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Mitochondrial Protein Synthesis May Be Involved in Long-Term Memory Formation[1]

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FRIDE, E., S. BEN-OR AND C. ALLWEIS. *Mitochondrial protein synthesis may be involved in long-term memory formation.* PHARMACOL BIOCHEM BEHAV **32**(4) 873–878, 1989.—The hypothesis that two qualitatively different stages of cerebral protein synthesis (PS) are required for the formation of long-term memory (LTM) for an active-avoidance task was investigated in rats. Cytoplasmic PS was inhibited with anisomycin (ANI—5.0 mg subcutaneously). When ANI was injected at 15 min pre- and 30 min posttraining, so that cerebral PS was inhibited by 90% for 2 hours starting just before training, LTM formation was prevented. When ANI was given after training, it was not effective. Mitochondrial PS was inhibited with chloramphenicol (CAP—1.5 mg intracisternally). Inhibition occurred 40 min after the injection. CAP interfered with LTM formation only when injected between 15 and 55 min after training. From these data it was concluded that two stages of PS are required for the formation of LTM. The first one takes place in the cytoplasm, starts with the commencement of training and is independent of newly synthesized mRNA. The second stage takes place in mitochondria starting approximately 25 min after training and is dependent upon newly formed mRNA.

Amnesia Rats Chloramphenicol Anisomycin Mitochondria Long-term memory Protein-synthesis inhibition

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THE possibility that protein synthesis in the brain is necessary for the consolidation of long-term memory (LTM) has been the subject of much speculation and many experimental studies. The evidence which supports the hypothesis is derived mainly from experiments in which the inhibition of protein synthesis has been shown to prevent LTM formation. Many authors seem to consider this evidence very strong (8, 21, 22, 42). The inhibitors which have been used are puromycin and acetoxycycloheximide (in the early studies) and cycloheximide (CXM) and anisomycin (ANI) in later studies.

In order to obtain LTM impairment, the administration of the inhibitor must be timed so that the rate of brain protein synthesis is greatly diminished during or shortly following training. When this is done, acquisition is not affected and memory persists for 3–6 hours after training before decaying (2, 3, 16, 38). It is usually inferred from experiments of this kind that protein synthesis during or shortly after training is an essential step in the sequence of neurochemical events which leads to the formation of a stable memory trace some hours later.

An important contribution to this problem was made by Bennett *et al.* (4). By using anisomycin, a rapidly-acting protein synthesis inhibitor, they found evidence which suggested that the inhibitor-sensitive step in protein synthesis, which was essential for LTM formation, was based on a messenger RNA template which was already in existence at the time of training. It thus appears that ongoing protein synthesis based on existing (not training-specific) mRNA is required during or shortly after training in order for LTM to be formed some time later.

On the other hand, de novo mRNA synthesis has been shown to be essential for LTM formation. This was done by using RNA synthesis inhibitors [camptothecin in goldfish (35) or 2,6-diaminopurine (DAP) in rats (29)]. In the DAP studies it was found that in order to block LTM formation, RNA synthesis had to be inhibited by 60% during the period lasting from 15–60 minutes after training. The newly synthesized mRNA formed during this period presumably participates in LTM formation by acting as a template for protein synthesis. However, the protein synthesis inhibitors mentioned above failed to prevent LTM formation if they were given so as to effectively inhibit protein synthesis occurring after the start of the DAP-sensitive period (2, 3, 16, 38). Thus, the postulated stage of de novo protein synthesis which is dependent on DAP inhibitable mRNA synthesis seems to be insensitive to CXM, AXM and ANI.

In order to resolve this paradox, we formulated the following working hypothesis. Two distinct temporally and spatially separated phases of protein synthesis are involved in LTM formation.

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The early phase takes place on cytoplasmic polyribosomes and does not require newly synthesized mRNA as its template. The second phase takes place on mitochondrial ribosomes and must be preceded by de novo mRNA synthesis.

Cytoplasmic protein synthesis is known to be inhibited by CXM (25,28) and ANI (24), which were used in most studies of LTM formation, whereas mitochondrial protein synthesis in known to be inhibited by chloramphenicol (CAP) but not by CXM (9, 25, 28, 39).

If our hypothesis is correct, CAP administration should produce LTM amnesia only if it is effective during the period that follows the period of sensitivity to DAP; when given earlier (so as to effectively inhibit mitochondrial protein synthesis only during or shortly after training) it should be ineffective.

In this study we compared the amnesia-inducing effects of ANI and CAP which inhibit cytoplasmic and mitochondrial protein synthesis respectively, at different times during and after training.

METHOD

Animals

Male rats of the local SABRA strain weighing 120–150 g were used. The intracisternal (IC) injection volumes were always 10 μl. Control animals received 10 μl of saline (29). Anisomycin [ANI, 2-p-methoxyphenyl-3-acetoxy-hydroxy-pyrrolidine] (5 mg) was injected subcutaneously at the back of the neck in 2 ml of saline (17).

Drugs

Anisomycin, 2-p-methoxyphenyl-3-acetoxy-hydroxy-pyrrolidine (ANI, a generous gift from Charles Pfizer Co., Groton, CT), was dissolved in an equimolar amount of HCl and the pH adjusted to between 6 and 7 (17). Chloramphenicol (CAP, synthomycetine succinate, Abic Ltd., Ramat Gan, Israel) was dissolved in 0.9% NaCl. [4,5-^{3}H] L-leucine (2 Ci/mmol) and [1-^{14}C] L-leucine (60.4 mCi/mmol) were obtained from the Nuclear Research Centre, Negev, Israel. All solutions were freshly prepared on the day of the experiment.

Behavioral Apparatus and Procedures

The shock avoidance apparatus described by Frieder and Allweis was used (19). It consisted of a wooden box 91 × 25 × 20 cm high divided into two equal compartments by a vertical sliding door. One half of the box was painted black and the other painted white. On the bottom of the box there was a grid floor which could be electrified. The current used was a 0.23 mA (RMS) 50 cps sine wave.

At the start of training, the rat was placed in the white side with the door closed. After a one-minute delay, the door opened automatically and the rat was then transferred to the black side by hand. Seven seconds later, a relay closed and the rat received a one-second electric shock. This trial was scored as a failure. [If the rat did not run to the safe (white) side after the first shock, further shocks of 1-sec duration were given at 1-second intervals until it did so.] Subsequently, running to the safe side within 7 seconds of being transferred to the black side, and thus avoiding shock, was scored as a successful response. Training was continued till the rat achieved a series of five successful responses out of five trials (5/5 criterion). The number of trials to reach the criterion TTC (excluding the 5 successive successful trials) was scored. Rats which during training had TTCs less than three or greater than 16 (22% of the animals trained) were excluded from the experiment. Pretraining injections—saline or drug—did not affect acquisition as compared to untreated rats. The average training TTC for all animals trained in this project was 7.8 ± 3.1 SD (n = 450). Each control or treatment group consisted of 12 rats.

Test Procedure and Statistical Treatment of Behavioral Data

Testing for memory retention was done in the same manner as training, 90 minutes or 5 hours later. Test TTCs of treated animals and controls were compared using the Mann-Whitney nonparametric one-tailed U-test to determine if memory was impaired by the treatment or not.

The behavioral data were standardized to eliminate the effect of unavoidable variations from experiment to experiment. The performance of the experimental group was expressed as % Memory Effectiveness (% ME) by relating the test data ($TTC_{exp.}$) to their controls in accordance with the formula:

$$\%\,ME = \frac{TTC_{naive} - TTC_{experimental}}{TTC_{naive} - TTC_{control}} \times 100$$

where TTC_{naive} is the average training TTC for untreated animals (which was 7.6 ± 3.1, n = 202), $TTC_{control}$ represents the average test TTC for the control (saline-treated) animals and $TTC_{experimental}$ represents the test TTC for the drug-treated animals in a particular experiment.

It may readily be seen that this formula positions experimental groups along a dimensionless scale. If the experimental procedure has no effect on retention then the experimental group will be scored as 100% retention. If the experimental procedure prevents retention completely then the experimental group will be scored as zero% retention. Partial effects of the experimental procedure on retention will result in scores distributed proportionally along this scale. Small variations in the absolute values and in the decrease in TTC due to training between different control groups are corrected for by this procedure. It is similar to the well-known "percent savings" score often used in behavioral research.

Biochemical Procedures

In order to assess the inhibition of mitochondrial protein synthesis under the influence of CAP, the incorporation of amino acids into mitochondrial proteins in vivo was determined by a double labeling method. For each experimental point, 4 animals were included: one pair of animals (the "control pair") were both injected with saline and each received a different label. The two brains were pooled and their mitochondria were then isolated. One of the remaining 2 animals (the "experimental pair") was injected with saline, and the last rat received CAP. Each member of this experimental pair received a different label, which was alternated with each successive experiment. The dosage of CAP and time of measurement were those which had been shown to produce amnesia; 1.5 mg of the drug given 15 min after the end of training. Ten or 55 min after the intracisternal injection of CAP, [4,5-^{3}H] L-leucine and [1-^{14}C] L-leucine (20 μCi/10 μl/rat and 5 μCi/10 μl/rat respectively) were injected intracisternally.

Thirty minutes after receiving the tracer, the animals were killed by cervical dislocation and their forebrains were immediately removed and placed in cold (4°C) 0.32 M sucrose in 10 mM tris-buffer (pH 6.5–7.4). This tissue was immediately homogenized by 10 up and down strokes in a Dounce homogenizer using an "A" pestle with a clearance of 0.025–0.040 mm. The tissue was then further homogenized with 10 strokes of a "B" pestle (0.013 mm clearance).

For the incorporation assay on whole forebrain tissue, 0.5 ml aliquots of the homogenate were precipitated with cold 10% $HClO_4$ and centrifuged in a Sorvall RC-5 superspeed centrifuge (8000 × g for 10 min). From the supernatant, 0.5 ml aliquots were

counted in 10 volumes of toluene containing 4 g/l PPO, 0.5 g/l POPOP and 330 ml/l Triton. The precipitate was drained for 60 min and then dissolved in NaOH (final concentration 0.1 N). Aliquots were removed for protein determination (31) and incorporation of labelled amino acid into protein was measured in 10 volumes of scintillation fluid.

Forebrain mitochondria were isolated by centrifugation on a discontinuous sucrose gradient. Perikaryal (PE) and intraterminal (I) mitochondria were isolated separately using the method of Whittaker and Barker (41) with modifications (25,33). After sampling for whole tissue determinations the remaining homogenates from two differently labelled brains were combined and centrifuged at 1000 × g for 11 min. The supernatant was removed and the pellet was washed twice by resuspension in 0.32 M sucrose and resedimentation at the same speed. The combined supernatants were divided into two equal parts for the isolation of fractions PE and I. For the PE fraction, the supernatant was centrifuged at 17000 × g for 60 min yielding a pellet which was suspended in 0.32 M sucrose and subsequently layered over a sucrose gradient consisting of equal volumes of 1.2 M and 0.8 M sucrose which was prepared 3–4 hours in advance. The gradient was then spun at 53000 × g for 120 min (Beckman Model L2-75B ultracentrifuge using a SW27 rotor). The pellet (PE) fraction was suspended in 0.32 M sucrose. For fraction I, the supernatant from the triple 1000 × g sedimentation was spun at 12000 × g for 120 min. The pellet was dispersed in water and the suspension was layered over a gradient consisting of equal volumes of sucrose, 1.2 M, 1.0 M, 0.8 M, 0.6 M and 0.4 M. After centrifugation at 63000 × g for 120 min in a SW 25.1 rotor, the I pellet was suspended in 0.32 M sucrose. Proteins of both pellets were precipitated and assayed for amino acid incorporation and protein content as was done for whole forebrains.

The specific activity (SA) of the protein in dpm/mg, following correction for the different specific activities of the injected amino acid and the variation in the SA of the precursor amino acid pool in different brains is a measure of protein synthesis.

The free-labelled amino acid values were used to correct for interindividual variations in the availability of free amino acids in the brain (12) according to the method introduced by Hyden and Lange (27). This method was originally used for small brain areas and subsequently for large brain regions (37) and for whole brain (12). Application of this correction factor which was referred to as "amino acid concentration" presumes a constant ratio between free-labelled amino acids and the measured specific activity (27). In our study we also found a linear relationship between free labelled amino acids and specific activity in whole brain (not shown). Hence inhibition of protein synthesis was calculated as outlined below, using the following formulae sequentially:

Specific Activity

$$SA = \frac{\text{DPM Bound (mitoch)}}{\text{mg Protein (mitoch)}}$$

Relative SA:

$$RSA = \frac{SA}{\dfrac{\text{DPM free leucine in whole brain}}{\text{mg protein in whole brain}}}$$

Amino-acid incorporation

A) Control Pair:

$$INC(C) = \frac{RSA(^3H,SAL)}{RSA(^{14}C,SAL)}$$

B) Experimental pair:

$$INC(E) = \frac{RSA(^3H,SAL)}{RSA(^{14}C,CAP)} \text{ or, } INC(E) = \frac{RSA(^{14}C,SAL)}{RSA(^3H,CAP)}$$

Incorporation ratio control/experimental

$$INC(E) = \frac{INC(C)}{INC(E)}$$

$$\%\ \text{Inhibition} = [1 - INC(C/E)] \times 100$$

For example, if the specific activities of the control pair of ^3H and ^{14}C-leucine-injected rats were 0.24 and 0.28 respectively; the specific activities of the control and CAP-injected rats of the experimental pair were 0.26 and 0.20 respectively and the ^3H and ^{14}C-leucine 'concentrations' were 1.2 and 0.7 respectively, then:

$$INC(C) = \frac{0.24/1.2}{0.28/0.7} = 0.50$$

$$\text{and } INC(E) = \frac{0.26/1.2}{0.28/0.7} = 0.76$$

Hence, % Inhibition = (1–0.50/0.76) × 100 = 34%.

RESULTS

Biochemical

With respect to the effect of ANI on cerebral protein synthesis we relied on the data reported by Bennett *et al.* (5), and that of Flood *et al.* (17) who showed that, within 15 min after a single subcutaneous injection of ANI in rat (5 mg) and mice (0.5–3 mg), brain protein synthesis is inhibited by 90% for 1–2 hours. This inhibition may be prolonged in mice by giving an additional injection of ANI (17) and we assume that this applies equally well to rats.

The degree of mitochondrial protein synthesis inhibition following CAP administration is shown in Table 1. It may be seen that 40 min after CAP administration, amino acid incorporation into perikaryal mitochondria is inhibited by 44%; for intraterminal mitochondria the inhibition was 36%. Eighty-five min after CAP no inhibition was seen; rather, an augmentation of amino acid incorporation was observed.

Behavioral

Figure 1 describes % ME as a function of the time of injection, where zero-time denotes the end of the 15-min training period.

The Effect of ANI on LTM and MTM

It has been shown that a single injection of ANI 15 min before the start of training results in a 90% inhibition of protein synthesis starting during training and lasting for one hour (5). From Fig. 1 it can be seen that a similar injection (i.e., −30 min in Fig. 1) caused a moderate impairment of LTM (ME = 63%), which was not statistically significant. In order to prolong the period of protein synthesis inhibition, two injections of ANI were given; the first one 15 min pretraining and the second 30 min posttraining (i.e., at −30 and +30 min). This treatment resulted in a significant impairment of LTM (ME = 32%, $p<0.01$).

The fact that ANI has to be active during training in order to interfere with LTM consolidation was shown by giving 2 injec-

TABLE 1

THE EFFECT OF CAP ON THE IN VIVO INCORPORATION OF ^{3}H LEUCINE INTO PERIKARYAL (PE) AND INTRATERMINAL (I) MITOCHONDRIA

Time		Relative SA* ± SEM [4,5^{3}H] Leu + Sal	[1^{14}C] Leu + Sal	[4,5^{3}H] Leu + CAP	[1^{14}C] Leu + Sal	% Inh
40 min	PE	0.48±0.03 (4)	0.25±0.02 (3)	0.20 (1)	0.18±0.01 (2)	44
	I	0.44±0.03 (4)	0.25±0.02 (3)	0.20 (1)	0.16±0 (2)	36
85 min	PE	0.33±0.02 (3)	0.15±0.02 (4)	0.42±0.13 (2)	0.36 (1)	−72
	I	0.28±0.03 (3)	0.14±0.03 (4)	0.29±0.04 (2)	0.33 (1)	−35

*See the Biochemical Procedures section for definitions of Relative Specific Activity (RSA) and % Inhibition.

The number of animals used for each determination is given in parentheses below the relative SA figures. Measurements were made at 40 and 85 minutes after the injection of CAP. The last column gives the % inhibition.

tions of ANI 15 and 75 min after training. As seen in Fig. 1, this treatment did not affect LTM formation (ME = 108%). These results, indicating that LTM formation is impaired only if ANI inhibits protein synthesis during training, are in accordance with those obtained by others (15,16).

In order to determine whether the amnestic effect of ANI is specific for LTM, we gave two injections of ANI (15 min pre- and 30 min posttraining), but tested for memory 90 min after training when only medium-term memory (MTM) is present (19). In that experiment there was a trend toward impairment of MTM formation (ME = 43%, not significant).

The Effect of CAP on LTM

CAP was injected 55, 35, 25 or 15 min before training or 0, 15, 35, 55, 75 or 105 min after training at doses of 0.5 mg or 1.5 mg. The only times which resulted in a significant impairment of memory were 15, 35 and 55 min after training. A larger dose (4.5 mg) neither increased the extent of amnesia, nor did it prolong the period of susceptibility to CAP.

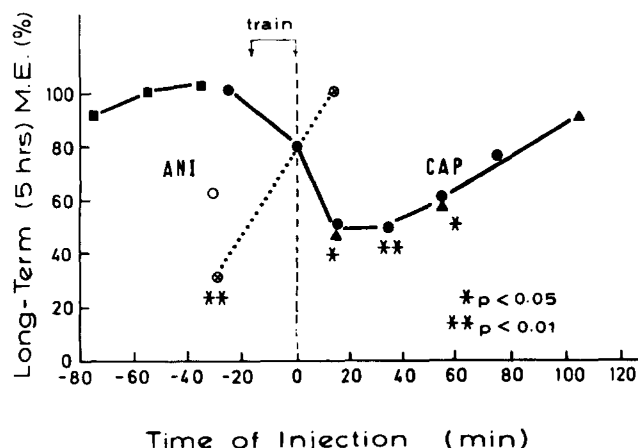

FIG. 1. The susceptibility of LTM to injections of ANI or CAP as a function of the time of injection. Doses used: ○–5 mg ANI; ⊗—2 × 5 mg ANI given with an interval of 1 hr between injections; ■—0.5 mg CAP; ●—1.5 mg CAP; ▲—4.5 mg CAP.

The Effect of CAP on MTM and Performance

The specificity of CAP's effect on LTM was tested in the following two experiments. CAP (1.5 mg) was injected at a time which had been shown to result in impairment of LTM (15 min after training), However, in this experiment, animals were tested for MTM [90 min after training (19)]. It appeared that MTM was not affected by this treatment (ME = 90%). CAP's effect on performance was tested by administration of 1.5 mg of the drug at a time after training when LTM formation was no longer susceptible to CAP (135 min after training). The injection-to-test interval was the same as the amnesia-inducing experiments (275 min). The results showed that performance was not affected (ME = 116%).

DISCUSSION

The observation that CAP inhibited amino acid incorporation into mitochondrial protein 40 min after injection is in accordance with a previous report on mitochondrial protein synthesis after intracranial injection of CAP in chicks (20).

Cerebral mitochondria synthesize 15–25% of their protein themselves, while the remainder is imported from the cytoplasm (13,32). Barondes and Cohen (1) found a reduction in mitochondrial protein after intracerebral injection of cycloheximide (85%) and puromycin (57%) in mice. Since cycloheximide inhibits only cytoplasmic protein synthesis (25,28), the decrease in mitochondrial protein after cycloheximide must have reflected an inhibition of cyctoplasmic protein synthesis.

Puromycin, on the other hand, inhibits both cytoplasmic and mitochondrial protein synthesis (39). Hence, the reduction in mitochondrial protein after puromycin probably resulted from the combined inhibition of both classes of protein synthesis. Thus, overall, Barondes and Cohen's data cannot determine the relative contributions of cytoplasmic and mitochondrial protein synthesis in LTM formation.

The observation that the inhibition (36–44%) we observed after CAP administration was about 25% greater than the anticipated 15–25% suggests that CAP acted through more than one mechanism. It is known that CAP also inhibits mammalian DNA synthesis (43). However, the possibility that CAP exerted its amnestic effect through DNA synthesis inhibition may be excluded since it has been shown that DNA synthesis is not involved in memory formation (6,12).

It has been noted that CAP interferes with cell respiration by inhibiting NADH oxidation (18) and that the synthesis of the cytochromes a, b and c, which are essential for cell respiration and which are all mitochondrial products, is inhibited by CAP (14). It is therefore possible that interference with cell respiration is a secondary effect of mitochondrial protein synthesis inhibition. Whether CAP affects LTM through interference with mitochondrial protein synthesis alone and/or through decreasing cell respiration cannot be determined from the data obtained in this study. However, the CAP stereoisomer D-threochloramphenicol also inhibits respiration, but does not inhibit mammalian mitochondrial protein synthesis (18). Therefore this stereoisomer might be used to determine whether CAP affects LTM through protein synthesis inhibition (in which case, the isomer will not produce amnesia) or by interfering with cell respiration (in which case it will produce amnesia).

We confirmed previous reports that in order for ANI to prevent LTM formation it has to effectively inhibit cerebral cytoplasmic protein synthesis during training (15). CAP, on the other hand,

interferes with LTM consolidation only if it inhibits mitochondrial protein synthesis by 30–40% starting after training. CAP has no effect on LTM if it is active during training.

We interpret these results as indicating that there are two phases of protein synthesis which are required for LTM formation. These phases of protein synthesis differ qualitatively from each other: the first one takes place on the cytoplasmic ribosomes, whilst the second phase takes place on mitochondrial ribosomes. It would appear that both phases are essential for LTM formation since when only one of them is inhibited, LTM formation is impaired. After this work was completed, a study was published (7) which also reported that posttraining injection of CAP inhibited LTM formation in rats. No attempt was made in that study to determine the critical time of CAP injections with respect to training on LTM formation or protein synthesis. However, since CAP was given just after training, the sensitivity of LTM formation to this treatment could well correspond to the period of CAP's effective inhibition of mitochondrial protein synthesis in our experiments.

The necessity for two stages of protein synthesis for the formation of LTM, the first one occurring during training and the second one later, has been proposed previously (23, 26, 34). However, we suggest that the second stage revealed in those studies is qualitatively different from the second stage of protein synthesis on mitochondrial ribosomes, which we are proposing.

Thus, Hyden isolated a soluble protein fraction 8–24 hr after training (26). However, proteins produced by mitochondria are insoluble (10, 13, 36). Matthies *et al.* (34) also differentiated between two stages of protein synthesis. During the first phase, occurring during acquisition, soluble proteins were formed, whereas 6–8 hours after training, insoluble peptides were synthesized. Thus, the second stage could have included mitochondrial protein formation. However, the contribution of protein synthesis by the mitochondria to that of total brain tissue is only 2–5% (10,32), whereas the incorporation studies by Matthies *et al.* were performed on whole, nonfractionated tissue. Therefore, changes in protein synthesis rates, confined to the mitochondria, would probably have gone undetected in their studies. Moreover, in a later study (23), it was reported that both stages of protein synthesis were sensitive to ANI. Hence, it is clear that their second phase of protein synthesis does not correspond to the second stage which we have observed in our experiments and which we ascribe to mitochondrial protein synthesis.

Kobiler and Allweis (29,30), using the same behavioral paradigm and DAP to inhibit RNA synthesis, showed that RNA synthesis is required for LTM formation and that it normally takes place between 15 and 60 minutes after training. This RNA synthesizing phase thus follows the CXM-ANI sensitive phase of protein synthesis, but probably precedes the mitochondrial CAP-sensitive protein synthesizing phase which is also required for LTM formation as revealed in this study. We therefore speculate that RNA synthesized between 15 and 60 minutes after training is used immediately afterwards by the mitochondria in order for them to produce proteins required for LTM consolidation. The requirement for newly synthesized RNA for memory-related mitochondrial protein synthesis is supported by the observation that in mammalian mitochondria, CAP inhibits newly-induced protein synthesis rather than ongoing protein synthesis (40).

[Since the present study was completed, Giuditta *et al.* (Pharmacol. Biochem. Behav. 25:651-658; 1986) reported decreases in DNA specific radioactivity in the mitochondrial fraction isolated from brain tissue of rats immediately after they had learnt a reverse handedness task. Although these data may not have any relevance to the formation of long-term memory, the involvement of mitochondrial activity in a cognitive process is consistent with our findings.]

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