the ^{3}H-leucine concentration determined locally allowed all specific activities to be compared at uniform free ^{3}H-leucine concentration.

In an earlier study (Hydén and Lange, 1968*b*), the incorporation of ^{3}H-leucine in the CA3 nerve cell protein fractions 4 and 5 (Fig. 4) was evaluated on the fifth day of training, i.e. on the linear, increasing part of the performance curve. The specific activities of these protein fractions were significantly greater in trained rats compared with control rats of the same age ($p < 0{\cdot}005$), and there was some evidence for higher incorporation in the hippocampus contralateral to the training paw.

Protein fractions 4 and 5 presumably each contain several species of proteins, and there is no reason as yet to believe that the qualitative characteristics of the protein formed during training is specific for the process since no data as to the composition of these proteins exist. Nevertheless, it is pertinent to ask whether the increased synthesis of fractions 4 and 5 is specific for the training.

This we attempted in the present study by measuring fractions 4 and 5 in rats given 5, 7, and 10 training sessions according to the following schedule. A group of 24 rats were given 5 days of training. Five of these (Group I) received ^{3}H-leucine prior to the last training session and the CA3 hippocampal nerve cell material was taken for analysis as described above. The remaining animals were placed in cages, and given food and water *ad libitum*. After fourteen days, they were all subjected to two training periods of 25 minutes each; five of these animals (Group II) were given ^{3}H-leucine and the CA3 nerve cell material was taken for analysis. The remaining rats (Group III) were returned to their cages for fourteen additional days, then trained for three days with two training periods per day (each of 25 minutes),

TABLE 5

Average number of successful reaches per day for rats using the nonpreferred paw to retrieve food pills from a narrow glass tube

	No. of rats	Reaches
Group I (Performance on day 5)	24	100
Group II (Performance on day 14)	19	90
Group III (Performance on day 30)	14	90

and, after ^{3}H-leucine injection, their hippocampal brain cells were taken for analysis. The controls were untrained rats of the same age of which 50 per cent were littermates of the experimental animals.

The performance of the rats in the three groups are shown in Table 5. Table 6 demonstrates that the specific activites of protein fractions 4 and 5 were significantly increased after 5 and 7 training days but not after 10. The corrected specific activites (counts per minute per microgram) of protein fractions 4 and 5 differ from the corresponding values in a paper recently published (Hydén and Lange, 1968*b*); this is due to a more refined separation technique which allowed a better separation of smaller amounts of the protein sample. The values found for the unseparated protein were, of course, not affected. The unseparated protein of the CA3 pyramidal nerve cells behaved like that of fractions 4 and 5 in showing higher incorporation values in Groups I and II, but not in Group III.

TABLE 6

Corrected specific activities of hippocampal CA3 nerve cell proteins, both the unseparated and fractions 4 and 5

	Fractions 4 and 5			Unseparated protein	
	No. of rats	No. of gels	Corrected spec. act. cpm ± S.E.	No. of samples	Corrected spec. act. cpm ± S.E.
Group I (training 5 days)	5	10	3·3 ± 0·40	10	14·20 ± 1·90
Group II (resumed training day 14)	5	10	3·9 ± 0·48	10	15·50 ± 1·90
Group IIA (half training time)	2	5	13·0 ± 0·60		
Group III (resumed training day 30)	14	35	1·8 ± 0·17	28	5·10 ± 0·58
Control	10	24	1·5 ± 0·16	20	6·00 ± 0·92

Following a chance observation, we made a study of the incorporation of ^{3}H-leucine by two rats which were subjected to half the initial training time allowed the other rats. Whereas Group I had 10 training sessions

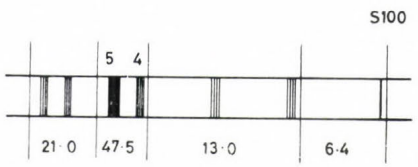

Fig. 5. Gels 400 μ in diameter, containing separated ^{3}H-labelled pyramidal nerve cell protein from the CA3 region, were cut in four pieces and the radioactivity was determined as counts per minute after combustion. Note that the radioactivity in protein fractions 4 and 5 is relatively great.

(2 × 25 minutes × 5 days), these 2 rats had 5 (2 × 25 minutes for 2 days, 1 × 25 minutes on day 3), and on day 14, they were given only a single 25 minute training session before being killed for analysis. On training day 3 they had made 120 reaches, and in the final session 100 reaches. These animals, Group IIA in Table 6, gave a greater protein synthesis response than those receiving the longer training.

Since the incorporation of ^{3}H-leucine into the 4 and 5 protein fractions increased significantly during the training, it became important to know the relation of these fractions to the rest of the nerve cell protein in terms of ^{3}H-leucine incorporation. Protein of the CA3 nerve cells from Group I rats (trained for 5 days) was therefore separated on polyacrylamide gels, divided in four parts, and the radioactivity was determined in each part; as can be seen from Fig. 5, the radioactivity of protein fractions 4 and 5 is relatively high.

(B) Increased synthesis of S100 protein

Both the electrophoretic pattern of the soluble CA3 nerve cell protein isolated in the experiment just described and micro-densitometer recordings (Fig. 7) made of 75 protein separations stained with brilliant blue showed two protein bands at the front in the trained rats compared to only one in the controls (Figs 6 and 7, Table 7). This protein fraction of the controls gave a positive immunological reaction when treated with antiserum against the S100 protein. Figure 7 shows that the amount of protein contained in the two anodal bands of trained rats was greater than the amount of protein contained in the one band of controls. Furthermore, when gel cylinders from experimental rats with two anodal front bands were immersed in satu-

TABLE 7

Frequency of single and double front anodal protein fractions in the electrophoretic pattern of 75 polyacrylamide gels from 23 rats (7 controls, 4 resumed training on 14th day, 12 resumed training on 14th day and on 30th day)

	No. of protein bands	
	One	Two
Control	20	0
Group II	5	10
Group III	20	20

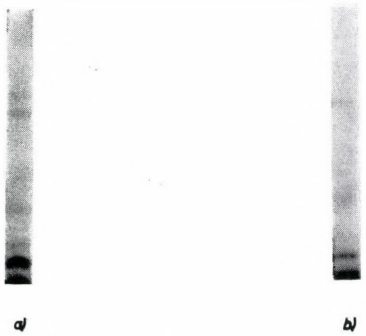

Fig. 6. Photographs of nerve cell protein of the hippocampus, CA3 region, separated on polyacrylamide gels 400 μ in diameter and stained by amido black. (*a*) From control rat, (*b*) from rat on day 5 of training with the nonpreferred paw. The acidic proteins migrate toward the bottom of the gel.

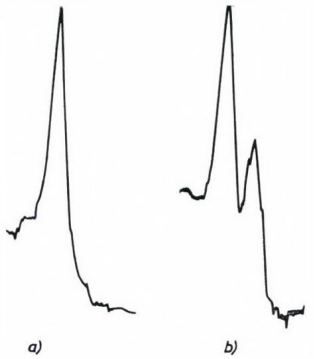

Fig. 7. Microdensitometric recording of the anodal front protein fractions shown in Fig. 6*a*, control (*a*) and in Fig. 6*b*, trained animal (*b*).

rated $(NH_4)_2SO_4$ solution for 20 minutes, the band closest to the anode disappeared, identifying it as S100. These facts – the electrophoretic localization of the new protein fraction, its disappearance in saturated $(NH_4)_2SO_4$ and the increased amount of front protein in the trained rats – suggest that brain-specific S100 protein increased in amount during training. This S100 protein was presumably localized in the nuclei of the hippocampal neurones.

At this point it seems appropriate to comment on the protein changes during the intermittent training over a period of one month.

The rats performed well both on the 5th, 14th, and 30th day, i.e. when they had received training for 5, 7, and 10 days. If the increased synthesis in the hippocampal nerve cells had been an expression of increased and sustained neural function, then the increased incorporation values on the 5th day of initial training and on the 14th day of resumed training would have presumably been found also when the rats were subjected to 3 days of resumed training 30 days after the initial training sessions. The fact that the incorporation values did not at that last stage differ from those of the controls is a strong indication that the observed increase in synthesis is correlated to learning processes occurring during the training. We would like to suggest the interpretation that when the novelty of the task has passed, the hippocampal nerve cells ceased to respond with increased synthesis of this type of protein. A response may well occur in other parts of the brain.

It is even more striking that the S100 protein increased in amount during the learning to reverse hand since it is a brain-specific protein and thus can be expected to mediate specific brain functions.

Our interpretation of the result given above is that the increase of the S100 protein during reversal of handedness specifically relates the S100

protein to the learning processes. However, as we pointed out above, training involves several factors not related to learning *per se*. In the reversal of handedness experiments, the unspecific factors have been eliminated or reduced to a minimum. The motor and sensory activity, attention, motivation, and reward are equated between the experimental and control animals, and the stress involved in reversal of handedness is minimal. In view of these considerations, we used a technique which specifically related the S100 protein during reversal of handedness to learning *per se*.

A group of 8 rats were trained during 2×25 minutes per day for three days. Between the first and second training session of the fourth day, half of the rats were injected intraventricularly on both sides with 2×25 μg of antiserum against S100 in 2×25 μl. The other half of the group was similarly injected with the same amount and volume of antiserum against rat γ-globulin. The rats were slightly anaesthetized with fluothane. The animals were trained a second session on the fourth day 45 minutes after the injections. After this treatment, the rats were trained for three further days with 2×25 minutes per day. The results are presented in Fig. 8. The number of reaches is plotted against the number of training days. Before injection of antisera, all rats followed an identical performance curve. After the injection of antiserum against rat γ-globulin, these rats followed a performance curve which was an extrapolation of the performance curve before the injection. The same was the case with a rat which was injected with the same volume of physiological NaCl.

By contrast, the rats injected with antiserum against S100 protein did not increase in performance, i.e. the number of reaches per day remained

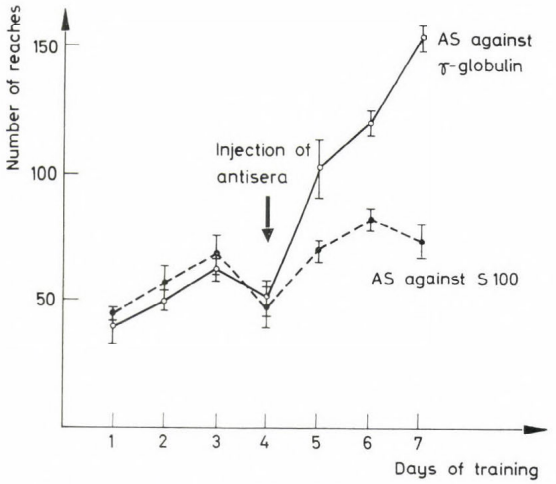

Fig. 8. Performance curves of two groups of rats, 6 experimental, 5 controls. One group injected with antiserum against S100, the other with antiserum against rat gamma-globulin on the fourth day of training.

at the same values as those immediately before the injection. As is seen from the curves in Fig. 8, the difference in number of reaches between the two groups of injected rats is clearly significant.

Another way to present the results is the following. For each rat, the sum of reaches for the first three training days is calculated as is also the sum of reaches for the last three training days. The number of reaches during the day of injection are thus not included in these sums. The ratio between the first and second sum is determined. The averages of this ratio are $0{\cdot}73 \pm 0{\cdot}053$ for the rats injected with S100 antiserum (6 rats), and $0{\cdot}42 \pm 0{\cdot}033$ for the control rats (5 rats). The difference between these ratios is highly significant ($p < 0{\cdot}001$). It is obvious that the experimental rats show a decrease in learning capacity.

If instead the difference between the second and the first sum is calculated, you obtain for the rats injected with S100 antiserum 78 ± 18, and for the control rats 206 ± 19 reaches. The difference between these numbers (128) is highly significant ($p < 0{\cdot}001$) leading to the same conclusion as above that the experimental rats show a decrease in learning capacity. Thus, the S100 protein is specifically correlated to learning processes.

EXPERIMENT 2: SENSORY CONDITIONING TEST IN RATS: INCORPORATION OF ^{3}H LEUCINE INTO NEURONAL PROTEIN

This experiment was designed to limit participation of such factors as motivation, motor activity and reinforcement in an experiment involving change of behaviour (Hydén et al., to be published). For that purpose we measured protein synthesis in rats subjected to paired and unpaired tone and light stimuli, and to light stimulus alone. A total of 80 rats were used in pilot tests, behavioural checks and for final experiments. The rat was placed in a cage with a wired floor in a sound absorbing dimly lit room for 20 minutes prior to the experiment. One group of rats received an acoustic signal of 1,000 Hz (conditioning stimulus) followed by a visual (unconditioned) stimulus. The tone and light stimuli both lasted 0·2 sec and were presented automatically at a frequency of 6 per minute. Another group of rats was used as behavioural controls. In this group the tone and light stimuli were followed by an electric shock; in ten trials the rats learned to jump up to a shelf to escape the electric shock (criterion 8 out of 10) when the tone-light stimuli were presented. This test, a type of sensory conditioning described and discussed by Morrell (1967), demonstrated that a linkage had been formed between the two sensory areas. A third group of rats received tone and light stimuli distributed at random. A fourth group received only light stimulus at a frequency of 6 per minute.

The experimental rats were first bilaterally injected with ^{3}H-leucine intraventricularly during light fluothane anaesthesia, then given sound-light or sound-light at-random stimulation for 15 minutes. The time lapse from the last injection to sampling of brain material was 40 minutes.

Both types of stimulation *increased* the incorporation of ^{3}H-leucine into the neuronal protein of the hippocampus but *decreased* it in the visual cortex

TABLE 8

Tone-light conditioning in rats.
Incorporation of ^{3}H-leucine into protein isolated from hippocampal nerve cell and visual cortex of rats. Data in cpm total protein/cpm total supernate

Stimuli	No. of samples	Incorporation of ^{3}H-leucine	
		Visual cortex	Hippocampus
Paired	8	$3{\cdot}49 \pm 0{\cdot}23$	$3{\cdot}43 \pm 0{\cdot}41$
Unpaired	8	$3{\cdot}50 \pm 0{\cdot}23$	$4{\cdot}45 \pm 0{\cdot}56$
Light only	12	$3{\cdot}48 \pm 0{\cdot}18$	
Control	8	$4{\cdot}46 \pm 0{\cdot}41$	$2{\cdot}86 \pm 0{\cdot}41$

(Table 8). Control animals given light stimulus alone showed decreased incorporation in the visual cortex.

There are two findings in this sensory conditioning experiment which seem to exclude the possibility that the protein changes were correlated with learning processes during the conditioning. The first is the fact that light stimulus alone gave the same incorporation values of the visual cortex as did the paired and unpaired tone-light stimuli. The second circumstance is the fact that the tone-light stimuli distributed at random gave the highest incorporation of ^{3}H-leucine into the protein of the hippocampal nerve cells. Therefore, the conclusion is that the protein changes observed during the conditioning presumably are expression of increased attention or orientation reflexes. The finding that the incorporation values for the cells of the visual cortex in all three types of sensory experiments were lower than those of the controls, agrees with electrophysiological observations.

DISCUSSION

The aim of the studies reported here has been to correlate protein changes in nerve cells (and glial cells) in particular parts of the brain with learning processes which occur during training. It seems evident that mapping the areas that respond with defined changes in protein fractions during behavioural experiments is prerequisite for a comprehensive theory on the mechanisms relating macro-molecules in brain cells to storage and retrieval of information. The observations relating behavioural responses to synthesis and composition of RNA in nerve and glial cells (Hydén and Egyházi, 1962, 1963, 1964; Hydén and Lange, 1965; Shashoua, 1968), taken in conjunction with the observations on protein reported here, may be considered a beginning which may eventually form the basis of such a theory.

It is interesting that the immunological study reported here brought out such clear differences in the antigen compositions of neurones and glia. This finding brings into question the matter of transfer of RNA from glia to neurone for which view there exists some evidence (Hydén and Lange,

1966). Such transfer could still take place even if the neuronal protein programmed by the glial RNA was not antigenic for rabbits challenged by our technique. S100 protein for instance, is not antigenic unless injected under special circumstances.

This S100 protein seems, however, to be definitely linked to learning, as is demonstrated especially well in the experiment where antiserum against S100 impaired learning while that against γ-globulin did not (Fig. 8). All factors including that of stress were identical for the control and experimental rats in this study. Before and after the antisera injections all were subjected to the same training program and the injections into the brain ventricles were carried out under identical conditions. Additional food was supplied to rats receiving S100 protein antiserum to compensate for the different amounts of reinforcement obtained. The result showing that only the S100 protein antiserum inhibited further learning seems clearly to link this brain-specific protein, S100, to learning processes occurring during training.

As for the nerve cell proteins 4 and 5, these are acidic even though their composition is still unknown. Their response during intermittent training spread over one month seems significant and is pertinent for the interpretation that the synthetic response was linked to learning processes within the training. The fact that the hippocampal nerve cells did not respond with increased synthesis of these proteins after the last training sessions (a month after the initial training) excludes the possibility that the increased protein synthesis during the two previous training sessions was merely an expression of increased motor activity, sensory activity, attention, or change in age.

The protein changes in the transfer of handedness experiment, a case of instrumental learning, can be compared with those in the sensory-sensory experiments, a case of classical conditioning. The instrumental case is a complicated type involving i.a. motor-sensory activities, motivation and attention. The acidic—including S100—protein in hippocampal nerve cells rises during acquisition of behaviour in this case, but during sensory conditioning no systematic change related to learning can be seen in either the hippocampal or the cortical cells. The protein synthetic response of hippocampal cells in this type of classical conditioning cannot therefore be equated with that taking place during instrumental learning. In sensory conditioning, the direction and magnitude of hippocampal protein synthesis changes seem only to follow the response of the cells to the sensory input, and to have no relation to the learning factors involved.

Thus both the light-tone and the at-random stimuli gave high incorporation values of ^{3}H-leucine into the hippocampal nerve cell protein. At visual cortex the incorporation values were equal and lower compared to the controls for all conditions of stimulation. It may therefore be tentatively concluded that in hippocampal cells the biochemical mechanisms taking place during instrumental learning differ from those during classical sensory conditioning.

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DISCUSSION

G. Ungar: The significance of all the experiments dealing with chemical changes depends on their correlation with learning or "mere" neural activity. This seems to be an almost insoluble problem because we cannot isolate

learning from all the concomitant neural processes. A good example of this signal to noise interrelationship is the investigation in which an isolated catfish head has been submitted to various olfactory stimuli and the subsequent changes in brain RNA have been estimated. Most stimuli produced changes in base ratios irrespective of whether they were relevant to the fish or not. For example, both menthol and shrimp extract produced similar changes although the former is certainly a novel stimulus, while the latter is part of the normal environment of the fish. Did learning take place in both cases or in neither? It is impossible to say. This is, therefore, a serious conceptual obstacle to the correct interpretation of all the results obtained by Dr. Hydén and others who were looking for chemical changes in brain RNA and protein.

H. HYDÉN: The experiments I have talked about here were all performed on mammals. A few years ago we published the results of the experiments in which we obtained base ratio changes of RNA in the motor neurones of fish by extensive motor activity. Similar results were never achieved in analogue experiments with mammals. We stressed that both morphology and organization of nucleic acids, morphology and composition of nerve cells of invertebrates and fish—not to speak about the glia—differ from those of mammals in this respect. I do not think therefore that conceptual difficulties were involved here. I believe that it is clear that one should be catious in equating the results obtained from cold-blooded animals with the effects received on mammals.

G. HORN: Dr. Hydén, with regard to your last remark, did you not refer in your talk to Shashoua's results on goldfish?

H. HYDÉN: Yes, I alluded to his results, too. He used a system where he tried to change the whole nervous system and then to analyse the changes by trying to separate motor activity and stress factors.

G. HORN: Dr. Ungar has raised an important issue, namely that of distinguishing between neural activity which is thought to be both necessary and sufficient for learning from neural activity which is thought to be necessary but not sufficient. It is difficult to see just what controls can be used to distinguish the two kinds of activity (but see Horn et al., *Nature*, in press, 1971). One approach that is commonly used is to allow the experimental animal to form a conditioned association and to use as the control an animal which is not allowed to form the association. However, the two animals will probably learn *something*, though each may learn something different. Another complication is that the two procedures will evoke very different behavioural responses in the two animals and they will almost certainly be subjected to different degrees of frustration and stress. Non-specific stress may have an effect on neural activity (see Semiginovsky et al., *Life Sciences* **2**, 1169–1180, 1970). Recently I was interested to read of findings that corticosterone is selectively taken up by cells in the hippocampus (McEwan et al., *Nature* **220**, 911–912, 1968), an effect which may also be relevant to your results.

H. HYDÉN: I agree with your last comments about the background noise. Even the use of the determination of the 17-ketosteroids in the urine of rats is insufficient for the removal of old doubts about the stress factors. As far

as attention and motivation are concerned, of course, there is no learning in an animal experiment except where the factors of unspecific attention have been eliminated. It seems to me that when one speaks about the "quiet environment" or the "stimulating environment" as in Rosenzweig's experiments, if one speaks about naive animals, controls and cage-animals, it is essential to define exactly the conditions of these animals, too, and not only those of the experimental animals. Therefore, I think a discussion about the controls is very much needed.

DISK ELECTROPHORETIC ANALYSIS OF SOLUBLE BRAIN PROTEINS IN THE BRAIN OF MONKEYS SUBJECTED TO VISUAL DISCRIMINATION TRAINING

by

Lj. Mihailović, Lj. Kržalić, B. Petrović and D. Čupić

Institute of Pathological Physiology, Faculty of Medicine,
University of Belgrade, Yugoslavia

An appreciable body of work, largely inspired by Hydén and reviewed by him at this conference (p. 69), has provided a reasonable support to the concept that RNA and especially proteins, with their conformational capacities, are "the strongest candidates as executive molecules" involved in the mechanisms of storage and retrieval of information.

In view of the possibility, heralded by our previous studies (Mihailović and Janković, 1965; Mihailović et al., 1969; Mihailović and Hydén, 1969) that by applying the immuno-neurological methods one could not only attempt to study the physiological role of these macromolecules in the processes of learning and memory but even approach their ultimate identification, a series of investigations has been devised encompassing a number of simultaneously combined psychophysiological, biochemical, immunological and electrophysiological experiments oriented towards this obviously most complex and difficult goal. The first encouraging results to be briefly summarized below are the preliminary biochemical part of this more general project.

The present experiments have been undertaken, therefore, to investigate protein changes in the brain of monkeys subjected to visual discrimination training and, in particular, to search for the possible presence in the brain of a hypothetical protein specific for the process of learning and the information storage.

Three groups of animals (Rhesus monkeys) were used. Subjects from the first group were taught (in Wisconsin General Testing Apparatus—WGTA) (Fig. 1) to perform pattern discriminations to the criterion of 90 per cent correct responses in two successive days for each of 24 successive tasks. The animals were subsequently trained to discriminate in succession a series of different objects (300 pairs) until the establishment of the learning set.

In order to control for nonspecific, motor, sensory and other behavioural concomitants of the learning process which have been shown to increase the RNA synthesis (Hydén, 1967; Pevzner, 1966) thus possibly influencing the protein synthesis as well, each of these animals had its counterpart in the subject assigned to the second group of animals, which was exposed to identical experimental situation, presented with the same visual discrimination tasks and given exactly the same number of trials both, per day and total, but in such a way as to retain the chance performance (Fig. 2).

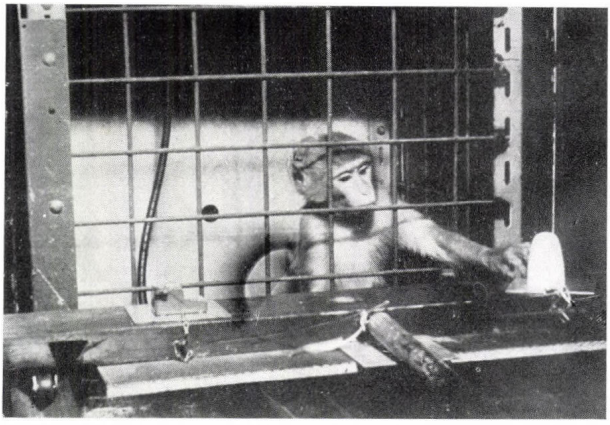

Fig. 1. Monkey in WGTA performing object discrimination task.

This was made possible by the use of two Gellerman schedules enabling random association of the reward (a cube of apple) to the assigned pattern. It should be emphasized, however, that these animals, although deprived of regular reinforcement remained highly motivated to test throughout the long-lasting experiment.

The third group of animals consisted of subjects not exposed to visual discrimination training but kept and maintained under the same general laboratory conditions. Before sacrificed, the animals from the first two groups were retested on the last pattern discrimination task and presented a series of ten pairs of entirely new objects, respectively.

Proteins in sucrose-Triton x-100 extracts of the brain tissue from the inferior temporal gyrus, gyrus principalis and hippocampus were then deter-

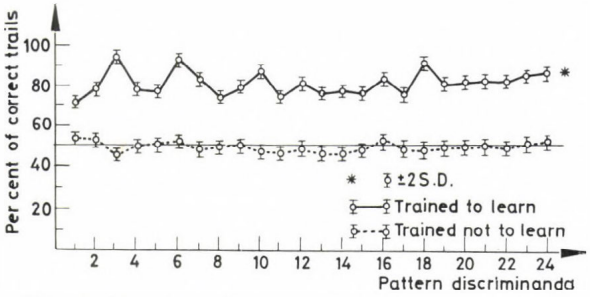

Fig. 2. Showing the correct trial score of total responses for each of 24 different patterns. Note the chance level of correct responses in animals trained not to acquire visual discrimination capacity as compared to the high percentage of total correct responses in animals which did learn pattern discrimination.

mined according to the method of Lowry et al. (1951) and electrophoretically separated on 7 per cent polyacrylamide gels according to the method of Davis (1964), under rigorously standardized conditions (200 μg protein/gel, using a current of 2·5 mA for 90 min). The listed brain regions were selected to be analysed for the following reasons: the inferior temporal gyrus for its repeatedly demonstrated crutial and specific relevance for visual discrimination learning (Chow, 1952; Mishkin and Pribram, 1954), gyrus principalis for it has not been shown to be indispensible for this type of learning (Weiskrantz et al., 1962; Weiskrantz, 1964), and hippocampus for its amply demonstrated importance for the recording any current experience and the establishment of the new behaviour (Scoville and Milner, 1957; Penfield, 1958; Grastyán, 1961).

Following densitometry, only the prealbumin—anodal—part of the gels, in which the protein fractions were quite clearly distinguishable and gave densitometric records precisely and reliably analysable, was subjected to semi-quantitative analysis. Another reason was the finding that during the establishment of the new behaviour it was the synthesis of these acidic proteins (which at electrophoresis move near the separation front) that was significantly changed (Hydén and Lange, 1968). The integrated densitometric values of the square areas below individual peaks were quantified and expressed relative to band I (see diagram in Fig. 3), which was found to contain insignificantly different amounts of protein in the samples

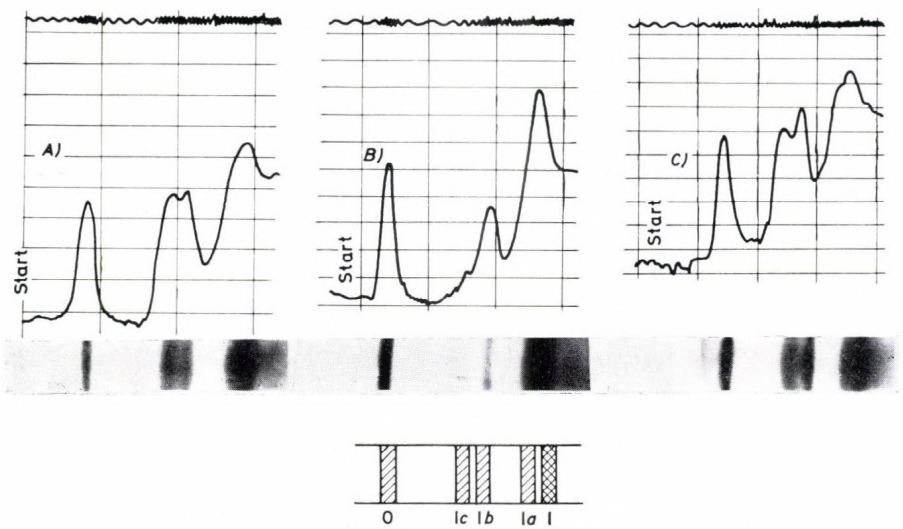

Fig. 3. Representative electrophoretic patterns with the corresponding densitometric records of soluble brain proteins extracted from the inferior temporal gyri of experimentally naive monkeys (*A*); monkeys which were trained but did not learn visual discrimination (*B*); and monkeys which did learn visual discrimination. Note in *B* the remarkable decrease in the amount of protein contained in the band Ic as designated arbitrarily on the diagram at the bottom of the Figure.

obtained from all the different brain regions from both experimental and control groups of animals. Thus obtained data were *t* tested.

Analysis of the results revealed significant differences in the amounts of protein in the two prealbumin bands (O and Ic) in different groups of animals as well as in various regions of the same animal. The main and most consistent finding, however, was that the amount of protein contained in band Ic separated from the extracts of both the inferior temporal gyrus and hippocampus but not of the gyrus principalis, was significantly smaller in the animals which were trained not to acquire visual discrimination than in naive controls. The corresponding values in animals which did learn visual discrimination and experimentally naive, untrained controls were, however, not statistically different. The results concerning the changes found in the inferior temporal gyrus are illustrated in Fig. 3 and presented in Table 1.

TABLE 1

Inferior temporal gyrus

Group of animals	No. of animals	Electrophoretic fractions			
		O	Ic	Ib	I
Trained to learn	4	9·3 ± 1·12	6·5 ± 2·64	10·0 ± 1·58	23·0 ± 3·39
		$p > 0{\cdot}05$	$p > 0{\cdot}05$	$p > 0{\cdot}05$	$p > 0{\cdot}05$
Controls	4	7·5 ± 1·22	7·0 ± 1·87	8·3 ± 1·53	21·75 ± 5·14
		$p > 0{\cdot}05$	$p < 0{\cdot}05$	$p > 0{\cdot}05$	$p > 0{\cdot}05$
Trained not to learn	4	8·8 ± 0·55	3·5 ± 1·8	8·8 ± 1·34	22·5 ± 2·06

It seems highly relevant just to mention in the present context that the RNA base ratios in the cells from the inferior temporal gyrus of the same animals [determined in Prof. Hydén's laboratory, according to the micromethod of Edström (1964) and employing the double blind procedure] exhibited also statistically significant change of the same type as originally observed by Hydén and Egyházi (1964) in rats subjected to the transfer of handedness and confirmed subsequently by Shashoua (1968) in the goldfish trained to acquire a new swimming skill. In our monkeys which did learn visual discrimination G + C : A + U values were significantly lower than the corresponding ratios in animals both trained not to learn visual discrimination and experimentally naive controls.

Another interesting and statistically significant finding was an increase of protein in the front band O separated from the hippocampal extracts of monkeys which learned visual discrimination as compared to the corresponding value in experimentally naive controls.

The results presented challange discussion in many directions and consideration of great many problems such as the adequacy of controls, proper timing of sample taking during the learning experience, the distribution of findings etc. The results are considered, however, too few and perplexing at the present moment to permit any interpretation in terms of functional significance, which would only involve the danger of contributing just another speculation to the many which so abundantly overflow this still rather nebulous field of research. Further studies which are under way in our laboratory involving short term experiments of the same general type including similarity of the controls, will presumably throw more light on the nature of the changes described in the present paper, and enable their plausible interpretation. It seems reasonable to assume, however, that the protein changes described do occur in conjunction with the process of learning. They do by no means tell us anything about the specificity of changes with respect to the acquisition of the given set of information. An antiserum against the homogenates of the inferior temporal gyrus of animals which learned visual discrimination is being made in our laboratory in a hope that, following the absorption with homogenates of the corresponding brain regions of both naive and trained controls, it might possibly retain an antibody specific to the macromolecules involved in the acquisition of visual discrimination learning and facilitate our efforts in approaching their identification. Until this be done, however, it is only fair to say that the results obtained are encouraging indeed, although they emphasize even more all conceptual, phenomenological and semantic difficulties commonly encountered and recognized in this complicated field of research.

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DISCUSSION

H. RØIGAARD-PETERSEN: Just two questions: (1) How long did it take you to remove the areas of the brain you used for gel-electrophoresis? (2) When you had applied too much protein on your gel and did not get all the fractions out, could the reason be that bigger molecules filled the pores in the gel and thereby stopped the smaller molecules?

LJ. MIHAILOVIĆ: (1) We, of course, did the removal of the brain as fast as we could. In any case it did not take longer than 10 minutes. (2) As far as your second question is concerned, this – we think – could be the reason, but we do not believe that the use of another spacer gel would have made much difference.

R. GALAMBOS: As we all know, it is difficult to be sure that antibodies will be prepared specially to some brain proteins. Do you have any special technique to assure that antibodies will be indeed prepared to such substances as the S100 complex in the trained and untrained brains which you are using as antigens?

LJ. MIHAILOVIĆ: As yet we have no special techniques. I think that it is not an erroneous hope and assumption that the S100 in the brain might be linked to some higher molecular weight proteins capable of inducing antibody formation.

F. ROSENBLATT: Can you estimate the amount of protein present in your fractions and can you describe any of the physical characteristics, such as solubility or molecular weight?

LJ. MIHAILOVIĆ: It is, of course, possible to cut out the fraction and estimate the amount of protein by means of available micromethods. So far, however, this has not been done. We also cannot describe at the present moment the physico-chemical properties of proteins involved.

H. HYDÉN: The experiment we heard about is an adequate control. We have here a control as good as we can get at the present time for a given discrimination test. Getting the same material as Dr. Mihailović and his colleagues used, we found the same type of base ratio changes in the nerve cells which were isolated. I must underline that we have found the same results using quite another test, the handedness reversal experiment.

LJ. MIHAILOVIĆ: I consider Dr. Hydén's words rather a comment than a question. I must say that in order to control all possible non-specific concomitants of behaviour, such as motor activity, sensory input, etc. we tried to set up an adequate model. But, of course, it can also be subject to certain criticism.

EFFECT OF HYPOPHYSEAL PEPTIDES ON MEMORY FUNCTIONS IN RATS

by

B. Bohus

Department of Physiology, University Medical School, Pécs, Hungary

It has become evident that adaptive responses consist of an inseparable unit of behavioural and endocrine events. In addition to the fact that endocrine responses are controlled by the central nervous system, it is also known that hormones may affect central nervous function, that is, the behavioural reactions may be hormonally conditioned.

Attention has recently been focussed on the effect of pituitary-adrenal hormones on conditioned avoidance behaviour. The influence of anterior pituitary adrenocorticotropic hormone (ACTH) appeared in a delay of extinction of conditioned avoidance response (CAR) in rats when the peptide was given throughout the extinction period (de Wied, 1966; Bohus et al., 1968). This effect of ACTH did not depend on the presence of the adrenal cortex. Observations indicate that ACTH fragments which contain the 4–10 amino acid sequence of the ACTH molecule are still capable to delay the extinction of a CAR (de Wied, 1966; Greven and de Wied, 1967). Furthermore, it was shown that if the L-phenylalanine in position 7 of ACTH 1–10 peptide is changed to its D-isomer (ACTH 1–10/7-D-Phe) rapid extinction of CAR takes place after the administration of peptide (Bohus and de Wied, 1966). Thus, a close relation between the structure of the peptides and the behavioural responses were demonstrated by the above-mentioned experiments.

It was observed, however, that the effect of lysin-vasopressin or pitressin (posterior pituitary extract) appeared to be similar to that of ACTH and related peptides (de Wied, 1965). Therefore, it was considered to be of interest to investigate whether the effect of pitressin and of ACTH-like peptides on the extinction of the CAR involves the same mechanisms despite the structural difference of these peptides.

INFLUENCE OF PITRESSIN AND α-MSH ON EXTINCTION AND RETENTION OF A SHUTTLE-BOX AVOIDANCE RESPONSE

To study the mode of action of pitressin and of α-MSH, and ACTH-like peptide male albino rats were trained to avoid the unconditioned stimulus of an electric foot shock (1·8 mA) delivered through the grid floor of a shuttle-box (de Wied and Bohus, 1966). The conditional stimulus was the sound of a buzzer which lasted for 5 sec before the unconditioned stimulus was

given. Ten trials were presented daily with a fixed intertrial interval. Acquisition period was run till the rats achived the criterion of learning — 24 or more avoidances during 3 consecutive sessions. Then the rats were subjected to an extinction procedure. The non-reinforced trials were given daily for 14 days. Twenty-one days after this first extinction period the retention of CAR was tested in a second extinction training. Ten non-reinforced trials were given daily for 3 days.

Pitressin tannate in oil (Parke, Davis and Co.) in a dose of 1 IU/rat, synthetic α-MSH (Ciba) in a dose of 10 μg as a long-acting zinc phosphate preparation or a placebo was administered subcutaneously. The first injection was given 18 hours before the first acquisition session and subsequently every other day till the criterion of learning was reached.

No effect of the peptides on the acquisition was found. Administration of pitressin, however, markedly affected the performance of CAR during the first extinction period (Fig. 1). Extinction of CAR did not occur. In contrast, treatment with α-MSH did not affect the response performance during the first extinction period. Thus, the CAR became extinct like in placebo-treated rats. Significant retention of the response 21 days after completion of the first extinction period was found in rats treated with pitressin during the acquisition. Rats treated with α-MSH or with placebo exhibited almost no retention of the CAR.

Thus, long-term effect of pitressin was observed in these experiments. Pitressin treatment preserved the response performance even 35 days after the last injection of the posterior pituitary extract. In contrast, administration of α-MSH, an ACTH-like peptide had no influence on the response performance either during the first or the second extinction period. In accordance with the observations mentioned in the introduction it is suggested that ACTH-like peptides and pitressin affect basically different mechanisms to delay the extinction of a CAR. While the influence of ACTH-like peptides is relatively short-term since it persists only during the presence of peptides, pitressin, a posterior pituitary extract may promote the fixation of the earned response. This extract may contain peptide(s) which interfere with

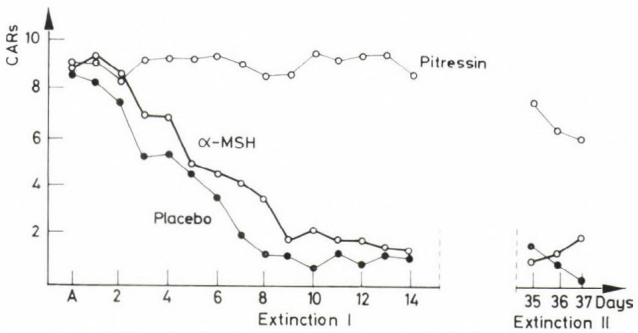

Fig. 1. Effect of pitressin and α-MSH administered during acquisition of the extinction and retention of a conditioned avoidance response.

long-term memory storage of the learned response and its effect is doubtless without the presence of the peptide(s).

Since a number of reinforcements required to develop a conditioned avoidance response the question arose whether the long-term effect of pitressin appeares when the number of reinforcements is less. Therefore, the influence of pitressin and of ACTHβ 1–24, a synthetic ACTH peptide, on the retention of a one-trial learning task in a passive avoidance situation was studied.

EFFECT OF PITRESSIN AND ACTH β 1–24 ON THE RETENTION OF A PASSIVE AVOIDANCE TASK

The experiments were performed in a box of two compartments, one of them being bright and big, and the other small and dark with a grid floor. The rats were allowed to explore for 180 seconds and the time spent in the small compartment was recorded by an electric timer. The rats preferred to stay in the small compartment in order to avoid the brightness of the other one. Learning trial consisted of an unavoidable intermittent foot shock of 0·5 mA for 60 sec in the dark compartment. Retention of the task was represented by a decrease in time spent in the small compartment. Retention was assessed 24, 48 and 240 hours after the learning trial. Male albino rats were treated randomly with 1 IU of pitressin, 10 μg of ACTHβ 1–24 (Organon) and a placebo 18 hours before the learning.

As seen in Fig. 2, rats treated with pitressin retained the passive avoidance task 240 hours after the learning trial while administration of ACTHβ 1–24 did not led to a similar effect. Table 1 shows that the time spent in the small compartment was significantly less in pitressin-treated rats. No differences were found 24 or 48 hours after the learning trial.

This experiment stresses that pitressin may promote the fixation of a one-trial learning task as well. Thus, the long-term effect of the posterior pituitary extract did not depend on the number of trials in avoidance situations.

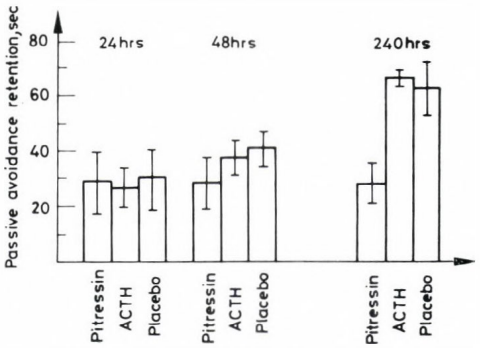

Fig. 2. Effect of pitressin and ACTH on the retention of a passive avoidance task.

TABLE 1

Effect of pitressin and ACTH on the retention of a passive avoidance task

Treatment	No. of rats	Retention		
		24 hrs	48 hrs	240 hrs
Pitressin	12	28·6 ± 10·5*	29·4 ± 8·6	29·6 ± 7·5**
ACTH β 1–24	10	27·5 ± 6·8	37·5 ± 6·8	66·8 ± 3·2
Placebo	11	30·4 ± 10·7	40·9 ± 6·9	62·8 ± 11·2

* Mean ± S. E. of time (in sec) spent in the dark compartment.
** $p < 0.01$.

EFFECT OF PITUITARY PEPTIDES ON THE EXTINCTION AND RETENTION OF A POLE-JUMPING AVOIDANCE RESPONSE

Since pitressin with high pressor activity may contain other peptides than vasopressin it seemed worthwhile to study the influence of pure vasopressin on the retention of an avoidance response. In addition, the influence of the anterior pituitary thyreotropic hormone (TSH) and of posterior pituitary oxytocin was also tested. The hormones and saline as placebo were given daily either during acquisition or first extinction.

Male albino rats were trained to avoid the unconditioned stimulus of an electric shock (1·0 mA) by jumping onto a pole. As conditional stimulus the light of a 35 W bulb was presented for 5 sec before the US of foot shock. Ten trials were given daily with a fixed intertrial interval for 3 days. Those rats which scored 8 or more CARs during the third acquisition session were subjected to an extinction procedure. A total of 30 non-reinforced trials were given during three sessions of 10 trials each. The retention of the CAR was tested 10 days later in a second extinction period consisting of 10 trials.

The effect of pituitary peptides on the extinction and retention of the CAR is summarised in Table 2. Daily administration of ACTHβ 1–24, TSH and oxytocin had no effect on response performance during either the first or the second extinction period if the peptides were given during acquisition. Administration of lysin-vasopressin (Ciba) during acquisition resulted in a high response performance during the first extinction and in a significant retention 10 days later.

If the peptides were administered daily during the 3 days of the first extinction the total number of CARs scored during this extinction period was high in rats treated with ACTHβ 1–24 but no retention of the response was observed. Vasopressin treatment resulted in a delay of extinction and a significant retention of the CAR occurred. Like in previous experiment, TSH and oxytocin had no influence on the extinction and retention.

These experiments reveal that vasopressin is responsible for the long-term memorization effect of a posterior pituitary extract. The influence of

TABLE 2

Effect of pituitary peptides on extinction and retention of a pole-jumping avoidance response

Treatment	No. of rats	First extinction		Second extinction	
		Trials	CARs	Trials	CARs
(1) During learning					
ACTH β 1–24	8	30	14·6 ± 0·9*	10	2·1 ± 0·7
TSH	10	30	12·9 ± 1·8	10	1·0 ± 0·7
Oxytocin	8	30	13·0 ± 1·8	10	2·0 ± 1·0
Vasopressin	12	30	22·8 ± 2·1**	10	8·0 ± 0·5**
Placebo	10	30	13·5 ± 1·0	10	2·0 ± 0·2
(2) During extinction					
ACTH β 1–24	8	30	24·6 ± 2·1**	10	2·4 ± 1·0
TSH	12	30	13·8 ± 2·0	10	1·9 ± 0·6
Oxytocin	10	30	12·7 ± 0·9	10	1·7 ± 0·4
Vasopressin	14	30	23·4 ± 2·0**	10	8·1 ± 0·7**
Placebo	10	30	14·0 ± 0·7	10	1·9 ± 0·4

* Mean ± S. E.
** $p < 0{\cdot}01$.

vasopressin is highly specific from structural point of view since oxytocin which differs only in two amino acids from the vasopressin had no influences on the avoidance behaviour of rats. Short-term effect of ACTHβ 1–24 in the presence of the peptide could be demonstrated again in these experiments.

Recent observations on memory transfer have raised the possibility that peptid-like material(s) may bear the specific information (Ungar and Oceguera-Navarro, 1965; Rosenblatt et al., 1966*a*, *b*). Since the molecular weight of vasopressin is near the range of that of the information-bearing peptide-like materials (Rosenblatt et al., 1966*b*) and this material contains an amino acid residuum less than 10, some similarities between vasopressin and the information-bearing brain extract may be predicted. One should, however, equalize the two materials. There are several dissimilarities between their behavioural influences. Thus, in contrast to the brain extract the influence of vasopressin does not

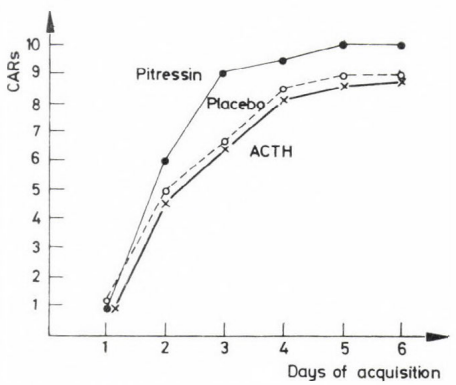

Fig. 3. Influence of pitressin and ACTH pretreatment on the acquisition of a pole-jumping conditioned avoidance response.

appear immediately after the administration. Enhancement of learning was not observed in rats treated with vasopressin and even the number of rats reaching the criterion of learning may be less in pitressin-treated groups than in ACTH or placebo-treated ones. Open-field activity of pitressin-treated rats is also diminished. Thus, the influence of pitressin appears after a transient period and seems to be unspecific in the aspect of the situation as seen in the next experiments.

EFFECT OF PRETREATMENT WITH PITRESSIN AND ACTH β 1–24 ON THE ACQUISITION OF A POLE-JUMPING AVOIDANCE RESPONSE

Male albino rats were treated with a single dose of pitressin, of ACTH β 1–24 as long-acting zinc phosphate preparation and a placebo. Seven days later the rats were trained in a pole-jumping avoidance situation as described previously. A total of 6 acquisition sessions of 10 trials each was given.

Figure 3 depicts the learning curve of the rats. Acquisition of the CAR was more rapid in rats pretreated with pitressin in comparison with the ACTHβ 1–24 or placebo-pretreated rats. If one computes the total number of reinforced responses (RRs) required to reach a learning criterion of 24 or more avoidances during 3 consecutive sessions significantly less RRs were observed in rats pretreated with pitressin than in those with ACTH 1–24 or placebo. The total number of CARs scored during criterion learning was also less in pitressin-pretreated rats indicating that less trial was necessary to reach the criterion (Table 3).

TABLE 3

Effect of pitressin and ACTH pretreatment on the acquisition of a pole-jumping conditioned avoidance response

Pretreatment	No. of rats	CARs	RRs
Pitressin	8	$27{\cdot}2 \pm 1{\cdot}9$*, **	$15{\cdot}2 \pm 0{\cdot}6$**
ACTH β 1–24	8	$31{\cdot}2 \pm 1{\cdot}4$	$20{\cdot}8 \pm 2{\cdot}7$
Placebo	8	$32{\cdot}5 \pm 2{\cdot}2$	$20{\cdot}0 \pm 1{\cdot}4$

* Mean $\pm$ S. E.
** $p < 0{\cdot}05$.

These experiments suggest that the influence of pitressin may appear without any specific connection with the given situation. Therefore, the effect of pitressin may be regarded as an unspecific enhancing influence on the fixation of avoidance responses.

GENERAL CONCLUSIONS

Present experiments indicate that vasopressin of posterior pituitary origin has a long-term, while ACTH or ACTH-like peptides bear a relatively short-term influence on the response performance in avoidance situation. If a response is learned under the influence of vasopressin the avoidance task appears unextinguishable and significant retention occurs in spite of the massive extinction procedure. These observations may indicate that vasopressin affects those processes which are involved in the formation of long-term memory storage. The fact that pitressin pretreatment may enhance learning without the presence of vasopressin suggests that the peptide may even prepare certain CNS mechanisms involved in avoidance learning. ACTH-like peptides, on the other hand, are effective only during the administration and their central nervous influence presumably involves short-term memory processes.

Since it is well known that emotional stresses like avoidance conditioning result in a release of vasopressin from the posterior pituitary the influence of the peptide on the fixation of the memory may be of physiological significance. However, the complexity of hormonal conditioning of CNS processes may involve counteracting influences as well. Thus, it has been observed that corticosteroids which are secreted in high amounts in stressful situations may inhibit short-term and long-term memorization. This dynamic character of hormonal conditioning may assure a very large scale of behavioural responsiveness of the individual living beings.

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DISCUSSION

J. Székely: How do these agents influence the process of acquisition?

B. Bohus: We have already described that ACTH has only slight, if any, enhancing effect on the acquisition of an avoidance response. Pitressin had no effect on the rate of acquisition in a shuttle-box avoidance task but the number of "non-learner" rats was regularly higher than in groups of untreated animals.

G. Horn: Have you studied the effect of hypophysectomy on extinction? If pitressin promotes fixation, one might expect learning to be adversely affected by this operation. Is there any evidence on this point?

B. Bohus: There is evidence presented by de Wied that posterior lobectomy — while leaving acquisition unaffected — results in a failure of resistance to extinction.

J. A. Dyal: Have you conducted experiments to determine if the effect of pitressin in increasing resistance to extinction is specific to the avoidance response which is learned, or is it perhaps due to an increase in fear of the CS. For example, have you tested the animals in an open field and measured the amount of freezing behaviour which occurs?

B. Bohus: Open-field activity of pitressin-treated rats is decreased in all the behavioural scores but the rats did not seem to freeze in this situation. On the basis of other studies with ACTH or corticosteroids it does not seem reasonable to suggest that pitressin simply increased the fear-level in avoidance situation.

G. Ungar: Have you tried to do similar experiments with positive reinforcement, that is, by rewarding the animals rather than punishing them. This would eliminate the factor of fear, which at present seems to be common to all these experiments?

B. Bohus: Several observations have been made in our laboratory on higher mammals suggesting the influence of pituitary-adrenal hormones on different rewarded behavioural responses. However, from the physiological point of view it seems reasonable to study the effect of these hormones on behaviour because they are indeed released endogenously in fearful situation.

RECENT LEARNING DEMONSTRATIONS AND SOME BIOCHEMICAL CORRELATES IN PLANARIANS AND PROTOZOANS*

by

W. C. Corning

Department of Psychology, University of Waterloo, Ontario, Canada

INTRODUCTION

Several invertebrate preparations have come to occupy potentially significant places in certain areas of biopsychological research. The strategy behind their use is straightforward—given the enormous complexity of the vertebrate brain, easier solutions might be attained with simpler systems. The logic is clear enough, and there are many parallels in other areas of biological research to provide hope of success. In the neurosciences, the squid axon, crustacean stretch receptor, snail nerve-muscle preparation, *Limulus* lateral eye, and many other systems have provided us with much of our basic data and concepts. Do we have equally fruitful preparations being developed in research on memory mechanisms?

In the time permitted I will not have the opportunity to review all preparations of possible value and will therefore emphasize my own research on planarians and some related research of others on protozoans. This bias is not intended to slight other simple systems being used in memory research. Several reviews are available that cover a broader spectrum (Bullock and Quarton, 1966; Corning, 1968; Corning and Ratner, 1967; Eisenstein, in press; Kandel and Spencer, 1968; Kennedy, 1967; McConnell, 1966; Thorpe and Davenport, 1965; Wiersma, 1967). In the case of both planarians and protozoans the research findings have engendered considerable controversy. There seems to be little disagreement concerning the relevance of certain studies in research on the biology of memory *providing* it can be agreed that learning has indeed been achieved at these phylogenetic levels. The problem is further complicated by certain definitional difficulties over what we mean by "learning". For example, in a summary of a conference reviewing the application of simple systems in memory research, Bullock and Quarton (1966) write that "the psychologists appeared to agree that they disagree; there are many definitions, but none has become canon (p. 206)". This perennial debate creates problems when attempting to convince scientists that the lowly flatworm learns, particularly when much of our experimental operations and data are based upon investigations of rats in surrealistic environs. However, in spite of disagreements over definitions I believe it is possible to construct a case for the existence of what would generally be considered "learning" by most behavioural scientists in both protozoans and flatworms.

* Some of the author's research reviewed in this paper has been supported by grants from the National Aeronautics and Space Administration (NSG 475; NGR 33-012-009).

The controversy over whether planarians can learn has been dealt with in considerable detail elsewhere (Corning and Ratner, 1967; Corning and Riccio, 1969; Jacobson, 1965; Jensen, 1965; McConnell, 1965, 1966). The major arguments of the critics have revolved around two points:

1. There is the possibility that in the light-shock classical conditioning paradigm first reported by Thompson and McConnell (1955), planarians are being sensitized to light (CS) by the shock (US) and that the response enhancement to light is not due to learning.
2. There have been attempts to replicate basic planarian findings such as classical conditioning, maze learning, retention after regeneration, and cannibalistic transfer, that have either failed to repeat the original results or have uncovered confounding factors that offer equally feasible explanations of the various findings.

With respect to the first point, it has recently been shown that most of the serious criticisms regarding the possibility of sensitization have been answered (Corning and Riccio, 1969). For example, Jacobson (Jacobson, 1967; Jacobson et al., 1967) has demonstrated that neither random nor backward presentation of the CS and US will produce the kind of response modification obtained with an orthodox CS-US pairing. Furthermore, Jacobson has also shown that differential conditioning is possible, i.e., that the response enhancement to light is stimulus-specific and is not the result of a generalized sensitization of the organism. These findings, along with those demonstrating operant conditioning (Best, 1965; Crawford and Skeen, 1967; Lee, 1963), maze learning (Best and Rubinstein, 1962; Humpheries and McConnell, 1964; Wells, 1967) and instrumental avoidance (Ragland and Ragland, 1965) should be most convincing to those willing to be objective about the existing literature.

As for the second point, the failure to replicate, we have previously noted that in no case has there been a serious effort to duplicate any particular study (Corning and Riccio, 1969). Deviations have involved species, shock source, training containers, housing conditions, response measures, trial sequences, and culture medium. In some cases it appears that the experimental design was arranged so that a "failure to replicate" was inevitable. In attempting to replicate some of our early work on retention after regeneration and the effects of ribonuclease on retention, Brown (1964, 1967) used a different species, a more intense light source, a different shock source, a different learning criterion, and then used a method of alternating anodal and cathodal trials that others (Barnes and Katzung, 1963) had shown would not lead to conditioning. Brown has also found that after regeneration, anterior portions are more sensitive to light than posterior portions at low light intensities, but at higher intensities, they displayed equal sensitivity. These observations were used to explain the equal retention shown by anterior and posterior regenerates in the original McConnell work where high intensities were involved (McConnell et al., 1959), and the differential

sensitivity obtained by Corning and John (1961) in trained regenerates that had been in ribonuclease and were exposed to a low intensity source. Brown's comments ignore the fact that in the Corning and John study, regeneration controls were used that showed the light sensitivity in anterior and posterior halves to be equal.

In subsequent research we have demonstrated that anterior and posterior regenerates display equal retention of a right-left maze discrimination habit (Corning, 1966). In this situation no light was involved in the task, indicating that the retention after regeneration phenomenon is not peculiar to light-associated training situations. A summary of these investigations is presented in Fig. 1. We have also begun an analysis of specific factors that may be producing the discrepancies between laboratories. One factor critical in determining light sensitivity is the slime deposited by planarians as they crawl about. We have found that in our cultures of *Dugesia dorotocephala*, light reactivity is almost halved by the presence of slime (Riccio and Corning, 1969). We have also shown that planarians will prefer the slimed portions of their environment when environmental conditions are adversive. These studies are summarized in Table 1 and in Figs 2 and 3.

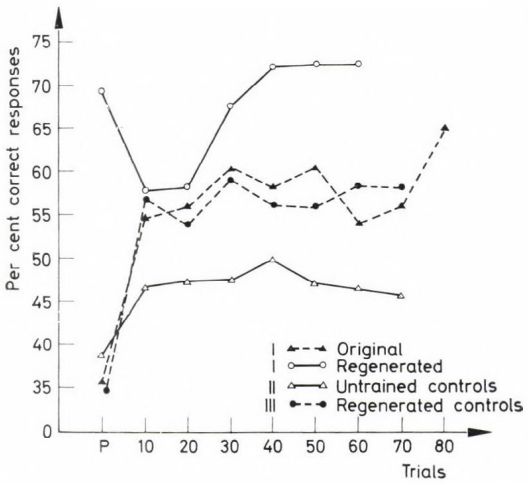

Fig. 1. Average per cent correct response performance of planarians in a "T" maze. All Ss were first given 10 preference tests (P) in the maze. Group I subjects were trained to a criterion of 9 out of 10 choices of the arm opposite to that selected during the preference tests. Group II subjects were permitted to take either arm to escape the maze. Group III contained newly regenerated subjects that were trained to criterion. It can be seen that the regenerates of Group I demonstrated retention of the habit during their post-regeneration preference tests and during retraining. The scores of the anterior and posterior regenerates were the same and are combined in this figure (from Corning, 1966).

In research carried out in collaboration with Dr. Simon Freed at Brookhaven National Laboratories, one of our initial concerns was the development of training apparatus and techniques that would ensure stable conditions for all subjects and would obviate or considerably reduce the possibility of sensitization accounting for any response modifications. We were interested in obtaining information concerning the effects of training treatments on the biochemistry of planarians. This interest stemmed from the finding that interference with RNA during regeneration can affect retention (Corning and John, 1961) and that a "transfer" of memory can be effected by injecting the RNA obtained from trained animals into naive animals (John, 1964; McConnell, 1962; Zelman et al., 1963). In Fig. 4 is the design

TABLE 1

Average per cent response to light per 20-trial session under slimed and non-slimed conditions for large and small subjects

		Subject no.	Slime	Non-slime
Large Ss (20–28 mm)	Group IA	1	11·5	36·5
		2	15·0	38·0
		3	11·5	15·0
		4	11·5	16·5
		5	13·5	23·5
		6	8·5	33·5
		7	18·0	31·5
		8	13·0	20·0
			$\bar{x} = 12{\cdot}8$	$\bar{x} = 26{\cdot}8$
	Group IC	1	30·0	40·0
		2	17·5	20·0
		3	22·5	80·0
		4	15·0	30·0
		5	10·0	5·0
		6	35·0	42·5
			$\bar{x} = 21{\cdot}7$	$\bar{x} = 36{\cdot}3$
Small Ss (9–13 mm)	Group IB	1	18·0	50·0
		2	20·0	60·0
		3	28·5	56·5
		4	43·5	65·0
		5	10·0	33·5
		6	20·0	38·0
		7	26·5	30·0
		8	16·5	23·0
			$\bar{x} = 22{\cdot}9$	$\bar{x} = 44{\cdot}5$
	Group ID	1	27·5	55·0
		2	40.0	47.5
		3	20·0	80·0
		4	50·0	45·0
		5	30·0	42·5
		6	25·0	92·5
			$\bar{x} = 32{\cdot}1$	$\bar{x} = 60{\cdot}4$

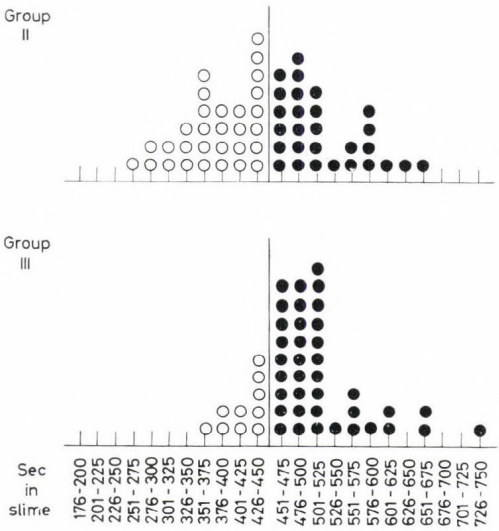

Fig. 2. Slime preferences in planarians. Group II animals received slime preference test under normal conditions; Group III represents the prefrences exhibited by subjects exposed to pH of 5·7 and 8·3, and UV light. Each circle represents the total time spent on the slimed side in a 900 second test period. Open circles: 450 seconds or less on slimed side; solid circles: 451 seconds or more on slimed side (Riccio and Corning, 1969).

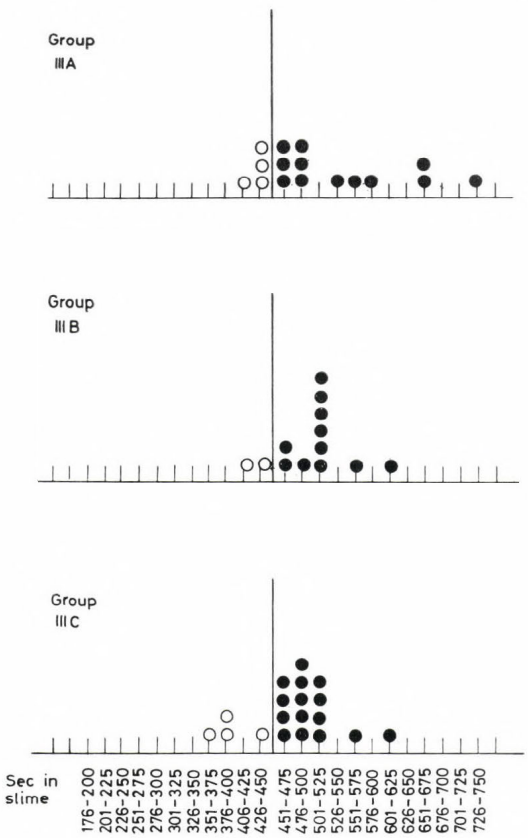

Fig. 3. Slime preferences under three adverse conditions. Group IIIA: pH of 8·3; Group IIIB: pH of 5·7; Group IIIC: UV light (Riccio and Corning, 1969).

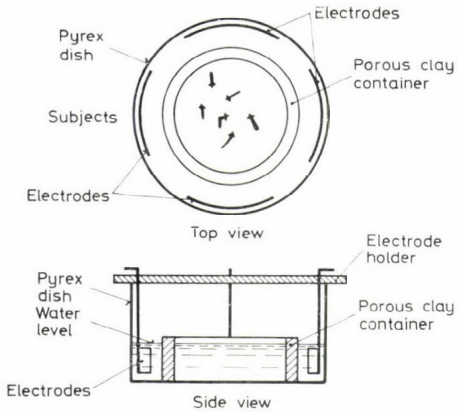

Fig. 4. A container for mass conditioning planarians using light as a CS and shock as a US (Corning and Freed, 1968; Freed, 1966).

of the container used to classically condition planarians with light (CS) and shock (US). This type of container has several advantages: (*i*) It permits several animals to be trained at the same time. (*ii*) The porous clay ring prevents animals from climbing on the electrodes and yet does not impede current flow. (*iii*) By keeping the animals in a central position a more uniform exposure to the light and shock stimulation is achieved. (*iv*) By randomly alternating the voltage drop between electrode pairs the subjects are prevented from assuming an orientation in the field that would allow them to receive a less than adequate shock. With the circuit schematized in Fig. 5 we were able to deliver experimental and control treatments to several bowls at the same time. With the simultaneous presentation of con-

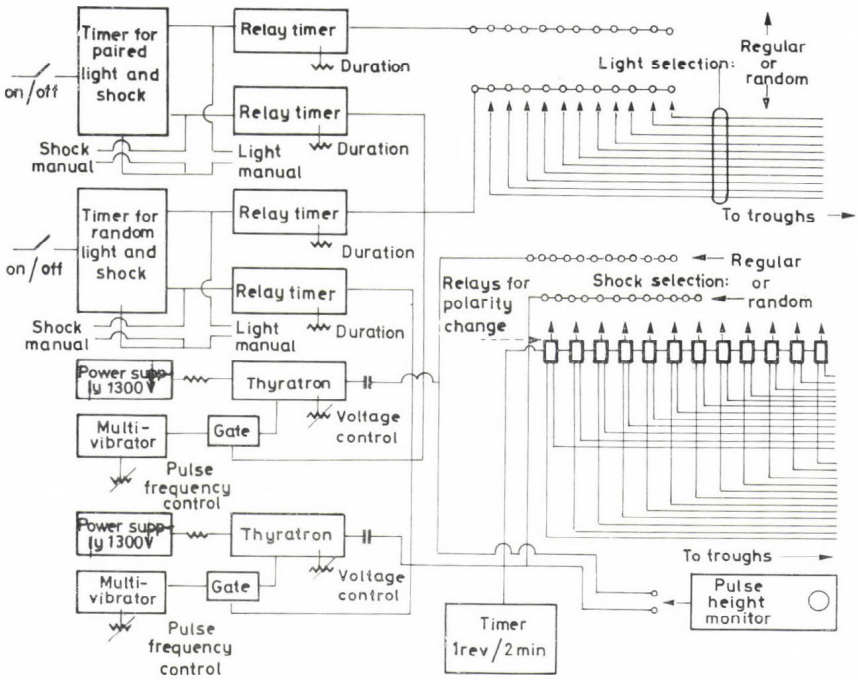

Fig. 5. Scheme of circuitry used to control experimental treatments in mass training apparatus (Freed, 1966).

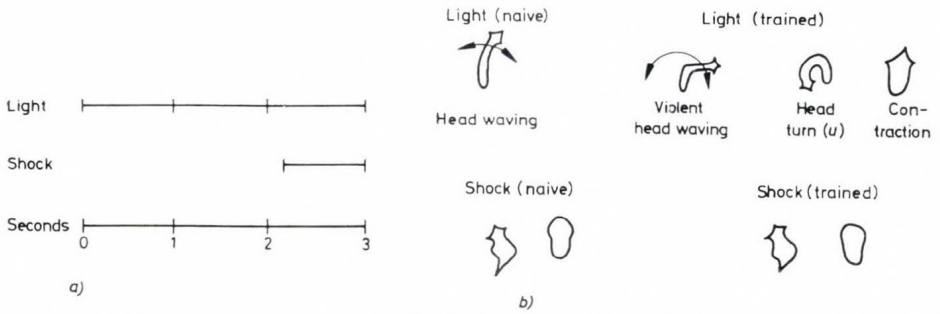

Fig. 6. (*a*) Duration and relationship of light and shock stimuli used to classically conditioned planarians. (*b*) Types of responses to light and shock observed in planarians

ditions to all subjects of the study we avoided biochemical and behavioural variations due to diurnal factors, temperature shifts, experimenter changes, etc., and we were able to run groups sufficiently large for adequate statistical analyses.

To assess the efficacy of this apparatus in the conditioning of planarians three groups of 10 *Dugesia tigrina* each were presented the following treatments:

Group LS was classically conditioned by pairing light (CS) with shock (US). Each trial consisted of 3 sec of light overlapping with 1 sec of shock as shown in Fig. 6. A total of 480 such pairings was presented.

Group LO was given 480 trials of light alone.

Group RSL was a sensitization control and was presented the same amount of light and shock stimulation in any one session as Group LS but the light and shock were never paired. The time between any two consecutive stimuli varied from 5 to 80 sec. On the average, there were two stimuli each minute.

Experimental sessions consisted of two 20-trial periods per day with the second period beginning 4 hours after the first. The interval between trials was approximately 1 min. For the first three days all groups were habituated to the light stimulus in order to reduce the innate reactivity to light. Each day during the habituation period the groups were given two 20-trial periods of light alone. All treatments were administered with the training containers housed separately in 1 of 12 compartments of a cupboard. The cupboard was light-proof and was bolted to the wall to minimize vibrations.

At specific stages during the experimental treatments, 6 subjects were randomly selected from each group and given their particular trial conditions individually in the training container. During these test trials the subjects' reactions to light were recorded. Three response classifications were used: A head waving ("*t*"); a head turning ("*u*"); and a contraction ("*c*"). These responses are depicted in Fig. 6. The stages selected for sampling were: after habituation to light, and after 40, 160, 240, 320, 400, 440 and 480 trials.

The frequency and types of responses to light that were recorded are summarized in Table 2 and the average per cent response over the trial

TABLE 2

Frequency and types of responses to light in planarians*

Stage	Group LS			Group LO			Group RSL		
	t	*u*	*c*	*t*	*u*	*c*	*t*	*u*	*c*
Habituation	13	2	0	14	2	2	12	7	2
40 trials	14	7	2	15	2	5	28	3	5
160 trials	28	9	5	9	2	2	18	6	7
240 trials	29	19	4	4	3	1	27	9	7
320 trials	32	16	4	11	1	2	20	4	2
400 trials	43	22	11	6	3	2	12	5	2
440 trials	64	30	12	10	3	1	12	2	4
480 trials	51	32	21	10	1	1	24	1	3
Totals	274	137	59	79	17	16	153	37	32

* Data based on 20 test trials given each of six Ss removed from each group at the various stages.

treatments are shown in Fig. 7. It can readily be seen that the paired presentation of light and shock produced a light-responsivity that was markedly higher in the later training stages than the response levels observed in both the sensitization control (Group RSL) and the light controls (LO).

These results coincide nicely with the findings of Jacobson's group (Jacobson et al., 1966) and satisfied us that the apparatus and techniques we had developed for the mass training of planarians did not "sensitize" animals and were an efficient way of training large numbers of animals at

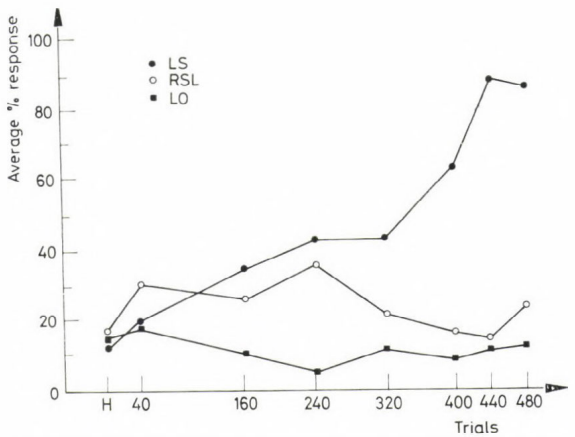

Fig. 7. Average per cent response to light in trained and control groups. See text for explanation (Corning and Freed, 1968).

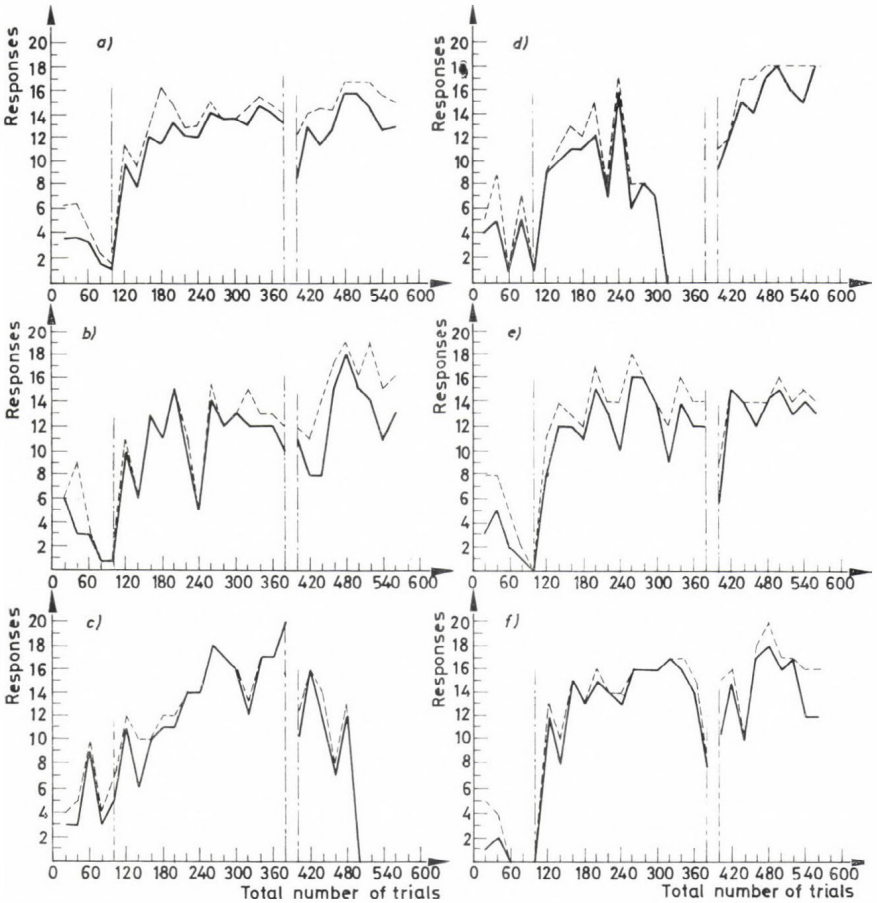

Fig. 8. Total (broken lines) and valid (solid lines) responses to light in 6 worms during classical conditioning. The vertical line at 100 trials marks the end of the pre-training habituation to light. The interval between the vertical lines at 380 and 400 trials was 39 days. The drop in performance of subject *d* at 320 trials was due to fissioning (Freed, 1966).

one time. Claudia Ferguson, working in Freed's laboratory at Brookhaven, has obtained evidence that animals trained in this apparatus will demonstrate long-term retention (Freed, 1966); after 39 days her subjects were still at a high level of responsiveness, additional evidence that "sensitization" is the least likely explanation (Fig. 8).

PROTOZOAN LEARNING: RECENT EVIDENCE

Prominent in the recent history of research on protozoan learning is the Gelber–Jensen debate over whether paramecia can acquire an association between a wire and food. Gelber (1952) had outlined procedures which at

first seemed to provide convincing evidence that *Paramecium aurelia* was capable of learning to approach a wire to obtain food (*Aerobacter aerogenes*) that was coated on the wire. Basically, the training procedure was to present the food-coated wire for a certain number of trials and count the number of animals that approached the wire. In a final test, a bare wire lowered into the culture was found to elicit a greater number of attachments when compared to control cultures that had been exposed to a wire without food. The interpretation of these findings was disputed by Jensen (1957*a*, *b*) who believed that "learning" had not been demonstrated conclusively and that other interpretations were equally likely. Jensen's major points were that the repeated dipping of a wire coated with bacteria will produce a bacteria-rich area, that paramecia will be attracted to this area, and that the presence of bacteria will increase attachment responses in paramecia. Thus, the creation of a food-rich zone accounted for the increased number of animals at the place where the wire was dipped rather than any "association" between wire and food. If Jensen's interpretations are correct, then the homogenization of the culture fluid prior to the final test with a bare wire should provide a critical test. Katz and Deterline (1958) performed this test and observed that the approach response to the wire in "trained" cultures disappeared.

The criticisms of Jensen and the findings of Katz and Deterline would appear to have settled the issue of approach learning in paramecia, but subsequent research of Gelber has kept viable the possibility of conditioning in her cultures (Gelber, 1954, 1956*a*, *b*, 1957, 1958*a*, *b*, 1962*a*, *b*, 1965; Gelber and Rasch, 1956). In a reply to Jensen, Gelber (1957) points out that (*i*) wire-clinging will occur when food reinforcement is placed elsewhere in the culture drop; (*ii*) the frequency of exposure of the wire-food combination is more critical than amount of food; (*iii*) in all experiments the culture fluid was stirred before the final test; (*iv*) paramecia do not necessarily approach a bacteria-rich area; and (*v*) that in his own experiments, Jensen utilized an excessively high concentration of bacteria. Furthermore, Gelber has demonstrated in later experiments that cultures placed in a clean medium prior to testing still retain the learned behaviour (1965), the response undergoes extinction (1958*b*), there is retention for up to 3 hours (1958*a*, 1962*b*, 1965), and that the acquired response is transferred to daughter cells (1965). These findings are impressive, and providing that food-induced thigmotropism can be ruled out as a confounding variable,* an interpretation of "learning" is certainly tenable.

Recent investigations attempting to show avoidance conditioning in protozoans have also been controversial. Both Best (1954) and Mirsky and Katz (1958) were unable to confirm that the earlier procedures of Bramstedt (1939) had unequivocally produced avoidance learning in paramecia. Bramstedt's procedures were to expose one-half of a well to light and heat while keeping the other half darkened and cooled. He observed that the paramecia would avoid the lighted half and that this avoidance persisted

* Katz and Deterline (1958) placed food in the culture dish of one of their control groups (IV) and found that the incidence of wire approach rose.

even when the temperature of the two halves was equalized. Best (1954) found that heat will induce a light-avoidance independently of its association with light, and Mirsky and Katz (1958) were unable to obtain a light avoidance either through the conditioning procedures of Bramstedt or through the modification of presumed environmental factors.

Fortunately, past difficulties in obtaining evidence for conditioning in protozoans has not discouraged further attempts. Sten Bergström (1968*a*, *b*, 1969) has obtained evidence that strongly suggests avoidance learning in the ciliate *Tetrahymena*. Bergström exposed one group (Group E) to paired light-shock trials, a second and third group to shock alone (Group S) and light alone (Group L), and used a fourth group as a base control (Group N). During testing sessions, when all groups had access to lighted and darkened areas of a test chamber, he found that Group E subjects spent less time in the lighted portions than any other group. During the 15-minute test period the proportion of Group E animals that were in the light dropped while the proportion of control subjects in the light rose slightly (Table 3).

TABLE 3

Proportion of animals in light after training and control treatments (from Bergström, 1968*a*)

Time from start of test period (min)	Group E	Group S	Group L	Group N
0	0·285	0·296	0·302	0·305
3	0·253	0·298	0·303	0·312
6	0·234	0·304	0·309	0·313
9	0·248	0·309	0·318	0·319
12	0·239	0·311	0·326	0·333
14·5	0·243	0·323	0·321	0·321

If habituation is included in the category of learned behaviour, then there is more conclusive proof that protozoans learn. Habituation in *Stentor coeruleus* was attained by Harden (1969) using mechnical stimulation. The dish containing the culture was located over a speaker cone; activation of the cone with pulses produced a rapid displacement of the dish and a contraction of organisms attached to the bottom. A camera photographed the Ss just after a stimulus presentation. Four of Harden's groups provided a convincing demonstration of habituation:

Group A Ss constituted a base line control: No stimulation was given the Ss and photographs were made at 1 min intervals.

Group B was given 30 stimuli with an inter-trial interval of 60 sec. One minute after the final stimulus the group was exposed to a flash of light in order to test for general fatigue, i.e., if the reponse diminution was due to fatigue, then there should be no reaction to light as well as the mechanical stimulus.

Group C was run for 9 successive days with 30 trials per day to test for retention after a 24-hour period.

Group D animals were given 4 runs of 30 stimuli each with 1 hour between each run. This group gave an estimate of short term retention.

The results presented in Fig. 9 indicate that with repeated mechanical stimulation, the responsivity of *Stentor* dropped over a 30-trial run. In Group B, stimulation of the Ss in another modality demonstrated that the response cessation was not due to fatigue; a flash of light still elicited contractions in animals that had been habituated to mechanical stimulation. The habituation curve for Group C was almost identical to that of Group A. This curve represents the responses recorded on the 9th day of habituation and indicates that there is little retention from day to day. The data of Group D would, however, indicate retention after 1 hour. The curve of Fig. 9 represents the per cent contraction recorded on the 4th run, 1 hour after the previous 30-trial session. It can be seen that the base rate of response was achieved much sooner in this group. Other groups used by Harden ruled out alternative explanations such as the accumulation of toxic substances in the culture medium.

Applewhite and Morowitz (1966) have also had success in habituating a protozoan (*Spirostomum*) to mechanical stimulation. In Table 4*a* are the results of an earlier experiment where it was found that with strong stimulation, there was some retention of the habituated response for 60 sec after criterion had been achieved. Criterion for habituation in these experiments was no response for two successive stimulations. The observation that *Spirostomum* returns to pre-habituation levels after several minutes eliminates injury as an explanation for the response diminution. Fatigue can also be excluded because the habituated subjects can still contract to more intense stimuli and to different stimuli such as electrical shock or UV (Applewhite et al., 1969). Other studies in Applewhite's laboratory have demonstrated phenomena similar to that obtained in multicellular organisms.

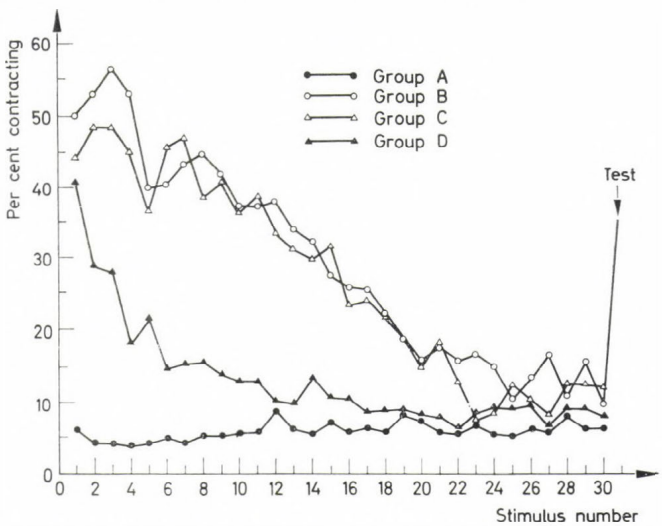

Fig. 9. Contraction responses of *Stentor* (Harden, 1969). See text for description of groups. The "test" at the completion of habituation for Group B was a flash of light.

TABLE 4

Habituation and associated phenomena in *Spirostomum*

(*a*) Habituation with weak and strong stimuli (Applewhite and Morowitz,1966)

	Weak stimuli	Strong stimuli
Initial trials to habituation	3·6 ± 1·6	10·0 ± 3·6
Retention 15 sec	1·6 ± 0·8	3·1 ± 2·0
Retention 60 sec	3·2 ± 1·1	7·4 ± 2·2

(*b*) Retention of habituation after transection (Applewhite, 1968)

Whole Ss	9·0 ± 6·9	—
Naive anterior sections	7·0 ± 4·1	—
Naive posterior sections	9·6 ± 6·1	—
Habituated anterior sections	3·1 ± 2·9	—
Habituated posterior sections	3·6 ± 3·1	—

(*c*) RNA turnover during habituation (Applewhite and Gardner, 1968)

Stimulus	% Ss contracting	CPM in habituated Ss	CPM in habituated controls
1	65 ± 9	7 920 ± 920	8 160 ± 938
20	42 ± 8	9 140 ± 950	8 053 ± 970*
50	22 ± 6	12 676 ± 1378	12 634 ± 1300
Retention test 4 min later	69 ± 8	26 036 ± 2040	27 730 ± 1981

(*d*) Protein turnover changes during habituation (Applewhite et al., 1969)

Stimulus	CPM in habituated Ss	CPM in control Ss
20	11 220	11 840
50	24 340	20 374**

* Experimental and control groups were significantly different (0·001) after 20 stimuli.

** Experimental and control groups were significantly different (0·01) after 50 stimuli.

If habituated animals were cut in half and tested 15 sec later, it was found that both halves exhibited savings of the habituation and that the degree of savings was approximately equal in the two portions (Applewhite, 1968*a*). Retention in *Spirostomum* can also be improved by cooling the animals immediately after criterion is reached (Applewhite, 1968*b*).

While earlier investigations had implied a biochemical basis for learning in planarians (Corning and John, 1961; McConnell et al., 1959), it was the demonstration of what appeared to be a cannibalistic transfer of memory that markedly aroused interest in the planarian as a preparation for research on memory mechanisms. This discovery also led to a proliferation of transfer studies in a wide variety of animals including crabs, fish, rats and starfish. In planarians it was found that this transfer effect could be obtained with a fraction containing RNA (Zelman et al., 1963). It remained to be determined whether there was actually a change in planarian nucleic acids during training. Dr. Simon Freed and I decided to examine the effects of classical conditioning on planarian nucleic acid quantity and turnover.

In order to gain a more complete picture of what nucleic acid changes accompanied the development of a conditioned response, we performed whole-body analyses of worms at several stages of training: after they had been habituated to light, and after 160, 340 and 460 training trials. We performed whole-body analyses because both the cannibalistic transfer studies and retention after regeneration studies indicated a non-local storage of information in the planarian. Two groups of *Dugesia tigrina* were used: Group I received the paired light-shock classical conditioning treatment and Group II was presented with random light and shock. Because the Ss of Group I began to contract more frequently as training progressed (both to the light and shock), the Ss of Group II were given an equivalent amount of contraction experience by tapping their dish an appropriate number of times. Estimates of nucleic acid turnover were obtained by using ^{32}P. Previous studies had shown that when radioactive inorganic phosphorus was placed in the culture medium, there was rapid incorporation. Electrophoretic and chromatographic separations established a nucleotide location of the tracer in the nucleic acid fractions. Extraction procedures were based upon those described by Scott et al. (1956). To keep exposure to the isotope at a minimum, the Ss to be loaded were not placed in a ^{32}P medium until 3 days before each of the stages. Samples containing 18–24 worms were removed from each of the main groups and placed in separate bowls containing ^{32}P (20 μC/ml of pond water). During the next three days, half of each sample received its usual experimental treatments (*A* samples), while the other half was placed in the training bowls but received no stimulation (*B* samples). No ^{32}P was present in the water contained in the training bowls. At the end of the 3 days the samples were killed by immersing them in liquid nitrogen. The main groups and the samples received their experimental treatments simultaneously. Further procedural details have been presented elsewhere (Corning and Freed, 1968; Freed, 1966).

We found that there were no significant differences between groups with respect to RNA and DNA quantities and for RNA/DNA ratios. However, the ^{32}P data did yield interesting differences between the trained and control groups. When RNA/DNA specific activity ratios were calculated, the conditioned Ss of the *A* samples showed significantly lower specific activity ratios at 160 and 340 trial stages when compared to all other groups includ-

ing the conditioned *B* samples. At the terminal stage (460 trials) the ratios of the various samples were again similar and not significantly different from those observed at the beginning of training. These data are summarized in Fig. 10.

These findings suggest that the biochemical consequences of different modes of stimulus presentation (paired light-shock vs. random light and shock) occur before there is any dramatic behavioural change. They also show that the changes in specific activity ratios produced by paired light and shock are transient, i.e. the LS-*B* samples did not demonstrate the differentiation observed in the *A* samples. The implications of these results will be discussed further by my research colleague, Dr. Simon Freed, in the ensuing discussion.

Analyses of single cells in trained and pseudoconditioned planarians by H. Hydén, E. R. John and co-workers have failed to yield any significant differences in RNA base ratios. These analyses were made at the completion of training and did not include any attempt at estimating turnover rates (E. R. John, personal communication). Crawford's group at Florida State have found some evidence that training produces differences in amino acid content (Crawford et al., 1965) but a follow-up study failed to replicate (King et al., 1965).

Biochemical investigations in the protozoan *Spirostomum* have yielded some interesting results, some of which correspond to our planarian research (Applewhite and Davis, 1969; Applewhite and Gardner, 1968; Applewhite et al., 1969*a*). Examination of the effects of various metals (sodium, potassium, calcium, magnesium, and manganese) on habituation indicated that magnesium and manganese impaired acquisition of habituation but did not affect contractibility. Subjects were also tagged with ^{3}H-5-uridine in order

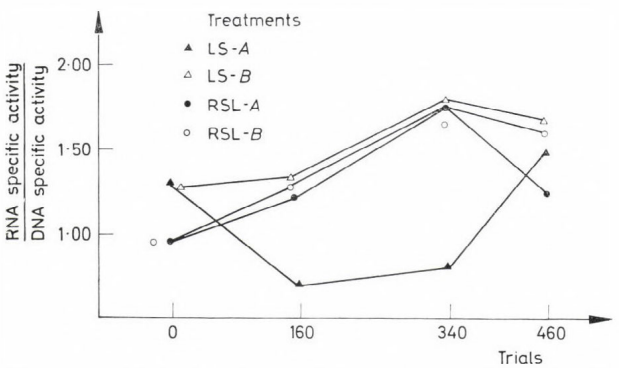

Fig. 10. RNA/DNA specific activity ratios at four stages of conditioning in planarians. LS, groups receiving paired light and shock; RSL, groups receiving random light and shock. The *A* samples were presented their usual stimulus conditions during the isotope loading period while the *B* samples were not (Corning and Freed, 1968; Freed, 1966).

to investigate RNA turnover during habituation. These studies are summarized in Table 4*c*. It can be seen that the experimental and control samples removed after 20 stimuli displayed significant differences in radioactivity. However, after 50 stimuli and at 4 minutes after the last stimulus the experimental and control samples were the same. As in our planarian work, these findings indicate that RNA turnover changes precede any overt behavioural change. Protein turnover changes were found to occur at a different point in habituation (Applewhite et al., 1969*a*). Whereas the RNA changes occurred early in habituation, protein turnover differences between experimental and control groups did not occur until the terminal stages (Table 4*d*).

Further experimentation has begun to localize the cellular structures subserving habituation (Applewhite et al., 1969*b*). *Spirostomum* were centrifuged so that the macronuclei were displaced to one end. They were then cut transversely, yielding a portion that had no macronuclei and 30 per cent less protein. These portions could still be habituated and would also demonstrate retention. It is speculated that the infraciliature is probably the locus of habituation.

CONCLUSIONS

With respect to the biology of memory it appears that research on protozoans and planarians has been extremely fruitful. Aside from the specific findings discussed in this paper, research on these organisms, especially protozoans, may force a restructuring of hypotheses about the physiological bases of learning. It is clear that learning is not a capacity restricted to vertebrates or even to multicellular systems. This does not mean that the physiological substrates are necessarily the same for all animals. At present, my own bias is towards assuming that there are probably certain cellular strategies involved in learning that are common to all organisms, but that the actual molecular species may differ. For example, adaptive enzyme formation may be a common mechanism underlying learning but the enzymes utilized and the conditions regulating their production may vary considerably from phylum to phylum. With this assumption, my interest in invertebrate learning mechanisms has at least a temporary justification.

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DISCUSSION

J. A. Dyal: I would agree that random presentation of the CS and the UCS is an appropriate control condition for any sensitization effects which may be involved in the classical conditioning of planarians. However, I have never quite understood the logic of the differential conditioning experiment as a control for sensitization. It would seem possible that those people who would apt for a sensitization interpretation could argue that the differential conditioning experiment represents sensitization to the S^+ and habituation to the S^-. How could you answer this argument?

W. C. Corning: By sensitization we mean always an unspecific phenomenon. In the literature if one speaks about sensitization, differentiation is excluded. The existence of differential conditioning is just the appropriate test to prove that the unspecific sensitization has developed into a real learning process.

ENDOGENOUS BIOCHEMICAL CHANGES DURING CONDITIONING PROCESSES

by

S. FREED

Departments of Biochemistry and Neurology
New York Medical College, U.S.A.

The results of the experiments with planarians together with the biochemical analysis given by Dr. Corning in the previous discussion may be summarized in the combined graphs of Fig. 1. More detail has been recently published (Corning and Freed, 1968). The black triangles of the upper graph show the increased frequency with which the planarians responded to light after they had been presented a sequence of paired light and shock stimuli. The black circles represent their responses to light after they had been subjected to randomly related stimuli of light and shock of the same number and intensity as in the paired stimuli. In the paired light-shock sequence the planarians achieved an efficiency of over 80 per cent in associating

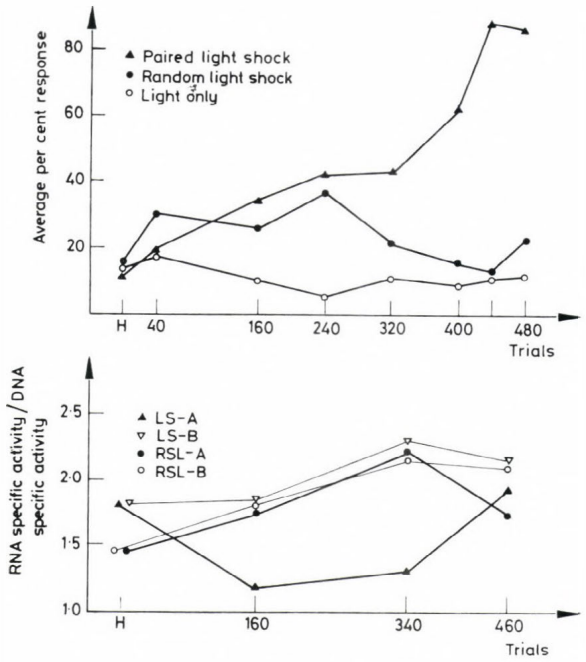

Fig. 1.

light with shock, while in the random light-shock sequence no trend occurred in the strong responses to light as the number of trials was increased.

The lower portion of the graph shows with increasing trial number the course of the ratios of specific activities of RNA to the specific activity of DNA. The planarians had been exposed to radioactive inorganic phosphorus, ^{32}P, in their ambient water three days before the training periods while, during training, the water contained only non-radioactive phosphorus. Turnover or synthesis of nucleic acids during training would entail, then, a reduction in their specific activities.

It is to be noted that in the planarians being conditioned, the ratio of RNA sp. act./DNA sp. act. has become less than the original value as well as less than in the control group; that is, those exposed to randomly related light and shock stimuli. Relative to DNA sp. act., the RNA sp. act. has become less, and consequently a greater synthesis of RNA has occurred during conditioning (when the animal was in the presence of non-radioactive phosphorus). The random stimuli serving as control provided correction for any facilitation of light responses because of electrical stimulation.

The differences in the ratios between the learning planarians and the control group at 160 trials and at 340 trials is statistically significant with p values less than 0·01. On the other hand, it may be noted that at 160 trials, the subjects have shown virtually no signs of learning and at 320 trials, the conditioned behavioural change is scarcely beginning to emerge, to a degree below statistical significance.

Biochemical changes clearly precede behavioural changes in conditioning. At the end of training the ratios of the specific activities of the successfully trained animals have reverted back to the value at the beginning of the training or of the controls. That is, no further change in ratio is required for the maintenance of the elaborated conditioned behaviour.

The thin lines of the lower graph represent the biochemical behaviour of the animals which had not been stimulated at the given stage but had been exposed to ^{32}P in their water in the ususal way. Their responses would represent persistence, if any, of biosynthesis of nucleic acids induced by stimulation at the previous stage. The fact that their ratios of specific activities of the nucleic acids were not significantly different from the corresponding ratios of the control group indicates that chemical changes induced by the conditioning process disappear in a few days.

The average per cent deviations from worm to worm in the magnitude of RNA sp. act. and of DNA sp. act. proved to be about two times the average of per cent deviation of the ratios, RNA sp. act./DNA sp. act. The lower values of these deviations imply dependence between the values of RNA sp. act. and of DNA sp. act. at each stage of training. A change in RNA sp. act. induced by improved response frequency in paired light and shock would then be accompanied by a change in DNA sp. act. of the subjects.

The mechanism which proposes itself first to account for such change in DNA is cell proliferation. On the other hand, it has long been regarded as proved that nerve cells do not multiply. Authors fully aware of this general acceptance have reported contrary experimental evidence (Rampan, 1962).

Recently a number of reports have appeared describing proliferation of microneurons (Altman and Das, 1965).

Dr. Henry Quastler of the Brookhaven National Laboratory where our work was done found by autoradiography that DNA was synthesized in the cytoplasm of planarian cells. A possible localization is in mitochondria where DNA is known to be present and where it is possibly involved in RNA synthesis with protein production.

The suggested involvement of cellular proliferation in conditioning would bring closer the analogy repeatedly referred to in the symposium between the neuronal processes of conditioning and of immune reactions.

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CONCLUDING REMARKS

by the Chairman

J. SZENTÁGOTHAI

The papers presented at this session and the ensuing discussions were certainly of considerable interest and have initiated various trends of thoughts. The way Dr. Mihailović and collaborators designed their visual discrimination tests for the study of changes in brain proteins were most elegant and appropriate with respect to the great difficulties of the task. The major difficulty with the protein mechanisms of memory and learning is that we have no useful notion as to how the electric events might be related—in both ways for fixation and for readout—to the protein changes. Electric events, i.e. generation of spike potentials, do not by themselves lead to specific changes in protein composition. We can drive—as shown beautifully by Dr. Hydén—certain neurones and this might mean that passive function does not cause any specific qualitative change in the proteins. The difference between passive relay through a synapse and the physiological events that may occur at the same site in situations of learning or recognition is that there are many other influences impinging upon the same neurone in the latter case. Thus, we would have to count with a variety of local processes—both inhibitory and excitatory—occurring simultaneously in various regions of the postsynaptic membrane. Irrespective of whether or not a propagated impulse of the neurone is generated, these local processes of the subsynaptic membrane might be of crucial importance for retaining the traces of any specific question in which the given neurone has been involved.

Section III

THE "CHEMICAL TRANSFER" PROBLEM

INCUBATION EFFECTS IN TRANSFER OF TRAINING IN RATS

by

A. M. Golub, F. R. Masiarz, Trudy Villars and J. V. McConnell

Mental Health Research Institute, The University of Michigan, Ann Arbor, Michigan, U.S.A.

In 1965, investigators in four independent laboratories (Babich et al., 1965; Fjerdingstad et al., 1965; Reinis, 1965; Ungar and Oceguera-Navarro, 1965) reported transfer of experiential information from trained to untrained mammals by injection of material extracted from the brains of the trained animals. These successful reports of "memory transfer" were followed by a series of negative reports (Byrne et al., 1966; Gross and Carey, 1965; Luttges et al., 1966) that did not substantiate the validity of the effect. Since that time experiments in more than 14 laboratories would seem to suggest that an effect of some sort does exist (Jacobson, 1967; Tunkl, 1968; Schutjer, 1968); yet, in light of the apparent difficulty in replicating these experiments in some laboratories, it would appear that the basic phenomenon may be highly elusive. In the present experiments we attempted to define more adequately one of the critical factors, the strength of donor training, that we believe must be controlled for the effect to occur.

We used the same basic paradigm first reported by Dyal and his associates (Dyal et al., 1967; Dyal and Golub, 1968) and replicated successfully in our laboratory (Golub and McConnell, 1968). In those experiments one group of donor animals received 8–10 days of continuously reinforced bar press training, followed by three days of experimental extinction, followed in turn by three days of reacquisition of the bar press response. A second group of donor animals served as controls for handling, activity, and sensitization. Following the training sessions, the donors were sacrificed and brain homogenates prepared from their brains and injected intraperitoneally into naive, recipient rats. "Memory transfer" effects were consistently reported for animals that had received material from trained, but not from untrained, donors.

In our replication and extension of the previous work, reported below, we used 60–90-day-old, Sprague-Dawley male rats maintained on 22·5 hr food-deprivation schedules; this procedure was carried out throughout all these experiments.* Grason-Stadler operant chambers were modified so

* Detailed procedures are available to investigators interested in repeating these studies. The authors thank J. Shelby and L. Stein for their assistance in carrying out this work. Supported, in part, by grants MH 16392-01 and MH 07417 from the U. S. National Institute of Mental Health, and by grant 5-T01-GM00187-09 from the U. S. Department of Health, Education and Welfare, Public Health Service Training Grant.

that the lever was moved to the side of the chamber opposite the food cup. Lever presses were cumulated on counters. Twelve donor animals received eight, 30 min sessions of bar pressing in which each bar press was reinforced by a 45 mg Noyes food pellet delivered into the food cup. Twelve additional donor animals served as quiet controls. These animals were handled each day, but were not trained. The eight days of bar press training given the experimental donor animals were followed by three daily extinction sessions in which bar presses were not reinforced with food, followed in turn by three daily sessions of bar press reacquisition. All sessions were 30 min in duration.

Within 15 min after the final training session, the donor animals were sacrificed by decapitation. The brain, excluding the olfactory bulbs and cerebellum, was removed and stored on dry ice. These brains were then coded and the remainder of the experiment performed using "blind" procedures in which personnel actively engaged in carrying out the study were unaware of from what groups the brains had been taken and to which groups the recipient animals had been assigned. Whole tissue homogenates were prepared by adding 1·0 ml of 0·154 M saline per brain and homogenizing gently with 15 strokes with a motor-driven pestle and with an ice bath surrounding the homogenizer. Recipient animals were each injected with 3·2 cm^3 of the homogenate.

Recipient animals were placed on a 22·5 hr food-deprivation schedule beginning six days prior to injection and were 23 hr food-deprived at the time of injection. This procedure was followed throughout all the present experiments. Twenty-four hrs following injection, all recipient animals were given the first of nine, daily (30 min) bar pressing sessions in which each bar press was reinforced with one 45 mg Noyes food pellet. Bar presses were automatically cumulated on counters.

Figure 1 presents the mean number of bar presses emitted by the experimental and by the control recipients on each of the nine days of training. The differences between these two groups on the final two days of training are statistically reliable by the Mann-Whitney U test ($p = 0{\cdot}02$). Thus, the control recipients were reliably superior to the experimental recipients, as measured by bar pressing. This result was entirely unexpected, especially when considered in light of five previous replications of the basic paradigm (Dyal et al., 1967; Dyal and Golub, 1968; Golub and McConnell, 1968) in which experimentally-injected recipients were shown to be superior to control-injected animals.

There was one major difference between the present study and previous experiments. In this study, due to an error in ordering recipient animals, training of the experimental donors was discontinued for one week following the three days of extinction, so that recipient animals would be available when the donors were sacrificed. During this seven-day period, the donors were maintained on the deprivation schedule, but were not trained. It occurred to us that the seven-day period of no training could conceivably have served the function of "incubating" the training experience just preceding it (i.e., the extinction training) so that extinction was the most likely aspect of the training to "transfer". We decided to test this notion by carrying out

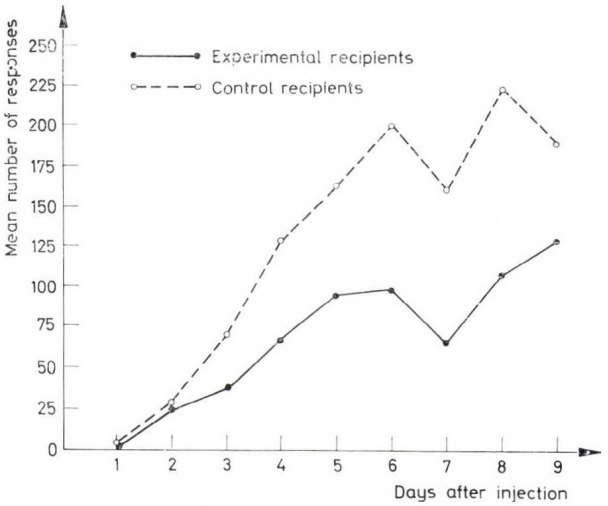

Fig. 1. Mean number of bar presses on each of nine daily sessions for recipient animals injected with brain homogenate either from the experimental or from the control donor groups.

additional experiments in which periods of no training (rest periods) were interposed during the donor training regime.

Twenty-four donor rats were given eight sessions of bar press training, three sessions of extinction, and three sessions of bar press reacquisition training, using the same training procedures outlined previously. Twelve of these animals received a seven-day rest period after acquisition training, but prior to extinction (Acq-Rest-Ext-Reacq); the remaining twelve animals received a seven-day rest period following extinction, but prior to bar press reacquisition training (Acq-Ext-Rest-Reacq). We hypothesized that if rest periods did indeed serve an incubatory function of consolidating the learning experience just preceding them, recipient animals receiving brain material from donors given their incubation period following acquisition training (Acq-Rest-Ext-Reacq) should be superior in their rate of acquisition of the bar press response to recipient animals injected with material from group Acq-Ext-Rest-Reacq. A third group of donor animals ($n = 12$) served as quiet controls and were not trained.

Following the final day of donor training, all donor animals were sacrificed by decapitation, and the procedures reported earlier were used for removing their brains and preparing the homogenates. Eighteen recipient rats were each injected intraperitoneally with 3·2 cm^3 of homogenate either from Group Acq-Rest-Ext-Reacq ($n = 6$), Group Acq-Ext-Rest-Reacq ($n = 6$) or from the quiet control group ($n = 6$). Twenty-four hrs later they were given the first of eight daily (30 min) bar pressing sessions for 45 mg Noyes food pellets on a continuous reinforcement schedule.

Our results were consistent with the incubation hypothesis. Animals receiving material from Acq-Ext-Rest-Reacq trained donors were inferior

to both control-injected and Acq-Rest-Ext-Reacq injected recipients, and recipient animals injected with material from Acq-Rest-Ext-Reacq donors were superior to all other groups on the bar press measure. We repeated this experiment two additional times with the same results. It thus appeared to us that "transfer of training" effects could be manipulated through incubation periods. We then decided to carry out a fourth replication of the paradigm, on this occasion making a preliminary attempt to isolate the chemical substrate mediating the effect. Also, we wished to explore the possibility that an RNA fraction would yield the effect.

We trained two groups of donor rats, using the same procedures reported earlier. One donor group ($n = 7$) was given a seven-day rest period following acquisition training (Acq-Rest-Ext-Reacq); the second group ($n = 5$) received a seven-day rest period following extinction (Acq-Ext-Rest-Reacq). A third group of animals ($n = 5$) served as untrained control donors. Following the training sequence, these animals were sacrificed as reported previously and an RNA extract prepared from their brains. Sacrifice of the donor animals, RNA extraction, and injection and testing of the recipient animals were all performed using "blind" procedures to avoid introducing experimenter bias.

RNA was obtained by a cold phenol extraction procedure; all procedures were carried out at 0–4 °C unless otherwise stated. For each gram of brain tissue, 10·0 ml of 0·154 M saline were added. The brains were allowed to thaw partially and were then homogenized for 1·0 min using a motor-driven teflon pestle. An ice bath jacketed the homogenizer during this process. Following homogenization, 0·5 volume of 88 per cent phenol was added and the homogenate stirred with a magnetic stirring bar for 30 min. The resulting mixture was centrifuged at 20,000 × g for 60 min. The top aqueous phase was withdrawn and transferred to clean test tubes. Then 0·10 volume of 1·0 M $MgCl_2$ was added and the solution stirred gently. Following this, 2·50 volumes of cold 95 per cent EtOH were added and stirred. This mixture was stored at −20 °C for 2 hrs. The resultant precipitate was centrifuged at 1400 × g for 30 min. The RNA pellet was resuspended in 6·0 ml of 75 per cent EtOH and resedimented by centrifugation at 1400 × g for 15 min. The pellet was again resuspended and centrifuged. This procedure was repeated three times. The RNA pellet was dried by evaporating the EtOH in an air stream. The RNA was then dissolved in 0·60 ml of 0·154 M NaCl per brain equivalent of RNA.

A pellet consisting of the yield of one donor brain reserved for assay was dissolved in 10·0 ml of gradient buffer containing 0·01 M acetate, pH 5·1, 10^{-3} M EDTA, and 0·1 M NaCl. An ultraviolet absorption spectrum of this solution is presented in Fig. 2. Two ml of the RNA sample were layered on a 28 ml linear gradient of 10–40 per cent sucrose in the gradient buffer and centrifuged at 25,000 RPM for 15 hrs at 0 °C in an SW 25·1 rotor of a Spinco L2-65B ultracentrifuge. The gradients were analysed for distribution of 260 mμ absorbing material using an LKB flow cell modified for use in a Beckman DU spectrophotometer with continuous recording on a Sargent recorder.

The density gradient optical density profile (Fig. 3) indicated the presence of both ribosomal RNA and transfer RNA. The material appeared to be

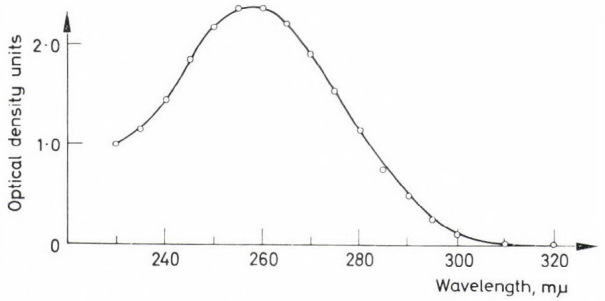

Fig. 2. Ultraviolet absorption spectrum of one brain equivalent of RNA extract dissolved in 10·0 ml of 0·01 M acetate buffer, pH 5·1 with 10^{-3} M EDTA and 0·1 Mt NaCl.

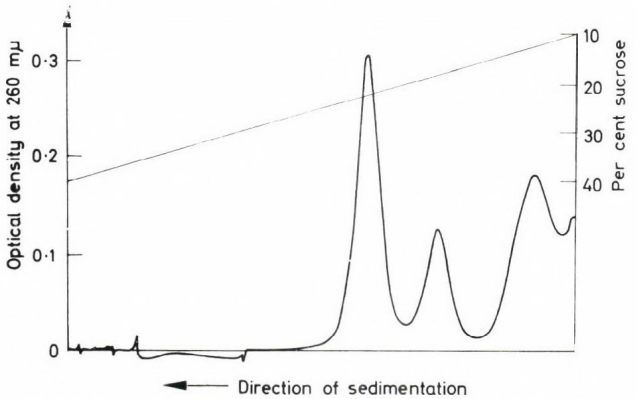

Fig. 3. 15–40 per cent linear sucrose density gradient sedimentation profile of one fifth brain equivalent of RNA extract. The gradient contained 0·01 M acetate buffer, pH 5·1, with 10^{-3} M EDTA, and 0·1 M NaCl and was centrifuged in a Spinco L2-B ultracentrifuge in an SW 25·1 rotor.

undegraded, as indicated by the fact that the ribosomal RNA 28S : 18S peak height ratio was greater than 2·0. (Further analyses are currently in progress.)

Twelve rats served as recipients and were given subdural brain injections of the RNA extract.* Four were injected with RNA obtained from the brains

* Prior to the injection of the RNA, each animal was anaesthetized with an intraperitoneal injection of sodium pentobarbitol. An incision was made along the line of suture sagittalis between the animal's eyeballs and the skull was exposed. A dental drill with a 1 mm bur was used to make a hole in the suture sagittalis directly between the eyeballs (i.e. above the space between the olfactory bulbs). The hole was drilled through the skull. A 250 mμ tuberculin syringe with a 30 g, 1/4-in-long needle was used to inject the RNA. This syringe had been kept in a refrigerator and was loaded with 0·15 ml (0·25 brain equivalent) of RNA solution immediately before the injection. The needle was kept at a 45° angle with the frontal plane, 2 mm deep from the outside surface of the skull. The RNA solution was injected into each recipient over a 5 min period (Lindberg and Ernster, 1950).

of the quiet control donor animals, four with RNA from the brains of group Acq-Rest-Ext-Reacq, and four with RNA from the brains of group Acq-Ext-Rest-Reacq. One of the recipients injected with RNA from group Acq-Rest-Ext-Reacq died shortly after being injected, and thus no data are reported for that animal.

Twenty-four hrs following these injections, the recipient animals were given the first of eight daily (30 min) bar press sessions, during which each bar press was reinforced by a Noyes pellet. Figure 4 presents the mean number of bar presses emitted by each of the recipient groups during each of the eight sessions. The means during the initial three days of training were submitted to an unweighted-means solution analysis of variance (Winer, 1962). The results of this analysis indicated that the groups were significantly different in their mean rate of bar pressing ($p < 0{\cdot}05$) and that there was a significant interaction effect between sessions and treatment ($p < 0{\cdot}05$). Individual comparisons between the three groups indicated that group Acq-Rest-Ext-Reacq was significantly superior to either of the other groups ($p < 0{\cdot}05$) and that the recipient control group was reliably superior to group Acq-Ext-Rest-Reacq in mean number of responses over the first three days of training ($p < 0{\cdot}05$).

These results suggest that incubation periods may be critically important in obtaining positive "memory transfer" effects. We have evidence that, unless donor animals receive prolonged training (overtraining), investigators obtain "transfer" effects with their paradigms only when their animals are trained five, but not seven days a week (Ungar and Reinis). It is therefore

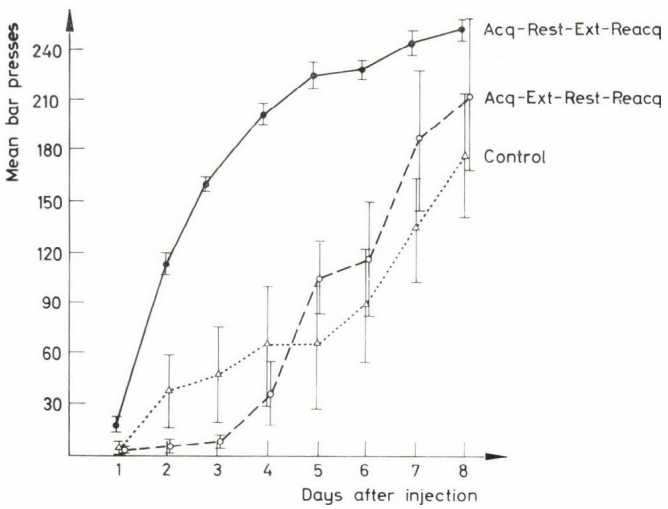

Fig. 4. Mean number of responses emitted by the recipient groups on each of eight days following injection of brain RNA. The RNA was extracted from donor animals trained on either Acq-Rest-Ext-Reacq, Acq-Ext-Rest-Reacq, or from quiet control animals. The vertical bars represent one standard error of the mean.

possible that the failure of certain investigators (Byrne, 1966; Gross and Carey, 1965; Luttges et al., 1966) to replicate the positive reports of "transfer of learning" may reflect a failure to incorporate incubation periods into the training regime of their donor animals. We do not know why these incubation periods are necessary, but we do have additional evidence, presently in preparation, that they dramatically reduce the time necessary for donor animals to acquire a particular response in laboratory learning situations.

Finally, since the extract used in the previous experiment contained impurities, the present results should not be taken as evidence that RNA is necessarily the active chemical mediating the present effect. Enzyme digestion studies are currently in progress to provide evidence regarding the contribution of RNA as the substrate for the present effect.

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DISCUSSION

G. Ádám: It is common experience in Pavlovian laboratories that the overtraining of animals leads to a pessimum effect. For instance, dogs cannot be trained twice a day in classical conditioning experiments. As far as the weekends are concerned, the intermission always enhances learning: dogs perform better on Monday than on Friday or Saturday. These well-known observations underline and support Professor McConnell's findings, as well as our earlier published data about the pessimum transfer effect of donors, which were overtrained (Ádám and Faiszt, *Nature* **216,** 188, 1967).

J. V. McConnell: I am glad to know this.

J. Székely: Experiences are the same with verbal human learning. Sleeping and rest improve performance invariably.

J. V. McConnell: Thank you for your comment.

O. L. Wolthuis: In general the points you made clear may be true, but nevertheless I would like to put a small hole in this way of reasoning. We have done one trial avoidance experiment (in a Bures 2 compartment setup) in which we gave the animals 6 days of rest after the test and before decapitation. We got no transfer effect. On the other hand, in other experiments we used a repeated training 5 times per day during 5 days in which we got a positive transfer.

J. V. McConnell: I apologize, I did not know that Dr. Wolthuis waited 6 days. My hypothesis fails. As far as the five days training are concerned, in some cases, five days of donor training are enough. In many cases, however, five days are not enough unless a rest period is interposed.

F. Rosenblatt: As you know, we have found that inversion of effects frequently results from changes in dosage. Have you tried different dosages in the paradigms which gave positive and inverted effects? Is it possible that this difference was due to a difference in effective dose?

J. V. McConnell: Our effect is not due to dosage, since we used the same dose in all cases.

CHEMICAL NATURE OF THE TRANSFER FACTORS; RNA OR PROTEIN?*

by

G. Ungar and E. J. Fjerdingstad

Baylor College of Medicine, Houston, Texas, U.S.A.

There are good historical reasons why most of the early experiments of chemical transfer of acquired information were started out to prove that memory, like genetic information, is stored in nucleotide sequences. This was suggested by the brilliant achievements of biochemical genetics and by Hydén's findings of base ratio changes in the nuclear RNA of neurons involved in learning (Hydén, 1959). On closer examination, however, there were valid logical arguments against RNA being directly responsible for the transfer effects. Assuming that acquired information is stored in RNA sequences, we still have to postulate a protein molecule whose synthesis is directed by them and which ultimately controls the processes of consolidation and retrieval. The existence of such "executive molecules" (Hydén, 1969) is implicit in all the molecular hypotheses of learning and memory.

There are other reasons which make it much less likely that transfer of the learned behavior from donors to recipients is mediated by the RNA template rather than by its protein translation. If we would wish to "transfer", for example, a hormone or an enzyme action, we would extract the hormone or the enzyme rather than its RNA template. Intraperitoneally injected RNA would have to go through several barriers in order to direct the synthesis of the enzyme: it would have to penetrate into the appropriate cells after having passed across capillary walls and, in the case of the brain, through the blood-brain barrier. There is evidence to show that nucleotide sequences of sufficient length can be stopped by any or all of these boundaries (Luttges et al., 1966).

Postulating that the transfer factors are peptide sequences does not deny the role of nucleic acids in the storage of acquired information. The permanent or at least durable nature of memory must assume a self-replicating molecule for storage and only nucleic acids can fulfil this condition.

EXPERIMENTAL EVIDENCE

Besides the theoretical arguments just mentioned, there are reports in the literature which indicate that peptide linkages are essential for the activity of the transfer factors. In the first transfer experiments published from our laboratory (Ungar and Oceguera-Navarro, 1965) it was found

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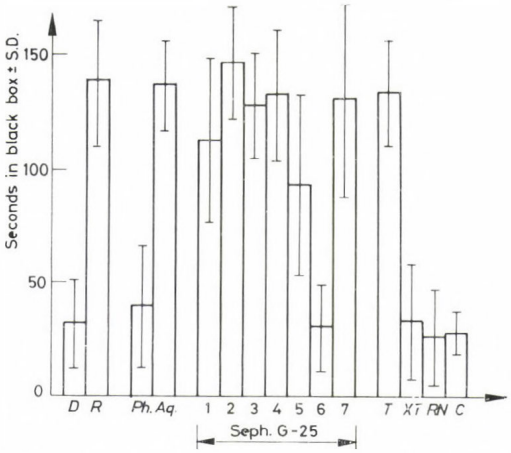


Fig. 1. Chemical properties of the transfer factor of dark avoidance. Effect of the purification and characterization procedures on the activity of donor brain extract in recipients. Ordinate: time in dark box + S.D. (the shorter the time, the higher the activity). *D*, dialyzate; *R*, retentate after dialysis of homogenate against 20 volumes of distilled water for 20 hrs at 2 °C; *Ph.*, phenol phase; *Aq*, aqueous phase after partition of the dialyzate in equal volumes of 80 percent phenol and water. Separation on Sephadex G-25 column (each tube contained 3 ml): fr 1, tubes 25–35; fr 2, 36–44; fr 3, 45–51; fr 4, 52–60; fr 5, 61–70; fr 6, 71–90; fr 7, 91–110. Incubation with enzymes: *T*, crystalline trypsin, 0·5 mg/ml; *XT*, crystalline chymotrypsin, 0·5 mg/ml; *RN*, pancreatic ribonuclease, 0·4 mg/ml; *C*, control, treated like the trypsin sample but without enzyme added.

that the active material was dialyzable, passed into the phenol phase on phenol partition, was destroyed by chymotrypsin and not by RNase, and gel filtration suggested a molecular weight of around 1,200. The material responsible for the transfer of morphine tolerance exhibited similar properties (Ungar and Cohen, 1966).

The material studied more recently (Ungar et al., 1968), which induces dark avoidance in the recipients was also found to be dialyzable, hydrolyzed by trypsin, unaffected by RNase and its elution characteristics suggested the same comparatively low molecular weight (Fig. 1). This material has now reached a purification stage at which it is active at 0·1 μg per mouse and, on thin layer chromatography, corresponds to a definite spot detectable by the ninhydrin reaction.

A fourth active substance was extracted from the brain of rats trained to avoid stepdown from a platform. Its properties are similar to the three others just mentioned but it is destroyed by both trypsin and chymotrypsin (Fig. 2). The existence of chemical differences between transfer factors, as suggested by different enzyme specificities (Table 1), is supported by the observation that there is no cross-transfer between dark avoidance and stepdown avoidance (Ungar, 1970*a*).

The data just summarized indicate that the factors active in the transfer of morphine tolerance, habituation, dark avoidance and stepdown avoidance have a comparatively low molecular weight and their activity depends on the integrity of some peptide linkages. We have encountered other situations (brightness discrimination, conditioned avoidance) in which the material responsible for transfer was not dialyzable and whose properties suggest a more complex molecule (Ungar, 1967).

Observations made by other workers have also indicated that transfer factors may be peptide sequences (Rosenblatt et al., 1966; Giurgea et al., 1969; Zippel and Domagk, 1969; Golub et al., 1969). This is also suggested

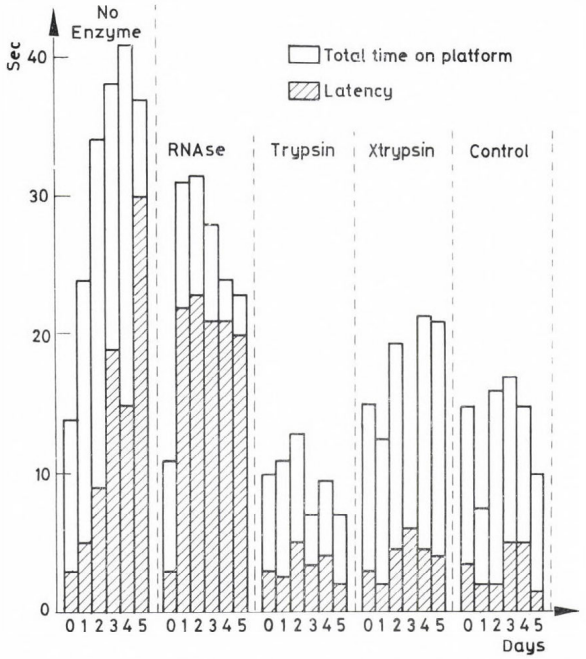


Fig. 2. Effect of enzymes on the activity of brain extracts taken from rats trained for avoidance of step-down from platform. Abscissa: days of testing before (0) and after injection of the extracts; ordinate: time in sec of latency of step-down and total time spent on platform. The higher the values the more active the extract is. The first set of columns (no enzyme) shows the effect of extract from trained donors which was not treated by any enzyme but which was incubated under the same conditions as the enzyme-treated samples (RNase, trypsin, chymotrypsin). The last columns show the effect of a control brain extract taken from untrained donors.

TABLE 1

Effect of proteases and ribonuclease on transfer factors of morphine tolerance (MT), sound habituation (SH), dark avoidance (DA) and step-down avoidance (SA)

Factor	Enzyme		
	Trypsin	Chymo-trypsin	RNase
MT	+	0	+
SH	+	0	+
DA	0	+	+
SA	0	0	+

+ = activity present; 0 = activity destroyed.

by the finding that actinomycin D administered to recipients inhibits the transfer effect (Reinis, 1968). Since actinomycin D is an inhibitor of the transcription for DNA to RNA, it should not interfere with transfer if it s caused by a direct effect of donor RNA.

The difficulty raised by the passage through the blood-brain barrier of nucleotide chains does not exist for peptides and moderately sized proteins. Many pharmacologically active peptides have central nervous effects after systemic administration and proteins of the size of γ-globulins have been shown to cross the blood-brain barrier (Ungar, 1970*b*).

The evidence for nucleic acids is based on the fact that RNA extracted from the brain by the usual procedures has shown definite activity. It should be noted, however, that none of these preparations was protein-free. It is also true that the peptide extracts are contaminated by nucleotides but these are limited to short sequences which are unlikely to carry the information necessary for the transfer of behaviour. Faiszt and Ádám (1968) found the activity in ribosomal RNA which has no information content at all. This is hardly compatible with the observations indicating specificity of transfer (Ungar, 1970*a*; Rosenblatt, 1970).

RNA-BOUND PEPTIDES

In an attempt to reconcile the contradictory findings, we took advantage of the presence in this laboratory of proponents of the RNA (Fjerdingstad, 1965) and of the peptide (Ungar and Oceguera-Navarro, 1965) hypotheses. We used a pool of brains taken from rats trained for dark avoidance and made two preparations: (*i*) a "peptide" extract from which most nucleic acids and larger proteins were eliminated by dialysis, phenol partition and acetone precipitation (Ungar et al., 1968) and (*ii*) an "RNA" extract made according to Røigaard-Petersen et al. (1968). Both extracts, when injected intraperitoneally to groups of mice, showed approximately equal activity. After incubation with trypsin, chymotrypsin and RNase, both preparations were inactivated by trypsin and not by the other enzymes.

The results suggested that the active material may consist of peptides bound to RNA. Since the transfer factor was found to be basic when passed through ion exchange columns (Dowex 1 and 50), it seemed possible that it would form complexes with RNA, somehow in the manner of histones with DNA.

This assumption prompted us to try a dissociation of the complex by dialysis at various pH levels. Figure 3 shows the results of these experiments. It is seen that at pH 7·5, 7·0 and 6·5 the activity is retained in the non-dialyzable fraction but at 6·0 and 5·5 tends to pass into the dialyzate. At pH 5·0 and 4·5 the dialyzability reverses again but at pH 4·0 and below (down to pH 2·5) all the active material becomes dialyzable.

It seemed reasonable to explain the non-dialyzability observed at pH 4·5 and 5·0 by the formation of a complex between the active substance and some acidic material which has its isoelectric point at these pH levels. In another series of experiments we adjusted the pH to various levels

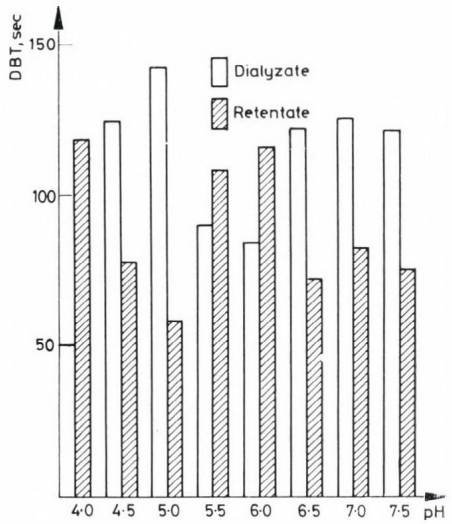


Fig. 3. Dialysis of "RNA" preparations at different pH levels. Abscissa: pH; ordinate: time spent in dark box by recipient mice injected with dialyzates or retentates obtained after dialysis at pH indicated. The lower the value, the higher the activity of the fraction.

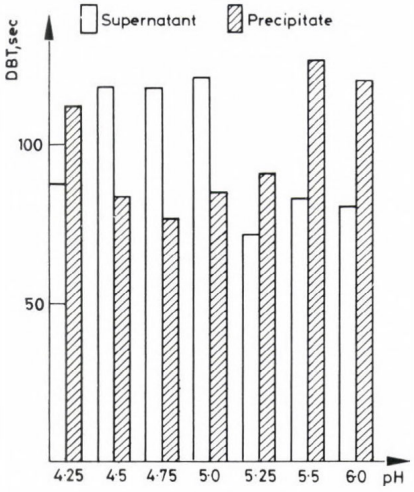


Fig. 4. Solubility of the active material at different pH levels. Abscissa: pH; ordinate: time spent in dark box by recipients injected with supernatant or precipitates obtained after adjusting pH to levels indicated and centrifugation at 25 000 × g for 1 hr.

between 4 and 6, and after stirring for 1 hr at low temperature, centrifuged the samples at 25 000 × g for 30 min. Supernatants and precipitates were tested and the results are summarized in Fig. 4. It shows that between pH 5·0 and 4·5 the active material is in the precipitate and at pH 4·25 starts appearing in the supernatant.

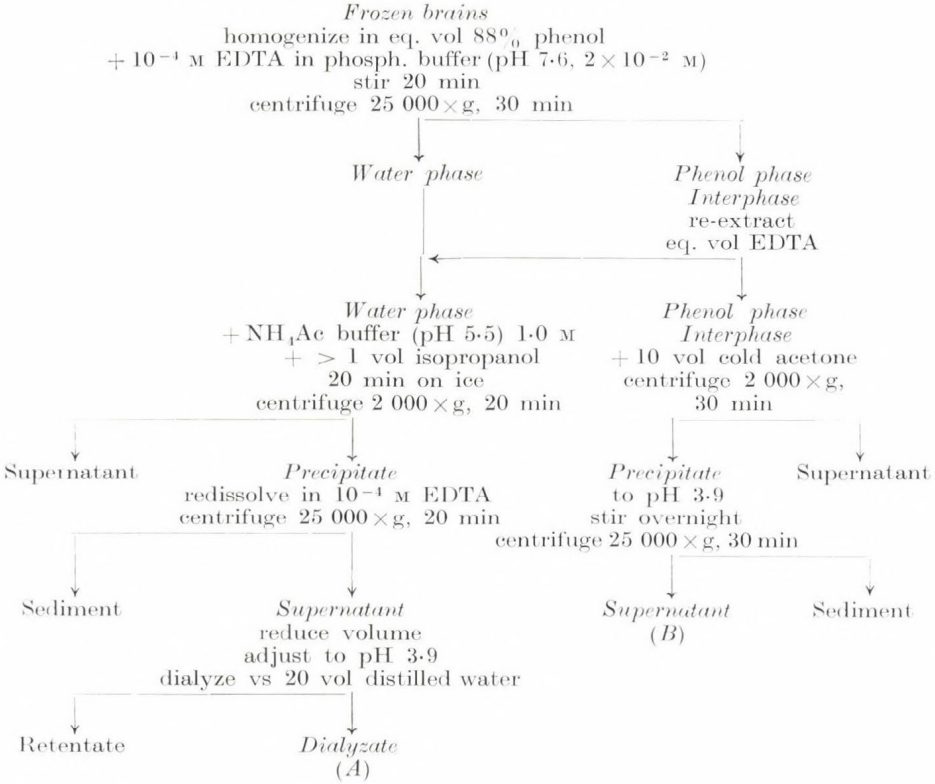
As a result of these experiments we adopted the procedure outlined in Table 2 to prepare the transfer factor of dark avoidance. We find about 2/3 of the activity in fraction *A* and 1/3 in fraction *B*. We have no definite evidence that *A* and *B* are identical although their enzyme affinities and elution properties on gel filtration are the same. We assume now that there is, for each situation, a single peptide in which the relevant information is encoded but this peptide has a tendency to form complexes with RNA and other acidic material (proteins or carbohydrates). The complexes formed both with RNA and the acidic substances can be dissociated at appropriate pH levels. Of course, the final proof for this assumption will only be forthcoming when the factors are isolated and their structure determined.

CONCLUSIONS

The results of the experiments just summarized tend to explain the fact that both RNA and peptide extracts of brain can transfer learned behaviour. They suggest that the active material consists of specific peptide sequences

TABLE 2

Purification scheme of transfer factor for dark avoidance (active fractions in italics)



bound to RNA. It is likely that these peptides exist in two forms: the RNA-bound form, present probably in the cytoplasm, and the free form, perhaps partly bound to acidic substances that may be located on the surface of the cell, especially at synaptic junctions. The most urgent task at present is to isolate and identify at least one of the transfer factors.

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DISCUSSION

J. V. McConnell: Did you incubate the brain material with RNase before or after dialyzing?

G. Ungar: We did it both before and after dialysis.

H. Hydén: I have one remark and one question. Even if RNA is injected intraventricularly or intracranially the destructive activity is quite high. If one looks at the preparative papers at the present time and at the results of the preparation of RNA, one can find signs of breakdown of the RNA in almost any paper which deals with brain macromolecules. The destructive activity of the cerebrospinal fluid, for example, is quite high too. Wouldn't you say that your material would be broken down at least to oligonucleotides or perhaps to nucleosides?

G. Ungar: Yes, I agree with you. The only possible case where RNA could have been intact and active is the planarian experiments. I do not know exactly the details, perhaps Dr. McConnell can say something about that. But I do not think that in any of the mammalian experiments the brain extract injected intraperitoneally or intracranially would act as intact RNA.

TRANSFER OF BEHAVIOURAL BIAS AND LEARNING ENHANCEMENT: A CRITIQUE OF SPECIFICITY EXPERIMENTS*

by

J. A. Dyal

University of Waterloo, Waterloo, Ontario, Canada

This symposium on "The Biology of Memory" should be viewed as a part of the current effort in the two-thousand-year-old search for the physical basis of mind. You will recall that Aristotle had placed 'mind' in the heart and that a few hundred years later Galen took the mind out of the heart and put it in the head. About 1800 years later Karl Lashley summarized his 30 year effort by saying that his research had "yielded a good bit of information about what and where the memory trace is not. It has discovered nothing directly of the real nature of the engram. I sometimes feel in reviewing the evidence on localization of the memory trace, that the necessary conclusion is that learning is just not possible" (Lashley, 1950).

Around the turn of this century William James anticipated the current interest in the molecular basis of memory by writing, "every smallest stroke of virtue or of vice leaves its never so little scar. The drunken Rip Van Winkle, in Jefferson's play, excuses himself for every fresh dereliction by saying, 'I won't count this time.' Well! He may not count it; but is being counted nonetheless. Down among his nerve cells and fibres the molecules are counting it, registering and storing it up to be used against him when the next temptation comes" (McGaugh, 1967).

How molecules go about registering and storing information resulting from experience is still an intriguing mystery. However, several different approaches have been taken toward the solution of this problem and have resulted in at least circumstantial evidence that indeed molecular registering and storage of experience does occur. The appropriate interpretation of the results of all these varied approaches has generated considerable controversy. But certainly the most controversial of the approaches to the physical basis of learning and memory has been the one which is our present concern, namely "memory transfer".

Following the publication of the initial positive reports in 1965 (Babich et al., 1965; Fjerdingstad et al., 1965; Reinis, 1965; and Ungar and Oceguera-Navarro, 1965), there ensued a period of intense research aimed at determining the reliability of the phenomenon. Our laboratory began work on the problem in the spring of 1966, when it was beginning to appear that the

* The research was supported by grants from the Texas Christian University Research Foundation and from the University of Waterloo. Participation in the meeting was facilitated by a travel grant from the National Research Council.

transfer effects were simply not replicable. The procedure which Golub, Marrone and I used in this first series of experiments was similar to that of Jacobson in that we attempted to transfer an approach response to a compound auditory and visual stimulus which was paired with the delivery of food in the original training of the donors. (Dyal et al., 1967). The experimental donors were trained for 10 days to press a bar on a CRF schedule in a Skinner box, they were then extinguished for 4 days, and then given three more days of reinforced training on a CRF schedule. During the reinforced sessions when the rat pressed the bar, a light dimmed, a click occurred and a food pellet was delivered into the food cup. A control group for the effects of stimulus sensitization was yoked to the experimental group so that whenever the experimental animal pressed the bar the light-click stimulus occurred in the control box but no food was delivered. Brain homogenates were injected IP into the recipients and they were tested 24 hours later in the Skinner box; the 'light-click' stimulus was pulsed every 60 sec and the number of times the animal stuck his nose into the food magazine in 30 minutes was recorded on counters. The results of the first two experiments can be seen in Fig. 1. The differences between the experimental group (AER) and the yoked control group were significant in each case. Recognizing that in an area such as this one we need more than a couple of statistically significant experiments to make a point, we repeated this experiment a total of 6 times (Dyal and Golub, 1968; Dyal et al., 1969). Three of these have yielded a statistically significant difference between the experimental and control groups and the other three although not significant were all in the appropriate direction. If you use the procedure recommended by Winer (1962) for obtaining a combined probability value for a series of experiments testing the same hypothesis then you find that the probability is less than 2 in 100 that the results from the total set of experiments are due to chance. In addition, our procedure has been replicated with significant positive results by Golub and McConnell (1968). Thus we feel that the positive memory transfer observed with our procedure is both statistically reliable and experimentally reproduceable.

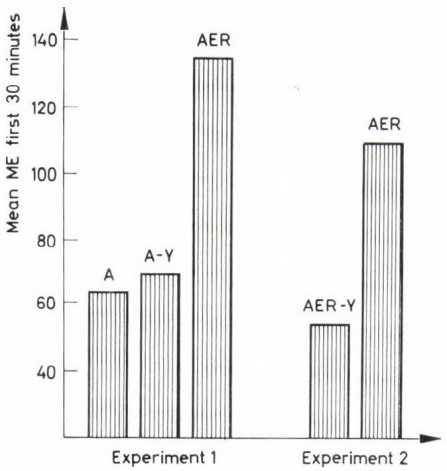

Fig. 1. Mean number of magazine entries made by experimental and control groups in the first two experiments (Dyal et al., 1967).

Furthermore, I believe that a close examination of the 60 papers which have obtained positive results and the 30 papers which have reported negative results will convince an unbiassed reader that transfer of behavioural bias through injection of substances from the brains of trained animals is a *reliable*, *real*, *valid* phenomenon.

Assuming the validity of the phenomenon, two other general questions present themselves: (*i*) the question of specificity – how is the transfer of behavioural bias related to the training of the donors? (*ii*) the question of chemical basis – what is the chemical nature of the active transfer agent? We have already heard some stimulating research reported by Professor Ungar on the latter topic. I want to devote the remainder of my paper to an analysis of the problem of specificity and a critique of those experiments which have attempted to demonstrate specificity.

There are of course many different referents for the term "specificity" in the several languages which are relevant to the biology of memory. Previous papers in this symposium have referred to "system specificity", "molecular specificity", "information specificity" and several other unspecified specificities. I want to discuss the problems associated with yet another kind of specificity namely "behavioural specificity". By behavioural specificity I mean simply that the behaviour of the recipients in a transfer of behavioural bias experiment is dependent upon the *training* given the donors. In order to show that the behaviour is dependent upon the training of the donors we must use appropriate control procedures to eliminate motivational factors as reasonable alternative interpretations. The appropriateness of motivational interpretations may be evaluated by testing the recipients in test situations that permit measurement of activity level. Several investigators have done this and have rather consistently failed to find that the obtained transfer effects could be due to differential activation. Perhaps a preferable approach to this problem is to circumvent it altogether by using a transfer task which is known to be uneffected by motivational variables (e.g. a discrimination task).

You will note that I have preferred to use the term transfer of "behavioural bias" rather than transfer of learning or memory. Transfer of behavioural bias may turn out to be quite dependent on the variables which are well known to influence learning such as CS-UCS interval in Pavlovian conditioning and aspects of the response-reinforcement contingencies in instrumental conditioning. If such dependency is demonstrated then it seems appropriate to designate the effect as a transfer of learning or memory. On the other hand, such a high degree of informational complexity may not be involved and the transfer may still be specific to a sensory modality or a response system and may be the result of passively driving the system. If repeated stimulation results in an increased responsivity to the stimulus it is often referred to as stimulus sensitization. It may also be called pseudoconditioning in that the increased performance is not dependent upon a contingency between the CS and the UCS or between the response and a reinforcing event. As recently pointed out by Rescorla (1967) the most appropriate control group for sensitization and pseudoconditioning in a Pavlovian conditioning is a random presentation of the CS and the UCS. Other less adequate but usable controls are "CS only" groups or "UCS only" groups. In instrumental conditioning the appropriate control is one in which the contingency between the discriminative stimulus and reinforcement is disrupted as in yoked control procedures.

Another effect which should be differentiated from transfer of a specific

behavioural bias is the transfer of response potentiation. I will use this term to refer to the case in which an injection of a substance selectively potentiates responses of one type but not of another. As an example of the problems of interpretation created by response potentiation let me remind you that Cook et al. (1963) had appeared to have demonstrated enhancement of learning in a pole climbing avoidance task as a result of injections of yeast-RNA. However, later research by Wagner et al. (1966) demonstrated that the facilitation was a result of the yeast-RNA potentiating the pole climbing response in the experimental group. The control group on the other hand never made the response and thus it could not be reinforced. The appropriate controls for non-specific response potentiation would be sensitization and motor activity control groups.

It should be noted that stimulus sensitization, pseudoconditioning and response potentiation may all be dependent on donor training but not upon the contingency between CS and UCS or between a response and a reinforcer. It is possible that these forms of behaviour bias may involve different physiological and biochemical mechanisms from those involved in classical and instrumental conditioning. Whether or not a reinforcing event at the behavioural level is relevant at the neurophysiological and biochemical level is an empirical question which can be answered only if we maintain the conceptual distinction long enough to determine if it is empirically meaningful and theoretically useful.

Assuming that the transfer of learning is a valid phenomenon we may distinguish three types of transfer of conditioning:

(1) *Conditioned Stimulus Bias.* In this case the performance difference between the experimental and control groups is due to an increased sensitivity of the donor to the critical stimuli and that this increased sensitivity is a direct result of the reinforced training given the donors. A basic design from which to infer conditioned stimulus bias is one in which two groups of donors are conditioned; one is conditioned to make response 1 to stimulus 1 and the other to make response 1 to stimulus 2. The recipients are then tested by having both S1 and S2 presented and the frequency of response to each stimulus is measured (Fig. 2). In order to infer that the transfer represents true conditioned stimulus bias, it is necessary to run both a stimulus sensitization control group and an untrained-control group. If the experimental group shows a greater effect than the sensitization group then conditioned stimulus specificity may be inferred. However, if the sensitiza-

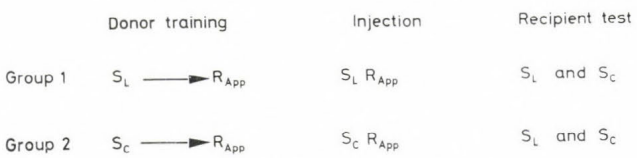

Fig. 2. The Jacobson stimulus bias paradigm. The recipient test was a non-reinforced test of the number of approaches to S_L and S_C by the recipients of each extract (Jacobson et al., 1965).

tion group is also greater than the untrained group we know that stimulus sensitization has *also* been transferred.

(2) *Conditioned Response Bias.* Conceptionally similar to conditioned stimulus bias is conditioned response bias. The design necessary to isolate the possible transfer of a response bias requires that two groups be trained to make quite different responses in the same stimulus situation. The recipients are then tested with both responses available and the relative frequency with which each response is made is measured. If the R_1 injected animals make more R_1 than the R_2, injected animals and similarly the R_2 injected animals make more R_1 responses, and if they also differ from a response potentiation control group then a sound basis is provided for the inference of transfer of response bias.

(3) *Conditioned S-R bias.* It is of course possible that memory transfer involves not only the coding of information on both the input or/and output sides, but it may preserve the relationship between the stimulus complex and a particular reinforced response. This would be the most complex and the most specific of the alternatives. Whether or not S-R specificity is able to be demonstrated it does not seem unreasonable that the transfer of learning effects could be specific to the behaviour biases of the first and second types. I would tend to agree with Booth who, in an excellent article on vertebrate brain RNA and memory, has commented as follows: "Avoiding incoherent conceptualizations, it is difficult to imagine a more direct mechanism for chemical transfer of behavioural information than the relatively specific biassing of the recipient towards taking in or putting out certain sorts of information . . . testing recipients without reinforcement can demonstrate changes . . . in sensory preference or motor differentiation." (Booth, 1967).

It is one thing to admit the possibility transfer of some specific stimulus or response bias and quite another thing to demonstrate it. Now let us examine those experiments which have been offered as evidence for specific transfer of behavioural bias. One of the first papers to deal with the problem was reported by Jacobson et al. (1965). The paradigm is represented in Fig. 2. They trained two groups of rats to approach a food cup; one to a discriminative "click" stimulus and one to a discriminative "light" stimulus. One group of recipients was injected with the "click" brain extract and another with the "light" extract. Both groups were then tested by determining the number of cup responses made to 25 click stimuli and 25 light stimuli randomly presented. From Table 1 it may be seen that the click injected group responded to the click more than the light and the light injected group to the light more than the click.

These results appear to be a good demonstration of conditioned stimulus specificity, but are they? The answer is no. The reason is that the experiment does not contain a control for stimulus sensitization.

There have been *two* attempts to replicate these results, one by Halas et al. (1966) who included untrained controls but no sensitization controls. They obtained no differences between any of the experimental or control groups in number of responses to the click stimulus; unfortunately they tested with the click stimulus only and thus we do not have the same test situation as

TABLE 1

Total number of responses per animal on the 25 test trials with click and on the 25 test trials with light*

Stimulus		Score (C-L)
Click	Light	
	Injection with RNA-C	
2	3	−1
3	4	−1
5	2	3
5	2	3
6	1	5
7	1	6
7	0	7
11	2	9
	Injection with RNA-L	
0	7	−7
0	7	−7
0	3	−3
1	3	−2
0	2	−2
0	2	−2
0	1	−1
7	5	2

* Jacobson et al. (1965).

used by Jacobson whether or not this made a difference in the results is unknown but experiments by Grice and Hunter (1964) suggests that it could have. de Balbian Verster and Tapp (1967) also repeated the Jacobson experiment and found that both the click injected group and the light injected group made significantly more responses to the click than to the light. They thus concluded that there was no differential transfer. It would appear that Jacobson's demonstrations of specificity cannot be accepted until further replications are conducted. Also since the rats appear to be biassed to prefer the click it may not be an ideal test situation for specificity.

Using what he calls a cross-transfer design, Ungar has reported data which he interprets as demonstrating that transfer of habituation of a startle response is specific to the stimulus used to habituate the donors. The design of the experiment is represented in Fig. 3. In one group of mice the startle response was habituated to an air puff; a second group was habituated to a loud noise. The recipients were injected with either air puff or sound habituated brain extract. Half of each injection group was then habituated to the sound and half to the tone. As you can see from Fig. 4, habituation of the recipients was greatly facilitated by injection of brain homogenates from the donors who were trained to the same stimulus.

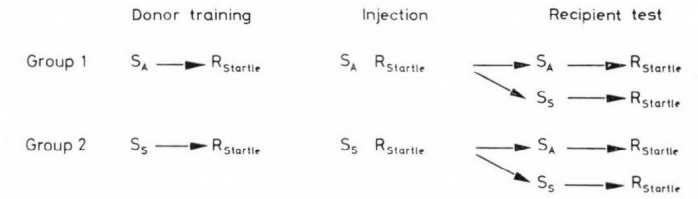

Fig. 3. The Ungar cross-transfer of habituation paradigm (Ungar, 1966).

There are several questions which must be raised in deciding whether or not stimulus specific transfer of habituation is a valid phenomenon. First, and foremost, is the question of replicability. To my knowledge no one has attempted to replicate the effect. I would urge that this important experiment be repeated several times in every laboratory interested in the problem of specificity of transfer. Second, even if the results of the Ungar experiment prove to be highly replicable, the experimental design does not permit a strong statement about the degree of specificity involved. Ungar chose two stimuli which were quite different along many relevant dimensions. It is thus surely not a very compelling argument for a high degree of specificity that habituation in the recipients was not facilitated by injection of the cross-modal extract. A stronger degree of specificity would be demonstrated by testing within a single dimension. Third, if you will re-examine

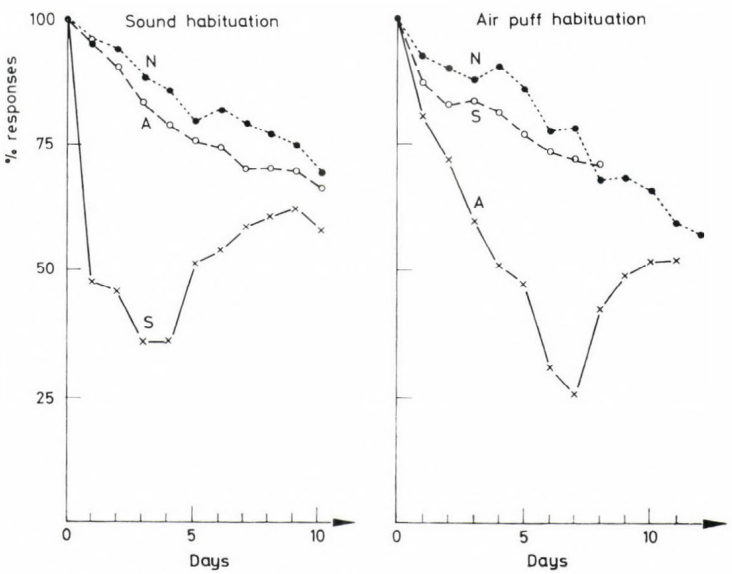

Fig. 4. Rate of habituation by recipients as a function of type of injection.

Ungar's data you will note that both the "air-puff-injected, sound habituated" group and the "sound-injected, air-puff habituated" group habituated faster than the groups injected with untrained brains. If this aspect of the results proves to be real then we would appear to have both general and specific effects being transferred. The non-specific effects could reflect either a transfer of stimulus generalization or a transfer of conditioned response bias. Fourth, if stimulus specific habituation proves to be a replicable phenomenon in this situation, we must ask if it is peculiar to this particular stimulus–response system. It could be that habituation transfer doesn't hold for other response systems. If this seems implausible let me remind you again that the learning enhancement which Cook et al. (1963) demonstrated to result from chronic injection of yeast-RNA turned out to be dependent upon the type of task which was used (Wagner et al., 1966).

In the light of the foregoing considerations, I believe that we must conclude that Ungar's experiment represents an important line of research which must be vigorously pursued; however, the stimulus specificity of transfer of habituation processes is not yet established.

Ungar (1969) has used the cross-transfer design to test for specificity in two passive avoidance tasks. The two tasks were the dark-box avoidance task of Gay and Raphelson (1967) and the step-down platform. As prescribed by the design (Fig. 5), half of the mice which were injected with dark-box extract were tested in the dark-box and half were tested in the step-down box. The step-down injected recipients were similarly split. The results may be seen in Fig. 6. It is apparent that significant positive transfer is obtained when the recipients are tested in the situation corresponding to their injections but little or no transfer when they were tested in the other situation. Professor Ungar concludes he has again demonstrated specificity of the transfer. I would agree but I would reiterate and elaborate my contention that the cross-transfer design is a weak and ambiguous demonstration of specificity. In any learning situation there is an extensive set of stimulus elements which are conditioned to an equally extensive set of response elements. If no transfer is observed when recipients are tested in a stimulus situation which is quite unlike the donor's training situation and which requires a different kind of response, the most one can conclude is that there is at least one behaviour in one situation which is not activated

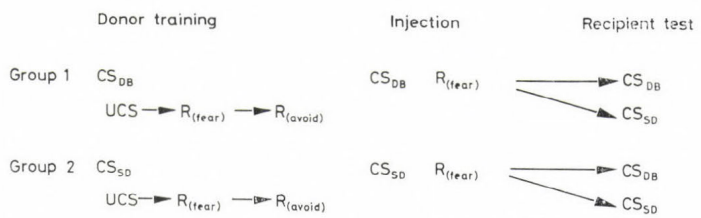

Fig. 5. The Ungar cross-transfer of conditioned fear paradigm. The recipient test was a non-reinforced avoidance in either the "dark-box" or "step-down" situation (Ungar, 1969).

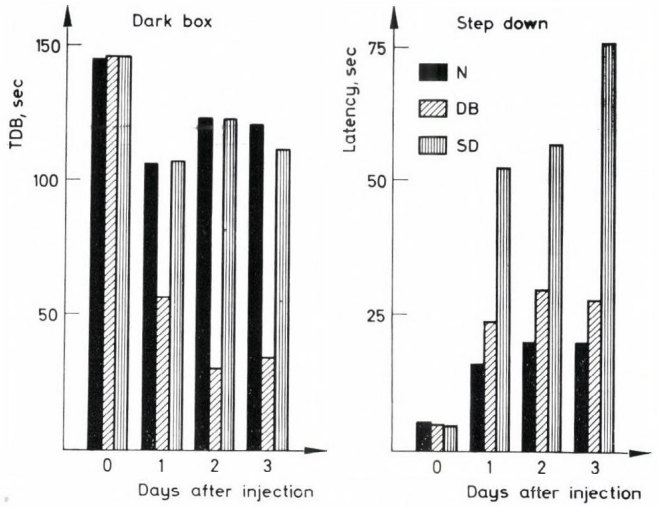

Fig. 6. The results of Ungar's cross-transfer of passive avoidance.

by the transfer agent. Hardly an earth shaking inference! In other words, the strength of the argument for specificity of transfer increases as the number of stimulus and response elements increase between the training and the test situation. The strongest test of specificity then is in a test situation only slightly different from that of the donors. The test must involve controlled manipulation of the degree of similarity between the two situations. Again, the strongest test for specificity is within a single stimulus or response dimension, i.e., specificity must be tested by means of generalization gradients. For example, the stimulus specificity of a conditioned fear of the dark-box could be tested by varying the brightness of the dark side from black to light grey.

The design recommended by Rosenblatt and Miller (1966*a*) as a preferred behavioural assay technique is represented in Fig. 7. As you can see, it is

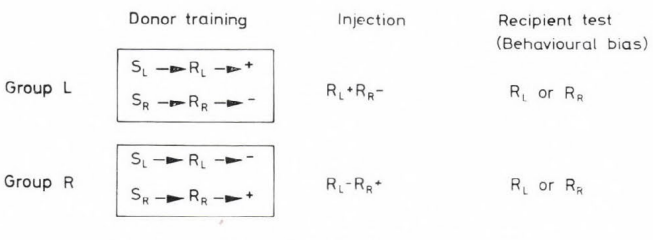

Fig. 7. The Rosenblatt and Miller transfer of discrimination paradigm. The behavioural bias tests were conducted with either both responses reinforced or both responses non-reinforced (Rosenblatt and Miller, 1966*a*, *b*).

a between groups design based on a comparison of discrimination scores for the left injected group and the right injected group. They have used four basic discrimination tasks and tested with non-reinforced tests. They made 21 independent tests of various extraction procedures, dosage, and injection sites. When one considers the total scores for all test sessions we find that only 3 of the 21 experiments yielded significant positive transfer; and in 8 of the 21 the discrimination scores were in the negative direction. However, when the data from all 21 experiments were pooled and a U-test was applied, it was found that overall the left injected animals made more left responses than the right injected animals with a one tailed probability of 0·021.* Rosenblatt and Miller also present data on the 'best' session of the six test sessions and as one would expect from such data selection the p values are higher but even then only four of the 21 comparisons show significant positive transfer and 6 of the 21 comparisons show substantial (but not quite significant) negative transfer.

In another series of symmetrical choice tasks Rosenblatt and Miller (1966*b*) found that a Y-maze tends to give negative transfer with low dosages while the two-bar box tends to give positive transfer. However, in the two-bar box only one of six independent comparisons over all test sessions was significant. What may we conclude regarding specificity from Rosenblatt's research? First, it should be noted that in none of these discrimination experiments has a non-associative control group been trained. Thus it is not possible to determine if the obtained effects are properly thought of as stimulus sensitization, response potentiation or a specific transfer of behavioural bias. Nonetheless, the discrimination task seems to offer a powerful test of a specific behavioural bias. However, again the question of replicability haunts us; undoubtedly, Rosenblatt has repeated the 2 or 3 procedures from the Rosenblatt and Miller series which looked promising but the results of such systematic efforts to establish one of these as a reliable behaviour assay have not been forthcoming. Perhaps we will hear more about that shortly. Nonetheless, on the basis of the work thus far published, we must characterize the Rosenblatt and Miller series as promising but not conclusive demonstrations of specific behavioural bias.**

Golub and I have used the extension of the discrimination design represented in Fig. 8 to test for transfer of response specificity. We used two responses which were morphologically quite different from each other. As you can see Group 1 was rewarded for pressing the bar (BP) but not for magazine entry (ME) whereas Group 2 was rewarded for magazine entry

* For the one-tailed probability an alpha value of 0·025 would normally be required to reject the null hypothesis. Since many researchers in this area have proposed that there is a phenomenon of negative transfer, all statistical decisions should be based on *two*-tailed probability values of 0·05 or one-tailed values of 0·025 or 0·975.

** The new research presented by Rosenblatt at this symposium represents a quite impressive extension of his previous program and at this juncture constitutes the most convincing demonstration of behavioural specificity thus far available. It is to be hoped that this research paradigm and behavioural task will be pursued in other laboratories since it seems to offer the most promise for a repeatable behavioural assay technique.

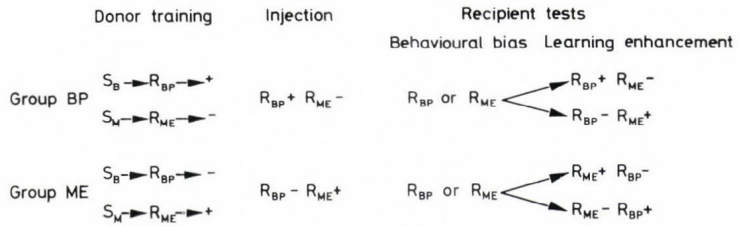

Fig. 8. The Dyal and Golub transfer of response specificity paradigm. The behavioural bias tests were conducted under non-reinforced conditions while in the learning enhancement test one *or* the other (BP or ME) response was reinforced (Dyal and Golub, 1969).

(ME) but not for bar pressing (BP). The magazine entry response was that of sticking the nose into what would normally have been the food cup. Food pellets were delivered into another part of the box. The logic of the design required two test periods. The first test period is a non-rewarded free choice situation in which we measured the number of BP and ME made in a 30 min test session, with the secondary reinforcer of "light-click" (see Dyal et al., 1967) presented automatically every 60 sec. The second test consisted of five sessions of reinforced training on either the bar press or the magazine entry. In the first test, if the transfer results in a behavioural bias which is response specific, then the recipients which received homogenate from donors trained to bar press should exhibit a greater increase in bar presses over baseline than those which were injected with homogenate from magazine trained donors. Similarly those recipients injected with magazine homogenate should increase their magazine entries more than the bar press injected recipients. The results only partially confirm the transfer of response bias hypothesis. Those recipients which were injected with bar press homogenate increased their bar presses over baseline, while those which we injected with magazine entry homogenate decreased their bar pressing (Table 2). On the other hand, the magazine injected recipients showed no tendency to increase their magazine entries, in fact, as you can see in Table 3, the post-injection score was somewhat lower than their pre-

TABLE 2

Mean number of BP during 30 min perference test (Dyal et al., 1969)

Injection received			
BP		ME	
PRE	POST	PRE	POST
7·45	10·15	8·87	6·29

TABLE 3

Mean number of ME during 30 min preference test (Dyal et al., 1969)

Injection received			
BP		ME	
PRE	POST	PRE	POST
57·8	58·5	55·4	46·2

injection score. The bar press injected recipients did not change their ME level on the post-injection preference test.

In the second test, we would expect that if there is an enhancement of learning, the animals injected with homogenates from donors trained in the same way (BP-BP and ME-ME) should learn faster than the comparable groups which were trained opposite their injection (ME-BP and BP-ME). Considering first those recipients who were reinforced for bar pressing we found that those subjects which were injected with homogenate from bar press donors made significantly more bar presses than those which were injected with magazine homogenate in the first reinforced session. This result, taken with the preference test data, suggests that the transfer is specific to the particular response which has been trained.

However, this conclusion is somewhat weakened by the fact that, as in the case of the preference data, the magazine injected recipients showed no increase in magazine entries. Furthermore, even the apparent specificity exhibited by the bar press injected recipients is called into question by the fact that, during the second 30 min test, this group made more ME responses than the magazine injected recipients. It could thus be argued that all of the differences are due to a general activation by the bar press homogenate which for some reason did not occur for the magazine homogenate. Although this interpretation cannot be completely refuted, there are some correlational data which argue against the transfer effect being primarily due to activation; nonetheless the case is not a strong one. Furthermore, since none of the other test sessions revealed significant effects, we feel that response specificity has not been demonstrated clearly by this experiment.

Attempts to establish stimulus specificity of transfer in a brightness discrimination have been made by Jacobson's laboratory (Jacobson et al., 1966); however, his statistical analysis has been criticized (Barber, 1966; Worthington and MacMillan, 1966), furthermore, Hoffman et al. (1967) have failed to replicate the results. Experiments by Kimble and Kimble (1966) and by Golub and myself (Dyal and Golub, 1968) have also failed to obtain evidence for a stimulus specific transfer of a brightness discrimination. Similarly, Allen et al. (1969) have failed to find evidence to support specific stimulus in a pattern discrimination.

Essman and Lehrer (1967) used a discrimination design similar to that of Rosenblatt and Miller except they used a learning enhancement test rather

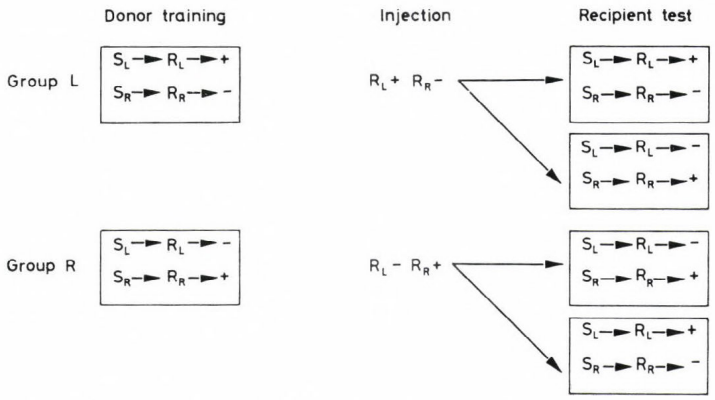

Fig. 9. The Essman and Lehrer response specificity paradigm. The recipient test involves reinforced discrimination learning with straight transfer and cross-transfer groups. Four control groups were also trained: non-discriminative motor training; untrained controls; yeast-RNA, and normal saline (Essman and Lehrer, 1967).

than a behavioural bias test. Their experiment is, in my mind, the best controlled of all the experiments which have been conducted in memory transfer research. They trained mice to choose either the left side or the right side of a single choice water maze. Of the four experimental groups of recipients, two were injected with right trained RNA extracts and two with left trained extracts; half of each of these injected groups were four control groups; one was injected with extracts from donors given non-discriminative motor training, one was injected with untrained brains, one injected with yeast RNA and one with normal saline. There were no significant differences among any of the control groups in trials to a learning criterion. All experimental groups combined made fewer errors than all control groups combined indicating the presence of at least a general enhancement of learning effect. Comparisons among the experimental groups supported the proposition that the enhancement was specific to the responses which were trained in the donors, e.g., left-trained recipients given extracts from left-trained donors made fewer errors than right-trained recipients of the same extracts.

Now let me summarize the implications of this paper as follows:

(1) The total evidence available thus far strongly supports the contention that through injection of brain extracts it is possible to modify the behaviour of a recipient animal in a way that appears to be related to the training of the donors.

(2) That this transfer effect is dependent upon and specific to the *learning* of the donors has not yet been clearly demonstrated.

(3) As may be seen in Fig. 10, the most powerful paradigm to use to investigate specificity is one based of discrimination learning in the donors; which includes pretraining preference tests of the donors; preinjection pref-

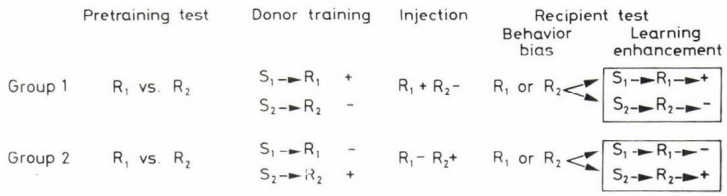

Fig. 10. A proposed paradigm for testing specificity of transfer of behavioural bias. By making the pre-training test of the donors identical to the behavioural bias test which the recipients will receive, it would be possible to use a saving score as a measure of transfer in the learning enhancement test phase.

erence tests for the recipients; a post injection behavioural bias test (non-reinforced or reinforced) plus a series of learning enhancement tests for the recipients. The basic score would be a pre vs. post injection discriminating score (%Lpre-%Lpost) i.e., a discrimination score based on savings. These change scores would then be used to compare left-injected recipients with right-injected recipients. Motor-trained control, untrained control groups should be run to determine if differences in the experimental groups are due to transfer of approach responses to the positive stimulus or transfer of avoidance responses to the negative stimulus.

I want to conclude with a quote from George Ungar as follows: "The concept of chemical transfer of learning is still certainly in need of bigger and better experiments to gain general acceptance. But, I believe acceptance will come as the many variables which influence the effect become better known and controlled. In the meantime, the only reasonable and truly 'scientific' attitude is a skeptical but open mind. There have been too many precedents in the history of science of 'absurd' ideas becoming ultimately the universally recognized truth." (Ungar, 1969.) Through the continued collaborative efforts of biochemists, biologists and psychologists, it may be that the memory transfer experiments may become one of the major avenues of the researcher who would continue the quest for the holy engram.

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DISCUSSION

W. Essman: The data cited from experiments by Essman and Lehrer were interpreted by us as a non-specific facilitation rather than one of specificity. "Motor training" extracts given to naive recipients gave a comparable degree of facilitation of maze acquisition, compared to mice given "untrained" extracts. When liver extracts were given, the same results were obtained as occurred with brain extracts; i.e. reduced maze errors after "trained" extracts as compared to "untrained" extracts. We also saw that yeast RNA, brain RNA and liver RNA led to increases in plasma uric acid, which alone could account for facilitation without resulting in altered locomotor activity.

J. A. Dyal: It was the most recent paper by Essman and Lehrer (1967) entitled "Facilitation of Maze Performance by 'RNA extracts' from maze trained rats" to which I referred. The concluding sentence of the abstract of that paper says: "The data suggest that 'RNA extracts' from the brains of donor mice trained to a specific task facilitate learning by naive animals on that task." Furthermore, the data are quite consistent with that interpretation since "left-trained recipients given extracts from left-trained donors showed more criterion errorless performance than right-trained recipients given the same extracts ($\chi^2 = 13{\cdot}08$; $p < 0{\cdot}001$) and right-trained recipients of extracts from right-trained donors showed greater errorless performance than left-trained recipients of the same extracts ($\chi^2 = 5{\cdot}28$; $p < 0{\cdot}05$)". In this experiment no difference was obtained between motor-trained and untrained recipients but the . . ."Recipients from trained donors showed greater errorless performance as compared with recipients of extracts from motor-trained or untrained donors". In this experiment you did not give injections of liver extracts; the experiment can stand on its own and its results are certainly quite compatible with a specific facilitation interpretation. When you refer to getting the same results with liver RNA as brain RNA, you are undoubtedly referring to the first study (1966) in which the data showed that there were fewer errors for trained and motor-trained groups than for untrained groups in the case of both brain extracts and liver extracts. Now I would contend that the reliability of the transfer effect based on liver extracts is quite questionable at this point since other investigators, who have been able to obtain rather consistent transfer effects with brain extracts have obtained no effects with liver extracts (Ungar, personal communication and Reinis, S. *Activ. Nerv. Super.* **7**, 167, 1965).

W. C. Corning: Of the many "transfer" experiments that have been run, how many have shown positive effects, zero effects, and effects in the opposite direction? If the transfer phenomenon were a random event, then we should expect to observe negative transfer as much as positive transfer.

J. A. Dyal: I do not have a frequency count on the number of experiments which have shown significant positive transfer effects and significant negative transfer effects. But having reviewed, quite recently, all of the research which is summarized in the bibliography it is clear to me that the number of significant negative effects does not come even close to the number of

significant positive effects. While the question which you raise is an extremely important one, and one which has given me some concern, I find no evidence in the published or unpublished literature to make me think that we are indeed sampling from a random set of events.

It should be noted furthermore that Rosenblatt has pointed to variables such as dosage level and type of training situation, which tend to increase the likelihood of a significant reversed transfer effect. At this point I would say that we must consider that reversed transfer may be a real effect and attempt to isolate the variables controlling it.

W. C. CORNING: In how many cases do we go back and throw out a study that did not turn out right and continue to collect data until we get the right results? We can always go back and find reasons for a negative result but how often do we look for spurious factors that might cause positive results.

J. A. DYAL: This point also is an extremely good one and one which I believe can be answered by reference to the bibliography which I have provided. You will note that it is divided into published and unpublished studies both positive and negative. Having been in the "transfer game" for several years now, I felt that I knew of most (but undoubtedly not all) of the investigators, who had done work in the area. I wrote letters to all of these people, making essentially the same point that you made and asking them to be especially careful to report to me any unpublished negative studies. Although I have not received a complete return, I would expect that about 90 per cent of the experiments which have ever been conducted on memory transfer are included in the bibliography. If this is correct, then I see no reason to believe that there is a substantial or differential number of unpublished reports which have obtained negative results. On the contrary, I believe that the researchers in this area have been quite sensitive to this problem and have made unusual efforts to let all of their research become known by colleagues.

G. HORN: You referred to Rosenblatt's experiments in which he obtained 3 significantly positive results out of 21 experiments. This is not very much higher than a chance level of success.

J. A. DYAL: Yes, this is true, but should not be taken as a serious criticism for two reasons: (*i*) In this set of experiments Rosenblatt was exploring a variety of parameters to see which ones might give an effect. The results thus should not be viewed in the same light as if they had been 21 experiments using a single procedure for which there was strong reason to believe positive results should be obtained. (*ii*) Dispite the fact that only a small proportion of the experiments yielded statistically significant effects the general tendency of the set was in the positive direction and the combined probability over all experiments was significant.

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Note. The bibliography referred to by Dyal was not reproduced because of space limitations. The interested reader may obtain the bibliography by writing directly to the author.

MEMBRANE SPECIFICITY AND MEMORY TRANSFER. THE FATE OF ^{3}H-LEUCINE-LABELLED HOMOGENATES INJECTED INTRAPERITONEALLY INTO RATS*

by

E. F. Stillwell,[a] R. J. Porter[b] and W. L. Byrne[c]

Department of Biochemistry, Duke University, Durham, North Carolina, U.S.A.

In an attempt to understand the behavioural modifications resulting from the injection of brain material from trained donors into naive animals (Byrne, 1969; Quarton, 1967), so called "memory transfer", a series of experiments were initiated using isotopically labelled homogenates of specific organs and in one instance the olfactory lobe was injected separately from the rest of the brain. ^{3}H-leucine was selected to label the donor animals since there is increasing recognition of the importance of proteins in membrane structure (Korn, 1966; Green et al., 1968) and the ability of non-identical subunits to interact selectively in a complex environment (Gerhart and Schachman, 1965). For those components which are specific to a given organ it seemed possible that following injection they might preferentially localize in the corresponding organ or region of an organ. This preferential localization is a necessary but not sufficient corollary of a proposed explanation for the "memory transfer" phenomenon (Byrne, 1969; Byrne and Hughes, 1967). Previously, Ebert (1954) clearly showed selective transfer of radioactivity from adult chicken spleen and kidney grafts, using labelled ^{35}S-methionine, into the corresponding tissues of host chick embryos. Further, Walter et al. (1956) showed a preferential localization of injected adult liver and heart homogenates into the homologous tissues of embryonic chicks. It was suggested that these effects might be due to the transfer of specific protein moieties or specific components of protein molecules larger than amino acids (Mahler et al., 1956).

The present report deals with the incorporation of injected ^{3}H-leucine-labelled homogenates of adult rat brain, lung, liver, and kidney into the organ counterparts of recipient rats. A comparison was also made of the amount and specificity of localization of homogenates which were heated in a boiling water bath prior to injection. A separate experiment involved injection of ^{3}H-leucine labelled donor homogenates of olfactory lobe, brain

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[a] Present address: Department of Biology, Old Dominion College, Norfolk, Virginia.

[b] Present address: University Hospital of San Diego County, University of California School of Medicine, San Diego, California 92103.

[c] Present address: Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38103.

(without the olfactory lobe), liver, and kidney into recipients which also were injected with unlabelled leucine.

Prior to injection into the donor rats, ^{3}H-leucine was diluted in 16 per cent gelatin to give a more uniform release over a period of hours (Kostyo, personal communication). Seven grams of gelatin (Knox No. 1) were dissolved in hot distilled water, final volume 44 ml, and 0·32 g NaCl added to achieve isotonicity. The 4·5 ^{3}H-leucine was purchased from New England Nuclear Copr. in 0·01 N HCl and 2·5 ml (2·5 mc) were mixed with 9·5 ml of the gelatin. This solution was stored at 3 °C and liquified in a warm water bath at 45 °C when needed for injection. The donor rats (male, Holtzman, wt. 174–182 g) were kept in individual cages in the fume hood, with food and water *ad libitum*, and 0·6 ml of the leucine-gelatin solution was injected subcutaneously at 12-hr intervals for a period of three days in the region above the right scapula with a disposable 1 cc Tomac syringe and a No. 22 G needle. The last injection was given 12 hours before they were sacrificed by decapitation and the brains, lungs, livers, and kidneys removed. The brain included olfactory lobe, stem and cerebellum. The pooled organs from three rats were weighed and cold 0·25 M sucrose added equal to twice the pooled organ weight (1 g per 3 ml). The organs were thoroughly homogenized in glass Potter-Elvehjem homogenizers with motor-drive pestles. For the "boiled" homogenates 6·4 ml of each homogenate were placed in a boiling water bath for 10 min in the glass homogenizer tube. Following the heat treatment, the "boiled" homogenates had to be redispersed by homogenization. Aliquots of the homogenates were frozen and stored for counting. The average incorporation of leucine for one set of donor animals is given in Table 1.

TABLE 1

Incorporation of ^{3}H-leucine into donor organs following subcutaneous injection over a period of 72 hours

Organ	Average weight	DPM per gram	Per cent uptake of injected leucine*/organ
Brain	1·6 g	1,310,000	0·12
Liver	9·8 g	1,850,000	1·00
Kidney	2·7 g	2,120,000	0·32
Lung	2·0 g	1,210,000	0·13

* 0·83 mc ($1{\cdot}8 \times 10^{9}$ DPM) injected.

The recipient rats (male, Holtzman, 170–180 g) were injected intraperitoneally with 1·8 ml of homogenate using a disposable 5 ml syringe and a No. 19 gauge needle. They were sacrificed 18 hours after injection by decapitation. The organs from the recipients were removed, weighed and processed individually. They were homogenized as described above and stored at −15 °C. Variations from this procedure, if used, are specified.

TABLE 2

Incorporation of radioactivity into recipient organs 18 hours after intraperitoneal injection of ^{3}H-labelled donor homogenates

Type of homogenate and DPM injected (1·8 ml)	Organ counted	Average weight	Average DPM per g	Per cent uptake of injected homogenate per organ
Liver	Brain	1·8	3 560	0·57
1 110 000 DPM	Liver	8·1	8 040	6·06
(n = 3)	Kidney	1·5	7 370	1·01
	Lung	1·3	4 980	0·55
Kidney	Brain	2·1	3 790	0·58
1 272 000 DPM	Liver	7·2	8 700	4·89
(n = 3)	Kidney	1·4	13 040	1·43
	Lung	0·9	5 840	0·39
Brain	Brain	2·0	2 575	0·64
788 000 DPM	Liver	7·7	7 830	5·10
(n = 3)	Kidney	1·7	4 580	0·98
	Lung	1·2	3 520	0·53
Lung	Brain	1·9	2 430	0·63
726 000 DPM	Liver	7·8	6 200	6·63
(n = 3)	Kidney	1·6	4 980	1·10
	Lung	1·2	5 810	0·96
Boiled Liver	Brain	1·8	1 310	0·20
1 110 000 DPM	Liver	7·9	5 880	4·16
(n = 3)	Kidney	1·6	2 180	0·31
	Lung	1·2	1 420	0·15
Boiled Kidney	Brain	2·2	1 870	0·31
1 272 000 DPM	Liver	7·8	12 030	7·34
(n = 3)	Kidney	1·5	6 640	0·78
	Lung	1·0	2 730	0·21
Boiled Brain	Brain	1·8	1 890	0·42
788 000 DPM	Liver	7·6	7 230	6·93
(n = 3)	Kidney	1·5	5 140	0·97
	Lung	2·1	2 650	0·70
Boiled Lung	Brain	1·8	880	0·21
726 000 DPM	Liver	7·8	4 080	4·34
(n = 3)	Kidney	1·5	2 240	0·46
	Lung	1·2	1 440	0·23

For counting, duplicate 0·1 ml aliquots (33 mg tissue) of each homogenate were added to 2·0 ml of Nuclear Chicago solubilizer in glass scintillation vials (20 ml) and shaken overnight at room temperature. To this was added 10 ml PPO-POPOP cocktail (0·5 per cent PPO, 0·01 per cent POPOP per 2 litres toluene). Samples were counted in a Beckman Liquid Scintillation Counter, model LS-150, at a maximum of 5 per cent error with the variable discriminator module using a 40–200 setting. This setting was used to eliminate chemiluminescence. Sample counts were corrected for background and quench by the external standard channels ratio method, and activity was expressed as disintegrations per minute per gram tissue (wet wt.).

The results of injecting ^{3}H-leucine-labelled homogenates are given in Table 2 and Table 3 expresses these same results in terms of the incorporation into a specific organ relative to the incorporation in the liver of the same animal. The relative specific activity in Table 3 is given as a mean along with the standard deviation.

TABLE 3

Relative specific activity in recipient organs 18 hour after injection with labelled tissue homogenates (each figure represents the mean and standard deviation)

Relative specific activity	Injection			
	Liver	Kidney	Lung	Brain
DPM per g/DPM per g				
Liver/Liver	1·0	1·0	1·0	1·0
Kidney/Liver	0·99 ± 0·30	1·72 ± 0·87 *($p < 0·005$)	0·81 ± 0·09	0·88 ± 0·09
Lung/Liver	0·66 ± 0·20	0·76 ± 0·28	0·94 ± 0·13 ($0·005 < p < 0·01$)	0·63 ± 0·11
Brain/Liver	0·48 ± 0·17	0·41 ± 0·13	0·40 ± 0·07	0·48 ± 0·08 (N.S.)
	Boiled Liver	Boiled Kidney	Boiled Lung	Boiled Brain
Liver/Liver	1·0	1·0	1·0	1·0
Kidney/Liver	0·35 ± 0·10	0·55 ± 0·07 (N. S.)	0·53 ± 0·21	0·68 ± 0·14
Lung/Liver	0·26 ± 0·05	0·21 ± 0·01	0·36 ± 0·14 (N. S.)	0·38 ± 0·14
Brain/Liver	0·24 ± 0·03	0·13 ± 0·04	0·21 ± 0·10	0·25 ± 0·10 (N. S.)

* p is a probability value expressing the significance of the incorporation of homogenate into homologous tissue compared with the three non-homologous tissues.
N. S. means no significant difference.

Significant quantities of the radioactivity were present 18 hours after the injection and the per cent uptake per organ for the homogenates (Table 2) was several fold greater than the per cent uptake of the free amino acid into the donors (Table 1). The "boiled" brain homogenate showed a higher incorporation into liver and lung, but a more typical result was a marked decrease in incorporation into brain, lung and kidney and approximately the same incorporation into liver. The results in Table 3 were expressed as activity ratios in an effort to correct for inherent varations in uptake by individual animals. The data were subjected to an analysis of variance to obtain levels of confidence. The relative specific activity of kidney was highest ($p < 0{\cdot}005$) when kidney homogenate was injected, and the relative specific activity of lung was highest ($p < 0{\cdot}01$) when lung homogenate was injected. However, brain showed no significantly higher relative specific activity when brain homogenate was injected. In this experiment, therefore, preferential localization of labelled homogenates into the organs of origin has been demonstrated with kidney and lung but not with brain.* The results in Table 3 indicate that the specificity for kidney and lung was destroyed by the heat treatment and this lability to temperature is suggestive of a preferential localization which would depend upon a protein–protein interaction. The sensitivity to heating of the individual components in the homogenates is, of course, unknown but it is interesting to note that for the "boiled" homogenate, the highest per cent uptake into brain (Table 2) occurred with the injection of "boiled" brain homogenate. This apparent specificity is not significant, however, if the results are expressed as an incorporation relative to liver (Table 3).

The lack of specific localization for the brain homogenate was not surprising in view of the blood brain barrier, but there was a sizeable incorporation of radioactivity. The next experiment was therefore designed to test for relative specificity within the brain and was also designed to minimize the labelling due to the utilization of any leucine released from the homogenate by enzymatic breakdown. The olfactory lobe was selected as a separate entity which could be compared to the remainder of the brain since it could be readily dissected out in the donor and the recipients, and in addition to being anatomically distinct, it is a relatively primitive region. The size of the olfactory lobe made it necessary to use the pooled homogenate from three donors for a single recipient; the amount of tissues (0·34 g vs. 0·60 g) and the amount of radioactivity injected was still somewhat lower than the other samples. Unlabelled leucine, 25 mg in 16 per cent gelatin, was injected i.p. into each recipient two hours before and seven hours following the injection of the labelled homogenate in an attempt to dilute any ^{3}H-leucine released from the homogenates. Table 4 summarizes the results of intraperitoneal injection of labelled homogenates of olfactory

* Prior to the present experiments, qualitatively similar findings were observed by Porter (unpublished results) in this laboratory using donors labelled with ^{14}C–CO_2 and ^{3}H-leucine. In the case of the ^{14}C–CO_2-labelled tissues, the donors were labelled by continuous exposure to ^{14}C–CO_2 for 72 hours prior to sacrifice, and the specific localization of labelled material in this case could be explained by a variety of components including proteins, glycoproteins, etc.

TABLE 4

Relative specific activity in recipient organs 18 hours after injection with labelled tissue homogenates and 50 mg cold leucine
(each figure represents the mean and standard deviation)

Relative specific activity	Injection			
	Liver	Olfactory lobe	Kidney	Brain
DPM per g/DPM per g**	$n = 3$	$n = 1$	$n = 3$	$n = 2$
Liver/Liver	1·0	1·0	1·0	1·0
Olfactory lobe/Liver	0·13 ± 0·03	0·69 ± 0·05 *($p < 0·0005$)	0·13 ± 0·04	0·22 ± 0·03
Kidney/Liver	0·88 ± 0·03	0·72 ± 0·05	1·11 ± 0·04 ($p < 0·0005$)	0·64 ± 0·13
Brain/Liver	0·30 ± 0·04	0·34 ± 0·09	0·21 ± 0·01	0·35 ± 0·07 ($0·01 < p < 0·025$)

* p is probability value expressing the significance of the incorporation of homogenate into homologous tissue compared with the three non-homologous tissues.

** 33 mg tissue samples (28 mg for olfactory lobe) were digested in 2 N NaOH for 1 hour at 80 °C; 2·5 ml Bio-Solv (Beckman) + 10 ml butyl PBD–PBBO cocktail were added and counted 50 min (5 per cent error) in a Beckman Scintillation counter.

lobe, the remainder of the brain, liver, and kidney into recipient rats with simultaneous injections of unlabelled leucine. Labelled homogenates of olfactory lobe, kidney and brain all showed preferential localization into the corresponding organs of origin 18 hours after injection into recipients. The effect was most pronounced with olfactory lobe and least with brain. The preferential localization of injected olfactory lobe and kidney can also be seen by comparison of per cent uptake per organ (Table 5). It should be emphasized, however, that the data for the localization of injected olfactory lobe is based on a single animal.

Yoffey and Courtice (1956) have shown that substances including intact cells injected into the peritoneal cavity are taken into the lymphatic vessels via the diaphragm. For example, a major portion of a labelled protein introduced into the peritoneal cavity of a rat was recovered in the thoracic duct. The data reported here demonstrate a preferential localization of intraperitoneally-injected, leucine-labelled homogenate into all corresponding organs of origin studied, namely in the olfactory lobe, kidney, lung and, to a lesser extent, brain. This preferential localization in kidney confirms the earlier reports described above (Ebert, 1954; Walter et al., 1956; Mahler et al., 1956) for kidney, heart and spleen in a different species under different conditions. The mechanism of the apparently specific reincorporation is not known and may be related to the observations of Lilien and Moscona (1967) on the specific aggregation of embryonic cells, but the homogenates are presumably cell-free, and it seems more likely that the mechanism would be one which somehow mimics or integrates itself with the normal process of turnover and *de novo* synthesis in the young adult animal. The emphasis on protein localization as a consequence of using ^{3}H-leucine labelling does

TABLE 5

Comparison of per cent uptake of injected homogenates into olfactory lobe and kidney

Type of homogenate and DPM injected	*n*	DPM/g	Olfactory lobe per cent uptake/organ	Percentage of olfactory lobe homogenate uptake
Olfactory lobe (155 000 DPM)	1	650	0·06	100
Brain (463 000 DPM)	2	312	0·01	17
Liver (808 000 DPM)	3	468	0·007	12
Kidney (1 007 000 DPM)	3	780	0·008	13

Type of homogenate and DPM injected	*n*	DPM/g	Kidney per cent uptake/organ	Percentage of kidney homogenate uptake
Kidney (1 007 000 DPM)	3	10 980	1·92	100
Brain (463 000 DPM)	2	2 100	0·91	47
Liver (808 000 DPM)	3	6 060	1·09	57
Olfactory lobe (155 000 DPM)	1	860	0·84	44

not preclude future studies using other types of labelling as well as the fractionation of the homogenate prior to injection.

These results are preliminary, but they suggest that organ-specific components can localize with some specificity when injected into an intact animal and this apparently includes localization within a specific region in the central nervous system. The relevance of these results to the "memory transfer" phenomenon is yet to be determined, but the behavioural changes would seem to require that the injected materials have the capability of relocating in a specific region, presumably a region analogous to the location in the donor animal. Byrne and Hughes (1967) suggested "that long term memory was based on synaptic modification as a result of the synthesis of protein subunits which modify the synaptic membrane (interaction of non-identical subunits) of a 'prewired' (Sperry, 1966)* nervous system".

* Sperry has pointed out the time importance of an orderly pattern of specific cytochemical affinities which regulate a highly selective, chemotactic growth of specific nerve fibre pathways and synaptic connections. He extended the concept of chemoaffinity to include development patterning of central fibre systems and brain pathways as well as synaptic connections by demonstrating that regenerating teleostoptic fibres preferentially select different central pathways in order to reach their proper synaptic zone. The term prewired has been used to describe this concept of the nervous system.

One possible mechanism for the behavioural modification* would be a mechanism whereby the injected molecules or molecular complexes would mimic the proposed consolidation process and modify specific components of the recipient animal's brain.

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* A statement of the current status of "memory transfer" (Byrne, 1969) seems to indicate that there are a variety of behavioural changes which can be transferred, and the active material or materials is yet to be defined.

EFFECTS OF TRAINED BRAIN EXTRACTS ON BEHAVIOUR

by

F. Rosenblatt and S. Rosen

Section of Neurobiology and Behaviour, Cornell University, Ithaca, New York, U.S.A.

Since 1965, experiments on the behavioural effects of extracts from the brains of trained rats have remained highly controversial, despite the large number of experiments which have been reported (Fjerdingstad et al. 1965; Ungarn and Oceguera-Navarro, 1965; Babich et al., 1965; Byrne et al., 1966). The history of these experiments has been reviewed elsewhere (Rosenblatt, 1969). In our laboratory, early experiments were concerned, first, with replication attempts based on the Babich–Jacobson design (Rosenblatt et al., 1966); second, with the development of improved behavioural techniques for the assay of extract effects (Rosenblatt et al., 1966; Rosenblatt and Miller, 1966); and third, with the refinement of biochemical procedures for the separation of active components from brain. This has led us to consider the use of antibodies to brain extracts, both as an analytical tool for the localization and tracing of active components, and as a possible agent for influencing behaviour, either by interacting with antigens present in the brain or by mimicking the effects of antibody-like substances participating in the "transfer" phenomenon. This paper will first summarize the results of our behavioural studies, in order to provide the necessary background for a consideration of the antibody experiments. We will then describe the preliminary results obtained from our antibody work.

COMPARISON OF BEHAVIOURAL DESIGNS

From the outset, we have considered it methodologically desirable to use experimental designs in which experimental and control groups both received brain extracts from donors which differed only in the specific content of their experience, or learning task. We wanted to avoid extracts from donors differing in activity, emotional experience, motivation, physiological state, or other variables which might introduce spurious differences in the composition of experimental and control extracts. Discrimination tasks in which one group of donors was trained to one alternative and a second group to a different but similar alternative, such as left vs. right choices in a maze or Skinner box, seemed most likely to be free from possible artifacts. We wished to avoid discriminations between such alternatives as dark and light, or visual patterns to which rats might have an

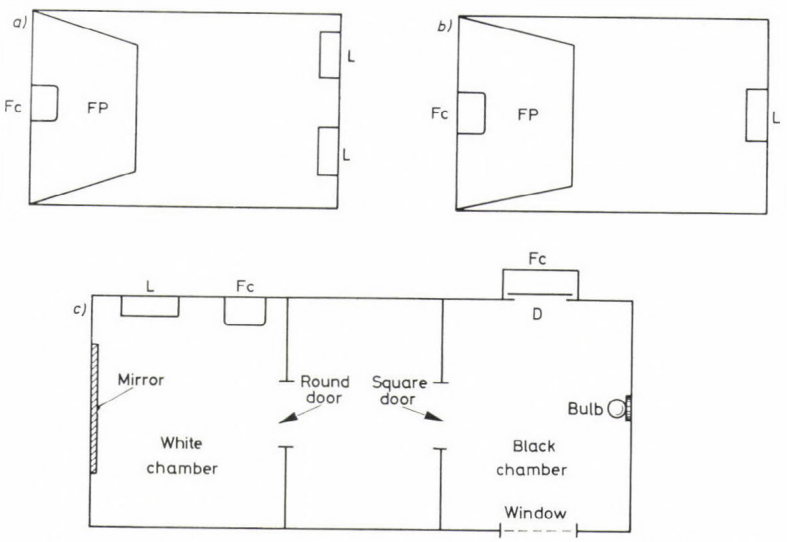

Fig. 1. Apparatus used for training and testing of rats. (*a*) Two-bar box; (*b*) one-bar box used for pretraining of recipients; (*c*) bar-door box, used in asymmetric discrimination experiments. Fc, foodcup. The assymmetric box (*c*) has a transparent ceiling over the white chamber and an opaque ceiling over the black chamber. L, lever; FP, floor pedal; D, door.

innate difference in preference or emotional response. After experimenting for over a year with various alternatives, we decided that a left/right discrimination task was the one which best met our criteria (Rosenblatt and Miller, 1966; Rosenblatt, 1966), and for the next several years we concentrated almost exclusively on this design. The apparatus (Fig. 1*a*) consists of a modified Skinner box with two bars at one end, and a food cup at the opposite end. Donor rats are trained to operate either the left or right bar in order to obtain a food pellet. Recipients are first preconditioned in a one-bar box (Fig. 1*b*) in which they learn to press the bar for food, but do not acquire any side preference. They are then injected and tested in the two-bar box, being fed regardless of whether they press the left or right bar. This guarantees that they will continue to respond over a series of tests, but no systematic left or right preference is taught. The per cent of left responses is computed for each rat, and the difference between the mean per cent of left responses for a group injected with "left-trained extract" and the mean per cent of left responses for a group injected with "right-trained extract" serves as a measure of effect in these experiments.

In all of our standard experiments, extracts are obtained by a method which has been described in detail elsewhere (Rosenblatt, 1969). Donor rat brains are pooled, and an aqueous extract obtained by three successive extractions with distilled water. This is then precipitated with acetone, and the precipitate which comes down between 50 and 75 per cent acetone

is retained as the active fraction. All experiments described in this paper employ an identical extraction procedure.

The two main questions which we set out to answer, initially, were (*i*) Is there an effect of trained brain extracts on behaviour, which depends upon the training of the donors? (*ii*) How specific is this effect to the experience of the donors, or to the task which they are required to learn? The answer to both of these questions was made far more difficult by our finding that the behaviour of recipient rats might correspond either directly or inversely to that learned by donor rats in a left/right discrimination task, depending strongly upon the exact dosage of extract employed in an experiment (Rosenblatt and Miller, 1966; Rosenblatt, 1969). Such inversion effects, which had been noted occasionally by previous investigators (Nissen et al., 1965), seemed to be particularly prevalent in the carefully balanced left/right choice situation.

In an attempt to determine whether a reliable relationship could be found between the training of the donors and the observed bias of the recipients, a large number of experiments were performed over a two-year period, with replications by William Herblin at DuPont Laboratories. In these experiments we tested the dosage range from 0·013 to 0·050 brain equivalents per recipient rat, and tried to demonstrate the existence of a maximum (positive effect) in the dose-response curve at a dose of 0·025 brain equivalents. Both at our laboratory and at Herblin's, it appeared that such a maximum did exist, but that it was exceedingly unstable and difficult to replicate. It seemed to be sensitive to the slightest variation in experimental technique, condition of the rats, or variations in equipment. Some of these results are illustrated in Figs 2*a* and 2*b*, which show the dose

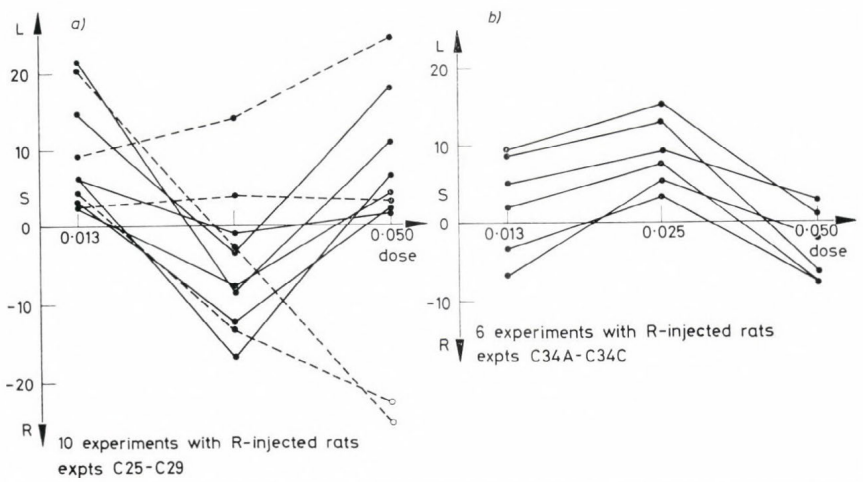

Fig. 2. Comparison of dose-response curves in two series of consecutive experiments. All rats were injected with right-trained brain extract, from donors trained in the two-bar box (Fig. 1*a*). S, shift from pretest, in mean percentage of left bar-pushing responses. Dose in brain equivalents.

response curves for two different sets of consecutive experiments, with rats injected with right-trained extracts. While the earlier series shows a maximum right-bias appearing consistently at 0·025 brains, the later series shows a maximum left-bias appearing at the same dosage. Moreover, a pool of all data obtained on left-right discrimination experiments to date revealed that the net effect (averaging over all doses) was an inversion effect; i.e., whatever consistent effect emerged indicated that the principal effect of the extract was either to interfere with the task learned by the corresponding donors or to promote the opposite behaviour (Rosenblatt, 1966).

When, at the end of two years, we were still unable to replicate the left-right dose-response curve reliably, it seemed that the time had come to re-examine the basic assumptions which had led to this choice of experimental design. In earlier experiments, where we had obtained generally positive results (Rosenblatt et al., 1966), and in experiments at other laboratories where positive results were reported, there was less emphasis on symmetry, and the differences between experimental and control groups were much greater than in our left-right design. It seemed possible that the left-right choice, in which rats were tested for only one relatively minor aspect of what they learned in the apparatus, might be particularly subject to conflicting effects and instability. Donor rats gain considerable experience pressing both bars (not merely the correct one); they must learn to suppress bar-pressing on the wrong bar, as well as to press the proper bar; and the sensory signals to which both groups learn to respond are identical. Moreover, it seemed likely that the sets of neurones involved in left and right bar-pressing behaviour would be largely identical, and that any differences would probably involve symmetric, and chemically related types of neurones. Thus a very slight change in balance, favouring excitatory over inhibitory factors, or favouring one cue over another, might result in a change from positive to inversion effects in a "transfer experiment".

Thus we were led to design a new set of training-boxes, in which similarities between the two tasks would be minimized, and a maximum number of distinct perceptual cues would be available to characterize each alternative. The likelihood that sensitivity to different stimuli was easier to transfer than preference for different responses (Rosenblatt, in press) was a further consideration. In the new "asymmetric design" (Fig. 1*c*), donor rats had a choice of a black or white compartment, differing in operant tasks, lights, windows, and other incidental cues. One group of donors was trained to operate a lever in a white compartment for a food reward, while a second group was trained to push a door for food in a black chamber. Experiments with groups trained for eight days and ten days (15 minutes per day), with and without the introduction of a two-day "rest period" during training (as suggested by McConnell) show very similar results. Twenty-three groups of recipients have been tested with these extracts, each group consisting of 30 "bar recipients" and 30 "door recipients". Injections were done intravenously, after three unreinforced sessions in the apparatus, and data are obtained from a single 5-minute test 24 hours after injection, without reinforcement. The results for these 23 experiments are shown in Fig. 3. No significant inversion effects were obtained, and 20 of

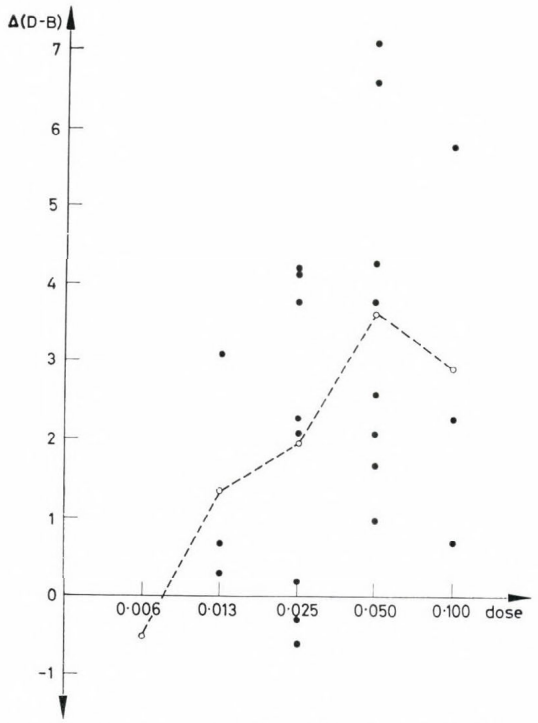

Fig. 3. Dose-response curve for 23 consecutive experiments with asymmetric bar-door problem (Fig. 1*c*). Each point represents the difference (Δ) between the mean percentage of time spent in the door chamber by a group of 30 door-injected rats and by a group of 30 bar-injected rats, on their first postinjection test. Dose in brain equivalents. Data are adjusted for differences of pretest scores by means of a covariance correction.

the experiments showed a positive effect. (The probability of 20 out of 23 being positive is 0·0003 by a binomial test, and 4 of the individual experiments were significant at $p = 0{\cdot}05$). The percentage of time spent in the bar-chamber was found to provide a better measure of effect than the ratio of bar to door-presses, and tests on subsequent days show a steadily diminishing effect. The points shown in the figure represent the difference (for each experiment) between the mean percentage of time spent in the bar-chamber by bar-injected rats and by door-injected rats. This is adjusted statistically to eliminate effects of covariance between pre-injection scores and post-injection scores. (Correlations between test and pretest tend to be about 0·30; unadjusted scores show a very similar distribution, but with slightly greater variability.) There is a generally increasing effect with higher doses, although the dose-response curve has not yet been explored

sufficiently to determine whether there are local maxima or minima, or whether the effect peaks at some dosage. Seven additional groups of rats were tested with an extract from donors trained for six days, and showed a weaker effect (three groups showing non-significant inversion effects, and four showing positive effects).

In a subsequent series of experiments, the boxes were modified slightly, the colours at the two ends being interchanged, and the opaque ceiling being replaced by a wire-grille and transparent plastic ceiling, in order to eliminate the preference which we had found in favour of the door-end of the apparatus. While this treatment has not entirely eliminated the bias, it has reduced it considerably, and most groups of rats are about evenly divided between preference for the white and dark end by the time of their second pretest. In addition, the operant tasks were replaced by automatic feeding of the donors (one pellet every 20 seconds). Six out of seven groups run to date with this modified design have shown positive effects (the seventh being a non-significant inversion).

We are still concerned, however, about the possibility that an inherent bias towards one alternative or the other may be an important factor in these experiments. We have found, for example, that rats recently shipped from the supplier have a much stronger dark-preference than rats who have been kept for a week or more before use; the day after arrival, practically 100 per cent of our rats prefer the black to the white chamber. It is easy to see that an extract which induces or simulates a nervous state or a relaxed state in a recipient rat might drastically affect the bias measured in our experiments. The observed bias due to the extracts is much smaller than the bias due to various intervals from shipment dates, degrees of starvation, or other factors which affect the exploratory behaviour of the rats. Thus the main question remains one of specificity: do the different extracts actually differ qualitatively in their effects upon the recipient animals, or are we merely observing differences in degree of some common effect? Control experiments which will attempt to answer this question are now in progress. In general, what must be shown is that a number of different effects can be produced by different extracts which differ in *opposite directions*, relative to a neutral control group. It is not enough that they differ from one another, or even that they go in opposite directions from pretest scores, since shifts from pretests might be composed of a summation of a positive extract effect with a negative spontaneous shift. Furthermore, a complete dose-response curve must be studied to demonstrate that one extract is not simply producing a phase-shifted effect, due to a lower or higher concentration of the same active factor.

Although the above steps must be completed in order to prove that the two extracts being tested both contain task-specific factors, there seems little doubt at this time that they at least differ significantly in one factor, which influences the behaviour of recipients (in an asymmetric situation) in such a way as to favour the behaviour learned by the donors.

On the assumption that the active constituents of our brain extracts in the experiments described above were some kind of protein or polypeptide, it seemed possible that we might be able to obtain antibodies which would be useful in characterizing and localizing them. Moreover, since the extracts themselves seemed to produce differential effects on behaviour, it seemed possible that antibodies against the extracts might also affect the behaviour of recipient rats into whose brains they were injected. The work of Mihailović and Janković (1961) demonstrated that specific brain nuclei could be affected by antibodies against these nuclei. Janković et al. (1968) have also demonstrated the abolition of a defensive conditioned reflex by antibodies directed against a protein fraction of naive cat brain. D'Monte and Talwar (1967) have shown that antibodies to occipital, motor, and sensory cortex of the monkey differ in their electrophoretic pattern and that the cross-reactivity of homologous antigen-antibody systems has a much higher haemagglutination titre than heterologous systems.

Since our antibody experiments were begun while we were still concentrating on left-right discrimination tasks, we decided to attempt to form antibodies against left-trained and right-trained rat brain extracts, prepared as described above. Since these "transfer experiments" are themselves difficult to reproduce reliably, and are particularly subject to inversion effects, as we have just seen, results obtained from corresponding antibody experiments must be considered tentative, and subject to reinterpretation after the experiments have been repeated with a consistently positive procedure, such as the asymmetric bar-door choice.

Antibodies were obtained by preparing a left-trained and right-trained brain extract by the acetone precipitation method described above. These extracts were used as antigens, for two successive groups of rabbits. In the second group, in which the rabbits were stimulated and bled on a systematic schedule, the procedure was as follows: The rabbits were injected at multiple subcutaneous sites with two brain equivalents of extract each, emulsified in Freund's complete adjuvant. A first bleeding was taken 11 days later. At 20 days, the rabbits were bled again (second bleeding). At 22 days they were restimulated, with multiple subcutaneous injections in F.C.A. Additional bleedings were taken at roughly 10 day intervals. A total of 11 bleedings were collected from the rabbits of Group II. The rabbits were restimulated twice more (1 brain equivalent per rabbit) subcutaneously, with F.C.A., at 3 week intervals. The schedule of injections and antibody titers obtained over an 80 day period (using a passive hemagglutination technique) are shown in Fig. 4. A gamma-globulin fraction was prepared from the rabbit sera by the following procedure: The serum is precipiated three times with saturated ammonium sulfate to a concentration of 50 per cent. (In this high concentration γM tends to be precipitated.) The precipitate is dissolved in saline and dialyzed against 0·01 M tris-saline buffer, pH 7·4. The gamma-globulin concentration is determined by optical density at 278 mμ. Precipitin tests showed that precipitating antibody was present, and several tests using Ouchterlony plates to compare reactivity with brain,

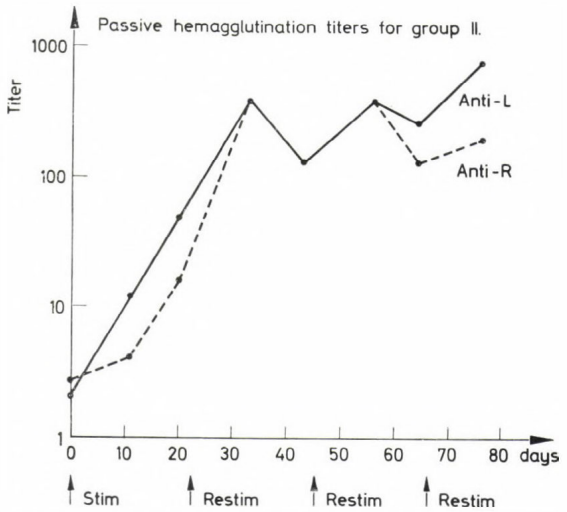

Fig. 4. Antibody levels in sera obtained from rabbits of Group II, as determined by passive hemagglutination. Titers are for the pooled sera from three L-injected and three R-injected rabbits.

kidney, and liver have suggested that the antibody contains a mixture of components, with different reactivities for the different tissues.

To evaluate the behavioural effects of these antibodies, groups of naive rats were injected intraventricularly with anti-L and anti-R gamma-globulin, and tested in a two-bar box (Fig. 1*a*), as in a regular transfer experiment. Table 1 summarizes the results of these experiments, for the initial test session (generally 12 to 14 hours after injection). Altogether, 16 groups of rats were tested with antibodies from the rabbits of Group I, and 9 groups were tested with antibodies from Group II. In each experiment there were 10 to 15 anti-L recipients and 10 to 15 anti-R recipients. Dose is given in milligrams of gamma-globulin, and performance measures (delta) are given as the mean per cent of left responses for the anti-L injected group minus the mean per cent of left responses for the anti-R injected group. Thus a positive delta represents a bias similar to that of the original donor rats, while a negative delta (which we continue to call an "inversion effect") represents a bias opposite to that of the donors. In other words, a positive delta means that anti-L induces rats to push the left bar and anti-R induces them to push the right bar. Probabilities are obtained by a single-tailed Mann-Whitney U-test.

In experiment 1, recipient rats were pretrained to push the left or right bar, and each of these groups was divided into two subgroups, one of which received anti-L and the other anti-R antibody. Since we originally expected that the most likely effect of the antibody would be interference with the learning or performance of the homologous response, we expected to find

TABLE 1

Summary of antibody experiments. First test session

Group I (4 Rabbits)					Group II (6 Rabbits)				
Bleeding	Expt.	Dose	Delta	Prob.	Bleeding	Expt.	Dose	Delta	Prob.
1	1 (L-trained)		−11·6	0·948	1	A	1·3 mg	−8·6	0·924
	(R-trained)		0·6	0·712		B	5(IV)	2·2	0·388
2	2	5(IV)	−15·3	0·913		C	5(IV)	−8·2	0·794
		5(IC)	−11·5	0·797	2	A	1·3	11·4	0·029*
3	3	5 mg	−12·9	0·870		B	1·3	18·1	0·002*
		15	−16·7	0·900		C	1·3	6·1	0·182
	4	5	31·1	0·000 3*	3	A	1·3	−2·1	0·603
	5	5	10·3	0·056		B	1·3	−7·2	0·725
4	6	1·5	17·9	0·025*	4	A	1·5	−7·1	0·796
	7	1·5	16·7	0·009*					
	8	1·5	− 6·4	0·774					
5	9A	1·5	2·1	0·327					
	9B	4	− 5·1	0·734					
	9C	1·5	0·6	0·532					
6	10A	1·5	− 9·8	0·532					
	10B	1·5	7·4	0·210					

* Prob. of 5 out of 25 experiments significant at 0·03 level = 0·001.

negative deltas in this experiment. This was, indeed, the main effect observed (although non-significant) in the early bleedings from both Group I and Group II. The only significant results obtained, however, were the five experiments marked with asterisks in the table. Each of these corresponded to a "positive" effect, and all were concentrated in the middle series of bleedings (the third and fourth bleeding of Group I, and the second bleeding of Group II). The distibution of scores (percentage of left responses) for the rats in these two consecutive runs of positive experiments is shown in Fig. 5. While these two groups of experiments are clearly significant when considered by themselves, we must still show that the effect is significant when considered as part of the complete series of experiments run. The most conservative test that can easily be used for this is the binomial probability that at least 5 out of 25 experiments will be found to be significant at the 0·03 level (corresponding to the weakest of the five significant cases). This probability is 0·001. (The probability of finding either 5 significantly positive or 5 significantly negative experiments at this level is 0·002.) We conclude, therefore, that there is strong evidence for a behavioural effect induced by antibodies to trained brain extracts, which mimics the training of the donor animals.

In later bleedings, no significant effects were found. It is also noteworthy that the main effect was found in the initial test of a series of six, while with the injections of brain extract rather than antibodies, effects were

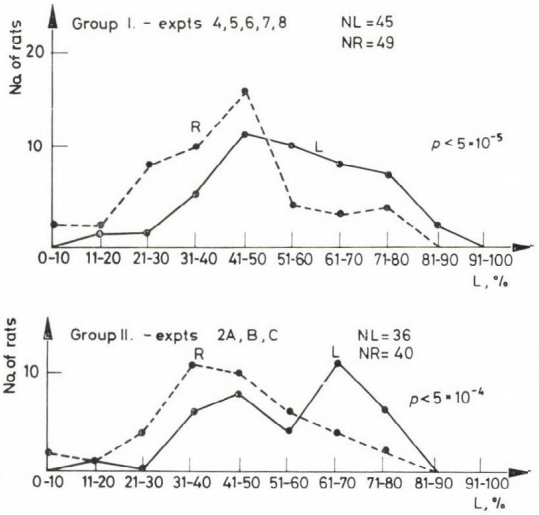

Fig. 5. Distributions of percentage of left responses for rats injected with anti-L and anti-R antibody, for two consecutive series of experiments with Group I and Group II rabbit sera. Probabilities are obtained by a Mann-Whitney U-test.

minimal on the first session and tended to increase progressively in later sessions (Rosenblatt, PNAS). This suggests that the antibodies may be mimicking the effect of a second-order product, synthesized in the recipient rats with a time-lag of over 24 hours in response to brain extracts. The possibility that the transfer phenomenon which was previously observed might be a manifestation of an immunological reaction to the brain extracts has been considered. Two experiments were run with immunosuppressants (6-mercaptopurine) administered together with the brain extracts. While the only significant effects found in these experiments were in the control groups (which received brain extract without 6-MP), the results are insufficient to demonstrate that 6-MP actually interfered with the transfer effect. We have also sought to demonstrate an immune response of rats to our brain extracts, by precipitin tests, passive hemagglutination, and by skin tests. While none of these have given any evidence of antibody production they may be insufficiently sensitive and are not conclusive.

Some theorists, including Szilard (1964), have speculated upon the possibility that the mechanisms of neurological and immunological memory might be closely related. In this case, antibodies prepared against brain extracts might mimic the phenomena of memory in some fundamental sense, and the effect of the brain extracts when injected directly might furnish immunological inducers to the recipients which are generated in the donors during their learning process. The results obtained in our experiments are not inconsistent with this hypothesis. On the other hand, since the primary

effect of left/right-trained brain extracts upon the behaviour of rats seems to be an inversion or interference effect, it is still possible that the seemingly "positive" effect of the antibodies is actually an interference with the molecular processes which would have favoured inversion. The best way to resolve this question seems to be to repeat the antibody experiments with the bar/door design, which we have shown to be free from inversion effects. If the antibodies continue to produce a "positive" effect in this design, then the first of these hypotheses would seem to be upheld.

Apart from the implications of the behavioural results in these experiments, the finding that we can obtain antibody with different properties for left-trained and right-trained brain extracts strongly supports the view that the left and right bar-pressing tasks are characterized by distinctive macromolecules. It also provides an analytic tool which may be used in studies of localization of the corresponding antigens, both macroscopically and subcellularly, and for purification of the different antigens by cross-reaction techniques. Further work in our laboratory is directed toward investigating the feasibility of some of these methods, as well as a more careful characterization of the antibodies which we have obtained.

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DISCUSSION

J. SZENTÁGOTHAI: Experimental neuroembryological evidence has shown that neuroblasts receive, already in relatively early stages of development, specific cues with respect to what direction—oral or caudal, dorsal or ventral—their axons should grow. In spite of the reversal of the axis orientations of various parts of the early brain *anlage*, many axons find their appropriate goals correctly. Strangely there is little if any evidence that the neuroblasts would have any cues that would specify them as belonging to the left or the right side. Most nerve tract crossings are not brought about by some inherent tendency of the axons to reach the other side, but rather as a consequence of the bilateral symmetry of the CNS producing transversally oriented micellular structures in the medullary tube commissures (Székely and Szentágothai, 1956, *Acta Biol. Acad. Sci. hung.* **6**, 215–229) that in turn induce most fibres to cross that arrive in their vicinity. Thus there may be very little specific difference between the chemical characteristics of the same kind of neurones belonging to the right and the left side. This may be the reason why your results are not very conclusive.

F. ROSENBLATT: I believe your explanation is very plausible. We may simply have made a poor choice of a discrimination task, if our purpose is to find a problem which differs strongly in the chemical characteristics of the neurones involved. If the cells responsible for a left or right choice are indeed chemically similar, then very slight secondary influences or details about our training boxes which we have failed to control adequately might become the main determinants of bias in our extract. On the other hand, the fact that inversion effects seem to predominate consistently in the left-right task, rather than a completely neutral mixture of positive and negative effects, and that this had been found in Y-mazes as well as two-bar Skinner boxes, does seem to indicate that there is some kind of chemical specificity characterizing the left-trained and right-trained rats, even though it is easily overpowered by other factors and is difficult to obtain reliably. The antibody experiments also seem to indicate a definite difference between the two kinds of extract.

W. C. CORNING: Have you observed the complex dose-response curve in naive animals? What if you were to observe such a curve in naive animals?

F. ROSENBLATT: It is not clear how any dose-response curve could be observed in "naive animals", if this means animals receiving no brain-extract at all. If you mean animals who are injected with different doses

of extract from untrained donor rats, then one might indeed observe differences in left or right bias at different doses. We have found that almost any treatment, including naive brain extracts, and even injections of large doses of distilled water, is likely to alter the bias of a rat. Thus if rats in a particular box exhibited a general tendency to shift towards the left or the right (as might occur if one bar was slightly easier to press than the other), I would expect this bias to be influenced by almost any type of treatment. We have seen repeated indications that our differential left-right effect is superimposed upon the effect of non-specific factors, of which there may be a large number. I would therefore consider a dose response curve obtained for animals injected with naive extract as a demonstration of the effects of these non-specific factors (or perhaps factors specific to the experience of the "naive" donor rats). It is for this reason that we have emphasized the use of differential techniques, comparing the effects of a left extract against a similarly trained and similarly handled right extract, rather than against the hypothetical construct of a "naive rat".

R. Galambos: Your report of a positive result upon behaviour of anti-brain antibody is the first I know of in the rat, and you are to be congratulated for having succeeded in this demonstration. Have you such controls on this as negative results after injection of antibody to such rat tissues as normal brain, or liver?

F. Rosenblatt: We have used only anti-brain antibodies in our behavioural experiments, although we have completed a preliminary check for cross-reactivity with other tissues, and found evidence for a mixture of brain-specific and non-specific antibodies. We have also run groups of saline-injected controls in our first series of experiments. These groups came out between the anti-L and anti-R groups.

G. Ungar: Dr. Rosenblatt's steadfastness in sticking by the left-right experiments is admirable but at the present stage of the transfer problem it risks to confuse the issues. We need at this point clear-cut situations which are not accompanied by reversal and do not exhibit multiphasic dose-response curves. The problem of inversion may be interesting to study at some future stage outside the context of the transfer experiments.

F. Rosenblatt: The problem of finding a good task for use in "transfer experiments" is to find one in which it is likely that chemically different kinds of neurones are involved (hopefully from the same sensory modality), but in which the different alternatives do not differ in attractiveness, or in emotional quality, or difficulty of learning. Any of these factors might result in differences in hormonal composition of the extracts, due to differences in stress or emotional experience of the donors, and such differences in composition might affect the recipients' behaviour in a manner entirely independent of memory or learning. It is not clear to me how the inversion phenomenon could be studied "outside the context of transfer experiments", since it is specifically a transfer phenomenon.

CHEMICAL TRANSFER OF COLOUR AND TASTE DISCRIMINATION* IN GOLDFISH *(CARASSIUS AURATUS)*

by

G. F. Domagk and H. P. Zippel

University of Göttingen, G.F.R.

We wish to report upon a series of experiments we began about a year ago in which we have been able to demonstrate a transfer of an acquired information from trained goldfish into naive recipient animals.

Our fish are kept in groups of two in the training tanks (size $130 \times 20 \times 30$ cm). During the conditioning phase the animals are fed Tubifex worms at either small side of the basin through plastic funnels with small holes in it. The vibrations occurring when the fish bites or swims against the funnel can be registered as either "left" or "right" by a two-channel automatic recorder (Zippel, 1970).

As Hafen (1935) had found in her experiments performed with minnows (*Phoxinus laevis*), our fish showed a strong innate preference for red light; green light, which initially was repellent, was chosen as the conditioning stimulus (CS). During the first 10 to 15 training sessions green light was given for 5 minutes at one side of the tank, followed by a short period of CS plus feeding of Tubifex (= unconditioned stimulus, US) at the same side. After the fish had learned to swim to the green light, a red light was shown during the training period on the opposite side of the tank. For a few days the fish preferred the red light to the green, but after a total of 40 to 60 trials, a conditioned reflex to green light was manifested.

Fish thus trained served as the donors of brain; using the technique described by Ungar et al. (1968), we homogenized the pooled brains in a Potter-Elvehjem homogenizer, dialyzed the whole homogenate against 20 volumes of distilled water, and collected after 24 hours the outer liquid with the low molecular weight material, which then was concentrated by lyophilization. The small residue was dissolved in Ringer's solution and injected intraperitoneally into naive recipient animals whose preference for red light had been tested before (Fig. 1). About 12 hours after the injections the animals began to swim to the green light. This preference for the green light offered randomly at the left or right side of the tank (red light being shown at the opposite side) could be observed for about one week. A statistical analysis of our results, which have been published recently (Zippel and Domagk, 1969), is given in Table 1.

* This investigation has been supported by a grant from the Deutsche Forschungsgemeinschaft.

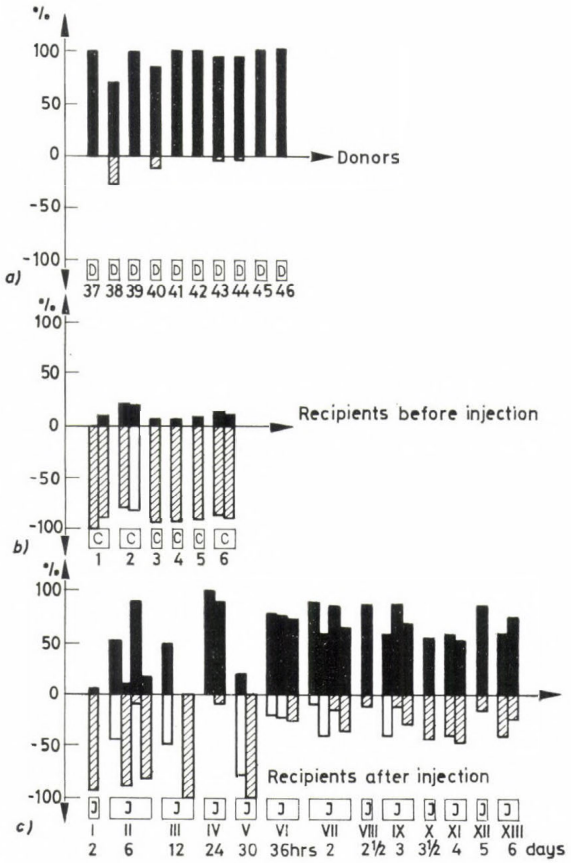

Fig. 1. Chemical transfer of colour discrimination in goldfish. Preference to green light (black blocks) as compared to red light (striated blocks) or no colour offered (white blocks). (*a*) Behaviour of donor group exposed to green and red light after 37 to 46 training sessions. (*b*) Behaviour of recipient group exposed to green and red light without previous training. (*c*) Behaviour of recipient group from two hours to six days after i.p. injection of brain extract prepared from donor group *a*.

These preliminary experiments looked quite encouraging; the only trouble was the long training time (6 to 8 weeks) needed for the donor groups. We therefore tested the animals' ability of taste discriminations. Using the training tanks described above solutions of various chemicals were dropped into the feeding funnels, a stream of water being offered at the opposite side of the basin in order to avoid artefacts due to the dropping. We found solutions of glucose to be attractive and solutions of either acetic acid or

TABLE 1

Statistical analyses of colour discrimination experiments performed with 4 fish groups (Wilcoxon matched pair signed rank statistics)

	Before injection	0 to 24 hours after injection	24 to 48 hours after injection	2 to 6 days after injection	Training after forgetting
Recipients	$p < 0{\cdot}02$ for red light ($n = 7$)	$p > 0{\cdot}05$ ($n = 5$)	$p < 0{\cdot}05$ for green light ($n = 5$)	$p < 0{\cdot}02$ for green light ($n = 6$)	$p > 0{\cdot}05$ ($n = 10$)
	$p < 0{\cdot}005$ red light ($n = 8$)	$p > 0{\cdot}05$ ($n = 6$)		$p < 0{\cdot}05$ green light ($n = 5$)	$p > 0{\cdot}05$ ($n = 9$)
Control recipients	$p < 0{\cdot}005$ red light ($n = 9$)	$p > 0{\cdot}05$ ($n = 5$)	$p > 0{\cdot}05$ ($n = 5$)	$p > 0{\cdot}05$ ($n = 6$)	$p < 0{\cdot}02$ green light ($n = 10$)
	$p < 0{\cdot}005$ for red light ($n = 8$)	$p > 0{\cdot}05$ ($n = 6$)		$p < 0{\cdot}05$ red light ($n = 5$)	$p > 0{\cdot}05$ ($n = 9$)

quinine to be repellent for the fish (Bieck and Zippel, 1969). Therefore we chose the latter as the CS. Within one or two weeks of training all fish would learn to go for the previously disliked chemical, since food was offered on the same side of the tank after the testing phase. When the side of the CS was changed during the testing period, the fish soon swam to the new position of the CS. Figure 2 shows that an acquired preference

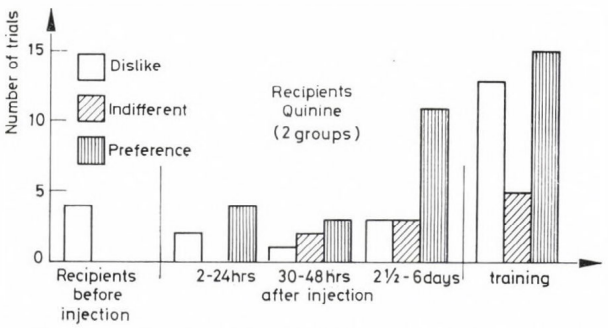

Fig. 2. Behaviour of goldfish exposed to quinine solution before and after injection of brain extract prepared from quinine adapted donors. Training was given to recipient animals 2 weeks after forgetting.

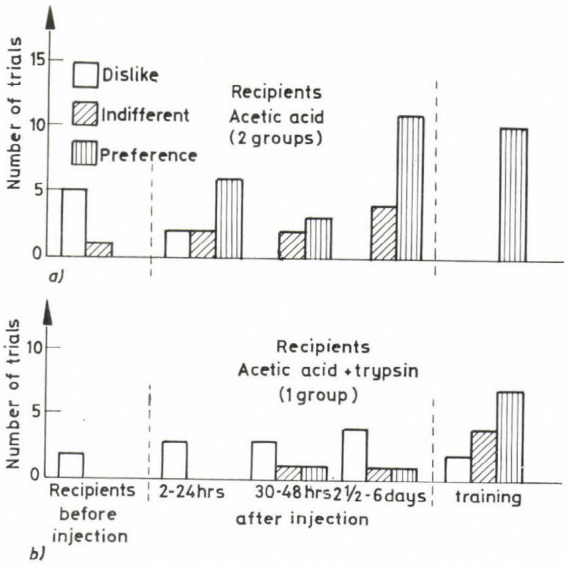

Fig. 3. Transfer efficiency of brain extracts from acetic acid adapted donors before (*a*) and after (*b*) treatment with trypsin.

for quinine can be transferred by an i.p. injection of low molecular weight brain extracts prepared in the manner described above.

By a further experiment we were able to give support to Ungar's idea of the peptide nature of the material responsible for the transfer phenomenon. One half of the brain extract obtained from acetic acid adapted fishes was treated with trypsin for 30 minutes. As can be seen from Fig. 3, this treatment with a protease destroyed the transfer factors, whereas the undegraded control extract gave positive transfer effects.

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DISCUSSION

G. Ungar: These are very beautiful experiments and should bring us closer to the solution of the specificity problem. Could you tell us how many animals were involved in each of the experiments?

G. F. Domagk: About a dozen of goldfish had been used for the colour discrimination training, and for memory transfer 2:1 ratio of donor to recipient brain equivalents had been injected. For the experiment with acetic acid adapted animals, 8 fish were used, and one half of the dialyzed material had been degraded by a 30 min incubation with trypsin. In these transfers which gave positive results with the undergraded material a 1 : 1 ratio (donor: recipient brain equivalents) had been used.

EFFECT OF BRAIN EXTRACTS ON THE FIXATION OF EXPERIENCE IN THE RAT SPINAL CORD*

by

J. Daliers and C. Giurgea

Pharmacological Department,
UCB Dipha, Brussels, Belgium

INTRODUCTION

Several years ago a particular form of memory at spinal level was described by Di Giorgio (1929) who demonstrated that centrally (lesion) induced asymmetry in the rear limbs of several species of animals may persist after spinal cord transection.

Later Chamberlain et al. (1963) using rats, demonstrated that asymmetry induced from cerebellar lesion persist after the spinal cord is transected, provided that they have been maintained in asymmetry for a sufficient length of time, namely about 45 min. This phenomenon has been interpreted by a permanent change occurring in the neuronal structure of the spinal cord.

Spinal fixation time (SFT) i.e. the minimal time needed by the spinal cord to maintain the postural asymmetry after section, may also be used for pharmacological studies of learning (Chamberlain et al., 1963; Giurgea and Mouravieff-Lesuisse, 1969).

These studies, which took into account the number of treated animals maintaining their asymmetry when transected at 35 minutes after the onset of asymmetry (time at which all saline injected animals lost their asymmetry), show that SFT reacts rather specifically to drugs that are presumed to enhance learning and memory. In the above mentioned papers from our laboratory it was shown that no modifications of SFT were obtained with various psychotropic drugs such as stimulants, tranquillizers, hallucinogens, antidepressants, etc. Since SFT seems to be derived of any motivational factor in the normal sense of the word, it is understandable that this model should not react to psychotropic drugs.

The aim of our study has been to investigate whether the acquisition of this new behaviour by the rat, i.e. the asymmetry, can be modified by brain extracts supplied by animals which were surgically rendered asymmetric.

MATERIAL AND METHODS

Details on SFT technical procedure have already been described elsewhere (Chamberlain et al., 1963; Giurgea and Mouravieff-Lesuisse, 1969); we summarize here the experimental method in order to help the comprehension of the results.

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(1) Cerebellar lesion

The 270 animals used are Wistar albino rats of 250–350 g. They are anaesthetized with 40 mg per kg i.p. Nembutal, and the left half of the anterior cerebellar lobe is removed by suction (Fig. 1); when emerging from anaesthesia asymmetry consists in an ipsilateral tonic flexion and a contralateral hypotonic extension of the hind legs. This moment is considered time zero.

Asymmetric animals are used either as donors of extract or as subjects for SFT.

(*a*) *Donors.* Rats are maintained in asymmetry without any spinal section for several, usually 17, hours. They provide the material to be extracted.

(*b*) *Recipient (SFT) test.* Rats are injected intraperitoneally with the extract either the day before (17 hours) or on the day of SFT test. In this latter case, extracts are given simultaneously with Nembutal, thus 2 to 3 hrs before the awakening of the animals. Each animal receives the extract from two rats.

Their spinal cords are transected (level T 7) 35 minutes after the onset of asymmetry; some minutes later the persistence or the lost of asymmetry is measured and animals are classified as positive (persistence) or negative (lost).

(2) Extracts

Rats are stunned, decapitated and their whole brains removed and frozen with dry ice as quickly as possible. This step must not take more than one minute. The material may then be stored at −18 °C. After defreezing the

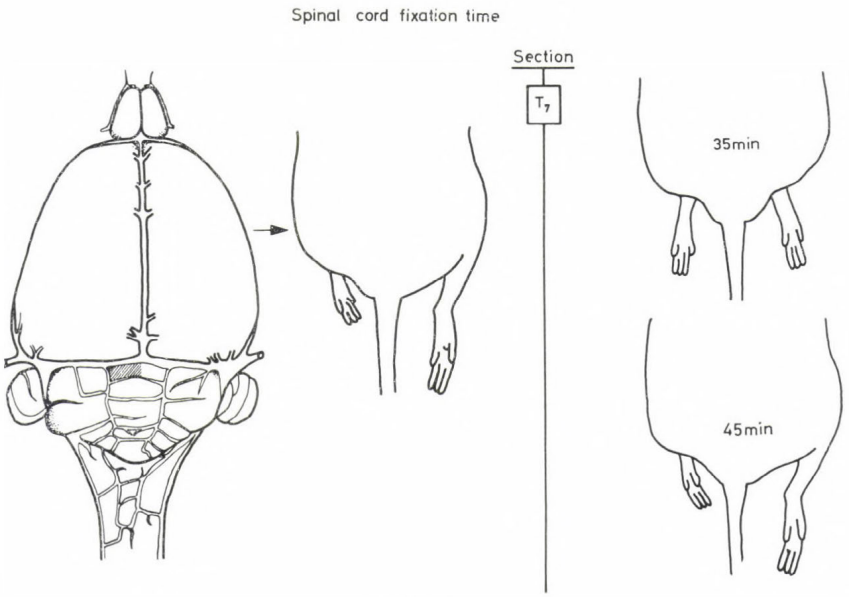

Fig. 1.

brains are homogenized (Mixer J & K, 25,000 rev. per min) and the solvent is added according to the chosen extract procedure.

(*a*) *Chloroform-methanol-HCl extract* (C-M-HCl extract). 160 ml chloroform-methanol mixture 2 : 1, containing 1 per cent concentrated HCl, is used for 40 brains. After keeping for 30 min at zero °C, the mixture is centrifuged; the upper aqueous layer is easily removed while the organic layer and the insoluble material (interphase) are again extracted with 40 ml water.

The aqueous layers are pooled, first partially evaporated under vacuum and then lyophilized. The resulting dry material is easily dissolved in water and the pH is brought up to 8·5; after keeping this material at zero °C for at least 60 minutes, the insoluble material is removed by centrifugation and the clear supernatant, after pH adjustment to 7, is ready to be injected. All the treatments of the extract are done between zero and 10 °C.

(*b*) *Phenol extract* (RNA extract). The RNA is extracted according to the classical method described by Littauer and Eisenberg. This procedure has been slightly adapted and already described in detail (Daliers, 1968). It consists mainly in obtaining the aqueous layer of a phenol extract (using EDTA sodium dodecyl sulfate and Na acetate).

Two volumes of 98 per cent alcohol are added to this solution and the precipitate is collected after a night at zero °C.

After drying under vacuum the white powder is finally slowly dissolved in water and ready to be injected.

RESULTS

(1) Positive effect on SFT

Table 1 shows that animals injected with brain extracts from "cerebellar" animals maintain their asymmetry after the spinal cord transection. In the same conditions (35 minutes time interval) all saline injected rats lost their asymmetry.

TABLE 1

Effect of the injection of brain extracts in naive animals on spinal fixation time (Extract prepared after 17 hrs asymmetry)

Extract injected	Donor	No. of asymmetric animals / No. of tested animals after section at 35 min	Conclusion	Fisher-Yates probability test
Saline (NaCl 0·9%)		0/24	Control: inactive	—
Aqueous layer of chloroform-methanol-HCl extract	Cerebellar lesion	8/10	active	$p < 0{\cdot}005$
	No lesion	0/5	inactive	—
Aqueous layer of phenol extract	Cerebellar lesion	8/12	active	$p < 0{\cdot}005$
	No lesion	0/5	inactive	—

Control extracts, from unlesioned rats (no cerebellar lesion) are inactive. The activity is found in both phenol and C-M-HCl extracts.

(2) Control extracts

(*a*) Table 2 presents results of an experiment performed on C-M-HCl extract in order to study the specificity of the correlation between the lesion and the activity. The first experiment is performed with an extract obtained after a unilateral temporal lesion. That lesion produces no asymmetry of the paws in the donor. This material is inactive; we may thus conclude that not all lesions are able to produce an active extract.

TABLE 2

Specificity of CNS lesion (Chloroform–methanol–HCl extract prepared after 17 hrs asymmetry)

Donor	No. asymmetric animals / No. of tested animals after section at 35 min	Conclusion	Fisher-Yates probability test
(1) Cerebellar lesion	8/10	active	$p < 0{\cdot}005$
(2) Temporal lesion	0/5	inactive	—
(3) Cerebellar lesion + spinal cord lesion	4/4	active	$p < 0{\cdot}005$
(4) No lesion	0/5	inactive	—

(*b*) The second control experiment is performed with an extract supplied by animals in which the spinal cord has been transected immediatly after the onset of the cerebellar induced asymmetry. These animals in spite of their cerebellar lesion, are thus deprived of any postural asymmetry since their hind legs are paralysed.

Table 2 shows that this extract is active. Consequently asymmetry in itself and its proprioceptive feedback, here interrupted by the section, are not necessary for the biosynthesis of the active material into the brain.

(3) Dynamics of activity

C-M-HCl extracts were prepared removing the brains at various lapses of time after the onset of asymmetry. Table 3 shows that more than 2 hours are needed for the brain to elaborate a sufficient amount of active material.

TABLE 3

Activity of the extract in correlation with the time spent in asymmetry by the donor (C–M–HCl extract)

Time elapsed in asymmetry	No. of positive animals / No. of tested animals after section at 35 min	Conclusion	Fisher-Yates probability test
45 min	0/5	inactive	—
2 hrs	0/4	inactive	—
6–7 hrs	4/5	active	$p < 0{\cdot}005$
17 hrs	8/10	active	$p < 0{\cdot}005$

CONCLUSIONS

The experiments with habituation to sound (Ungar and Oceguera-Navarro, 1965; Daliers and Rigaux-Motquin, 1968) and in particular those on morphine tolerance (Ungar and Cohen, 1966) allow to think that motivation *stricto sensu* is not a critical factor on which the success of a transfer experiment depends. The non-necessity of motivation was already proved concerning the acquisition of a classical conditioned reflex (Giurgea and Raiciulescu, 1959; Doty and Giurgea, 1961). The present work contributes to the view that motivation does not necessarily interfere with the transfer mechanism.

From the chemical point of view, it is of some interest that the transfer is obtained both with the phenol and the C-M-HCl procedure. As most of the macromolecules are theoretically absent from this last extract, since they are precipitated by methanol and HCl, we may expect that in our case activity is not supported by RNA molecules.

The other—phenol—extract, rich in DNA and RNA contains also some peptides and polysaccharides (Kirby, 1956). Thus we may conclude that activity may be supported by other molecules than RNA, most probably, by some relatively small molecules such as oligopeptides.

This kind of molecule was already proposed by Ungar as being responsible for transfer (Ungar et al., 1968).

The study of this particular point is to be continued using enzymatic incubation of the brain extracts.

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DISCUSSION

R. Galambos: (*i*) Would you elaborate on the test applied to the recipient? (*ii*) To test whether this is a specific effect of a cerebellar lesion, have you removed the entire cerebellum in the donor?

J. Daliers: (*i*) The recipient animals are submitted to the test of SFT, i.e. it is measured whether their asymmetry persists after spinal cord section performed at 35 minutes after the onset of asymmetry, when control animals do never show any persistence of the asymmetry. (*ii*) We have not performed this experiment; I think that it would be a good control experiment and that we would try to perform it.

G. Horn: (*i*) Have you ever tried injecting spinal cord extracts obtained from animals with combined cerebellar and spinal lesions? (*ii*) Asymmetry of the legs persists if the cord is transected some 45 min after a cerebellar lesion, suggesting that the hypothetical brain changes have taken place in that time. Brain extracts from animals with cerebellar lesions are not effective, in the transfer situation, at this time, at least 6–7 hours must elapse (Table 3). Have you any suggestion to make about this apparent discrepancy?

J. Daliers: (*i*) We never tried this kind of experiment but we have a preliminary result which can be of some interest.

We have used spinal cord phenol extract from asymmetric animals and this material is also active but only if we used the same amount as of the brain extract. We have injected the material from 10 spinal cords per animal. This was performed only on two rats. (*ii*) I think that there is no real discrepancy between these two facts. Indeed as Dr. Ungar has showed in the experiment with dark avoidance (Ungar et al., *Nature*, **217**, 125, 1968), the animal has learned the avoidance from his first trial. This behaviour is stable and long-lasting; nevertheless after this first trial, if you make an extract, it will be inactive. The same seems to be true for the lesioned animals in our experiments. I think that this time being between 45 minutes and 6–7 hours is one more proof that memory consolidation is a time dependent process.

G. Ungar: These experiments illustrate the central problem of all transfer experiments. They show that animals submitted to a given experience develop some sort of a chemical equivalent of this experience in their brain. The development of this material can be tested by administering an extract of the brain to naive recipients. Whether the experience can be called learning or not is a secondary point and which has no important bearing on the problem.

K. Kelemen: The background of your transfer experiments is not quite clear to me. I should like to know whether the asymmetry is lacking in all control animals 35 min after transection and is present in all of them after

45 min? What I really wish to know is whether the positive transfer effects might not be caused by statistical chance.

J. DALIERS: When transected 35 minutes after the onset of asymmetry, all the control animals lost their asymmetry (24/24).

At 45 minutes only one animal out of 24 lost its asymmetry.

That means that if you compare, for instance, the values 0/4 and 4/5 of Tables 1 and 2 (the less significant result) by the Fischer-Yates exact probability test, you will have a $p < 0{\cdot}005$.

G. HORN: If the hypothetical brain changes continue over several hours, as Dr. Ungar suggests, one might expect the duration of leg asymmetry to increase the longer the interval between a cerebellar and a spinal lesion. Does this happen?

J. DALIERS: In fact, it so happens that even after only 45 minutes of asymmetry, the spinal cord may be transected without loss of asymmetry and asymmetry persists for several hours. This fact has been described by Chamberlain et al., (*J. Neurophysiol.* **26,** 662, 1963) and also confirmed in our laboratory.

THE MEMORY TRANSFER EFFECT: AN UNSPECIFIC PHENOMENON?

by

J. Faiszt and G. Ádám

Department of Comparative Physiology,
Eötvös Loránd University, Budapest, Hungary

Some three years ago in 1966, a rather unusual event occurred in our laboratory. Our team working for many years on several electrophysiological and biochemical problems of conditioning was taken aback by four different reports published independently of one another on the inter-animal memory transfer in mammals. Our first reaction was, of course, healthy scepticism. Nevertheless, being in possession of a rather great variety of conditioning techniques we decided to repeat the published experiments. Although we appreciated Professor Hydén's data on RNA-changes in neurones and Professor McConnell's findings on memory transfer in planarians, we did not believe in the possibility of inter-mammal transfer. Thus, our scheduled series of transfer experiments has been guided exclusively by curiosity and scepticism! Our operant behaviour was consequently identical with the behaviour of most of the colleagues of the 22 laboratories busy with transfer problems.

In our first series, published in *Nature* in 1967, we got a surprising positive transfer effect on rats and tried to clear up some preliminary conditions of this phenomenon. Under our experimental conditions of avoidance training the optimal effect occurred when donors were trained for about 10 days, i.e. 12 experimental sessions, and when adult recepients were not habituated to the conditional stimulus only to the unconditional one.

The results of our second series have been published in *Nature* in 1968. As some of you might know, in this series we compared the effect of ribosomal, messenger and soluble RNA-fractions and found that the ribosomal RNA gave the best transfer effect of the avoidance response.

In the present paper we would like to give account of some new data concerning two main questions widely debated:

(1) We obtained some data about the specificity, or more exactly the unspecific character, of the transfer phenomenon.
(2) We tried to follow up some components of the RNA-fractions responsible for the successful memory-facilitation effect.

The methods applied were identical with those already published. Wistar-Rehbrücke rats performing in 100 per cent an avoidance task – jumping up on a shelf – in response to an auditory stimulus served as donors. The recepients had been pretrained to jump on the shelf in the experimental

box, but they remained naive with respect to the conditional stimulus.

As regards the unspecific character of the transfer phenomenon the results of our experiments will be summed up in which our donor rats received the CS (ringing of a bell) and the US (electric shock) not in the form of paired, associated stimuli, but according to a special schedule, independently from each other.

(1) In the first series (group 1 in Table 1) the bell and the shock were administered alternatively at 30 sec intervals, 5 stimuli each. The donors were treated in this manner for 12–15 days until the avoidance responses to the auditory stimuli decreased to a 5 per cent level. Figure 1 represents the avoidance behaviour of this group of animals. Usually, the motor reactions diminished not lower than to a 5 per cent level; in this special case this occurred on the 10th day probably owing to intersignal responses. Whole brain RNA-extract of the donor (using the phenol extraction procedure) was injected intraperitoneally to naive recipients pretrained to perform the avoidance task to electric shock. Figure 2 represents the performance of 40 recipients during 4 experimental sessions receiving the RNA-extracts of 20 pseudoconditioned and 20 control donors. The CS (5 in each session 4, 8, 12 and 26 hours after the injection) was administered for 5 sec. If the animal performed the defensive response, shock was not applied; if the rat did not jump, reinforcement was administered. The performance of the recipients treated with pseudo-conditioned donors' brain extract was significantly better than that of the control recipients (Wilcoxon test: $p < 0{\cdot}01$).

(2) In the second series (group 2 in Table 1) the pseudo-conditioning of the donor group was carried out by the random administration of the CS

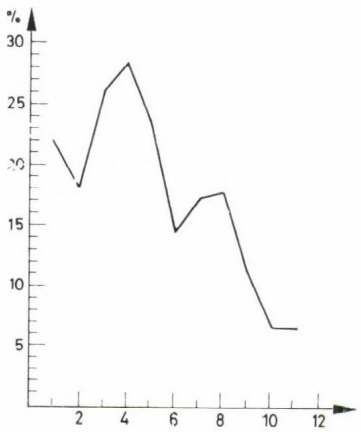

Fig. 1. Avoidance behaviour of a group of rats which received CS and UCS alternatively. Abscissa: experimental days; ordinate: percentage of avoidance motor response to 5 acoustic stimuli.

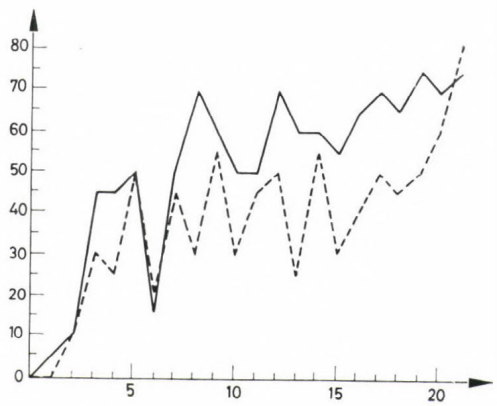

Fig. 2. Learning curve of 40 recipient rats receiving RNA-extracts of 20 pseudo-conditioned (solid lines) and 20 control donors (broken lines). Abscissa: no. of CS; ordinate: percentage of positive avoidance responses.

TABLE 1

Group		Number of donors		Number of recipients	Significance
		Naives	Trained		
Pseudo-conditioned	1. Alternative fix schedule (CS-US-CS-US-CS-US-CS-US-CS-US)	20	20	40	$p < 0{\cdot}01$
	2. Random fix schedule (US-CS-US-CS-CS-US-US-CS-CS-US)	20	20	40	$p < 0{\cdot}01$
	3. Random variable schedule	60	60	120	$p < 0{\cdot}02$
4. Homogeneous stimuli (CS or US)		20	20	40	—
Total		120	120	240	

and the US, but always in the same sequence: US-CS-US-CS-CS-US-US-CS-CS-US. Figure 3 shows the learning curve of 40 recipients. The facilitating effect of the pseudo-conditioned donors' brain extract is significant ($p < 0{\cdot}01$).

(3) In the third series (group 3 in Table 1) the pseudo-conditioning of the donors was carried out according to a variable schedule in each session in 3 different subgroups. Figure 4 sums up the results of 60 treated and 60 control recipients. The facilitation of learning of the group treated with

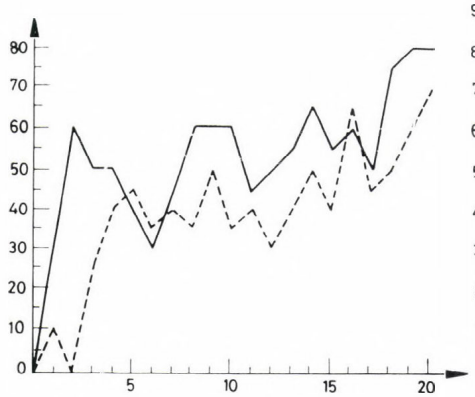

Fig. 3. Learning curve of 40 recipient rats receiving RNA-extracts of 20 pseudo-conditioned solid line and 20 control donors (broken line). For legends cf. Fig. 2.

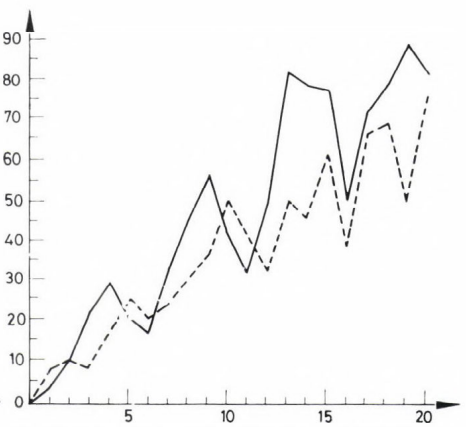

Fig. 4. Learning curve of 120 recipient rats. Legends as in previous figures.

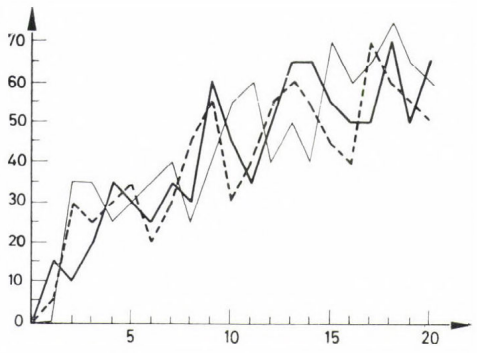

Fig. 5. Learning curve of 40 recipient rats treated with RNA-extracts from the brains of 20 donor animals receiving homogeneous stimuli (solid line) and from 20 controls (broken line).

pseudo-conditioned donors' RNA-fraction is obvious (significance: $p < 0{\cdot}02$).

(4) In the last, fourth series (group 4 in Table 1) of this pseudo-conditioning programme the donor groups received during 12 sessions 10 times in each session either the auditory stimulus, or the electric shock, but never the two together. As shown by Fig. 5, there was no difference between the experimental and control recipient groups.

Summing up the results of these four series of pseudo-conditioning experiments, it seems that the real conditioning treatment of the donors is not the prerequisite of the successful transfer effect. The CS and US administered in a random sequ ence do not enhance the donors' avoidance learning, but the brain extract of these donors does facilitate the conditioning of the recipients.

Two possibilities can be presumed:

Firstly, the facilitation of learning by brain extracts is not a specific transfer phenomenon. Or, at least the situation created in the donor animals' environment by the random administration of the two stimuli CS and US evoked a so-called "state dependent" learning, the molecular agents of which can be transmitted to the recipient rats.

The second possibility of some kind of peculiar specificity cannot be excluded either. If the donors received homogeneous stimuli, i.e. auditory or electric stimuli, the facilitatory effect did not occur. The donors' brain extract accelerated the recipients' learning only if the so-called pseudo-conditioning was carried out by the administration of two heterogeneous stimuli acting on two distract populations of neurones in the brain. According to Pavlovian rules the preliminary condition of learning is the effect of at least two stimuli evoking two distinct neuronal events; the monotonous stimulus leads to inhibition.

In the following our second experimental programme will be described related to some components of the RNA-fractions which might be responsible for the successful transfer effect. We compared the effect of the so-called "complete" ribosomal RNA fraction (found successful in our published experiments) with that of a high molecular and a low molecular weight fraction. The gel filtration has been carried out by Sephadex G50 separating the fractions above and below 10,000 molecular weight (Table 2).

(1) In the first series of this programme (groups I to IV) we made a comparison between the effect of the high and low molecular weight fractions and the brain extract of untreated control donors. From the four groups of this series we found a slight positive effect only in groups I and III, but not in groups II and IV.

TABLE 2

Group No.	Numbers of donors: Naives — Complete RNA-extract (1)	Numbers of donors: Trained — Complete RNA-extract (2)	Numbers of donors: Trained — RNA-extract MW below 10,000 (3)	Numbers of donors: Trained — RNA-extract MW above 10,000 (4)	Number of recipients	Significance* p
I	⑳		20	⑳	60	$1\text{–}4 < 0{\cdot}05$
II	20		20	20	60	
III	⑳		⑳	20	60	$1\text{–}3 < 0{\cdot}05$
IV	20		20	20	60	
V	⑮	⑮	15	15	60	$1\text{–}2 < 0{\cdot}02$
VI	㉕	㉕	25	25	100	$1\text{–}2 < 0{\cdot}02$
VII	20	20		20	60	
VIII	⑳	⑳		20	60	$1\text{–}2 < 0{\cdot}02$
IX	⑳	⑳		⑳	60	$1\text{–}2 < 0{\cdot}02$ $1\text{–}4 < 0{\cdot}05$
X	⑳	⑳		20	60	$1\text{–}2 < 0{\cdot}02$
XI	⑳	⑳	20		60	$1\text{–}2 < 0{\cdot}05$
XII	⑳	⑳	20		60	$1\text{–}2 < 0{\cdot}05$ $1\text{–}3 < 0{\cdot}05$
XIII	⑳	⑳	20		60	$1\text{–}2 < 0{\cdot}02$
XIV	20	20	20		60	
Total	280	200	200	200	880	

* The significance has been calculated between the performance of animal groups appearing in the table in circles.

(2) In another series (groups V and VI) we compared the effect of high and low molecular weight fractions not only with control donors, but with the trained donors' complete RNA-extract. Surprisingly enough in both groups (V and VI) the complete RNA-fraction proved to be significantly effective, while the high and low molecular weight fractions turned out to be without any facilitating influence.

(3) The same unexpected result was found in the third series, in groups VII to XIV. The gel filtered fractions had somehow "lost" their enhancing effect, whereas the complete RNA-extract was unequivocally effective.

In Fig. 6 the average learning curve of 200 recipient rats injected with trained donors' complete RNA-extract is represented as compared with 200 control

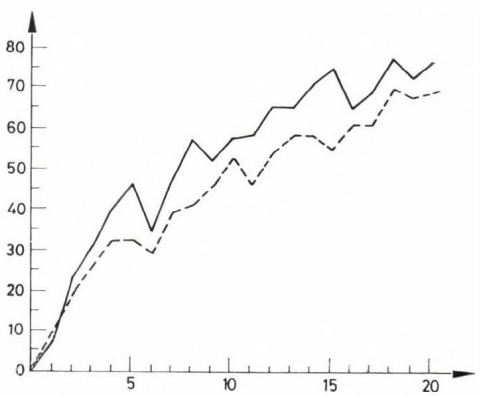

Fig. 6. Average learning curve of 200 recipient rats injected with trained donors' complete RNA-extract as compared with 200 control recipients.

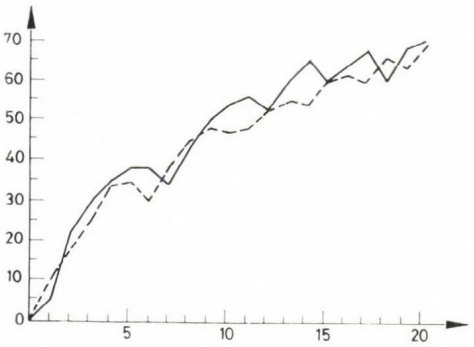

Fig. 7. Average learning curve of 200 recipient rats injected with trained donors' low molecular weight fraction (below 10 000).

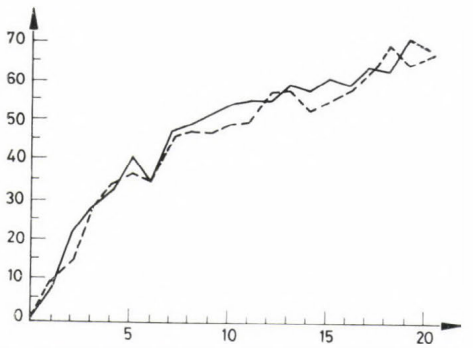

Fig. 8. Average learning curve of 200 recipient rats injected by trained donors' high molecular weight fraction (above 10 000).

recipients. The difference is obvious and statistically significant. On the contrary, neither the low molecular weight fraction (Fig. 7) nor the high molecular weight extract (Fig. 8) showed any facilitating influence.

In conclusion, the chemical approach of the fraction responsible for the successful transfer effect has caused some difficulty for us. The complete RNA-extract is in most cases effective, whereas its two Sephadex-fractions become ineffective. Principally this fact can hardly be explained: the gel filtration is a very fine method causing no structural alterations in the molecular structure and a minimal loss of the brain tissue. According to Professor Ungar's findings, the effectiveness of dailysable transfer agent depends on the integrity of its peptide linkages and has a tendency to form complexes with RNA. It is not unlikely that our RNA-extract contained this factor in a loose bond which might explain its effectiveness.

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DISCUSSION

G. Ungar: I agree with Dr. Faiszt's and Dr. Ádám's conclusion concernnig the loss of the active material in course of gel filtration. The low molecular substances must have come out in their procedure. As far as the specificity problem is concerned, I do not believe that it can be solved by expe-

rimental designs in which the recipients are reinforced. In this case all you can say is that the learning rate is accelerated. We know that this can be done by many non-specific substances (drugs, yeast, RNA, etc.). The problem of specificity can be attacked only if the recipients are not reinforced so that they do not learn anything during the test. This has been done with conditioned avoidance and with many other situations. All our experiments of the last three years were done without reinforcing the recipients.

G. Ádám: Thank you for your comment, Dr. Ungar, but as you heard, we used a quite different approach from yours. In a previous work published in *Nature* we demonstrated that the non-reinforced recipients—at least in our experimental situation—were less effective than those animals who were familiar with the experimental setup, i.e. who were reinforced. To avoid such discrepancies between experimental situations, a standard design should be worked out among all laboratories engaged in transfer research.

F. Rosenblatt: How "pseudo" was your pseudo-conditioning? If I were a rat in a situation in which I could escape from a shock by jumping to a shelf and a sound occurred at intervals, I think I would tend to associate that sound with the shock-situation.

G. Ádám: I agree with you, Dr. Rosenblatt. As we have mentioned in our lecture, if the donors received homogeneous stimuli, i.e. auditory or electric stimuli, the facilitatory effect did not occur. The donors' brain-extract enhanced the recipients' learning only in the case if the stimuli had been administered heterogeneously: e.g. auditory and electric stimuli in a random sequence. Thus a peculiar kind of "learning" cannot be excluded.

F. Klingberg: Did you observe intertrial responses, that means between the presentation of stimuli during the pseudo-conditioning, and how did you evaluate them?

G. Ádám: I guess you are thinking of our donor animals' intertrial responses. In these rats the preliminary condition of finishing their training had been the lack of spontaneous intertrial jumpings.

J. Szentágothai: I wish to put forth a question which I think is essential: Why do not people while working with transfer phenomena try to use not only brain-extracts, but also extracts from other organs? I believe that this would be an essential control. Do we have any information on this?

G. Ungar: Yes, we used muscle and liver extracts in our experiments and never obtained positive results with any other tissue except the brain.

G. Ádám: So far we have performed a body of experiments with an average of about three thousand recipients in our laboratory and under certain conditions found a positive so-called "transfer" effect, i.e. an enhancing or facilitating influence. We also tried liver-extract: it was ineffective.

G. Horn: Brain homogenates are usually used in transfer experiments. Has anyone used control injections containing more restricted parts of nerve tissue: autonomic ganglia, peripheral nerve, spinal cord, or various parts of the sensory pathways?

F. Rosenblatt: We have reported an early experiment (using the Jacobson technique) in which we observed a stronger effect from cerebral extract

than from brain-stem extract. However, the complexity of the dose-response curve and the presence of inversion effects in our later experiments, made it essential to find some more adequate technique which gives a reliable dose-response diagram. These comparative experiments should be repeated with our new techniques.

J. V. McCONNELL: I am glad that the experiments on mammals proved to be successful. I must remind you that in my own studies with planarians specific transfer effect occurred cannibalistically. Donors trained to go to a special alley in a maze were fed to untrained cannibals. One group of cannibals were fed with donors trained to go to the dark alley: this was the best recipient group. Another group of planarians was trained to go to the light alley: the cannibals fed by them constituted the second best group. The third group of recipients has been fed with both light-trained and dark-trained donors: this group was significantly inferior to the other two groups. I cannot interpret this experiment in any other way, but thinking of some specific memory transfer effect.

G. ÁDÁM: I think the main problem in all our papers presented here or published in the literature, is the existence of a great variety of experimental situations. As you know, there is a controversial discussion in the literature about the difference in the brain mechanism between the classical (type I) and the operant (type II) conditioning. Now, as we have heard from Dr. Ungar, Dr. McConnell and other colleagues, we can speak about type III, type IV, etc. conditioning approaches. Our laboratory has been dealing with several conditioning techniques for other purposes in the last 10 years. But for the problem under discussion we have chosen the avoidance training, because it is a very simple, reproducible and easily discriminable task. As I mentioned earlier, it would be advisable if we could agree upon a common and very simple test which could be repeated in different laboratories represented here at this meeting. It would be desirable that an agreement should be achieved on some tests on specificity. I do not think, e.g. that the right-left situation which was so heroically trained with several thousands of animals in Dr. Rosenblatt's laboratory, is the most suitable test. It seems to me too sophisticated. We ought to choose a very simple situation for the animals: an approach training or an avoidance conditioning in a very unequivocal manner. This training programme should be repeated in a stereotyped manner by several laboratories to decide the elementary and very important basic question of the specificity of this "transfer" effect.

F. KLINGBERG: Both Professors Szentágothai and Ádám raised the question about the specificity of the transfer effects. We are in possession of a great body of facts about the biochemical basis of different sensory systems and several motivational processes. This might be the specificity of not only synapses, but also of different parts of the brain. The main question is: have we any evidence that memory contents or concrete information can be transferred? I have never made such experiments, but I suppose that the reported results reflect only a gross specificity, that means rather a facilitating effect on the system which has been involved during the training of the donors.

There are some questions unresolved, as for example why the transfer of homogenates from the whole brain does not provoke a great variety of behavioural effects, why is the effect restricted to the type of reaction which the experimenter expects to see. All this leads to the suggestion that this phenomenon is not a real transfer of some memory content of concrete experiences or information. The report of Dr. Faiszt and Professor Ádám may throw some light on the problem. It is very important to pay attention to the level of activity in the different situations of several systems and parts of the brain. We all know that this level of activity is maintained by biochemical mechanisms or vice versa: certain biochemical substances may cause non-specifically a certain state of general behavioural activity. I feel that the transfer experiments show evidently a general biochemical background involved in learning situations but the concrete content of these plastic processes depends on very concrete neuronal circuits and nets.

F. Rosenblatt: First of all I would like to comment on Dr Ungar's and Dr. Domagk's experiments. In each of these cases we have an example of highly polarized situations. For instance, in cases involving the training of the animal to do something in the red light which the fish strongly prefers and in a green light which the animal definitely does not prefer. In each of these cases we are really not dealing with a discriminative situation, but rather with the choice between doing something which the animal does not normally do or not doing it. The successful transfer effects that have been reported by Ungar, for example, have generally been in the direction of facilitating a trend which is normally present in these groups of animals, that is to say a bias which is likely to occur in any case. He selected his groups of recipients for a strong direct bias. These groups which are selected in this manner have a natural tendency subsequently to move somewhat in the direction of the light. Now what George Ungar is really doing in this case is to accentuate that natural tendency. Again I think that in the case of the fish experiments we must presume a very strong initial bias, which later tended to appear even in the control groups. In each case of these highly polarized situations when one tried to transfer the opposite effect, apparently there is difficulty and one finds that the controls and the experimental groups look very much alike. In non-polarized situations, as in our left-right experiments, we do not find such a gradient but again even a slight asymmetry can produce very profound disturbances in the results. I would also like to comment very briefly on Professor Ádám's suggestion that perhaps one should try for the simplest possible behaviour in transfer experiments. We have done in fact several experiments with simple Pavlovian conditioning of avoidance reflexes and things of this sort. We have not been very lucky in these attempts. I think that this is an empirical question: we do not know what is being transferred. We do not know how complex the optimum behaviour should be. Perhaps we needed a very simple test, and alternatively we could need a test with as many different situations and as much learning and information as possible, in order to optimize the results. The only way we can answer this question is to try a spectrum of different possibilities and find out empirically which is the effective one.

R. Galambos: A most useful outcome of this Symposium would be the description of a transfer experiment that would almost certainly give positive results in the hands of any competent experimenter anywhere in the world. I realize that a number of the experts collected here have for some two years now been attempting to design and agree upon such an experiment, and I wonder whether they can, at this time, put it on record for us.

CONCLUDING REMARKS

by the Chairman

H. Hydén

We are fortunate to have here at this meeting a group of people with many years' experience in the study of the transfer of behaviour. We have heard much about the necessary caution that should be taken in designing experiments and about the danger of bias. We have been advised about various experiments and their reliability. It seems to me, after all this, that the real importance of this meeting could be to agree upon experiments that would meet all requirements and could also be repeated in every laboratory. It would be worth while, and of great importance, if we could reach such an agreement.

No uniform opinion has been formed today regarding the specificity of the transfer phenomenon; we have heard several objections, and several unsolved problems have been put forward.

"I have a grand memory for forgetting"—this is a quotation from a novel leading us to the ultimate conclusion that in the end we have a condensed memory, a skeletonized memory of the experience.

It is to be hoped that this discussion will help us to get nearer to the truth about experimental design and specificity—at least.

Section IV

THE ROLE OF TRANSMITTER SUBSTANCES IN LEARNING AND RETENTION

THE ROLE OF BIOGENIC AMINES IN MEMORY CONSOLIDATION*

by

W. B. Essman

Queens College of the City University of New York, Flushing, U.S.A.

Over the past several years we have been concerned with the relationship between alterations in brain biogenic amines and the extent to which such changes are related to the memory consolidation process. The process of memory consolidation has been generally defined as the interval of time following training or learning, within which those cellular or multi-cellular events which lay down a more permanent trace for storage of the acquired experience, are susceptible to the disruptive effects of a variety of agents and/or events. The consolidation period has been demonstrated experimentally in a variety of paradigms, within which the duration of this period has been considered to last from several seconds to several hours. Differences in the definition of this temporal interval may well reside in differences between the species of animal utilized, the learning or training technique employed, the stimulus parameters employed in training, and the parameters associated with the disruptive agent or event. A temporal gradient for retrograde amnesia has usually been taken as good evidence for a consolidation period; such a relationship implies that, as the interval between learning or training increases, there is a decrease in the efficacy with which specific events cause a disruption in memory consolidation. Such disruption has usually been determined on the basis of a retention test or tests given at a time where the effects of the disrupted event are not longer in evidence. The relationship between brain biogenic amines and memory consolidation has been explored in our laboratory in several respects; published data have indicated that pharmacological agents, capable of modifying the brain level of 5-hydroxytryptamine (serotonin) can effectively antagonize or reduce the amnesic effect of electroconvulsive shock (ECS) given within a few seconds following learning (Essman, 1967). This observation was related to the finding that ECS elevated the whole brain serotonin level, while decreasing the whole brain level of ribonucleic acid (RNA). When the synthesis of brain RNA was accelerated and its level increased in mice by treatment with tricyanoaminopropene, the amnesic effect of post-training ECS was significantly reduced, and the extent to which RNA level was regionally affected by ECS in the drug-treated animals was considerably less marked than the predicted decrease in regional level observed

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in control animals (Essman, 1966). With the same compound, a relationship between drug dosage, change in brain RNA level, and the magnitude of post-training ECS was demonstrated; at a greater current intensity, ECS produced a greater incidence of retrograde amnesia in control animals, whereas the degree of antagonism toward the amnesic effect of electroshock was related to the extent to which RNA level was increased by the drug and the extent to which RNA level was decreased by ECS in experimental animals (Essman and Golod, 1968).

In an instance wherein a drug, specifically affecting the level of metabolism of brain serotonin, was utilized, the effect of ECS still resulted in a well negatively correlated change in brain RNA and serotonin; ECS led to an increase in brain serotonin turnover time, whereas under drug treatment conditions, where the post-training amnesic effect of ECS was significantly reduced, turnover time was decreased by ECS from a drug-induced elevation in this measure of brain serotonin metabolism. It was also determined that under the experimental conditions utilized, brain norepinephrine was not consistently altered by electroshock, nor was the activity of brain monoamine oxidase affected (Essman, 1968*a*).

Several pharmacological agents, generally classed as antidepressant compounds, have also been shown to reduce the incidence of retrograde amnesia produced by post-training ECS. These compounds, while they affect the turnover of brain serotonin differentially under control conditions, depending upon their central mode of action, generally had the same effect upon turnover following ECS, as described previously; where inhibition of MAO was not involved, turnover time, in the absence of ECS, was increased and, subsequent to ECS administration, turnover time was significantly decreased. Under control treatment conditions, as observed previously in other studies, turnover time was increased (Essman, 1968*b*).

The relationship between pharmacologically-induced changes in RNA synthesis and the effects upon regional changes in brain serotonin metabolism have been further explored to the extent where such changes are brought about by treatment with tetracyanopropene; the degree to which post-training ECS resulted in retrograde amnesia was reduced as a function of the duration of such treatment. For specific regions of the mouse brain, serotonin turnover rate was increased, specifically in the corpus callosum and hypothalamus, as RNA synthesis and level were increased in these regions. In single cells of the diencephalon ECS reduced RNA concentration by approximately 21 per cent, whereas for this same region, the turnover time of serotonin was increased. The reduction in serotonin turnover time, paralleling these drug treatment conditions, as observed in the corpus callosum and hypothalamus, implicated a possible functional role of glia in accounting for such changes, and further reinforcing the possible functional interrelationship between RNA and serotonin as a basis for dealing with the memory consolidation process on a molecular level (Essman and Essman, 1969).

An example of the extent to which whole brain RNA level and serotonin level are affected by ECS may be seen in Fig. 1, where these concentrations were obtained from mice ten minutes following the application of a trans-

corneal current of 10 mA. As previously indicated, ECS led to a decrease in RNA level of a correlated increase in serotonin concentration.

The apparatus utilized for the behavioural portion of the experiments consists of a double chamber apparatus, with a clear lucite vestibule adjoining a larger, opaque, box, the floor of which consists of grids, wired in series through a sham-operated grid scrambler to a 700 V power supply. Contact by the mouse between any two grids on the floor of the inner chamber provides for a foot shock of 3 mA for three seconds, following which the animal is removed from the apparatus. The training trial consists of placing the animal into the outer chamber, from which it enters the inner chamber through a 2-inch diameter hole, usually within ten seconds, into the more preferred darkened box, where foot shock is administered. Electroconvulsive shock is given following this training trial, usually at ten seconds, and a single testing trial for retention of the avoidance response is given 24 hours later. In untreated control animals there is usually a low initial latency to respond by stepping into the darkened chamber and, on the testing trial, avoidance behaviour is characterized by significantly elevated latencies to respond by entering the chamber in which foot shock was previously given, or a complete failure to make such an entry response by remaining in the outer vestibule. In the animals given post-training ECS, a retrograde amnesia is usually observed on the testing trial; this is characterized by a failure to show avoidance, and the animal usually re-enters the chamber in which foot shock was previously given, showing a latency to respond, which is essentially comparable to that shown on the training trial. Animals not given foot shock on the training trial re-enter on a testing trial, showing comparable response latencies. This technique has been shown to result in a remarkably stable degree of avoidance behaviour, which is still apparent in animals tested at periods of several months following a single conditioning

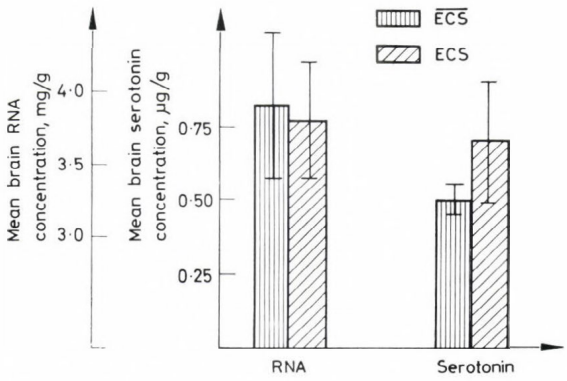

Fig. 1. Mean ($\pm\sigma$) whole brain serotonin of ribonucleic acid (RNA) and 5-hydroxytryptamine (5-HT) with 20 minutes following the administration of electroconvulsive shock (ECS) or sham-shock treatment ($\overline{\text{ECS}}$).

trial. In the mice given post-training ECS, the retrograde amnesia is relatively permanent in that avoidance behaviour does not appear to be reinstated when a retention trial is given at later times.

An initial series of experiments was concerned with the extent to which changes in brain serotonin level, as approximating those produced by ECS, would affect the incidence of conditioned response acquisition. In this study, 5-hydroxytryptamine was administered systemically in doses which have previously been shown to result in increases in brain concentration of approximately 20 to 30 per cent. At lower doses, the amine does not effectively cross the blood-brain barrier. Animals were given either physiological saline, or 5-HT in doses of 75 or 100 mg/kg, i.p., one hour prior to being given a single training trial in the apparatus, previously described. Under each of the drug or saline conditions, foot shock was administered at either 3 mA or 6 mA, and a single testing trial for the retention of the avoidance behaviour was given 24 hours later. Independent assessment of the effects of 5-HT treatment, as described, upon threshold to foot shock at the same current levels used in conditioning, indicated that such thresholds were not affected. The results, indicating the per cent incidence of conditioned responses established under these conditions, are summarized in Fig. 2. It may be seen that there was only a very slight reduction in the incidence of conditioned response acquisition at both 5-HT doses, where the foot shock was 3 mA (10 per cent). At the higher foot shock intensity there was a 22 per cent reduction in conditioned response acquisition in the mice given 75 mg/kg of 5-HT, and a 62 per cent reduction in response acquisition in mice given 100 mg/kg of 5-HT. These findings suggest that higher intensity foot shock, which is less susceptible to the amnesic effect of ECS, is affected to a greater extent by increases in brain 5-HT level, resulting from systemic injection. This finding may also suggest that a more stable conditioned avoidance behaviour, as produced by a more intense conditioning stimulus, is consolidated less efficiently where 5-HT brain levels are elevated at the time of conditioning. Brain 5-HT levels were not affected by foot shock under any of the experimental conditions described.

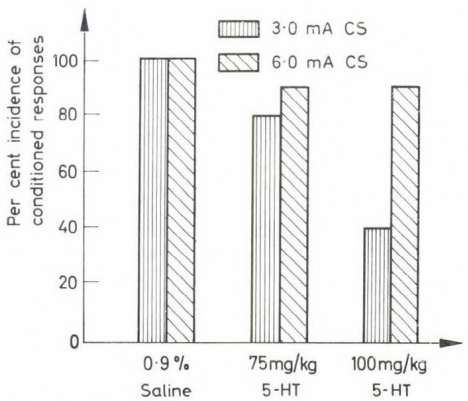

Fig. 2. Per cent incidence of conditioned response acquisition in serotonin (5-HT) and saline-treated mice trained for response acquisition at two stimulus intensity levels.

In a further study of the relationship between changes in brain 5-HT level and the susceptibility of mice to the amnesic effect of post-training electroshock, groups of animals were given i.p. doses of the serotonin precursor, 5-hydroxytryptophan (5-HTP). These doses varied from 3·12 to 100 mg/kg and were administered one hour prior to a single training trial, followed ten seconds later by ECS (10 mA). A 3 mA, 3 second foot shock was utilized

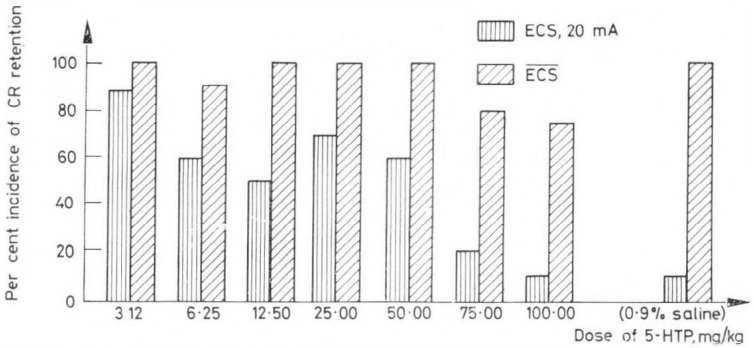

Fig. 3. Per cent incidence of conditioned response retention following ECS or $\overline{\text{ECS}}$ in 5-hydroxytryptophan (5-HTP)-treated mice.

on training, and control animals, under each of the drug and saline treatment conditions, were given sham ECS ($\overline{\text{ECS}}$), consisting of application of the corneal electrodes only, with no current passed. The results of this experiment are summarized in Fig. 3, showing the per cent incidence of conditioned response retention under ECS and $\overline{\text{ECS}}$ conditions for drug- and saline-treated animals. It may be observed that saline-treated animals receiving sham ECS showed a predicted high incidence of conditioned response retention, whereas for the saline-treated animals given ECS the incidence of conditioned response retention was reduced to approximately ten per cent. At doses of 5-HTP, above 50 mg/kg, the incidence of acquisition of the conditioned response was reduced and the amnesic effect of post-training ECS was highly effective. The surprising finding in this study was the failure of post-training ECS to significantly reduce the incidence of conditioned response retention in the animals treated with low doses of 5-HTP. This is particularly apparent in the animals treated with 3·12 mg/kg. In order to assess this finding more carefully, the experiment, as described above, was replicated with mice given either 3·12 mg/kg of 5-HTP or an equivalent volume of physiological saline. One hour following drug treatment, animals were given either ECS or $\overline{\text{ECS}}$. ECS was given at either 10 mA or 20 mA following a single training trial and retention tests were given 24 hours following training. It was also established, under these conditions, that there was no alteration in foot shock threshold produced by 5-HTP treatment.

The results of the behavioural portion of this study are summarized in Table 1, from which it may be observed that saline-treated animals, given $\overline{\text{ECS}}$, showed the predicted high incidence of conditioned response retention, and that the 20 mA ECS in the saline-treated animals resulted in a higher incidence of retrograde amnesia than did the 10 mA ECS. The 5-HTP-treated mice given ECS showed the same high incidence of conditioned response retention, again indicating that these drug-treatment conditions

TABLE 1

Median response latency difference and per cent incidence of conditioned response retention and maximum testing trial latency for saline- and 5-hydroxytryptophan- (5-HTP)-treated mice given ECS (10 or 20 mA) or sham ECS (ECS) following training

Treatment		Median response latency difference (testing-training)	Per cent incidence of cond. resp. retention	Per cent incidence of maximum testing trial latency ($\geq$ 30 sec)
Injection	Post-training			
5-HTP (3·12 mg/kg)	$ECS_{10\ mA}$	27·0	88	67
5-HTP (3·12 mg/kg)	$ECS_{20\ mA}$	19·0	88	47
5-HTP (3·12 mg/kg)	$\overline{ECS}$	25·0	100	95
Saline (0·9%)	$ECS_{10\ mA}$	2·0	30	15
Saline (0·9%)	$ECS_{20\ mA}$	−3·5	28	0
Saline (0·9%)	$\overline{ECS}$	24·5	100	100

did not alter conditioned response acquisition. Significant attenuation of the amnesic effect of ECS was again apparent in mice treated with 3·12 mg/kg of 5-HTP. This occurred at both ECS intensity levels, with a slightly greater degree of antagonism toward amnesia achieved at the lower ECS intensity.

Table 2 summarizes the statistical analysis of the comparisons between saline and drug treatment and ECS intensities insofar as the incidence of criterion conditioned response retention is concerned. Table 3 summarizes the statistical analysis for the incidence of maximum response latency incidence on the retention testing trial. In general, these findings indicate that at a low dose of 5-HTP the amnesic effect of post-training ECS is significantly reduced and that this reduction appears to be an inverse function of the intensity of the amnesic stimulus.

TABLE 2

Chi-square analysis of incidence of criterion conditioned response retention in saline- and 5-hydroxytryptophan- (5-HTP)-treated mice given ECS at 10 mA or 20 mA

Comparison	*df*	χ^2	*p*
Between Groups			
($Saline_{10\ mA}$, $5\text{-}HTP_{10\ mA}$, $Saline_{20\ mA}$, $5\text{-}HTP_{20\ mA}$)	3	25·56	$<0·001$
$Saline_{10\ mA}$ vs. $5\text{-}HTP_{10\ mA}$	1	13·19	$<0·001$
$Saline_{20\ mA}$ vs. $5\text{-}HTP_{20\ mA}$	1	12·08	$<0·001$
$Saline_{10\ mA} + 5\text{-}HTP_{10\ mA}$ vs. $Saline_{20\ mA} + 5\text{-}HTP_{20\ mA}$	1	0·29	n.s.

TABLE 3

Chi-square analysis of incidence of maximum response latency ($\leq$ 30 sec) for saline- and 5-hydroxytryptophan-(5-HTP)-treated mice given ECS at either 10 mA or 20 mA

Comparison	*df*	χ^2	*p*
Between Groups ($Saline_{10\ mA}$, $5\text{-}HTP_{10\ mA}$, $Saline_{20\ mA}$, $5\text{-}HTP_{20\ mA}$)	3	23·82	$<0{\cdot}001$
$Saline_{10\ mA}$ vs. $5\text{-}HTP_{10\ mA}$	1	11·63	$<0{\cdot}001$
$Saline_{20\ mA}$ vs. $5\text{-}HTP_{20\ mA}$	1	8·74	$<0{\cdot}01$
$Saline_{10\ mA} + 5\text{-}HTP_{10\ mA}$ vs. $Saline_{20\ mA} + 5\text{-}HTP_{20\ mA}$	1	3·45	$>0{\cdot}05$

The mean whole brain concentration of serotonin and the turnover of this amine in the brain are summarized in Table 4. It may be observed that the effect of 5-HTP treatment was, as expected, to elevate whole brain 5-HT level under $\overline{ECS}$ conditions; the effect of ECS was to elevate brain 5-HT level with a small, statistically insignificant difference, depending upon the current intensity of the ECS. 5-HIAA level was also increased by ECS in both the saline control groups as well as in the drug-treated groups. As observed in previous studies with other pharmacological agents in doses which serve to attenuate the amnesic effect of electroshock, drug treatment led to an increase in brain 5-HT turnover time, and the increase in turnover time produced by ECS appeared to be a function of the current intensity; i.e., a greater increase in turnover time occurred at the 20 mA level and at the 10 mA level.

TABLE 4

Mean ($\pm\ \sigma$) brain serotonin (5-HT) concentration and turnover in saline- and 5-hydroxytryptophan- (5-HTP)-treated mice given ECS (10 or 20 mA) or sham ECS ($\overline{ECS}$)

Determination	Treatment					
	5-HTP			Saline		
	$ECS_{10\ mA}$	$ECS_{20\ mA}$	$\overline{ECS}$	$ECS_{10\ mA}$	$ECS_{20\ mA}$	$\overline{ECS}$
5-hydroxytryptamine (Serotonin — μg/g)	1·09 (0·47)	1·12 (0·16)	0·85 (0·11)	0·71 (0·22)	0·78 (0·14)	0·50 (0·06)
5-hydroxyindoleacetic acid (μg/g)	0·60 (0·09)	0·61 (0·10)	0·46 (0·08)	0·39 (0·07)	0·42 (0·09)	0·29 (0·05)
5-HT turnover rate (μg/g/hr.)	0·61	0·58	0·51	0·45	0·42	0·35
5-HT turnover time (min.)	107	116	110	94	111	86

These findings indicate, in general, that a low dose of the 5-HT precursor, 5-HTP, leads to an increase of approximately 70 per cent in whole brain 5-HT level, an increase in 5-HT turnover rate of approximately 33 per cent, and an increase in 5-HT turnover time of approximately 30 per cent. This would suggest that at this low dose of 5-HTP the synthesis and degradation, as well as the level of brain 5-HT are increased. Within 20 minutes after ECS administration, one hour following drug injection, turnover time in experimentally-treated animals was less markedly increased than it was in control animals.

Another approach to the question of differences in memory consolidation, as a function of differences in the metabolism of brain serotonin, was explored in an experiment in which two discrepant age populations of mice were utilized. Laboratory-bred mice were sexed at three days of age and housed (5/cage) until they reached either 23 or 43 days of age, at which time baseline locomotor activity levels were again obtained, and animals were given a 3 mA foot shock for one minute in the activity box. This technique serves, in general, to suppress locomotor activity and represents another type of single trial avoidance conditioning technique. Animals were tested for 15 minutes of locomotor activity 24 hours following the training session, and brain tissue was obtained from all experimental animals and control animals, and from non-behavioural participant mice from the same age groups. Figure 4 shows the extent to which locomotor activity was suppressed as a function of age. Basal activity levels were approximately 63 per cent higher in the 45-day-old animal as compared with the 25-day-old animal, and activity levels in the 45-day-old group were still 13 per cent higher than in the 25-day-old animals when tested 24 hours after the foot shock. The younger mice showed conditioned activity suppression of only 17 per cent, as compared with the 42 per cent suppression in locomotor activity shown by the older animals.

Fig. 4 shows that the whole brain concentration of 5-HT and 5-HIAA are somewhat higher in the older mice, and a comparison of behaviourally

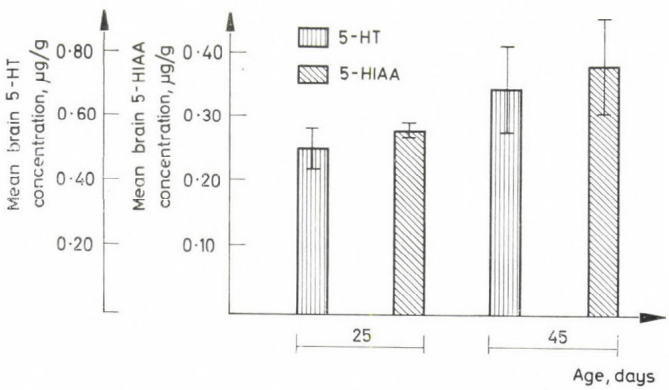

Fig. 4. Mean whole brain serotonin of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in mice of 25 and 45 days of age.

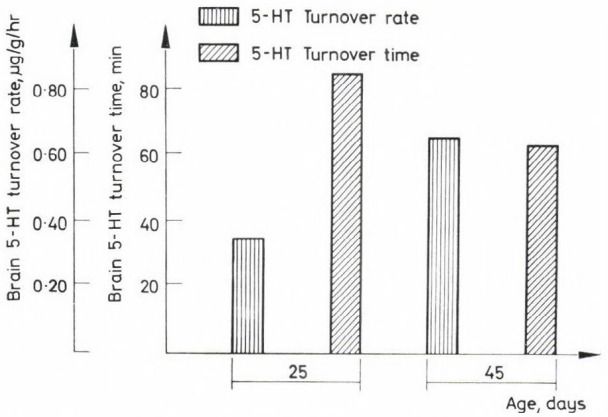

Fig. 5. Brain 5-hydroxytryptamine (5-HT) turnover in mice of 25 and 45 days of age

utilized mice, with non-behavioural participants, yielded essentially no difference in amine level for each of the respective age groups.

Figure 5 shows that turnover rate at 25 days of age is lower, and turnover time is higher than these measures of 5-HT synthesis and metabolism at 45 days of age. These findings suggest that the synthesis and degradation of brain 5-HT is greater in older animals, and this finding may possibly relate to the behavioural differences observed under these discrepant age conditions. These findings are related to our previous studies, inasmuch as they may reflect differences in capacity to acquire and express new behaviour, as related to brain 5-HT metabolism.

A further consideration of age differences in learning and, specifically, memory and its bearing to the role of brain serotonin was considered during early development in the mouse. Mice, ranging in age from 15 to 30 days,

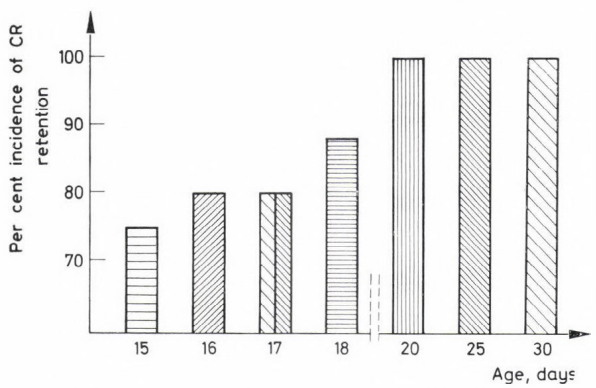

Fig. 6. Per cent incidence of conditioned response acquisition in mice at different ages

were tested for their capacity to acquire the one-trial conditioned passive avoidance response, previously described. A relatively high incidence of acquisition was noted among these groups of animals with 75 per cent, or more, of animals in all age groups showing stable response acquisition. These findings are summarized in Fig. 6.

In another series of experiments, groups of mice, ranging in age from 15 to 30 days, were given a single conditioning trial, followed either immediately (0 = within 10 seconds), 10 minutes (10), 20 minutes (20), or 60 minutes (60) later by a single transcorneal ECS of 10 mA. These animals were tested for retention of the conditioned avoidance response 24 hours following training, and the results are summarized in Fig. 7.

There are two very apparent findings which emerge from these data; one is a clearly indicated temporal gradient for the amnesic effect of electroshock, such that as the training-ECS interval is increased, the per cent incidence of retrograde amnesia, produced by ECS, becomes decreased. The most apparent amnesic effects of ECS, therefore, are seen when this stimulation is given whithin approximately ten minutes following the training trial, although at 20 minutes there is still some apparent degree of amnesia, as compared to the 60-minute group in which 100 per cent retention is shown on the 24-hour test. The second finding apparent in these data is the obvious departure from the age trend in susceptibility to the amnesic effect of ECS at all training-ECS intervals; this clearly occurred in mice of 17 days of age in which, particularly in the 0 and 10 minute group, there is a statistically significant reduction in the incidence of ECS-induced retrograde amnesia. The group given ECS immediately following training showed 80 per cent retention on the testing trial, whereas at all other training-ECS intervals, 100 per cent of these animals showed retention of the avoidance behaviour.

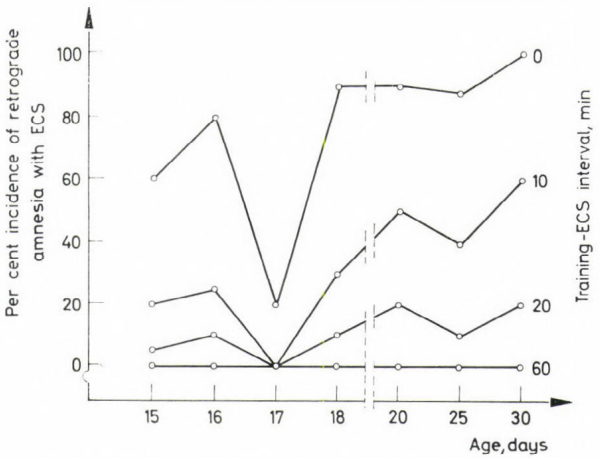

Fig. 7. Incidence of conditioned response retention in mice at several ages given post-training ECS at several intervals following a single training trial.

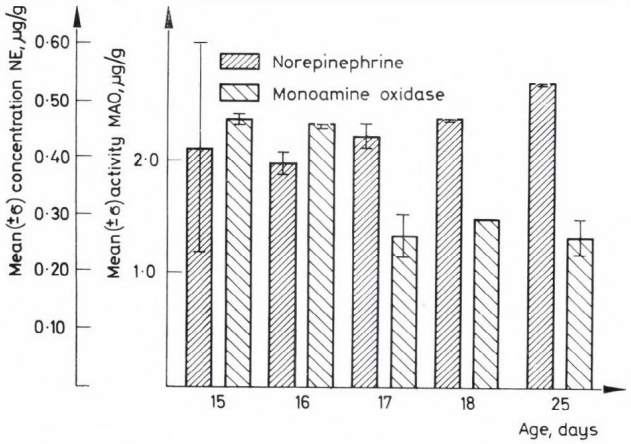

Fig. 8. Mean brain serotonin of norepinephrine and monoamine oxidase activity in mice of several ages.

Amine levels were measured under identical age differences up to 25 days of age, and as may be observed in Fig. 8, whole brain norepinephrine level did not vary within this age range, as a function of age. Brain monoamine oxidase activity decreased from 16 to 17 days of age and remained lower up to 25 days of age; however, specific differences, except for the 16 to 17 day change in this enzyme, were not apparent.

Brain serotonin and 5-hydroxyindoleacetic levels are summarized in Fig. 9, from which it may be observed that the amine and its metabolite

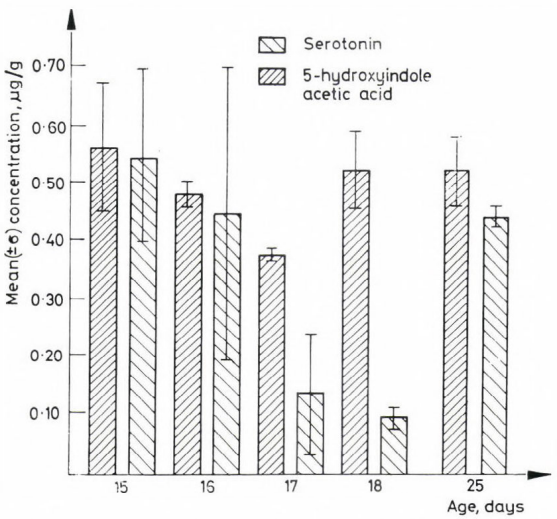

Fig. 9. Mean brain 5-HT and 5-HIAA levels in mice at several ages.

decrease gradually from 15 to 17 days of age, and after 17 days of age, 5-HIAA is again elevated, although 5-HT remains low by 18 days of age. The turnover of whole brain serotonin was also measured, and it may be seen that within the age range studied, 17-day-old animals have the highest turnover rate and the lowest turnover time, as compared to prior and subsequent ages. It may, therefore, appear that 17 days of age, in the male CF-1 mouse, may represent a critical age at which the synthesis and metabolism of brain serotonin is such that the effects of ECS are only minimal in producing those changes which are capable of disrupting such metabolism to the degree to which this becomes necessary to account for an interruption of the memory consolidation process. The well-correlated change at 17 days of age in serotonin concentration, turnover, and resistance to the amnesic effect of electroshock leading to retrograde amnesia suggest another level of analysis on which the role of serotonin in the memory consolidation process may be considered.

Another means by which differences in brain serotonin concentration and metabolism may be obtained is through differential housing. Prior studies (Essman, 1969) have indicated that there are regional differences in brain 5-HT concentration and turnover which are brought about as a result of isolation housing. Figure 10 summarizes some of these differences in brain norepinephrine, serotonin, and 5-hydroxyindoleacetic acid in isolation-housed mice (28 days of isolation, commencing at 25 days of age), as compared to aggregation housing (housing 5/group for 28 days, commencing at 25 days of age). It may be observed that 5-HT and 5-HIAA are somewhat elevated as a consequence of isolation. Differences in turnover rate (Fig. 11) and turnover time (Fig. 12) for several regions of the mouse brain indicate generally that isolation housing leads to reduced turnover time, a finding which, in the light of previous studies, would allow for the prediction that

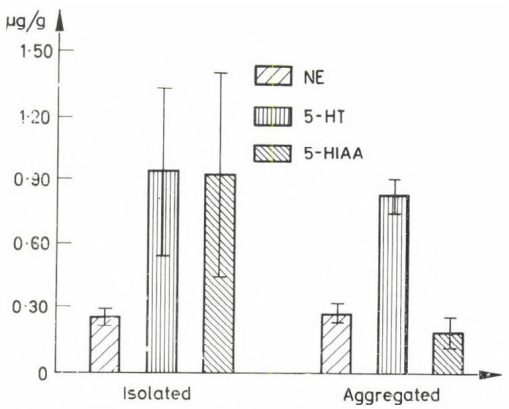

Fig. 10. Differences in brain serotonin of norepinephrine, 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid in isolated and aggregated mice.

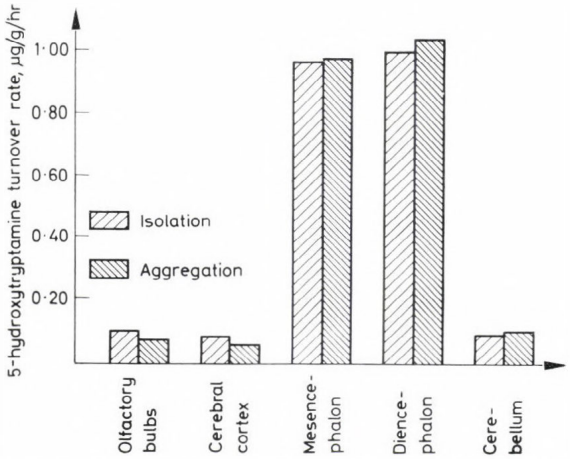

Fig. 11. Brain serotonin turnover rate in isolated and aggregated mice.

under these conditions one might expect a reduced rate of learning or a more poorly consolidated memory trace, or a memory consolidation process which may be more highly susceptible to the amnesic effects of post-training conditions. This hypothesis was tested, utilizing the single trial avoidance conditioning technique, previously described, and the results are summarized in Fig. 13. It may be observed that under ECS conditions isolation-housed animals showed approximately 30 per cent less conditioned avoidance retention, suggesting that the acquisition of the conditioned avoidance response was less efficient in this group. Taking this discrepancy into account

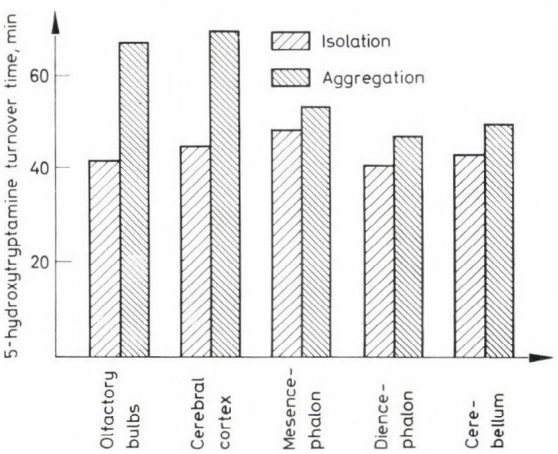

Fig. 12. Brain serotonin turnover time in isolated and aggregated mice.

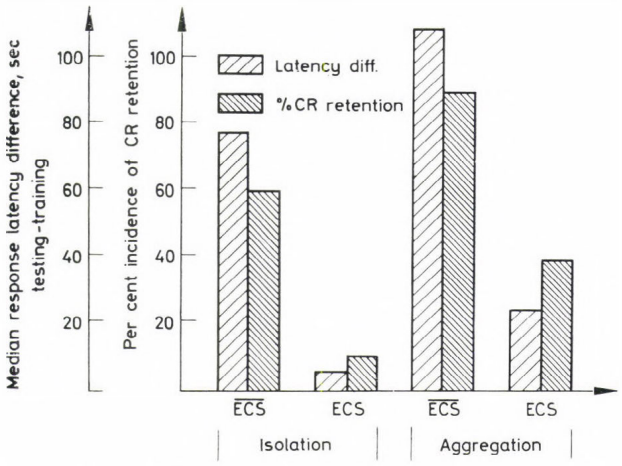

Fig. 13. Effect of post-training ECS or $\overline{\text{ECS}}$ upon conditioned avoidance response retention in differentially-housed mice.

in evaluating the amnesic effect of ECS administered 10 seconds after the training trial, it may be observed that the greater incidence (approximately 20 per cent more) of retrograde amnesia was produced by ECS in the isolation-housed group. These findings support the hypothesis that the changes in serotonin level and metabolism, produced by isolation, are consistent with a reduced incidence of response acquisition and an increased incidence of ECS-induced retrograde amnesia.

Considering the possibility that ECS might well exert different effects as a function of differential housing, and that these effects may well relate to processes affected by both isolation housing as well as ECS, the role of RNA in this interaction was specifically considered. It has been previously shown that isolation housing did not lead to any systemic changes in whole brain RNA level; however, in view of the intercorrelation between changes in RNA and serotonin, and changes in serotonin and RNA, the interaction of these molecules might allow for a prediction that conditions which provided for increased serotonin levels could, possibly, affect the synthesis and level of RNA. This relationship will be supported by both physical evidence and *in vivo* studies presented later in this paper. However, to treat this investigation in a chronological fashion, the RNA content per cell for different regions of the mouse brain was compared under $\overline{\text{ECS}}$ conditions and ECS conditions for isolation- and aggregation-housed animals. The change in RNA content per cell, as a consequence of ECS, is summarized for several areas of the mouse brain in isolation- and aggregation-housed animals, as shown in Fig. 14. A general decrement in RNA content per single cell may be observed to have occurred as a consequence of ECS in isolation-housed animals; the only possible exception to this finding occurred in the diencephalon, where the decrease in single cell RNA was approximately

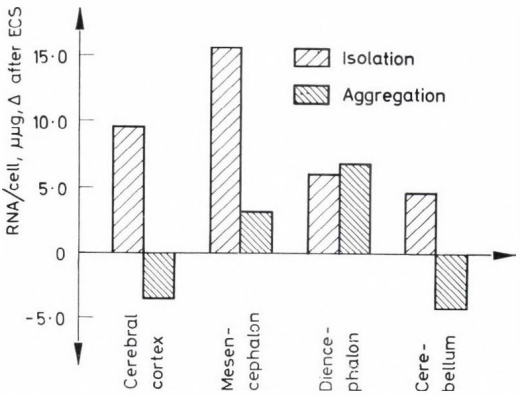

Fig. 14. Changes in RNA content per cell in several regions of the mouse brain following ECS administration in differentially-housed mice.

the same under both conditions of differential housing. These findings are consistent with the hypothesis that conditions leading to concentration and turnover changes in brain serotonin, which correlate with reduced learning and/or increased suscepibility to the amnesic effects of electroshock, also lead to an increase in the degree to which ECS results in an RNA decrement in single cells from several areas of the brain.

In an earlier study (Essman, 1969) it was indicated that, either as a result of prolonged isolation, or perhaps because of increased age, behaviours which developed as a consequence of isolation tend to become minimized, whereas, to some extent, the biochemical changes attending prolonged isolation tend to remain preserved. In order to consider this question in the light of the present theme, studies were carried out in mice subjected from weaning (21 days) to two discrepant periods of prolonged isolation, either 138 days or 152 days. Behavioural and biochemical determinations were obtained for these animals. Training and testing for retention of conditioned avoidance behaviour under ECS and $\overline{\text{ECS}}$ conditions was carried out, utilizing the previously described single trial technique, with retention trial given at 24, 48, 72 and 96 hours after training. The behavioural results are summarized in Table 5. As may be observed, $\overline{\text{ECS}}$ conditions resulted in a stable and high incidence of conditioned response acquisition and retention, whereas the animals isolated for 138 days showed an initially low incidence of conditioned response retention as a consequence of ECS; however, by 96 hours post-training, 60 per cent of these animals showed retention, indicating that the degree of retrograde amnesia produced by ECS was unstable over time, and reinstatement of a significant degree ($p < 0{\cdot}01$) of conditioned response retention was possible under these conditions. For the mice isolated for 152 days a high, stable degree of conditioned response retention was observed, indicating that ECS under these

TABLE 5

Median response latency (sec) and per cent incidence of conditioned response retention for mice following two periods of prolonged isolation housing

Duration of isolation (days)	Condition	Retention trial (hrs post-train.)			
		24	48	72	96
138	$\overline{\text{ECS}}$	120·0 (100)	120·0 (100)	120·0 (80)	120·0 (100)
	ECS	9·5 (20)	22·0 (20)	24·5 (40)	44·0 (60)
152	$\overline{\text{ECS}}$	120·0 (100)	120·0 (100)	120·0 (100)	120·0 (100)
	ECS	30·6 (75)	67·5 (75)	51·0 (75)	56·5 (75)

conditions was less effective in leading to a retrograde amnesia than under previously observed conditions. In Table 6, the concentration of serotonin and 5-hydroxyindoleacetic acid, and turnover data for brain serotonin are summarized for several regions of the mouse brain. It may be observed that increased turnover time for serotonin occurred in the cerebral cortex and diencephalon of mice isolated for 152 days. There was also a significantly lower level of 5-HIAA in the diencephalon of the 152-day isolates. These observations are consistent with the general prediction that such changes should lead to an attenuation of the amnesic effect of ECS and do, in fact, agree in this regard with the behavioural data.

TABLE 6

Mean ($\pm \sigma$) regional concentration (μg/g) of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), with turnover rate (μg/g/hr) and turnover time (min) of brain 5-HT for mice under two durations of prolonged isolation housing

Region	138 days				152 days			
	5-HT	5-HIAA	5-HT turnover		5-HT	5-HIAA	5-HT turnover	
			Rate	Time			Rate	Time
Cerebral cortex	0·17 (0·04)	0·18 (0·02)	0·14	46·62	0·25 (0·05)	0·17 (0·03)	0·20	70·20
Mesencephalon	0·46 (0·06)	0·27 (0·03)	0·37	82·20	0·46 (0·07)	0·36 (0·11)	0·37	61·20
Diencephalon	0·51 (0·04)	0·54 (0·02)	0·43	47·76	0·51 (0·06)	0·28 (0·02)	0·41	87·60

The effect of ECS on brain serotonin level and metabolism is summarized in Table 7 for the same regions of the mouse brain. It may be observed that whereas 5-HT level in the diencephalon was significantly increased as a consequence of ECS, the magnitude of this increase was appreciably lower in the 152-day isolates; the magnitude of the increase in turnover rate of brain 5-HT was also appreciably lower in the 152-day isolates, as was the change in turnover time, resulting from ECS. It appears, on the basis of the present findings, that possible differences in the incidence of stability of retrograde amnesia may be accounted for by differences in the magnitude of the change produced by ECS in 5-HT level and turnover, particularly as observed in the present study for the diencephalon. A further implication of these findings is that changes in brain serotonin level and metabolism and the effect of ECS upon these changes, as well as the ability of ECS to produce retrograde amnesia, appears to be a function of age, duration of isolation housing, and possibly of the interaction of these two variables. In any case, further detailed study of this phenomenon appears warranted.

In order to assess the effect of a reduction in brain amine level upon acquisition of avoidance behaviour and retention of an avoidance response, experiments were carried out in which: (*i*) training for acquisition of conditioned avoidance behaviour and the presentation of post-training ECS were given under conditions of reduced levels of brain norepinephrine and dopamine in mice; and (*ii*) training and post conditioning ECS were given to mice, in which brain serotonin levels were reduced.

Mice were given i.p. injections of 100 mg/kg of alpha methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase, the enzyme involved in the rate-limiting step in the formation of DOPA, which has been shown to lead to a reduction in brain level of both dopamine and norepinephrine. At several intervals following drug injection (immediately = 0, 4, 6, or 8 hours) groups of mice were given a single training trial for acquisition of the passive avoidance response, as previously described, and one group of animals at each of the respective post-injection times was given a single transcorneal

TABLE 7

Regional per cent change in 5-HT, 5-HIAA, and 5-HT turnover resulting from ECS given to mice under two durations of prolonged isolation housing

Region	138 days				152 days			
	5-HT	5-HIAA	5-HT turnover		5-HT	5-HIAA	5-HT turnover	
			Rate	Time			Rate	Time
Cerebral cortex	18	28*	14	11	16	0	15	16
Mesencaphalon	11	−11	11	26*	26*	−6	24*	33*
Diencephalon	118*	−15	107*	145*	49*	29*	46*	14

* $p < 0{\cdot}02$.

ECS (10 mA), while the remaining group, at each respective time, was given sham-ECS ($\overline{ECS}$). Testing trials were given at 24 and 48 hours following training. Under parallel conditions, mice given similar injection-training-ECS or $\overline{ECS}$ conditions were sacrificed within ten minutes following the completion of this sequence, and brain levels of norepinephrine, dopamine, and 5-hydroxytryptamine were determined from brain tissue. The results of this study are summarized in Table 8, from which it may be observed that the maximum degree of norepinephrine and dopamine depletion occurred four hours following injection, although these amines were depleted at six and eight hours following injection; however, to a lesser degree. Tissue obtained from animals at the zero time did not show any appreciable change from baseline levels observed in other studies, as might be expected. Brain serotonin level was unchanged as a function of drug treatment; however, as may be observed at the zero time, 5-HT level was elevated as a consequence of ECS, closely paralleling those observations reported earlier in this paper. The behavioural data closely parallel the biochemical data in one respect. The incidence of conditioned response acquisition, as measured by the testing trial given 24 hours following training, indicated that the acquisition of this avoidance response was impaired as a function of the degree to which brain norepinephrine and dopamine were depleted, i.e. the lowest incidence of CR retention in $\overline{ECS}$-treated mice occurred at four

TABLE 8

Median response latency difference, per cent incidence of conditioned response retention and mean brain amine levels in mice treated with alpha-methyl-p-tyrosine (100 mg/kg, i.p.), and trained with post-training ECS or $\overline{ECS}$ at various postinjection times

Time post-inj. (hrs)	Condition	Median response latency diff. (test.-time.)	Per cent incidence of CR retention		Mean ($\pm\sigma$) brain amine level (μg/g)		
			24 hrs	48 hrs	Norepinephrine	Dopamine	5-HT
0	ECS	110·0	10	10	0·54 (0·13)	1·03 (0·12)	1·01 (0·16)*
	$\overline{ECS}$	−2·0	100	100	0·48 (0·18)	0·98 (0·11)	0·83 (0·11)
4	ECS	55·0	65*	60*	0·18 (0·14)	0·34 (0·14)	0·88 (0·12)
	$\overline{ECS}$	1·0	20*	39*	0·21 (0·11)	0·36 (0·11)	0·76 (0·10)
6	ECS	111·0	22	44*	0·29 (0·09)	0·46 (0·13)	0·81 (0·12)
	$\overline{ECS}$	3·0	80	75	0·31 (0·06)	0·48 (0·11)	0·83 (0·09)
8	ECS	108·0	25	17	0·38 (0·14)	0·73 (0·09)	0·79 (0·13)
	$\overline{ECS}$	−2·0	95	80	0·37 (0·10)	0·76 (0·12)	0·84 (0·11)

* $p < 0{\cdot}02$.

hours following injection, with a slight reduction in CR retention at six hours, and no appreciable reduction at eight hours.

The amnesic effect of ECS, taking the reduced incidence of acquisition into account in evaluating this phenomenon, was significantly less pronounced in the four-hour group on both of the retention testing trials, and for the six-hour group, on the 48-hour retention testing trial. It is apparent that the effect of the drug treatment did not influence the retention test at either 24 or 48 hours, as indicated by the result of the zero-hour groups. It, therefore, appears that a reduction in norepinephrine and dopamine level can, as a function of the degree to which such depletion occurs, reduce the incidence of acquisition of avoidance behaviour. This observation has been made in other studies (Rech et al., 1968). One further observation is of interest with respect to the present data, and that is that the amnesic effect of ECS is significantly reduced in conditions wherein brain norepinephrine and dopamine levels are appreciably lowered in the brain. The possible relationship of this finding to the data reported earlier with respect to changes in brain serotonin may be of interest in this respect. It may be noted that brain serotonin levels in animals with reduced norepinephrine and dopamine concentrations were not obviously affected by ECS; the only condition under which there was an ECS-induced elevation of brain serotonin occurred in the zero-time group, where catecholamine levels were not yet affected. Under all other conditions, ECS had no apparent effect upon brain serotonin level, suggesting the possibility that one requirement for the amnesic effect of ECS is its ability to alter brain 5-HT level and, perhaps, such alterations are dependent upon the extent to which brain catecholamine levels are depleted. It should be further noted that the lowered levels of catecholamines had no effect upon foot shock threshold or upon the occurrence of a full clonicotonic convulsion produced by passage of a transcorneal current of 10 mA. It would, therefore, appear that any explanation of the results in terms of foot shock or electroshock threshold would not be warranted.

In another study, the effect of lowered brain 5-HT levels upon the acquisition and retention of conditioned avoidance behaviour was assessed. A reduction in brain serotonin level has been shown to follow the administration of p-chlorophenylalanine (PCP) through the inhibition of tryptophan hydroxylation (Koe and Weissman, 1966). We have previously shown (Essman, 1967) that such a drug-induced depletion of brain serotonin and 5-hydroxyindoleacetic acid results in a smaller elevation of brain 5-HT following the administration of ECS. The purpose of the present experiment, therefore, was to determine whether reduced levels of 5-HT and a small reduction in the degree to which 5-HT elevation occurred following ECS, might possibly play a role in the acquisition and retention of avoidance behaviour. It has been previously suggested (Woolley, 1965) that lowered levels of brain 5-HT lead to an improvement in maze performance by mice. The nature of the behavioural technique employed in our studies is such that acquisition differences probably cannot reflect any improvement in acquisition inasmuch as this appears to be a relatively all-or-none event in a single-trial conditioning situation; however, differences in the strength

of the acquired conditioned response should be reflected in the ability of post-training ECS to disrupt the retention of such behaviour.

Mice were injected with p-chlorophenylalanine, in doses of 100, 200 and 300 mg/kg, or with an equivalent volume of 0·9 per cent saline, 72 hours prior to being given a single training trial in the apparatus previously described. One group of animals under each of the respective injection conditions, was given a single ECS ten seconds following the training trial, whereas other groups for each of the respective conditions were given $\overline{\text{ECS}}$. A single testing trial was given 24 hours following training, and the incidence of CR retention was determined. Brain norepinephrine and 5-hydroxytryptamine levels were determined under parallel conditions following either ECS or $\overline{\text{ECS}}$ administration. The results of this study are summarized in Table 9, and indicate that brain 5-HT levels were depleted as a function of PCP dosage. It may be noted that the extent to which 5-HT depletion occurred in mice was appreciably less marked than the extent to which this has been reported in rats (Koe and Weissman, 1966). Brain norepinephrine levels appeared essentially unchanged under these conditions. The extent to which ECS resulted in elevated brain 5-HT level was less marked among PCP-treated mice than observed in the saline-treated controls; however, some degree of elevation occurred as a result of ECS under each condition. The behavioural data indicate that a reduction in brain 5-HT level did not alter the incidence of conditioned response acquisition and retention, and that the effect of post-training ECS was to result, in every case, in a sig-

TABLE 9

Median response latency difference (testing-training trial), per cent incidence of conditioned response retention, and mean brain amine levels in mice treated with p-chlorophenylalanine 72 hours pretraining

Treatment	Post-treat. cond.	Md. response latency diff. (sec)	% incidence of CR retention	Mean ($\pm\sigma$) brain amine level (μg/g)	
				NE	5-HT
Saline (0·9%)	ECS	0·0	5	0·62 (0·12)	0·94 (0·10)*
	$\overline{\text{ECS}}$	112·0	95	0·56 (0·14)	0·78 (0·11)
PCP (100 mg/kg)	ECS	—3·0	5	0·58 (0·10)	0·72 (0·14)*
	$\overline{\text{ECS}}$	115·0	95	0·54 (0·11)	0·65 (0·18)*
PCP (200 mg/kg)	ECS	—1·0	0	0·53 (0·11)	0·63 (0·11)*
	$\overline{\text{ECS}}$	111·0	100	0·57 (0·13)	0·56 (0·13)*
PCP (300 mg/kg)	ECS	—4·0	10	0·54 (0·12)	0·54 (0·13)*
	$\overline{\text{ECS}}$	114·0	95	0·53 (0·11)	0·47 (0·12)*

* $p < 0{\cdot}02$.

nificant degree of retrograde amnesia. These findings again support our previous observations regarding alterations in brain 5-HT by ECS and the amnesic effect of ECS. The present findings also suggest that a reduction in brain 5-HT level has no effect upon the amnesic properties of ECS, nor does brain norepinephrine appear to be dependent upon reduced brain 5-HT levels, either in terms of the response of this amine in the brain to ECS or to alterations in brain 5-HT, produced by ECS.

Because of the apparent consistency with which several regions of the mouse brain showed alterations in brain serotonin level and metabolism is response to ECS, several further attempts to characterize the changes in this amine on a cellular level, in response to ECS, were made.

Mice, ranging in age from approximately 35 to 40 days, were given a single transcorneal ECS of 10 mA or ECS which, from previous data, may be expected to elevate regional as well as whole brain serotonin levels, as determined within 10 to 20 minutes after such treatment. Since areas within the diencephalon and limbic system appear to be implicated insofar as the amine changes are concerned, and on the basis of other experimental and clinical evidence, to be involved in the memory process, these areas of the brain were removed and prepared for fractionation. Utilizing a technique wherein discontinuous gradients of sucrose and ficoll were employed with successive centrifugation steps following mincing and mesh extrusion of the tissue, a neuronally-enriched and glial-enriched fraction was obtained. Animals were sacrificed one hour following ECS in order to determine the extent to which persisting alterations in brain 5-HT level occurred. Each of the fractions from each of the experimental conditions was homogenized, extracted, and carried through a microspectrofluorometric procedure for estimation of 5-HT and 5-HIAA concentration. The results are summarized in Table 10. The level of 5-HT and 5-HIAA in the glia-enriched fraction was appreciably higher, although the difference did not reach a satisfactory level of statistical significance. The effect of ECS, as reflected in the two fractions, is of interest to note. At one hour following ECS, the 5-HT level

TABLE 10

Mean ($\pm \sigma$) concentration of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in neuronal and glial fractions from mouse brain following ECS or sham-ECS ($\overline{\text{ECS}}$)

Condition	5-HT	5-HIAA
Neuronal fraction		
$\overline{\text{ECS}}$	1·173 (0·007)	0·269 (0·124)
ECS	0·804 (0·001)	0·201 (0·020)
Glial fraction		
$\overline{\text{ECS}}$	1·253 (0·002)	0·402 (0·020)
ECS	1·734 (0·017)	0·617 (0·022)

in the neuronal fractions was significantly reduced, whereas at this same time, 5-HT levels, as well as 5-HIAA levels in the glia fraction, were significantly elevated. These findings are summarized in more detail in Table 11. There were highly significant differences in both 5-HT and 5-HIAA in the ECS-treated animals between the neuronal and glial fractions, suggesting that by one hour following ECS there is a persistent elevation of 5-HT in a glia-enriched fraction obtained from structures of the diencephalon and limbic system, and that the magnitude of this change is considerable in this fraction as compared to that observed in neurons.

In considering the possibility that elevated cellular levels of serotonin can persist for as long as one hour following ECS, and that a one-hour difference between a training experience and ECS is unlikely to result in a retrograde amnesia, it seems apparent that central changes associated with ECS-induced retrograde amnesia may possibly involve ongoing processes which relate to memory storage. In order to assess this question, several studies were performed. An initial study was concerned with the extent to which increases in available 5-HT concentration would affect protein synthesis. The synthesis of cerebral protein has been associated with memory processes in several regards and, under certain conditions, the inhibition of protein synthesis has been shown to lead to memory deficits which emerge at times considerably beyond the initial learning experience (Flexner, 1967). In the present study, protein synthesis in synaptosomes prepared from mouse cerebral cortex and limbic system tissue was measured. Synaptosomes were prepared from this tissue according to the techniques described by Austin and Morgan (1967), and the rate of incorporation of ^{14}C leucine into the synaptosomal fractions was determined. The purity of the synaptosomal fractions was verified by enzymatic determinations of succinic dehydrogenase and acetylcholinesterase activity. Addition of 5-HT to the incubating fractions was based upon concentration differences resulting in brain 5-HT as a result of (*i*) ECS, and (*ii*) systemic administration of 100 mg/kg of 5-HTP. In the former case, 5-HT differences averaged 3×10^{-7} M, whereas in the latter case an almost equal level emerged (2×10^{-7} M). Addition of the first 5-HT concentration resulted in a significant degree of inhibition of protein synthesis, as determined from changes in the rate of incorporation

TABLE 11

Per cent change (%Δ) in 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in neuronal and glial fractions from mouse brain and level of statistical significance (p) for ECS and $\overline{\text{ECS}}$ treatments

Comparison	5-HT		5-HIAA	
	% Δ	p	% Δ	p
$N\text{-}G_{ECS}$	+ 6·82	>0·70	+ 49·44	>0·70
$N\text{-}G_{\overline{ECS}}$	+115·67	<0·001	+ 206·96	<0·001
$N\text{-}N_{ECS}$	— 31·46	<0·001	— 25·28	>0·80
$G\text{-}G_{ECS}$	+ 38·39	<0·001	+ 53·48	<0·02

TABLE 12

Rate of ^{14}C leucine incorporation into synaptosomes from mouse cerebral cortex and limbic system structures and per cent protein synthesis as affected by 5-hydroxytryptamine (5-HT)

Condition	Rate of incorporation ($\mu\mu$ M leucine/mg Protein/hr)	% Synaptosomal protein synthesis
Control (Cerebral cortex)	34·29	100
Control (Limbic system)	36·60	100
+5-HT (3×10^{-7} M) (Cerebral cortex)	26·06	76
+5-HT (3×10^{-7} M) (Limbic sytem)	24·89	68

of ^{14}C leucine. These findings are summarized in Table 12. It may be observed that there was a slightly higher rate of ^{14}C leucine incorporation in limbic system synaptosomes (~6 per cent), and the degree to which protein synthesis was inhibited was greater in the synaptosomal fraction from the limbic system structures.

We have previously reported that intraventricular administration of 5-HT can serve to rapidly impair the acquisition of one-trial passive avoidance behaviour. This has been observed with concentrations of approximately $1{\cdot}17\times10^{-2}$ M, which is considerably above the concentration difference resulting either physiologically or through precursor administration. The introduction of 5-HT at this concentration in incubating synaptosomes, from which ^{14}C leucine incorporation into brain was determined, indicated that protein synthesis was inhibited in excess of 90 per cent.

A further series of studies was concerned with the changes associated with elevated brain serotonin, where such elevation was produced by conditions which have been shown to impair conditioned response acquisition and/or retention of such behaviour. Groups of mice were injected intraventricularly with either 5-HT ($1{\cdot}17\times10^{-2}$ M) or 0·9 per cent saline, and several other groups of animals were injected i.p. with 100 mg/kg of 5-HTP. The mice given intraventricular injections of 5-HT were sacrificed 30 minutes following such injection, whereas those animals given systemic injection of 5-HTP were sacrificed at one hour. Whole brain RNA and total protein under these conditions, were estimated, utilizing standard fluorescence and absorption techniques. A mean value for total protein, based upon precipitated material from equal aliquots of a 20 per cent brain homogenate, was estimated at 6·84 mg ($\pm$0·74). Protein level in the brains of 5-HTP-treated mice was reduced by approximately 35·8 per cent to a value of 4·39 mg.

In the 5-HT-treated animals, total protein was reduced by approximately 32 per cent to a value of 4·56 mg. These findings are somewhat consistent with our previous observation that synaptosomal protein synthesis is inhibited by addition of 5-HT. The degree to which such inhibition occurs is very consistent with the extent to which total protein in the mouse brain was reduced in the present study. Whole brain ribonucleic acid was also estimated under these experimental conditions, and from a mean whole brain level of 2·20 mg/g (±0·30) RNA level in animals injected intraventricularly with 5-HT was reduced to 1·40 mg/g. Those animals given systemic injections of 5-HTP had a whole brain RNA level of 2·00 mg/g, 60 minutes following such injection. These small changes in total RNA are partially consistent with our previous findings regarding the negative relationship between brain 5-HT level and RNA content under several physiological and pharmacological conditions which regulate the degree to which memory consolidation can be disrupted. Preliminary findings have indicated that with differential gradient fractionation yielding a glia-enriched fraction from cerebral cortex and sub-cortex, excluding olfactory bulbs and cerebellum, total protein levels are slightly affected under conditions paralleling those described above; there is a slight trend toward an increase in total protein in cortical glial fractions (36·8 per cent) 30 minutes following intraventricular 5-HT administration, whereas total glial protein, 60 minutes following systemic administration of 5-HTP, was not altered. This preliminary finding may possibly indicate a reciprocal interaction between neurones and/or their endings with adjacent glia in the mediation of changes related to the process of memory consolidation as influenced by brain 5-HT. Further studies, now in progress, are directed toward answering some of these relevant questions.

A final series of studies has been concerned with physical evidence regarding the interaction of serotonin with nucleic acids and nucleotides. Some preliminary data (Bittman et al., 1969) has indicated a hierarchy of nucleic acid and nucleotide binding to free 5-HT under several conditions. Further investigation has shown that the formation of a complex between 5-HT and RNA occurs. Binding, as measured by the quenching fluorescence at pH 7, indicates that 5-HT and RNA result in quenching of 18 per cent, as compared to a free 5-HT baseline. Recent studies (Essman, 1970) have indicated that mice, treated with lithium salts, showed reinstatement of retention or a passive avoidance response following apparent retrograde amnesia produced by electroshock. Paralelling this phenomenon were increases in brain magnesium level and short-term alterations in brain 5-HT turnover. A possible mechanism by which this observation may be accounted for is that an increase in the ionic strength of magnesium may affect the interaction between 5-HT and nucleic acids. Physical evidence for this interaction was obtained and, as a function of increased ionic strength of magnesium, the quenching of 5-HT fluorescence was increased, such that at a 5-HT concentration of 4 μg/ml and addition of 2·5 M magnesium, fluorescence was quenched by approximately 18 per cent (approximately equal to the degree to which RNA was quenching 5-HT fluorescence). At the same ionic strength of magnesium, the 5-HT-RNA complex was

quenched by approximately 32 per cent. Addition of lithium ions over a range of varying ionic strengths did not quench the fluorescence of either 5-HT or the 5-HT-RNA complex. This evidence indicates that 5-HT and RNA bind to one another, that magnesium ions bind to the 5-HT molecule, and that the binding of 5-HT to RNA is markedly increased in the presence of magnesium ions. Additional studies, now in progress, have been undertaken to further elucidate the relationship between these interactions observed in physical studies and their bearing upon similar interactions hypothesized *in vivo*.

The studies summarized in this paper, and the data presented, suggest an important role for 5-hydroxytryptamine in the mediation of at least one phase of the memory consolidation process. The relationship between changes in this amine in whole brain, regional sites within the brain, and on a cellular level, and other events implicated in other phases of the memory consolidation process (RNA synthesis, protein synthesis, and their interdependency), have been shown, under several experimental conditions; these relationships may possibly constitute a model within the framework of which 5-hydroxytryptamine may possibly play an important role in the mediation of memory consolidation and in those conditions wherein the memory consolidation process is impaired.

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DISCUSSION

R. Yu. Ilyutchenok: Did you see amnestic effect of high doses of serotonin without ECS? Dr. Devoino from the immunological laboratory of our Institute published data about the inhibition of immunological memory by serotonin, reserpine and MAO inhibitors.

W. B. Essman: That was shown on the first slide. We must remember that we used two stimulus intensity levels. Otherwise it has been reported by other workers that in pigeons the impairment of learning is associated with an increased serotonin level in the telencephalon.

CHOLINERGIC BRAIN MECHANISMS AND MEMORY

by

R. Yu. Ilyutchenok

Laboratory of Neurophysiology and Pharmacology of Behaviour,
Institute of Physiology, Siberian Department of the U.S.S.R. Academy of Sciences,
Novosibirsk, U.S.S.R.

In recent years attempts have been made to use a number of drugs for improving and blocking memory. Figure 1 is a diagrammatic representation of the results of these studies. It was found that drugs acting upon the cholinoreactive structures and stimulants have the strongest influence upon memory. The acceleration of the consolidation of memory under the effect of nonconvulsive doses of stimulants is perhaps also due to the facilitation of synaptic intraneuronal transmission.

The formation of memory is, to some extent, related to the structural changes in the synapses leading to the facilitation of the passage of impulses in the neuronal systems along corresponding channels of communication. In the reverberation of the impulses in the neuronal chains, in the potentiation and in the changes in the number of presynaptic vesicles (i.e. in the changes of contact between the pre- and postsynaptic neurones) the cholinergic mechanisms are primarily involved. The further activation of the synthesis of nucleic acids and proteins may also contribute to the processes of memory by augmenting the enzyme systems facilitating the synthesis of acetylcholine and also by changing the configuration and the synthesis

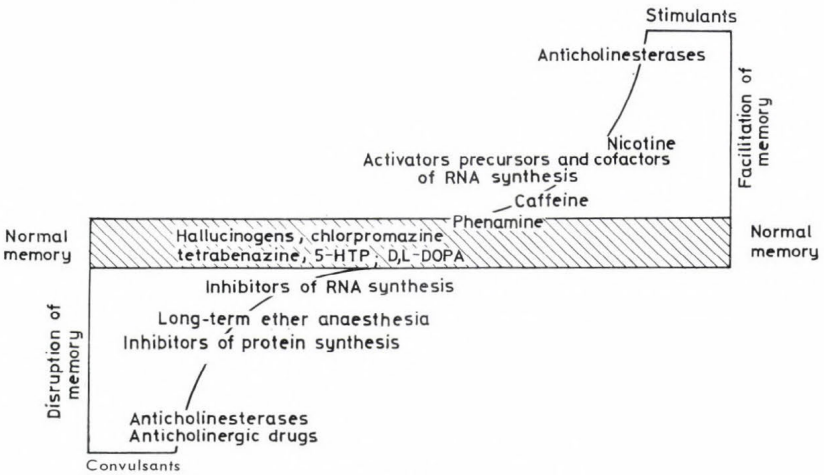

Fig. 1. Action of drugs on memory.

of the receptor proteins with a corresponding alteration in the sensitivity of the cholinergic receptor (Barondes, 1965; Meerson, 1967). Beritashvili (1967) considers that RNA is involved in the formation of active protein that acts upon the synaptic membrane and, consequently, facilitates the transmission of impulses.

All these processes are included into the concept of the intrinsic mechanisms of memory. But the final step in the formation of memory depends not only upon the intrinsic mechanisms of memory. Such nonspecific systems as the limbic and the ascending reticular activating system also play an important role in memory formation. These systems comprise a powerful cholinergic mechanism.

Hence it is feasible to interfere with the intrinsic as well as the regulatory mechanisms of memory by modifying the activity of the brain cholinergic structures.

Synaptic transmission can be altered by the stabilization of acetylcholine with anticholinesterases and by blocking the cholinergic receptor of the subsynaptic membrane with anticholinergic drugs.

Conditioning would be facilitated if just the amount of information-bearing acetylcholine necessary for the response were protected from destruction. Actually, an increase in the acetylcholine level up to a definite point brought about by anticholinesterases does accelerate the acquisition of conditioned responses. When the amount of acetylcholine surpasses the optimum level, a synaptic blockade may ensue as the result of acetylcholine excess. The transfer of information against the background of large doses of drugs that stabilize the mediator and intensify its release is similar to the transfer of information in the presence of noise in definite channels of communication. Both the increase of mediator quanta incoherent with the signal and the presence of noise will result in a decrease in the amount of information that can be transferred through a synapse.

It seems that differences in the effect of large doses of anticholinesterases at various times after administration can be explained by the same mechanism. Learning was inhibited completely if learning was started later, when there was an excess of acetylcholine and synaptic blockade had developed.

Deutsch (1966) has proposed an interesting hypothesis: as a result of learning the initially nonfunctioning synapses are modified and start to emit a transmitter. But the increase in acetylcholine released at such synapses is gradual. It is suggested that even under the significant blockade of anticholinesterase, acetylcholine never accumulates in excess at the synapses. For this reason, at the early stages of conditioning anticholinesterases lead to the facilitation of synaptic transmission by preventing the destruction of acetylcholine and thus promote conditioning. If forgetting were also due to the decrease of the transmitter at the synapse, then this would provide a plausible explanation why memory restoration is facilitated when anticholinesterases are administered 28 days after training, when the conditioned response had disappeared in the controls.

The deterioration of the formation of new memory traces during the blockade of the cholinergic brain mechanisms is attributed to the impairment of

short-term memory (Herz, 1960; Bureš et al., 1964; Meyers, 1965; Bohdanečký and Jarvik, 1967). The impairment of registration as well as the consolidation of memory traces may underlie this deterioration. The experiments of Yeliseyeva and Loskutova in our laboratory have shown that this amnesic effect of muscarinic anticholinergic drugs is not determined by their influence upon registration. The administration of an adequate dose of the anticholinesterase galanthamine can abolish the effect of this anticholinergic drug. In these experiments the blockade of the cholinergic receptors in mice was limited in time by the registration stage (Fig. 2). The effect of benzacin given 5 minutes before the experiment was abolished by galanthamine just after the conditioned response had been acquired after one-trial conditioning. The anticholinergic drug did not exhibit its amnesic effect here.

It, therefore, can be assumed that the deterioration of conditioning as well as retrograde amnesia under the effect of anticholinergic drugs are related to the impairment of the initial stages of consolidation.

Anticholinergic drugs, however, do not prevent conditioning in all the cases. The intensity of stimulation and the duration of training are of paramount significance in conditioning against the background of the action of anticholinergic drugs. Anticholinergic drugs block conditioning when the number of trainings is small; when the number is increased, their blocking effect is attenuated.

One of the possible mechanisms of the anticholinergic drugs is the blockade of the transmission across cholinergic synapses in neuronal chains. Such a blockade should prevent the subsequent consolidation of memory. But the possibility of acquiring a conditioned response during the blockade of the cholinergic receptors by anticholinergic drugs is at variance with such an explanation.

Yeliseyeva has made an interesting observation in our laboratory. In cats conditioned fear was elaborated against the background of the effect of anticholinergic drugs; the stimulus was paired 10 times with unavoidable electric shock. Against the background of benzacin (1–20 mg per kg) fear was not displayed, but in the consecutive days, when the

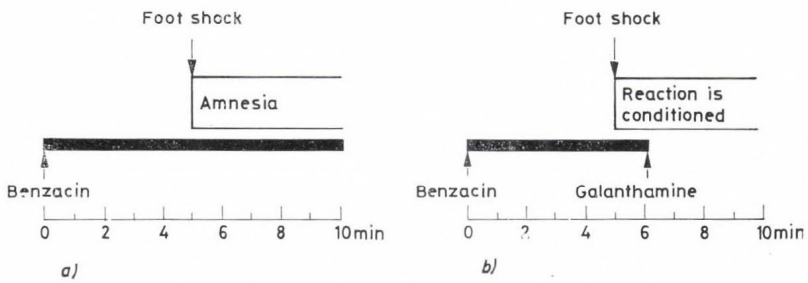

Fig. 2. Influence of benzacin on fear conditioning in the cat. (*a*) Benzacin administered 5 min before electroshock blocks the elaboration of fear response. (*b*) Anticholinergic effect of benzacin is abolished 1 min after electric shock by the anticholinesterase galanthamine; the amnesic effect of benzacin is not displayed.

animals were tested without the drug, conditioned fear was clear-cut. This observation is consistent with the data in the literature (Ricci and Zamparo, 1965; Meyers, 1965; Oliverio, 1968).

Learning, therefore, is possible without the repetition of correct specific responses. Memory traces can be registered and retained when response performance is blocked in the process of training, although the retention time of conditioned fear in animals treated by anticholinergic drugs is shortened.

We believe that the outcome of the struggle for the receptor between anticholinergic drugs and endogenous acetylcholine released in the course of the reaction is decisive. It seems reasonable to assume that if acetylcholine wins in the competitive struggle for the receptor, the response is performed. The deblocking of the receptor occurs, probably, when large amounts of endogenous acetylcholine are released as, for instance, during repeated trainings, and, in particular, when repeated electric shocks are unescapable. When large doses of anticholinergic drugs are delivered, the deblocking of the receptor will not take place and, in consequence, the response will not be acquired.

According to another hypothesis, memory traces under normal conditions are registered and retained in the cholinergic structures, but when they are blocked, the traces can be retained in the cholinergic structures as well (Bureš et al., 1964; Meyers, 1965; Bohdanečký and Jarvik, 1967). It might be presumed that the latter are adrenergic. But the possibility of conditioning cats against the background of the simultaneous blockade of the cholinergic and adrenergic structures demonstrated by Yeliseyeva (although it was necessary here to increase the strength of the current to such an extent as to elicit the unconditioned defense response) refutes such an explanation. What are these noncholinergic mechanisms remains so far another puzzling aspect of memory. It may be speculated that some type of nonsynaptic reserve mechanism is switched into action.

The emotional state at the moment of registration or just after this stage has a strong influence upon memory formation. It is conceivable that emotional excitation cannot only improve, but also accelerate consolidation and, on the other hand, the inhibition of emotions will deteriorate this process.

In our studies we have compared the influence of anticholinergic drugs on short-term and long-term memory (Ilyutchenok and Yeliseyeva, 1966; Ilyutchenok, 1968; Ilyutchenok and Chapligina, 1970). In dogs with well-established food reflexes, fear response to the feeder was elaborated by delivering an electric shock when they tried to eat from it. The fear response was maintained for several months which is in agreement with results of Beritashvili (1968).

A single intramuscular administration of the muscarinic anticholinergic drugs amisyl (benactizine) or benzacin (0·5 mg per kg) 10–15 minutes after fear conditioning did not produce its stable disappearance. Only the repeated administration of these drugs for 2–3 days (1 or 2 times daily) promoted the total effacement of fear. It is much harder to achieve the complete disappearance of this memory of fear emotion when anticholinergic drugs

are given 2·5 weeks and especially 2–3 months after training. When anticholinergic drugs are given so late, fear disappears only after large amounts of the drugs have been administered repeatedly (for 3 days, 1 mg per kg 4 times daily or 10 mg per kg 2 times daily). Fear usually supports defense behaviour and, therefore, it is not extinguished but is retained for a considerable time. When fear response is blocked, conditioned defense response cannot be maintained for a long time, this is what we observed in our experiments. Perhaps it was difficult to elaborate the defense response against the background of anticholinergic drugs because the attempts to elaborate it were undertaken when the emotional response was inhibited. The prolonged blockade of the cholinoreactive brain structures leads to the complete obliteration of emotional memory, and to its structural disruption. Possibly emotional memory has different mechanisms from those of other types of memory, for the other types of long-term memory are not effaced by anticholinergic drugs. The mechanisms of emotional fear are probably included into a functional system with cholinergic neurones. It seems to be the muscarinic cholinergic mechanisms of the *li*mbic system.

The changes in the activity of hippocampal neurones induced by anticholinesterases and by cholinomimetic drugs (Salmoiraghi and Stefanis, 1965; Biscoe and Straughan, 1966; Ilyutchenok and Pastukhov, 1968) evidences cholinergic reception in a limbic system. It is highly interesting that theta-rhythm in the hippocampus and in the septum are retained during the administration of anticholinesterases and cholinomimetics, when the reticular formation is ablated through premesencephalic section (Ilyutchenok and Bannikov, 1968). This suggests that the limbic system possesses its own muscarinic cholinergic mechanism.

The effect of anticholinergic drugs is displayed better in animals in which the response is not elaborated stable enough. These are the instances when emotional responses and reticular influences are the most strongly involved. When these influences are abolished artificially by anticholinergic drugs, the consolidation of memory trace, probably, deteriorates. Well-trained animals have enough information to perform the response (Simonov, 1967). The response then can be performed with no emotions, and the anticholinergic drugs will have no amnesic effect.

The activity of the ascending reticular activating system is of significance in memory formation. The excitation of this system results in the activation of cortical neurones (Bremer, 1961) and in the differentiation of the cortex into independent, asynchronously functioning neuronal groups (Lindsley, 1958). In our experiments (Ilyutchenok and Gilinsky, 1969) the first effect was reflected by the frequency increase of 45·4 per cent of cortical neurones during high frequency stimulation of the midbrain reticular formation. As to the second effect, the reticular inhibition of 27·1 per cent of the neurones plays an important role in the differentiation of neuronal constellations. The weakening of the connections between the sensory units in the cortex improves sensory discrimination because it allows a more contrasting perception of information ascending from the receptors. Reticular inhibition may be of importance in the mechanisms of spatial distribution of signals in neuronal constellations.

It appears that the activation of the reticular formation reduces the number of reverberating circuits which correspond to the sensory signal, while there may be an acceleration of the pulses in the circuits. It may be possible to influence different stages of memory in the course of the changes in the activity of the reticular activating system. Anticholinergic drugs in this case may exert the strongest effect.

Since in this system muscarinic cholinergic structures dominate both at the level of the brain stem and in the cortex (Rinaldi and Himwich, 1955; Ilyutchenok, 1962; Smirnov and Ilyutchenok, 1962; Krnjević, 1967), the influence of anticholinergic drugs on memory through reticular mechanisms may consist, on the one hand, in the decrease of the ascending flow of reticulo-cortical impulses, and, on the other hand, in the blockade of the cholinoreactive cortical inhibition (Ilyutchenok and Gilinsky, 1969). The effect of anticholinergic drugs amounts to an increase in the number of reverberating circuits with concomitant decreased activity within each of them.

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DISCUSSION

S. Freed: Did you carry out experiments with approach reflexes rather than defensive reflexes?

R. Yu. Ilyutchenok: The experiments were carried out by the method of Beritashvili. At first dogs were trained to conditioned food reflexes. After that, when attempting to eat from one of the feeders, they received an electric shock. Fear reaction elaborated after 1–2 electric shocks was maintained for several months.

F. Klingberg: Many drugs have antagonistic effects depending on their doses, which is a well-known fact. How do you explain, e.g. the facilitating effect of cholinergic substances (Nivaline, Eserin) on conditioning in low doses and its blocking effect in higher doses?

R. Yu. Ilyutchenok: Increase of acetylcholine to a definite level facilitates synaptic transmission. If only the amount of information-bearing acetylcholine providing the response were protected from destruction by anticholinesterases, this would facilitate learining. When acetylcholine level exceeds the optimum under the effect of large doses of anticholinesterase, the synapses may be blocked in consequence of acetylcholine excess. Information transfer under sufficiently high doses of anticholinesterases may be compared with information transfer in the presence of noise in a channel of communication. It leads to the decrease in the amount of information transferable across the synapses.

J. KNOLL: Some psychopharmacological agents have a dual action. They facilitate, e.g. a conditioned response in small doses, and inhibit the same in high doses. This depends on the method used.

R. YU. ILYUTCHENOK: Small doses of anticholinergic drugs do not block the synaptic transmission or in general the excitatory mechanisms, but the excitatory mechanisms as well as the reticulo-cortical system can be blocked by higher doses of the same drug.

A PSYCHOPHARMACOLOGICAL APPROACH TO A POSSIBLE SYNAPTIC MEMORY MECHANISM

by

J. KNOLL, K. KELEMEN and BERTA KNOLL

Department of Pharmacology, University Medical School, Budapest, Hungary

Learning and memory seem to us quite clear in their meaning in spite of the fact that very little if any of their real physiological and biochemical bases are known. Our poor knowledge regarding these physiological processes is excellently reflected in the shallowness of the current definition of learning according to which it is a more or less permanent change in behaviour resulting from experience.

Starting from this general definition a specific type of processing and retention of information in the rat will be described.

The simple device used in our experiments was the modified jumping test developed by us (Knoll, J. and Knoll, B., 1964), which proved to be very useful for screening purposes in psychopharmacological research work (Knoll, 1967). The essence of the method is the following:

The rat is placed on a hot plate under a 30 cm high glass cylinder and we measure the time until the animal jumps from the warm plate to the upper rim of the cylinder.

The interval between the beginning of the test and the onset of the escape reaction when the rat jumps to the rim, which varies from one animal to the other, is taken to reflect the capability of the animal to escape and is expressed in arbitrary units from zero to ten, as shown in Table 1. Mark

TABLE 1

Onset of escape reaction (min)	Units
0 – 10	10
11 – 20	9
21 – 30	8
31 – 40	7
41 – 50	6
51 – 60	5
61 – 90	4
91 – 120	3
121 – 240	2
No jumping within 4 hours	1
Death without jumping within 4 hours	0

ten is given when the animal jumps within 10 minutes and the lowest mark, zero, is given when the animal is unable to escape and dies on the hot plate in hyperthermia.

If the temperature of the plate is high enough, the unconditioned avoidance reaction sets in within a very short time and a conditioned response develops rapidly. Using, e.g. a plate temperature of 60 °C, a conditioned escape response is built up after a few trials, which might persist for weeks or months without reinforcement (Knoll, 1969). If, instead of 60 °C, a plate of 45 °C temperature is used, a quite different aspect of learning can be followed in the rats. Every rat escapes even from this low temperature plate by jumping onto the rim of the cylinder but, independently of the duration of the training the development of a conditioned reaction was never to be observed (Knoll, 1965). A peculiar type of learning can be demonstrated in these animals. This is shown in Figs 1 to 3.

It is evident from the figures that a remarkable change in the behaviour of animals develops during training.

Five mg per kg morphine (Fig. 1, column 2), 15 mg per kg tetrabenazine (Fig. 2, column 2) and 15 mg per kg chlorpromazine (Fig. 3, column 2) completely inhibits the escape response in untrained animals but not in the trained ones. The last columns in Figs 1 to 3 demonstrate that on the 21st day of the training procedure the psychodepressants investigated lose their ability to inhibit the unconditioned avoidance reaction. The data representing the animals' performance on the 7th and 14th days of training, respectively, show, on the one hand, that only as light improvement in the escape reaction is to be observed, especially on the 7th day of training and, on the other, the loss of sensibility towards morphine and the major tranquillizers developing gradually during training.

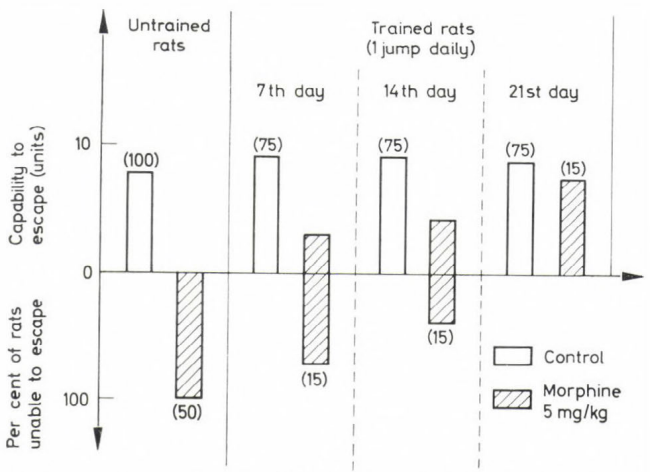

Fig. 1. Change in the effect of morphine in trained rats. Morphine was administered subcutaneously 30 minutes before onset of measurement. Number of animals in brackets.

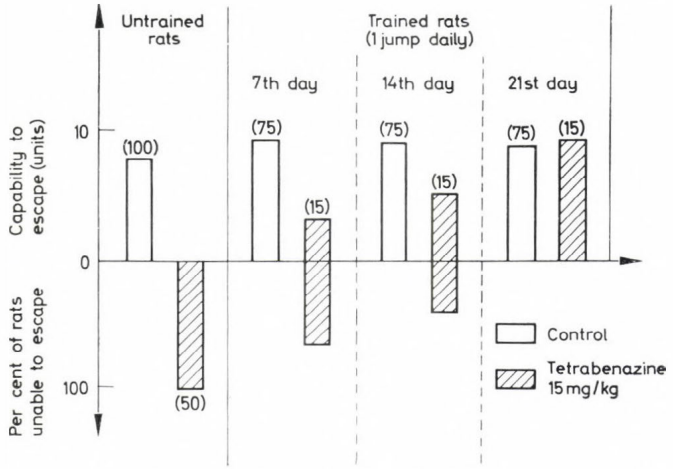

Fig. 2. Change in the effect of tetrabenazine in trained rats. Tetrabenazine was administered subcutaneously 1 hour before onset of measurement. Number of animals in brackets.

Figures 4 to 6 show some important characteristics of the peculiar type of learning and retention demonstrated in Figs 1 to 3. These figures demonstrate the sensitivity of the stored long term memory traces towards electroconvulsive shocks (ECS). Electroshock by itself does not influence the unconditioned escape reaction in untrained or in trained rats (cf. the 2nd and 4th columns in Figs 4, 5 and 6) but deleates completely the effect of training.

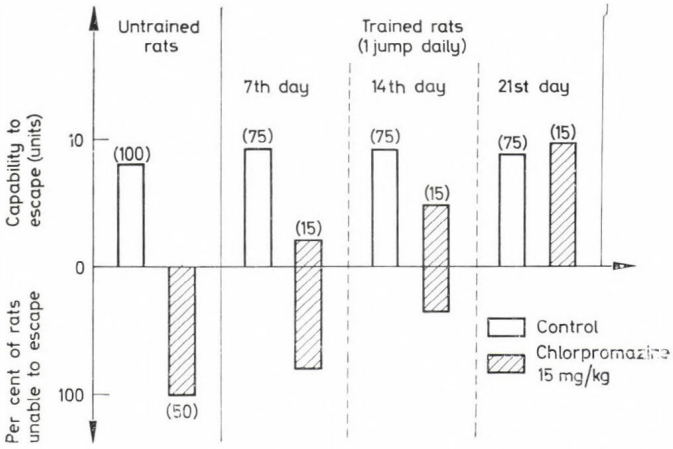

Fig. 3. Change in the effect of chlorpromazine in trained rats. Chlorpromazine was administered subcutaneously 1 hour before onset of measurements. Number of animals in brackets.

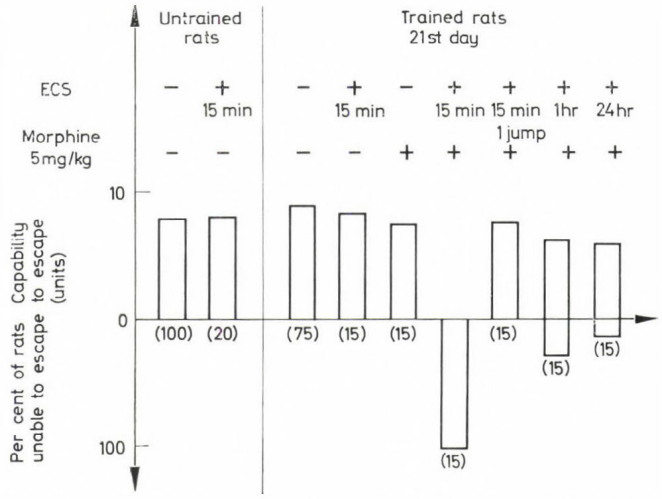

Fig. 4. Effect of morphine in trained rats treated with electroshock. Training = 1 jump daily. ECS = Delivery of two shocks (25 mA, 0·2 msec) within 15 minutes for evoking the tonic hindlimb flexor component of maximal electroshock seizure. Morphine was administered subcutaneously 30 minutes before onset of measurement. Time interval between the 2nd ECS and morphine administration is indicated on the figure. Number of animals in brackets.

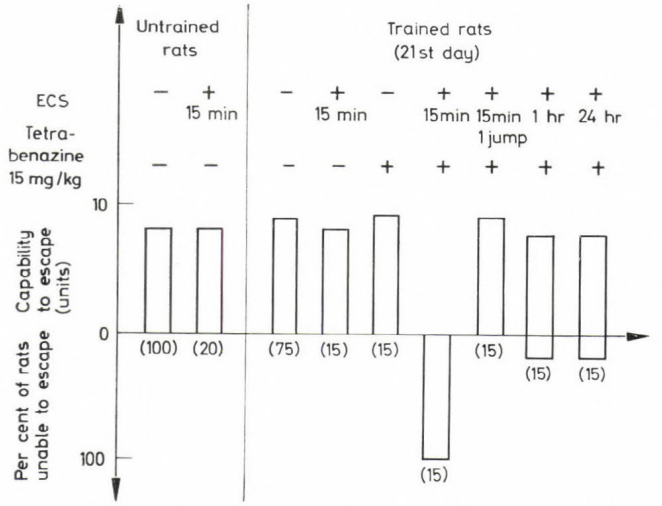

Fig. 5. Effect of tetrabenazine in trained rats treated with electroshock. Tetrabenazine was administered subcutaneously 1 hour before onset of measurement. Otherwise the same as in Fig. 4.

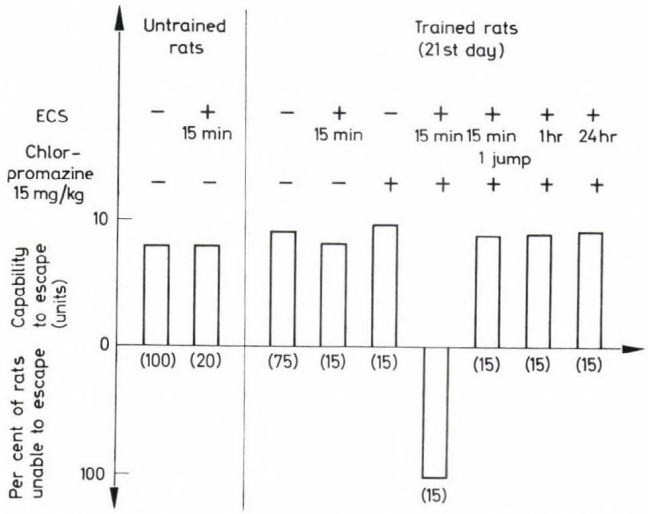

Fig. 6. Effect of chlorpromazine in trained rats treated with electroshock. Chlorpromazine was administered subcutaneously 1 hour before onset of measurement. Otherwise the same as in Fig. 4.

The trained animals stimulated with ECS react to morphine, tetrabenazine and chlorpromazine like untrained ones (cf. the 6th column in Figs 4, 5 and 6). One jumping reaction after ECS stimulation immediately restores the memory patterns (cf. the 7th column in Figs 4, 5 and 6). A fairly rapid retrieval even spontaneously was observed after ECS treatment (cf. the last two columns in Figs 4, 5 and 6).

Our data clearly show that a peculiar type of learning and retention occurs in rats under appropriate experimental conditions. This type of memory can be characterized mainly by its specific sensitivity to ECS and remarkable insensitivity towards such doses of morphine and major tranquillizers which completely inhibit all types of conditioned reactions and even a great number of unconditioned responses.

A detailed analysis of the unconditioned escape reaction in the setup shown in this presentation led us in earlier works to the conclusion that a gradually increasing special excitatory state in a specific neuronal network underlies the physiological basis of the animals' capability to perform the escape response. We defined this specific type of central excitatory state, named active focus, by the aid of its physiological characteristics (Knoll, 1969).

On the other hand, we have also learned from our earlier work that the rise of excitation in this neuronal network is most efficiently blocked by the highly active groups of psychodepressants, of which morphine, tetrabenazine or chlorpromazine can be called characteristic representatives, and which are all well known for their ability to interfere in one way or other with transmission processes in certain types of central synapses.

It might be, therefore, supposed that during training a type of synaptic modulation develops in the neuronal network which renders the animal capable to perform the unconditioned escape reaction. This modulation might then provide normal transmission between the neurones even in the presence of such concentrations of psychopharmacological agents that in untrained animals completely block the connections. The material basis of the stored long-term memory would then consist of the gradual development of this acquired modulation of synaptic transmission insensitive to the narcotic analgesics (e.g. morphine) and major tranquillizers (e.g. tetrabenazine of chlorpromazine) and specifically sensitive to ECS. Be as it is, however, the physiological nature and biochemical basis of this peculiar ECS-sensitive type of processing and retention of information needs further detailed analysis.

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DISCUSSION

O. FEHÉR: Did you see any change in the behaviour of the animals after morphine or chlorpromazine? It seems to me that these drugs may have abolished orientation reaction and this prevented the animals from learning a new behaviour.

J. KNOLL: Dr. Fehér is perfectly right saying that the lack of orientation, caused by morphine or chlorpromazine, may prevent the learning of a new behaviour. It is a well-known fact that central depressants inhibit learning and learned reactions. The question arising in connection with our data is just the opposite one: why the depressants do not inhibit (in doses completely abolishing all types of conditioning) the repeated jumping reactions. The memory underlying conditioning is sensitive to these drugs. This is the principal proof that the learning process we are dealing with is different from conditioning.

G. UNGAR: Have these animals received morphine or chlorpromazine every day during training?

J. KNOLL: No, the animals received one single dose of the drug at the trial and never before in their life. So this drug resistance was not caused by any tolerance phenomenon but by a specific learning process.

R. YU. ILYUTCHENOK: Attenuation of defensive reaction under high doses of chlorpromazine is related not to the blockade of the mechanisms of fear,

but rather to the inhibition of mechanisms through the motor-vegetative reaction displayed in consequence of general reactivity reduction, including the attenuation of response to noxious stimuli. We have data showing that other beta-adrenoblocking agents have no isolated effect either; neither on the established fear reaction, nor on its elaboration.

J. KNOLL: Our results cannot only be explained by the effect of chlorpromazine on the motor-vegetative reactions. These components of the behaviour are essentially the same in untrained and trained animals after ECS treatment. The untrained animals and the trained ones treated with ECS, however, are unable to jump under the same dose of chlorpromazine which leaves the behaviour of normal trained rats unchanged. Thus, training evokes a change in the CNS which cannot be detected by simple observation of behaviour, only by the modified drug sensibility, and this change is eliminated by ECS.

F. KLINGBERG: I would like to comment on the role of electro-convulsive shock on memory traces. Dr. Pollack showed that after electro-convulsive shock in human subjects very long traces of hypersynchrony appear in the EEG accompanied by amnesia. Such results are known also from animal experiments. In my report, I pointed out the impairment of learning and memory traces by processes accompanied by hypersynchrony.

J. KNOLL: We have no data on the EEG changes accompanying these reactions, yet. We are planning to do such experiments, maybe they will provide some further information.

J. SZÉKELY: Have you controlled the effect of electroshock in the other form of escape reaction from the hot plate?

J. KNOLL: Yes, we have. ECS by itself does not inhibit either the unconditioned or the conditioned escape from the hot plate. Under our experimental conditions the only detectable effect of ECS is the restoration of the drug sensitivity, i.e. the elimination of this special effect of training.

I. MADARÁSZ: I would like to know if you ever did try to apply more than two ECS's consecutively to the trained rats?

J. KNOLL: Yes, we did. But the frequent repetition of ECS leads to a quite different problem because it might inhibit the unconditioned escape by itself.

CONCLUDING REMARKS

by the Chairman

J. V. McConnell

The word "memory" is as difficult to define, and hence as meaningless, as is the term "life". We all come to the study of "memory" with old prejudices and biases which prevent us from looking at the problem objectively. For instance, we are all anthropocentric—we are more interested in man and the higher animals than in the lower organisms, yet 95 per cent of all living organisms are invertebrates. We think that learning occurs in the brain, because man has a brain, yet experiments with planarians show memory is stored throughout the body, and even simpler animals that have no brains (and no nerve cells) are capable of learning simple tasks.

In a similar fashion, we tend to overemphasize the importance of electrical activity as a measure of learning, yet if electrical activity is important, it must be a "late arrival" phylogenetically since unimportant electrical phenomena occur in very simple animals that can learn. Hence, a chemical basis for memory must have occurred in evolution prior to the appearance of any pronounced electrical activity at all. But it is easy to put an electrode into a brain and very difficult to measure chemical changes so most of us prefer to look for memory where it probably does not exist.

Thus we need a new approach to the study of memory, an approach that does not blind us to the realities of nature. By giving up our prejudices and biases, we may eventually learn what learning is all about.

Section V

ELECTROPHYSIOLOGICAL CONCOMITANTS OF ELEMENTARY LEARNING

DATA ON THE MECHANISM OF THE ACOUSTIC HABITUATION

by

O. Fehér

Department of Animal Physiology, József Attila University, Szeged, Hungary

Although a survey of recent literature of the memory research convinces the reader that most new and interesting results come from biochemical laboratories, and that electrophysiological studies have lost importance, I am of the opinion that further results can only be expected from the united effort of these different branches. The chemical changes which occur in the nerve cells in the course of repeated use provide a basis for the organizational changes, recordable by electrophysiological methods. The simplest forms of the electrical signs in cerebral cortex are the evoked potentials, which signify the arrival of an afferent volley. The analysis of these simple, or apparently simple, signs enables us to clear up the elementary organizational patterns of the cortex. The evoked potentials are the most frequently recorded phenomena in the course of the elaboration of conditioned responses. The consistent changes of the electrogram during the conditioning process reflect modifications in central nervous function.

One of the well known changes which ensue during repetitive stimulation is habituation. In our experiments we have been dealing with the habituation of acoustic click stimuli and electrical stimuli delivered to the medial geniculate body (MGB), as it could be recorded from the primary acoustic area of the cat's cerebral cortex. The evoked potentials elicited in this way were recorded from a depth of 1,500–1,700 μ, always from the zone of maximal amplitude. Although habituation manifested itself in each phase of the evoked potentials, my report will include only changes of the negative phase.

The evoked potentials elicited by acoustic clicks or electric stimuli to the MGB showed a marked decrease with increasing stimulus frequency. This is the most prominent sign of habituation (Fig. 1).

The amplitudes obtained at various frequencies plotted against the log time separating the stimulating pulses yield

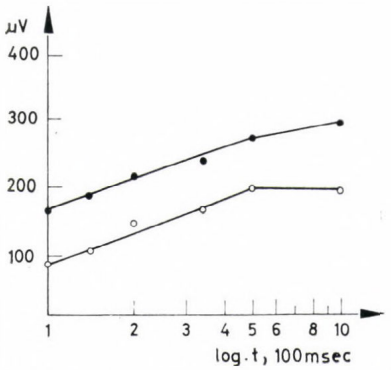

Fig. 1. Frequency dependence of the amplitude of the potentials evoked by the electrical stimulation of the MGB with sub-maximal (lower curve) and supermaximal (upper curve) strength.

a straight, or almost straight, line in case of both acoustic clicks and MGB stimuli. In other words, the evoked potential amplitude is directly proportional to the logarithm of the time between the successive stimuli. From zero up to 1·0–1·6 cps the amplitude fails to change remarkably. In a frequency range from 2·0 to 10·0 cps, however, a marked habituation is observable, at both maximal and submaximal stimulus strengths. The tangents of the straight lines do not reveal any difference.

The individual potentials show of course a remarkable dispersion around the mean, characteristic of the repetition frequency, but the averages calculated from series of 25 evoked potentials fit in with the logarithmic relations very well.

This form of habituation can be analysed on the basis of two preliminary assumptions: (*i*) the synapses are at least partially fatigued by the repetitive use and (*ii*) the afferent impulses activate some form of inhibition in the cortex, which reduces the amplitude progressively with increasing frequency.

It seems very plausible that both mechanisms may play a role in this habituation, but I tried to explain it mainly by the synaptic fatigue or more correctly by the effect of repeated use on synaptic efficacy.

The evoked potentials are built up mainly from single excitatory postsynaptic potentials elicited by afferent impulses in the dendrites and somata of the cortical pyramidal cells. Therefore, their amplitude is a function of the transmitter quantity released at the presynaptic terminals. According to Takeuchi the amplitude of the PSP's depends on the membrane potential of the presynaptic terminals and consequently on the amount of the transmitter released. In our experiments, however, the variation in the amount of the transmitter was not caused by polarization but supposedly by the repetitive use alone.

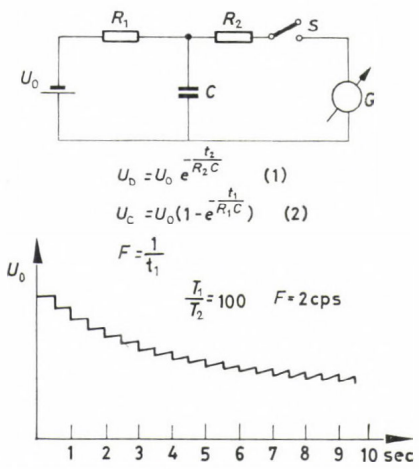

Fig. 2. The top diagram represents a simple circuit, constructed for the modelling of transmitter dynamics (cf. text). The lower graph visualizes the sequence of potential changes of condenser *C* when switch *S* is turned on and off rhythmically. The time of shortcircuiting is 1 msec, the time of recharging, 500 msec. This corresponds to a stimulus frequency of 2 cps. On the ordinate the charge of the condenser appears in arbitrary units.

It is well known since Perry's work on sympathetic ganglia that each impulse liberates the same fraction, the same proportion of the transmitter being stored momentarily. At higher stimulation frequencies each impulse liberates smaller doses of the transmitter and so the evoked PSP's would be smaller. This leads to a partial depletion of the terminals, because the synthesis and restoration of the transmitter are considerably slower processes than liberation.

For modelling these events in the presynaptic terminals we constructed a mathematical model of a simple electrical circuit which can simulate functionally the processes described qualitatively above (Fig. 2).

The battery U_0 charges the condenser C through resistance R_1 to a potential U_0. Now, at the moment when switch S is turned on, the condenser begins to discharge through resistance R_2 and its momentary potential can be measured by instrument G. After opening the discharging circuit, condenser C stops discharging and begins to regain its charge from battery U_0. The equations of discharge (1) and recharge (2) shown in the figure represent simple exponential functions. In this model, battery U_0 represents the synthesizing power of the terminals. Condenser C is the capacity of the terminal, whose momentary charge is proportional to the quantity of the transmitter stored. $\frac{R_1}{R_2}$ expresses the relation between the respective rates of transmitter refilling and release. The potential difference shown by instrument G is analogous with the evoked potential. By choosing the parameters of the circuit suitably one can simulate the habitution of evoked potentials correctly also quantitatively (Fig. 3*a*).

If we choose the values of $\frac{R_1}{R_2} =$ from 100 to 1000, the time of short-circuiting $t = 1$ msec, and repeat it many times, with an arbitrary value of U_0,

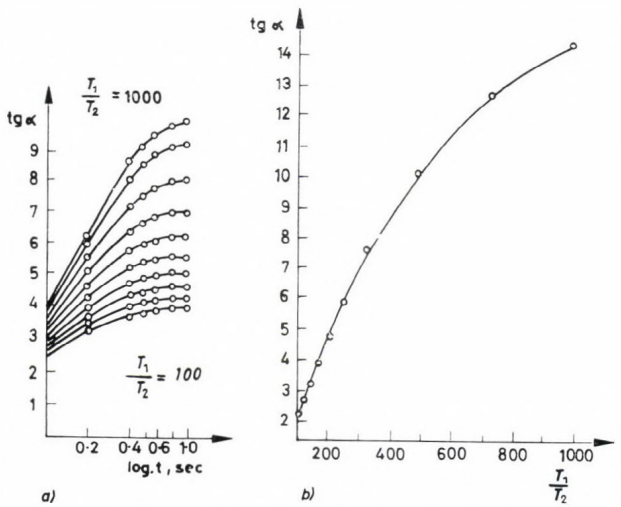

Fig. 3. (*a*) Curves obtained by continuous operation of the model shown in Fig. 2 at different stimulus frequencies and varying ratios of time constants between 100 and 1000. Ordinate: the limiting values of the condenser discharges belonging to various stimulus frequencies as computed after many cycles of the model. (*b*) Correlation between the tangents of the curves in (*a*) and the ratios of time constants.

we obtain a sequence of potential changes like this. As you can see in this figure, at 2 cps repetition frequency, the total charge of the condenser decreases stepwise and tends to a limiting value; the voltage of the single discharges also tends to some limit which remains practically constant and characteristic for the repetition frequency. At various frequencies this limiting value is directly proportional to the log time separating two subsequent impulses. The circuit and the whole sequence of events was simulated by an electronic computer type Minsk 22 and described also analytically. As a result of computer simulation we obtained these curves showing the amplitude changes in the frequency range examined in the experiments. The initial section is almost parallel with the abscissa, the second, decaying part is very nearly straight. Varying the respective time constants of the charging and discharging circuits so that their quotients fall into the range from 100 to 1000, we obtain curves whose tangents are of the same magnitude as those of the experimental curves. Considering that the only factor on which the tangent values depend is the quotient of the time constants and resistances, from a given experimental curve one can calculate the ratio between the rate of the transmitter refilling and release. The correlation between the ratio of the time constants and the tangent of the second straight part of the curve is also shown in Fig. 3*b*.

As an alternative explanation it could be said that an inhibition which is synchronously activated by afferent impulses would be able to modify the amplitude exactly in the same manner. This, however, could be excluded with certainty upon evidence from experiments on strychninized cerebral cortex. Strychnine, as is well known, is a potent inhibitor of synapses working on the basis of postsynaptic inhibitory mechanism in the central nervous system. Therefore, strychnine causes to appear on the cortex enormous paroxysmal discharges showing signs of irradiated excitation in the neuropile of the upper cortical layers. Such strychnine potentials can be provoked on the auditory area by both acoustic clicks and MGB stimuli (Fig. 4). The strychnine potentials elicited by repetetive stimulation of the MGB obeyed the logarithmic regularity much better than did the evoked potentials. Therefore it seems that postsynaptic inhibition does not play a considerable role in the mechanism of this type of habituation. Postsynaptic inhibition could better be made responsible for the random variations in the evoked potential amplitude, because after strychnine this becomes apparently reduced.

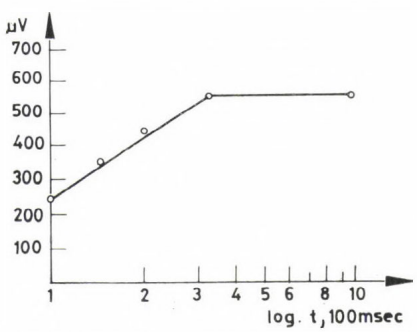

Fig. 4. Frequency dependence of strychnine potentials elicited by supermaximal MGB stimuli and lead off from the surface of the gyrus ectosylvius anterior.

One may suppose that instead of postsynaptic, presynaptic inhibition may be involved. This form of inhibition, however, has not been proved to work in the mammalian cortex. I think that our model, based upon the

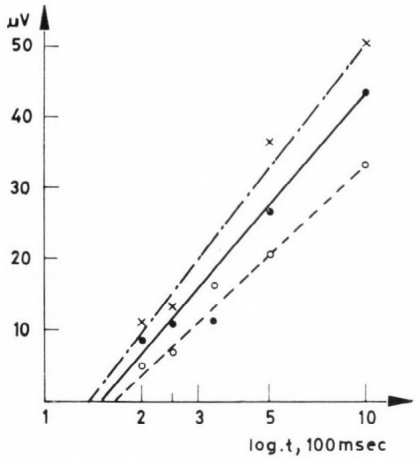

Fig. 5. Frequency dependence of cortical potentials lead off from a depth of 1 700 μ of the gyrus ectosylvius anterior, elicited by supermaximal MGB stimulation. Continuous line: control, dotted line: during 400 μA hyperpolarization, broken line: during 400 μA depolarization.

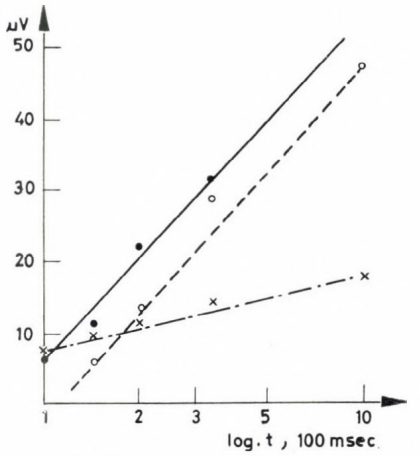

Fig. 6. The effect of locally applied 0·5 per cent (broken line) and 2·0 per cent (dotted line) potassium chloride on the frequency dependence of evoked potentials by MGB stimulation. The cortical potentials were lead off from the gyrus ectosylvius anterior by depth recording from 1 800 μ. Continuous line: control.

dynamics of transmitter release and restoration, offers a better explanation than any other assumption. The results of our model experiment seem to be supported by those of other types of experiments (Fig. 5).

As it has been mentioned earlier, hyperpolarization enhances transmitter liberation, and depolarization has the opposite effect. All these agents exert their effects by modifying the membrane potentials of the presynaptic endings. As it can be seen in Fig. 5, hyperpolarization increased and depolarization decreased the tangent of the frequency function obtained by MGB stimulation. This means that the rate of transmitter release was enhanced at the cost of restoration in the case of hyperpolarization, and the opposite happened during depolarization. One might object that these changes in amplitude, which can be predicted on the basis of our model, could be caused also by the changes in the membrane potential of the postsynaptic elements. In this latter case, however, we should have obtained no change in the slope of the curve, but a displacement parallel to the original curve. In addition, the amplitude of the EPSP is far less influenced by changes in postsynaptic membrane potential than by those occurring in presynaptic membrane polarization, as it has been proved by Takeuchi on neuromuscular junction and on sympathetic ganglion cells.

That in depolarization real membrane potential changes must have ensued is supported also by the action of potassium chloride (Fig. 6). This salt applied locally in 2 and 0·5 per cent concentrations on the cortex changed the slope of the frequency curve in the same direction as depolarization.

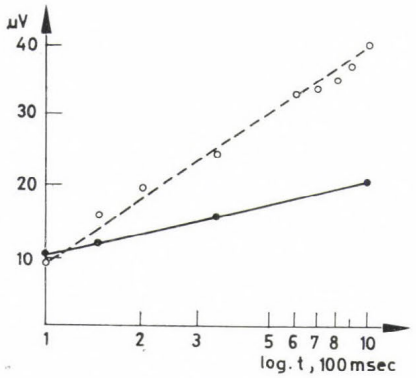

Fig. 7. The effect of 2 per cent GABA solution, applied locally to the auditory cortex, on the evoked potentials by MGB stimulation. Continuous line: control, broken line: after application of GABA.

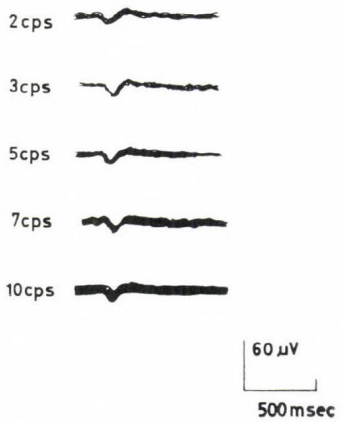

Fig. 8. Evoked potentials lead off from the MGB at auditory click stimuli of different frequencies applied to the opposite ear. Each record has been made by superposition of 10–20 faint traces. No sign of habituation occurs.

GABA in 2 per cent concentration exerted an effect analogous with that of hyperpolarization (Fig. 7), which permits to assume that the hyperpolarizing effect of this substance observed in invertebrates affects, in the mammalian cortex, only presynaptic endings. It is remarkable that GABA enhanced not only both kinds of evoked potentials but also the recruiting potentials.

Taking records from the MGB at various frequencies of auditory click stimulation, no sign of habituation was observed (Fig. 8).

Summing up our experimental results and theoretical considerations, I should say that when we search after mechanisms of habituation, before adopting more sophisticated explanations, one must take into account the changes in synaptic efficacy which ensue regularly after repeated use. It seems very probable to me that such a partial exhaustion gives rise to those structural and functional improvements in presynaptic terminals to which Professor Szentágothai referred in his paper (p. 21).

DISCUSSION

J. Székely: Why is not there any habituation at the level of the medial geniculate body?

O. Fehér: I have not said that there would be no habituation in the MGB at all, but I failed to observe it in the frequency range applied to the cortex in our experiments.

I. Madarász: On the basis of chronic habituation experiments, it is generally accepted that habituation—at least in the intact CNS—must be essentially connected with some sort of memory function. The rather specific

nature of habituation, which means that by changing the stimulus parameters, dehabituation occurs at the very first application of a novel stimulus, speaks—as almost generally accepted—for the inhibition-type mechanism of the phenomenon. May I ask you whether you have any explanation, on the basis of your experiments, for the specificity? May it be that this molecular environment, or molecular picture, as Dr. Sebestyén calls it, would be a primitive sort of memory function?

O. FEHÉR: I do not think that this type of habituation would be specific only for the auditory system, or only for this experimental situation. The phenomenon studied is, no doubt, a component of habituation, but does not fulfil all its criteria. I am convinced that the changes ensuing in the terminals during habituation are connected with further, more persistent biochemical or even morphological modifications. And this is where our subject is related to the problem of memory.

F. KLINGBERG: (*i*) Did you measure the recovery cycles? (*ii*) You spoke about a possible mechanism of fatigue at the presynaptic membrane. Did you take in account that there might be some kind of accommodation? The process might be influenced by the participation of dendrites. Were these axosomatic or axodendritic synapses? (*iii*) If you compare results after electrical stimulation in the medial geniculate body and after click stimuli, you should consider the fundamental difference between the two cases. In the latter case, 4–5 neurones are involved in the chain. The response to the click is a multichannel one, some neurones are not immediately involved, others drop out, others come into the process later on. So there are many influences at different levels and several channels and processes involved which are not represented in your simple model. Events in only one cell may be quite different in relation to the whole process of habituation. (*iv*) Have you any explanation for your finding that responses in the medial geniculate body did not change. Such changes were observed under different conditions in the lateral geniculate body by Fernandez-Guardiola, Naquet, Scherrer and others.

O. FEHÉR: The recovery cycles ranged from 600 to 1000 msec. In the generation of evoked potentials the axosomatic synapses are supposed to play the main role, whereas in the case of the strychnine potentials, the apical dendrites would be the site of origin. The stimuli applied were almost always supermaximal. This prevented a major variation in the units taking part in a response. The differences between click responses and MGB stimulus responses were, to my surprise, small as regards the habituation. This means that some of the peculiarities of the evoked potentials observed may be ascribed to the cortical synapse.

To your last question I may repeat what I have said previously: in a range from 1 to 10 cps no habituation in the MGB could be observed.

HABITUATION AND MEMORY

by

GABRIEL HORN

University of Cambridge, Department of Anatomy,
Cambridge, England

INTRODUCTION

Any system which can be modified by an event in such a way that the original event can be reconstructed may be said to exhibit a specific memory. Clearly if there is no after-effect there can be no memory: and if the after-effect is common to a wide variety of stimuli, it will provide little information about the nature of a particular stimulus. A widely held view concerning the nature of the putative after-effects of experience is that a given environmental change activates specific populations of neurones. This strengthening, commonly visualised as a growth of synaptic linkages, is considered to be the cellular basis of memory. Since growth is likely to involve changes in protein synthesis, which is controlled by RNA, and which in turn is controlled by DNA, it follows that changes in these substances may be expected as a consequence of experience. Changes in nucleic acids have long been postulated in relation to memory (Horn, 1952; Hydén, 1960; Hydén and Egyházi, 1962). These are no longer thought of as representing "taped records" of the environmental change, but to be necessary steps in the modification of synaptic connectivity.

All this is eminently reasonable. But it needs to be emphasised that these arguments are based on slender evidence. Our notions of the neural basis of acquired memory are little more than logical constructs made from observed behavioural changes. As a result there is a danger of confusing the construct with reality. There is also a danger of overlooking the possible role of known after-effects of neural activity because they do not fit in with our preconceived ideas.

One of the known after-effect of stimulation appears as a negative trace of the antecedent neural activity. In this paper I wish to consider the possibility that this after-effect may play a role in information storage, a memory that is basically and, perhaps, paradoxically a weakening rather than a strengthening of synaptic linkages.

SOME AFTER-EFFECTS OF STIMULATION

1. TIME COURSE

Many cells in the central nervous system of both vertebrates and invertebrates respond each time a stimulus is applied to a given array of receptors. The response of such cells is commonly stable whether the stimulus is applied

as frequently as 5 per sec or only once every 5 sec. There are other cells, however, which do not give such stable responses. If a stimulus is applied once every few seconds, the evoked discharge decreases (habituation). An example of a unit behaving in this way is given in Fig. 1. This unit, which was recorded from the mid-brain of a rabbit, responded with a vigorous discharge of spikes when a brief puff of air was applied to its cutaneous receptive field, located over the animal's right shoulder. The response to repeated applications of the stimulus applied at intervals of 3 sec was irregular, but was maintained over the first 13 presentations: thereafter the number of spikes in the evoked discharge declined until, by the twentieth presentation or so, there were no more spikes present when the stimulus was applied than would be expected from the background firing rate.

The rate at which the response of such a unit declines depends on several factors, including the frequency of stimulation and the number of times the stimulus has been presented in the past. The effects of varying these two factors are shown in Figs 2 and 3 respectively. The responses plotted are of single units recorded from the tritocerebrum of locusts. In each case, as in all studies of these neurones described in this chapter, the stimulus

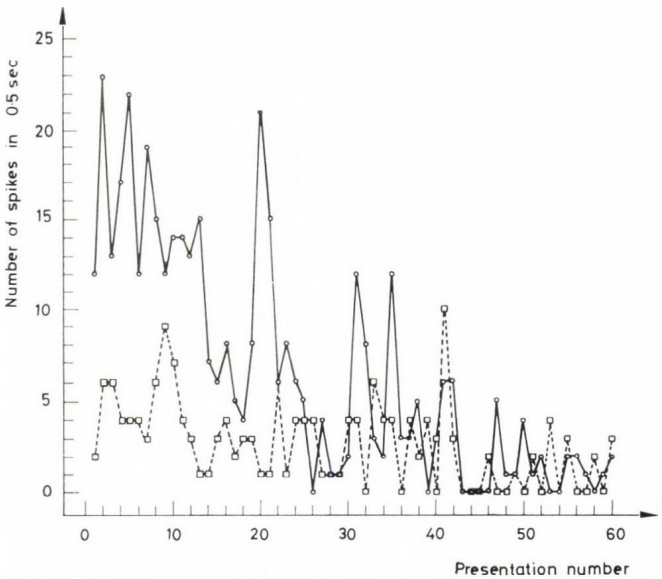

Fig. 1. Effects of stimulus repetition on the response of a unit recorded from the tectotegmental region of the rabbit mid-brain. A brief puff of air was applied at 3 sec intervals to the cutaneous receptive field located over the right shoulder. The number of spikes present in the 0·5 sec before(background activity, open squares and broken lines), and during (open circles and solid lines) the first 0·5 sec of stimulation are plotted against stimulus presentation number (Horn and Hill, 1966)

was a black disk moved across the visual field. The effects of varying the interval between successive movements are shown in Fig. 2. The shorter the interstimulus interval (i.s.i.), the more rapidly the response declined and the smaller it became. Thus there was only a small decrement when the i.s.i. was 120 sec and a much larger one when the i.s.i. was 5 sec.

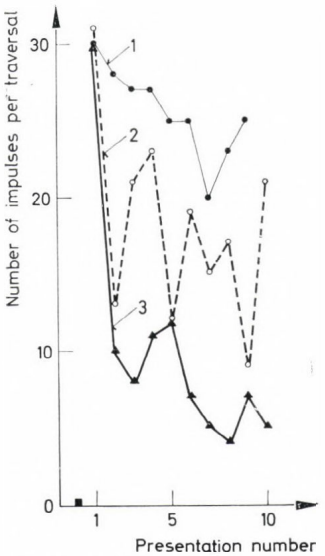

Fig. 2. Effect on response decrement of varying the interstimulus interval. Tritocerebral unit of locust. Disk (4° diameter) moved downward (40°) in visual field in 1 sec. A rest period of several minutes intervened between the different groups of trials. Curve 1, i.s.i. 120 sec; curve 2, i.s.i., 40 sec; curve 3, i.s.i., 5 sec. The filled black square in this and in other figures represents the number of spikes which could be expected in the time taken for the disk to move across the field, calculated from the background firing rate (Horn and Rowell, 1968).

The influence of differences in the amount of prior stimulation on responsiveness is illustrated in Fig. 3. In one experiment the disk was moved six times across the visual field, the i.s.i. between movements being 10 sec. The stimulus was then withdrawn for 300 sec and another group of six stimuli presented. A total of five such groups of movements (short series) was made. Five groups, each containing 60 stimuli (long series) were then presented, the interval between stimuli and between groups of stimuli was the same as for the short series. Responses to the first six stimuli of each of the last four groups in each series are plotted (Fig. 3). It may be seen that, for each group, the first six responses in the long series (open circles) are much weaker than the six responses of the short series (closed circles), there being only one point of overlap. As groups of 60 stimuli were successively applied, the responsiveness of the cell to the first six stimuli of each group gradually declined, an effect that is shown by the falling response to the initial stimulus. Two hours after the last stimulus of the long series had been given the cell had recovered its responsiveness (Fig. 3, last curve).

If a cell is allowed to rest after a period of stimulation, its capacity to respond usually increases. In cells behaving in this way, the magnitude of recovery is usually a relatively simple function of the rest interval. Such a relationship is illustrated by the set of curves plotted in Fig. 4. In many cells in the anaesthetised mammalian brain and spinal cord a period of 3 to 10 min of rest is usually adequate to restore responsiveness to its original level (Fig. 5). Invertebrate neurones often take longer than this to recover: indeed recovery may not be achieved even after a rest period of several hours (Fig. 6). The responses plotted on this figure were obtained from a cell in the locust brain and were evoked by an upward movement of the disk in the visual field. The response to successive movements gradually declined over 10 presentations from an initial value of 71 spikes to

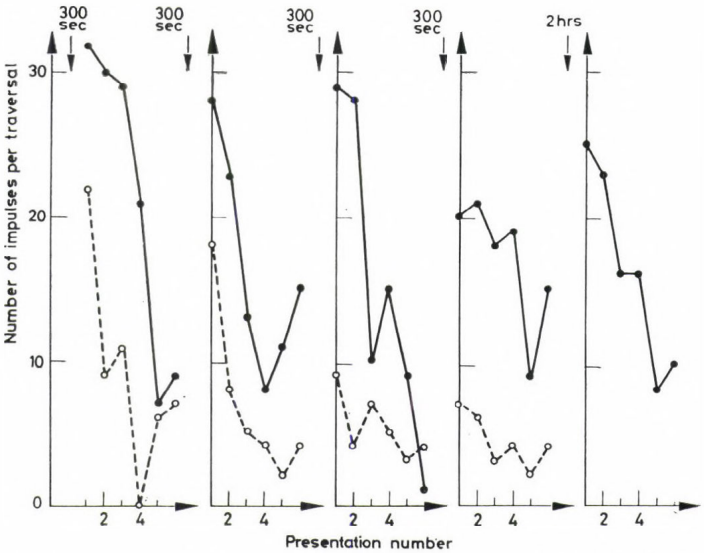

Fig. 3. Effect of previous stimulus exposure on responsiveness. The short series (closed circles) are responses to groups of 6 movements of the black disk separated by intergroup intervals of 300 sec. The responses to the first 6 of a group of 60 movements (long series) are plotted as open circles. After the last of the long series of movements had been made the cell was allowed a rest period of 2 hr. The stimulus was then presented and the responses evoked are plotted in the last curve on the right. In all curves the i.s.i. was 10 sec (Horn and Rowell, 1968).

a final value of 16. The cell was then left unstimulated for 5 hr after which the disk was again moved in the visual field. The initial response (25 spikes) was only slightly greater than the response to the last movement of the previous series. Obviously it is necessary to consider whether such a prolonged depression is a result of a general deterioration of the preparation. This is unlikely since the cell responded (63 spikes) to a forward movement presented immediately after the second set of upward movements, almost as briskly as it had done (66 spikes) before the first set of upward movements had been presented.

An interesting form of response modification consequent on prior exposure to a stimulus is shown in Fig. 7. Responses were recorded soon ($<$1 hr) after the locust had been immobilised. The responses to the first group of 9 movements, though variable, showed no consistent trend (Fig. 7, curve 1) and the responses to the second and also the third (curve 2) group of stimuli waned only slightly. The responses to all subsequent groups of stimuli were different, although the response to the first movement following a pause of 5 min or more was always brisk. Instead of giving maintained responses to repeated stimulation, the responses waned. This effect is shown in

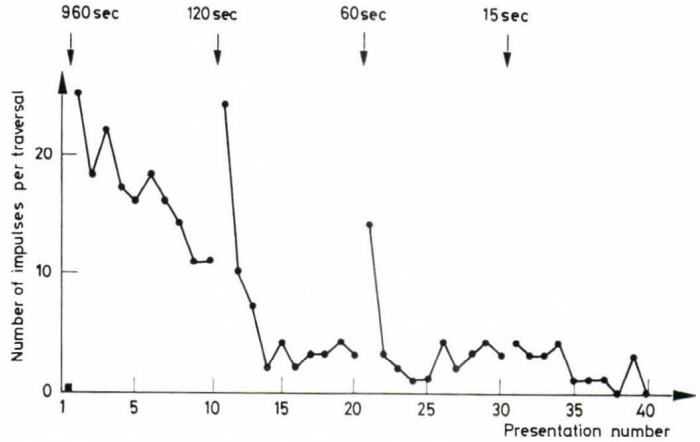

Fig. 4. Effect of varying the interval between groups of 10 stimuli. Each stimulus was a forward movement of a black disk in the visual field. The i.s.i. was constant at 10 sec. The number above each arrow is the duration of the intergroup interval. Each series after the first follows directly after the preceding one (after Horn and Rowell, 1968).

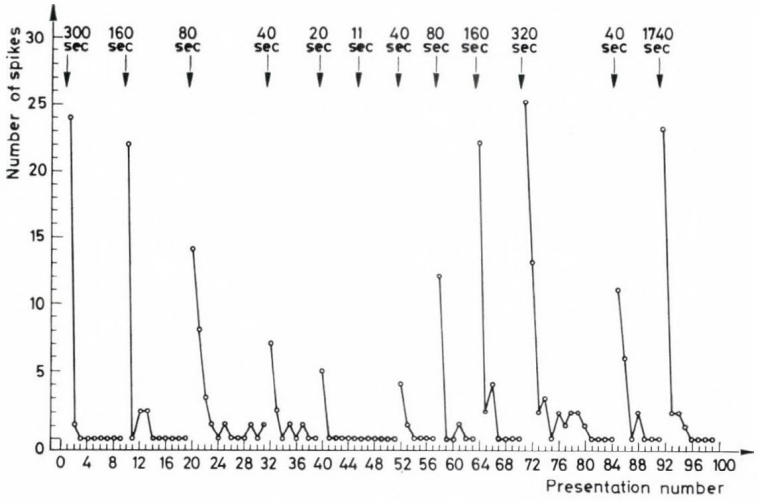

Fig. 5. Attenuation of response to a repeated movement across the visual field and recovery following a lapse of time. Unit recorded from rabbit mid-brain. The i.s.i. was 4 sec. The number of spikes evoked by each movement is plotted against stimulus presentation. Numbers above the arrows are the times between groups of movements. Initial responses to movements which followed a rest of 300 sec or more were approximately constant (after Horn and Hill, 1966).

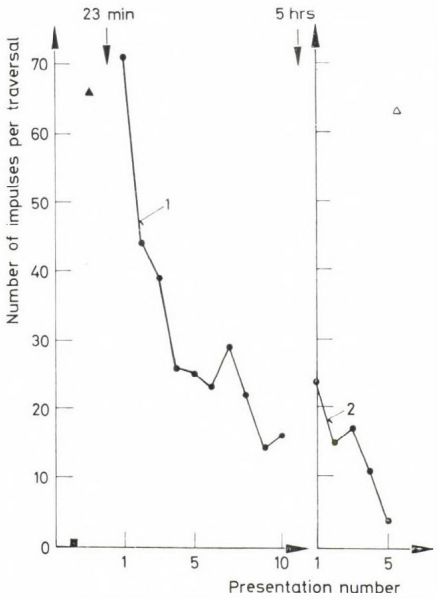

Fig. 6. Prolonged depression of transmission. Tritocerebral unit of locust. Each filled circle represents the number of impulses evoked by an upward movement of a black disk in the visual field. i.s.i., 10 sec. An interval of 5 hr elapsed between the first (curve 1) and second (curve 2) groups of movements. Movement in a forward direction, immediately after the second group of upward movements evoked almost as many spikes (open triangle) as were evoked before (closed triangle) the first set of upward movements was presented (after Rowell and Horn, 1968).

curve 3, which represents the responses to a group of six movements made after a rest period of 90 min. The rate of attenuation was extremely rapid and by the third movement the evoked discharge had fallen to a very low level. Such a change, from a non-decremental, to a decremental form of responding, may not be reversed even after a rest period of 10 hrs (Horn and Rowell, 1968). Indeed, Rowell (1970) finds that such changes may persist in a given cell until the end of an experiment which may last for several days.

The reason for the failure to demonstrate such long-term changes in neurones in the mammalian brain is not clear. It may be that such neurones do not exist in these brains. There are other explanations however. Thus there is a possibility that long-term effects are not seen because the drugs used to immobilise the animal may selectively depress such units. For this reason, or because the cells may be particularly small, they may not be detected by conventional microelectrode recording techniques. In addition, the activity of single units in the mammalian brain is not commonly recorded for prolonged periods of time.

2. SPECIFICITY

It may be seen from Fig. 6 that there is an element of specificity in the unresponsiveness that results from stimulus repetition. When the response to an upward movement had waned to a low level, the cell still responded maximally to movement in the vertical plane. Examples of more specific forms of response decrement can also be demonstrated in the locust. Thus (Fig. 8), once the response to a movement in a given plane had become habituated, a brisk discharge was evoked in the cell by moving the disk along a line parallel with, but a few degrees below the original one. The unit whose responses are shown in Fig. 9 also shows intramodality specificity, but was recorded from the mammalian mid-brain. The cell responded to a 1000 Hz tone. When this was presented at intervals of 1·5 sec, the

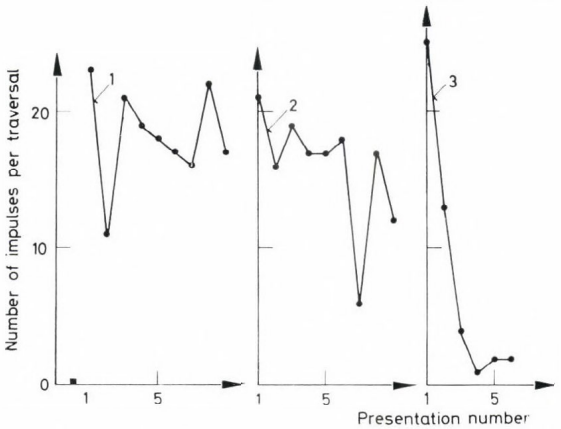

Fig. 7. Changes in response characteristics of a tritocerebral unit. The disk was moved downward at i.s.i. of 5 sec. The responses plotted in curves 1 and 2 are respectively the discharges evoked by the first and third sets of 9 movements in the experiment. A rest interval of 30 min preceded the third group of stimuli. The responses plotted in curve 3 followed an intergroup interval of 90 min (after Horn and Rowell, 1968).

response waned and that evoked by the 15th stimulus (record 15) was barely greater than the background discharge. Following a 5 min pause the stimulus evoked a brisk response (record 1). This gradually declined and was not greater than the background firing discharge

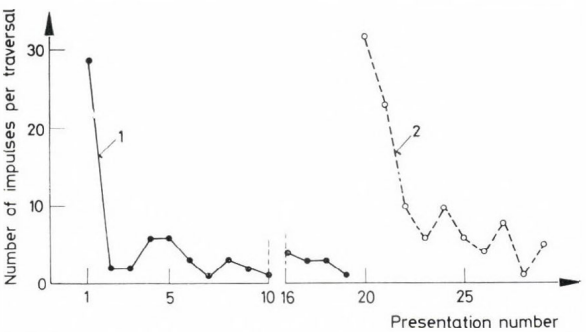

Fig. 8. Specificity of habituation. Tritocerebral unit. The disk was moved repeatedly (i.s.i., 10 sec) in a horizontal plane and the responses to successive movements are shown in curve 1. In the 10 sec interval between the nineteenth and twentieth presentation, the disk was displaced downward 12° and moved along a line parallel with the original one. A brisk discharge was initially evoked which gradually declined (after Horn and Rowell, 1968).

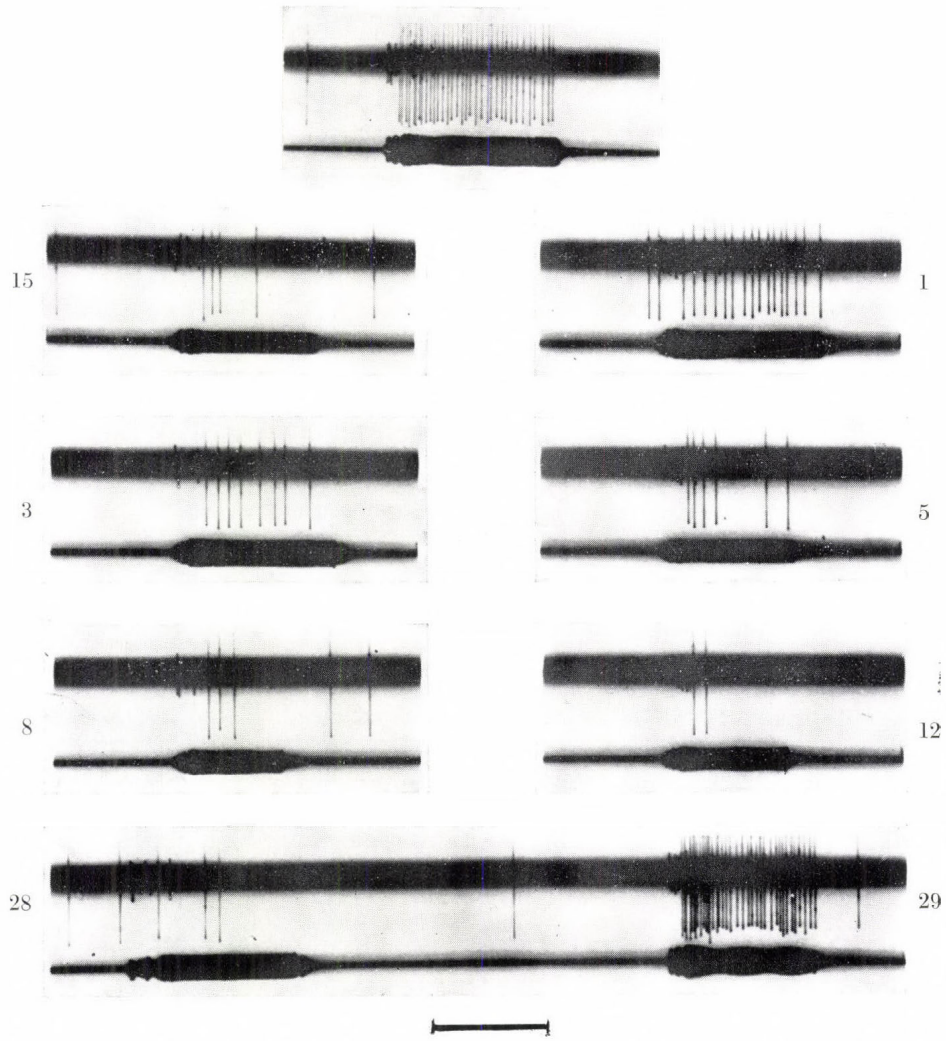

Fig. 9. Specificity of habituation in a unit recorded from the rabbit mid-brain. Interstimulus intervals between tones in a series was approximately 1·5 sec. Scale 0·5 sec. Presentation number shown in margins (after Horn and Hill, 1964).

at the eight and subsequent presentations. A 1500 Hz tone was substituted for the twenty-ninth presentation. The new stimulus evoked 32 spikes in the first 0·5 sec. This compares with 28 spikes evoked in 0·5 sec by the 1500 Hz tone presented before the habituating sequence (top record). Thus the failure of response to the 1000 Hz tone was not accompanied by any failure of response to the 1500 Hz tone.

In effect, repeated exposure to a given stimulus causes a functional

ablation of some of the response capacities of the cell. The ablation may be highly specific to the stimulus, though the correspondence is rarely exact. This effect may be shown by observing the responses to two stimuli before and after presenting one of them repeatedly. The results of such an experiment are shown in Fig. 10. The unit, recorded from the rabbit mid-brain, initially responded to tones of 1000 Hz and 950 Hz (Fig. 10, trace 1). The 1000 Hz tone was then delivered at intervals of 1·5 sec until the "response" was no greater than that expected from the background discharge (trace 2). The unit was excited by tones of 900 Hz (traces 2 and 4) and 1050 Hz (trace 5), but not by the 950 Hz tone (trace 3). Habituation of the response to the 1000 Hz tone had thus generalised so that the response to the 950 Hz tone was eliminated.

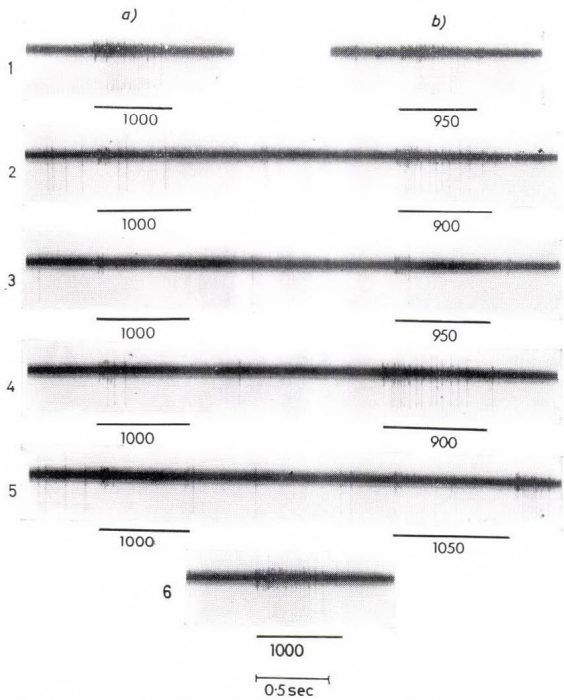

Fig. 10. Stimulus generalisation exhibited by a unit recorded from the mid-brain of a rabbit. The responses are to tonal stimuli the frequencies of which are given below the horizontal bars, and the duration by the lengths of these bars relative to the time scale. Traces 2 to 5 are continuous. The responses shown in the records in trace 1 are controls for the 1000 Hz and 950 Hz tones and were presented before the 1000 Hz tone was repeatedly applied. The response to the 1000 Hz tone shown in trace 6 was recorded several minutes after trace 5 was recorded (Horn and Hill, unpublished data).

Another interesting feature of the experiment from which the records in Fig. 10 were taken is that the cell, although excitable, remained unresponsive to the 1000 Hz tone. This was not due to any lasting impairment of its capacity to respond to this tone which, when presented several minutes after the end of the experiment, evoked a discharge of spikes (Fig. 10, trace 6). During the stimulus sequence therefore the cell had, in some sense, acquired a memory not to respond. The behaviour of three units showing a similar property are shown in Fig. 11. The cells were recorded simultaneously by a single microelectrode in the rabbit mid-brain. The units were activated by a spot (Fig. 11, Sp) of light moved across the receptive field

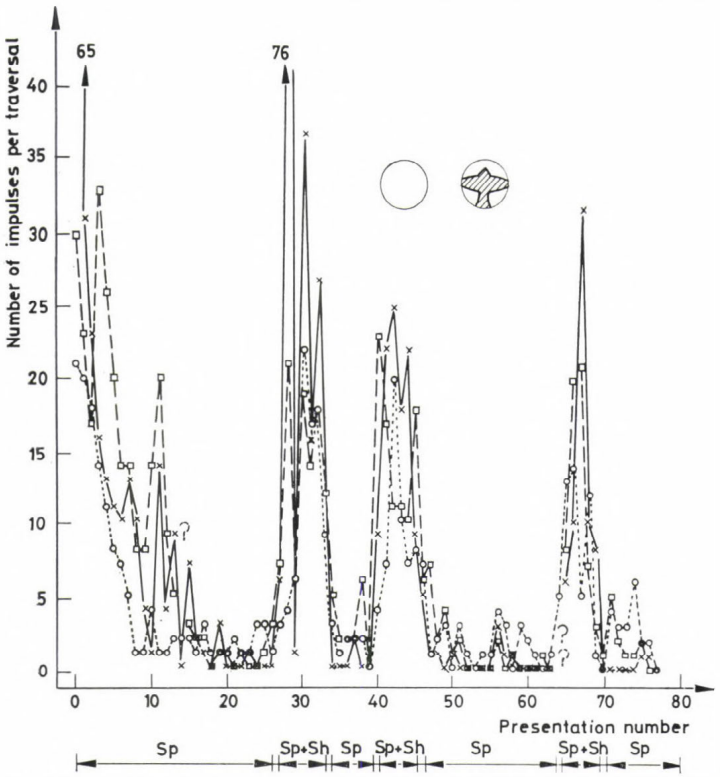

Fig. 11. An example of three units, each of which exhibits a memory not to respond to a previously iterated stimulus. The units were all recorded simultaneously through a single microelectrode in the superior colliculus of the rabbit, and their responses separately identified and plotted. Repeated movement of a spot of light (Sp, left hand inset) 1° in diameter, across the receptive field at intervals of 4 sec evoked discharges which rapidly attenuated. When the figure (Sp + + Sh, right hand inset) was substituted for the spot, the unit responded. It did not respond when the spot (Sp) was substituted for this figure (Horn and Hill, unpublished data).

at intervals of 4 sec. By the twentieth presentation the movement evoked between 1 and 2 spikes. Following the twenty-ninth presentation of the spot a complex figure (see right inset, Fig. 11) was presented, the rate of stimulation being kept constant. The new stimulus (Fig. 11, Sp + Sh) evoked a brisk discharge, and continued to do so for several presentations. When the original spot was substituted, the cell remained unresponsive. It responded again when the complex stimulus was re-substituted and again failed to respond when the spot alone was moved across the receptive field (Fig. 11, presentations 46–63).

3. MECHANISMS

At first sight this seemingly elaborate pattern of activity might be expected to require an elaborate neuronal network. In the sense that the neurones make complex discriminations the network is indeed elaborate. However, in the sense that the response of a cell to a particular stimulus wanes, the neuronal mechanisms may be quite simple and an elaborate network may be uneccessary. Such failure would occur in the system of neurones leading from the receptor surface to the recorded cell gradually ceased to transmit a response to the repeated stimulus: and the failure would be specific if the system of neurones activated by the stimulus were also specific. If some other stimulus were presented which activated a different neuronal pathway to the recorded cell, anatomically independent of the depressed pathway, the cell would be activated once again. These possibilities are illustrated in the simple diagram in Fig. 12*a*. The recorded cell R is activated by stimulus A over a pathway represented by A–R. Let transmission breakdown at the junction in this pathway shown by the filled circles. When this happens, stimulus A will fail to excite cell R. If stimulus B is then applied, it will excite cell R since the activity it sets up in the network reaches R over a pathway (B–R) which is independent of that originally excited by stimulus A.

If the pathways excited by stimulus A overlap with those excited by B, there will be some interaction of response decrement. Such a system is illustrated in Fig. 12*b*. The recorded cell R is excited by 1000 Hz and 950 Hz tones. The 1000 Hz tone excites cell 7 and hence cell R; the 950 Hz tone excites cell R through cell 8. Assume that cells 7 and 8 will be excited only if they receive input from >2 feeder cells (i.e. cells 1–6): assume also that, if the 1000 Hz tone is repeatedly presented, transmission breaks down at the synapses containing the terminals of cells 1–4. When this happens cells 7 and R will cease to respond. If the 950 Hz tone is now presented, cells 3, 4, 5 and 6 will fire. Transmission across the synapses of cell 8 with cells 3 and 4 is depressed, however, because of previous exposure to the higher tone. The condition that cell 8 will fire if it receives input from >2 feeder cells is not satisfied: so that cells 8 and R do not respond to the 950 Hz tone.

Most of the known decremental properties of neurones described above can be understood in terms of a self-generated depression of transmission (SGD) in a system of neurones connected to the recorded cell and activated

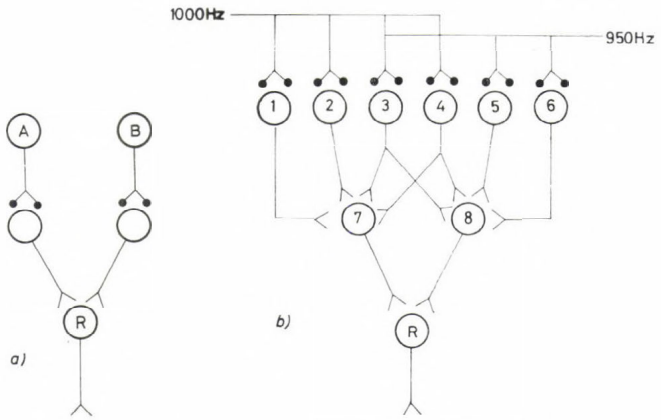

Fig. 12. (*a*) A simple network showing highly specific response decrement in a recorded neurone R. Stimulus A excites a set of neurones that excite cell R over a pathway represented by A–R. When stimulus A is repeatedly applied the synapses of cell A with the next cell gradually fail to transmit so that cell R gradually ceases to respond to this stimulus. Cell R responds however to stimulus B which excites cell R over the network represented by B–R and which is anatomically independent of A–R. (*b*) A network exhibiting stimulus generalisation. For further discussion see text (after Horn, 1967)

by a particular stimulus (Horn, 1967). The SGD may be brought about in a number of ways but two possibilities stand out quite clearly. On the one hand the SGD may result from a build-up of inhibition. As each burst of spikes, evoked by successive stimuli, traverses a chain of neurones, the activity of inhibitory neurones gradually builds up, depresses and finally blocks transmission in the chain. Recovery of transmission depends upon the dissipation of the inhibitory effects. There is some evidence for this view (Holmgren and Frenk, 1961; Wickelgren, 1967*b*; Wall 1970), but as yet it is not strong. Thus it has been remarked by many observers (for example, Bell et al., 1964; Horn and Hill, 1966) that the spontaneous activity of the recorded cell rarely changes even when the response to a repeated stimulus has fallen to a low level: when this happens the cell may remain fully responsive to other stimuli. Furthermore, Spencer et al. (1966*b*) found that excitatory postsynaptic potentials (EPSPs) recorded from motoneurones in the spinal cord gradually waned on repeated stimulation of an afferent nerve, and that the motoneuronal membrane was not hyperpolarised. Similar observations were made by Segundo et al. (1967) in their studies of neurones in the bulbar reticular formation. The above results suggest that the recorded cells are not inhibited: the results do not, however, exclude the possibility that response decrement is brought about through the inhibition of a cell which is always "upstream" from the

recorded neurone, the inhibited cell for some reason (e.g. size) not being detected by the microelectrode.

On the other hand it is possible that transmission breaks down at certain synaptic junctions without the involvement of internuncial cells, which are essential for the inhibitory build-up theory. In the absence of these cells transmission failure (synaptic depression) might be brought about by changes in the capacity of the synaptic terminals to liberate transmitter substance (Bruner and Tauc, 1966), or of the postsynaptic membrane to respond to it. There are a number of pieces of experimental evidence which suggest that such mechanisms may indeed be implicated, at least at some junctions. Thus, when a drop of water falls on the head of *Aplysia*, an EPSP can be recorded from the giant cell of the left pleural ganglion. If the stimulus is repeated at intervals of 10 sec or even longer, the size of the EPSP gradually declines. This sequence of changes also occurs when the afferent fibre to the neurone is stimulated electrically, transmission occurring across what is thought to be a single synapse (Bruner and Tauc, 1966). The latter experiment suggests that the changes do not involve internuncial neurones. This view is supported by observations on the isolated stellate ganglion of the squid. Decremental transmissions across this ganglion can be demonstrated, but internuncial cells have not been described (Young, 1939). The presynap-

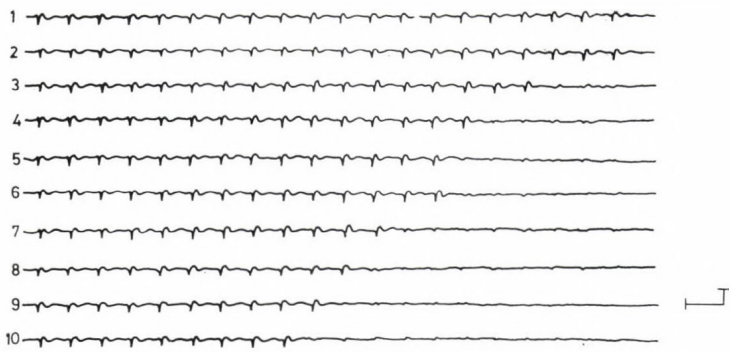

Fig. 13. Response decrement at the giant synapse of the squid. Isolated stellate ganglion preparation. The mantle connective (pre-nerve) was stimulated by a train of 20 shocks. Each shock was 0·1 msec duration, and delivered at a rate of 40 per sec for 0·5 sec. Ten such trains were given. The interval between trains was 10 sec. The response of the giant axon in the last stellar nerve (post-nerve) was recorded using extracellular platinum hook electrodes. The responses to each train of shocks are shown. A postsynaptic spike is elicited by each of the 20 shocks in the first and second trains (records 1 and 2). Subsequent trains failed to elicit 20 spikes. The pattern of response decrement was consistent, the later shock of the train failing progressively to evoke impulses in the post-nerve, although a graded response (synaptic potential) persisted. Scale 25 msec and 3 mV (downward deflection is negative) (Horn and Wright, 1970).

tic fibres in the pre-nerve are monosynapticly connected to a giant axon in the post-nerve. When the pre-nerve was excited by a train of 20 shocks in 0·5 sec (Horn and Wright, 1970), a response of the postsynaptic giant fibre to each shock was recorded (Fig. 13, record 1). The trains were repeatedly applied every 10 sec, and the response of the postsynaptic giant axon gradually waned (records 3 to 10). If the system is allowed a period of rest, some recovery of transmission will occur, the degree of recovery depending on the duration of the rest period (Fig. 14).

The most likely cause of this breakdown in transmission (for discussion see Horn, 1970) is a progressive reduction in the amount of transmitter substance liberated by the repeatedly activated synaptic terminal. This mechanism would be extremely economical of neurones, since internuncial cells are not required to choke off a response, and would have the great advantage that the changes are synapse-specific.

Whatever the mechanism of the transmission breakdown, there are clear instances where it appears to be a two-stage process. If repeated stimulation results in a response falling to a level at which decremental and restitutive processes are in balance, then a given period of rest should restore the response to a given level, however long the period of repeated stimulation. This expectation is rarely fulfilled (Horn and Rowell, 1968; Rowell, 1970). The evidence suggests rather that a response which has dropped to a maintained low level soon ceases to be a steady-state response. If synaptic depression is brought about by a reduction in the amount of transmitter released, maintained repeated stimulation may act further back on this process, perhaps by decreasing the mobilisation or synthesis of transmitter substances.

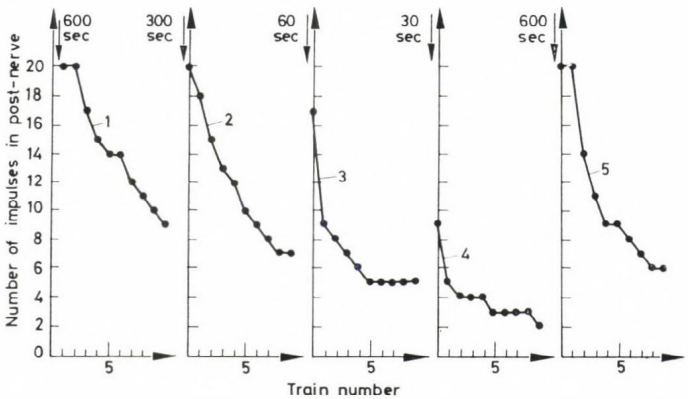

Fig. 14. Response decrement and recovery following a lapse of time at the giant synapse of squid. The experimental details are given in the legend to Fig. 13. The interval between groups of stimuli are given beside the arrows. (Note the similarities with the sets of curves shown in Figs 4 and 5) (Horn and Wright, 1970).

SHORT- AND LONG-TERM CHANGES AND THEIR RELATION TO BEHAVIOUR

In the intact organism some synapses, when activated within the physiological range, have a high factor of safety and continue to transmit whenever the presynaptic fibre fires. Such synapses are found commonly in the primary sensory pathways and elsewhere, for example the synapse of the monosynaptic stretch reflex in the spinal cord (Prosser and Hunter, 1936; Spencer et al., 1966*b*) and the neuromuscular junction. Other synapses, such as those described above, have a low factor of safety, rapidly fail to transmit and recover only slowly. There is thus a very wide range of safety factors in junctional transmission and also a wide range of recovery with an upper limit, for some cells (see Figs 6 and 7), that has not been identified. It is not difficult to visualise possible mechanisms for these long-term effects. Thus the synaptic terminals of a neurone may contain only a limited amount of transmitter substance and be incapable of manufacturing more locally. If this takes place at the cell body, then it may take hours or days before sufficient of the substance has passed to the terminals to re-establish full transmission, depending on the substance involved, the rate of synthesis, the rate of transport along the axon and the length of the axon. Another possibility is that some terminals have a fixed amount of transmitter and when this is used up, transmission across the synapse involved ceases for good.

It is of some interest to consider the possible uses to which these changes in transmission may be put. If the response of a cell to a repeatedly applied stimulus rapidly wanes, the cell may be a novelty detecting unit. That is it will "recognise" as familiar the repeated stimulus, and not respond to it, though it will respond to other stimuli. Certain behavioural responses have similar characteristics. These may be observed in simple behavioural responses [e.g. the flexion reflex of the spinal mammal (Prosser and Hunter, 1936; Spencer et al., 1966*a*), the tentacular contraction response (Bruner and Tauc, 1966; Bruner and Kehoe, 1970) and the gill withdrawal reflex of *Aplysia* (Kandel et al., 1970)] and in complex (e.g. the orientation reaction of mammals) behavioural responses. With regard to the former the correlation between the change in behaviour and the change in neuronal activity is good. Thus habituation of the flexor reflex is brought about by a gradual withdrawal of excitation from motoneurones (Spencer et al., 1966*b*) due to habituation at synapses within the polysynaptic reflex arc (Wickelgren, 1967*b*): habituation of the gill withdrawal reflex appears to be brought about by a gradual depression of transmission across an identified synaptic junction (Kandel et al., 1970). It is somewhat more difficult to obtain direct experimental evidence relating to the neural mechanism of the more complex units of behaviour, but there is a reasonably good fit between the parametric features of habituation of the orientation reaction and of neuronal habituation in the brain-stem.

In all these cases it may be supposed that the memory not to respond consists of a breakdown in transmission across synaptic junctions. Such prolonged after-effects of activity, though specific, relate to a somewhat

special form of memory (that of habituation). Could similar neural mechanisms be involved in other forms of memory, such as that implicated in associative learning? This is certainly a logical possibility, though it may be no more than that. The essential requirement is to use a decremental process in a system in such a way that the output of the system increases. This is done in the circuit illustrated in Fig. 15*a*. In this figure it is assumed that cell 5 is excited by cell 3 and inhibited by cell 4. When cells 3 and 4 fire together there is no output from cell 5 since the two inputs exactly balance. Assume that when cell 4 is repeatedly excited transmission across the junction between it and cell 5 is depressed, but that such depression does not occur between cells 3 and 5. If cells 3 and 4 are simultaneously and repeatedly activated, cell 5 will begin to fire because the inhibiting effect of cell 4 will gradually disappear. Ultimately cell 5 will be driven by cell 3 and will continue to be driven in this way for as long as cell 4 is ineffective. Such a circuit could be used in a network that exhibits some of the properties of classical conditioning. One such network, designed by my colleague M. J. Wright and myself, is illustrated in Fig. 15*b*. In this network only single cells are shown; these could of course be regarded as representing large numbers of neurones. An unconditioned stimulus evokes an unconditioned response by exciting through cell 1 the two command neurones R_1 and R_2. Cell 1 simultaneously drives excitatory cell 3 and inhibitory cell 4 evoking in them $\lesssim n$ spikes per sec. The activity of these two cells summates algebraically on cell 5. Assume that transmission betwen cells 3 and 5 is always stable, and that betwen cells 4 and 5 is stable only if the number of spikes traversing cell 4 is $\lesssim n$ per sec. Thus the unconditioned stimulus alone will not excite a discharge in cell 5. The conditioned stimulus is considered to excite cell 2. This also evokes $\lesssim n$ spikes per sec in cells 3 and 4 and hence no output from cell 5. This conditioned stimulus (more strictly, at this

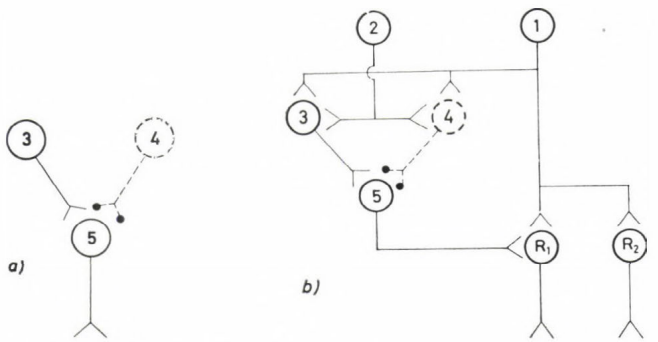

Fig. 15. (*a*) A mechanism by which the response of an output neurone (cell 5) may increase as a consequence of a breakdown in transmission (at the junction between cells 4 and 5). (*b*) A neuronal network which could mediate the known properties of classical conditioning. The memory in the system consists of a breakdown in transmission of the junction between cells 4 and 5. For further discussion see text.

stage, the neutral stimulus) does not evoke a behavioural response since it has no effective access to the command neurones R_1 and R_2 (see Horn, 1970). When the conditioned and unconditioned stimuli are paired, cells 1 and 2 are active and we suppose that together they excite $> n$ spikes per sec in cells 3 and 4. When this happens transmission across the synapse between cells 4 and 5 will be depressed. As this happens cell 5 will begin to discharge. Initially it will be capable of evoking a weak discharge in command cell R_1. As the inhibitory effectiveness of cell 4 falls to a low level, cell 5 will be driven by cell 3. If now the unconditioned stimulus is withdrawn and the conditioned stimulus alone applied, it will evoke $< n$ spikes per sec in cells 3 and 4. Cell 4 will have no effect on cell 5, so this will discharge $< n$ spikes per sec. In turn cell 5 will drive command cell R_1 and so evoke a conditioned response. With the arrangement shown, the conditioned response will be different from the unconditioned response, but contained within it, a feature that is characteristic of the relationship between the two responses. The conditioned response will persist so long as transmission across cells 4 and 5 is depressed. If transmission recovers, the conditioned response will be "forgotten".

If activity in cell 2 also led to a weak hyperpolarisation of cell 4 terminals it is possible to see how a conditioned response would become extinguished by presenting the conditioned stimulus alone. When the synaptic terminals of cell 4 are depressed, but not otherwise, hyperpolarisation of its terminals due to activity in cell 2 might gradually restore transmission (see Horn, 1970) and so lead to the extinction of the response. Type II conditioning could be fitted into a similar scheme, the reward system controlling synaptic depression by acting on the terminals of cell 4.

CONCLUSIONS

The memory in the system illustrated by Fig. 15 is, essentially, the synaptic block and as this is removed the association between conditioned and unconditioned stimuli disappears. I do not wish to argue that such synaptic changes *are* involved in associative learning; only that they *could* be, and that they need not entail changes in the synthesis of RNA or protein. The fact that these somewhat far-fetched arguments can be made at all shows, quite forcibly, that the need in the immediate future is for the descriptive analysis of neural changes in well controlled learning situations. This does not mean that we should have no theories: only that they should not be regarded as substitutes for hard experimental evidence.

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DISCUSSION

R. Galambos: If I understand your idea of presynaptic "ablation" you are suggesting that some synaptic junctions are secure, capable of transmitting at high frequency for long time periods, while others are not like

this. The second class fail rapidly, even though required to act infrequently, and these are the ones responsible for habituation.

G. Horn: Yes, that is correct.

F. Rosenblatt: (*i*) It is very attractive to consider models for memory which are based on selective blockage of synapses. I have found it somewhat easier, from a molecular standpoint, to consider such mechanisms as occurring through postsynaptic effects, however, (for example, by the masking of a receptor site by a macromolecule). I wonder if you could elaborate on your reasons for preferring a presynaptic to a postsynaptic interpretation of your results. (*ii*) Do you think your observations might be related to those of Wiesel and Hubel on loss of response in the visual system of light-deprived kittens, whose decremental performance seems to occur in the *absence* of patterned inputs, and the cells involved seem to be "taken over" by active sources of stimulation at the expense of inactive sources? Their observations suggest loss of performance due to lack of stimulation in the presence of rival sources, rather than due to *increased* level of stimulation.

G. Horn: With regard to the first question, I have reviewed the reasons elsewhere (Horn, G.: Changes in neuronal activity and their relationship to behaviour. In: Horn, G. and Hinde, R. A. [Eds]: *Short-Term Changes in Neural Activity and Behaviour*. Cambridge University Press, London, 1970). The chief point is that it is possible in the giant synapse of the squid at least, to restore transmission across a synapse depressed by stimulus repetition, by manipulating the properties of the presynaptic terminal, for example, by hyperpolarising its membrane. Of course at other synapses different mechanisms may operate and the postsynaptic membrane may be involved. With regard to your second question, there is no evidence one way or the other, but it would surprise me if the decremental change I have described bear any relationship to the observations of Wiesel and Hubel (Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. *J. Neurophysiol.* **28**, 1029–1040, 1965). Their work was done on kittens and it is possible that in a developing nervous system sensory stimulation is necessary for the maintenance of normal growth. In the absence of such stimulation neurones might regress or fail to develop their full synaptic arborizations. If the latter happened, the cells might appear functionally to regress relative to normally developing cells. It would certainly be interesting to know whether the changes Wiesel and Hubel describe are found in the adult cat, or whether they can only be demonstrated in kittens. I do not know whether such experiments have been conducted.

J. Szentágothai: You mentioned that it is lamina IV of the dorsal horn in the neurones of which this effect can be observed. The dendrites of these neurones are synaptically engaged in the multineurone network of the substantia gelatinosa. Would it not be more reasonable to assume, therefore, that this effect of habituation—here at least—is caused by a build-up of inhibition in the substantia gelatinosa network and by the removal of facilitation that is excercised upon the lamina IV dendrites by the network?

G. Horn: This is certainly possible, but there is as yet no direct evidence to support this view.

F. KLINGBERG: When we speak about an animal's behaviour we have to consider that behavioural acts are integrative, involving several different processes, structures and levels. The fundamental process of behavioural habituation may not be the cellular change which you have described. Other cells involved in habituation may behave in quite another way. But I agree with your conclusion that habituation is a kind of learning. Let me recall our experiences with freely moving rats with chronic electrodes implanted in different brain structures. An absolutely novel stimulus applied to the waking but relaxed animal causes an evoked potential (EP) in the primary projection area of the cortex with an amplitude, let us say of 100 per cent. But it does not provoke any behavioural reaction if it has no component which would act as an unconditioned stimulus eliciting an unconditioned reaction. Only by repetition under different conditions, or similarity to other stimuli which are known by the animal, does the stimulus cause behavioural activation. At this time the EP's have lower amplitudes. We call this the disturbance phase of learning. The EP's can even disappear in the electrical background activity, i.e. their amplitudes can fall far below 50 per cent of their initial level. Further repetition of the stimulus causes an increase of EP amplitudes even up to 150 per cent and more, while the animal displays less motor activity and more attention. We call this the adaptation phase of learning. Further repetition of the stimulus causes less and less behavioural changes and the EP's fall back to the 100 per cent level, which is relatively constant when the animal is again in a state of a relaxed wakefulness. We call this the phase of well-adapted behaviour. The same sequence of events is observed during the elaboration of conditioned responses, the only difference being that the end result, the well-adapted behaviour, was the behavioural response. The extinction process, too, shows the same phases. The phases are influenced by the kind of reinforcement or reward, that is by the motivational state of the animal. The existence of these phases of learning has been shown using many different parameters. These are described in our recently published monograph (Pickenhain and Klingberg, *Hirnmechanism und Verhalten* etc. Fischer, Jena, 1968).

G. HORN: I agree with you that in the intact organism many processes may interact. Indeed it could well be that many forms of behavioural habituation involve both incremental and decremental effects (Hinde, R. A., Behavioural habituation. In: Horn, G. and Hinde, R. A. [Eds]: *Short-Term Changes in Neural Activity and Behaviour*. Cambridge University Press, London, 1970). At the cellular level such interactions may also occur, but the basic decremental process may be cellular habituation of the kind I have described: this process may interact with incremental processes and so, by acting on various command neurones, give expression to more complex forms of behaviour than a "purely" decremental one.

ON NEUROPHYSIOLOGICAL MECHANISMS OF SHORT-TERM MEMORY

by

T. N. Oniani

Institute of Physiology, Georgian Academy of Sciences, Tbilisi, U.S.S.R.

The reverberation and post-tetanic potentiation of the impulse transmission in the neural circuits of the brain are supposed to have an important role in the organization of recent memory (Hebb, 1949, 1961; Eccles, 1953; Konorski, 1961; Beritashvili, 1968). Keeping this point in mind, we have attempted to elucidate the role of the limbic system in the regulation of delayed conditioned reactions, the duration of which seems to be defined by recent memory (Konorski, 1961; Konorski and Lowicka, 1959; Beritashvili, 1968). The limbic system is thought to be, on the one hand, a highly organized circle of certain nervous structures (Papez, 1937), and an essential regulator of motivational emotions, on the other hand (Kaada, 1951; Gelhorn and Loofbourrow, 1963, Magoun, 1958).

A simultaneous study of behaviour and the EEG have shown that during conditioned and non-conditioned motivational emotions a sharp increase in the hippocampal theta rhythm (Green and Arduini, 1954; Grastyán et al., 1966; Elazar and Adey, 1967; Parmeggiani, 1967) and the appearance of burst activity of sinusoidal waves with a frequency of 35–40 per sec in some limbic structures (Gedevanishvili, 1948; Galeano et al., 1964; Oniani et al., 1968) are observed.

METHODS

Delayed reactions to conditioned signals served as a behavioural test for short-term memory. Experiments were carried out on cats in a special experimental cage having two chambers (Fig. 1). The smaller chamber was a starting place, the larger one on the right and left sidewalls had windows beyond which feeders were placed.

In the first series of experiments alimentary conditioned reflex was elaborated only to one feeder. A tone (500 Hz) served as a conditioned signal. 4–5 seconds after the signal the door of the starting chamber was opened and the cat was allowed to enter the larger chamber and to take with a forepaw a piece of meat from the feeder. It is natural that during such a manipulation conditioned response was elaborated to complex stimulation, i.e. to a tone plus the opening of the door. It is clear that the opening of the door without a tone also caused activation of food-motor reaction and, in these conditions, it was impossible to measure the duration of delayed

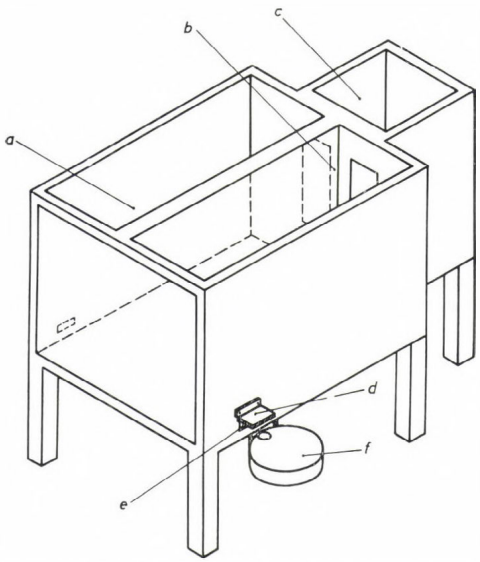

Fig. 1. Schematic drawing of the experimental cage. *A*, front chamber; *B*, starting chamber; *C*, door of the starting chamber; *D*, feeder; *E*, suspending doors; *F*, a small window.

responses. For this reason, conditioned reaction to the opening of the door has been further on specially extincted through non-reinforcement. After a solid differentiation of the tone from the opening of the door, we turned to measuring the duration of delayed conditioned response. A conditioned tone was presented for 10–20 seconds and, at different intervals after its cessation, the door was opened and the cat was allowed to perform foodmotor reaction.

The changes in the electrical activity of different neocortical areas and the limbic structures were studied through the chronically implanted steel electrodes. Spectral analysis and integration of rhythms comprising the background activity of different brain structures were made with a two-channel analyser "Sanei". The principle the analyser worked on was as follows: the background activity was divided into definite rhythms of 2–4 Hz (delta), 4–8 Hz (theta), 8–13 Hz (alpha), 13–20 Hz (beta first) and 20—30 Hz (beta second). The individual rhythms were then, for a definite span of time, summated by an integrator as the mean values. In our case the time of integration was 10 sec. Figure 2 illustrates an example of spectral analysis of the background activity in the auditory cortex (trace 1) and the dorsal hippocampus (trace 2). The rest of the channels registered separate spectra of the recordings and finally integrated values of the rhythms had been registered for 10 seconds. The first five inflections show the values of rhythms from the auditory cortex and the other five from the dorsal hippocampus.

When the cat sat in the experimental cage in a drowsy state, both in the auditory cortex and the hippocampus slow waves of 2–4 Hz and 4–8 Hz prevailed. During electrical stimulation of the reticular formation the animal aroused and this time, owing to desynchronization, the slow waves of 2–4 Hz, 4–8 Hz and 8–13 Hz were greatly suppressed in the neocortex. As to the hippocampus, the 2–4 Hz waves underwent suppression, while the 4–8 Hz waves were markedly augmented. Such a state was maintained as an after-effect for a certain time after the cessation of reticular stimulation.

RESULTS

The duration of delay in our cases was found to depend upon the satiation of the animal, i.e. upon its appetite. Hungry cats appeared capable of food-motor performance 1–2 minutes after the cessation of the conditioned

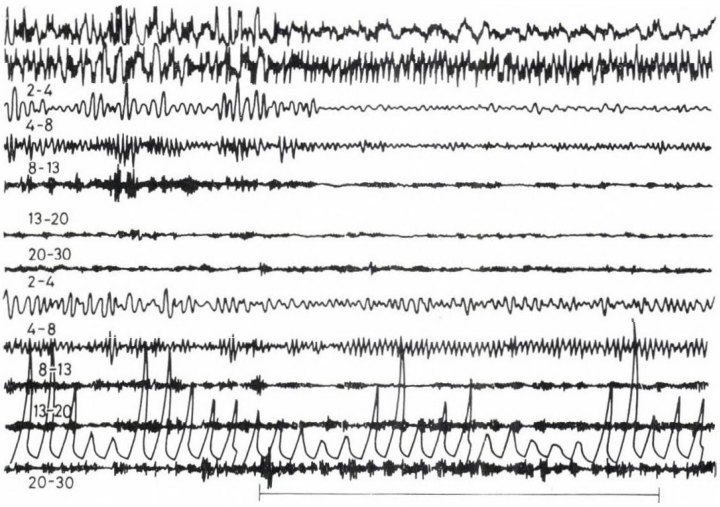

Fig. 2. Changes in electrical activity of the visual cortex and the dorsal hippocampus caused by electrical stimulation of the reticular formation. Spectral analysis of both summated activity and integrated values of separate rhythms are shown (2–4 Hz, 4–8 Hz, 8–13 Hz, 13–20 Hz, 20–30 Hz). Time of integration is 10 sec. The first 5 deflections show integrated rhythms. Calibration 200 μV; time 1 sec.

signal, whereas in half-satiated animals the delay did not exceed 10–30 seconds. It was noticeable that the duration of delay in these experiments was correlated with that of the alertness of the animals produced by the conditioned signal.

In response to a conditioned stimulus and in the course of delay regular changes were observed in the background electrical activity. They manifested themselves in the increasing of amplitude and frequency of theta rhythm in the hippocampus, in the arising of "burst" activity of sinusoidal waves with the frequency of 35–40 per second in the amygdala, pyriform cortex, the anterior hypothalamus and the olfactory bulb. Figure 3 illustrates changes in the electrical activity of the neocortical auditory area (trace 1), the pyriform lobe (trace 2) and the symmetric points in the dorsal hippocampus (traces 3 and 4) during application of the conditioned signal and after its cessation. During the wakefulness of the cat sitting in the starting chamber (beginning of Fig. 3*A*) the background activity of the neocortex and the pyriform lobe was desynchronized, while in the hippocampus delta waves and theta rhythms predominated.

In response to conditioned signals in the pyriform cortex "burst" activity appeared, while in the hippocampus theta rhythm sharply increased and delta rhythm was suppressed. The ensuing changes in the electrical activity persisted for a certain time (10–30 sec) after the cessation of the conditioned signal. This time the cat was alert. As the animal calmed down the

"burst" activity in the pyriform cortex ceased (Fig. 3*B*). In the hippocampus delta waves were restored, while theta rhythm decreased up to the background level. Against this background, the opening of the door caused only a weak orientation reaction expressed in the suppression of slow activity in all the structures recorded, without burst activity in the pyriform cortex. As to the theta rhythm, it did not change or was slightly suppressed in response to the opening of the door (Fig. 3*B*).

The changes in the EEG which were produced during the action of a conditioned stimulus, as well as during its after-effect, are considered by us an electrical expression of reverberation of impulses in the neural network of the brain.

Thus, these experiments showed that duration of delay in the conditions of one feeder must be dependent on reverberation of impulses in the neural circuits of the brain. As a result of such reverberation in the neocortical structures suppression of all the main slow waves is observed, whereas in the limbic structures (amygdala, pyriform cortex, anterior hypothalamus and olfactory bulb) there is a "burst" activity of sinusoidal waves with a frequency of 35–40 per sec. At this time, in the hippocampus a marked augmentation of the amplitude and regularity of theta rhythm with suppression of other waves are observed.

In the second series of experiments a conditioned food-motor reaction was elaborated using two feeders. In response to a tone (500 Hz) the cat

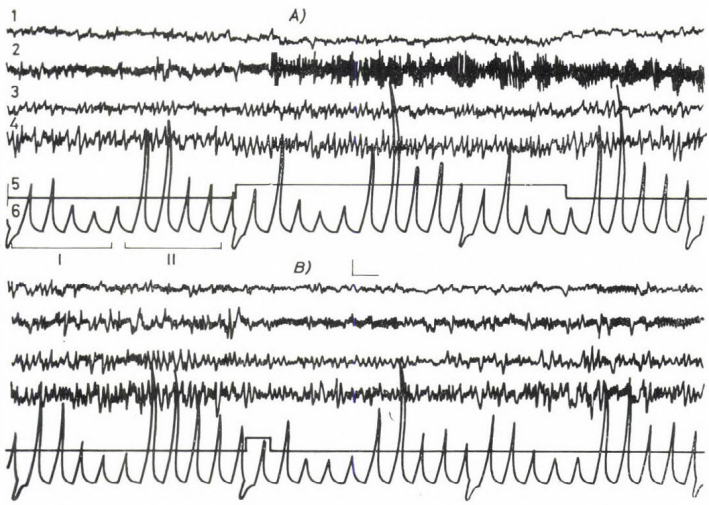

Fig. 3. Changes in the background electrical activity of the neocortex (1), the pyriform lobe (2), the dorsal hippocampus (3, 4) (*A*) to a preparatory conditioned stimulus and (*B*) to the opening of the door, with one feeder. 5, Marking of stimulation and, 6, record of integrated values of separate rhythms of the hippocampus. Calibration 100 μV; time 1 sec.

approached the right feeder and on the clicks, the left one. 4–5 sec after the conditioned signal the door of the starting chamber was opened and the animal performed food-motor behaviour. The conditioned signal ceased as soon as the cat received a reinforcing portion of food. The measuring of delayed reaction was started only after a 90–100 per cent differentiation of conditioned sounds had been achieved.

Thus, conditioned food-motor reaction was elaborated to complex stimuli consisting of two components. The first component of the complex was a tone or a click and the second one was the opening of the door. In each case these components may be classified as a preparatory and a triggering stimulus. Conditioned delayed reaction was measured in the following way: a conditioned preparatory signal (tone or click) was delivered for 10–20 seconds. After its cessation the door of the starting chamber was opened at different intervals of time and the maximum of delay measured.

Under these conditions, it is natural to suggest that a correct decision of the task is determined by those traces that have been retained in the brain from the preparatory stimuli. The localization of traces of different preparatory stimuli differ from each other, and one and the same triggering stimulus activates that circuit in which the trace is more recent.

In response to preparatory conditioned signals the background electrical activity in the neocortical structures and in the limbic system changes in the same manner as it has been shown in the first series of experiments, where delayed reactions have been studied with one feeder.

Figure 4 demonstrates changes in the background activity of the auditory cortex (trace 1), the pyriform lobes (trace 2) and the symmetrical points in the dorsal hippocampus (traces 3, 4) produced by conditioned stimulation and during delay after its cessation (Fig. 4*A*, *B*). It is to be seen that the conditioned signal elicits "burst" activity in the pyriform cortex, and augments the hippocampal theta rhythm. These changes persist for a certain time after the conditioned stimulus has been ceased, and are well correlated with the alertness of the animal. If after the cessation of the conditioned signal the animal is released from the starting chamber during "burst" activity and the augmented theta rhythm, the decision of the task is correct.

However, as distinguished from the first series of experiments where conditioned alimentary reaction has been elaborated only to one feeder and where the duration of delayed reaction seemed to be determined only by that of reverberating excitation within the neural circuits of the brain, in this series the cats appeared to be able to decide the task correctly not only during the reverberation, but also after its cessation. We suggest that the cessation of reverberation, particularly in the limbic system is expressed in the disappearance of those changes which had been produced by the conditioned stimulus. With the disappearance of "burst" activity in the above named limbic structures and the decrease of the theta rhythm to the background level, the alertness of the animal passes. The cat calms down and may fall into a drowsy state and show in all the structures slow activity characteristic of such a state (Fig. 4*C*).

If some time after the cessation of the preparatory stimulus (3–10 min) the door is opened, the cat gives a correct reaction. At this time, desynchro-

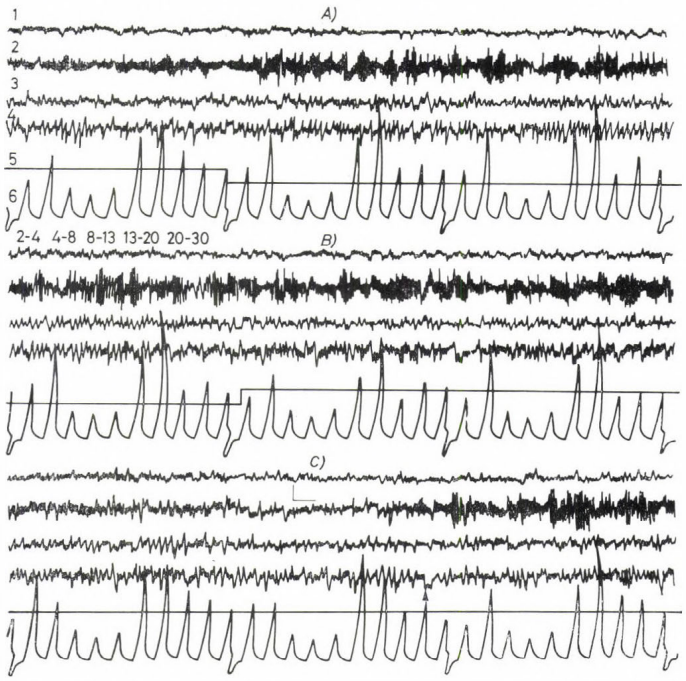

Fig. 4. Dynamics of the background electrical activity of the neocortex (1), the pyriform lobe (2), the dorsal hippocampus (3, 4) to a preparatory conditioned stimulus (*A*, *B*) and to a triggering stimulus (*C*, an arrow). 5, Signal line; 6, record of integrated values of separate rhythms (2–4 Hz, 4–8 Hz, 8–13, Hz, 13–20 Hz, 20–30 Hz), the first 5 deflections of the left and other 5 deflections of the right hippocampus. The time of integration is 10 sec. Calibration 100 μV; time 1 sec.

nization in the neocortex and "burst" activity with augmentation of theta rhythm (Fig. 4*C*) in the limbic structures are obtained, i.e. the reverberation of impulses starts in the neural network.

Thus, if in the case of one feeder the duration of delay can be suitably explained by the reverberation of impulses, then from the second series of experiments it has become clear that reverberation alone cannot account for the maximal duration of delayed conditioned reaction. After the cessation of reverberation, produced by the preparatory stimulus, there apparently remain other kinds of traces on the basis of which the triggering stimulus can produce not only the realization of conditioned food-motor behaviour, but also sound discrimination. On the basis of up-to-date electrophysiological knowledge of synapses of the central nervous system, the post-tetanic potentiation seems to be the reason for such a trace (Eccles, 1964).

As pointed out above, maximal duration of delay in our experiments was the time after the lapse of which the cat's decision of the task was correct and did not exceed 10–15 per cent errors (though the preparatory condi-

tioned stimulus had ceased). The maximum of delay appeared, depending upon the individual capability of the animal, to fluctuate from 3 to 10 minutes; at greater delays the cat left the starting chamber and in response to a triggering stimulus made not for the signalled feeder, but for the other one, i.e. committed an error. However, in erroneous cases at supermaximal delays the same change in the background electrical activity of the limbic structures was observed as in the correct ones within the range of delay (Fig. 5).

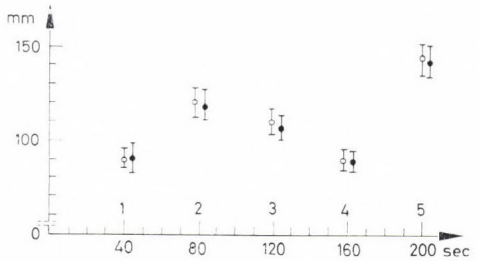

Fig. 5. Dynamics of the hippocampal theta rhythms during correct (open circle) and incorrect (black circle) delayed reactions. Ordinate: amplitude abscissa; time. 1, The cat is sitting in the starting chamber; 2, during the presentation of preparatory stimulus; 3, 4, during delay after the cessation of conditioned stimulus; 5, during the presentation of triggering stimulus.

Very often in the satiated animal a conditioned stimulus caused a certain increase in the amplitude of the hippocampal theta activity, but conditioned reflex activity was not yet realized, the animal would not leave the starting chamber upon opening the door. However, if against this background the lateral hypothalamus were electrically stimulated, it would induce alimentary behaviour in the satiated animals, the cat would rise and perform conditioned food-motor reaction. It should be pointed out that in such tests cats usually reacted correctly.

Changes in the electrical activity of different brain structures in an experiment of this kind is illustrated in Fig. 6. A conditioned signal (a tone of 500 Hz) produced a negligible increase in the hippocampal theta rhythm, while delta waves were considerably suppressed; 5 sec after the beginning of the conditioned signal the door was opened, but the cat did

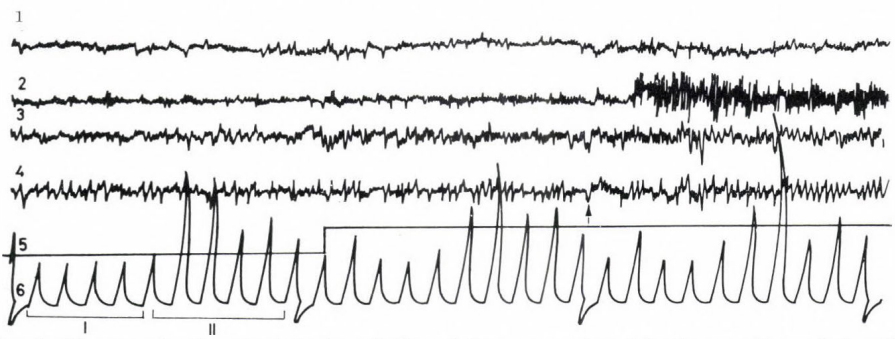

Fig. 6. Changes in the electrical activity of the neocortex (1), the pyriform lobe (2), the dorsal hippocampus (3, 4) in response to a conditioned signal, electrical stimulation of the lateral hypothalamus (arrow) and during performance of conditioned food-motor behaviour. 5, Signal line; 6, record of integrated values of separate rhythm (2–4 Hz, 4–8 Hz, 8–13 Hz, 13–20 Hz and 20–30 Hz), the first 5 deflections of the visual neocortex and the rest of the dorsal hippocampus.

not give any reaction and remained in the starting chamber; 10 sec after the application of the conditioned signal the lateral hypothalamus was electrically stimulated causing an intensive increase of the hippocampal theta rhythm and the appearance of "burst" activity in the pyriform cortex. In response to electrical stimulation the cat went directly to that feeder to which conditioned reaction to a tone had been elaborated.

Traces of the conditioned stimulus causing no food-motor performance in the satiated animal must have persisted for a certain time after its cessation. If in the satiated animal the lateral hypothalamus were stimulated not during the conditioned signal (Fig. 7*A*), which had failed to cause activation of food-motor performance, but 40–60 sec after its cessation (Fig. 7*B*), the animal would preferentially go to the feeder to which food-motor performance had been elaborated in response to the given signal.

The results which had been obtained by studying the effect of electrical stimulation of the hypothalamic centres regulating the alimentary behaviour on delayed reactions of hungry animals are worth mentioning. It was found that if electrical stimulation of the lateral hypothalamus, usually causing activation of alimentary behaviour in the satiated animals and augmentation of the hippocampal theta rhythm (Fig. 7*B*), is delivered in the interval between the preparatory and triggering conditioned stimuli, then the maximal delay will be considerably increased (by 50–70 per cent). This apparently

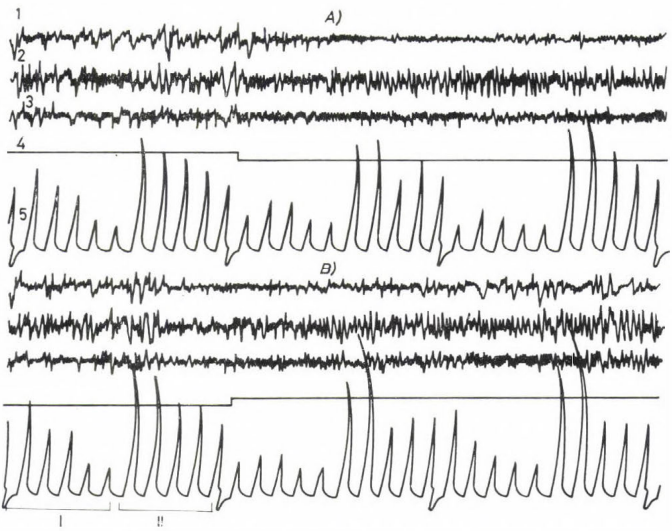

Fig. 7. Changes in the background electrical activity of the sensorimotor cortex (1), the dorsal (2) and the ventral (3) hippocampus. In the satiated cats a conditioned stimulus (*A*) and electrical stimulation of the lateral hypothalamus (*B*) elicited the conditioned alimentary reflex. 4, Marking of stimulation; 5, record of integrated values of different rhythms of the neocortex (the first 5 deflections) and the dorsal hippocampus (the other 5 deflections).

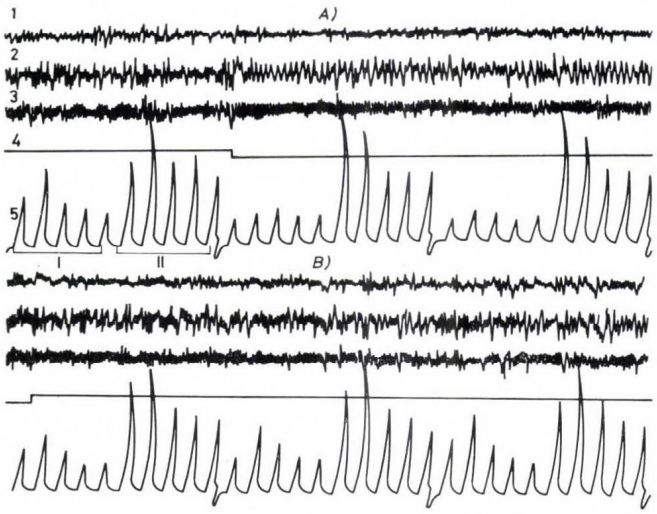

Fig. 8. Changes in the electrical activity of the neocortex (1) and dorsal hippocampus (2, 3) in response to electrical stimulation of the ventromedial hypothalamus (*A*) and after the cessation of stimulation (*B*). 4, Marking of stimulation; 5, record of integrated values of separate rhytms of the neocortex (the first 5 deflections) and the dorsal hippocampus (the other 5 deflections). Calibration 100 μV; time 1 sec.

can be accounted for by the reverberation caused by a preparatory conditioned signal being increased and prolonged owing to stimulation of the centres of "hunger". Accordingly also the duration of post-tetanic potentiation in the reverberating network would increase, which is likely to underlie the lengthening of the delay maximum.

Electrical stimulation of the ventro-medial hypothalamic nuclei, which are considered the centre of "satiation" (Anand, 1961), has an opposite effect on the duration of delayed reaction. In our experiments, very often, electrical stimulation of these nuclei with a moderate strength delivered shortly after the cessation of conditioned signal led to the inhibition of alimentary behaviour and shortening of the delay in case the animal was hungry. On such occasions, in the dorsal and ventral hippocampus a considerable increase in the delta waves was observed (Fig. 8*A*, *B*). As to the theta rhythm, it either did not change or its amplitude slightly decreased.

A negative effect of electrical stimulation of the ventro-medial hypothalamus upon the duration of delayed food-motor reactions may be explained by the cessation of reverberation of impulses arising in response to a preparatory conditioned signal, due to a weakening of alimentary motivation. Changes in the background electrical activity of such an important structure of the limbic system as the hippocampus also points to this. It seems that this change is quite the contrary to that observed during alimentary behav-

iour in response to electrical stimulation of the centre of "hunger", i.e. the lateral hypothalamus, like the response to a conditioned alimentary signal.

The entity of the limbic circle is a determining factor for the optimal reverberation of impulses. This is evident from the effect of lesions in some limbic structures on delayed conditioned reaction. In our experiments in collaboration with Koridze (Koridze and Oniani, in press) a bilateral ablation of the gyrus cinguli evoked a sharp shortening of the duration of delayed reaction to conditioned stimuli. Figure 9 shows the percentage of correct and incorrect responses during different delays between the preparatory and triggering stimuli. As is seen, in the first days, after a 100 per cent discrimination of the preparatory conditioned stimuli has been reached in intact animals, the percentage of correct reactions is significant only with a 10 sec delay (Fig. 9*A*). In the subsequent days the percentage of the correct responses gradually increases and after 8–10 days the maximum is established. This time the animal's decision of the task with a 10 sec delay is 100 per cent, but with delays of 1 and 5 min the correct responses approach 95 and 90 per cent, respectively (Fig. 9*B*).

After bilateral ablation of the gyrus cinguli the discrimination of the conditioned stimuli is not disturbed if the triggering stimulus is given during the presentation of the preparatory one. However, if the triggering stimulus is given after the cessation of the preparatory one, the animals give an erroneous response. Figure 9*C* shows the percentage of correct and incorrect reactions with 10 sec, 1 min and 5 min delays three months after the operation. As is seen, the delayed reactions are completely disturbed and after a long exercise the preoperative delayed reactions are restored only within 10–60 sec (Fig. 9*D*).

The above-described facts can be understood if we suppose that one of the main determining factors in the organization of neurophysiological basis

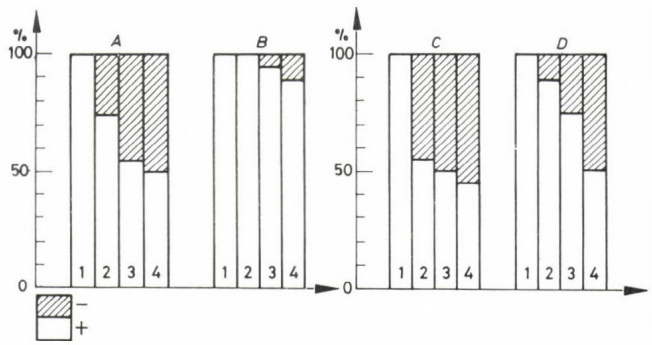

Fig. 9. Percentage of correct and incorrect responses during different delays of foodmotor reactions. The first column shows the percentage of the total number of sessions, the rest of the columns indicate the ratio of correct (+) and incorrect (−) responses with 10 sec (2), 1 min (3) and 5 min (4) delays. *A* and *B* before, and *C* and *D* after ablation of the gyrus cinguli.

of recent memory is the reverberation and post-tetanic potentiation in the functional nervous circuits of the brain. In particular, reverberation of impulses and facilitation of the conduction in the neural network of the limbic system seem to have an important role in the regulation of delayed alimentary conditioned reactions.

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DISCUSSION

F. KLINGBERG: Have you any idea what is reflected by the "burst" activity in the prepyriform cortex? We had similar results recorded from the olfactory bulbs in rats. There is evidence that this has something to do with the sense of smell and related emotional processes.

T. N. ONIANI: It is well known that one can observe burst activity in the limbic structures of cats during the development of emotional reactions, e.g. fear or avoidance. However, the origin of this burst activity still remains unknown. It was supposed that there must be a triggering structure either in the olfactory bulb (Adrian and others) or in the amygdala (MacLenon and others). The relationship between this burst activity and the function of smell receptors is also a problem to be discussed. Now the most important fact for us is the coincidence of this burst activity with the conditional signal probably eliciting it.

G. HORN: It is a big step to argue from changes in electroencephalic activity to the underlying cellular mechanisms. Have you any more direct evidence for reverberating circuits?

T. N. ONIANI: The conditional signals elicit a burst activity in the limbic system and the theta rhythm becomes more expressed in the hippocampus. These changes last a few minutes after the cessation of the signal. I think that such a long lasting after-effect can be explained only by reverberation of impulses. This is, of course, a hypothesis which must be proved.

G. HORN: What structures are involved in this putative reverberation? Do you, for instance, implicate the so-called Papez-circuit? If so, does cutting it at some point interfere with your EEG changes?

T. N. ONIANI: The Papez-circuit comprises the main structures of the limbic system. I suppose that there must be various sub-systems or networks in this complicated structure. Evidently, the lesion of the limbic circuit as a whole or of some sub-system must have an expressed effect on short-term memory.

G. HORN: What are your reasons for implicating post-tetanic potentiation? Has it been demonstrated in the structures you studied?

T. N. ONIANI: The phenomenon of post-tetanic potentiation has been proved to occur also in brain synapses. Sir John Eccles in his monograph on the physiology of synapses gives a clear evidence of this problem.

J. SZÉKELY: Hungarian workers, e.g. Grastyán and his colleagues, have made similar observations on the role of hippocampal and neocortical activity in the elaboration of conditional reactions. I am also very glad to hear about your interpretation of reverberating circuits. In our experiments with Professor Ádám we have found similar phenomena proving indirectly the role of self-reinforcing reverberating events.

HYPERSYNCHRONY AND LEARNING

by

F. KLINGBERG

Department of Clinical Neurophysiology, Karl Marx University
Leipzig, G.D.R.

The analysis of the information content of the so-called electrical "background activity" is one of the most difficult technical problems. Since the 1958 Moscow Colloquium there was only little progress in the interpretation of rhythms, synchrony and desynchrony. The electrophysiological approach to the very complex integrative processes of the brain by analysis of evoked potentials and by microtechnics was more effective. Our experiences on correlations between electrophysiological phenomena and the behaviour in freely moving animals were summarized in a monograph published recently (Pickenhain and Klingberg, 1969). The present study is restricted to some results on certain rhythmical electrocortical processes which more than twofold exceed the background activity and may be called "hypersynchronized rhythms".

Figure 1 shows three types of such rhythms. The first is confined to the visual projection areas and is triggered by visual stimuli. The phenomenon is known from many species and is called photically evoked afterdischarge (PhAD). The second type is localized in the anterior parts of the cortex,

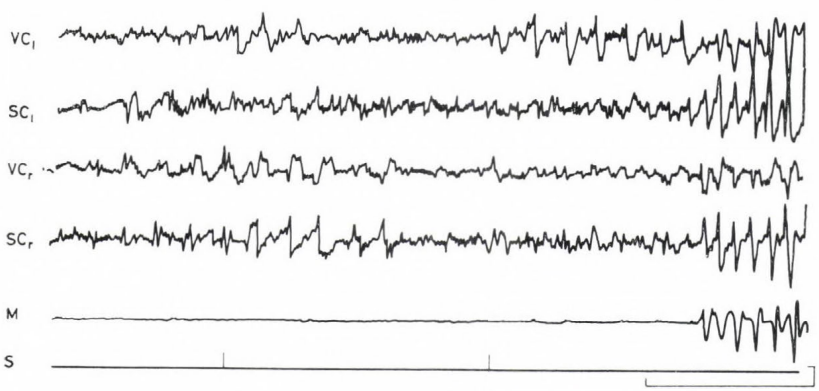

Fig. 1. Electrocorticogram of the visual cortex (VC) and sensorimotor cortex (SC) from the left and right hemisphere in a rat 25 days after implantation of a cobaltagar pellet into the right sensorimotor cortex. Compare PhAD, Sp and Co-Sp. M — Movement registration, S — stimulus marks (light flashes). Calibration: 1 sec, 200 μV (after Klingberg et al., 1969).

has higher frequency and amplitude than the PhAD and can be triggered by electrical stimuli in the thalamus, caudate, septum and other nuclei (Kohler and Klingberg, 1969). In some rats it appears spontaneously and is known from other species under the name "spontaneous spindle activity" (Sp) (Klingberg and Pickenhain, 1968). Both types have common properties: they are generated by recurrent inhibitory circuits in thalamic nuclei, the PhAD the lateral geniculate body, the Sp in unspecific nuclei as, for instance, in the nucleus ventralis thalami. The third type is produced by injury after implantation of cobalt-agar pellets (containing maximum 2·5 mg cobalt powder) in cortical areas (Klingberg et al., 1969). To avoid the term "epileptogenic" or "epilepsy" we called it "cobalt-induced spindle activity" (Co-Sp). Our knowledge about its generating mechanism is still scanty. All three types have analogous spike-wave-like potential forms and are immediately suppressed or interrupted by strong activating stimuli as by the electrical reinforcement in avoidance conditioning (disturbance phase of learning, see Pickenhain and Klingberg, 1969). The duration of the suppression is shortest on the Co-Sp and longest on the Sp.

Figure 2 demonstrates interesting correlations between avoidance conditioning and appearance of PhAD. The elaboration of the conditioned avoidance response (CAR) in this experiment included some difficulties for the animal: a ten sec delay of the reinforcement after onset of the 1 per sec light flash series; the CS was never interrupted before the tenth flash, it had only weak activating effect on the behaviour even before habituation but could enhance the synchronization of the ECoG; the 40 V, 10 per sec electrical stimulus was relatively weak. The animals had no pretraining of US and did not know the place for escape before onset of conditioning. Under these conditions only 50 per cent of the animals showed 70 per cent CAR performance (escape to a hanging rod). Their values were averaged as group I, those of the other animals as group II. The reaction time of CAR was significantly longer in group II ($p < 0{\cdot}001$). The number of PhAD was counted and related (intraindividually) to the values before conditioning, i.e. after a short habituation to the flash series (relaxed wakefulness, 100 per cent). During the first session there was scarcely any difference between the two groups, but further on the rats of group II showed more signs of behavioural inhibition, less motor activity and more arrest reactions. Before and after the CAR and especially during non-performance (negative reactions) the number of PhAD was significantly higher in group II and reached the level of before conditioning. At the onset of extinction, group II had a large and immediate increase of PhAD.

As published earlier, the appearance of PhAD represents a state of relaxation or behavioural inhibition (Pickenhain and Klingberg, 1969). Their greater renewed increase in group II in the avoidance experiment fits well to the more intense behavioural inhibition, lower performance and longer reaction times of CAR in this group. Since the second type of hypersynchronized rhythms, the Sp, is easily and for a long time suppressed by motivating stimuli, only a small number of rats produced Sp during avoidance conditioning. These animals did not exceed 20 per cent of CAR performance. Intraperitoneal application of 100 mg per kg 2,3-dioxoindoline (Isatin),

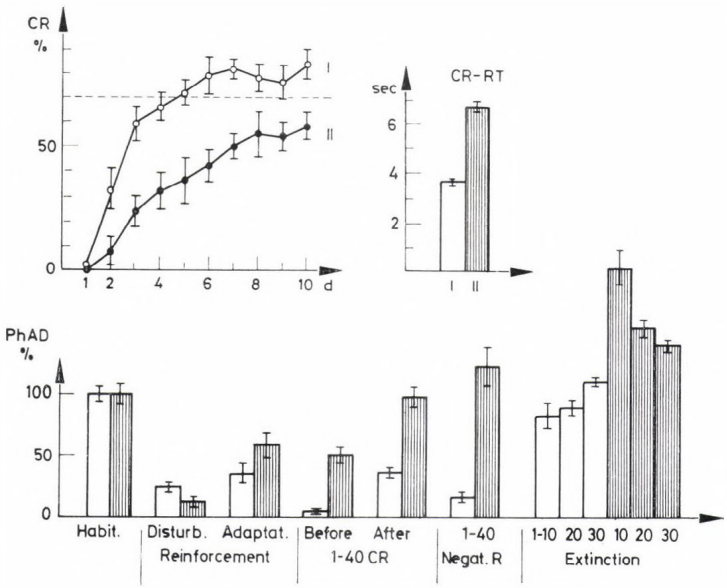

Fig. 2. Correlation between the elaboration of a conditioned avoidance reflex (escape) and the appearance of photically evoked afterdischarges (PhAD) during a ten sec light flash series (1/sec) used as the conditioned stimulus. Mean values and standard errors. Left upper diagram: CR as a percentage of trials during ten subsequent days (*d*, ten trials a day per animal); I (white circles): group of animals reaching the 70 per cent CR performance; II (black circles): group of animals not reaching 70 per cent (6 rats in each group). Right upper diagram: Average reaction times of fourty consecutive CR's in group I (white columns) and group II (shaded columns). Lower diagram: Appearance of PhAD during different experimental situations related to the values (intraindividual) after habituation for a short time (relaxed wakefulness, 100 per cent). Group I shows a significantly greater decrease of PhAD during the elaboration of CAR than group II (shaded columns), but smaller increase during extinction.

which induces the spontaneous appearance of both types of hypersynchrony (Klingberg and Müller, 1968), reduced CAR performance, decreased motor reactivity and prolonged the CAR reaction times (Hirschfeld et al., 1969). The lower dosis (40 mg per kg), which did not induce Sp, had no effect on CAR.

Our next step was to investigate the influence of the most stable type of hypersynchronized rhythms, the Co-Sp, on acquisition and retention of CAR (Kunz and Klingberg, 1969). In these experiments we used a 1·5 kc per sec tone as CS and reinforced it by 50–70 V alternating current after 1·5 sec. Both stimuli were switched off, when the rat escaped. Figure 3 shows CAR performance beginning 10 days after cobalt implantation into the

right areas 7–18 of the neocortex in comparison with a control group and a third group with electrocoagulation of the same areas. The cobalt-group did not learn at all in spite of the fact that the morphological lesion (2 mm diam.) was half as large as in the electrocoagulated group. Not even the unconditioned escape could be observed in the first three sessions. The control goup, when they had learned the CAR, was used for a retention test 8 weeks after cobalt implantation. The performance and re-learning now was zero for 8 sessions.

Figure 4 shows the same result of a retention test with another group of rats before and ten days after cobalt implantation. Four and nine months after the implantation of the cobalt-agar pellet in the right hemisphere with different localization, avoidance conditioning in 8 sessions resulted in a maximum of 25 per cent performance. Only one rat, which displaced electrophysiological changes after the injury but never Co-Sp, reached the criterion of 70 per cent when tested 4 months after implantation. All the other rats showed Co-Sp in the primary or secondary focus, and an increased PhAD in the other hemisphere in a larger area. Figure 5 demonstrates the triggering of Co-Sp by the CS (9 months after cobalt implantation) and well expressed photically evoked recruiting and continued arrest behaviour during CS. Figure 6 is an example of increased hypersynchrony mainly on the focus side and muscle tremor during hanging on the rod (*a*) and another example of slowed and prolonged PhAD in the left area 17 just after performance of a CAR (*b*).

The results proved to be independent of the localization of the cobalt focus in the cerebral cortex. On the other hand, the implantation of cobalt-agar pellets into the cerebellar cortex did not influence CAR (Langer and

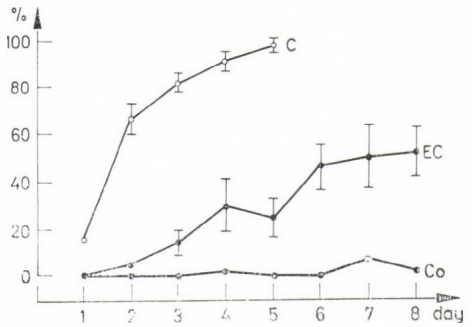

Fig. 3. Comparison of CAR performance as a percentage of trials, beginning 10 days after cobalt implantation, 10 days after electrocoagulation in the same area (EC) and in a control group (C) (after Kunz and Klinberg, 1969).

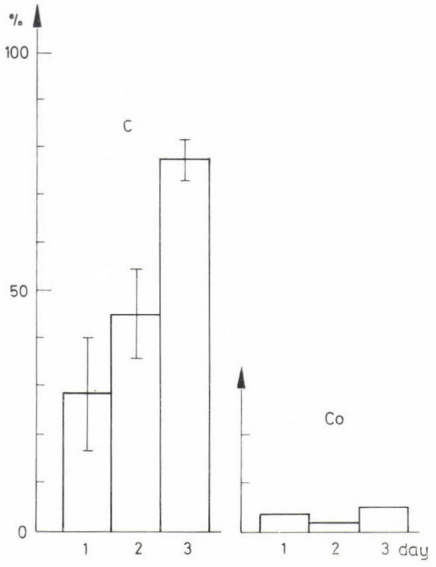

Fig. 4. CAR performance before (C, three columns on the left) and 10 days after cobalt implantation (Co, columns on the right) (retention test) (after Kunz and Klingberg, 1969).

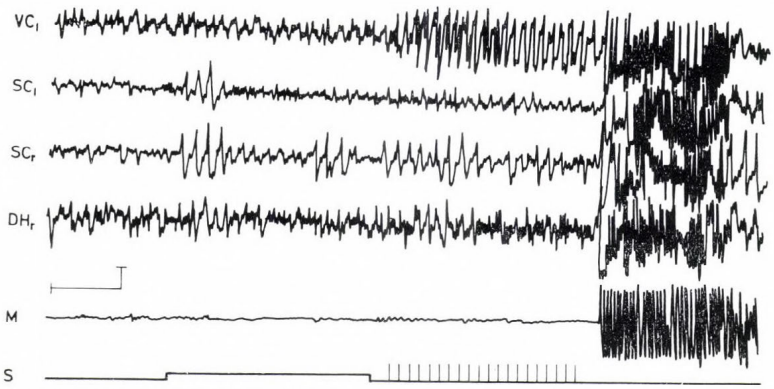

Fig. 5. Triggering of Co–Sp by the CS (tone): no motor response (M) during CS. Cobalt focus in the right sensorimotor cortex (9 months after implantation), propagated into the left sensorimotor cortex and the dorsal hippocampus (DH). Photical evoked recruiting just before electrical reinforcement. Calibration: 1 sec, 200 μV (after Kunz and Klingberg, 1969).

Klingberg, 1969) and was never accompanied by increased synchrony in the cerebral cortex. No such influence resulted from cobalt implantation into the olfactory bulb.

Impairment of learning and retention by neocortical epileptogenic foci in monkeys was reported by Stamm et al. (1961*a*, *b*, 1963). Similar results have been published in connection with other forms of hypersynchrony, e.g. "audiogenic epilepsy" (Nováková et al., 1957, 1958, 1962; Essman and Hamburg, 1962), generalized hippocampal self-sustained after discharges (Kreindler, 1960; Delgado and Sevillano, 1961; Flynn et al., 1963) or clinical forms of epilepsy. Some analogies between Co-Sp and *petit mal* (bilateral synchrony) are of considerable interest. The latter is accompanied not only by hypersynchrony in the thalamocortical structures, but also by disturbance of memory (Jus, 1963; Jus and Jus, 1963; Mirsky and Van Buren, 1965), of the time sense (Holubář and Machek, 1962) and prolonged reaction times (Megrabyan et al., 1962; Tizard and Margerison, 1963). Ethosuximide, which is an effective drug in *petit mal* therapy, suppressed PhAD, Sp (Kästner et al., 1969), but also the Co-Sp and improved the CAR performance after cobalt implantation (Kästner, Müller, Hirschfeld, personal communications). Cobalt focus gives rise to long-lasting changes in the ferment activity of its surroundings, principally to a lower activity of various oxydases (Luppa, personal communication). This may initiate and maintain for a long time changes in neurone excitability, from which increased firing will result. The most important consequence of the existent focus is functional, i.e. the compulsive influence on other parts of the brain, and if this reaches thalamocortical or cortical chains, it may be transformed into hypersynchrony.

In view of our experimental results and those in the literature, it may be concluded that the more or less extended hypersynchrony in thalamocortical

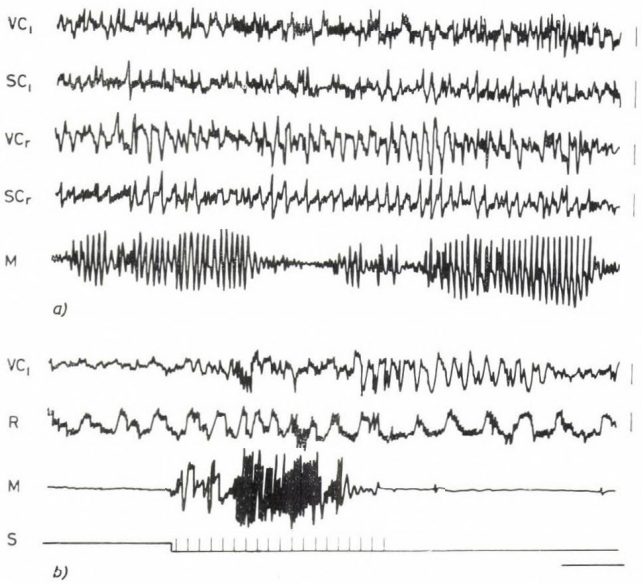

Fig. 6. (*a*) The same rat as in Fig. 5. hanging on the rod displaying muscle tremor (M) and Co–Sp. (*b*) Prolonged and slow PhAD in the visual cortex contralateral to the cobalt focus, in this case just after a conditioned escape to the rod. R, respiration record. Calibration: 1 sec, 200 μV (after Kunz and Klingberg, 1969).

neurone chains has a general inhibitory effect on motivated nonautomatized motor behaviour as this is controlled by higher levels. Herein is included the inhibitory effect of stimulation in the caudate, thalamus, and especially in the recruiting systems, but also the different kinds of sleep induction, because sleep spindles and delta waves may be regarded as a form of hypersynchrony.

In totally synchronized neurone pools the analysis of incoming information is impossible and processes of short-term memory when localized in such a neurone pool are immediately interrupted. But certain synchrony would be necessary in the brain to restrict the information flow and analysis to the biologically optimum dimensions. Therefore the highly complicated changes in the degree of synchronization of the different groups of neurones represent an important factor of learning and memory.

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DISCUSSION

O. Wolthuis: The changes you report on are very interesting and are obvious to all of us. But I suppose it would be more convincing to have your control implanted with control agar pellets, because the operation itself might lead to an epileptogenic focus.

F. Klingberg: We did such controls and there were only very little changes in the electrical activity directly on the focus, but hypersynchrony was never induced. The impairment of learning was insignificant.

O. Fehér: Do you think that recruiting potentials are always in correlation with inhibition? In anaesthetized cats evoked potentials fall sometimes into the period of a spindle and they seem to be depressed. But this is not always so. Do you think that recruiting potentials may contain also activating impulses? One can differentiate between spindle waves and seizure potentials by application of GABA locally on the cortex. Recruiting potentials are enhanced by GABA, whereas seizure potentials are reversed and depressed.

F. Klingberg: The recruiting response is a general phenomenon. We now know that most of the thalamic nuclei have a mechanism with recurrent inhibition. But in different cases of stimulus localization different structures and projections to the cortex are involved, which causes the differences in parameters and functions of the recruiting process. That may be reflected by different termini as "augmenting" and "recruiting". But the processes in general are determined by the recurrent inhibition. If the evoked hypersynchrony reaches for instance the motor and sensory motor cortex or the related associative area one can observe motor inhibition as described in experiments by Grastyán, Sakhiulina, Weinberger and others. The content of activation processes in the recruiting phenomena firstly maintains or propagates the process of hypersynchrony itself. On the other hand, in some cases processes in brain structures not involved may be released, when the hypersynchronized structures have had a strong influence on them before. The seizures you speak about have quite another mechanism. Drug effects as abolishment of PhAD and S were also effective in abolishing Co-Sp, but the duration of the effect in the last case was shorter and weaker on the primary focus activity. This means that functional analogues exist between the three types of hypersynchronization I have reported here.

I. Madarász: I understand that your cobalt focuses were of a small diameter and they invariably impaired CAR, irrespective of their position in the cortex. Did you make any attempt at the surgical removal of these foci?

F. Klingberg: Such experiments were known from Morrell and others, and were done 10–15 years ago. We did not want to repeat them. Concerning the question that surgical processes could be responsible for the appearance of hypersynchrony, I can say that in several hundreds of rats, neither surgical injury nor electro-coagulation caused such type of hypersynchrony. But such injuries can be responsible for changes in the thresholds of evoking or inducing hypersynchrony.

I. Mészáros: What is your opinion about the tremor induced by the cobalt? Is it an extended motor influence, possibly?

F. KLINGBERG: We have no concrete facts to explain it in detail. Firstly, the motor control by the higher levels is changed or abolished during hypersynchrony. Secondly, one can expect that secondary centrifugal influences reached the thalamus, caudate and also pallidal and nigral structures. The different frequencies of tremor and hypersynchrony allow to deny a direct influence. Investigations on the biochemical changes, such as the enzyme activity, are still in progress.

S. FREED: Is cobalt specific for the epileptiform hypersynchronicity and does it depend on the state of subdiversion?

F. KLINGBERG: It is not entirely specific. Other metals are also active. This was investigated by Kopeloff and co-workers beginning in 1940. Cobalt was found to be the best inducer of the process in rodents. Even a solid piece is active. The smaller the grains of the metal-powder, the more effective it is.

S. NARIKASHVILI: Why do you speak in this case of "spindle" and why not of "seizure discharge"?

F. KLINGBERG: I think the difference in terminology is not so essential at the moment, but we have to avoid clear clinical terminology when we have not the clinical symptoms fitting the terminology. As far as I know, the 3 per sec spike-wave in humans is not called seizure discharge either.

QUANTITATIVE ASPECTS OF DELAYED CONDITIONAL EVOKED POTENTIALS

by

J. I. Székely and G. Ádám

Department of Physiology, University Medical School and
Department of Comparative Physiology, Eötvös Loránd University,
Budapest, Hungary

It must be noticed that in some of our several series of experiments on delayed conditional evoked potentials, conditional waves could not be detected visually. From the very beginning we supposed that in our so-called "unsuccessful" experiments the conditional potentials had really existed, but were too small for the visual signal/noise detection, being covered by the background activity.

The aim of the present experiments has been the control of the existence of these electrical learned phenomena in a quantitative form. The statistical analysis enabled us to draw some conclusions about the optimum parameters of this type of conditioning and about the possible mechanism of this peculiar kind of memory storage.

Our acute experiments were performed on cats using chloralose anaesthesia. The experimental approach was identical with that used in our previous series: the electrical stimulation of the splanchnic and sciatic nerves were associated. The intensity of the conditional splanchnic stimulus was 1 V, and that of the following unconditional sciatic stimulus 10 V. The delay between the CS and US was 200, 300 or 4000 msec. The intertrial interval was 2, 3, 4 or 5 sec. The primary evoked potentials were recorded on the gyrus ectosylvius anterior or lateralis. The preamplified waves were averaged by means of a 256 channel computer.

The programme of the experiments has been the following: first we gave 20 sciatic stimuli and determined that critical portion on the screen where the primary answer could be seen. This 50 msec wide area contained, besides the primary response, some further components of the evoked potential. Then 20 control splanchnic stimuli followed. After these controls we began the training procedure which consisted of 40 pairings and 20 applications of the CS without sciatic reinforcement each. The obtained 20 conditional responses were averaged by means of the 256 channel computer. During one experimental session 400 pairings and 200 isolated applications of the CS were performed altogether. This training procedure was followed by extinction: the CS was applied 400 times by itself, without sciatic reinforcement.

In order to characterize, in a quantitative manner, all the events of our conditioning procedure and to screen out the background noise from the conditional response, we proceeded in the following manner: by using the method of integral approach we determined the magnitude of all the poten-

tials in the so-called "critical portion". This area was expressed as the percentage of the potentials detected in the 50 msec segments preceding and following the critical portion. This value obtained before the associations was regarded as the control of the given experiment. In the course of the conditioning procedure the value of this quotient was determined after 40, 80, 120 . . . 400 associations and extinctions, respectively. If a delayed conditional evoked potential really existed, this learned response would appear just in the critical portion: i.e. this indicator should increase. More exactly: in this case the approximative integral in the critical portion would increase as compared with the background activity in the neighbouring portion. Obviously, the condition of the increment of this indicator was not only the appearance of conditional evoked potentials, but their exact timing and delay. It must be mentioned that all data obtained by the computer in numerical form have been processed by means of ICT-type computers.

Figure 1 shows the magnitude of the conditional responses as a function of associations and extinctions, respectively. The above-mentioned indicator is plotted on the ordinate and the number of associations and extinctions on the abscissa. The broken line indicates the control value before pairings and the average value of the data measured in the course of the training procedure. As it can be seen, initially the curve rises sharply, then it becomes rather flat. The augmentation of the indicator is not marked, but statistically significant. Of course the curve declines in the course of extinction. This learning curve seems to prove the existence of conditional delayed potentials in a quantitative form.

Figure 2 shows the rate of conditioning as a function of some parameters of the training procedure. In this series the whole experimental group was subdivided according to the parameters of the associations. The height of each column shows the average per cent increase of the indicator expressed. On the bottom the delay between CS and US and the intertrial interval of each group is indicated. The observed changes were significant only in groups 4 and 5, in which the delay between CS and US and the intertrial interval had been the shortest. The optimum effect of the shortest intertrial interval and delay may indicate the PTP-like mechanism of this type of conditioning, but some other form of facilitatory event cannot be excluded either. The last column represents the results of the "pseudo-conditioning" experiments. These have been practically unsuccessful. Pseudo-conditioning means that unconditional sciatic stimuli are paired with each other. The

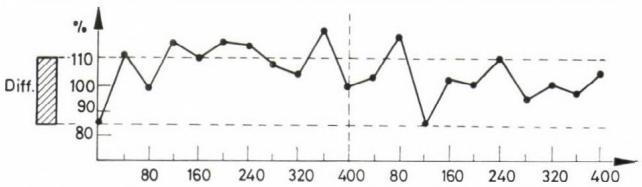

Fig. 1. The learning and extinction curve of conditional delayed evoked potential (cf. text).

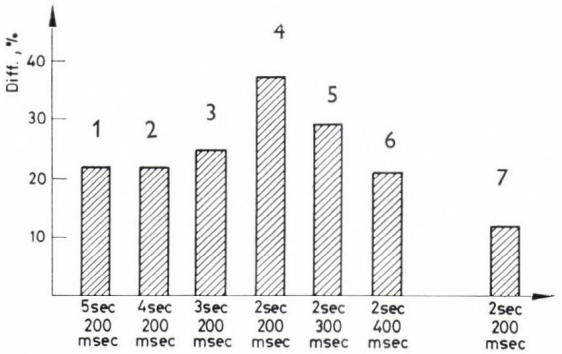

Fig. 2. Mean intensity of conditional evoked responses of several animal groups which have been conditioned with different training parameters (cf. text).

failure indicates that the preliminary condition of learned potentials is the association of two different kinds of stimuli acting on distinct populations of central neurones. Obviously, this is the case in classical conditioning, too. The failure of pseudo-conditioning may be explained also by occlu-

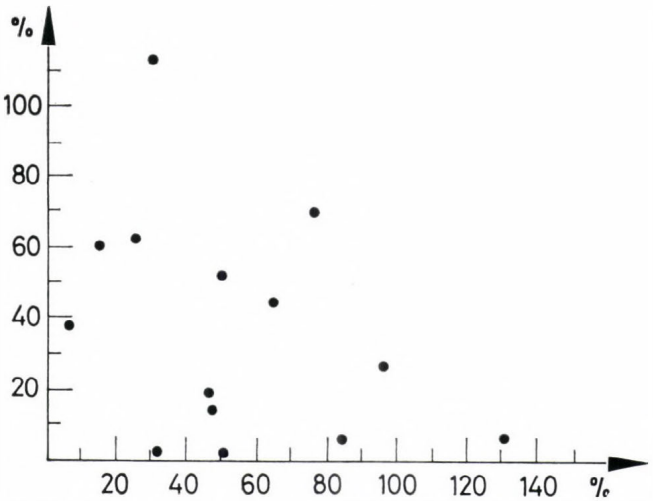

Fig. 3. Correlation between the amplitude of the first potential evoked directly by splanchnic stimulation and the second potential regarded as the conditional response, elicited by the same CS. Ordinate: mean area of the potential expressed as a percentage of the average control. Abscissa: the integral area of the first potentials evoked directly by the CS, expressed as a percentage of the unconditional evoked response evoked by the sciatic US. Each point represents a single experiment.

sion. In Fig. 3 the magnitude of the first unconditional potential evoked by splanchnic stimulus was expressed as a percentage of the sciatic response. The correlation is negative and, taking into account all the experiments, it is statistically significant. This means that if the primary potential evoked by the splanchnic stimulus was relatively great as compared with the sciatic stimulus, in the given experiment, the rate of conditioning was not considerable. This points to the possible negative role of occlusion in this learned phenomenon. Obviously, in case of the association of homogeneous stimuli, a more intensive occlusion can be anticipated than in the case of a real conditioning.

The statistical analysis of our data seems to show that reverberating circuits may have a role in this type of learning. In a special series of experiments on 12 cats we applied a training procedure similar to our other studies without, however, eliciting a conditional response by applying the CS after the pairings. In other words, the spontaneous activity was measured before and after the associations. The power spectra of these samples have been determined by means of Fourier-analysis of their autocorrelation function. By this we could detect the periodical components of this spontaneous electrocorticogram. Figure 4 shows the average spectrum of the 12 experiments. The reciprocal value of different frequencies, in other words the wave-length in logarithmic scale have been expressed on the abscissa and their participation in the composition of the spectrum on the ordinate. The white columns represent the initial values, the black ones those after the pairings and the third values obtained after the extinction. It can be seen that the participation of the 200 msec component has been nearly doubled after the associations and it decreased following extinction. In other, words a peculiar kind of driving can be observed: the spontaneous brain activity assimilated the rhythm of the associations. The participation of the subharmonic component diminished at the same time. The changes proved to be statistically significant. The assimilation of the

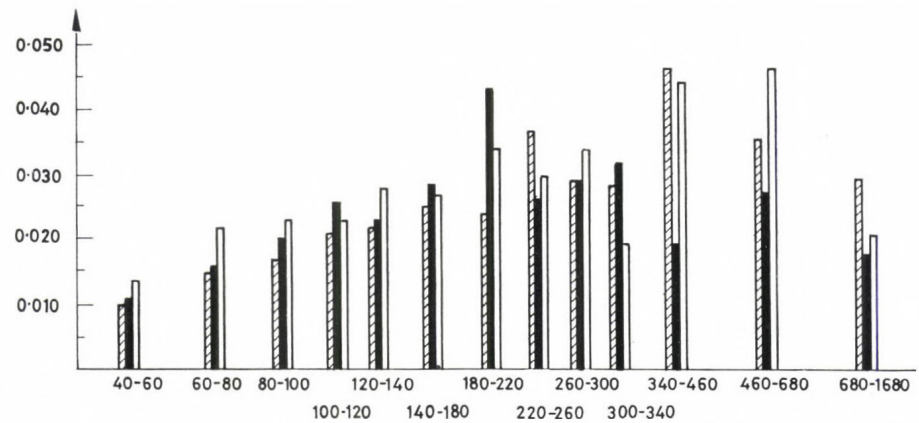

Fig. 4. Power spectrum determined by means of Fourier-analysis of the autocorrelation function of 12 experiments (cf. text).

rhythm observed by many workers ever since Livanov's pioneer experiments indicates the possible role of reverberating events also in this type of conditioning.

Summing up our data: a PTP- or facilitation-like mechanism seems to play some role in the delayed conditional evoked responses. Occlusion, on the contrary, is a pessimum condition in our training situation. The role of reverberating neuronal events cannot be excluded.

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CONDITIONED EVOKED POTENTIAL—A TRACE OF LEARNING IN MAN

by

I. MÉSZÁROS

Department of Comparative Physiology, Eötvös Loránd University, Budapest, Hungary

The methods by which conditioned evoked potentials can be studied in laboratory animals in the course of learning have been desribed by Prof. Ádám (p.321 in this volume). I would like to present the results of our observations undertaken in men. In these experiments it was possible to analyse those components of the learning process which are inaccessible in animal studies. Thus, for example, we were able to determine the effects on the establishing of conditioned reflexes of the concentration or diversion of attention; also, it was possible by hypnotic suggestion to effect the various factors involved in establishing the conditional response, and to analyse the firmness of the conditioned connections.

The conditioned (first) stimulus consisted of a brief click, which was reinforced by a stroboscopic flash 200 or 400 msec after the first stimulus. The difficulty encountered in this study was that the evoked deflection usually does not, or only slightly, exceed the amplitude of the spontaneous electrical activity; consequently, it could be revealed only by the technique of averaging. It is important, however, that temporary connections are usually extinguished if repeated in a sufficiently high number for averaging. The aim of our preliminary experiments had been to establish the optimal number of repetitions in the case when the conditional response is not yet extinguished and the evoked deflection can already be clearly distinguished from the background activity. In the majority of the experiments 40 tracings were averaged, but occasionally this number was increased to 100.

In each experimental subject the deflections evoked by single auditory stimuli and by the photic stimuli were recorded. Then the two stimuli were associated in groups of 40 or 100, with a delay of 200 or 400 msec. The stimuli were presented at a fixed, rhythmic rate of 0·5 c.p.s. Thereafter, an attempt was made to elicit the conditioned response. To avoid the time response to become established, the reflex was elicited always arrhythmically. Whenever necessary, reinforcement was repeated. The results were analysed by statistical methods, determining the frequency at which the various components of the conditional response appeared in the traces.

Below it will be shown how in a single experiment a conditioned evoked potential can be established, and the changes occurring in the uncondition-

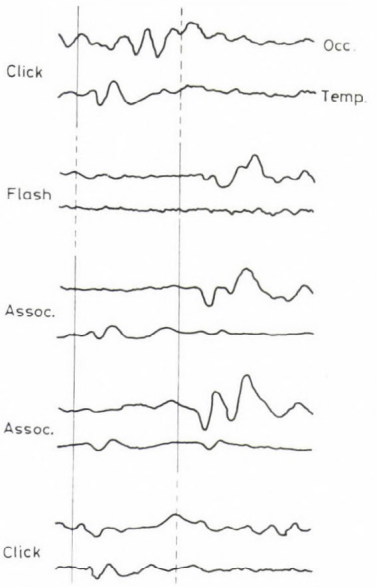

Fig. 1. Establishment of the conditioned evoked potential on subject Sz. K. (first experiment).

ed evoked deflection during the process of reinforcement will be discussed.

The delay between the auditory and photic stimuli was 400 msec (Figure 1). Occipital and left temporal activities were recorded. The number of traces averaged 100. The first vertical line indicates the acoustic stimulus, the second one the stroboscopic stimulus. Where the line becomes broken, no stimulus was presented. There is a response in the occipital lead too, prior to the reinforcement procedure, which has a protracted delay; the second part of the trace is quiet in both regions. The photic stimulus elicited practically no response temporally. In the course of reinforcement, however, there appeared a successively more and more marked light-elicited deflection in the temporal lead, which was regarded as a sign of the conditioned connection being established; finally, the acoustic stimulus. even when presented alone, brought about the evoked response in both leads, exactly with the same delay as the time lag between the two stimuli in the course of reinforcement.

A negative deflection was frequently observed in the occipital region, prior to the presentation of the photic stimulus. This deflection was termed x-wave. As this deflection was frequently recorded in our tracings, we have assumed that it is a phenomenon similar to Grey Walter's contingent negative variation. The experimental conditions are similar, and the deflection was invariably negative in our observations. It was, however, recorded by the usual EEG electrodes with a short time constant. Our subjects did not have to press a button at the time of the photic stimulation. These factors, in addition to the circumstance that the electrode in the present experiments was not placed on the vertex but on the occipital region, might provide a satisfactory explanation to the modification of the response.

Further attempts were made to find out whether it is possible under the above experimental conditions to record the CNV? Therefore, in 28 experiments the responses were recorded also with a long time constant, using the leads recommended by Grey Walter.

The subjects did not have to press a button in the moment of stimulation, but they were instructed to blink at the time of the photic stimulation. This motor response was recorded, too. This was important because the appearance of the motor response indicated the firmness of the conditioned reflex.

Figure 2 shows the conditioned evoked potentials recorded in 2 subjects in the occipital, vertical and temporal leads, and the accompanying motor

response. In both cases the CNV is apparent, and various manifestations of the conditional evoked potentials are well demonstrated in all cortical leads. In these experiments, 40 responses were averaged.

Figure 3 shows the average of 100 responses, recorded in the same leads with a delay of 200 msec, however, with an X–Y recorder. The top trace shows the average of the spontaneous activity. In the middle, the evoked potentials recorded in the course of reinforcement are represented. The motor response appeared with a delay of 50 msec. At the bottom the conditioned responses are shown. The conditional potential appeared in all three leads. In this case, however, the peak of the motor response appeared only about 10 to 15 msec after the expected stroboscopic flash.

The question now arises whether this conditioned response is sufficiently firm, and how long does it persist without reinforcement.

After a sufficiently high number of reinforcements amounting to several thousands, the response persists without further reinforcement for periods as long as 3 to 4 months (Fig. 4).

In a further series of experiment an attempt was made to establish the effect of hypnotic suggestion on the conditioned evoked potential. To approach this problem, part of the investigations was undertaken during a hypnotic state, by giving various commands to the subjects (Fig. 5). The subject was instructed to look out for the flash when tracings 1, 3 and 5 were

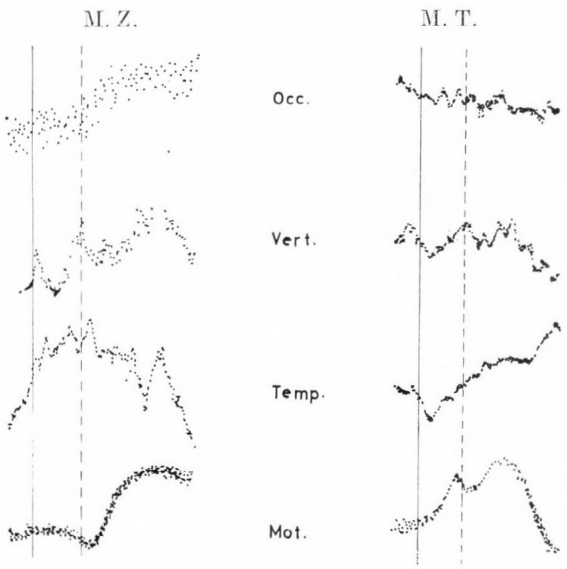

Fig. 2. Conditioned evoked potentials on different leads (two subjects). Appearance of the CNV in the vertical lead.

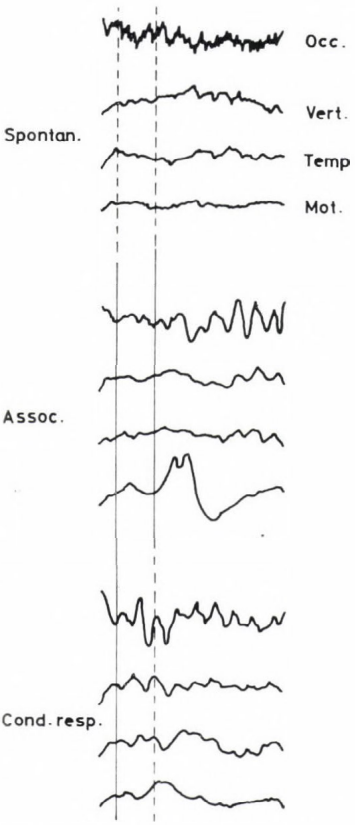

Fig. 3. Changes of motor responses during conditioning.

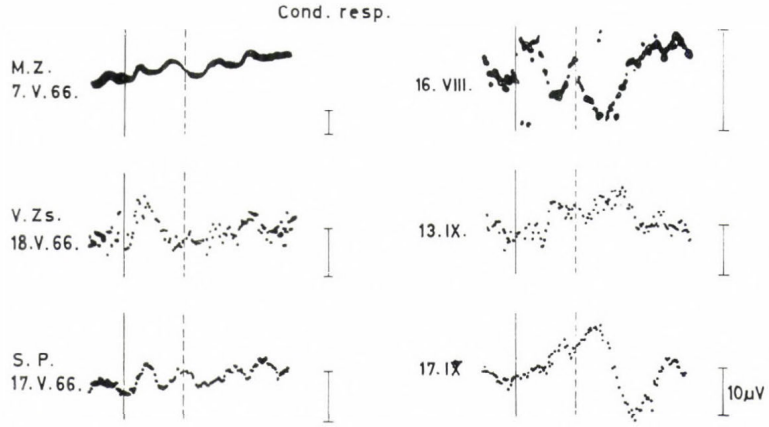

Fig. 4. Persistency of the conditioned evoked potentials in occipital lead.

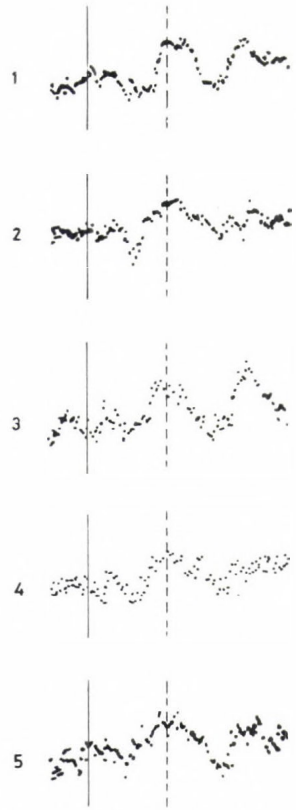

Fig. 5. Effects of different hypnotic suggestions on the conditioned evoked potential.

recorded. It should be emphasized that in these experiments only the acoustic stimulus was presented. While recording the second trace, the subject was informed that "there will be no flash". The amplitude of the evoked deflection decreased, nevertheless, occasionally we observed an increase of the deflection. On trace 4, the command was: "You have lost your sense of time". In this trace, the conditional response is practically absent indicating that we were able to eliminate the time factor, thereby abolishing the conditioned reflex.

CONCLUSIONS

(1) The results presented here indicate that a conditioned evoked potential can be established also in human subjects. It can be extinguished, but after a sufficiently high number of reinforcements it tends to persist over considerable lengths of time.

(2) In the course of the establishing of the response the evoked deflection recorded in various cortical regions undergoes characteristic changes. Evoked potential in response to the photic stimulus appears first of all in the temporal area.

(3) The fact that concurrently with the above process a response appears also in the associa-

tive areas has been repeatedly demonstrated by others. This is the reason why the establishment of the conditioned evoked potential is associated with the appearance of CNV.

(4) So far we have not been able to clarify the origin of the x-wave preceding the conditional deflection. The circumstance that it was frequently recorded also when the delay was increased to 400 msec renders it highly improbable that it would be a late phase of the sound induced deflection. Its connection with the CNV cannot be excluded. It is likely that it is due to some hitherto undefined component of the delay or attending mechanism.

(5) The conditioned evoked potential is associated almost invariably with a motor response, a blink, which appears with the delay used during reinforcement. This latter is usually confirmed earlier than the conditioned evoked response.

(6) On the basis of the experiments carried out in hypnotic suggestion it appears that the most sensitive component of the temporary connection is the timing of the delay. Any disturbance of the latter, namely, has been shown to considerably affect the appearance of the already firmly established conditioned reflex.

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THE ROLE OF TEMPORAL PATTERNS IN LEARNING AND RETENTION

by

G. Ádám, T. Kukorelli and Éva Markel

Department of Comparative Physiology, Eötvös Loránd University, Budapest, Hungary

In the following some results of our investigations carried out in recent years will be summed up. Some of the data have already been published (Ádám, 1967; Ádám, Adey and Porter, 1966; Ádám and Kukorelli, 1964; Ádám and Kukorelli, 1965; Székely and Ádám, 1968; Székely and Ádám, 1970), other results are relatively new. Details of some of our experiments are contained in other papers of this volume (Mészáros, Székely and Ádám).

The main question of our experimental programme has been the temporal sequence of events during learning. In other words how does the brain process the information received in form of exactly timed stimuli, i.e. in form of temporal patterns.

We have started out from data of classical conditioning, namely from two rather neglected phenomena: the so-called "time reflex" described by Pavlov in the early twenties and the "delayed inhibition" analysed somewhat later (Pavlov, 1951). As an experimental model in the study of temporal patterns we have applied a rather simple conditioning technique, which seemed to be adequate for the exploration of some basic, elementary problems.

The main point of our technique is the delayed conditioning of primary evoked potentials recorded from different brain areas. The method is actually a tracer technique. A conditional stimulus is applied, which evokes a primary potential in the cerebral projection areas. This stimulus is followed after a certain delay by a second stimulus, the reinforcement, which is actually a tracer stimulus in this case. After the repeated association of these two stimuli the CS evokes by itself a labelled response which can be distinguished from the background noise. This is, properly speaking, a delayed conditional potential. As a criterium of the learned response the fulfilment of the following two postulates was required: (*i*) The CS had to evoke by itself, i.e. without UCS, two potentials: in addition to its own response a second one corresponding to the previous UCS; (*ii*) This second potential, regarded as a learned response had to appear with the same delay as did the potential evoked previously by the tracer stimulus, the reinforcement. Thus the parameters of the delayed conditional evoked potential serve as the exclusive indices of learning and retention (Fig. 1). In all of our experiments single, brief stimuli served as the CS and the UCS; the evoked responses were stored and averaged by means of a 256

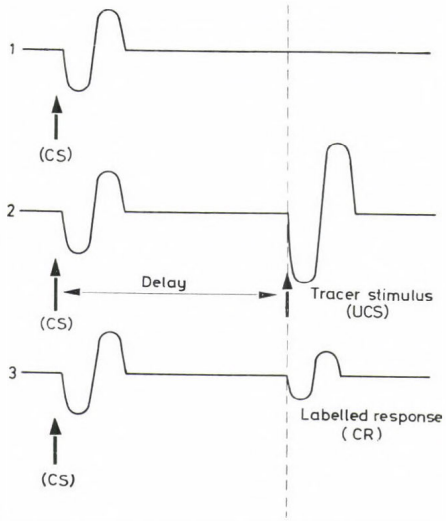


Fig. 1. Scheme of the conditioning procedure. Curve 1, evoked potential to the conditional stimulus (CS); curve 2, evoked responses to the conditional stimulus and to the unconditional stimulus (UCS) regarded as tracer stimulus; curve 3, the application of the CS by itself evokes, in addition to its own potential, a second response regarded as the conditional reaction (CR). This second potential appears with the same delay as the potential evoked previously by the tracer stimulus.

channel computer. Most experiments were carried out on anaesthetized cats using splanchnic and sciatic associations.

The most remarkable feature of this type of conditional potential has been the exact timing of its appearance (Fig. 2). The learned potential showed the same delay as the tracer stimulus on the basis of which it had been elaborated. The latency histogram in Fig. 3 clearly indicates the exactly timed delay when compared with the histogram of the associations. The phenomenon seems to be similar to the Pavlovian "time reflex", which occurs as follows: a dog fed every 15 or 30 minutes shows a surprisingly periodical salivation curve when feeding is stopped. This "time reflex" has been interpreted by most of the workers in this field by the "delayed inhibition", which in classical conditioning studies develops very slowly, with a remarkable inertia. Our results suggest, however, that the delay is an amazingly mobile process: the timing of the conditional potential is, from its very beginning, identical with the delay of the reinforcing stimulus, at least in the range between 80 and 500 msec investigated by us so far.

The so-called pseudo-conditioning, i.e. the association of homogeneous stimuli does not lead to the appearance of a conditional response (Fig. 4). It seems that a precondition of this type of learning, similarly to classical conditioning, is the association of heterogeneous stimuli, acting on two distinct afferent systems of the brain. Some quantitative aspects of this question are discussed in the paper of Székely and Ádám in this volume (p. 309).

The delayed conditional potential proved to be distributed over a rather wide area of the brain. In the cortex it appeared bilaterally on both projections of the afferent systems, e.g. auditory and somatosensory, the stimulation of which had been combined. It could be detected in the sepcific nuclei of the thalamus (Fig. 5) and even in the midbrain. A special series of experiment has been carried out to study the possibility of elaborating the conditional delayed potentials in the mesencephalon. The conditional evoked potential elaborated in the intact animal persists after decerebration (Fig. 6). It was even possible to obtain conditional delayed evoked poten-

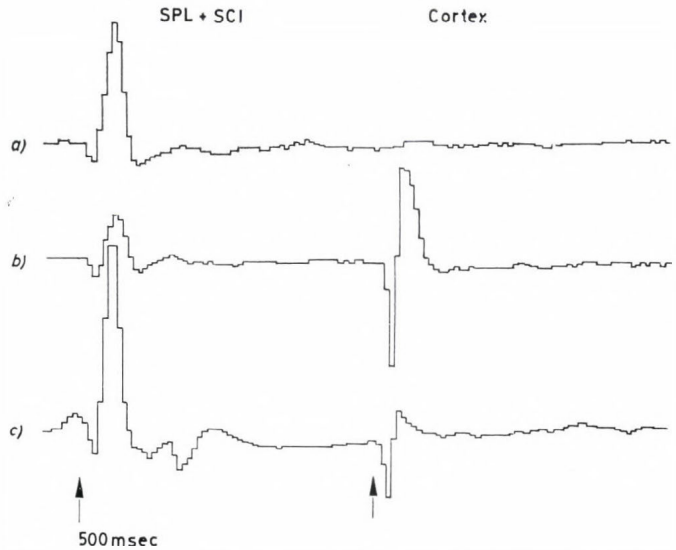


Fig. 2. Delayed conditional evoked potential. (*a*) Average of 40 potentials to left splanchnic stimulation; (*b*) association of splanchnic and sciatic stimulation with delay of 500 msec; (*c*) average of 40 splanchnic evoked potentials following 160 reinforcements. The conditional evoked potential appearing with the same delay as the previous sciatic reinforcement is clearly visible. Recording from the right sensory motor cortical area No. II of the cat. NTA 256 computer, X–Y-recorder applied.

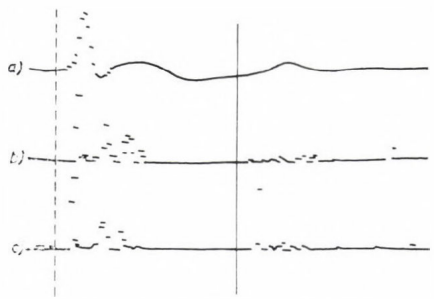


Fig. 3. Latency histogram of the conditional evoked potential. (*a*) Average curve of delayed conditional evoked potential to splanchnic stimulation with previous sciatic reinforcement; (*b*) latency histogram of the same learned response; (*c*) latency histogram of a series of stimulus associations (splanchnic + sciatic stimuli). The exactly timed delay of curve *b* is clearly detectable if compared with the delay of the second histogram on curve *c*. Dotted vertical line: splanchnic stimulus, heavy vertical line: time of application of the reinforcing sciatic stimulus. Delay, 200 msec. The NTA 256 computer adjusted to the 10 μV level of the negative polarity when recording the latency histograms.

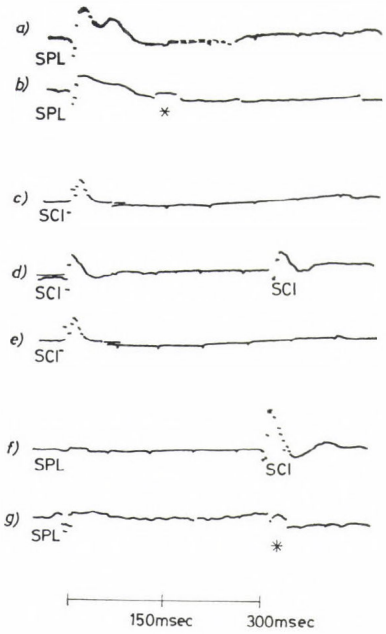


Fig. 4. The pseudo-conditioning experiment. The elaboration of a conditional delayed evoked potential with a delay of 150 msec (curve *b*) is followed by the association of homogeneous sciatic stimuli with a delay of 300 msec (curve *d*). This pseudo-conditioning procedure is characterized by the absence of a learned evoked response (curve *e*). The repeated association of heterogeneous stimuli (splanchnic + sciatic, curve *f*) leads in the same experiment to the appearance of a conditional potential (curve *g*) with a delay of 300 msec corresponding to the previous reinforcement.

tials in the midbrain cat, i.e. when the conditioning was begun after the transection of the brain stem (Fig. 7). In the figure the extinction can be seen as well.

Having observed a relatively easy extinction of this learned response, its stability became questionable. Considering that an elaborated condi-

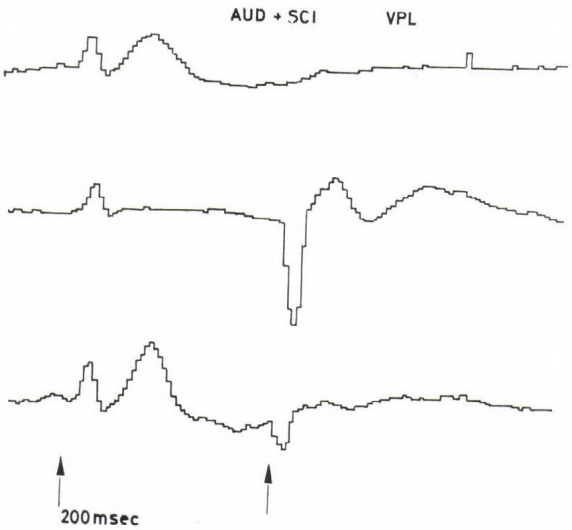
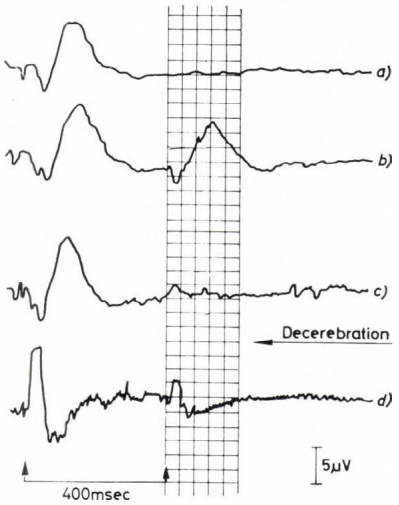


Fig. 5. Delayed conditional evoked potential recorded from the thalamic VPL nucleus. Delay, 200 msec. Legends the same as in Fig. 2, except that auditory stimuli (clicks used as CS) have been associated to sciatic stimuli (UCS).

Fig. 6. Conditional delayed evoked response in the midbrain. First arrow, moment of administration of the CS. Second arrow, time of application of the delayed UCS. *a*, Splanchnic shock (CS); *b*, association of splanchnic (CS) and sciatic (UCS) shocks, delay, 400 msec; *c*, the splanchnic shock (CS) evokes by itself two potentials, the second one is the conditional delayed response; *d*, conditional response after decerebration. Each curve represents the average of 50 evoked potentials. Vertical calibration of one quadrate, 5 μV.



tional potential can be extinguished in about thirty minutes, it seemed to be a labile, short-term phenomenon. A special series has been undertaken to investigate the stability and the consolidation of the conditional potential in chronic animals. Single clicks were associated with the administration of electric shock to the hind leg. When applying a systematic reinforcement day after day, the delayed learned potential proved to be a relatively long-lasting event. Thus, this electrical sign of learning seems to be a labile process only in its initial phase; when, however, the associations are continued (80 pairings each day), the conditional delayed potential turned into a consolidated phenomenon (Fig. 8).

Our data indicate that our approach differs essentially from other "sensory-sensory" techniques: the only and exclusive criterium of the existence of the conditional response is the detection of the delayed labelled potentials. The "assimilation of the rhythm" first described by Livanov and Poliakov in 1945 might have a similar neuronal background. The same can be supposed for Grey Walter's (1964) contingent negative variation. The time sequence of stimuli has been in the centre of our attention. The

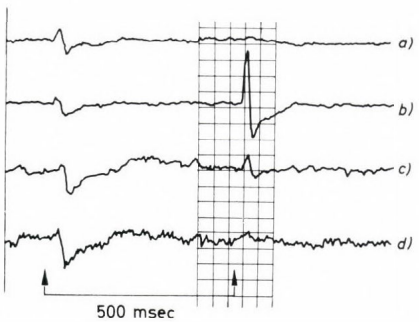


Fig. 7. Conditional delayed evoked potential in the decerebrated cat. Delay, 500 msec. Legends the same as in Fig. 6. *d*, Extinction of the conditional response after 100 isolated splanchnic stimulations.

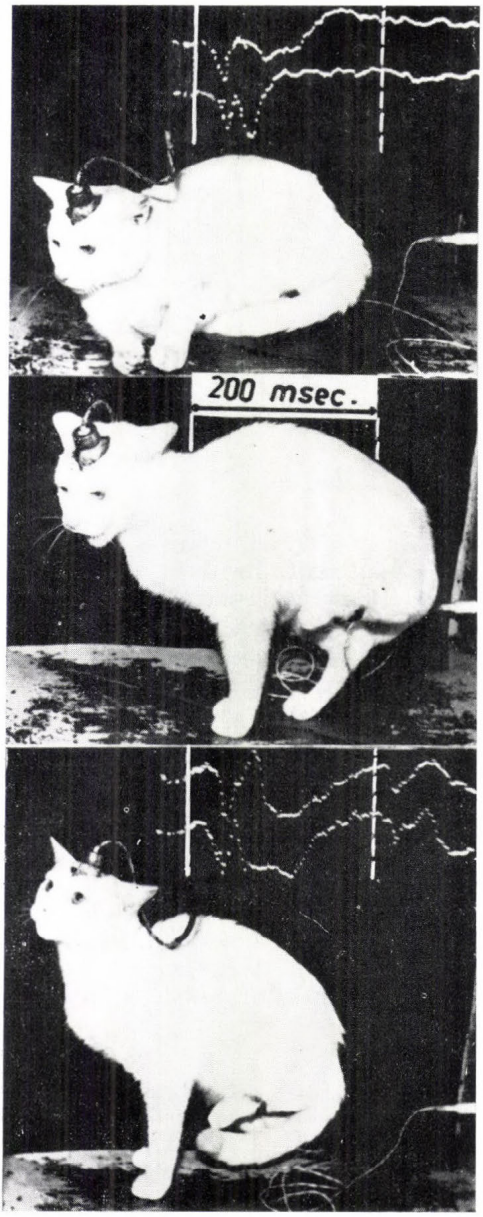


Fig. 8. Somatic and electrical signs of conditional delayed evoked responses. Vertical heavy and dotted lines mark the time of application of the CS and UCS. Unlike control (above) and conditional response (below), the associations (middle) are not represented by corticograms. Each curve represents the average of 40 potentials.

mechanism of delay of this exactly triggered process can hide different—yet unknown—mechanisms. In its initial phase it could be a reverberating neuronal phenomenon (cf. paper by Székely and Ádám in the present volume, p. 309). A special triggering centre, i.e. the existence of a population of

peculiar "pacemaker" neurones responsible for this timing, cannot be excluded either. Even a holographic resonance mechanism (cf. paper by Ladik and Greguss in the present volume, p. 343). might be thought of. On the basis of our data it can be stated that the time sequence of events during learning is one of the basic principles of this elementary plastic phenomenon.

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DISCUSSION*

R. Galambos: Have you excluded the possibility that the electrical response recorded to click alone after pairing click and flash might be due to eyeball rotation? You have created, I would guess, a conditioned eyeblink, and I would expect that the eye movement associated with the blink would appear at your recording electrodes.

I. Mészáros: I know very well Professor Galambos's work on the correlation between eye-movements and auditory evoked potentials. In the knowledge of these possible artifacts we made special efforts to separate the muscle potentials from the evoked brain responses.

G. Ungar: Have you thought of trying to transfer chemically your conditioned evoked potentials? They seem to be almost ideally suited for such experiments because they are so easy to quantitate. Its usefulness would, of course, depend on the possibility of training the animals so that they retain the electrical response for prolonged periods.

G. Ádám: No, we did not plan to apply the conditional evoked potentials in our so-called transfer experiments. This test seems to be too specific at this moment for the demonstration of this controversial phenomenon.

O. Fehér: In your opinion where is the site of the connection of these conditioned potentials? Can they be recorded from the cortex, the diencephalon and mesencephalon?

* A summary discussion of papers appearing on pp. 309 to 327.

G. Ádám: In our opinion every level of the mammals' central nervous system can be an appropriate structure for such associative processes. From the spinal cord up to the cerebral cortex, the possibility of elaborating elementary learned reactions cannot be excluded.

O. Fehér: I would suggest to try artificial hyperpolarization to enhance synaptic efficacy. Russinov in the Soviet Union and Morrell in the United States have demonstrated that hyperpolarization of the respective cortical areas improved the conditioned responses.

G. Ádám: Thank you for your suggestion.

F. Klingberg: Dr. Mészáros, did you evaluate the later cyclic changes after your photically evoked potentials? It would be of great interest to compare your human data with some results obtained on rats.

I. Mészáros: No, unfortunately we did not evaluate statistically the late responses.

I. Madarász: I should like to be informed whether the students in the course of hypnotic suggestions were aware of the whole experimental schedule, and of your preliminary supposition concerning the important role of temporal sequences in this sort of learning?

I. Mészáros: The students were aware of the general plan of our experiments, but they did not know anything about our presumptions or hypotheses.

I. Tomka: Could you get, Dr. Mészáros, in all the cases conditioned reactions in humans? What about the variability of these learned evoked reactions in man?

I. Mészáros: Yes, we could elaborate in all our subjects a more or less pronounced conditioned evoked reaction. In some of them, however, the learned electrical signs could be detected only by special statistical methods, but not visually.

K. Lissák: We all know that humans are very difficult subjects for such experiments. Have you made any controls with completely naive subjects instead of students who presumably were aware of what they can expect?

I. Mészáros: Yes, of course, we made some control experiments: the results have been the same.

F. Rosenblatt: Since the question of using these techniques as a basis for transfer experiments has been raised, I would like to mention that we have done a series of about 5 transfer experiments using conditioned eyeblink reponses in the rat, with a tone as CS. The results were disappointing. We obtained a consistent suppression of normal eyeblink rates in the recipients, rather than an enhancement. Since normal eyeblink rates in response to a tone were very low, we obtained little data, and this was abandoned as an unsatisfactory technique. We have also been interested in finding a suitable electrical response which might be used as a sign of transfer, and we have chosen to use a conditioned interhemispheric delayed response (IDR), described by Ratledge and Doty. This response is highly stable and closely localized at the contralateral homotopic point from the applied stimulus.

G. Ádám: Thank you for your comment, Dr. Rosenblatt. I think your findings accentuate my opinion on the inadequateness of the conditional electrical signs for transfer experiments.

G. Horn: This is a very interesting series of experiments, but it is not clear to me how far they advance the analytic study of learning mechanisms, compared, say, with recording from a muscle. Your explanation for the electrical changes observed involve, once again today, reverberating circuits and post-tetanic potentiation, but have these phenomena ever been demonstrated to be involved in learning?

G. Ádám: The advantage of our method has been the elimination of the inert efferent system, thus we could quantitatively evaluate the learning process by recording direct brain events. Our electrophysiological method is a type of the so-called sensory-sensory conditioning technique, which seemed to be very suitable for studying elementary plastic processes. As far as the mechanisms of reverberating circuits and post-tetanic potentiation are concerned, of course, we have no direct evidence on these processes. I think these elementary phenomena have never been demonstrated to be involved in learning. It is only a very plausible presumption, direct evidence depending on future technical development in planning and programming behavioural experiments.

THE EVOKED POTENTIALS OF DIFFERENTIAL INHIBITORY STIMULUS

by

Á. Sarkadi, and I. Tomka

Department of Physiology, Institute of Neurosurgery, Budapest, Hungary

In the past decade a great number of frequently contradictory data has been published and several conclusions have been drawn about the correlations between evoked potentials and conditioned reflex behavioural patterns. Interest has been turned, partly due to technical progress, to the components of evoked potential and their relationship to the variations of the background activity of a given structure. This time we should like to report on one of the initial results of our experiments launched in this direction.

Freely moving cats with chronically implanted electrodes were used. The epidural electrodes were spaced 2 mm apart at the most on standard points of the primary somatosensory, acoustic and optic and associative cortex by the stereotaxis method and with bipolar outlets. The test series were started after the perfect recovery of the animals, in a sound-proof chamber. The electrocorticogram was recorded by a polygraph type Elema-Schönander, the oscilloscope output of its four channels being coupled to the inputs of an amplitude-analyser type KFKI NTA-512. The system was calibrated with the aid of an oscilloscope and the polarity of the evoked potentials were checked through a reference electrode. The conditioning stimuli consisted of well audible clicks lasting 20 seconds of a phonostimulator (designed by our electronic laboratory) controlled by 2 per sec square-waves of a Disa Multistim. In order to obtain a differential (non-reinforced) stimulus only the frequency of the phonostimulator was varied, which means that the click differed only in quality for differentiation. We could average 40 evoked potentials obtained simultaneously from four cortical areas during the stimulus period, which were photographed and printed out in the decimal system. The animal's behaviour was first habituated to at least five kinds of clicks differing in quality. Then we proceeded to establish an alimentary conditioned reflex with 10 sec delay from the beginning of the stimulus. So we used the so-called overlapping conditioning process and were able to compute the average of 20 evoked responses from each cortical area, in the course of the delay period and of the post-reinforcement feeding time, respectively. In each daily conditioning session 12 to 18 trials were given at irregular intervals.

The evaluation of the individual phases of the development of temporary connections was based upon generally known and accepted criteria applying to behaviour and electrocorticography. The experiments were carried out

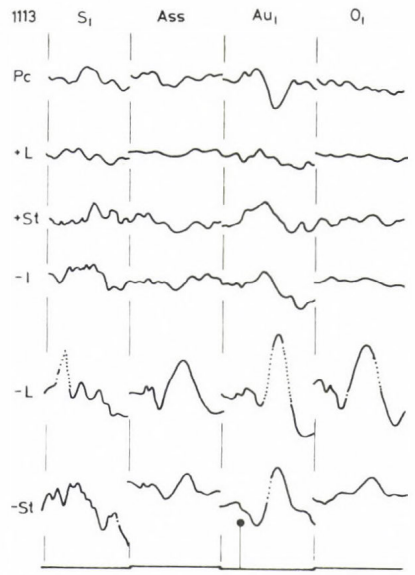

Fig. 1. Changes of simultaneously averaged evoked potentials ($N = 40$) of primary somatosensory (S_1) auditory (Au_1), optic (O_1) and "associative" (Ass) cortical areas in chronic experiment (see text). The length of a quarter of computer's memory is 102·4 msec. In this and all subsequent figures: bipolar recordings; time of stimulus is at the beginning the of traces; positivity is upwards; calibration (on the bottom line in the third quarter, retouched) is at 20 msec from the beginning of the record 100 μV for Au_1 and 50 μV for the others.

on five adult cats. To illustrate our results we shall describe the performance of one animal.

In the pre-conditioning period (*Pc* in Fig. 1) the most uniform and stablest response was given by the acoustic cortex. The evoked potential of the associative cortex of considerably smaller amplitude was more variable, while the response of the remaining two areas were still rather meaningless, and practically unassessable as electrical reactions. In the relatively short and unstable ($+L$) orienting phase of temporary connection there is complete attenuation. It is a characteristic feature of the stable ($+St$) reflex, that the response is limited to the acoustic cortex; it is moderately variable and its early positive component is bigger and the ensuing negative one smaller and more variable than those of the preconditioning response. The response received to the first differential stimulus ($-I$) is the same as given to the positive stimulus, but is more attenuated. When the animal fails to get up upon being presented the feeder ($-L$) for the first time, an irradiated high-amplitude, monomorphous response is obtained. After a distinct startle reaction the animal makes a few steps, stops suddenly, looks at the feeder with a fixed, tonic attention, then relaxes when the stimulus is over and returns to its previous activity. In the stabilization phase ($-St$) all this shows a tendency towards attenuation. This irradiated high-amplitude response does not appear if the animal continues its motor activities (e.g. washing himself, walking) started prior to the stimulus, after a very short attentive reaction.

The electrocorticogram during the positive, stable conditioned reflex (Fig. 2) consists of irregular low voltage fast activity over all the areas. This did not change in the intersignal periods, in the unstable phase. In the stable stage, in the course of the last daily trials we frequently noticed a diffuse intersignal activity of very high voltage with a regular 10 to 12 c.p.s. from the somatosensory area. At the beginning of conditioning there is a gross evoked potential corresponding to the startle reaction, which undergoes the known variations in the course of the experiment.

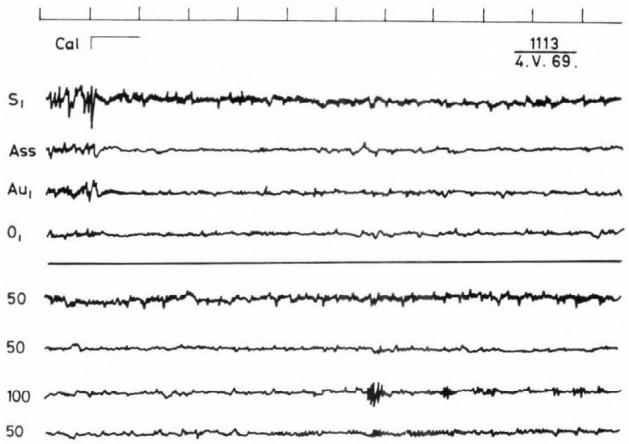

Fig. 2. Electrocorticogram during the stable phase of delayed alimentary conditioned reflex. Calibration μV and 1 sec. The calibration sign indicates the beginning of the conditioning stimulus.

Figure 3*a* shows the electrocorticogram of the unstable stage of differentation representing intensive, fixed, attention, accompanied by a minimal motion. The final phase of the stimulus brings forth, from the somatosensory area, an activity of higher, and more regular frequency (16 to 20 c.p.s.). This phase is getting longer in the course of subsequent tests (Fig. 3*b*). This phenomenon is not seen in the case of motor, non-goal-directed activities. The evoked potential appears first from the acoustic cortex, then irradiates to the association and finally to the optic cortex; the faster somatosensory activity appears now or later, or increases meanwhile, without altering the average amplitude or shape of the evoked responses.

In the stable phase, when the animal is indifferent to the stimulus or remains quiet, the very short desynchronization from the somatosensory cortex is first followed by a short, more regular and slightly increasing bout of faster activity, which is then replaced by a slower, diffuse rhythm, uninterrupted up to the intersignal period. This resembles the state of drowsiness. The evoked potentials are slightly more attenuated irrespective of the time-, regularity- and amplitude-conditions of this activity.

Summing up the aforesaid we find, joining other authors, that any kind of motor manifestation results in the abolishment or, at least, the attenuation of the evoked potentials, at an almost equal rate regarding its components, whatever the circumstances. In the phase preceding the reinforcement mainly the early components appear, distored by the quantity and quality of motor manifestations, while they are distinctly marked during the whole stimulus time only up to a negative component of 30 msec. In the differentation period, during the establishment of an active inhibition of the motor response, they take a gradually more distinct form and, as soon as the inhibition is complete, also the later components appear

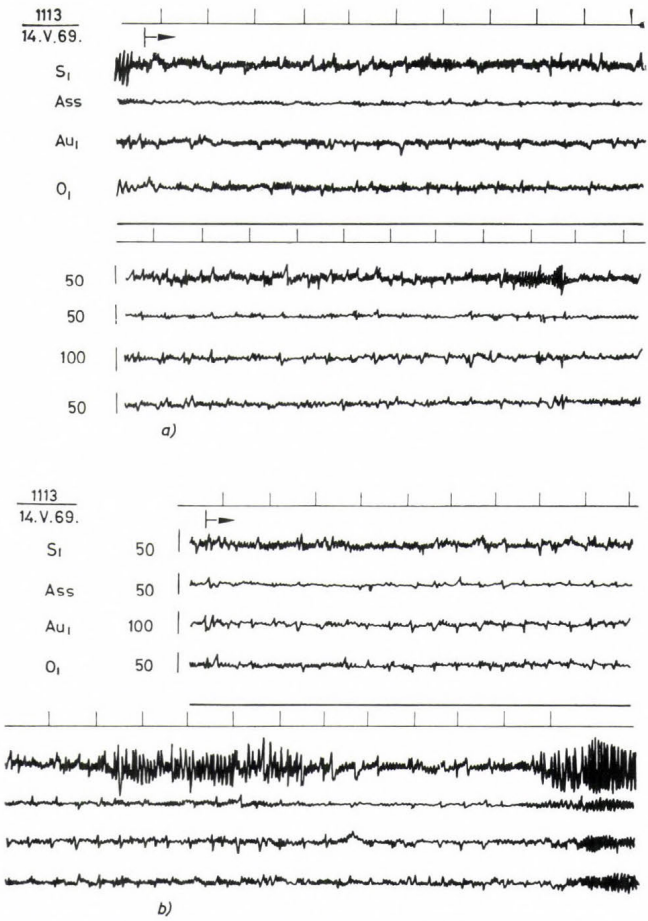

Fig. 3. Two different electrocorticograms characterizing the unstable phase of the development of differential inhibition.

with full intensity (Fig. 4). Adding to this the results obtained by other authors on distraction, as well as the data supplied recently in a conditioned reflex background, we wish to confirm the theory that the evoked potentials generally increase in conditions of inhibition of the somatic sphere, and decrease under the effect of organized, active, motor reactions. We may add, at the same time, that, in our opoinion, the decisive factor is the level of evoked attentivity in the alert internal inhibitory processes in the various stages of intensity and irradiation.

As regards the variations of background activity in the same regions and under similar conditions, our observations correspond to those of other authors. Accordingly, the inhibition of the conditioned response brings

forth the appearance of slow waves which, however, are not sufficient to explain such inhibition, and while its variations involving different forms of inhibitions are well discernible, their mutual relations are difficult to be interpreted by the physiological approach.

Nevertheless, from the pertaining experiments the conclusions can be drawn that apparently there is a certain parallelism between the intensification of internal inhibition, the late and slow components of cortical evoked potentials and the variations of the electrocorticogram. We would, therefore, follow the course of Jasper (1963), according to whom an appropriate application of the information theory and similar mathematical formulations may open more rational ways to the understanding of the cerebral mechanism of sensory perception influenced by the level of wakefulness and selective attention.

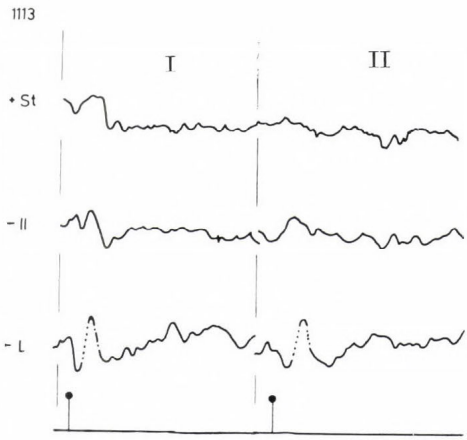

Fig. 4. The average of 20 evoked potentials from acoustic cortex in the delay period (I) and the post-reinforcement feeding time (II). The total length of each averaged response is 256 msec. The movement response attenuates the click-elicited potential ($+St$). There is no change in the responses for the second differential inhibitory stimulus ($-II$). The latest positive wave is followed by a large negative deflexion in the unstable phase ($-L$) of differential inhibition.

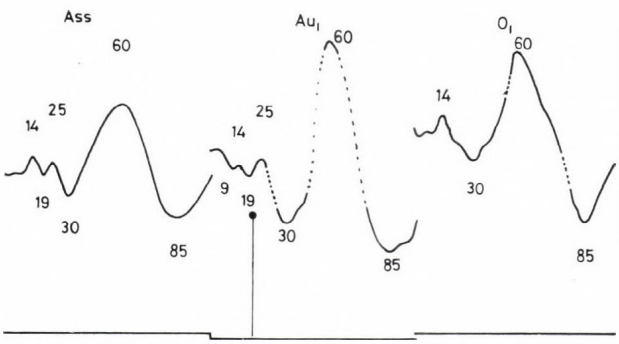

Fig. 5. The evoked potentials of differential inhibitory stimulus with time-relations in msec. The earlier components are relatively small, the later wave at 60 msec is the highest, and this and the slow negative deflexion at 85 msec irradiated most consequently.

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DISCUSSION

R. Galambos: Do you have any theoretical explanation for the remarkable similarity in the wave shapes of the evoked response at the auditory, association and visual cortex electrodes in your trained animals? What brain events are so similar in the three places?

I. Sarkadi: The similarity of the wave shapes is the most interesting fact, indeed. The association responses were recorded from the anterior middle suprasylvian area and on the basis of their behaviour during positive conditioning we can not suppose their origin from the so-called anterior suprasylvian auditory field (cf. Shaw, J. A. and Thompson, R. F., Dependence of evoked cortical association responses on behavioral variables. *Psychon. Sci.* **1,** 153–154, 1964; Shaw, J. A. and Thompson, R. F., Inverse relation between evoked cortical association responses and behavioural orienting to repeated auditory stimuli. *Psychon Sci.* **1,** 399–400, 1964; Thompson, R. F. and Shaw, J. A., Behavioral correlates of evoked activity recorded from association areas of the cerebral cortex. *J. comp. Pyshiol. Psychol.* **60,** 329–339, 1965). Really, the similarity exists only at the appearance of differentiation, related with the inhibited state of the conditioned reaction, and only in the late components. Taking into consideration the appearance of these large late responses in several areas in a time-lagged manner, the phenomenon might be explained by a tonic cortical inhibitory state, spreading relatively slowly from the primary receiving area of the differential inhibitory stimulus. It is evident that our results must be confirmed with changes of potentials of subcortical structures in the same circumstances and in the other inhibitory state, too.

CONCLUDING REMARKS

by the Chairman

R. GALAMBOS

Let me put before your thoughts two of mine. You recall that Professor Szentágothai told us that every neurone in the central nervous system is potentially connected with every other one. I think he gave us a maximum of five units required to connect anything with anything else. In a general way, I suppose what we were trying to do this morning was to decide as far as possible which of these unrealized but potential connections are created during the course of conditioning and learning. Is that not really the essence of the question today? We heard about efforts to resolve this problem in two different classical ways. First, the ablation attempts in which one destroys the possibility of making these connections and examines the question as to what can be realized thereafter. It seems to me that the seizure discharge approach, this kind of chemical effort of a special sort carried out by Dr. Klingberg, is not the most appropriate approach to resolve the fundamental question of neural connections during learning.

Now a few words about the second sort of effort, the electrophysiological types of approach, which ran through so many of the studies today, our efforts to discover what one means when he says reverberating circuit, or how it can be that connections realized between the nerve cells could be examined through this electrical method. Now, I do not know what your feeling is at the end of the day. These were very beautiful experiments that we heard about. But I am not sure that the electrophysiological approach by itself can answer our fundamental question. Most probably it must be combined with other methods.

My second point would be the following: I missed this morning, and I think that I missed in this whole Symposium, what to me sounds like the really essential question that we face when we study memory and learning. And that is: how it can be that these two major actions of the nervous system, one is the creation of this fantastic electric activity, ranging from spontaneous rhythms, evoked potentials, etc., and the other, namely, the intracellular chemical changes, are interrelated? In fact we have evidence about an electrical apparatus on the one hand, and a chemical factory on the other. It has been pointed out be several colleagues at this Symposium that there must be some kind of relation between these two fantastic activities in the brain cells. Somehow the electric activity, that we heard so much about today, as it shifts and changes during the course of acquisition and performance of behavioural responses, must be linked to the chemical ones that are going on in the neurones.

Section VI

THEORETICAL BIOLOGY AND MEMORY

POSSIBLE MOLECULAR MECHANISMS OF INFORMATION STORAGE IN THE LONG-TERM MEMORY

by

J. LADIK*

Physics Department, The City College of The City University of New York, U.S.A.

and

P. GREGUSS**

Department of Ophthalmology, New York Medical College, New York, U.S.A.

INTRODUCTION

Where and how does the brain store its memories? This is a great mystery, which can only be solved by research in more than one discipline. Communication across traditional boundaries, however, is retarded not only by differences in vocabulary, in research techniques and in the way a problem is subtly influenced by the subjects and materials employed by scientists in different disciplines but also by the fact that in the endeavour to eliminate side effects important relations are orverseen. This is partially the case in the interpretation of biological information processes, where the psychologists are preoccupied primarily with questions of *process*, whereas neurophysiologists and neurochemists with the question of how the brain achieves short-term and long-term *storage;* both are neglecting that information is always combined to a sort of energy. Energy, however, propagates mainly in wave form, i.e. the carriers of information are waves. This then means that information is always combined with one of the attributes of the information bearing wave: either with its frequency or with its amplitude or with phase, i.e. the biological input could be described exhaustively in terms of amplitude, frequency and phase. However, all known macroscopic energy (i.e. wave) receptors—organic and anorganic ones—are insensitive to the phase of the information bearing wave. Yet, phase is processed in the central nervous system since, e.g. there is a 3 D perception which is always linked to phase information.

Experimental evidence suggests that point-to-point peripheral to central sensory representation does not occur. The central nervous system appears to work with complex patterns of events which occur in large populations of cells. Mountcastle (1961) stated that such a mechanism may be located in a sensory receiving area of the cerebral cortex which is responsive to the spatial and temporal patterns of information concerning the peripheral event provoking it. The nerve impulses from each sensory receptor or group of receptors are at the same time apparently not kept isolated from other sensory nerve impulses. However, there is no doubt that the spatial and temporal patterns of nerve impulses provide nevertheless the basis of

* Permanent address: Central Research Institute for Chemistry of the Hungarian Academy of Sciences, Budapest, Hungary.

** Permanent address: Railway Scientific Research Institute, Budapest, Hungary.

sensory perception. For the degrees of freedom of encoded electrical information to remain invariant, transformations on both the amplitude and phase of the electrical information must be transmitted and processed. It is for this reason that the question of encoding of phase in the brain must be raised. Unfortunately the transmission of phase has not up to now been studied directly in the brain—except in one case (Greguss, 1967, 1968*a*, *b*; Aryapatians and Konstantinov, 1967).

BIOHOLOGRAPHY

Recently one of us has shown that the striking abilities of pattern recognition of animals using ultrasonic echolocation for hunting and orientation suggest strangely that these animals perceive not only the amplitude but also the phase of the ultrasonic wave; nevertheless, their receptors are also insensitive to the phase of the information bearing ultrasonic waves (Greguss, 1967, 1968*a*, *b*). This controversy can only be understood if these animals are using, in their information technique, the process called wavefront reconstruction of two-step imaging process discovered in 1947 by Gábor for electromagnetic and electron waves.

The basic idea of this process is that by using a so-called coherent reference background, the information combined to the phase of the signal bearing waves is transformed, in a *reversible* form, into amplitude information so that the sensory system which is only sensitive to the amplitude of the information bearing wave can record it. This two-step imaging process involves the recording of the Fresnel diffraction pattern of the information pattern (i.e. encoding) and then using this recorded pattern—called hologram—to reconstruct—decode—the original information pattern.

What makes the hologram unique as a recording and storage model is that every element of the input information is distributed over the *entire* recording media. The hypothesis that biological information processing and so memory, too, is based on the principle of holography is, among others, attractive because remembering or recollecting literally implies a reconstructive process—the assembly of dismembered *mnemic* events.

The first histological evidence for a coherent background has been found by Ayrapetyants and Konstantinov (1967), who did not, however, realize that this was the key for capabilities of these animals. They found, namely, that when the bat emits a signal bearing ultrasonic wave, the part of his brain which ordered to emit the wave sends a stimulus—an information—on this to that part of the brain where the reflected signal bearing wave—the information to be processed—will be received. The stimulus sent by the first part of the brain to the second represents the coherent reference background (Fig. 1).

There is reason to believe that the bioholographic concept is not restricted to cases where the signal bearing wave is produced by the living being itself. From the work of Sokolov (1960) it has become apparent that somewhere the sensory input is matched against a comparator before being processed further in the central nervous system. This does not mean, how-

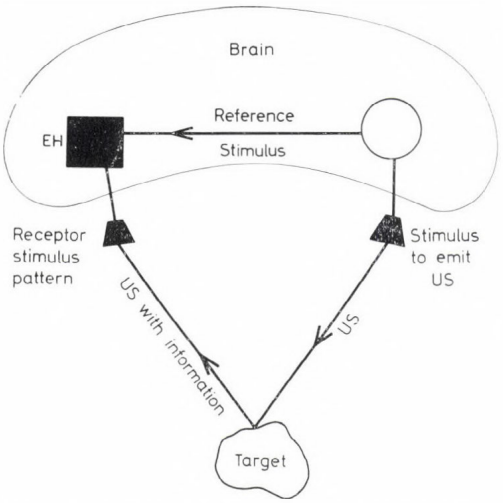

Fig. 1. The information processing mechanism in bats.

ever, that the central nervous system needs to store an infinite number of sensory "models" for recognition to take place. On the contrary, it is enough if a finite number of sensory "model elements" exists if these are stored in a holographic form. In the following, therefore, we shall first present evidence for the assumption that mnemic events are distributed in the brain, because the stimuli arriving at the membrane of nerve cells include the information pattern in holographic form, and then we describe how this can happen.

We would like, however, to emphasize that after our first biholographic model other holographic models (Longuet-Higgins, 1968; Gábor, 1968*a*, *b*) of temporal recall have been presented but—as Barrett (1969) has proved—these fail to adequately explain storage in the central nervous system: the notion of a signal, interacting with the delayed portion of the same signal and thus stored, may explain associative recall, but it does not explain the recognition of the signal itself.

GENERAL MODEL

The following description of our model contains only those function groups which are necessary for describing the generation of a stimulus whose intensity represents, in a reversible form, not only the amplitude bound-, but also the phase-bound information to be stored or recalled. Although this model does not claim morphological equivalents, its mechanism could be of help to neurophysiological or experimental psychological studies.

The model starts from the recognition that a good deal of impressions—information—of the external world must be eliminated during information processing, otherwise our memory would be unable to store the entire flow of sensory information. So, as can be seen from Fig. 2, a function group A acts as a gate at the beginning of the information processing and lets through only a stimulus pattern—depending on the information pattern—which can be described by the function $f(x, y)$ (x and y are the characteristic coordinates of an information pattern). At the same time, this information pattern induces an arbitrary stimulus pattern R from the all-neural activity and this serves as a reference background. According to the holographic principle, under the effect of these reference stimuli, a series of information

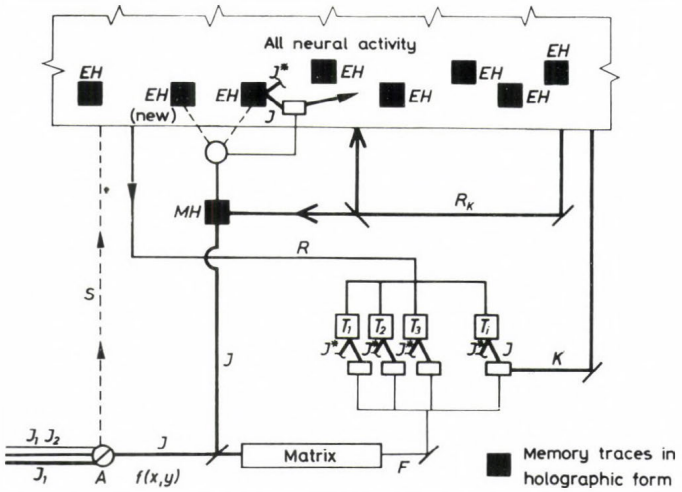

Fig. 2. The general model of the bioholographic information processing hypothesis (for legends see text).

elements is recontsructed from a finite number of "model elements" stored in the holographic forms T_i. In the meantime the Fourier transform F of the information pattern $f(x, y)$, which passed through the function group A, is formed in a group of neurones and this can convolute with the information elements T_i induced by R. As a result of this convolution a series of stimuli of the same frequency and phase, but of different amplitudes arises, which by further interference gives birth to a "code stimulus" K representing the original information pattern $f(x, y)$. (In other words the amplitudes of the different waves just add together.) This code stimulus then generates a specific reference stimulus R_K from the all-neural activity to which all engrams (EH)—memory traces—i.e. information patterns, stored in holographic form, belong.

At this stage of information processing two possibilities arise: either there is an engram which was formed with the specific reference background identical with the specific reference stimulus R_K or not. In the first case, according to the holographic principle, the original information pattern is reconstructed in the brain, i.e. the recall is performed. In the second case, however, the specific reference stimulus R_K interacts with the original information pattern $f(x, y)$ recording it in a holographic form (MH), which then will be a part of the all-neural activity of the brain. This stage of information processing is identical with the formation of the "short-term" memory. After several repetition circles it changes to "long-term" memory.

INFORMATION STORAGE

EXPERIMENTAL FINDINGS

The next question which arises is how the information carried by the stimuli arriving to the neurones is stored in the brain, or in other words what is the material realization of the holograms mentioned in the previous section.

In this connection it should be mentioned that in recent years it was reported that the base composition of RNA has changed in the brain of rats as a consequence of learning (e.g. Hydén, 1962). Further investigations have shown that by transplanting the RNA prepared from the brain cells of trained animals to the brain of other naive ones of the same race, these animals learned much more quickly than the control animals which were not subjected to this RNA transplantation (e.g. Quarton, 1967). Therefore it seemed plausible to assume that in the process of learning the structure of RNA (its base composition, and presumably also its sequence, although the latter was not shown directly) changes and in this way it may serve as a component of memory.

It is well known, however, that messenger or sRNA molecules in a cell (also in a brain cell) have not a long life and, therefore, they cannot serve as the basis of a really long-term memory. Therefore we are forced to look after some memory mechanism which is based on structural changes in DNA during the learning process. Since DNA carries the genetic information in the form of its base sequence, the most simple assumption is that a change occurs in the sequence of the DNA molecules. If this really takes place, the base composition and sequence of the RNA molecules coded by it will also change, and so the previously mentioned experimental results (Hydén, 1962; Quarton, 1967) can be explained in this way.

A change in the base sequence of DNA usually can occur, however, only during its duplication, most probably with the aid of the mechanism proposed for point mutations by Watson and Crick (1953) and placed on a physical basis by Lowdin (1965). On the other hand the division of nerve cells in mammals stops at a rather early age (in men at the age of 1·5 years) and from this time on the total amount of their DNA remains unchanged. Some recent investigations have shown, however, that there is DNA synthesis and decomposition in the brain cells. For instance, Morita et al.(1964) have found the incorporation of ^{32}P into the DNA of adult rat brain cells. More recently Sung (1968) has reported deoxyribonuclease activity in the brain cells of rats which was higher than the usual activity of this enzyme in other kinds of cells.

HYPOTHESIS FOR INFORMATION STORAGE

Between the two sides of a membrane of an unexcited nerve cell there is a potential difference as it is well known of $\sim$100 mV. Since the thickness of this membrane is 50–100 Å, this corresponds to a field strength of $\sim 10^5$ V/cm. Since many nerve cells have the shape of an ellipsoid, the

resultant of these field strengths in the neighbourhood of the nucleus (if we take it to be approximately in the middle of the cell) will be approximately zero. On the other hand, a stimulus arriving at a given region of the membrane surface will depolarize it, and so the potential difference between the two sides of the membrane will decrease to zero. Therefore also the field strength, due to this potential difference will vanish at the region where the stimulus has arrived and, therefore, a non-zero resultant field strength will occur at the place of the cell nucleus (Fig. 3).

In this connection it should be mentioned that if the cytoplasm between the cell membrane and nucleus were just an aquaeous solution of high ionic concentration, the space charge which would set up near the membrane would entirely eliminate the electronic field at distances beyond approximately 100 Å from the membrane and, therefore, the effect of changes in the membrane potential would not be felt at the site of the nucleus. We should not forget, however, that the cytoplasm is not just a strong electrolyte with high concentration of anorganic ions which possess a large mobility. On the contrary, since in the cytoplasm we have a high concentration of dissolved proteins and of many other organic substances, this solution must have a fairly ordered structure which prevents the free motion of ions considerably. It is, therefore, more correct to think of the cytoplasm as a "liquid crystal". Thus, while in a solid conductor or semiconductor we have high electronic (hole) mobility and low ionic mobility and in a strong electrolyte, high ionic and very low (practically zero) eletronic mobility, in a liquid crystalline sytsem, like the cytoplasm, we can expect an intermediate situation. Here the ionic mobility is much lower than in a strong electrolyte, but still higher than in a solid, and at the same time there is an increase in electronic mobility as compared to strong electrolytes.

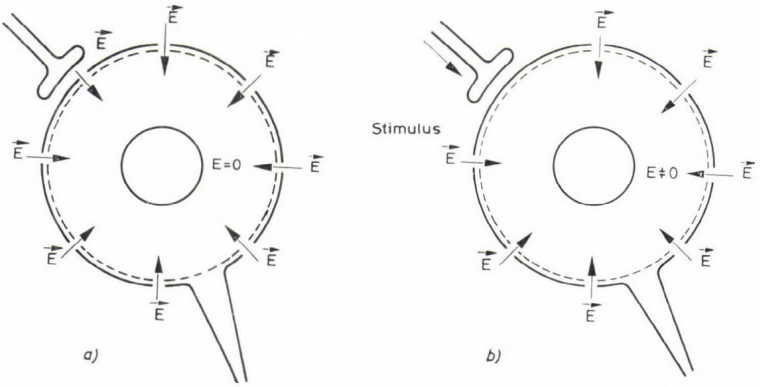

Fig. 3. (*a*) In the unirritated nerve cell the field strength due to the potential differences between the two sides of the membrane gives a zero resultant at the cell nucleus. (*b*) In consequence of the stimulus the field strength decreases to zero at the given synapsis and, therefore, there will be a non-zero resultant field at the nucleus. Both parts of the figure are strongly schematic.

Bearing in mind this intermediate position of cytoplasm between a solid and a strong electrolyte, we can expect that changes in the membrane potential *do have* an effect on the electric field also at the cell nucleus.

This assumption is supported also by the experiments of Loewenstein and Kanno (1963) who have found by measurements with a microelectrode that the depolarization of the cell membrane of *Drosophila flavoroplota* by mechanical damage causes also the complete depolarization of the undamaged membrane of the nucleus. Therefore, we cannot exclude the possibility either that the depolarization of a given region of the nerve cell membrane will result in the depolarization of a region of the nuclear membrane and thus will cause a change of electric field of the order 10^5 V/cm at a DNA molecule of the nucleus.

If there is a DNA duplication in the nucleus of the cell in which the depolarization of the membrane takes place, the ensuing change of electric field at the DNA molecule may increase the probability of point mutations during the DNA duplicatoin. Namely, according to Lowdin's (1965) theory, the probability of such a tautomeric rearrangement of the nucleotide bases in a base pair of DNA which can lead to a point mutation depends on the shape of the potential function acting on the protons in the hydrogen bonds of the base pairs. If this function is strongly asymmetric in the ground state of the base pair,* the probability of this "mutagenic" tautomeric rearrangement via proton tunnelling is small. On the other hand, if the potential function becomes symmetric due to excitation of a π electron of a base pair, or due to ionization, the probabilty of the tautomeric rearrangement via double proton tunnelling increases to a very great extent (Lowdin, 1965; Ladik, 1964), because the first level of the proton will already be a tunnelling level.

Lowdin (1965) has pointed out also the probability that strong local electric fields can strongly influence the shape of the potential surface acting on the protons in the base pair. According to the detailed calculations performed in different approximations for the shape of these potential functions (Ladik, 1964; Rein and Harris, 1964; Rai and Ladik, 1968) and potential surface (Rein and Harris, 1965, 1966; Lunnel and Sperber, 1967), the differences between the minima corresponding to the normal and "mutagenic" tautomeric forms of the base pairs are of the order of $\sim$1 eV. Since the distance between the minima is $\sim$1 Å, this means that an electric field strength in the order 10^8 V/cm is needed to distort the originally asymmetric potential functions (surfaces) to symmetric ones (Fig. 4).

Such enormous field strength of course cannot be produced technically and for energetic reasons it is very unprobable that it would exist even in a region of 10 Å, the size of a base pair. On the other hand, it can well happen that due to the change of the electric field strength at a DNA molecule, a positive (for instance K^+) ion comes with a distance of 1–2 Å

* Or more correctly, as it is discussed in detail by Lowdin (1965), the potential surface of both protons involved in the tautomeric rearrangement has a much deeper minimum in the normal tautomeric form of a base pair, than in the unusual, "mutagenic" form.

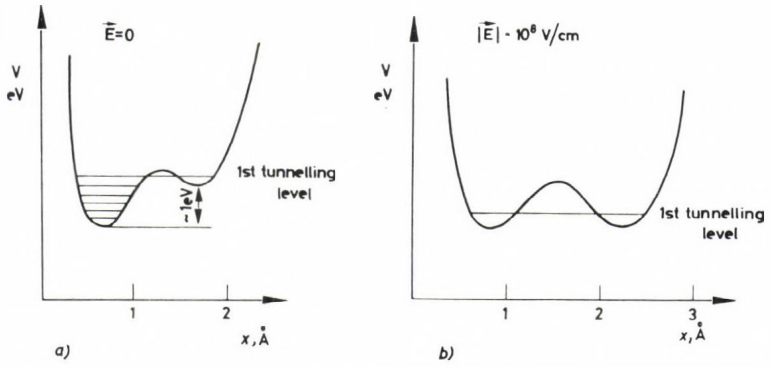

Fig. 4. (*a*) Schematic form of the double-well potential function of the hydrogen bond of a nucleotide base pair in the ground state of the base pair in the absence of electric field. (*b*) The same potential function in the presence of an electric field strength of $\sim 10^8$ V/cm.

from a hydrogen bond of a base pair. Since an elementary charge produces, at a distance of 2 Å from the charge, a field strength of $3{\cdot}6 \times 10^8$ V/cm, this extra positive ion can distort the asymmetric potential to a symmetric one. There is also the possibility that in consequence of the field a positive ion binds to a nucleotide base. If this ion takes away an electron from the elctronic system of the base pair, the base pair thus positively ionized will again have, according to the calculations of Rein and Harris (1965, 1966) and Lunnell and Sperber (1967), a symmetric potential. Therefore, we can conclude that if all this happens at the point of DNA duplication, the probability of changes in the original base sequence of the DNA molecule will increase considerably.

Further a homogenous field strength of the order of 10^8 V/cm can induce the breaking off of the nucleotide base pairs and thereby trigger the duplication of DNA molecule (Seprődi et al., 1969). In reality, however, it is very unprobable that such a high field can exist in the region of a whole base pair. However, the possibility might again be thought of that changes in the electric field of the order of 10^5 V/cm at the DNA molecules of the nucleus can influence the distribution of ions in such a way that at a given base pair the strong *inhomogeneous field* produced by the vicinity of an ion (ions) can induce the separation of the bases. (At the same time it seems rather probable that there exist such ionic configurations which can cause the breaking off of a base pair with an inhomogeneous field strength at the hydrogen bonds smaller than $\sim 10^8$ V/cm.) This means that if there is a change in the electric field of the order of 10^5 V/cm at a DNA molecule of the nucleus of a nerve cell, it can have a twofold effect. It can induce the duplication of DNA and at the same time it may greatly increase the probability of point mutations via the proton tunnelling mechanism in the DNA molecule. The probability of point mutations will be enhanced

of course also when a DNA molecule is already duplicating in the presence of the electric field.

We have seen previously that in consequence of the excitation of a nerve cell, the field strength decreases from $\sim 10^5$ V/cm to zero at the region of the cell membrane where the stimulus arrives. We have tried to show also that this may cause a change in the resultant electric field at the cell nucleus. We have emphasized that in the assumed transfer of the electric fields from the cell membrane to the nucleus the structure of the cytoplasm plays a key role. In this connection it should be mentioned that, according to some assumptions (Mishra, 1965), the stimulus arriving at a nerve cell first produces a change in the distribution of ions in the cytoplasm, which, in turn, causes changes in the cybotactic (liquid crystalline) structure of this aqueous solution. It is further assumed (Mishra, 1965) that these changes are responsible for short-term memory and that through these changes in the structure of the cytoplasm further changes in the cell are induced, constituting the basis of long-term memory. As we can see our train of thought is in accordance with this theory.

On the basis of the above considerations it seems probable that in consequence of the excitation of the nerve cells, the rate of DNA synthesis in their nuclei and at the same time the probability of the occurrence of point mutations in the newly synthetized DNA molecules will be increased. Our statement is indirectly supported by experimental results according to which the rate of RNA synthesis in the brain cells of mammals is increased upon excitation of these cells (Fevzner, 1966). Preliminary experiments which have shown that the number of divisions of chicken embryo heart cells has increased significantly (Bozóky et al., 1963) under the effect of electric fields are also in accordance with the assumption that an electric field may induce DNA synthesis in the cell nucleus.

The conclusion seems to be at hand that the electric field at the DNA molecules of the cell nucleus, arising in consequence of the excitation of a nerve cell, may cause changes in the original base sequence of these molecules. The changes caused by the original information in these DNA molecules will remain there for a long time and therefore they can serve as information storage in long-term memory.

Of course, in this case the long-term memory can function only statistically. This means: in the process of perception the stimulus pattern which contains, in a holographic form, the original information pattern acts on a large number of brain cells. Most of these stimuli will not cause any change in the base sequence of the DNA molecules, but in a fraction of the cells there will be changes in the information stored in their DNA molecules. Since, however, in holograms regarded as a recording medium, every element of the input information pattern is evenly distributed, the statistical character of this recording process does not make any difference. Not even if we assume that most probably the majority of these changes will occur in such regions of the DNA molecules from which the information cannot be recalled by the specific background stimulus pattern R_K. Some changes of the base sequence in some specific regions of some DNA molecules in a certain group of brain cells ensue, and these are specific in the sense that

they correspond to the process of storage of the information pattern. This model is all the more attractive because it is consonant with endeavours looking upon the brain as a statistically organized system which displays certain classes of lawful behaviour.

ALTERNATIVE HYPOTHESES

Finally, the alternative hypotheses of Fong (1969) and of Bradley (1968) should be mentioned. Fong assumes that the electric field caused by the nerve impulses interacts with the permanent dipoles of the stacked bases of RNA. Owing to this interaction, a base may rotate in the field. The energy gained in this way is enough for unstacking a base and the unstacked bases in an RNA chain stabilized by a protein bound to it form the physical basis of long term memory. Further, he assumes that the recall of the information recorded in this way occurs by the linear motion of the RNA chain in some (yet unspecified) "pick-up". The unstacked bases produce an electric field different from the stacked ones and also impulses with a field strength and time duration similar to those of the nerve impulses. Fong's hypothesis has thus the advantage of giving a mechanism also for the recall. Its physical basis, however, does not seem clear to us.

Bradley (1968) assumes that the electric field caused by a nervous impulse may change the ion concentration in the vicinity of an enzyme capable of RNA synthesis. The enzyme activated in this way will synthetize RNA. Since RNA is more suitable for binding ions than are the mononucleotides. the newly synthetized polymer might act as a reservoir for ions. If a second impulse arrives, the reactivated enzyme may catalyze RNA synthesis, or its degradation depending on the concentration ratio of polynucleotides/mononucleotides. If degradation occurs, the release of the bound ions could reinforce the electric effects of the signal, which could lead "to a greater synthesis of the polymer in post-synaptic nerve cells and thereby increase the probability that an impulse starting at the beginning of a pathway will travel through and activate the pathways in the neural net". As we can see Bradley's hypothesis has the advantage to connect mechanisms on the molecular level with mechanisms on the cell level. On the other hand, due to its complexity a great deal of work is needed until its validity can be proved or disproved.

RECALL OF STORED INFORMATION

It is easy to see that in case any one of the above-mentioned hypotheses for information storage in long-term memory proved valid, the proposed mechanisms must provide also for the recall of the stored information.

If the information is stored in DNA, repetition of the same stimulus can trigger the unwinding of DNA at the region where sequence changes have occurred in consequence of the first stimulus. This can give rise, through the synthesis of specifically changed RNA and protein molecules, to ion distribution changes in the cell. These, in turn, may produce such electric field changes at the cell membrane that are very similar to those produced by the original stimulus.

The two other alternative hypotheses include also possible mechanisms for the recall of the stored information (see p. 352).

We are of the opinion that the different mechanisms proposed for the explanation of memory are only the first very crude steps in our understanding of brain functions on the molecular level. We hope, however, that they will stimulate further more detailed experimental and theoretical investigations which may lead to a much better founded molecular mechanism of memory.

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DISCUSSION

G. Horn: How did you obtain a reading from your system?

P. Greguss: I think the reading out can be clearly seen from the second figure of our paper.

O. Fehér: I should like to raise two questions. Firstly, what does your model tell about subliminal electrical activity of the nervous tissue, about excitatory and inhibitory postsynaptic potentials? Their amplitude is sometimes very low: 1–2 mV on cerebral cortex neurones. Can they modify cell metabolism? This point is very important, because nervous function includes not only transmitted spikes, but PSP's too, which are essential factors in integrative function. Secondly, if impulses impinging upon the cell membrane are able to modify the nucleotide sequence of DNA, what is the guarantee that they do not modify the basic genetical code of the nerve cell thus altering its specific metabolism, distrubing its overall functions?

P. Greguss: It is difficult to answer Dr. Fehér's questions since it is a further step in the elaboration of our theory to take into consideration the subliminal electrical changes on the one hand, and the relationship between the basic genetical code and the individually stored memory, on the other.

G. Ungar: I suppose that according to your proposal when new information gets into the brain, this will change the structure of DNA in a large number of neurones and this is your holographic representation.

P. Greguss: Yes. The nerve impulse which is active stores the information in a holographic form.

G. Ungar: How do you explain that all memory traces are not permanent? Once you have produced a new DNA sequence, this must be there for ever, so you cannot forget anything. The second question is the following: We have to speculate in terms of concrete neural pathways. This is substantially the same question that Dr. Horn asked, namely, how do these new sequences of DNA modify the neural connections? There must be some intermediate mechanism as, after all, DNA is just a store. It has to produce different RNA's and corresponding proteins. How do you explain this sequence of events? Dr. Hydén said that a protein must be the executive molecule and in this case how does this executive molecule create the new connection necessary for the expression of learned behaviour and the retrieval of what you learn. Do you have any clear ideas about that?

P. Greguss: I have some ideas, but it is a bit too early to speak about them, because they have to be proved experimentally.

H. Hydén: It would be a good idea to have the information stored phase angle, as it was proposed. The information content could be rather high and the rather high frequencies could trigger any specific enzyme reactions, and they could initiate synthetic processes. In other words, this model could be a nice link between electrical phenomena and synthesis of molecules of some kind. But it sounded as if you had in mind that there would be storing molecules acting like a tape. Of course, this assumption would be too naive and would have to be discarded already from the beginning.

Such a hypothesis is too crude and cannot be conceived as a matter of fact. As far as DNA is concerned, recently an article has been published in *Nature* by J. Griffith and H. Mahler about the so-called ticketing model of memory. The hypothesis of these workers is in some respect similar to your assumption. As many times as the nerve cell is firing, the methylation of the DNA molecule represents a ticket, and the number of tickets would give information about the number of times the nerve cell could have fired.

G. UNGAR: I am just wondering about the so-called central dogma of the genetic code. DNA is a substance which is in the cell for life, in which all the genetic characters are encoded and if we go on playing around with DNA, it could be a fruitful beginning. I do not think that this dogma should never be touched, but I think that we do not have enough information at this stage to touch anything as sacred as that. And just one more point: in my understanding you spoke about some sort of random changes in the DNA molecule. I have difficulties in understanding how these random changes produced in a thousand different cells can produce any sort of order.

P. GREGUSS: You pointed to the main question of the holographic principle. If you take a holographic plate and cut it into different pieces of different shape, you obtain the information, the original information on each fragment.

G. UNGAR: But this is not random.

P. GREGUSS: It is quite random. This is just the principle of holography. Unfortunately, I have not enough time to discuss in detail the basis of this most fascinating theory. I agree with my discussants that our hypothesis must be proved and proved again not to vulgarize the whole principle of memory study.

A NEW NEURONAL MODEL WITH DISTRIBUTED MEMORY

by

F. Sebestyén

Department of Physiology, University Medical School, Pécs, Hungary

INTRODUCTION

As early as in 1958 the opinion was expressed that the organization of the brain and that of the computer must be basically different, first of all because the logical depth of the brain is much less than that of the computer (von Neumann, 1956, 1958). For this reason the computer of von Neumann's type, as a model, seems to offer few if any help in real-brain research.

More recently, for a greater resemblance, demands for a computer with distributed memory have arisen (Stark and Dickson, 1966), based on findings similar to those of Lashley.

The model (Sebestyén, 1968), to be described in this paper, is an attempt to cope with the problems and demands mentioned above and to help research on memory in general. First a particular case will be expounded to familiarize the rather unusual ideas. Then the model itself will be dealt with, and last some problems of memory will be discussed in connection with the application of the model.

THE ORGAN

The organ is shown schematically in Fig. 1. It consists of the pipe units labelled with the letters $a, b, c, \ldots, z, y$, and the common air medium in which the units are situated. The role of the air is not only to serve as the medium of the air waves generated by the organ itself, but to be a genuine component of the latter by which the interrelations between the pipe units, shown by the lines in Fig. 1, are realized.

THE STRUCTURE OF THE PIPE UNITS

The pipe units, 24 in number, have identical structures. The key to the capital letter symbols which appear in the magnified version of pipe unit i, is as follows (Fig. 1):

M_i = Microphone

R_i = Resonator, which consists of selective circuits tuned to the pitches of the pipes P_i ($i = a, b, c, \ldots$). It has one input formed by the parallel connection of the selective circuits, and a set of outputs, one from every selective circuit. They have two states "1" or "0", depending on the sounding of its proper pitch

C_i = Comparator, with two sets of inputs, one from the resonator R_i and one from the memory ME_i

ME_i = Memory with a set of outputs which can be in one of the two states: "1" or "0"

MS_i = Switch of memory which changes the state of the memory ME_i

V_i = Valve

B_i = Bellows

P_i = Pipe

The cause-and-effect relations between the blocks labelled with the capital letters are indicated by the directed lines in Fig. 1.

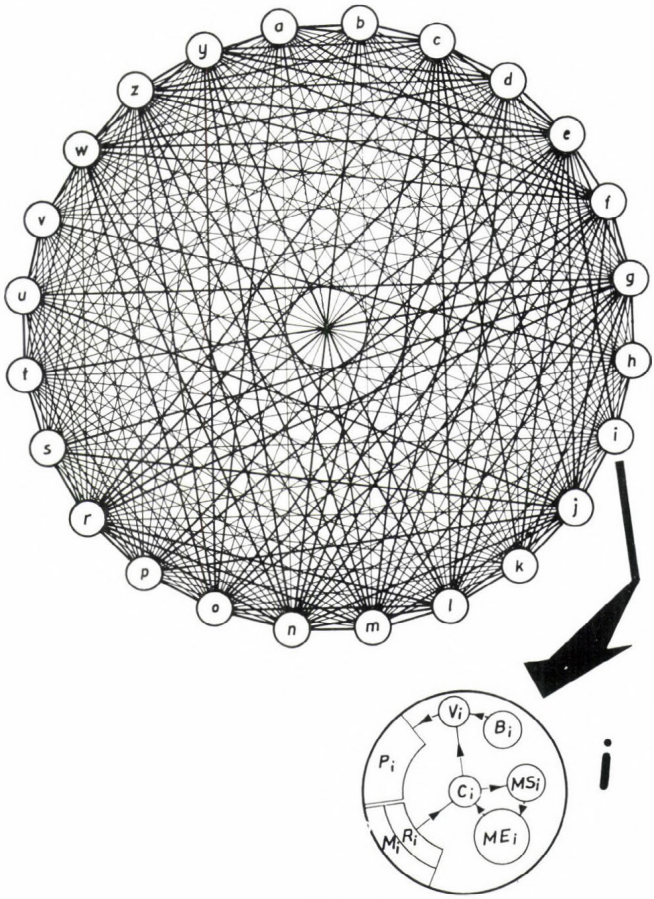

Fig. 1.

THE FUNCTIONING OF THE PIPE UNITS

If the organ is made to function, i.e. the pipes of the units give sounds as required by the piece of music to be played, the microphone M_i is impinged by sound waves, by an air signal which is transformed first into an electrical signal, then into simultaneous "1"s and "0"s, into the complex output of the resonator R_i.

The input signal of the comparator C_i coming from the resonator R_i is changing whenever any one of the pipe units starts or stops to give sound, while the one coming from the memory ME_i changes only after the sounding of its own pipe P_i and is waiting in the meantime.

If, and only if, the signal from R_i becomes equal to the signal from ME_i, the valve V_i is opened by the comparator C_i, after a certain delay to be explained later. Consequently a sound is generated by P_i. This, as a component of the air signal, impinges upon the microphones of all pipe units, among them upon the microphone of its own unit i too, and, as an electrical signal, changes the output of the respective resonant circuit into state "1"; the position of ME_i is also changed into the next one by the memory switch MS_i. Until the next event of "becoming equal", the pipe unit i remains silent; this is how all the pipe units which are the only kinds of constituents of the organ, function.

The event of "becoming equal" is the most important point in the functioning of the organ. For this reason it is practical to call it briefly coincidence.

THE DISTRIBUTED SETTING-UP OF THE ORGAN

While in the case of the organ, too, the functions of storing and reading out information, of timing, of controlling and supplying energy can be distinguished, there are no such material parts which would do these jobs in themselves.

If the organ is required to play some piece of music automatically, the proper information has to be stored in it beforehand. But as there is no such unit as a store, information has to be stored in a distributed form, if at all, in the totality of the memories $ME_i (i = a, b, c, \ldots, z, y)$ of the pipe units. To express that the parts stored belong together, the term" set of memory", *MES* will be used for them.

Again for the same reason, the reading-out of information has to be executed in the same distributed manner. This is the sum of the coincidences ensuing in the organ at different places and times. The point is that the action of the pipe units is determined by the pipe units themselves!

THE SET OF MEMORY (*MES*)

The elements of *MES* are the memories ME_i, as many in number as the kinds of the notes in the piece of music to be played. Again each ME_i has as many states as the number of notes characterizing the pitch of its P_i. As the coincidences result in sounds, in the simplest case of *MES*, every

state has to be proper to the sounding picture just preceding the sound due to occur. By a state of ME_i a permutation of "1"s and "0"s is meant (see the definition of ME_i).

For illustration, the first five bars of the Preludium and Fugue in C-major by J. S. Bach will be considered. Its conventional score is shown in Fig. 2. For the sake of convenience the technical version, which is similar to a punched paper tape (Fig. 3), will be used in the procedure resulting in the *MES* of the piece of music. Time is shown along the horizontal lines from left to right, and the different pitches along the vertical lines. The small letters labelling the lines are explained in Fig. 2. The black bars on the horizontal lines represent the notes; the longer a bar, the longer the time during which the respective pipe has to sound. On the horizontal axis the unit of time is equal to the time value of the note 1/16.

From Fig. 3, the number of the memories can be found out easily by counting down the horizontal lines: 24. The number of the states of e.g. the memory ME_e can be found out by counting the bars on horizontal line e: 11. And again, a state of ME_e belonging to, e.g. the 10th action of the unit e can be established by going back on the tape in time looking for any bars preceding the given bar on any of the lines '$f - o - w$'.

Timing, too, is a distributed function, so the relevant information should be stored in a distributed form in the memories ME_i. The exact beginning and the duration of action should be determined; it means that two time values belong to every state of memory. They can be found out from the tape, too. The duration is the length of the bars, which is two units of time at the 10th action of pipe unit e. The other information is the delay from the moment of coincidence to the beginning of action. On the tape the delay appears as the distance between the starting point of bar no. 10 and that of the preceding bar or bars. In this case it is one unit of time.

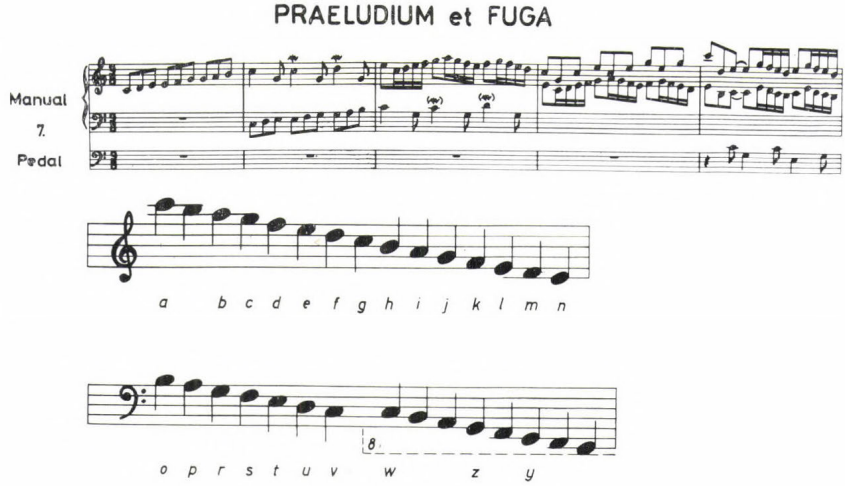

Fig. 2.

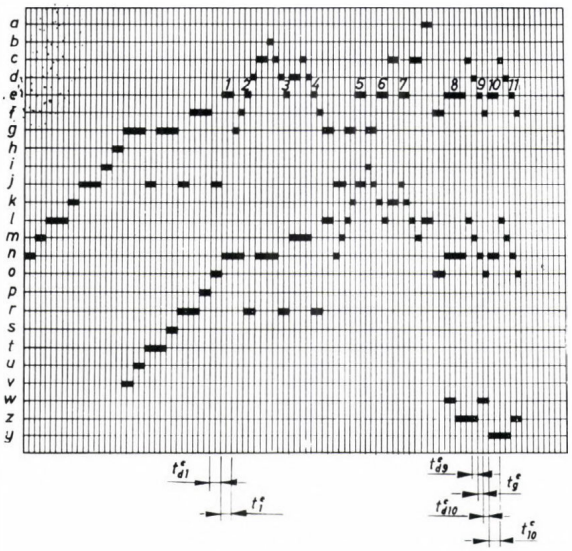

Fig. 3.

If this is done for all the bars, the procedure discussed can be considered as a coding of the information from the tape to the set of memory. Then the reading-out might be called automatic decoding. For proper functioning, the relation between the coding and the decoding has to be inverse. On the basis of the given coding procedure the inversity depends on the nature of the piece of music in question. If it is strictly logical the simplest possible coding (the one discussed) is satisfactory. If the piece of music chosen is not strictly logical, a more complicated coding is required, which takes into consideration a longer part of music backwards from the coincidences. More detailed discussion of the problem is unnecessary and beyond the scope of this paper.

THE FUNCTIONING OF THE ORGAN AS A WHOLE

The first problem is how to start the organ. Even the most trivial way, i.e. starting the organ by switching-on the power supply of every pipe unit, is worth mentioning. From the point of view of function, switching-on is distributed too, and, besides, this can be achieved by sounding the first sound (see version *b* below). One of the pipe units has to give sound first, so in the individual units the effect of switching-on is equivalent to the effect of the coincidence. The switching-on of the other pipe units except for the first one, on the other hand, is potential only, for they cannot give sound unless the proper coincidence occurs.

There are two other possibilities to start the organ:

(*a*) by silence,

(*b*) by providing the first sound of the piece of music from a foreign source of sound.

Naturally, in both cases the power supplies have to be switched on beforehand. In the first case, the memory of the pipe unit due to give the first sound has to be set into the "silent state", which is the permutation consisting of "0"s only. After all these conditions having been fulfilled, the organ begins to work at the first "silent state" of the common air medium.

No matter which way of start is chosen, the memories of every pipe unit have to be set into the states just preceding the first of their own actions before the organ is actually started.

In the explanation of the functioning, let the foreign (b) case of the start be chosen. According to the piece of music (supposed to be strictly logical for the sake of simplicity) some of the first states of memories found out from the tape (Fig. 3) are as follows. (The order from left to right and from top to bottom is proper to the course of music. Naturally, the outputs of all the memories have to be set into the state of '0', except for the ones in quotation marks whose states are "1".)

(1) ME_m : 'n' (waiting for the foreign pitch 'n')
(2) ME_l : 'm'(waiting for the pitch 'm')
(3) ME_k : 'l' (waiting for the pitch 'l')
(6) ME_h : 'i' (waiting for the pitch 'i')
(7) ME_g : 'h' (waiting for the pitch 'h')
(8) ME_v : 'h' (waiting for the pitch 'h' too!!)

When the foreign pitch is generated (say, by a common organ) the music starts to play immediately. Though all the units are under the influence of the sound waves, coincidence can be brought about in unit m only, resulting in the action of its own pipe P_m, which switches its memory into the next state. (This sound is the second of the piece of music: the first was the pitch 'n' generated by the foreign source!) Next, though all the units are under the influence of this sound again, owing to the proper setting of ME_l, a coincidence can come about only in unit l, resulting in P_l giving sound, which switches its memory into the next state, etc. In this way, the functioning of the organ is a type of "chain reaction". Events constituting the "chain reaction" could be any of the following four kinds:

(1) One sound due to one sound
(2) One sound due to more than one sound
(3) More than one sound due to one sound
(4) More than one sound due to more than one sound.

A peculiar property of the organ is that its functioning can be spoilt permanently by foreign pitches imitating sound pictures due to happen later though already being waited for by a memory (or memories) of a pipe unit(s). The explanation of this behaviour is that the sound generated before due time cannot be a constituent of the proper sound picture, for the memory of the proper pipe unit is already in the next state at the correct time.

For later interest a version of the organ which is playing cyclically for ever is described now. Let it be supposed that the first and last sound picture of a certain piece of music is the same. If the organ is intended to play it cyclically, then the states of memories have to change cyclically, too. Thus the last sound picture will find the memories still waiting for it, so that the playing can go on for ever.

THE ESSENTIAL GRAPH OF THE INTERRELATIONS

Through the common air medium, every unit is in relation with all the others, as shown by the lines between the pipe units in Fig. 1.

By definition, a relation will be called active if it is necessary for any of the coincidences and is passive otherwise. Relating to a set of memory the essential graph is the sum of the active relations. The essential graph of the piece of music is shown by the thicker lines in Fig. 1.

Since during the functioning proper to the set of memory actually stored, a relation can be active more than once and, besides, the order of their activation is lost in their sum, an essential graph can belong to more than just one set of memory.

In connection with the essential graph, the organ has two further properties worth mentioning. Firstly, it is a criterion of faultless functioning that the essential graph of the interrelations be contained by the actual interrelations. So if this criterion is not violated, the rests of the circuits are not required to be specific. Secondly, the functioning of the organ cannot be explained solely on the basis of the interrelations.

THE EQUIPOTENTIAL PROPERTY OF THE REDUNDANT ORGAN

Now a redundant version of the organ will be specified. It consists of, say, 100 of every kind of pipe units, which are evenly distributed in a 'slab' of space filled up with air. The dimensions of the space occupied by the pipe unit are small compared with the hearing distance of the units.

An interesting property is that the redundant organ can be divided at will into two parts without any change in the functioning of the parts, but for their sound volume, if the smaller part still consists of at least one complete set of the pipe units, i.e. of 24 different units. The procedure of division can be carried out by removing the two parts from each other placing them out of hearing distance or by putting one of them into a sound-proof chamber.

The procedure of division can be carried out at random with the same result, any part of the redundant organ consisting of more than one set of the pipe units is a functionally equipotential part compared with the whole or with any other parts.

By this property, some sort of similarity can be recognized between a holograph and a 'slab' of the redundant organ. And what is more, in a certain sense, the latter can be considered an active holograph.

THE GENERAL BIOLOGICAL MODEL (GEBIMO)

Below the properties of the General Biological Model (GEBIMO) will be discussed by pointing out the corresponding properties of the organ, which is a particular case of the former.

THE SET-UP OF GEBIMO

GEBIMO consists of two general parts intermingled with each other:

(1) An active part, i.e. a population of units each equipped with a separate power supply and able to influence the state (composition) of the passive part and to be influenced by it.
(2) A passive part, the medium through which the interdependence between the units and the relation of GEBIMO to other systems is realized.

In the organ the active part is the totality of the pipe units and the passive part is the common air medium. The organ is able to influence other systems by the common air medium, but itself is not allowed to be influenced by other systems if faultless functioning is to be maintained: therefore it is both an open and a closed system.

The direct interdependence of the member units or the relation of the units to other systems of the world depends on their sharing the passive part.

The more units share the passive part, the smaller the diameter of the graph picturing the interdependences. In the organ all the pipe units share the common air medium, accordingly the diameter of the graph picturing their interrelations is one (Fig. 1). That the passive part is shared by two units means that its state or composition (physical, chemical, etc.) can be under the influence of both and that also both units can be influenced by the common medium.

The units must be equipped with

(*a*) their own power supply;
(*b*) some sort (sorts) of device sensitive to the states (composition) of the passive part shared;
(*c*) some sort (sorts) of device capable to generate changes in the states (composition) of the passive part shared;
(*d*) some sort of a material configuration changing its state for the purposes of memory;
(*e*) some sort of a comparator device capable to compare the states (composition) of the passive part transmitted by the sensitive device and the state of memory.

The effect of device *c* depends on the result of the comparison executed by device *e*. Devices *a* to *e* are not necessarily separate material mechanisms, in the organ the set-up of the pipe units should be considered much more an example of the functioning than of the structure.

Generally, the comparator compares the state (composition) of the neighbouring passive part on the basis of more than one time function simultaneously. In other words, the comparison is dependent on space and time functions; figuratively, it depends on changing pictures of states and composition of the passive part (cf. the organ).

In the organ it is only the state of memory that changes. Generally, the content of memory is allowed to change, too. Compared with the organ playing perpetually mentioned above, a system with a set of memory changing in content, too, goes around a spiral instead of a circle. The cause of the content changing is the influence of both the functioning of the system itself and other systems related with it through the common passive part.

The GEBIMO is a distributed system, which will be proved below.

In a man-made system a certain job is done by a certain part of the mechanism and nowhere else. This way of organization is called concentrated, in contrast to the distributed organization. Or conversely, in a distributed system, it is usual to speak about a certain job, although there is not any particular part of the system doing just that job alone.

The two extremes of distributedness are

(1) Different functions being distributed in certain parts of the system or over the whole;

(2) A certain function being distributed in a part of the system. The organ is an example of the first extreme: the functions distributed are the reading-out of information, the storing of information, timing, power supply, etc.

Distributed systems are superior to the concentrated ones, for the latter are contained by the former as their special cases. Distributed systems belonging to the first type have no block diagrams in the usual sense.

CHARACTERISTICS OF THE FUNCTIONING OF GEBIMO

The most important steps in the functioning of this system are the coincidences occurring in the constituting units. The units become active due to neither the states (composition) of the passive part nor the states of their own memories, but to the coincidence of the two. In this way the units have great freedom to decide on doing anything. Approaching the same question from the other direction, it can be stated that the structure of the passive part is not a basis for the explanation of the functioning of GEBIMO.

The term 'structure' has several meanings. One of them is in connection with the set-up of a mechanism. The structure can be specific to the functioning or can be unspecific. For example, the structural nature of a net can be expressed by topological terms which, in turn, are not in direct connection with the function at all.

In the organ the structure of the interrelations realized by the common air medium is entirely unspecific to the functioning, and, what is more, the functioning is not explainable by the essential graph either (see p. 363).

Important characteristics of the functioning are its being parallel and simultaneous. These two words indicate that this type of system differs essentially from the serial (concentrated) systems where functioning occurs in successive steps which are in cause and effect related to each other (the von Neumann's type computer is a good example of the serial functioning).

The organized functioning is provided for by the memory set of GEBIMO. The elements of the set are the memories of the constituting units. The memory set of GEBIMO is allowed to change both in state and in content. The changing in content is in connection with learning properties (see p. 368).

THE APPLICATION OF GEBIMO TO THE CENTRAL NERVOUS SYSTEM (CNS)

The application will be called Distributedly Organized Memory Model Without Thermal Noise, DOMYNO.

In accordance with the cell theory there has to be a material component of the brain and, generally of the multicellular organisms, apart from the cells. This material component will be called extracellular material (EXMAT), irrespective of other designations in the different tissues.

The neurones and other cells of the CNS will play the role of the active part and the EXMAT will play the role of the passive part of the GEBIMO in the DOMYNO. Consequently, the cells are supposed to be able to change the molecular state and/or composition of EXMAT and to detect any such changes caused by themselves or other cells.

Our aim is not to give a full discussion of the problems involved but only to start a train of thought. Within the scope of a short paper we cannot enter into a detailed re-explanation of all of the knowledge gathered on the brain. With the organ and with GEBIMO we only wish to present a new approach to old problems and to furnish a few examples of application.

INTERCELLULAR COMMUNICATION IN DOMYNO

Starting out from what has been said about GEBIMO, EXMAT will be thought of as the medium of the interrelations between the cells (cf. the common air medium of the organ). After having excluded the possibility of any remote effect, actually the molecular composition (state) of the material surrounding the cells is the only factor on which cell action can depend.

The molecular state (composition) of EXMAT in the close vicinity of the cells will be called their molecular picture (MOPI) and will be regarded as the input signal of the cells in DOMYNO.

According to the common situation, it can be stated that EXMAT is changed by secretion from the cells, if the transmitter material of synapses is thought to be secreted too (but why not?). At half-way to the glandular (that is the classical) secretion, the neuronal secretion can be found.

In fact, perhaps with the only exception of the so-called electrical synapses, there is no "dry" communication between the cells. But even in this exceptional case, there is no difficulty of transmission: in this case the molecular pictures of the two cells are considered to have molecular parts in common—in contrast to the usual case when the material of the molecular picture belongs to neither of the cells.

The molecules which are the prospective constituents of the molecular pictures can reach the neighbourhood of the cells directly by being secreted there (for example into the synaptic cleft), or transported via the blood, lymphatic, etc. circulation. But in both cases, they might be driven by thermal agitation: for the change of MOPI of any cell, diffusion can be postulated. (It is self-explanatory in the case of the subsynaptic cleft, and acceptable in the capillaries and the larger vessels too, because of the laminar flow.) For this reason the communication system realized by EXMAT is free from thermal noise. The thermal agitation is the cause of the changes in the MOPI regularly and it is not a disturbance of communication. Again, in view of the input signal hypothesis, the interdependence between the cells and their MOPI is imagined to be similar to chemical reactions, and it is well known that the thermal motion of the molecules is the basic condition of these reactions.

COMPARISON OF THE INTERCELLULAR COMMUNICATIONS IN THE ELECTRICAL MODELS AND IN DOMYNO

In the electrically dominated models, attention is concentrated on neurones which have measurable electrical phenomena accompanying their actions. These cells are equipped with two sorts of elongated parts, the dendrites and the axons. In view of their electrical properties, these elongations can ideally be regarded as the wires of the neuronal nets with junctions at their synapses. Due to McCulloch's and Pitt's (1943) suppositions, the neurones in the nets are viewed as simple and inferior components of the nets themselves. Consequently, its behaviour is expected to be explainable on the basis of their own circuitries or wire-ins. The guiding principles of the explanations, in many instances, have been taken from the organization of the von Naumann's type computers (1961), or in other cases, as working hypotheses, from organizations of model systems of their own rights (Perceptron by F. Rosenblatt, 1962).

The topology of the graph of interrelations of DOMYNO is the same as the topology of the neuronal net of the electrical models. For it is a matter of convention only to speak about EXMAT being shared by two neurones, or about their synaptical junction. The main issue is that the two neurones are in interrelation with each other. The elongations of the neurones are factual things but to think of them as wires is dictated by the electrical models only. In the DOMYNO hypothesis the elongated shape of the neurones has been taken into consideration when the neurones are regarded as shareing a common extracellular material.

Now the question can immediately be asked: What is the justification for this somehow complicated determination of the same thing?

Naturally, there is no justification if only the interneural connections, that is the topology of the neuronal net, are considered. But if the problem of the "dry" and "wet" communication (distinction of the past) has to be tackled on the basis of *one* model, DOMYNO will be a suitable means. While an electrical phenomenon measured is quite incompatible with a hormone molecule being the messenger (Katz, 1966), the idea of MOPI as the input signal of the cells (not only that of the neurones!) does not make any distinction depending on the origin of the molecules in it. Some parts of the MOPI can be changed by the neuronal secretion, while other parts by other sorts of cells, and *vice versa*.

According to the idea of molecular picture, an action potential of an axon is not a signal of DOMYNO but only the sign of the prospective change in the proper part of MOPI. Though MOPI as such is not directly measurable, the idea can prove to be useful in the explanation of certain phenomena, and at the same time, indirect measurement is at hand by properly designed electrical, chemical, etc. instrumentation of the experiments.

SHORT-TERM MEMORY IN THE INTERCELLULAR COMMUNICATION OF DOMYNO

For physicists the term 'memory' means storage of information. Now, as far as the storage is concerned only, any material process can be its basis which has some after-effect influencing the next states of functioning of the system of which the process in question is a part. The signal which stores information can be static or dynamic in nature, i.e. constant or changing in time.

Indisputably, secretion has after-effects. Molecules secreted into some of the EXMAT will stay there for a time the length of which depends on the quantity of the molecules secreted and on the speed of elimination. The after-effect of the transmitter material secreted into a synaptic cleft is shorter in duration than that of some amount of hormone secreted by a gland into the blood stream.

In the electrically dominated neuronal models, the action potentials are to be imagined to be instantaneous phenomena. For this reason only reverberating circuits, closed paths of neurones could be held responsible for short-term memory. In DOMYNO, the phenomenon of secretion, with MOPI as the input signal, seems to be a good explanation of the mechanisms of some sort(s) of short-term memories.

THE PROBLEM OF TEMPORARY CONNECTIONS

The models which concentrate on the circuitry of the neuronal net try to explain different functions and also learning by supposing temporary connections at the synapses. They have no basic difficulties, for the connec-

tivity of the brain is so rich that to think that every neurone is in connection with all others is a better approximation than to think that only specific connections exist (specific to certain kind of functioning). Actually the diameter of the brain between peripheral points is about 10, which is the approximate number of neurones in the shortest path across the brain. In other words this means that there are a variety of paths between any two peripherial or other points of the brain.

But difficulties arise in the reasoning of why just only certain paths become activated and others do not and how new connections, that is new behaviours, come into being.

The coincidences, as the causes of action in the neurones and other cells, offer an answer to both problems. Accordingly, the circuitry is not specific to any function or behaviour, but to the sum of all past and future functions. In other words the neuronal net is the sum of all the interrelations necessary to the activity patterns which have been established and which are possible to be established by learning at all.

Now a certain activity pattern of the totality of the cells comes into being by coincidences according to the state of the set of memory distributed in the totality of the constituting cells and according to their preceding activity pattern. So learning is supposed to involve not the change of the circuitry but the content of the set of memory.

Naturally in the material theories of learning, that is information storage and retrieval, the problem is not the mere existence of the material changes, but the nature of the material traces of memory, the nature of the write-in and the read-out mechanisms.

As far as DOMYNO is concerned, the mechanism of learning is imagined to be distributed, that is the information of *an* experience for example is imagined to be stored by material changes occurring in the *entire*, or at least in a *great* part, of the set of memory distributedly, and the retrieval, or in other words the read-out, is supposed to take place by coincidences distributedly, too.

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CONCLUDING REMARKS

by the Chairman

K. Lissák

As interest had been turned to the function of the brain, the doctrine of the separate localization of mental functions gradually took form. In the nineteenth century Flechsig defined the association areas as distinct from sensory and motor representations in the cortex. According to Henschen's extreme theory, single ideas or memories would be linked to separate cells. Bechterew and Pavlov regarded higher nervous function as associations and as chains of conditioned reflexes. Pavlov came forward with the idea of analysers and refuted any attempt at the compartmentalization of brain functions. In recent years occurrences of various sorts have been reported in the nervous system during learning, e.g. changes in electrical activity or chemical composition; however, much of these changes may be irrelevant in respect to memory functions.

It seems to be a well-founded statement supported by ample evidence that the memory system of the central nervous system consists of a number of relatively simple modules and each records a different degree of consequences aroused by excitatory and inhibitory states. Semon (1904), who introduced the word "engram", did not define memory or mnemic faculty as a specific property of cell functions, he suggested the memory being a "formation of new connections". Studies on the mechanism of elementary learning processes have revealed that memory traces, at least of simple sensory-motor associations, are not laid down and stored within a particular association area of the brain and not within a restricted part of the brain supposedly concerned with each sensory modality. Lashley wrote in his famous paper "In Search of the Engram": 'memory traces are at first formed in the cerebral cortex, they are finally reduced and transferred by long practice to subcortical level'. This assumption is in accordance with the concept of Pavlov about the nature of conditioned reflexes, and has been confirmed even in recent studies.

There is no doubt that billions of neurones in the central nervous system are organized into a large number of systems and each system holds the traces of a number of memories. Lashley already emphasized that the neurones of each system may participate in different functions on the basis of different spatial and temporal distribution of impulse patterns. He called this feature of nervous function as "trace system". He also proposed that if a system in tonic activity dominates the brain field, this limits the organization of other systems. This assumption appears to be very similar to the theory of Uhktomsky of the dominant action. Both old and recent concepts of the nature of the memory function of the brain involve elements of the tremendous plasticity of nervous processes and avoid the separate learning capability of single cells.

Modern theories of memory function, based either on changes in ribonucleic acid composition of single cells after a number of impulses or on

neurophysiological and behavioural studies, must face the facts that, from the behavioural point of view, we must differentiate "reading-in" and "reading-out" in the memory functions, and most of the phenomena registered by students of learning processes are connected to such events of brain memorization. Interruption of brain circuits frequently interferes with the "reading-in" or -"out" capacity of the memory system and conclusions formed on this basis might be misleading.

Changes in the basic chemical composition of the neurones which occur during learning can be regarded as memory constituents and such discoveries contributed to the understanding of cellular processes underlying memory functions. On the other hand, biochemical changes of the cells seem to be unrelated to a particular memory but are involved in several kinds of memory function.

I feel that much of the evidence presented at this Symposium provided further contribution to our knowledge about mechanisms underlying elementary learning and memory function. Also, this Symposium proved that the methodology and technique of modern molecular neurobiology, including the pharmacological approach, are indispensable in our efforts to understand the interrelationships of brain and behaviour.

The word memory has been mentioned many times during this four-day conference; we have used it from the psychological and behavioural points of view, we have considered it as part of an objective system like a computer, and we have correlated it with electrical signs occurring during learning. I feel that all the speakers of this meeting, approaching the same problems from different points of view, contributed quite a lot to our understanding of the nature of the engram.