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NUCLEAR RNA CHANGES OF NERVE CELLS DURING A LEARNING EXPERIMENT IN RATS

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Communicated by A. E. Mirsky, June 1, 1962

As the nerve cells are rich in ribonucleic acid (RNA), the suggestion has been made that the RNA of the neurons may be linked with the capacity of the central nervous system to store information.

The RNA and protein synthesis and the enzymic activities of neurons have been found to be linked with neural function (review, see Hydén[1]). From these results the senior present author has proposed a thought mechanism model for intraneuronal molecular storage of information[1,2] which has been based on the production of specific proteins in each neuron following changes of the RNA base composition after adequate stimulation. The altered RNA base composition, which may persist for a long time, might arise through electrical patterns associated with sensory and motor activity.

Results are presented on the altered base ratio composition of nuclear RNA and cytoplasmic RNA from neurons of rats, subjected to a "trial-and-error" type of learning experiment during which a pattern of sensory and motor abilities was established in the rats. Concomitantly, the adenine/uracil ratio of the nuclear RNA increased significantly, and an increase of the total amount of the nerve cell RNA occurred. Requisite control experiments were carried out which excluded the possibility that the chemical changes observed were due to demands on the neural function *per se*.

The present paper, the first in a series, presents the results obtained from vestibular Deiters' nerve cells. These cells were chosen because they were directly involved in this learning performance, and because we wanted to use neurons from phylogenetically old parts of the brain in these first experiments and to avoid complicated cortical areas.

Material and Experimental Setup.—White rats of the Sprague-Dawley strain weighing 150–200 gm were used. A wooden cage (95 × 85 × 45 cm) with one side wall of glass was used for the experiments. At a height of 75 cm on one of the end walls, a small platform was arranged with a feeding cup. A 90-cm long steel wire, 1.5 mm in diameter, was strung between the floor and the platform. The experimental rats, kept on a minimal amount of food but with free access to water, were individually placed in the cage for 45 minutes daily. The only way for the animals to satisfy their food hunger was to learn how to balance up to the platform, an exceedingly difficult

task of the trial-and-error-type. These young rats took an average of four days to learn how to balance on the wire the full distance to the platform. On gaining the platform, a rat takes a small piece of food in his mouth and then walks down the wire in order to eat the food on the floor of the cage. On the first day, when they have learnt the performance, the rats usually balanced the whole way to the platform 3 to 5 times during the 45 minutes allotted, on the second day around 10 times, and on the third and fourth day around 20 times. The results presented in this paper are based on rats of the fourth day. In the tables these rats are designated *"learning."*

The control experiments were chosen to exclude the possibility that eventual changes observed were due mainly to the increased neural activity of the vestibular pathway of the rats. Two types of control material were used. As *controls*, rats of the same litter were used. These were kept in single cages, with free access to food and water (although on a low caloric diet). The cages were large enough to allow free movements on the floor.

As *functional controls*, rats of the same litter, stimulated in the following way, were used. The same part of the nervous system as in the learning experiments was stimulated with the difference that *the animal was passive* during the stimulation. The vestibular nerve was stimulated by rotating the animal through 120° horizontally and 30° vertically with 30 turns per minute and for 25 minutes twice daily for four days. The animal was taught to enter a small, tight-fitting box on the rotating disk with the head away from the center. After a few tests with the box they showed no avoidance reflexes during the course of the experiment. These animals will be designated *"vestibular stimulation."* The experimental setup has been used frequently in previous studies in this laboratory.[3]

Sampling of nerve cells: The animals were killed and the brain stem taken out as previously described.[3] The section through the lateral vestibular nucleus was always placed at the cranial border of the tubercula acustica. The big Deiters' nerve cells sampled by freehand dissection[4] from these sections project onto the cervical part of the spinal cord.[5] As Pompeiano and Brodal[5] have pointed out, the lateral vestibular nucleus is not only quantitatively more important but also more specific than the rest of the vestibular complex with regard to vestibular influence on the spinal cord.

The isolated nerve cells were treated with ice-cold 1 *N* perchloric acid for 5 minutes, absolute ethanol for 5 minutes, and chloroform for 5 minutes. In those cases where small pieces of the brain stem nucleus were fixed in Carnoy's solution, embedded in paraffine and sectioned, the de Fonbrune micromanipulator was used for isolating single cells. The amount of RNA per nerve cell was determined by extracting the RNA with a buffered solution of ribonuclease.[6] The RNA in the cell extracts was determined in micro-drops with a photographic-photometric method using radiation at 2,570 Å. Edström *et al.* have demonstrated that these treatments are effective preparative methods for determining the RNA base composition and content of nerve cells.[7] The method allows the determination of the amount of RNA down to 20 $\mu\mu$g with a variation coefficient of $\pm 5\%$. In all, 45 rats were used for a total of 345 analyses.

In the vestibular nucleus of rabbits and rats there are two types of big nerve cells, the Deiters' cells proper, and the lateral nerve cells which are slightly smaller.[3, 5] The Deiters' cells in rabbits have on an average 1550 $\pm$ 18 $\mu\mu$g of RNA per cell. The lateral nerve cells have 870 $\pm$ 17 $\mu\mu$g of RNA per cell.[1] Similar differences exist between these two types of cells in rats. Great care was taken that RNA values obtained on lateral nerve cells sampled by mistake did not cause an error in the final result.

Sampling and analyses of nerve cell nuclei: In order to precipitate the nuclear RNA and at the same time allow the nerve cell nuclei to be taken out by micro-dissection, a modified procedure of Harris[8] was used. The isolated nerve cells placed on a glass slide were briefly rinsed in cold isotonic NaCl solution, then treated with cold phenol-saturated water for 15 minutes, followed by cold abs. ethanol for 10 minutes and then covered by paraffine oil (*pro analysi*). The effect of this treatment is to cause the nuclei to contract slightly which is hardly noticeable at a magnification of 600×. The nucleus from each nerve cell was then removed with the aid of a de Fonbrune micromanipulator, using a glass instrument (Fig. 1). Twenty-five nuclei were used for each RNA analysis of base ratios.

Phenol was used solely for precipitation of RNA, and to check the efficiency of the method the following experiments were carried out. Deiters' nerve cells from a rabbit were isolated from the left and from the right side of the vestibular nucleus. The nerve cells from one side were pre-

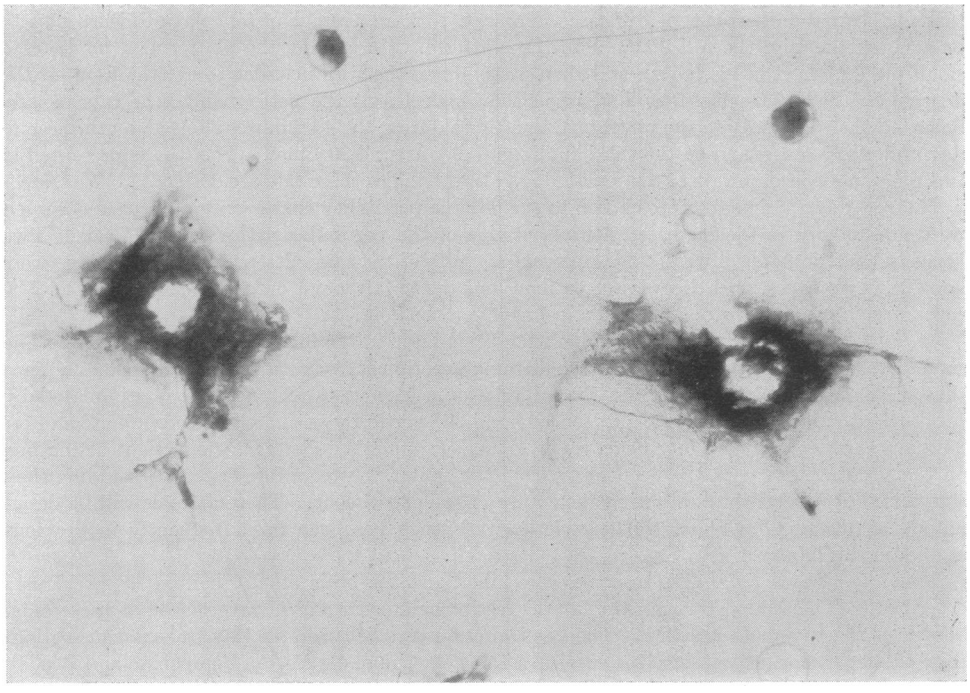

FIG. 1.—Two isolated Deiters' nerve cells cleaned from glia and treated with phenol-water. The nucleus from each cell has been taken out and the nuclei are seen situated above the nerve cells in the center of which is seen the corresponding hole. Photographed at 2,570 Å. Magnification 400 ×.

cipitated by cold 1 *N* perchloric acid, followed by cold absolute ethanol and chloroform, and those from the other side by cold phenol-saturated water followed by cold absolute ethanol. RNA of each cell was extracted with a buffered ribonuclease solution and the amount of RNA per cell determined as described above. With the perchloric acid precipitation the analysis gave 1560 ± 31 μμg/cell, and after phenol treatment 1565 ± 44 μμg/cell was obtained. The phenol procedure thus precipitates all RNA in nerve cells.

For purine-pyrimidine analysis (Edström[7, 9]) the pooled RNA, approximately 700 μμg, extracts from 25 nerve cell nuclei were used. The purines and pyrimidines were calculated as molar proportions in percentages of the sum. For 500 μμg of RNA the average values of the determinations showed a coefficient of variation of ±5%.[10]

Results.—Quantitative RNA changes in the learning experiment: The amount of RNA per nerve cell increased significantly during the course of the trial-and-error experiment from 683 μμg to 751 μμg of RNA per isolated nerve cell (Table 1).

Base ratio composition of RNA: (a) *Control nerve cells:* The nuclear RNA constituted approximately 4% of the cell's total RNA. The composition of the

TABLE 1

THE AMOUNT OF RNA/DEITERS' NERVE CELL OF RATS ON THE FOURTH DAY OF THE LEARNING EXPERIMENT

	Controls	V	Learning	V	P
RNA in μμg/cell	683 ± 17	7	751 ± 10	3	0.01
Number of nerve cells and analyses	97		69		
Number of animals	7		4		

P = probability after t-test.
V = variation coefficient, (S × 100)/mean.

nuclear RNA and the cytoplasmic RNA of the nerve cells from the control rats is shown in Table 2. The variation coefficient was satisfactory. The (A + G)/(C + U) ratio is 1.18; (A + U)/(G + C) = 0.64.

The base ratios of the nuclear RNA differ from the cytoplasmic RNA in having higher adenine and uracil values. There is a certain trend of complementarity, but the base ratio composition of the nuclear RNA does not agree with that of the DNA of rats.[17] The latter has the base ratios: A 28.6, G 21.4, C 20.4, T 28.4 with (A + T)/(G + C) = 1.33. This result will be commented on in the discussion. It is also seen that cytoplasmic RNA contains more guanine than cytosine whereas the reverse is the case with the nuclear RNA.[10] The same finding was made in Deiters' nerve cells of the rabbit.

TABLE 2

COMPOSITION OF NUCLEAR RNA AND CYTOPLASMIC RNA OF DEITERS' NERVE CELLS FROM CONTROL RATS

(Base ratios expressed as molar proportions in per cent of the sum)

	Whole cells		Nuclei	
	Mean	*V*	Mean	*V*
Adenine	20.5 ± 0.54	5.5	21.4 ± 0.44	5.4
Guanine	33.7 ± 0.33	2.2	26.2 ± 0.45	4.5
Cytosine	27.4 ± 0.34	3.0	31.9 ± 0.77	6.4
Uracil	18.4 ± 0.26	3.1	20.5 ± 1.01	13.0
Number	200		285	
Number of analyses	50		10	
Number of animals	5		7	

V = variation coefficient, (S × 100)/mean.

(b) *Cytoplasmic RNA in learning experiment:* From the results listed in Table 3 there is evident that no significant changes in the base ratios of the cytoplasmic RNA in the nerve cells could be observed during learning with the technique employed. The cytoplasmic RNA (A + G)/(C + U) ratios remained the same (1.18 and 1.22 respectively) and so did (A + U)/(G + C) ratios (0.64).

TABLE 3

COMPOSITION OF THE CYTOPLASMIC RNA OF DEITERS' NERVE CELLS FROM CONTROL RATS AND DURING LEARNING

	Controls		Learning	
	Mean	*V*	Mean	*V*
Adenine	20.5 ± 0.54	5.5	20.9 ± 0.19	2.6
Guanine	33.7 ± 0.33	2.2	34.0 ± 0.32	2.6
Cytosine	27.4 ± 0.34	3.0	26.8 ± 0.21	2.2
Uracil	18.4 ± 0.26	3.1	18.3 ± 0.13	2.1
Number of analyses	50		90	
Number of animals	5		8	

(c) *Nuclear RNA in learning experiment:* The results shown in Table 4 demonstrate that a *significant change* occurred in the base ratios of the nuclear RNA in the learning experiment, with the adenine increasing, and the uracil decreasing. The ratio of nuclear adenine/uracil changed from 1.06 ± 0.08 in the controls to 1.35 ± 0.10 in the learning animals with a P-value of 0.01. The nuclear RNA (A + G)/(C + U) ratio changed from 0.91 in the controls to 1.03 in learning. The nuclear (A + U)/(G + C) ratio remained at 0.72.

(d) *Functional controls with vestibular stimulation:* The neuronal amount of

TABLE 4

COMPOSITION OF THE NUCLEAR RNA OF DEITERS' NERVE CELLS FROM CONTROL RATS AND DURING LEARNING

(Amount of RNA per nucleus: 30 μμg)

	Controls Mean	Learning Mean	Learning V	P
Adenine	21.4	24.1 ± 0.39	4.3	0.001
Guanine	26.2	26.7 ± 0.87	8.6	
Cytosine	31.9	31.0 ± 0.95	8.1	
Uracil	20.5	18.2 ± 1.11	16.2	0.05
Number:				
of nuclei	285	243		
of analyses	10	8		
of animals	7	7		

RNA increased significantly, from 683 to 722 μμg per nerve cell, as a result of the rotatory vestibular stimulation.

The amount of RNA per nerve cell had thus increased as a result of stimulation. In this respect the nerve cells from the vestibular lateral nucleus did not differ from the corresponding cells of rabbit in previous studies in this laboratory.[2, 3, 11]

The base ratios of the nuclear RNA were next studied in these Deiters' nerve cells. The analyses of these functional controls showed no significant changes in the nuclear RNA base ratios (Table 5) as a result of vestibular stimulation during which the rats were passive.

TABLE 5

COMPOSITION OF THE NUCLEAR RNA OF DEITERS' NERVE CELLS FROM RATS SUBJECTED TO VESTIBULAR STIMULATION FOR 25 MIN/DAY AND FOR FOUR DAYS

	Controls Mean	Vestibular Stimulation Mean	Vestibular Stimulation V
Adenine	21.4	21.3 ± 0.69	6.5
Guanine	26.2	25.7 ± 0.46	3.6
Cytosine	31.9	31.3 ± 0.88	5.6
Uracil	20.5	21.7 ± 0.45	4.2
Number:			
of nuclei	285	173	
of analyses	10	6	
of animals	7	5	

Discussion.—The data presented thus link significant changes in the base ratio composition and production of nuclear RNA by neurons to the establishment of a sensory and motor pattern in rats during a learning experiment. The functional control experiment excludes, according to our view, the possibility that the qualitative chemical changes were caused by the unspecific stimulating factor on the vestibular pathway introduced by the demands of increased but "passive" activity.

The fact that the learning experiment is a more physiological stimulation than the vestibular experiment which is also stronger as stimulus on the vestibular apparatus than the learning experiment, supports the conclusion that the observed nuclear RNA changes are specific.

Nature of the nuclear RNA studied: The adenine and uracil changes in the nuclear RNA during learning are interesting considering recent characterization of the "messenger" RNA in *E. coli*.[12] According to current theory, genetic information in DNA is expressed via transcription into RNA messengers which in turn act as templates for protein synthesis (review, Brenner[18]). In our experiments no correspondence was found between the base ratios of the nuclear RNA and DNA

in the rat nerve cells. In the control rats the nuclear RNA showed a complementariness with respect to adenine and uracil. On the other hand, the guanine and cytosine values were higher, and their sum did not agree with that of DNA. The Deiters' nerve cells have a big nucleous rich in RNA,[13] and since the base composition of nucleolar RNA seems to agree with that of cytoplasmic RNA,[7, 14] it could be surmised that the nucleolar RNA should mask a chromosomal RNA if the latter was a copy of DNA; however, microchemical studies by Edström and Beerman[15] have shown that chromosomal RNA in Chironomus from Balbiani rings did not have a composition similar to that of DNA, nor had it a base symmetry. It is interesting that RNA extracted from different segments of the same chromosome differed in base ratios, as did RNA from different chromosomes. The nucleolar RNA in Deiters' nerve cells seems to comprise 25 per cent of the total nuclear RNA.[1] Therefore, nucleolar RNA can hardly be supposed to mask the base ratio composition of the rest of the nuclear RNA which is composed of chromosomal and nucleoplasmic RNA. The nuclear RNA analyzed in this study can therefore be assumed to be comprised primarily of chromosomal products.

Interpretation of the nuclear RNA changes observed: During learning, the adenine/uracil ratio of the nuclear RNA increased significantly. This indicates that a synthesis of fraction(s) of nuclear RNA with highly specific base ratios occurs during learning.

This newly synthesized RNA when transferred to the cytoplasm (containing 700 $\mu\mu$g of RNA) could only be detected by the present method if it were increasingly incorporated into the ribosomal RNA. The great number of analyses of cytoplasmic nerve cell RNA failed, however, to show significant base ratio changes in the learning experiments. The failure to detect an altered base ratio in the cytoplasmic RNA does not exclude the possibility that the specific nuclear RNA produced during learning is influencing or incorporated in small amounts into the ribosomal RNA. On the contrary, it brings to mind the characteristics of "messenger" RNA in bacterial systems.

In general terms the nuclear RNA changes presented in this study show that the neurons from an experienced animal are not the same biochemically as the neurons from an inexperienced one. A pertinent problem is the length of the period during which the nuclear RNA changes are observable. Preliminary data have shown than an increased adenine/uracil ratio persisted at least for 48 hours following the ending of the learning experiment (to be published in a subsequent study of the role of the nerve cells in the reticular formation during learning). This may mean that once the learning is accomplished and established, the production of specific nuclear RNA still occurs but presumably on a smaller scale. A permanent production or persistent change in the nuclear and cytoplasmic RNA of the nerve cell may persist at a molecular level, premanently affecting the protein synthesis in the cytoplasm. Further analyses of fractions of nuclear RNA of neurons may clear up this point. Such a mechanism for persistent storage of information in the nerve cell cytoplasm would require the existence of a self-perpetuating information transfer mechanism in protein synthesis. As an analogy it may be pointed out that indication for such a system has been found in genetic studies.[19] The presence of a Y chromosome segment in the oocytes of females of *Drosophila melanogaster*, resulted in a structural modification of a protein in their progeny. The modifica-

tion occurred even when the latter lacked the Y chromosome and was independent of the source of cytoplasm transmitted through the egg.

The importance of brain RNA in learning is also supported by experimental results obtained on planarians, the heads and tails of which regenerated in ribonuclease solution.[20] The intracisternal injection and incorporation of 8-azaguanine in brain RNA of rats has furthermore been shown to impair maze learning in rats significantly.[21]

A storage mechanism of information in the neurons, based on the data presented in this study, must be anchored in the genetic mechanism of cells.[2] The learning capacity would therefore depend on the potentialities of the species, with different chromosomes having a capacity to produce their own type of RNA—within the potentialities of the DNA of the species.

The specific regions of the chromosomes in the neurons involved during the learning and establishment of a performance may be momentarily more active. We would like to suggest that the observed changes in the base ratios in the nuclei of neurons during learning is caused by specific regions of the chromosomes producing their specific RNA.

Summary.—Purine-pyrimidine analyses were carried out on nuclear RNA and cytoplasmic RNA of neurons from rats subjected to a learning experiment during which there was established a pattern of sensory and motor abilities. Concomitantly, the adenine/uracil ratio of the nuclear RNA increased significantly and there are also an increased amount of RNA per nerve cell. Control experiments excluded the possibility that the chemical changes observed in the nuclear RNA of the nerve cell were due to demands on the neural function *per se*. The nuclear RNA changes during learning were interpreted as an activation of regions on the chromosomes to produce nuclear (chromosomal) RNA with highly specific base ratios.

These studies have been supported by the United States Air Force under Grant No. AF 61-(052)-248 and monitored by the European Office, Office of Aerospace Research, and by The National Multiple Sclerosis Society, New York.

The skillful assistance of Miss Margareta Pettersson is gratefully acknowledged.

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*AN HYPOTHESIS CONCERNING RNA METABOLISM AND AGING**

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Communicated by Milislav Demerec, June 25, 1962

Marked differences exist in the pattern of H^3-cytidine incorporation into RNA in tissues of young and old mice.[1] The results reported here confirm and extend the observations to animals of age groups not previously studied (Fig. 1). In hepatic cell nuclei, cytidine uptake first decreases and then increases considerably with increasing age of the adult mice. The incorporation pattern of cytidine in nuclei of dorsal root ganglia is similar to that seen in liver; in nuclei of cerebellar Purkinje cells, there is an age-related decrease of the incorporation. Various other tissues show trends of one or the other type; complete details will be reported elsewhere. The finding of opposing trends excludes any simple explanation such as general changes in distribution or availability of the exogenous precursor.

The data on utilization of tracer for RNA synthesis can be compared with data on RNA content of the tissues in question. In the wild rat, the RNA content of liver from old animals was found to be 10 per cent higher than in younger adults,[2] and in laboratory rats, nuclei of hepatic cells from old animals contain almost twice as much RNA as those from younger adults.[3] The RNA content of rat ventricular muscle decreases markedly between 15 to 150 days of age and rises slightly in rate at age 800 days or older.[4] Cytospectrophotometric measurement of the RNA content of cerebellar Purkinje cell cytoplasm[5] indicates a progressive loss of RNA with increasing age of the donor animal. These data on RNA content provide a pattern which parallels that of cytidine incorporation. However, the tracer uptake increases more, or decreases less, with age than the RNA content, so that the ratio of tracer uptake to RNA content increases with age.

The decrease in tracer uptake from infancy to youth is expected, but the increase with age in some tissues is puzzling. The following hypothesis is proposed; it is based on the assumed connection between aging and the occurrence of irreversible changes in molecular structure (e.g., refs. 6, 7).

1. As organisms grow older, the sites for RNA synthesis sustain damage with continual use.
2. The damaged sites produce defective messenger RNA which produces enzymes not capable of normal function.
3. The consequent accumulation of substrate molecules results in de-repression