*Pharmacology Biochemistry & Behavior*, Vol. 25, pp. 651–658, 1986. © Ankho International Inc. Printed in the U.S.A. 0091-3057/86 $3.00 + .00

# Synthesis of Rat Brain DNA During Acquisition of an Appetitive Task

ANTONIO GIUDITTA,[1] CARLA PERRONE CAPANO, GIUSEPPE D'ONOFRIO, CARLO TONIATTI, TONINO MENNA AND HOLGER HYDÉN*

*International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy*
*Department of General and Environmental Physiology, University of Naples*
**Institute of Neurobiology, University of Göteborg, Sweden*

Received 12 December 1985

GIUDITTA, A., C. PERRONE CAPANO, G. D'ONOFRIO, C. TONIATTI, T. MENNA AND H. HYDÉN. *Synthesis of rat brain DNA during acquisition of an appetitive task.* PHARMACOL BIOCHEM BEHAV **25**(3) 651–658, 1986.—We have examined the incorporation of [$^3$H-methyl]thymidine into DNA extracted from several brain regions of rats learning a reverse handedness task, of control rats allowed to use their preferred paw, and of control rats left in their home cages. In learning animals, decrements in percent incorporation were observed in the visual cortex, remaining brain, hippocampus and entorhinal cortex. In the latter two regions less marked decreases were present in the active control group. No variation occurred in the sensory-motor cortex. In learning rats the specific radioactivity of neuronal DNA was markedly decreased in the hippocampus and remaining brain. In the former region, a less marked decrease was present in active control rats. In subcellular fractionation studies it was observed that decreases in DNA specific radioactivity prevailed in the mitochondrial fraction isolated from the hippocampus and visual cortex of learning rats. Brain radioactive DNA was widely distributed among fractions differing in their degree of repetitiveness. Its pattern of distribution did not coincide with that of bulk DNA and differed significantly among behavioural groups. The results suggest a non random origin of newly-synthesized brain DNA and its involvement in learning.

Learning Appetitive learning Reverse handedness Brain DNA Neuronal DNA DNA synthesis

CONTRARY to a widespread belief, cerebral DNA undergoes an active process of turnover in the adult mammal. This finding was originally attributed to repair processes [18,19], but later results favored the alternative view of a true turnover of brain DNA [15,21]. DNA turnover also occurs in a purified fraction of neuronal perikarya prepared from rat cerebral cortex [21]. With regard to the possible significance of these findings, it is of interest that brain DNA synthesis increases in mice learning a passive avoidance task [22,23]. Comparable changes occur in concomitance with other plastic events in the nervous system (for a review, see [9]). Negative results were also reported. Cytosine arabinoside, an inhibitor of DNA synthesis, was shown to be without effect on the retention of an active avoidance reaction in the goldfish [5]. This early observation is in line with the opinion according to which the role of DNA in brain functions is limited to the transcription process. At the present time, however, it is well known that eukaryotic DNA may participate in a number of previously unsuspected functions [16] which may make it metabolically unstable. This has paved the way for an open-minded examination of the role of DNA in learning and other brain activities.

We have been interested in the possibility of a direct involvement of DNA in brain functions since our initial finding of an age-dependent accumulation of DNA in excess of the diploid value in the subesophageal region of octopus brain [6,11]. Working with higher vertebrates we have shown that an amnesic treatment, such as electroconvulsive shock, reversibly inhibits the incorporation of labelled thymidine into rat brain DNA [10]. More recently, we have started to examine brain DNA synthesis under a variety of training procedures. Our first experiment concerned the acquisition of an active avoidance task in the rat [25]. Under these conditions brain DNA synthesis was shown to depend on the degree of learning, as well as on the amount of stress. In later experiments carried out using the same training paradigm, we observed that, only in non learning rats, the concentration of brain DNA synthesized during training is inversely related to the amount of paradoxical sleep measured in the post-training period [1, 2, 13].

We now describe the effects of reverse handedness training on the incorporation of labelled thymidine into brain DNA. This appetitive task has previously been used in several investigations concerned with the effect of learning on the synthesis of brain protein and RNA [14]. The motivation, level of stress, type of reward and learned motor pattern associated with reverse handedness are substantially different from those of active avoidance learning. As such, they

[1]Requests for reprints should be addressed to Prof. A. Giuditta, International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Napoli, Italy.

seemed particularly appropriate to investigate whether the synthesis of brain DNA is generally involved in learning [7,20].

## METHOD

### *Training*

Female Sprague Dawley rats weighing 152±20 g (SD) were kept singly in standard cages with water ad lib. Training was carried out in a large, rectangular cage, one large side of which consisted of glass and the other of laminar wood. A 1 cm diameter open glass cylinder, slightly tilted upwards, protruded from a hole in one of the gavels, 5 cm above the floor. The glass tube was filled with 4 mm diameter protein pills (Astra-Ewos, Södertälje, Sweden) up to 3 cm from its opening. Rats were kept on a limited diet (hungry but with water ad lib) for 4 days before the start of the training period. In the first trial each rat was permitted 25 reachings which allowed its assignment to a left-handed or to a right-handed group. Only right-handed animals were kept for further experimentation. Two training sessions of 25 min each were scheduled daily at 10:00 a.m. and 3:00 p.m. One third of the daily allowance of food pills had to be grasped during the training sessions. The rest, maximally 4 g, was eaten in the housing cage. Water was kept ad lib. The same diet scheme was applied to the control rats. Body weight decreased somewhat in the pre-training period, but started to increase again during training. Rats were induced to use the non preferred paw by placing a Plexiglas wall close to and parallel to the left side of the glass cylinder. The number of successful reaches was recorded in each session. A second group of animals (active controls) were exposed to the same experimental treatment but were left free to use their preferred right paw to retrieve food. These rats experienced a similar amount of stress, motivation, motor activity and reward than the first group of rats, but were not required to learn the reverse handedness task. Rats of a third group (passive controls) were left in their home cages.

### *Injection*

Before the 4th training session each rat was lightly anesthesized with ether followed by fluothane and injected intracranially with [$^3$H-methyl]thymidine (20 Ci/mmol; Amersham International, Amersham, UK). The radioactive precursor (100 $\mu$Ci) was dissolved in 10 $\mu$l saline and was injected in the olfactory region of the cranium (50 $\mu$Ci) and in the lateral ventricles (25 $\mu$Ci in each ventricle). The latter sites were reached by inserting the needle of a 10 $\mu$l Hamilton syringe 2 mm deep at a point placed 2 mm caudally and 2 mm laterally with respect to bregma. To make the injection in the olfactory region, the needle was inserted at an angle of about 45° in the midline between the eyes and was pushed in the caudal direction until it reached the brain cavity. Injection at more than one site was preferred since it insured a more uniform distribution of the precursor. Soon after the injection the rat was placed back in its cage where it regained consciousness within a few minutes. Half an hour later it was exposed to the 4th training session and then killed by decapitation. Control animals of either group received the same amount of radioactive thymidine at the same hour of the day as their training mates.

### *Biochemical Procedures*

After killing, the brain of each rat was rapidly removed and chilled in ice-cold saline. Membranes and blood clots were removed and the brain was grossly divided into hippocampus, entorhinal cortex, sensory-motor cortex (2 mm laterally and 2 mm frontally with respect to bregma), and visual cortex (on the occipital pole of dorsal cortex). The remaining tissue (mostly other cortical regions and brain stem) was also saved. Samples were kept frozen at −20° until further processed. In one experiment the hippocampus and the remaining brain were fractionated according to the method of Satake and Abe [24] to yield a fraction of purified neuronal perikarya. In a separate experiment, the hippocampus and the visual cortex were fractionated into a crude nuclear fraction and a crude mitochondrial fraction. Each region was homogenized in cold 0.32 M sucrose (1:9 w/v) using a Potter homogenizer and a motor-driven Teflon pestle. The homogenate was centrifuged in rotor SS-34 of a refrigerated RC-2 Sorvall centrifuge at 3,000 rpm for 10 min to sediment the crude nuclear fraction. The sediment was resuspended twice in half volume 0.32 M sucrose and washed by centrifugation. The supernatant fractions were mixed and centrifuged at 9,000 rpm for 30 min to yield the crude mitochondrial fraction.

Tissue samples were homogenized with 9 volumes ice-cold water using a glass homogenizer. An equal volume of 2 M perchloric acid (PCA) was added and the mixture was kept at 4° overnight. The precipitate was collected by centrifugation and washed twice with 2–4 ml of cold 1 M PCA. The first PCA supernatant was mixed with the first washing and brought to a fixed volume with 1 M PCA (PCA-soluble fraction). The precipitate was resuspended in 0.5–1.0 ml 1 M PCA and heated at 80° for 30 min. After standing in ice for 15 min, any insoluble material was discarded by centrifugation. The supernatant fraction was saved while the sediment was hydrolyzed again with a fresh aliquot of 1 M PCA. The two hydrolysates were combined to yield the DNA extract. Suitable aliquots of the DNA extract and of the PCA-soluble fraction were mixed with a Triton-based scintillation mixture and counted in a Packard model 3385 spectrometer with an efficiency of approximately 40%. The content of DNA of the DNA extract was determined by a diphenylamine method [8]. Biochemical data were expressed as concentration of PCA-soluble radioactivity or radioactive DNA (dpm/tissue wet weight). Results were also calculated in terms of percent incorporation (radioactive DNA/total radioactivity × 100).

Several control experiments were made to test the reliability of the method of preparation of the DNA fraction. Radioactive brain homogenates and PCA-washed precipitates obtained therefrom were incubated with DNase I or RNase (Boehringer, Mannheim; 200 $\mu$g/ml; 60 min at 37°) in 50 mM Tris-Cl buffer pH 8.1 containing 5 mM $MgCl_2$. Under these conditions the amount of PCA-insoluble radioactivity was substantially decreased by DNase I (−81.1%), but not by RNase or by treatment with 1 N NaOH (60 min at 37°). A minor fraction of PCA-insoluble radioactivity remained insensitive to DNase hydrolysis (18.9%) and was not recovered in the DNA peak following centrifugation of a labelled brain homogenate in a CsCl gradient containing 0.2% SDS, 5 mM EDTA and 15 mM Tris-Cl buffer pH 8.1 (23%). A somewhat larger percent of PCA-insoluble radioactivity (27%) was not solubilized by repeated PCA hydrolysis (1 M PCA; 30 min at 80°). These results showed that the PCA-insoluble radioactivity which did not behave as DNA was not recovered in the PCA hydrolysate routinely prepared as our DNA fraction. In conclusion, the DNA extract appeared to contain all the radioactive thymidine incorporated into DNA but none of the spurious PCA-insoluble radioactivity.

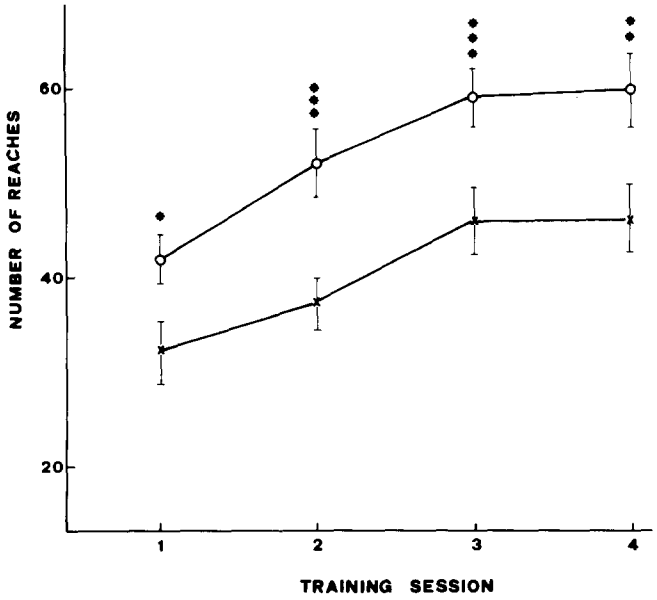


FIG. 1. Average number of reaches per session displayed by learning rats (○; n=33) and by active control rats (X; n=33). Vertical bars define standard error. By Student's $t$-test for paired data significant differences were present in the group of trained rats between the first and the second session ($t=2.215$, $p<0.025$) and between the second and the third session ($t=1.801$, $p<0.05$). Significant differences were likewise present in the group of active control rats between the first and the second session ($t=1.843$, $p<0.05$) and between the second and third session ($t=2.116$, $p<0.025$). The statistical significance of the difference in number of reaches observed in each session between trained rats and active control rats (Student's $t$-test) is indicated by $*p<0.025$, $**p<0.01$ and $***p<0.005$.

*DNA Renaturation Analyses*

DNA was purified from the remaining brain (our largest sample) by extraction with phenol and chloroform and enzymic hydrolysis with proteinase K and RNase. To obtain fragments of suitable length, purified DNA was dissolved in 66% glycerol and sheared in a Virtis model 60 homogenizer (30 min at 50,000 rpm) [4]. Sheared DNA had absorbance ratios of 1.8 and 2.2 at 260/280 nm and 260/230 nm respectively and an average size of about 350 nucleotide pairs, as determined by agarose gel electrophoresis. Renaturation analyses were carried out by sealing DNA samples in glass capillaries, heating at 100° for 5 min and incubating at 60° or 68° for the required period of time. The reaction was stopped by quickly chilling in ice and diluting in several volumes of 0.12 M potassium phosphate buffer pH 6.8. Separation of renatured DNA was carried out by hydroxyapatite chromatography [4]. Each reaction mixture was analysed in terms of radioactivity and absorbance at 260 nm. Results were expressed as relative specific radioactivity, that is the specific radioactivity of the renatured or single-stranded DNA fraction (cpm/$A_{260}$) was referred to the specific radioactivity of the two fractions combined. Each value was the average of 4–5 determinations.

*Statistical Analyses*

*In vivo* experiments were carried out on three different occasions during visits made by C.P.C. or G. D. at the University of Göteborg. The average values of percent incorporation calculated for each brain region within the same animal group remained essentially the same in the three experiments, as shown by statistical analysis. Incorporation data were therefore pooled together before statistical treatment. On the other hand, the experiments differed significantly from each other with regard to the values of PCA-soluble radioactivity and radioacitve DNA. The differences were most likely due to variations in the actual dose of radioactive precursor administered in each experiment. The following method was used to standardize these data. For each brain region of the group of active control rats we calculated the difference between the average value of PCA-soluble radioactivity (or radioactive DNA) measured in the experiment with the highest value and each of the other two experiments. These differences were added to the individual values of the other two experiments, irrespective of the experimental condition. Homologous data were then pooled together and spurious results excluded if they differed from the average by at least three times the variance value. The latter procedure led to the exclusion of only very few data from each group. Data were tested for variance homogeneity according to Bartlett's method. Factorial analysis of variance was carried out using an ANOVA program. Intergroup comparisons were made using Scheffé's test, Student's $t$test or Mann-Whitney U test [26,27].

## RESULTS

*Behavioral Data*

In learning rats the average number of successful reaches measured in each training session increased progressively up to the 3rd session, but showed no increment between the 3rd and the 4th session (Fig. 1). A comparable behaviour was observed in active control rats which, however, displayed a consistently lower number of reaches in each session.

*Biochemical Data*

*Whole tissue.* In each animal group the values of PCA-soluble radioactivity showed considerable variation among brain regions (Table 1). In the hippocampus, the PCA-soluble radioactivity was approximately 8-fold higher than in the sensory-motor cortex. Intermediate values were present in other brain regions. Comparable differences occurred in the concentration of radioactive DNA. This variability was attributed to an uneven distribution of the labelled precursor within the brain. The occurrence of additional variations in incorporation rates was born out, however, by comparing the values of percent incorporation within the group of passive control rats (Table 1). The highest and the lowest values occurred in the remaining brain and in the sensory-motor cortex, respectively. Either value differed significantly (Scheffé's test) from the values of percent incorporation measured in the other regions.

The values of PCA-soluble radioactivity were not significantly different in the three groups of rats. On the other hand, the amount of radioactive DNA was significantly lower in learning rats in comparison with passive control rats in the entorhinal cortex and in the visual cortex (Table 1). A similar decrease failed to reach significance in the remaining brain, while no change occurred in the hippocampus and sensory-motor cortex. Somewhat lower values of radioactive DNA were also present in active control rats in comparison with passive control rats in the entorhinal cortex, visual cor-

TABLE 1

CONCENTRATION OF PCA-SOLUBLE RADIOACTIVITY AND OF RADIOACTIVE DNA AND VALUES OF PERCENT INCORPORATION MEASURED IN DIFFERENT BRAIN REGIONS OF LEARNING RATS AND OF CONTROL RATS

| Brain Region | Condition | PCA-Soluble Radioactivity (dpm/0.1 g × $10^{-3}$) | Radioactive DNA (dpm/0.1 × $10^{-3}$) | Incorporation (%) |
|---|---|---|---|---|
| Hippocampus | PC | (8) 3908 ± 245 | (9) 133 ± 6.9 | (10) 3.6 ± 0.27 |
| | AC | (20) 3756 ± 246 | (20) 149 ± 8.1 | (20) 3.3 ± 0.18 |
| | L | (22) 4202 ± 201 | (23) 127 ± 6.6 | (20) 2.8 ± 0.11* |
| Entorhinal | PC | (24) 924 ± 52 | (23) 47.4 ± 3.8 | (23) 4.4 ± 0.17 |
| Cortex | AC | (31) 788 ± 44 | (32) 38.6 ± 2.1 | (29) 3.5 ± 0.16* |
| | L | (36) 892 ± 78 | (35) 34.3 ± 2.8* | (32) 3.0 ± 0.15* |
| Visual | PC | (22) 911 ± 55 | (22) 38.4 ± 3.9 | (20) 3.8 ± 0.30 |
| Cortex | AC | (30) 827 ± 48 | (30) 30.0 ± 2.2 | (26) 3.4 ± 0.16 |
| | L | (34) 897 ± 68 | (34) 27.3 ± 2.5* | (32) 2.6 ± 0.16*† |
| Sensory-motor | PC | (24) 446 ± 33 | (23) 13.9 ± 1.2 | (21) 2.4 ± 0.11 |
| Cortex | AC | (30) 518 ± 22 | (30) 16.2 ± 0.8 | (26) 2.3 ± 0.16 |
| | L | (36) 538 ± 30 | (37) 14.3 ± 0.9 | (32) 2.0 ± 0.09 |
| Remaining | PC | (11) 2262 ± 121 | (11) 150 ± 12 | (11) 6.1 ± 0.29 |
| Brain | AC | (21) 2042 ± 92 | (21) 126 ± 7.7 | (18) 5.3 ± 0.24 |
| | L | (23) 2224 ± 119 | (22) 117 ± 7.0 | (20) 4.4 ± 0.19*† |

Average values with standard error. Statistical analyses carried out with Sheffé's test.

* Different from PC ($p<0.05$); † Different from AC ($p<0.05$). PC, passive control rats; AC, active control rats; L, learning rats. In parentheses, number of rats.

TABLE 2

INCORPORATION OF [$^3$H]THYMIDINE IN THE FRACTION OF NEURONAL PERIKARYA PREPARED FROM THE HIPPOCAMPUS AND FROM THE REMAINING BRAIN OF LEARNING RATS AND OF CONTROL RATS

| Brain Region | Cellular Fraction | Condition | [DNA] (μg/0.1 g) | [$^3$H-DNA] (dpm/0.1 g × $10^{-3}$) | Specific Radioactivity (dpm/μg) |
|---|---|---|---|---|---|
| Hippocampus | Neuronal Perikarya | PC | (13) 35.7 ± 3.3 | (13) 32.5 ± 4.5 | (11) 978.0 ± 79.3 |
| | | AC | (10) 46.3 ± 2.9 | ( 9) 25.9 ± 2.0 | ( 8) 584.0 ± 28.3* |
| | | L | (12) 53.2 ± 3.5* | (11) 18.6 ± 3.0* | ( 9) 366.0 ± 49.3* |
| | Mixed | PC | (12) 36.8 ± 2.8 | (13) 34.2 ± 3.9 | (10) 1154.0 ± 57.1 |
| | | AC | ( 8) 24.8 ± 2.8 | ( 7) 22.1 ± 3.1 | ( 7) 886.0 ± 96.5* |
| | | L | (13) 33.7 ± 4.1 | (12) 17.6 ± 2.5* | ( 9) 466.0 ± 62.3*† |
| Remaining Brain | Neuronal Perikarya | PC | (13) 59.7 ± 2.3 | (13) 30.5 ± 4.4 | (12) 513.7 ± 67 |
| | | AC | (10) 68.9 ± 2.6 | ( 9) 25.8 ± 3.5 | ( 9) 421.2 ± 61.3 |
| | | L | (13) 76.0 ± 4.2* | (11) 15.9 ± 4.3 | (10) 160.1 ± 20.3*† |
| | Mixed | PC | (13) 30.8 ± 5.7 | (13) 17.4 ± 4.3 | (12) 553.4 ± 62 |
| | | AC | ( 9) 20.0 ± 2.1 | (10) 10.4 ± 1.1 | ( 9) 529.4 ± 53.3 |
| | | L | (12) 22.9 ± 4.6 | (12) 8.7 ± 2.4 | ( 9) 209.1 ± 39.4*† |

Details as in Table 1.

tex and remaining brain. These changes, however, remained below the level of significance. Several significant differences among groups were observed in the values of percent incorporation. In learning rats, the incorporation of labelled thymidine was significantly lower than in passive control rats in all brain regions, with the exception of the sensory-motor cortex. In addition, learning rats had significantly lower values of incorporation than active control rats in the visual cortex and remaining brain. In the latter two regions passive control rats and active control rats did not differ significantly from each other. As a result, the decrease occurred solely in the group of learning rats. On the other hand, in the entorhinal cortex the incorporation value of active control rats was significantly lower than in passive control rats and statistically similar to that of learning rats. In the hippocampus the percent incorporation of active control rats was not

TABLE 3

INCORPORATION OF [$^3$H]THYMIDINE IN THE NUCLEAR AND MITOCHONDRIAL FRACTIONS PREPARED FROM THE HIPPOCAMPUS AND FROM THE VISUAL CORTEX OF LEARNING RATS AND OF CONTROL RATS

| Brain Region | Subcellular Fraction | Condition | [DNA] ($\mu$g/0.1 g) | [$^3$H-DNA] (dpm/0.1g $\times 10^{-3}$) | Specific Radioactivity (dpm/$\mu$g DNA $\times 10^{-3}$) |
|---|---|---|---|---|---|
| Hippocampus | Nuclei | PC | (10) 103.3 ± 8.4 | (10) 93.8 ± 6.0 | ( 9) 0.97 ± 0.05 |
| | | AC | (11) 91.9 ± 5.8 | (12) 71.3 ± 9.9 | (12) 0.80 ± 0.11 |
| | | L | (11) 124.8 ± 6.8*†† | (11) 84.9 ± 7.8 | (11) 0.70 ± 0.05*** |
| | Mitochondria | PC | ( 8) 4.6 ± 0.7 | (11) 18.3 ± 1.4 | ( 9) 4.21 ± 0.50 |
| | | AC | (10) 3.9 ± 0.5 | (12) 16.1 ± 2.1 | (10) 4.08 ± 0.70 |
| | | L | (12) 10.9 ± 1.3***†† | (12) 17.4 ± 1.8 | (11) 1.78 ± 0.20***† |
| Visual Cortex | Nuclei | PC | (11) 53.2 ± 6.7 | (10) 15.2 ± 3.5 | (10) 0.30 ± 0.04 |
| | | AC | (11) 73.6 ± 4.2* | ( 9) 11.1 ± 2.0 | ( 7) 0.12 ± 0.02** |
| | | L | (12) 63.4 ± 6.6 | (10) 10.3 ± 1.3 | ( 8) 0.18 ± 0.05 |
| | Mitochondria | PC | (11) 2.7 ± 0.5 | (11) 3.2 ± 0.5 | ( 9) 1.32 ± 0.25 |
| | | AC | (11) 1.9 ± 0.3 | (10) 2.7 ± 0.4 | ( 9) 1.30 ± 0.21 |
| | | L | (13) 10.12 ± 1.8***†† | (11) 4.1 ± 0.7 | (10) 0.54 ± 0.12**† |

Differences from PC are indicated by *($p<0.05$), ** ($p<0.02$) and ***($p<0.002$); differences from AC by †($p<0.02$) and ††($p<0.002$). Mann-Whitney U test.

significantly different from either one of the other two animal groups.

The decrease in percent incorporation observed in learning rats in comparison with passive control rats was approximately the same in the entorhinal cortex (32%), visual cortex (31%) and remaining brain (28%) and only somewhat lower in the hippocampus (22%). In the former two regions the effect was largely due to a decrease in the concentration of radioactive DNA.

*Cellular fractions.* The involvement of neuronal DNA in reverse handedness learning was investigated in an experiment in which a fraction of neuronal perikarya was prepared from the hippocampus and from the remaining brain. A side fraction containing nerve and glia cells (mixed fraction) was also analyzed. As we have consistently observed [12, 21, 28], the neuronal fraction prepared according to the method of Satake and Abe [24] contained a large majority of well preserved neuronal perikarya and few large nuclei with light nucleoplasm and one or two nucleoli (not shown). These observations suggested that the degree of contamination by non neuronal cells was relatively low.

The DNA content of the fraction of neuronal perikarya prepared from either region was higher in learning rats and active control rats than in passive control rats (Table 2). Differences were significant in learning rats. Comparable changes were not observed in the mixed fraction. An opposite effect concerned radioactive DNA. In the neuronal fraction the amount of radioactive DNA was lower in learning rats than in passive control rats. A less marked decrease occurred in active control rats. The effect became significant only in the hippocampus of learning rats. Similar decrements were present in the mixed fraction, but the only significant difference occurred in the hippocampus between learning rats and passive control rats.

As a result of these changes, the specific radioactivity of the neuronal fraction was markedly lower in learning rats than in passive control rats in the hippocampus (−63%) and remaining brain (−69%). A less marked decrease was present in the hippocampus of active control rats (−40%) while the difference between active control rats and learning rats remained below the level of significance. In the remaining brain the specific radioactivity of the neuronal fraction was significantly lower in learning rats (−62%) than in passive control rats but did not differ in the comparison between active control rats and passive control rats.

The specific radioactivity of the mixed fraction was likewise considerably lower in the hippocampus of learning rats than in passive control rats (−60%) and only marginally lower in active control rats than in passive control rats (−23%). The difference between learning rats and active control rats (47%) was also significant. An analogous pattern was present in the remaining brain. In this region the specific radioactivity of the mixed fraction was significantly lower in learning rats in comparison with passive control rats (−62%) and active control rats (−61%), while no difference was present between active control rats and passive control rats.

*Subcellular fractions.* The influence of reverse handedness learning on the incorporation of radioactive thymidine into nuclear and mitochondrial DNA was investigated in an experiment in which nuclear and mitochondrial fractions were prepared from the hippocampus and from the visual cortex (see the Method section). In the hippocampus the DNA content of the nuclear fraction was significantly higher in learning rats than in passive control rats and in active control rats (Table 3). A similar effect was present in the mitochondrial fraction. In the visual cortex comparable data were obtained in the mitochondrial fraction, while in the nuclear fraction the DNA content was significantly higher in active control rats than in passive control rats. Significant effects were present in the values of specific radioactivity. In the hippocampus, learning rats had significantly lower values than passive control rats in the nuclear fraction (−28%) and in the mitochondrial fraction (−58%). On the other hand, in comparison with active control rats, the specific radioactivity of learning rats was significantly lower only in the mitochondrial fraction (−56%). No significant differences were noted between passive control rats and active control rats. In the visual cortex similar decrements were present in

TABLE 4

RELATIVE SPECIFIC RADIOACTIVITY OF RENATURED AND SINGLE-STRANDED DNA OBTAINED FROM THE REMAINING BRAIN OF LEARNING RATS AND OF CONTROL RATS

| DNA Fraction | Condition | Cot (M sec) 2 | 100 | 50,000 |
|---|---|---|---|---|
| Renatured | PC | 1.19 ± 0.06 | 1.09 ± 0.01 | 1.03 ± 0.01 |
| | AC | 0.90 ± 0.04* | 1.29 ± 0.03** | 0.95 ± 0.01** |
| | L | 0.92 ± 0.03** | 1.10 ± 0.03††† | 0.93 ± 0.01** |
| Single-stranded | PC | 0.98 ± 0.03 | 0.94 ± 0.02 | 0.83 ± 0.04 |
| | AC | 1.02 ± 0.01 | 0.88 ± 0.01* | 1.25 ± 0.06** |
| | L | 1.04 ± 0.02 | 0.94 ± 0.01†† | 1.75 ± 0.10**† |

Average values with standard error. Differences from PC are indicated by *($p<0.01$) and **($p<0.005$); differences from AC by †($p<0.025$), ††($p<0.01$) and †††($p<0.005$). Students t-test.

the mitochondrial fraction of learning rats whose specific radioactivity was markedly lower than in passive control rats (−59%) and in active control rats (−59%). A significant decrease was also observed in the nuclear fraction of active control rats in comparison with passive control rats (−60%).

*Renaturation analyses.* The nature of newly-synthesized brain DNA and the possible influence of reverse handedness learning were investigated by renaturation analyses of heat-denatured DNA (see the Method section). To obtain a sufficient amount of radioactive DNA, DNA was extracted from pools of remaining brain obtained respectively from passive control rats, active control rats and learning rats. Approximately ten animals were included in each pool. The values of percent incorporation measured in other brain regions of the same animals yielded data in full agreement with the results presented in Table 1.

In passive control rats the fraction of DNA renatured at Cot 2 (M sec) was 18.1±2.5% for radioactive DNA and 12.5±1.03% for bulk DNA. At Cot 100 the corresponding values were 43.5±0.95 and 35.0±0.5% and at Cot 50,000 they were 86.3±0.5% and 80.4±0.77%, respectively. The data showed that newly-synthesized brain DNA is widely dispersed among fractions differing in their degree of repetitiveness. In addition, the significant prevalence consistently observed in the percent recovery of renatured DNA when comparing labelled DNA to bulk DNA indicated that newly-synthesized DNA is more abundant in fractions with high and moderate degree of repetitiveness and cannot be considered a random copy of genomic DNA.

Significant differences among groups were observed at each Cot value in the relative specific radioactivities (RSR; see the Method section) of renatured DNA (Table 4). At Cot 2 and at Cot 50,000, learning rats and active control rats had lower values of RSR than passive control rats, while at Cot 100 the RSR of active control rats was significantly higher than in passive control rats and in learning rats. Complementary variations occurred in the fraction of single-stranded DNA. They reached significance at Cot 100 and at Cot 50,000. In addition, in the fraction of DNA which did not renature at Cot 50,000, the RSR of learning rats was significantly higher than in the two control groups. These findings indicated that the lower concentration of brain radioactive DNA noted in learning rats in comparison to control rats (Table 1) was not the result of a random process of inhibition of DNA synthesis but involved the fraction of highly repetitive DNA (up to Cot 2) and, to a lesser extent, the fraction of DNA with a low level of reiteration (from Cot 100 to Cot 50,000). Similar effects occurred in active control rats. Differential changes between learning rats and active control rats were present in the fraction of DNA renatured at Cot 100 and in the single-stranded fraction remaining at Cot 50,000. In the former fraction more labelled DNA renatured in active control rats than in the other two groups, while in the latter fraction considerably more labelled DNA remained single-stranded in learning rats than in the two control groups.

## DISCUSSION

Forcing rats to feed with their non preferred paw brings about significant decrements in the percent incorporation of radioactive thymidine into the DNA of several brain regions. In rats using their preferred paw, a decrease of lower magnitude is observed in the entorhinal cortex only. These results cannot readily be attributed to variations in the specific radioactivity of thymidine triphosphate, the immediate DNA precursor, since values of acid-soluble radioactivity remain remarkably stable. It is likely that the specific radioactivity of thymidine triphosphate remains equally unchanged. On this basis the results are presumed to indicate an effect on DNA synthesis or turnover. In any case a precursor effect may be excluded, since the nature of newly synthesized DNA (its degree of repetitiveness) also changes (see below).

Can these effects be attributed to learning? The acquisition curve of the learning group shows a progressive increment in the number of reaches, but no significant increase between the third and the fourth session (Fig. 1). Since the incorporation period occurs during the latter session, biochemical data may be thought to reflect performance rather than acquisition. The active control group, however, displays a similar lack of increment in number of reaches between the 3rd and the 4th session (Fig. 1). This behaviour is therefore common to all rats and, as such, cannot be taken to suggest that rats are not learning in the 4th session. It is more likely that the lack of increment in number of reaches reflects an unspecific damping effect induced by the anesthesia and the intracerebral injection made 30 min before training. This conclusion is strongly supported by repeated observations made on untreated rats whose feeding performance in the reverse handedness task is known to improve steadily up to and beyond the 10th training session [14].

The consistently higher number of reaches shown by learning rats in comparison to active control rats (Fig. 1) should not be considered an index of a higher degree of motivation or of a better motor ability. Rats of either group were equally deprived of food and had similar body weights at the beginning of the training period. In addition, rats using their non preferred paw had definitely more difficulty in retrieving food. The difference is rather to be attributed to the greater loss of protein pills which learning rats suffered through the grid floor as result of their impaired capacity to control the movements of the non preferred paw. In turn, this led to more reaches. However, the difference in number of reaches between learning rats and active control rats does not account for the differences in biochemical data. Indeed, the values of percent incorporation of each brain region were not related to the number of reaches made during the 4th training session, as shown by Spearman's analysis in either group of rats or in the two groups combined. It seems reasonable to conclude that the biochemical changes are not related to performance *per se*, but rather to learning, particularly in those regions (visual cortex and remaining brain) in which the values of percent incorporation are significantly lower in learning rats than in passive control rats and active control rats. On the other hand, rats feeding with their preferred paw cannot be considered as lacking any form of learning, since they progressively improve their performance during training. As a result, the decreased incorporation observed in the entorhinal cortex of active control rats might be related to learning, rather than to other unspecified factors.

The decreased incorporation brought about in brain by reverse handedness learning stands in contrast with the increments previously observed in mice acquiring a passive avoidance task [22,23] and in rats learning an active avoidance paradigm [3,25]. The discrepancy cannot be attributed to a species difference, and is likely to reflect differences in the nature of the learning task. In the reverse handedness paradigm the level of stress and the amount of locomotor activity are lower than in active avoidance learning. In addition, reward and motivation are of the appetitive rather than of the aversive type. One or more of these differences might account for the directions taken by the biochemical changes. It should be mentioned that in a study concerning non associative learning (behavioural habituation) the incorporation of labelled thymidine into DNA was likewise found to be reduced in some brain regions (C. Lamberti *et al.*, manuscripts in preparation). Since the level of stress is also low in the habituation paradigm, brain DNA synthesis may be assumed to decrease if learning involves a limited amount of stress. It may not be unreasonable to assume that some of the brain functions activated during learning may increase the level of radioactive DNA while other functions may decrease it. Under these conditions the overall effect would depend on the relative contribution of each of these functions. Whatever their nature will prove to be, the results of the present study strongly confirm the involvement of brain DNA in learning. They suggest furthermore that the incorporation of radioactive thymidine into brain DNA may be modulated in either direction by the nature of the learning task. To our knowledge, this may well be the first biochemical parameter to show this intriguing feature.

Our data provide the first indications on the cellular and subcellular localization of the DNA fraction involved in the learning effect. The involvement of neuronal DNA is suggested by the marked fall in DNA specific radioactivity observed in the fraction of neuronal perikarya prepared from the hippocampus and remaining brain of learning rats. The morphological purity of the neuronal fraction, consistently reproduced in our laboratory [12, 21, 28], supports this interpretation, rather than the alternative view that the results are due to contamination by glia cells. Indeed, the decrements observed in the mixed fraction (containing glia cells) are comparable to or even less marked than those present in the fraction of neuronal perikarya.

With regard to the subcellular localization of the learning effects, it is remarkable that the most marked, consistent and specific decrease in DNA specific radioactivity occurs in the mitochondrial fraction. Less marked and less specific decreases occur in the nuclear fraction. While it is premature to conclude that mitochondrial DNA is primarily involved (the mitochondrial fraction is heavily contaminated by other subcellular components) it is hard to immagine how these components could originate from nuclei, as the specific radioactivity of the nuclear fraction is lower than that of the mitochondrial fraction. An additional and unexpected observation regards the higher content of DNA recovered in the mitochondrial fraction of learning rats and the similar but more limited increase observed in the nuclear fraction of learning rats (hippocampus) and active control rats (visual cortex). Similar changes were also present in the fraction of neuronal perikarya prepared from the hippocampus and the remaining brain of learning rats. Since the content of brain DNA cannot be expected to appreciably change within one hour, the results are likely to reflect a higher recovery of DNA-containing structures, presumably due to variations of their physical properties. An alternative, and perhaps more intriguing, explanation might be based on the assumption that the colorimetric reactivity of brain DNA is enhanced as result of behavioural experience.

The wide distribution of radioactive brain DNA among fractions ranging in Cot values from 2 to 50,000 indicates that newly-synthesized brain DNA is not a copy of a single or of a few segments of the genome but is very heterogeneous. This excludes the possibility that the turnover of brain DNA [15,21] reflects the occurrence of amplification processes. At the same time, the relative prevalence of labelled DNA (with respect to bulk DNA) in the fractions of high and moderate degree of repetitiveness indicates that it is not a random copy of the genome. The significant variations observed in the relative specific radioactivity of renatured and single-stranded DNA in different animal groups argue in favour of a behaviour-modulated control of brain DNA synthesis. The latter conclusion supports the view that the incorporation of labelled thymidine in the adult mammalian brain and the subsequent loss of a considerable fraction of newly-synthesized DNA is better explained by a *bona fide* brain DNA turnover [9,21] rather than by repair reactions [18,19]. As judged by the values presented in Table 4, learning-dependent variations are likely to occur in the DNA fraction reannealed at Cot 100 and in the single-stranded DNA fraction remaining at Cot 50,000. Since these results are the outcome of relatively gross fractionation procedures, it remains possible that additional learning effects may be present in other DNA fractions.

ACKNOWLEDGEMENTS

The excellent technical contribution of C. Buono is gratefully acknowledged. We like to thank C. Di Fusco for her expert secretarial assistance. Financial support for this work was provided by the Ministero della Pubblica Istruzione, by the Università di Napoli and by the Progetto Finalizzato di Medicina Preventiva e Riabilitativa del CNR.

## REFERENCES


1. Ambrosini, M. V., R. Scaroni, C. Chiurulla and A. Giuditta. Influence of sleep on cerebral DNA synthesized during learing. *Neurosci Lett [Suppl]* **14:** S8, 1983.
2. Ambrosini, M. V., R. Scaroni, C. Chiurulla and A. Giuditta. Effetto del sonno sul DNA cerebrale sintetizzato durante l'apprendimento. *Boll Soc Ital Biol Sper* **60:** 596–601, 1984.
3. Ashapkin, V. V., G. A. Romanov, N. A. Tushmalova and B. F. Vanyushin. Selective DNA synthesis in the rat brain induced by learning. *Biokhimiia* **48:** 355–362, 1983.
4. Britten, R. J., D. E. Graham and B. R. Neufeld. Analysis of repeating DNA sequences by reassociation. *Methods Enzymol* **29E:** 363–418, 1974.
5. Casola, L., R. Lim, R. E. Davis and B. W. Agranoff. Behavioral and biochemical effects of intracranial injection of cytosine arabinoside in goldfish. *Proc Natl Acad Sci USA* **60:** 1389–1395, 1968.
6. De Marianis, B., E. Olmo and A. Giuditta. Excess DNA in the nuclei of the suboesophageal region of Octopus brain. *J Comp Neurol* **186:** 293–300, 1979.
7. D'Onofrio, G., C. Perrone Capano, C. Toniatti, H. Hydén and A. Giuditta. Effetto di un apprendimento di tipo appetitivo sulla sintesi del DNA cerebrale di ratto. Atti 28th Congr. Soc. Ital. Biochim 1982, pp. 469–470.
8. Giles, K. W. and A. Myers. An improved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* **206:** 93, 1965.
9. Giuditta, A. Role of DNA in brain activity. In: *Handbook of Neurochemistry*, 2nd edition, vol 5, edited by A. Lajtha. New York: Plenum Press, 1983, pp. 251–276.
10. Giuditta, A., P. Abrescia and B. Rutigliano. Effect of electroshock on thymidine incorporation into rat brain DNA. *J Neurochem* **31:** 983–987, 1978.
11. Giuditta, A., M. Libonati, A. Packard and N. Prozzo. Nuclear counts in the brain lobes of Octopus vulgaris as a function of body size. *Brain Res* **25:** 55–62, 1971.
12. Giuditta, A., B. Rutigliano and A. Vitale Neugebauer. Influence of synchronized sleep on the biosynthesis of RNA in neuronal and mixed fractions isolated from rabbit cerebral cortex. *J Neurochem* **35:** 1267–1272, 1980.
13. Giuditta, A., M. V. Ambrosini, R. Scaroni, C. Chiurulla and A. Sadile. Effect of sleep on cerebral DNA synthesized during shuttle-box avoidance training. *Physiol Behav* **34:** 769–778, 1985.
14. Hydén, H. and P. W. Lange. Modification of membrane bound proteins of the hippocampus and entorhynal cortex by change in behavior in rats. *J Neurosci Res* **9:** 37–46, 1983.
15. Kimberlin, R. H., D. B. Shirt and S. C. Collis. The turnover of isotopically labelled DNA *in vivo* in developing, adult and scrapie-affected mouse brain. *J Neurochem* **23:** 241–248, 1974.
16. Lewin, B. *Genes*. New York: Wiley, 1983.
17. Marmur, J. A procedure for the isolation of DNA from microorganisms. *J Mol Biol* **3:** 208–218, 1961.
18. Merits, I. and J. Cain. Rapid loss of labelled DNA from rat brain due to radiation damage. *Biochim Biophys Acta* **174:** 315–321, 1969.
19. Merits, I. and J. Cain. Loss of labelled DNA from rat brain following injections of precursors with high specific radioactivity. *Biochim Biophys Acta* **209:** 327–338, 1970.
20. Perrone Capano, C., A. Giuditta, A. Vitale and H. Hydén. Behaviour-dependent changes in cerebral DNA turnover. Abstr. 5th Europ. Neurosc. Congr. 1981, p. S244.
21. Perrone Capano, C., G. D'Onofrio and A. Giuditta. DNA turnover in rat cerebral cortex. *J Neurochem* **38:** 52–56, 1982.
22. Reinis, S. Autoradiographic study of $^{3}$H-thymidine incorporation into brain DNA during learning. *Physiol Chem Phys* **4:** 391–397, 1972.
23. Reinis, S. and R. W. Lamble. Labelling of brain DNA by $^{3}$H-thymidine during learning. *Physiol Chem Phys* **4:** 335–338, 1972.
24. Satake, M. and S. Abe. Preparation and characterization of nerve cell perikaryon from rat cerebral cortex. *J Biochem (Tokyo)* **59:** 72–75, 1966.
25. Scaroni, R., M. W. Ambrosini, G. B. Principato, F. Federici, A. Ambrosi and A. Giuditta. Synthesis of brain DNA during acquisition of an active avoidance task. *Physiol Behav* **30:** 577–582, 1983.
26. Snedecor, G. W. and W. G. Cochron. *Statistical Methods*, 6th edition. Ames, IA: The Iowa State University Press, 1967.
27. Siegel, S. *Nonparametric Statistics for the Behavioral Sciences*. New York: McGraw-Hill, 1956.
28. Volpe, P. and A. Giuditta. Biosynthesis of RNA in neuron- and glia-enriched fractions. *Brain Res* **6:** 228–240, 1967.