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Abstract

Mice were trained in an avoidance learning task. The incorporation of 3H-leucine into the hippocampal regions of trained mice was higher than that of control mice. When mice were injected with cycloheximide, a strong inhibitor of protein synthesis, impairment was evident in acquisition of learning. Cycloheximide produced morphological changes in mitochondria and microtubules of some brain axons. It is suggested that the cycloheximide-induced learning impairment may be due to the blocking of the synthesis of the specific protein necessary of neutral conductivity.

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THE EFFECT OF CYCLOHEXIMIDE ON MOUSE LEARNING

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Abstract. Mice were trained in an avoidance learning task. The incorporation of ^{3}H-leucine into the hippocampal regions of trained mice was higher than that of control mice. When mice were injected with cycloheximide, a strong inhibitor of protein synthesis, impairment was evident in acquisition of learning. Cycloheximide produced morphological changes in mitochondria and microtubules of some brain axons. It is suggested that the cycloheximide-induced learning impairment may be due to the blocking of the synthesis of the specific protein necessary for neural conductivity.

Many investigations have been performed in the past decade for biochemical correlates of learning and memory formation. Yanagihara and Hydén (1) recently reported an increase in specific protein in the hippocampal nerve cells of trained rats. Squire and Barondes (2) have presented some evidence that mice memory was impaired by inhibiting protein synthesis. Cycloheximide (CHX) and puromycin are potent inhibitors of protein synthesis and if given shortly before brief training, mice learn at a normal rate but forgetting occurs at a rapid rate after training (3). These drugs also have other effects on mice behavior (4). In contrast to the Squire and Barondes findings, Gervai and Csányi (5) reported that CHX did not produce an impairment of memory though protein synthesis was almost completely inhibited. Other investigators suggested that memory was impaired by inhibiting the synthesis of neuronal transmitters (6, 7).

The main purpose of the present invesigation was to examine whether protein is actually synthesized *de novo* during learning and how the drug affects learning.

MATERIALS AND METHODS

Chemicals. Cycloheximide (CHX) was purchased from Nakarai Chemicals Industries (Japan) and dissolved in 0.15 M saline.

Animals. ddN strain mice with high training scores were selected. Their offsprings were also selected by training scores until the fourth F generation. Animals weighing 20-30 g were used. They were habituated to handling for 3 min each day for 3 successive days before the experiments.

Adaptative behavior and body weight. Adaptative behavior was observed in a training apparatus with the floor area divided into nine squares. The number of squares mice entered (crossovers) during each 5 min epoch was counted. The animal body weight was measured after drug injection at the scheduled time for 7 consecutive days. The body weight was quantified as a percentage of initial weight.

Training apparatus and methods. The training box was divided into two sections with a common electric grid floor. Each section was $30 \times 30 \times 30$ cm and the walls were made of plastic board. An escape net was attached 10 cm above the floor and completely encircled one of the compartments. The second compartment did not have an escape net but was otherwise identical to the first compartment. A buzzer was attached to the outside of the box in the middle of the apparatus and a light was mounted above the buzzer and illuminated the two sections equally. Mice trained individually were given 5 min to explore the compartment and escape net. The number of crossovers during that time were recorded. The light and buzzer (conditioned stimuli, CS) were presented for 3 sec. CS termination was followed by the onset of electric current through the grid as shock. The current continued until the animal jumped from the floor to the escape net. The animal was allowed to remain in the net for 20 sec. The animal was then placed back on the grid floor for the next trial. The average interval from the start of one trial to the start of the next trial was approximately 30 sec. Animals were judged to have made an avoidance response if they jumped to the escape net before the onset of the electric shock stimulus. The apparatus and training method were essentially identical to those of Zemp *et al.* (8).

Two types of controls were used in the study of protein synthesis during the learning task. (a) A yoke control mouse was kept in the second compartment while the experimental animal was being trained. Yoke control mice were exposed to the light buzzer and electric current shocks at the same time as the trained animals and were handled the same amount of time but in a random manner. (b) Quiet control (non-shocked) animals were kept in individual cages and undisturbed as much as possible after injection. Training was terminated when trained animals had 60 trials which required 30 min. The second training period was performed 1 week after the first training period, the third training period 2 weeks after the first training period and the fourth training period 3 weeks after the first training period. The training scores were obtained by summing up the scores of each block of 10 trials and calculating the scores to percentages.

Drug injection. It is reported (6) that after subcutaneous treatment with 120 mg/kg CHX, mice developed amnesia of the learned behavior. Segal, Squire and Barondes (9) found learning impairment in mice by intracerebral injection of 200 μg CHX. In the present study mice were injected intraperitoneally with 0.5 ml of CHX (0.5 mg or 1.5 mg per 20 g body weight) before or after training. Intracerebral injections were performed under light ether anesthesia at the indicated times before or after training. The bregma was exposed and a small

hole was drilled 2.0 mm caudal to the bregma and 2.0 mm lateral to the sagittal suture on each lateral side. A fine needle was inserted perpendicularly through each hole to a depth of 3.5 mm, and 5 μl of 100 μg of CHX was slowly injected. The scalp was closed by surgical stitching. The animals recovered from anesthesia within 10 min after the procedure. CHX was administered 1 week, 2 days and 2 hr before the first training session, and just after the first training session or after complete learning had occurred. Control mice received the same volume of 0.15 M saline. Stereotaxic coordinates (10) were used to determine the site of injection.

Measurement of protein synthesis. Inhibition of cerebral and liver protein synthesis was estimated by determining the extent of ^{3}H-leucine incorporation into the brain or liver. Fifty microcuries of DL-[4, 5-^{3}H] leucine (22 Ci/m mol, Radio Chemical Centre, Amersham) in 0.5 ml of 0.15 M saline was injected intraperitoneally 5 min before training. Three animals (a trained mouse, a yoke control mouse and a quiet control mouse) were used in one test as a trio group. After a brief training session of 30 trials for 15 min, mice were killed by a guillotine apparatus, and the brain of each animal was removed from the skull in a cold room. The liver was also collected. The individual organs were homogenized in 5 ml of 10% trichloroacetic acid (TCA) containing 1 mM leucine. The precipitate was further homogenized in 5% TCA for protein determination and rate of amino acid incorporation. The aliquots finally obtained were analyzed for protein by the method of Wannemacher, Banks and Wunner (11). One milliliter aliquots were dissolved in 10 ml of Bray's dioxane scintilator solution (12) and counted in an Aloka liquid scintilator. Finally, specific activity (dpm/mg protein) was calculated.

Radioautographic preparation. Three animals, (a trained mouse, a yoke control mouse and a quiet control mouse) were used in one test as a trio group. The trained mouse was injected 50 μCi of ^{3}H-leucine in 0.5 ml of 0.15 M saline. Yoke control and quiet control animals received the same volume of ^{3}H-leucine at the same time. Five minutes after injection of ^{3}H-leucine, mice were trained in the avoidance learning task. After brief training of 30 trials for 15 min mice were killed by the guillotine apparatus, and the brain of each animal was removed for histological preparation. Brains were fixed in Carnoy's solution. Following fixation and dehydration, the brain was embedded in paraffin, sectioned horizontally and prepared for radioautography. The procedures followed were those of Dropp and Sodetz (13). The exposure time was 6 weeks. Coronal and sagittal sections were also obtained in the same way.

Electronmicroscopic observation. Mice were killed 1 week, 2 days or 2 hr after intracerebral injection of CHX. Brains were removed and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, and slices of the cortex or hippocampal regions were rinsed in buffer and cut into 1 mm blocks. The block was post-fixed for 1 hr in a 1% solution of osmium tetroxide in phosphate buffer. The tissue was embedded in Epon, the sections were double-stained on grids with uranyl acetate and lead citrate, and were examined by a Hitachi Hu-12 electron microscope.

Effect of CHX on cell division. For study of CHX effects on cell cycle and mitotic spindles, the drug was administered to sea urchins. Sea urchins were collected in summer. The injection of 0.5 ml of 0.5 M KCl solution into the body cavity of sea urchin caused a prompt discharge of gametes through the genital pores. A few drops of diluted sperm were added to freshly shed eggs suspended in filtered sea water. Five minutes later, the eggs were examined for formation of fertilization membrane. If fertility was lower than 98% in 200 eggs, the batch was discarded. CHX was dissolved and diluted in filtered sea water. The final drug concentrations were adjusted to 10^{-4} M, 10^{-3} M and 10^{-2} M. The eggs were treated at different stages of cell division. CHX was added at the time cleavage furrow occurred, when mitotic apparatus appeared and 10 min before insemination. The number of two cell stages was counted every 5 min after fertilization until the first division of control eggs (in sea water only) was completed.

RESULTS

Adaptative behavior and body weight. Fig. 1 and Table 1 show data obtained by administration of CHX by different routes. The intraperitoneal injection of 0.5

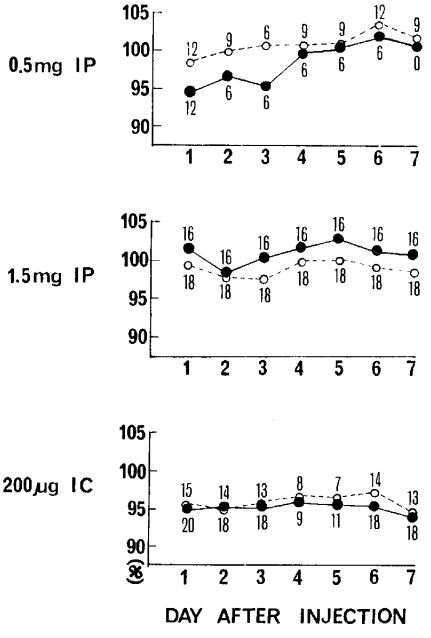


Fig. 1. Body weight changes after injection of CHX. The body weight after injection of CHX is shown as a percentage of initial weight. Top, After 0.5 mg of CHX per 20 g body weight was injected intraperitoneally. Middle, After 1.5 mg of CHX per 20 g body weight was injected intraperitoneally. Bottom, After 200μg of CHX per 20 g body weight was injected intracerebrally. Control mice were injected with the same volume of saline. ●, Saline mice; ○, CHX mice. The number indicates the animals tested per group.

TABLE 1. CROSSOVERS DURING THE 5 MIN EPOCH AFTER INJECTION OF CHX

Groups	Intact group	Route									
		Intraperitoneal dose						Intracerebral dose			
		0.5 mg CHX		Saline		1.5 mg CHX		Saline		200 μg CHX		Saline	
	N M±SD	N	M±SD	N	M±SD	N	M±SD	N	M±SD	N	M±SD	N	M±SD
No treatment	40 69±32.6												
Time after CHX													
2 hours		14	66±22.9	6	44±11.7	6	71±16.6	6	72±28.7	16	72±50.4	20	63±47.5
7 days		12	50±26.4	5	48±10.3	6	60±36.2	6	63±25.7	16	82±36.9	20	75±27.2

The volume of saline administered was the same as the CHX volume. The number of crossovers during 5 min epoch are shown.

TABLE 2. INCORPORATION OF ^{3}H-LEUCINE INTO BRAIN AND LIVER

	Treatment									
	Intact		CHX 2 day before		CHX 2 hours before		Saline 2 day before		Saline 2 hours before	
	N	M±SD	N	M±SD	N	M±SD	N	M±SD	N	M±SD
Brain										
Trained	16	805.3±90.3	5	1540.6±825.6	6	211.4±110.0	5	1239.1±164.0	5	1823.1±1445.8
Yoke	16	693.6±179.0	5	1139.9±224.5	6	173.0±81.0	5	1024.6±184.6	5	1359.4±261.3
Quiet	16	1055.7±415.6	5	1558.9±1052.9	6	181.7±52.8	5	1500.2±345.8	5	1483.1±342.5
Liver										
Trained	16	3587.9±1210.9	5	5303.3±2055.1	6	1107.5±606.1	5	6239.9±1968.1	6	7463.2±2549.3
Yoke	16	3156.7±1267.4	5	5566.1±1807.2	6	1207.9±789.3	5	5160,6±1851.4	6	5478.3±2986.7
Quiet	16	5549.7±2532.3	5	8428.9±2339.2	6	1135.7±280.7	5	7688.8±882.5	6	5548.9±2229.5

CHX or saline was injected intracerebrally 2 days or 2 hr before training session. Some mice remained untreated until training. Five minutes before training session mice in the trio group received an intraperitoneal injection of 50 μ Ci of ^{3}H-leucine. The specific activity (dpm/mg protein) of each group of N animals is shown.

mg or 1.5 mg of CHX produced a slight decrease in body weight within 3 days but recovery occurred to the initial level by 7 days. Mice injected with CHX intracerebrally did not differ in body weight from those injected saline. Administration of CHX did not produce a remarkable decrease or increase in the number of crossovers. Hyperactivity or hypoactivity might be related to the acquisition of learning. The crossovers and training scores of 150 animals were investigated. Crossovers during each 5 min epoch were counted, and mice were trained in the avoidance task. The results are shown in Fig. 2.

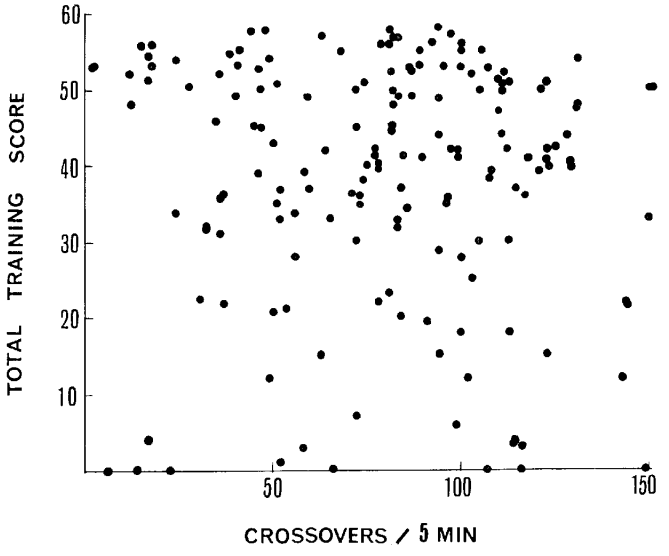


Fig. 2. Correlation between crossovers and total training scores. Adaptative behavior during 5 min was observed in 150 non-treated control animals. Each mouse was trained in a learning task of 60 trials.

Effect of CHX on avoidance task. Mice were divided into three groups. Mice in the first group received 0.5 mg of CHX intraperitoneally 1 week, 2 days or 2 hr before training and just after training. Animals in the second group were administered 1.5 mg of CHX intraperitoneally at the same time interval as the first group. An intracerebral injection of 200 μg of CHX per 20 g body weight was given to mice of the third group 1 week, 2 days or 2 hr before training, just after training and when learning was consolidated.

Mice injected 0.5 mg of CHX intraperitoneally 2 hr before the test (N=14) showed no difference in learning from the saline injected control mice (N=6) in the first training session (Fig. 3). They learned as well as the control mice in the second training session (CHX: N=12; saline: N=5) and in the third training session (CHX: N=6; saline: N=6). Six animals given 0.5 mg of CHX intra-

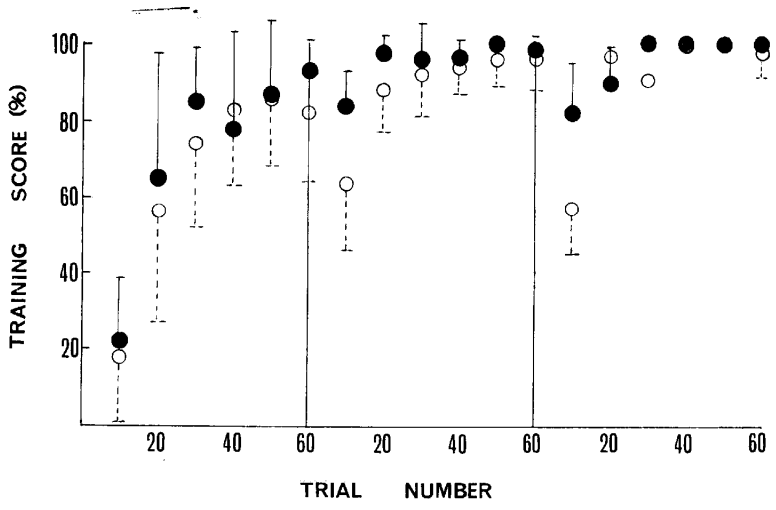


Fig. 3. Training scores of mice after intraperitoneal injection of 0.5 mg of CHX. Intraperitoneal injection of 0.5 mg of CHX was given, and 2 hr later the first training session was undertaken. Left, first training (CHX: N=14; saline: N=16). Middle, second training (CHX: N=12; saline: N=5). Right, third training (CHX: N=6; saline: N=6). ○, CHX; ●, saline. Bars indicate the upper or lower limit of SD.

peritoneally 2 days before the first training session and immediately after the first training session did not differ from the saline animals (N=6) in the first, second and third training sessions. When CHX (1.5 mg) was administered intraperitoneally to 6 animals 2 hr before the first training session, learning was significantly blocked (Fig. 4), but mice exhibited normal acquisition and retention 1 week after injection. Mice injected at the same dose of CHX (1.5 mg, intraperitoneally) 2 days before the first training session (CHX: N=6; saline: N=6) or just after training session (CHX: N=6; saline: N=5) showed learning at the normal rate in the first training and second training sessions.

Fourteen animals injected 200 μg of CHX per 20 g body weight, intracerebrally 2 hr before the first training session, did not learn in the first training session and showed impaired learning in the second training session, but 2 weeks after injection they learned at the normal rate (Fig. 5). Mice injected saline intracerebrally (N=19) also exhibited impaired acquisition of learning but had statistically higher training scores than CHX-injected mice in the first and second training sessions. Mice were injected intracerebrally with 200 μg of CHX per 20 g body weight at different time intervals. If injected 2 days before the first training session (CHX: N=6; saline: N=6), mice did not differ from saline-injected animals in the first, second and third training sessions. The same results were obtained from animals injected CHX 1 week before the first training session

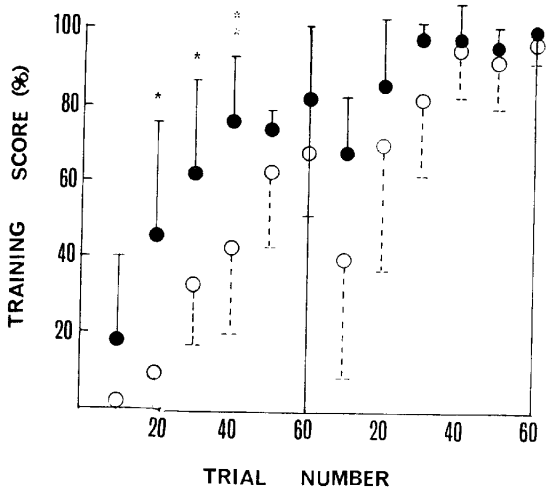


Fig. 4. Training scores of mice after intraperitoneal injection of 1.5 mg of CHX. Training scores were obtained from mice injected 1.5 mg of CHX intraperitoneally 2 hr before the first training session. Left, first training (CHX: N=6; saline: N=6). Right, second training (CHX: N=6; saline: N=6). ○, CHX; ●, saline. * $P<0.05$, ** $P<0.03$

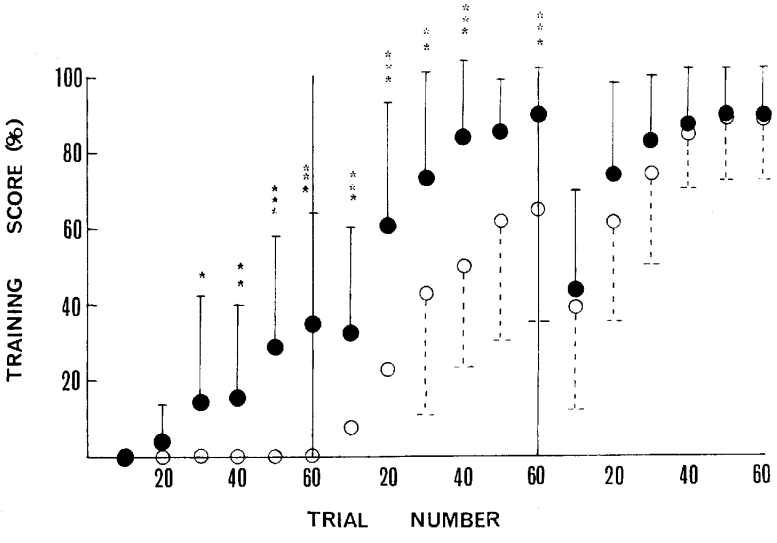


Fig. 5. Training scores of mice after intracerebral injection of 200 μg of CHX. Training scores were obtained from mice injected 200 μg of CHX intracerebrally 2 hr before the first training session. Left, first training (CHX: N=14; saline: N=19). Middle, second training (CHX: N=14; saline: N=19). Right, third training (CHX: N=19). ○, CHX; ●, saline. * $P<0.05$, ** $P<0.02$, *** $P<0.01$

(CHX: N=7; saline: N=7) and just after the first training session (CHX: N=10; saline: N=9).

Mice were trained several times until their training scores were over 50 at each training session. After learning appeared to have consolidated, the animals received the same dose of CHX intracerebrally (CHX: N=6; saline: N=6). The drug at this time did not affect learning in the subsequent training session at 1 week after injection.

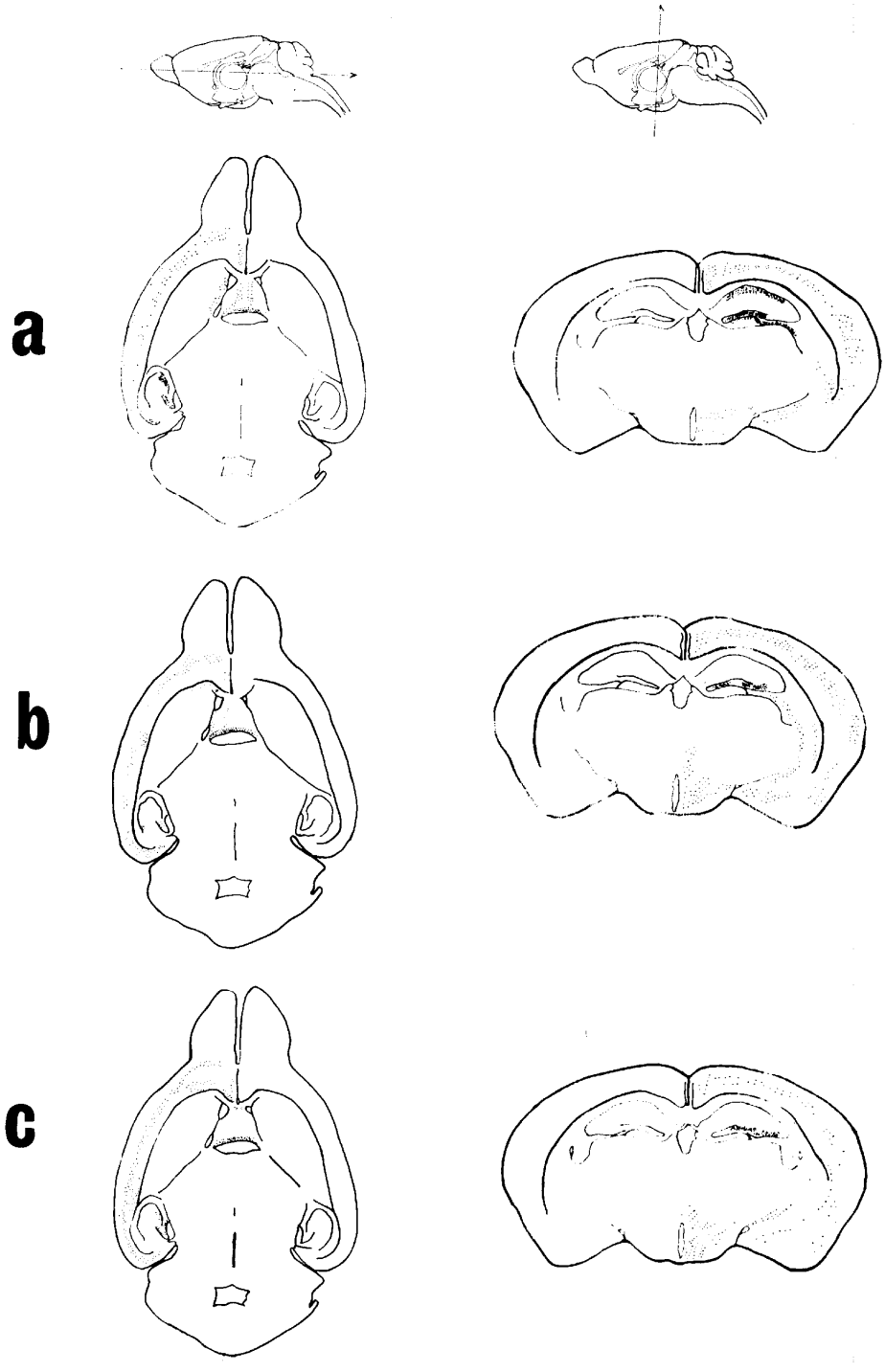
Incorporation of ^{3}H-leucine into brain and liver protein. Preliminary experiments indicated that radioactive leucine was incorporated into brain protein proportional to the dose administered (20 μCi-100 μCi) for 20 min after the intraperitoneal injection of ^{3}H-leucine. During the learning task, a trained mouse and a yoke mouse of the trio group received electric shock as unconditioned stimuli. The ratio of specific activity of incorporated ^{3}H-leucine in trained or yoke animals to that in quiet control animals was favorable because the electric stimuli received by mice in each trio group were non-uniform, varying with the learning performance of the trained mouse. The results are shown in Table 2 and Table 3. Intracerebral injections of CHX (200 μg) produced 80% inhibition of ^{3}H-leucine incorporation 2 hr after injection. During the learning task, the brain incorporation of ^{3}H-leucine in trained mice was higher than that in yoke animals. In animals neither treated by the drug nor saline (intact animals), the isotope ratio of trained or quiet animals to that of yoke animals was high, and the quiet animals

TABLE 3. ISOTOPE RATIO OF TRIO GROUP OF MICE

	Treatment									
	Intact		CHX 2 days before		CHX 2 hr before		Saline 2 days before		Saline 2 hr before	
	N	M±SD	N	M±SD	N	M±SD	N	M±SD	N	M±SD
Brain										
Trained vs Yoke	16	1.30±0.36	5[c]	1.39±0.39	5	1.44±0.44	5	1.23±0.24	5	1.60±0.61
Quiet vs Yoke	9	1.39±0.33	5[c]	1.62±0.44	6[a]	1.19±0.44	5	1.46±0.09[c]	5	1.30±0.29
Trained vs Quiet	9	0.83±0.19	6[a]	0.84±0.18	6	1.14±0.36	5	0.85±0.17	5	1.39±0.61
Liver										
Trained vs Yoke	16	1.07±0.23	5	1.33±0.75	6	1.27±0.71	5	1.27±0.39	6	1.37±0.59
Quiet vs Yoke	9	1.64±0.50	5	1.67±0.73	6	1.43±0.93	5	1.66±0.61	6	1.19±0.53
Trained vs Quiet	9	0.81±0.22	5[a]	0.74±0.14	6[b]	1.04±0.47	5	0.81±0.24[b]	6	1.31±0.55

a $P<0.05$ *b* $P<0.02$ *c* $P<0.01$

The radioactivity data of the brain or liver is shown as a ratio of the ^{3}H-leucine level in trained or yoke to quiet mice of each trio group tested at the same time.


a
b
c

incorporated more ^{3}H-leucine into brain protein than trained mice. The intracerebral administration of the drug or saline 2 days before the training session produced different values of isotope incorporation in yoke mice and quiet mice.

Incorporation into liver protein was also examined (Tables 2, 3).

Radioautographic investigation. Three animals of the trio group were examined for histological study during the learning task. Each trained mouse learned at the normal rate. Their training scores were over 10 in brief training of 30 trials. Fig. 6 shows schematic representations of brain slices. The labelled cells are shown as dots. The sections revealed pronounced labelling in the hippocampus, gyrus dendatus, several cortical areas and various structures in the peripheral regions of the ventricles (Fig. 7). Relatively high radioactivity was detected in the neurons and glial cells of the hippocampus of trained mice. (Figs. 8, 9). No significant differences were evident in the dorso-lateral, lateral and ventral cortices.

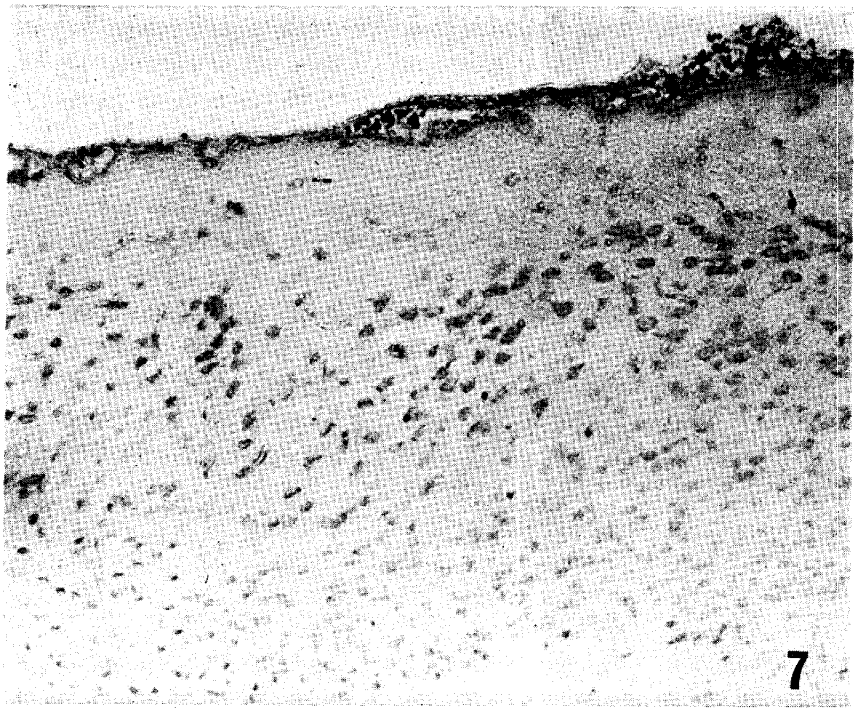
Electronmicroscopy. Electronmicroscopic observations of cells obtained from cortical and hippocampal regions revealed that mitochondria in axons became slightly swollen or elongated 2 hr-2 days after CHX administration (Figs. 10. 11), which was not observed 7 days later. Dispersions of microtubules or condensations of microtubules into irregular aggregates were noted in a few axons 2 hr-2 days after CHX treatment.

Effect of CHX on sea urchin. The mitotic apparatus appeared about 30 min after fertilization and the two-cell stage was observed within 45–75 min in control eggs. When CHX was added 10 min before insemination at a concentration of 10^{-2} M, cleavage was almost completely blocked, and the cells became fused so that the dividing cell appeared to be single celled. A smaller dose of the drug did not affect the first cleavage. If embryos were exposed to CHX at a later stage, just after the mitotic spindles appeared, the eggs showed delayed cleavage. This delay varied with the concentration of the drug administered. The cell division was not inhibited by CHX at the time of cleavage furrow.

DISCUSSION

The mice used in the present experiments showed rapid acquisition of learning. This result is probably due to their genetic constitution. We selected certain lines by sib matings of mice with high training scores. We obtained the ninth F generation in our colony. Squire and Barondes (2) found that the training scores

Fig. 6. Schematic representation of mice brains showing areas where ^{3}H-leucine was incorporated. Silver grains in the cells are shown by the dots. Left, horizontal sections. Right, coronal sections. a, sections from trained mouse; b, sections from yoke mouse; c, sections from quiet mouse. Arrows show the location of the section.



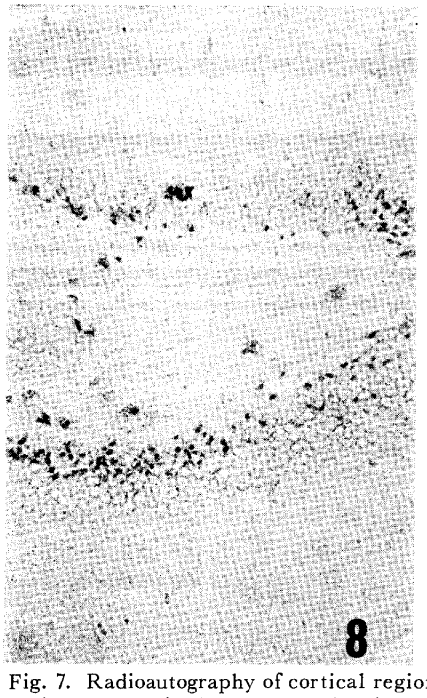


Fig. 7. Radioautography of cortical regions of mouse after ^{3}H-leucine injection. Silver grains are seen in the cells. H-E stain. ×450.
Fig. 8. Radioautography of hippocampal region. H-E stain. ×450.
Fig. 9. Higher magnification of Fig. 8. H-E stain. ×1500.

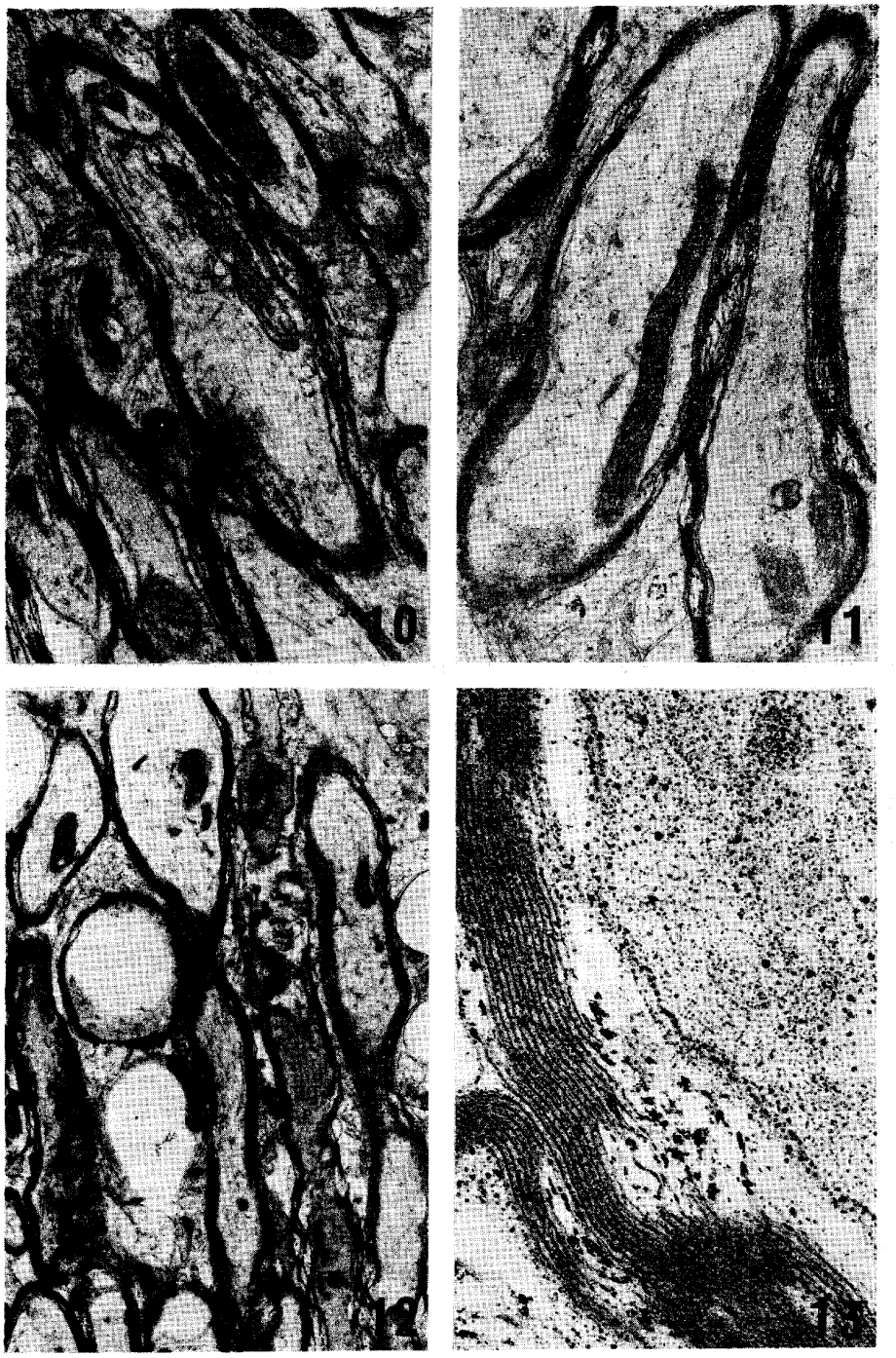


Fig. 10. Electron micrograph of axons of cortex. ×25,000.
Fig. 11. Electron micrograph of axons of hippocampal region. ×25,000.
Fig. 12. Electron micrograph of axons of hippocampal region. ×13,000.
Fig. 13. Electron micrograph of axon of hippocampal region. ×50,000.

of mice in their recent experiments on mice learning were lower than those of their previous study and attributed the discrepancy to differences in the population of the mice used. None of our mice showed any evidence of illness at the testing period after injection of CHX. The correlations between the training scores and crossovers were not significant. CHX administered 2 hr before the first training session impaired learning but once mice learned, they showed learning similar to the saline control mice 1 week after injection. It is suggested that CHX disrupted short-term memory but did not affect long-term memory.

The results obtained from the experiments of ^{3}H-leucine incorporation during the learning task suggest that *de novo* protein synthesis occurs during learning. Protein synthesis was inhibited by CHX and the electric current administered as unconditioned stimuli. MacInnes, McConkey and Schlesinger (14) reported that both CHX and electoconvulsive shock disrupted polysomes. Therefore, it is proposed that a trio group (trained, yoke and quiet animals) should be used in experiments where protein synthesis is measured in learning tasks with an electric shock.

Radioautographically, there was increased labelling of nerve cells in the hippocampal regions of trained mice. The observation indicates that the hippocampus may be the site responsible for short-term memory. This finding is consistent with the results of Pohle and Matthies (15) who administered the radioactive precursor of RNA and protein.

Electronmicroscopic investigation revealed that mitochondria in the brain axons became slightly swollen and elongated, and the microtubules were condensed or dispersed by treatment with CHX. These findings suggest that CHX inhibits microtubules and axonal flow in a similar manner to colchicine. Levitan, Ramirez and Mushynski (16) observed that the radioactivity of ^{3}H-leucine in the synaptosomes of trained rats was higher than that of control animals and suggested the involvement of axonal flow in learning. Metuzals and Mushynski (17) found the neurofilamentous network associated intimately with the cell membrane and nuclear pores in neurons of rabbit brain, while Hydén (18) referred to the involvement of neural surface protein and contractile protein network on the inside of the plasma membrane in neural conductivity. The results obtained from the experiments on the cell division of sea urchins showed that CHX inhibited not only protein synthesis after fertilization but also the synthesis of the specific protein necessary for furrow formation.

In conclusion, the results obtained in the present experments imply that the synthesis of specific protein during learning is inhibited by CHX. It is assumed that CHX inhibits axonal flow and the specific protein network and causes conformational changes of protein and finally produces impairment of learning.

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