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MEMORY IMPAIRMENT DURING PROLONGED TRAINING IN MICE GIVEN INHIBITORS OF CEREBRAL PROTEIN SYNTHESIS

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SUMMARY

When mice were given prolonged discrimination training to escape shock in an automated apparatus shortly after subcutaneous administration of the protein synthesis inhibitor cycloheximide (CXM), acquisition was normal during the initial (15–21) trials. Beyond this point, however, mice given CXM did not continue to improve as rapidly as mice given saline. Similar results were found when training occurred 4 h after intracerebral injection of CXM or puromycin. In contrast, isoCXM which depresses activity like CXM but does not inhibit cerebral protein synthesis had no effect on acquisition. Studies of the effects of different doses of CXM on activity, cerebral protein synthesis, the acquisition effect, and memory 24 h after brief training tended to associate the acquisition effect with inhibition of cerebral protein synthesis rather than to side effects of CXM. The results suggest that impairment in learning observed a few minutes after the beginning of prolonged training is due to inhibition of protein synthesis. Expression of normal memory may depend on cerebral protein synthesis within minutes after the beginning of training.

INTRODUCTION

When mice are given extended training shortly after subcutaneous injection of cycloheximide (CXM), acquisition is normal for about 20 trials but it is impaired beyond this point[7,9]. Impaired acquisition observed when training is continued beyond 20 trials could be due to inhibition of cerebral protein synthesis, which is believed to be required for development of long-term memory[1,6] or to some side effect of CXM on performance. The results of a number of previous experiments using subcutaneous administration of CXM suggested that side effects are not responsible. In the present experiments, isocycloheximide (isoCXM) and puromycin as well as CXM were used to explore further the basis of impaired acquisition. The results suggest that impaired

learning observed when training is continued beyond 20 trials is due to inhibition of cerebral protein synthesis and that cerebral protein synthesis is required for normal memory during prolonged training.

GENERAL METHOD

Subjects

Female mice hybridized from Balb-C females and C3H males were bred in our laboratory and introduced to the experimental situation at 8–10 weeks of age.

Drugs

CXM was obtained from Sigma Chemical Company. Isocycloheximide was synthesized by Dr. Francis Johnson, Dow Chemical Company. It was provided as a white crystalline compound with a sharp melting point. Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corporation. It was dissolved in 0.9% NaCl and titrated to pH 7 with NaOH immediately prior to injection.

Surgery

Intracerebral injections of drugs were performed under ether anesthesia. Injections were placed bitemporally 4 mm below the surface of the skull, 1 mm lateral to the sagittal sinus, and 1 mm anterior to the caudal suture. All drugs were dissolved in saline (0.9% NaCl) and administered in a volume of 10 μl/side. Following injection, openings in the skull were sealed with bone wax to prevent leakage. Subcutaneous injections were made on the backs of the mice using a saline solution containing varying amounts of CXM such that a 20 g mouse received a total volume of 0.24 ml.

Behavior

Learning was conducted in the Deutsch Carousel, an automated discrimination training procedure. The apparatus and training procedure have been described in detail elsewhere[7]. Briefly, a mouse is secured by the tail in the center of a circular platform, facing outward. The mouse is rotated in turn to each of 3 positions on the perimeter of the platform and at each position must touch the smaller of two objects to escape shock. A correct response is scored when the small object is touched before the large one. An incorrect response is scored when the large object is touched before the small one. The intertrial interval is 15 sec. The entire operation is controlled by a PDP/8L computer.

Locomotor activity was monitored in a sound-insulated chamber, 18 in. long, 18 in. wide, and 6.5 in. high, having a grid floor of 3/32 in. rods placed 3/8 in. apart. The floor was electronically divided into quadrants, and crossovers during each 2 min period were recorded automatically.

Protein synthesis inhibition

Inhibition of cerebral protein synthesis was estimated by determining the extent of incorporation of [^{14}C]leucine into brain protein. Four μCi of L-[^{14}C]leucine (51.2

mCi/mmole, Schwartz Bioresearch) was injected subcutaneously. Mice (4 in each group) were killed 30 min later. The brains were removed and homogenized in 5 ml water and a 1 ml aliquot was precipitated with 10% trichloroacetic acid (TCA), heated in a water bath at 90 °C for 15 min, cooled on ice for 1 h, and centrifuged at 10,000 $\times$ *g* for 10 min. One ml aliquots of the supernatant were added to 14 ml scintillant and counted. The insoluble fraction was washed in 5% TCA twice, then ether–ethanol (1:1) and acetone. The precipitate was dissolved in 0.1 ml of 1 *M* NaOH and counted. Results were expressed in terms of TCA insoluble radioactivity/TCA soluble radioactivity. This procedure helps to correct for differences in the specific activity of the precursor pool in the drug-treated and control mice, although the degree of inhibition of cerebral protein synthesis in the drug-treated mice is overestimated slightly[3].

EXPERIMENT 1

The finding[9] that CXM interferes with acquisition beyond 15–25 trials, but that prior acquisition is normal, raised the possibility that a deficit might be detectable earlier if the sensitivity of the experiment were increased. The chance of finding an early difference between CXM- and saline-treated mice was increased by examining the learning curves of a large group of mice given saline (n = 175) or CXM (n = 175) and trained in the Carousel for 15 trials. Other mice were given prolonged training to extend our previous observation that learning is markedly impaired after the initial trials.

Method

Mice (n = 175 in each group) were given 120 mg/kg of CXM or saline subcutaneously 30 min before training for 15 trials in the Deutsch Carousel. The resulting learning curves were divided into fifths (trials 1–3, 4–6, 7–9, 10–12, 13–15) and submitted to a two-way analysis of variance having repeated measures on one factor[10]. For other mice given CXM (n = 46) or saline (n = 45), training was continued until 69 trials were completed, which required about 25 min.

Results

There was no measurable difference between the learning curves of mice trained for 15 trials following CXM or saline (Table I). An analysis of variance revealed a significant trials effect ($F = 18.5$, $P < 0.01$), indicating that performance indeed improved during this brief training episode, but revealed no effect of drug ($F = 0.1$) nor of drug X trials ($F = 2.2$, $P > 0.1$). Thus, no measurable effect of CXM during initial learning could be demonstrated even when the number of animals per group was greatly increased. However, CXM clearly impaired performance when training was continued (Fig. 1). For all 69 trials, the effects of drug ($F = 10.2$) and of drug X trials ($F = 7.3$) are highly significant ($P < 0.01$). These results confirm our previous finding that learning during later trials is impaired by CXM and substantiates as well our observation that learning during the early trials is not measurably impaired by this drug.

TABLE I

EFFECT OF CYCLOHEXIMIDE OR SALINE ON LEARNING DURING THE FIRST 15 TRIALS

Mice (n = 175 per group) were given cycloheximide (120 mg/kg) or saline 30 min before training for 15 trials. The effect of drug ($F = 0.1$) or drug X trials ($F = 2.2$) was not significant ($P > 0.1$).

Trials	*Percent correct*	
	CXM	*Saline*
1– 3	45.9	46.7
4– 6	57.7	52.4
7– 9	54.2	56.6
10–12	55.2	53.0
13–15	59.2	64.8

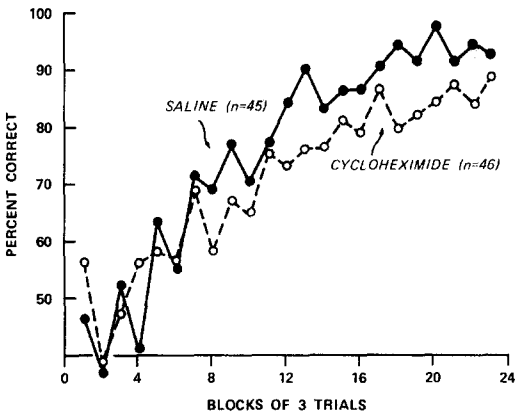

Fig. 1. Mice were given cycloheximide (120 mg/kg) or saline subcutaneously 30 min before training for 69 trials. For all 69 trials, the effects of drug ($F = 10.2$) and of drug X trials ($F = 7.2$) were significant ($P < 0.01$). During trials 1–23, the groups were not measurably different ($F = 0.8$, $P > 0.25$).

EXPERIMENT 2

Since subcutaneous injection of 120 mg/kg of CXM produces hyperactivity 10–40 min after injection[4,5,8] it seemed plausible that the impairment of acquisition with prolonged training might be related to this side effect. To evaluate this possibility we examined the relative effects of different doses of CXM on activity, the acquisition effect, cerebral protein synthesis, and memory 24 h after brief training.

Method

Mice were trained in the Deutsch Carousel as described previously. One group of mice were given CXM (1, 8, 30, or 120 mg/kg) or saline 10 min before training for 69 trials. A second group of mice were given CXM (30 or 120 mg/kg) 10 min before training for 21 trials and retention was measured 24 h later by retraining for an additional 21 trials. Activity effects of different doses of CXM (1, 8, 30, or 120 mg/kg) or saline were determined by injecting groups of mice 10 min before being placed in the

TABLE II

EFFECTS OF DIFFERENT DOSES OF CYCLOHEXIMIDE ON LEARNING, RETENTION, AND LOCOMOTOR ACTIVITY

Mice were given the indicated doses of cycloheximide (CXM) subcutaneously 10 min before training for 21 or 69 trials. Mice trained for only 21 trials were tested for retention 24 h later by training for an additional 21 trials. Activity was monitored by placing mice individually in a sound-insulated box from 10 to 40 min following injection. The data in each column were submitted to an analysis of variance having repeated measures on one factor[10]. Individual comparisons of CXM groups to the appropriate saline group were made with Dunnit's *t*-test[10].

Dose (mg/kg)	*69 Trials*	*21 Trials (n = 23 per group)*		*Crossovers/30 min (n = 7 per group)*
	Trials correct training (n = 45 per group)	*Trials correct training*	*Trials correct retention*	
120	48.9*	10.3	12.7*	159*
30	50.5*	11.7	15.6*	116
8	52.3	—	—	70*
1	53.0	—	—	57*
0	52.4	10.7	17.0	125

* Significantly different from the saline group ($P < 0.02$).

activity chamber for 30 min. Crossovers were monitored automatically during each 2 min period. In an additional group, inhibition of protein synthesis was determined at each of these doses of CXM. Mice were injected with CXM or saline and 10 min later were given [^{14}C]leucine. Thirty minutes later mice were sacrificed and inhibition of protein synthesis was determined as described above.

Results

Effects of CXM on acquisition with prolonged training and on memory 24 h later had similar dose–response relationships which could not be correlated with effects on activity (Table II). A dose of 120 mg/kg produced a deficit in original learning ($P < 0.01$), an impairment in memory 24 h after training ($P < 0.01$) and a marked increase in locomotor activity ($P < 0.01$). A dose of 30 mg/kg produced the learning deficit ($P < 0.02$) and the retention deficit ($P < 0.02$) but did not exert a measurable effect on activity ($P > 0.1$). Lower doses of CXM significantly *decreased* activity ($P < 0.01$) but had no effect on learning curves or on memory at 24 h. Comparison of the results obtained with 30 mg/kg and 120 mg/kg CXM suggested that hyperactivity was not closely correlated with the learning deficit or the retention deficit. The effects of these doses of CXM on cerebral protein synthesis are shown in Table III.

EXPERIMENT 3

This experiment sought to explore further a possible relationship between hyperactivity and the acquisition deficit by establishing conditions whereby extensive protein synthesis is established in the absence of hyperactivity. These conditions are

TABLE III

INHIBITION OF CEREBRAL PROTEIN SYNTHESIS BY SUBCUTANEOUS INJECTION OF CYCLOHEXIMIDE

Mice were given L-[^{14}C]leucine subcutaneously 10 min after the indicated dose of cycloheximide (CXM). The mice (4 in each group) were killed 30 min later.

Dose (mg/kg)	*Radioactivity/cerebrum (CPM)*		*TCA insol./ TCA sol.*	*Calculated inhibition (%)**
	TCA-insoluble	*TCA-soluble*		
120	265	3155	0.08	98.7
30	750	2940	0.26	95.5
8	1455	2915	0.50	91.4
1	3755	2200	1.71	71.4
0	8705	1510	5.76	—

* The TCA-soluble radioactivity estimates the specific activity of the leucine pool at the time of sacrifice, but overestimates the specific activity of this pool for the entire 30 min period after the injection of [^{14}C]leucine. Consequently, the degree of inhibition indicated is an overestimate[3].

satisfied by intracerebral administration of CXM, which is known to reduce locomotor activity, when measured 1–4 h after injection[5].

Method

Mice were injected in each temporal region of the brain with 100 μg CXM or saline in a volume of 10 μl. Four hours after injection, mice were trained for 69 trials in the Deutsch Carousel. An additional group was given saline subcutaneously 30 min before training to control for traumatic effects of the intracerebral procedure.

Results

The groups given saline intracerebrally or subcutaneously (Fig. 2A) were not significantly different ($F = 0.4$, $P > 0.25$), indicating that performance in this task is not affected by the surgical procedures involved in the intracerebral injection. Mice given CXM intracerebrally performed significantly below the level of the other two groups ($F = 10.6$, 12.7, $P < 0.01$), but learned normally during the first third of training (trials 1–23) ($F = 0.5$, $P > 0.25$). Since these CXM-treated mice are hypoactive rather than hyperactive when trained 4 h after intracerebral injection, these results indicate that impaired acquisition is dissociable from hyperactivity.

EXPERIMENT 4

The availability of a small quantity of isoCXM allowed a test of the possible relationship of hypoactivity to the learning deficit. IsoCXM is a stereoisomer of CXM which, when injected intracerebrally, inhibits locomotor activity to the same extent as it is inhibited by CXM, but does not inhibit protein synthesis, or exert amnesic effects[5]. If isoCXM were to impair original learning beyond 15–25 trials, then this impairment might be related to effects which CXM and isoCXM share and could

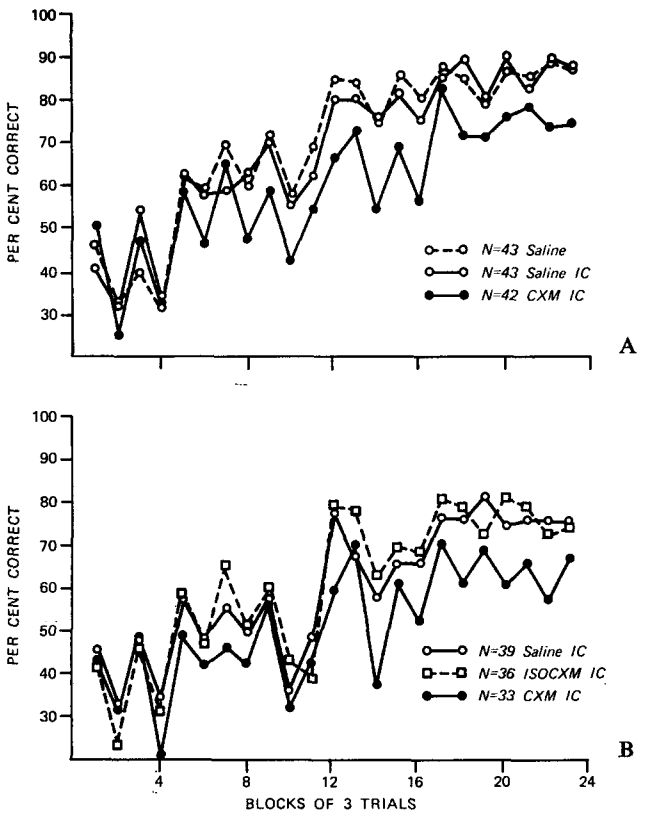

Fig. 2. A, Mice were given saline subcutaneously 30 min before training for 69 trials or 200 μg cycloheximide or saline intracerebrally 4 h before training for 69 trials. The groups given saline were not measurably different, but for all 69 trials the group given cycloheximide was impaired relative to both saline groups ($P < 0.01$). There were no measurable differences between any of the groups during the first third of training (trials 1–23), but during the second third ($F = 5.1$, $P < 0.05$) and the final third of training ($F = 9.9$, $P < 0.05$), the group given CXM was impaired relative to the intracerebral saline group. B, Mice were given saline, 200 μg cycloheximide, or 200 μg isocycloheximide intracerebrally 4 h before training for 69 trials. The groups given saline and isocycloheximide were not measurably different, but for all 69 trials the group given cycloheximide was impaired relative to both these groups ($P < 0.01$). There were no measurable differences between any of the groups during the first 23 trials, but during the second third ($F = 7.7$, $P < 0.05$) and the final third ($F = 4.5$, $P < 0.05$) of training, the group given CXM was significantly different from the group given saline ($P < 0.05$). The group given CXM was also significantly different from the group given isoCXM during the second third ($F = 4.4$, $P < 0.05$) and the final third ($F = 5.5$, $P < 0.05$) of training.

not be related exclusively to inhibition of protein synthesis. Alternatively, if isoCXM were to have no effect on original learning, then the impairment of original learning produced by CXM very likely depends on inhibition of cerebral protein synthesis rather than on hypoactivity or other side effects which CXM and isoCXM share.

Method

Mice were given saline, CXM, or isoCXM intracerebrally 4 h before training for 69 trials in the Deutsch Carousel. Drugs were administered intracerebrally, 100 μg

in each temporal region of the brain. To determine possible effects of isoCXM on retention after brief training in this task, other mice were trained for 21 trials 4 h after intracerebral injections of saline, CXM, or isoCXM and tested for retention 24 h later by training for another 21 trials.

Extent of inhibition of protein synthesis was also determined for these drugs. CXM, isoCXM, or saline were injected in each temporal region of the brain, as above. Mice were given [^{14}C]leucine 3.75 h after injection, killed 30 min later and the extent of inhibition of cerebral protein synthesis was determined as described above.

Results

IsoCXM and saline exerted similar effects on performance during 69 trials ($F = 0.6$, $P > 0.25$), whereas mice given CXM were significantly impaired relative to both these groups (compared to isoCXM group, $F = 10.9$; compared to saline, $F = 16.6$, $P < 0.001$ in each case) (Fig. 2B).

Retests 24 h after brief training indicated that mice given CXM exhibited the expected amnesic effect, but that mice given isoCXM or saline exhibited normal retention (Table IV).

IsoCXM had no effect on cerebral protein synthesis, and CXM inhibited 68% of whole brain protein synthesis when measured in the interval between 3.75 and 4.25 h after drug administration. The values for extent of protein synthesis inhibition produced by these drugs were confirmed in an additional experiment. In view of the finding (Table I) that more than 95% inhibition of whole brain protein synthesis is required to produce amnesia when CXM is given subcutaneously, it was unexpected that inhibition of only 68% of whole brain protein synthesis should produce amnesia when CXM is given intracerebrally. Presumably, with intracerebral injections, there is only limited diffusion of CXM away from the injection sites; indeed, 6 intracerebral injection sites have been used previously to inhibit more than 90% of overall cerebral protein synthesis[2]. It is expected that there is extensive inhibition of protein synthesis near the injection sites and little or none at distant sites.

TABLE IV

EFFECTS OF INTRACEREBRAL SALINE, CYCLOHEXIMIDE, AND ISOCYCLOHEXIMIDE ON MEMORY AND CEREBRAL PROTEIN SYNTHESIS INHIBITION

Mice were given the indicated drugs intracerebrally (100 μg/side) 4 h before training for 21 trials. Retention was tested 24 h later by training mice for an additional 21 trials. Extent of protein synthesis inhibition was estimated by subcutaneous injection of L-[^{14}C]leucine 3.75 h after the intracerebral injection and sacrifice 30 min thereafter.

Drug	*N*	*Trials correct training*	*Trials correct retention*	*Inhibition (%)*
Cycloheximide	19	8.9	10.5*	68
Isocycloheximide	19	9.8	14.6	0
Saline	19	10.5	14.4	—

* Significantly different from saline and from isocycloheximide groups, $P < 0.01$.

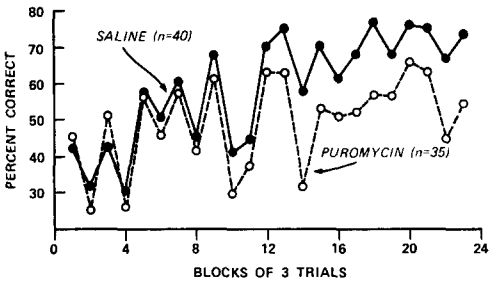

Fig. 3. Mice were given saline or 180 μg puromycin 4 h before training for 69 trials. For all 69 trials, the effects of drug ($F = 14.6$) and of drug X trials ($F = 6.7$) were significant ($P < 0.01$). During trials 1–23, the groups were not measurably different ($F = 1.0$, $P > 0.25$), but the groups were significantly different during the second third ($F = 7.5$, $P < 0.05$) and the final third ($F = 9.7$, $P < 0.05$) of training.

EXPERIMENT 5

As a further test of the possibility that impaired acquisition reflects some side effect peculiar to cycloheximide, mice were given prolonged training following injection of puromycin. Like cycloheximide, puromycin inhibits cerebral protein synthesis, but by a different mechanism of action and with different side effects[1,6].

Method

Mice were injected in each temporal region of the brain with 90 μg puromycin or saline. Four hours later, mice were trained for 69 trials.

Results

Mice given puromycin accumulated significantly fewer correct responses during training than control mice ($F = 14.6$, $P < 0.01$) (Fig. 3), although the groups were not different during the first third of training (trials 1–23, $F = 1.0$, $P > 0.25$). Puromycin-treated mice began to lose ground to control mice at about the same point on the learning curve as CXM-treated mice (Fig. 2A, B).

DISCUSSION

The present results confirm our previous findings that CXM-treated mice learn as well as saline-treated mice in a discrimination task for at least the first 15 trials, but are impaired beyond 15–25 trials. Since an impairment in acquisition could be related either to inhibition of cerebral protein synthesis produced by CXM, or to known side effects of this drug on activity, attempts were made to evaluate these alternatives. Hyperactivity found between 10 and 40 min after subcutaneous injection of 120 mg/kg CXM[4,5,8] does not appear to be responsible for impaired acquisition with prolonged training, since 30 mg/kg of CXM has no effect on activity but does impair acquisition with prolonged training. Furthermore, the acquisition deficit is observed

in the absence of hyperactivity following an intracerebral injection of CXM. Hypoactivity found 1–4 h after intracerebral injection of CXM or isoCXM[5] does not appear responsible for impaired acquisition because: (1) only CXM, not isoCXM, exerts an effect on acquisition; (2) acquisition is impaired by subcutaneous injections of CXM, when training is completed before hypoactivity begins[9]; and (3) acquisition is not impaired by subcutaneous injections of CXM when training is conducted during a period of hypoactivity, but after cerebral protein synthesis has partially recovered[9]. These considerations suggest that the deficit observed with prolonged acquisition may be a deficit in memory related to protein synthesis inhibition and not a side effect on performance. The finding that puromycin, another inhibitor of cerebral protein synthesis, also impairs acquisition strengthens this conclusion. Whereas it is never possible to attribute a behavioral effect of a drug conclusively to its major known pharmacological effect, the present results certainly support the hypothesis that impairment of acquisition beyond the initial trials of training is attributable to extensive inhibition of cerebral protein synthesis required for development of memory.

In the present experiment, the initial trials (1–21) are separated from a second block of trials (22–42) by only 15 sec, and performance in the second block is partially but significantly impaired relative to saline-injected controls (Figs. 1–3, also ref. 7). As the interval between the first and second block of trials is increased to hours, performance in the second block becomes progressively worse[7]. Indeed, when the second block is given 24 h after the first, no retention is detectable[7]. Whereas it is possible that the impairment in acquisition in the present experiment is unrelated to impairment observed at later times, it seems most likely that the acquisition deficit is early evidence of progressive impairment in long-term memory which is dependent on cerebral protein synthesis.

From this reasoning we contend that a 'long-term' memory process which is dependent on protein synthesis has not only been *established* within minutes after the beginning of training as suggested from previous studies[3], but is also *required* within minutes after the beginning of training for progressive improvement of performance. During prolonged training, continued normal improvement of performance may come to depend on memory of an increasing number of preceding trials, which may require the gradual development of an enduring memory process. It also seems reasonable that the proteins whose synthesis is required for normal performance during prolonged training are the same as those required for expression of memory 1 day after brief training.

The mechanism for maintaining partial memory for hours after briefly training CXM-treated mice in the present task[7] is still uncertain. Although it is possible that a short-term process independent of protein synthesis may persist in a form that is sufficient for partial expression of memory for hours after training, it is also possible that a weak long-term process, mediated by the small capacity for cerebral protein synthesis which escapes inhibition, is sufficient to maintain memory in partial form for several hours.

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REFERENCES

1 BARONDES, S. H., Cerebral protein synthesis inhibitors block long-term memory, *Int. Rev. Neurobiol.*, 13 (1970) 177–205.
2 BARONDES, S. H., AND COHEN, H. D., Cerebral protein synthesis and memory consolidation, *Brain Research*, 4 (1967) 44–51.
3 BARONDES, S. H., AND COHEN, H. D., Memory impairment after subcutaneous injection of acetoxycycloheximide, *Science*, 160 (1968) 556–557.
4 SCHNEIDER, C. W., AND CHENOWETH, M. B., Effects of cycloheximide on unrestricted behavioral patterns of mice, *Brain Research*, 25 (1971) 625–631.
5 SEGAL, D. S., SQUIRE, L. R., AND BARONDES, S. H., Cycloheximide: its effects on activity are dissociable from its effects on memory, *Science*, 172 (1971) 82–84.
6 SQUIRE, L. R., AND BARONDES, S. H., Inhibition of cerebral protein or RNA synthesis and memory. In J. GAITO (Ed.), *Macromolecules and Behavior*, Appleton-Century-Crofts, New York, 1972 pp. 61–82.
7 SQUIRE, L. R., AND BARONDES, S. H., Variable decay and recovery of memory in cycloheximide-treated mice, *Proc. nat. Acad. Sci. (Wash.)*, 69 (1972) 1416–1420.
8 SQUIRE, L. R., GELLER, A., AND JARVIK, M. E., Habituation and activity as affected by cycloheximide, *Commun. Behav. Biol.*, 5 (1970) 249–254.
9 SQUIRE, L. R., SMITH, G. A., AND BARONDES, S. H., Cycloheximide can affect memory within minutes after the onset of training, *Nature (Lond.)*, 242 (1973) 201–202.
10 WINER, B. J., *Statistical Principles in Experimental Design*, McGraw-Hill, New York, 1962.