# *CHANGES IN RNA CONTENT AND BASE COMPOSITION IN CORTICAL NEURONS OF RATS IN A LEARNING EXPERIMENT INVOLVING TRANSFER OF HANDEDNESS**

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In earlier attempts to study chemical correlates of learning in the brain, we have used rats which learned to balance on a thin steel wire.[1] Isolated, single neurons and their glia were analyzed from that part of the brain stem which was functionally involved in the establishment of this complex motor and sensory performance. The nuclear and cytoplasmic RNA of the big Deiters' neurons of the lateral vestibular nucleus were analyzed with respect to amount per cell and base ratio composition.

During learning, an increased synthesis of neuronal RNA was found, and the nuclear RNA showed a changed base composition with an increased adenine-to-uracil ratio. Similar, but not identical, glial RNA changes were found during learning.[2] Several control experiments were performed involving a stress experiment, vestibular stimulation, and also RNA analyses in a part of the brain outside of the vestibular nuclei. Only in the Deiters' neurons and glia were significant RNA base ratio changes found during learning, indicating a nuclear synthesis of small fraction(s) of RNA with highly specific base ratios.

In the present study, single cortical neurons were analyzed during transfer of handedness in rats. The advantage in this experiment is that the control material is obtained from the same brain.

*Experimental Setup and Material.*—White rats of the Sprague-Dawley strain weighing about 150 gm were used. The experimental setup consisted of a large wooden box with a glass cylinder (diameter 1 cm) placed 5 cm from the floor, into the opening of which the rat had to reach in order to grab small pieces of food. On the first day of the experiment the pieces of the food pellets (4 gm per day) were placed close to the opening, offering no difficulties for the animals to reach them with the preferred paw. Each rat was permitted to show by 25 reachings which hand it preferred. In this initial short test, 23 out of 25 reaches were demanded as a criterion for handedness. The conditions to be fulfilled in this respect have been studied earlier by Peterson[3] and Wentworth.[4] For our experiments, right-handed rats were forced to use the left hand to retrieve the daily ration of 4 gm of food per day (at 10 A.M. and 3 P.M., 25 min each time) from deep down in the glass cylinder. In order to force the hungry animals to transfer to the left hand, a wooden wall was arranged close to and parallel to the left side of the glass cylinder which was most effective in prohibiting the use of the right hand. In Figure 1 is plotted the number of reaches during the morning period of 25 min for 5 rats and for 5 days. On an average each rat performed 400–500 reaches during the 4-day period of the experiment. We also tested and confirmed the findings reported in the literature that once a shift in handedness had occurred by as few as 200 forced reaches, the animal proceeded with the "new" hand even when tested 9 months after the transfer.[4]

By using a stereotactic technique, Peterson and Devine[5] found indications for a critical area of the rat cortex controlling handedness which involved the cells in layers 5 and 6. These authors point out, however, that the critical area varies individually and probably encompasses a greater area of the cortex than the 0.5–1 $mm^3$ indicated by the results. We chose, however, neurons and glia of layers 5 and 6 from an area 2.7-mm lateral and 1.6-mm rostral from the bregma.[5]

These nerve cell bodies together with the first part of the dentrites contain a small amount of RNA, averaging 22 $\mu\mu g$. Therefore, about 10 nerve cells were used for each quantitative analysis.

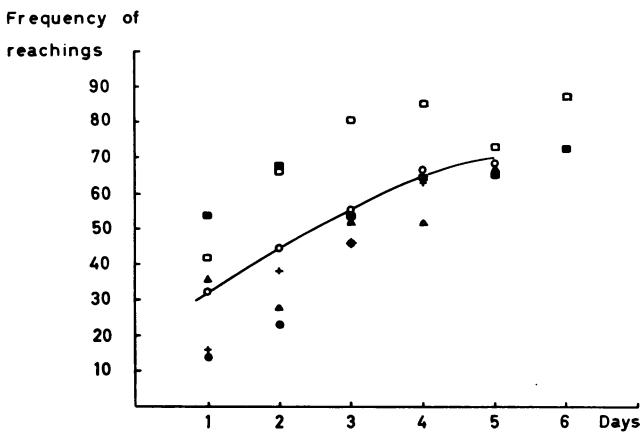


FIG. 1.—Number of reaches of six rats during the morning period of 25 min and for 5 days.

The cells and the glia surrounding the cell bodies were dissected from 30-$\mu$-thick sections using a de Fonbrune micromanipulator. The tissue had been fixed in Carnoy's solution, embedded, sectioned, deparaffinized with chloroform, and transferred to absolute ethanol, 70% ethanol, and 0.01 $N$ acetic acid. It has repeatedly been shown that Carnoy fixation and precipitation with cold perchloric acid preserves RNA and that RNAase treatment removes the RNA from nervous tissue prepared in such a way.[6] A review of the microtechnique for the quantitative determination of RNA from isolated cells and the electrophoretic separation of the RNA components has recently been published.[7] It deals with the technical procedure, application, and accuracy of these two methods. The random error in the determination of the RNA content in single ganglion cells was studied and an average $V$ value [$V$ = variation coefficient, ($S \times 100$)/mean] of 12.7% was found.[7] In calculating the random error, 4.1% of the variation was due to the method and 12.0% was the $V$ for the biological variation between single cells. In the application of the microelectrophoretic method, the average coefficient of variation of the analytical results was 5% for nerve cell RNA[8] and 7% for yeast RNA.[9]

The question has been raised whether this microelectrophoretic separation of hydrolyzed RNA in biological material gives the same result as do conventional macrochemical electrophoretic separations. One such case is offered by the analysis of nucleolar and ribosomal RNA of mature starfish oöcytes where the results were the same with both methods.[6, 10] In model experiments on purified samples of RNA the correspondence between macro- and microelectrophoresis is clear.[7]

The advantage of microelectrophoresis over macroelectrophoresis is the possibility of analyzing samples at the cellular level. This has proved to be a *sine qua non* for nervous tissue since its two cellular components, neurons and glia, differ in amount and composition of RNA. Even if as little as $10^{-6}$ gm of "gray matter" is taken, such a sample probably contains more glia than parts of neurons.

*Results.*—In all, 11 rats were carefully chosen for the microchemical studies. An average of 10 electrophoretic analyses were performed on each cortical sample listed in Table 2, equally divided on the right and left part of the cortex.

*Quantitative RNA changes in cortical neurons:* In all, 40 RNA determinations were carried out on neurons from the right side, here called *learning*, and 42 determinations from the left side, called *controls*. For each analysis 10 nerve cells were used.

TABLE 1

RNA CONTENT OF CORTICAL NEURONS IN TRANSFER OF RIGHT- TO LEFT-HANDEDNESS

| | Controls (left side) | Learning (right side) | $P$ |
|---|---|---|---|
| RNA $\mu\mu$g | 22 ± 2.3 | 27 ± 2.5 | 0.02 |

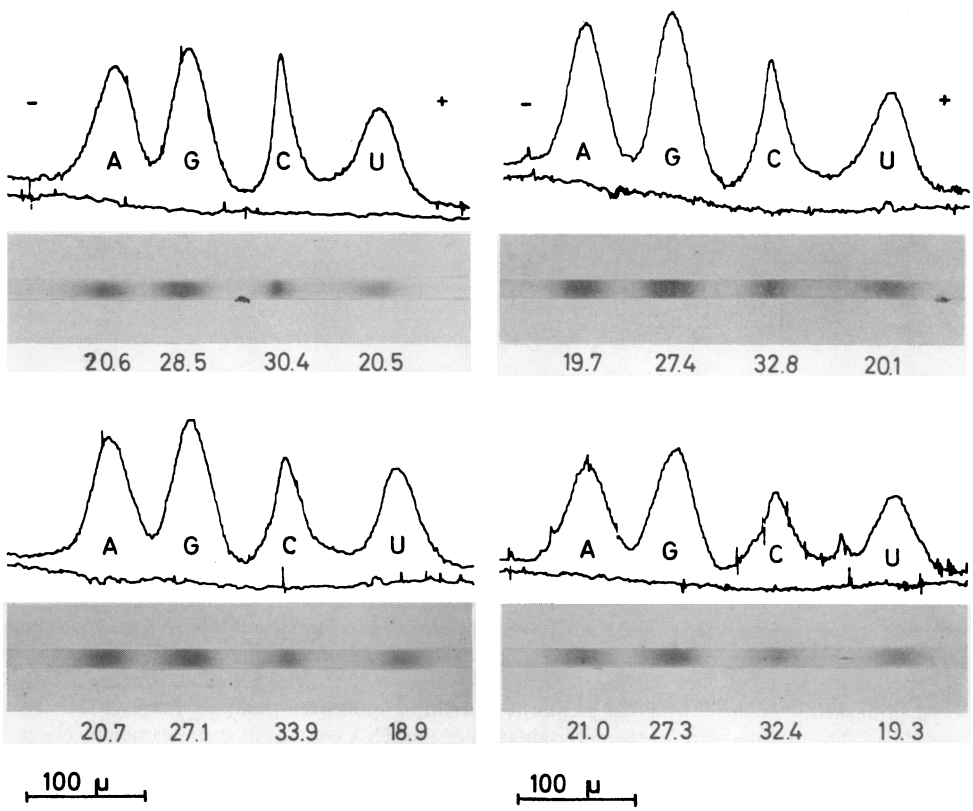


FIG. 2.—Microelectrophoretic separations of one RNA hydrolysate from cortical nerve cells. Each strip is about 20 $\mu$ in width and has been photographed at 2570 Å. Above each strip are the photometric tracings, and below are the quantitative values of the RNA components.

Thus, a significant increase of the amount of RNA per cortical neuron had occurred during the transfer of handedness.

*Qualitative RNA changes in cortical neurons:* As an example, demonstrating also the accuracy of the method, Figure 2 shows the result of four microelectrophoretic separations of one RNA hydrolysate from about 200 single nerve cells of the right side of the part of the cortex described above. Above each strip, showing the purine bases and pyrimidine nucleotides photographed at 2570 Å after the separation, are shown the photometric tracings. Below the microscopic strips are listed the quantitative values of the RNA components as molar proportions in per cent of the sum.

In Table 2 are listed the results of the analysis of neurons from five rats which were taken on the fourth day after a training period of 2 × 25 min per day.

Since the control material is taken from the contralateral side of the same brain, a Student's test was performed on the results. The difference between the purine-to-pyrimidine ratio of the control versus the learning cortex was found to be significant.

In Table 3 this difference is given in percentage and the $P$ values listed are based on the result of the Student's paired test. The coefficients of variation were for

controls and learning, respectively: A, 5.9, 2.5; G, 5.4, 4.7; C, 5.9, 5.5; U, 5.9, 6.4.

Thus, the analyses have shown that when a transfer occurs from right- to left-handedness, RNA increases in the neurons of that part of the cortex whose destruction removes the capacity of transfer. The purine-to-pyrimidine ratio was significantly increased in the RNA of the learning neurons compared to that of the controls.

*Further control experiments:* Three right-handed rats not subjected to transfer experiments were analyzed with respect to content and composition of RNA from the same neurons as analyzed in the transfer experiment. No significant difference between the right and left side of the cortex was found.

In another experiment, three right-handed rats were allowed to perform in the experimental setup with the same number of reaches for food and for the same time as was allotted in the transfer experiments. A slight increase of RNA in the left-side neurons was found compared to the RNA content of the right-side neurons. But no base ratio changes were found.

Transfer of information to the contralateral side is known to occur both with respect to visual memory[11] and discrimination by touch in monkeys.[12, 13] In our case, we have chosen to use neurons from the contralateral side of the same brain because of the obvious advantage for the biochemical analyses—and because handedness constitutes a favorable case, since the left side of the cortex already has been influenced by learning.

*Discussion.*—There has been great interest in the question of whether RNA or protein can be the chemical substrate for storage of information in the central nervous system. The microchemical technique of RNA determination per single cell provides a unique opportunity to analyze the problem at the level of the individual neuron.

The advantage in using experimental transfer of handedness lies in the fact that control neurons are available in the contralateral side of the same brain. The results showed that an increase of RNA occurs in the neurons of that part of the cortex engaged in the transfer of the complicated motor and sensory performance. The RNA produced during that forced experiment had an increased (A + G)/(C + U) ratio and a decreased (G + C)/(A + U) ratio over those of the control

TABLE 2

RNA BASE COMPOSITION OF THE CORTICAL NEURONS FROM THE CONTROL (LEFT) SIDE AND FROM THE LEARNING (RIGHT) SIDE

| Animal | 17 | | 27 | | 2:3 | | 2:12 | | 2:13 | | Mean | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Contr. | Learn | Contr. | Learn | Contr. | Learn | Contr. | Learn | Contr. | Learn | Contr. | Learn |
| Adenine | 19.0 | 20.0 | 16.6 | 20.2 | 19.2 | 20.6 | 19.0 | 20.6 | 18.2 | 19.3 | 18.4 ± 0.48 | 20.1 ± 0.11 |
| Guanine | 28.6 | 31.9 | 26.8 | 29.6 | 26.6 | 27.2 | 24.9 | 27.6 | 25.5 | 27.4 | 26.5 ± 0.64 | 28.7 ± 0.90 |
| Cytosine | 33.6 | 28.8 | 39.1 | 30.9 | 36.5 | 34.2 | 38.5 | 31.6 | 36.3 | 31.9 | 36.8 ± 0.97 | 31.5 ± 0.75 |
| Uracil | 18.8 | 19.3 | 17.5 | 19.3 | 17.7 | 18.0 | 17.6 | 20.2 | 20.0 | 21.4 | 18.3 ± 0.48 | 19.6 ± 0.56 |
| $\frac{A+G}{C+U}$ | 0.91 | 1.08 | 0.77 | 0.99 | 0.85 | 0.92 | 0.78 | 0.93 | 0.78 | 0.88 | 0.81 ± 0.027 | 0.95 ± 0.035 |
| $\frac{G+C}{A+U}$ | 1.65 | 1.54 | 1.93 | 1.53 | 1.71 | 1.59 | 1.73 | 1.45 | 1.62 | 1.46 | 1.72 ± 0.054 | 1.51 ± 0.026 |

TABLE 3

CHANGES IN THE RNA BASE COMPOSITION OF CORTICAL NEURONS FROM THE CONTROL (LEFT) SIDE AND FROM THE LEARNING (RIGHT) SIDE

| | Mean | | Change in per cent | $P$ |
|---|---|---|---|---|
| | Controls | Learning | | |
| Adenine | 18.4 ±0.48 | 20.1 ± 0.11 | +9.2 | 0.02 |
| Guanine | 26.5 ±0.64 | 28.7 ± 0.90 | +8.3 | 0.01 |
| Cytosine | 36.8 ±0.97 | 31.5 ± 0.75 | −14.4 | 0.01 |
| Uracil | 18.3 ±0.48 | 19.6 ±0.56 | +7.1 | 0.05 |
| $\frac{A + G}{C + U}$ | 0.81 ±0.027 | 0.95 ± 0.035 | +17.3 | 0.01 |
| $\frac{G + C}{A + U}$ | 1.72 ± 0.054 | 1.51 ± 0.026 | −12.2 | 0.02 |

neurons. Since the increase of RNA in each cortical nerve cell was moderate, and the ratio of nuclear to cytoplasmic volume was high, this means that the newly synthesized RNA had highly specific base ratios. The decrease of the ratio (G + C)/(A + U) suggests that the new RNA formed is of the messenger RNA type.

These results may be compared with those obtained on brain stem neurons in our earlier learning experiments on rats. In those experiments, the animals had to learn to balance on a 1.5-mm steel wire to reach food. In the big neurons of the vestibular nuclei clearly engaged in the process, production of nuclear RNA with an increased A/U ratio was found. By inference, on comparison with results of chromosomal RNA,[16] the conclusion was drawn that the neuronal RNA found was a chromosomal RNA and that the learning experiment caused stimulation of the genome of the neurons. In these balance experiments, the controls could not be taken from the same animal. Instead, another part of the brain stem was studied, and a stress experiment and a physiological stimulation involving the same vestibular nerve cells were carried out. In these control experiments, a neuronal RNA increase without base ratio changes was found. The conclusion from learning experiments involving neurons of the brain stem and cortex is that an acute learning situation with no precedence in the animal's life acts as a genomic stimulation, resulting in a production of RNA with highly specific base ratios in the neurons immediately involved.

This would imply the existence of a "mechanism of selection." In each of the millions of nerve cells involved, specific species of RNA and its end products would be produced after the learning had been consolidated, probably in minute amounts but present in each neuron whose activity is needed each time the complicated, learned task is performed.

In 1958, the senior author proposed a hypothetical "mechanism of instruction" involving the RNA of the neurons as a substrate molecule for storing information.[14] Modulated frequencies were thought to bring about base permutations of the RNA which would be stable. The specific protein being formed through this RNA should react with a complementary molecule in an antigen-antibodylike reaction activating the transmitter each time the same modulated frequency entered the neuron which once specified the RNA.

The problem of existence of an "instructional" or "selectional" mechanism involving RNA and storage of information remains. It is not difficult to imagine a parallelism between matching of antigenic information by the antibody mechanism, and by the neuronal mechanism in the case of nervous information. It is, there-

fore, interesting to note that Halac *et al.*[15] recently reported changes of RNA base composition occurring in a mixture of "immunologically competent cells" (mainly monocytes and lymphocytes) after contact with antigen.

*Summary.*—The RNA content and base ratios of cortical neurons were studied in rats during transfer of handedness. Corresponding neurons in the contralateral part of the same cortex served as controls.

The RNA content increased significantly in these neurons which have large nuclei relative to the cytoplasmic mass. The (G + C)/(A + U) ratio of the RNA decreased, indicating that the new RNA produced during learning is of the messenger type.

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# $H^p$ CLASSES OF HOLOMORPHIC FUNCTIONS IN TUBE DOMAINS*

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Let $B$ be an open connected set in real Euclidean $n$-space, $E^n$, and consider the tube $T_B$ in complex space $C^n$ whose basis is $B$, that is $T_B = \{z \mid z = x + iy, y \in B\}$. We say $F \in H^p(T_B)$, $0 < p < \infty$, if $F$ is holomorphic in $T_B$ and $\sup_{y \in B} \int_{E^n} |F(x + iy)|^p dx < \infty$; the usual modification is made for the case $p = \infty$. We shall be concerned with various results about the boundary behavior of functions in $H^p(T_B)$, and the proofs of some of those will be sketched below. Details will appear elsewhere.

1. *General Results.*—We recall that any $F$ holomorphic in $T_B$ has an analytic