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Reversal of Cycloheximide-Induced Amnesia by Adrenergic Receptor Stimulation[1]

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QUARTERMAIN, D., L. S. FREEDMAN, C. Y. BOTWINICK AND B. M. GUTWEIN. *Reversal of cycloheximide-induced amnesia by adrenergic receptor stimulation.* PHARMAC. BIOCHEM. BEHAV. 7(3) 259–267, 1977. – Amnesia for a multiple trial appetitive spatial discrimination habit induced by the protein synthesis inhibitor cycloheximide (CXM) was reversed by peripheral injections of both alpha (clonidine) and beta (isoproterenol) norepinephrine receptor stimulators. Stimulation of dopamine receptors with piribedil and acetycholine receptors with pilocarpine was ineffective in reversing amnesia. The clonidine-induced recovery was blocked by phentolamine and the isoproterenol recovery by propranolol. Examination of the temporal parameters of clonidine-induced recovery indicated that the amnesia was prevented if the agonist was injected either before training and CXM treatment, up to 1 hr after training and up to 3 hr prior to testing. Clonidine also alleviated amnesia induced by another protein synthesis inhibitor anisomycin, for a shock motivated brightness discrimination habit. These data suggest that the transient amnesia induced by CXM may be a consequence of disruption of adrenergic mechanisms and more specifically that norepinephrine may play an important role in memory retrieval.

Protein synthesis inhibition Amnesia Adrenergic receptors Memory retrieval Clonidine

A NUMBER of recent studies have indicated that inhibition of protein synthesis can frequently induce amnesias from which animals recover [4]. Recovery may be spontaneous (e.g., [17, 19, 21, 25]) or it may be induced by behavioral (e.g., [19]) and by pharmacological (e.g., [5, 17, 20, 22]) manipulations. Although the variables which determine recovery have not been identified, the phenomenon occurs with considerable reliability when amnesias are induced by cycloheximide (CXM) for multiple trial discriminated approach and avoidance habits [16,17]. These behavioral paradigms may therefore provide an experimental basis for the investigation of mechanisms which underlie memory retrieval as distinct from memory consolidation. The potential value of transient amnesias as a source of information about retrieval processes is that they provide an opportunity to attempt restoration of memory by pharmacological stimulation of neurotransmitter systems. The reestablishment of retrieval processes following acute memory loss by stimulation of neurochemical systems, may provide insight into the mechanisms which normally operate during retrieval of stored memories.

Studies by Flexner and his colleagues [10, 20, 22] first suggested that amnesias could be alleviated by stimulation of neurotransmitter systems. They showed that amnesias induced by both puromycin and acetcxycycloheximide (AXM) could be reversed by agents which stimulated the adrenergic system. Recent studies from our laboratory have shown that amnesias induced by CXM can be reversed by treatment with agents which increase catecholamine (CA) activity. Amnesia for a one-trial inhibitory avoidance response can be alleviated by treating amnestic mice with two monoamine oxidase inhibitors pargyline and pheniprazine [5]. Similarly, amnesia for a multiple trial appetitively motivated spatial discrimination habit can be reversed if pargyline, pheniprazine or d-amphetamine is administered prior to the retention test [17]. All of these studies suggest that pharmacological activation of central CA mechanisms can reestablish retrieval following memory loss. The experiments reported in this paper were designed to investigate some of the behavioral and pharmacological determinants of recovery from amnesia.

EXPERIMENT 1

One specific issue which requires clarification is the relative importance of NE and DA in mediating recovery from amnesia. Monoamine oxidase inhibitors increase intraneuronal concentrations of both NE and DA, and amphetamines releases both amines so that no information on the role of specific transmitters is available from our previous studies. By the use of specific agonists it should be possible to obtain direct information on the importance of NE and DA in recovery from amnesias induced by protein synthesis

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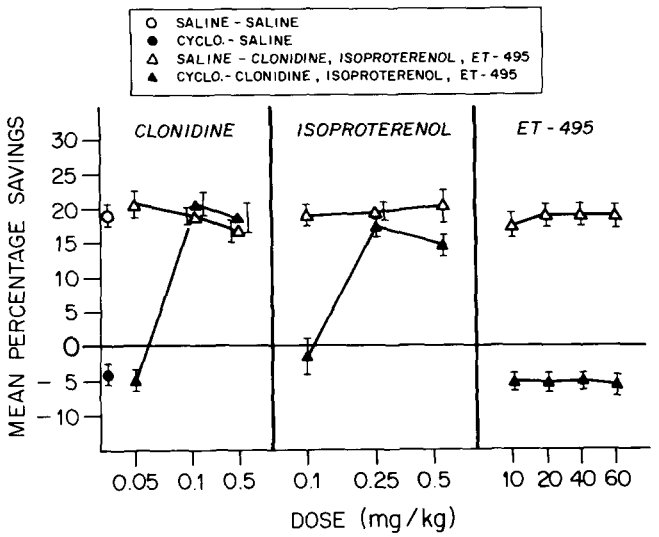

FIG. 1. Mean percentage savings for all groups in Experiment 1. ○ – SAL pretraining and SAL preretention test; ● – CXM pretraining, SAL pretest; △–––△, SAL pretraining, clonidine, isoproterenol or ET–495, pretest; ▲–––▲ CXM pretraining, clonidine, isoproterenol or ET–495 pretest. Dose levels for each receptor stimulator are shown on the abscissa. Each SAL group contained between 8 and 11 mice; and each drug group contained between 10 and 16 mice.

inhibitors. The object of the first experiment therefore was to determine whether recovery from CXM-induced amnesia could be induced by pretest treatment with adrenergic receptor stimulators, clonidine (alpha) and isoproterenol (beta) or with the dopamine (DA) receptor stimulator piribedil.

Method

Animals. Animals for all the experiments reported in this paper were male C57BL/6J mice 10 weeks old and approximately 22 g in weight. Mice were individually caged for 3 days prior to the beginning of the experiments and had free access to water at all times. Food was available ad lib up to the day prior to the beginning of the experiment.

Apparatus. Unless otherwise indicated the apparatus for all of the experiments in this study was a T-maze, 7.6 cm wide × 8.9 cm high. The center alley was 29.2 cm long and each arm was 17.8 cm long. The initial 8.9 cm of the center alley served as a start box, separated from the rest by a guillotine door. Guillotine doors at the start of each arm prevented retracing. The entire maze was painted flat black and covered with clear Plexiglas lids.

Procedure

The standard procedure used throughout the experiments was as follows: after 24 hr of food deprivation, each mouse was given a 10 min adaptation session in the maze which had food in both arms. Following adaptation, mice were fed 1.5 g of food in the home cage. On Day 1 mice were trained to go either to the right or to the left arm to obtain food. All animals were given a total of 20 trials. The intertrial interval was approximately 20 sec and a non-correction procedure was employed. On Day 2, all mice were trained to the side opposite that which had been correct on Day 1 until they had attained a criterion of 17 correct choices. On Day 3, mice were tested for retention of the reversal. We adopted this reversal procedure after a systematic study of the training parameters for this task [17]. We have shown that this combination of Day 1 and 2 training results in the greatest magnitude of amnesia when retention is tested 24 hr following reversal. Reversal training is not a precondition for amnesia: we have shown that lesser magnitudes of amnesia can be obtained using conventional training criteria without a reversal. It is of interest to note that Gleitman [13] has reported that forgetting in rats is greatly accelerated when a reversal procedure is employed.

Experimental Design

Mice were injected with CXM (120 mg/kg) or saline (SAL) SC 30 min before reversal training on Day 2. On Day 3, 1 hr before the retention test, both CXM and SAL treated mice were injected with either 0.9% SAL, the alpha NE agonist clonidine, the beta NE agonist isoproterenol or the DA agonist piribedil (ET–495). The dose levels of the receptor stimulators and number of mice in each group are given in the legend to Fig. 1.

RESULTS

Results are shown in Fig. 1. Savings were calculated by using the formula; errors on the training day minus errors on the test day divided by errors on the training day. The typical amnesic effect of CXM is shown at the left of panel 1 (CXM vs. SAL, $t = 5.52$, $p<0.001$). Results of an ANOVA carried out on the clonidine data indicate a significant drug effect (SAL-clonidine vs. CXM-clonidine), $F(2,68) = 14.3$, $p<0.001$; and a significant dosage effect, $F(2,68) = 20.65$, $p<0.001$. An ANOVA of the isoproterenol data indicates a significant effect for drug group (SAL-isoproterenol vs. CXM-isoproterenol) $F(1,67) = 83.58$, $p<0.001$, and a significant drug × dosage interaction, $F(2,67) = 11.60$, $p<0.001$. These data indicate that neither clonidine nor isoproterenol influenced the retention scores of SAL-treated animals but both agonists significantly facilitated retention in mice previously treated with CXM. The significant interaction results from absence of recovery in CXM treated animals at the lowest dose level of each agonist. In contrast piribedil failed to reverse CXM – induced amnesia over a wide range of dose levels (10–60 mg/kg).

A question raised by these data concerns the specificity of the recovery effect. Since clonidine administration results in enhanced retention performance, an interpretation of the results in terms of toxic side effects is obviously ruled out. The possibility that enhanced retention reflects a general facilitation of learning and/or motivation rather than recovery of a specific memory has to be entertained. Although we have previously shown that pheniprazine-induced recovery from CXM amnesia cannot be accounted for in terms of general learning enhancement [17,18], an experiment to evaluate the specificity of the clonidine reocvery effect was carried out. Mice (N = 22) were injected with CXM 30 min prior to reversal training and 23 hr later all mice were injected with 0.5 mg/kg of clonidine. One hour following clonidine injection, 10 mice were tested to the same side that was reinforced during reversal and 12 mice were retrained to the opposite side. We have previously shown [17, 18, 19] that under these testing conditions SAL treated mice require significantly more trials to reach criterion when retrained to the opposite than when they are

TABLE 1

EFFECT OF REVERSING S^D DURING RETRAINING ON RETENTION IN CLONIDINE TREATED MICE*

Groups	Training	Retention	
		Same Side	Opposite
SAL-SAL	26.1 ± 0.52	22.5 ± 0.24	30.2 ± 0.11
CXM-SAL	28.2 ± 0.14	28.8 ± 0.17	26.2 ± 0.34
CXM-CLON	27.2 ± 0.31	25.4 ± 0.17	28.4 ± 0.22

*Numbers are mean trials to criterion ± SEM. The SAL-SAL and CXM-SAL data are included for comparison.

retrained to the same side. On the other hand CXM-treated mice require significantly fewer trials to reach criterion when they are retrained to the opposite side than when they are retrained to the same side. This enhanced rate of reversal learning presumably results from an absence of proactive interference and rules out the possibility that the poor test performance of amnestic animals evident on the conventional test results from debilitating side effects of CXM. By the same reasoning, if clonidine is inducing recovery of the reversal habit, mice tested to the side opposite that which was correct on reversal training should require significantly more trials to relearn than clonidine treated mice retrained to the same side. The results of this experiment are shown in Table 1.

Results of a 3 × 2 ANOVA carried out on the retention data indicate a significant interaction between type of retention test (same vs. opposite side) and drug treatment, $F(2,55) = 44.56$, $p<0.001$. This interaction indicates that mice treated with only CXM perform significantly better when tested on the reversal measure than when tested to the same side. Saline treated animals show the opposite effect; their performance is significantly worse when a reversal measure of retention is employed. The retention performance of CXM mice treated with clonidine is similar to that of SAL controls. The demonstration that the retention scores of CXM-clonidine mice (like those of SAL controls) can be increased or decreased by changing the measure of retention strongly suggests that clonidine is facilitating recovery of a specific spatial response.

Biochemistry

In order to confirm that piribedil was stimulating DA receptors [7] the effect of this agonist on DA turnover was estimated by studying the decline of NE and DA following tyrosine hydroxylase inhibition [1]. Mice were injected with piribedil (60 mg/kg IP) 15 min prior to an injection of α-methyl-para-tyrosine-methyl ester (250 mg/kg IP). Animals were sacrificed at 3 hr after alpha MPT and whole brain (minus cerebellum) was dissected at 4°C and stored on dry ice. Brains were homogenized in ice cold 0.4 N perchloric acid, centrifuged for 10 min at 10,000 Xg in a Sorvall refrigerated centrifuge and CA's isolated by alumina chromatography. NE and DA levels were determined using spectofluormetric techniques [2,15]. The results (Table 2) indicate that alpha-MPT induced depletion in whole brain DA levels was significantly reduced by piribedil treatment. Similar results were obtained with the prototype DA agonist apomorphine. The data indicate that NE turnover is increased by DA receptor stimulation. This result is in agreement with previous findings by Corrodi [7]. These findings indicate that DA receptor activation induced a reflex reduction in dopamine turnover and thereby demonstrate that piribedil was pharmacologically active as a DA agonist in the behavioral experiments.

EXPERIMENT 2

In Experiment 1 and in our previously published studies, we have demonstrated that CXM-induced amnesia can be reversed when adrenergic stimulating agents are administered prior to the retention test. These studies were specifically designed to investigate the role of adrenergic mechanisms on retrieval processes. Recent studies have indicated that amnesias can be reversed when recovery-inducing agents are given both before and after training. For example, Stein, Belluzzi and Wise [23] have shown that diethyldithiocarbamate (DEDTC) induced amnesia can be reversed by intraventricular injections of NE administered immediately, but not 2 or 5 hr after training. Serota *et al.* [22] demonstrated that acetoxycycloheximide (AXM) – induced amnesia could be reversed when metaraminol (a false transmitter which releases NE) is administered 30 min before training, 30 min to 2 hr after training and immediately to 2 hr before testing. No restorative effects occurred if metaraminol was administered either 2.5 hr

TABLE 2

EFFECT OF DA RECEPTOR STIMULATORS ON DECLINE OF BRAIN CA LEVELS AFTER TYROSINE HYDROXYLASE INHIBITION BY a-MPT

	NE		DA	
	μg/g	Percent Change from control	μg/g	Percent change from control
Control	0.49 ± 0.032		1.62 ± 0.207	
a-MPT	0.32 ± 0.001	−35	1.02 ± 0.033*	−37
Piribedil-aMPT	0.24 ± 0.040‡	−51	1.23 ± 0.040†	−24
Apomorphine-aMPT	0.28 ± 0.001	−43	1.74 ± 0.178	+ 7

Values are means and standard errors. Each group was composed of four mice.
*a-MPT vs. control $t = 2.41$, $p<0.05$.
†Piribedil vs. a-MPT $t = 3.83$, $p<0.01$.
‡Piribedil vs. a-MPT $t = 9.24$, $p<0.001$.

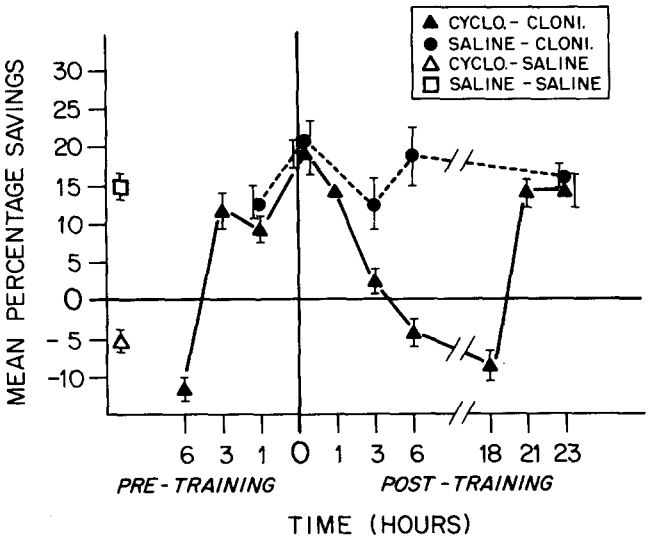

FIG. 2. Mean percent savings for both CXM and SAL mice injected with 0.5 mg/kg clonidine at times both pre- and posttraining indicated on the abscissa. The standard reference groups which received CXM and SAL only are shown on the left.

after training or 2.5 hr before testing. The present experiment is designed to investigate in more detail the temporal characteristics of clonidine-induced recovery from CXM-induced amnesia.

Design

Adaptation and Day 1 training were as previously described. For all groups CXM was injected 30 min before reversal training and retention was tested 24 hr later. Different groups of mice were injected with clonidine (0.5 mg/kg) at the following times with reference to reversal training: 3 hr (N = 15) and 1 hr (N = 10) before; immediately (N = 8), 1 hr (N = 8) 3 hr (N = 8), 18 hr (N = 10), 21 hr (N = 10) and 23 hr (N = 12) after. The effect of clonidine on saline treated mice was assessed at the following time points: 1 hr before (N = 10), immediately (N = 8), 3 hr (N = 8), 6 hr (N = 10), and 23 hr (N = 12) after reversal training. In addition the standard reference groups treated only with CXM (N = 10) and saline (N = 12) administered 30 min before reversal training were also included.

RESULTS AND DISCUSSION

The results of this experiment are shown in Fig. 2. The typical amnestic effect of CXM is indicated by the differences between the CXM and SAL reference groups, $t = 4.32$, $p<0.001$. It is evident from Fig. 2 that the retention scores of SAL treated mice are not significantly influenced by clonidine. None of SAL-clonidine groups is significantly different from the standard SAL control group. On the other hand, time of clonidine administration exerted a significant influence on degree of retention in CXM treated mice. A one way ANOVA revealed that there were significant differences in degree of retention among the CXM-clonidine groups, $F(9,88) = 13.13$, $p<0.001$. Individual Sheffe-pair-wise comparisons were carried out on the CXM-clonidine data. The results show that groups injected with clonidine 6 hr before, and 3, 6 and 18 hr after training were significantly ($p<0.001$) different from all of the other groups, none of which differed significantly from each other. These data indicate that clonidine does not block CXM-induced amnesia when it is injected either 6 hr before training, or 3, 6 or 18 hr after training. At all other times clonidine treatment, blocked the amnestic effect of CXM. A trend analysis carried out on the CXM-clonidine data indicates a pronounced cubic trend in the time course of the recovery, $F(1,88) = 88.29$, $p<0.001$. These data thus indicate that clonidine can alleviate CXM-induced amnesia when it is administered both before and shortly following training, as well as before testing.

It is likely that the effects of clonidine at these two time periods are mediated by different processes. In the situation where clonidine is administered before or shortly following learning, the subsequent recovery of memory may reflect the prevention of the development of amnesia. There is some evidence that amnesia induced by protein synthesis inhibition develops gradually, reaching its maximum 3–6 hr after training [3]. The decline in the effectiveness of posttraining clonidine may parallel the development of amnesia for this task. The effectiveness of pretesting clonidine administration may reflect the effects of adrenergic stimulation on retrieval mechanisms. One hypothesis suggested by the present data is that when the amnesia is fully developed, restoration of memory can only be achieved by stimulating adrenergic receptors before or during the retention test. Pretesting receptor stimulation is not necessary for restoration if clonidine is administered before the amnesia is fully developed. This hypothesis would predict that recovery from amnesia would occur for the 3 and 6 hr posttraining groups if retention was tested 1 hr later in each case.

The possibility that clonidine administered before CXM was reducing the level of of inhibition of protein synthesis was evaluated. The effect of clonidine (0.5 mg/kg, IP) on the incorporation of ^{3}H-tyrosine into protein was determined in CXM (120 mg/kg SC) treated mice. Clonidine was injected into mice 30 min prior to CXM. L-tyrosine – 3,5 ^{3}H (48 Ci/mmol) 20 μC, obtained from New England Nuclear, was injected IP 15 min after CXM and animals sacrificed by cervical disslocation 30 min later. Brains were dissected at 4° C, homogenized in ice cold 0.4 N perchloric acid (PCA) and homogenates centrifuged for 20 min at 10,000 Xg. The PCA precipitate was dissolved by use of a tissue solubilizer NCS (Amersham), Aliquots of the PCA supernatant (soluble fraction) and PCA precipitate (insoluble fraction) were dissolved in 15 ml of aqueous counting solution and radioactivity measured in a Searle liquid scintillation counter. Estimate of protein synthesis was obtained by calculating the ratio of ^{3}H-tyrosine associated with the PCA precipitate to that associated with the PCA soluble fraction [26]. The ratios in control (N = 6), CXM (N = 12), and CXM-clonidine (N = 9) mice were 0.333, 0.042, 0.041, respectively. These data indicate that CXM inhibits mouse brain protein synthesis by 87.4%. The level of inhibition in the CXM-clonidine group was 87.7% indicating that clonidine had no effect on CXM induced inhibition of protein synthesis. Thus, it is improbable that clonidine administered prior to CXM, attenuates CXM induced amnesia by reducing the magnitude of protein synthesis inhibition at the time of training.

EXPERIMENT 3

In order to determine whether the NE agonists clonidine and isoproterenol antagonize CXM induced amnesia by

their direct action on receptors, an attempt was made to selectively block recovery of memory by the use of specific adrenergic blocking drugs. Both propranolol (a beta antagonist) and phentolamine (an alpha antagonist) were injected prior to the administration of both clonidine and isoproterenol. It would be predicted that if the isoproterenol effect was mediated by beta receptor activation it would be blocked by propranolol and not by the alpha receptor antagonist phentolamine. Similarly if the clonidine effect was mediated by alpha-receptor activation, it should be blocked by phentolamine and not by propranolol.

Design and Procedure

Adaptation and Day 1 training were the same as previously described. On Day 2 CXM was injected 30 min before reversal training, and retention was tested 24 hr later (Day 3). On Day 3 all mice were injected with either clonidine (0.5 mg/kg) or isoproterenol (0.5 mg/kg) 1 hr before the retention test. Varying doses of propranolol (2.5, 5.0, 10.0 mg/kg) phentolamine (1.0, 5.0, 10.0 and 20.0 mg/kg) or SAL were injected 20 min prior to the administration of the two receptor stimulators.

RESULTS AND DISCUSSSION

Results of this experiment are shown in Fig. 3.

CXM treated animals show the typical amnestic effect when compared with SAL conrols, $t = 5.11$, $p<0.001$. In the absence of blocking drugs, both clonidine, $t = 3.94$, $p<0.001$, and isoproterenol, $t = 4.45$, $p<0.001$, significantly improve the retention scores of CXM treated mice. In order to statistically assess the effect of the blocking agents, each phentolamine dose group was compared with the CXM-clonidine reference group, and each propranolol dose group was compared with the CXM-isoproterenol reference group. The results of these tests indicated that the clonidine induced recovery was effectively blocked by phentolamine at 5, $t = 5.53, p<0.001$, 10, $t = 6.66, p<0.001$ and 20 mg/kg $t = 5.71$, $p<0.001$, but not at 1.0 mg/kg, $t = 0.88$. Propranolol did not significantly influence the clonidine-induced recovery from amnesia. The isoproterenol-induced recovery on the other hand was significantly attenuated by 5.0 mg/kg, $t = 4.35$, $p<0.001$, of propranolol and completely blocked by 10 mg/kg, $t = 6.19$, $p<0.001$. Propranolol at 2.5 mg/kg was ineffective in blocking the amnesia. Neither dose of phentolamine significantly blocked the isoproterenol induced recovery. The results of this experiment demonstrate the pharmacological specificity of clonidine and isoproterenol and suggest that these agonists are probably exerting their behavioral effects by direct action on specific adrenergic receptors.

EXPERIMENT 4a

In all of the proceeding experiments as well as in previously reported studies we have used performance on a food motivated spatial discrimination task as the measure of retention and pretraining CXM treatment as the amnestic agent. The purpose of the present experiment was to determine whether clonidine-induced recovery of memory is independent of the particular training and testing conditions employed in our previous studies. In the present experiment therefore, a shock motivated brightness discrimination task was employed, the amnestic agent anisomycin (ANI) was injected immediately posttraining. Clonidine (0.5 mg/kg) was administered 30 min prior to the retention test which occurred 5 days after training.

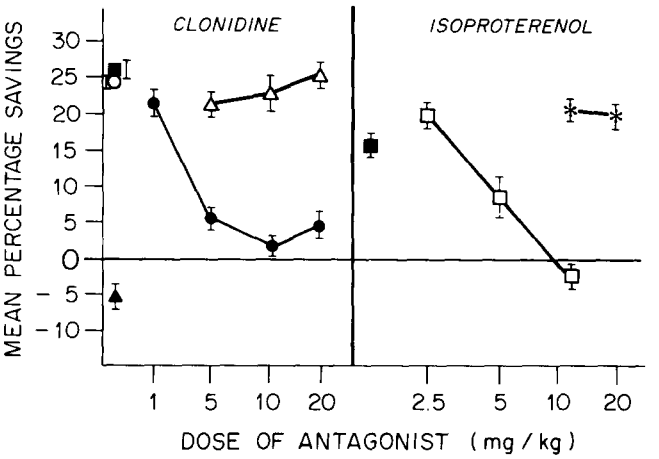

FIG. 3. Mean percentage saving and SEMs for all groups in Experiment 3. Clonidine and isoproterenol were injected 1 hr prior to retention test at a dose of 0.5 mg/kg. Doses of propranolol and phentolamine are shown on the abscissa. ○ – SAL pretraining, SAL pretest; ▲ – CXM, pretraining, SAL pretest; ■ – CXM, pretraining, clonidine or isoproterenol pretest; ● – CXM pretraining, phentolamine and clonidine pretest; △ – CXM pretraining, propranolol and clonidine pretest; □ – CXM pretraining, propranolol and isoproterenol pretest; X – CXM pretraining, phentolamine and isoproterenol pretest.

Apparatus

The apparatus was a Y-maze constructed of Black lucite and covered with a clear Plexiglas top. Each arm was 22 cm long, 10.2 cm wide, and 18 cm high. The arms were separated by guillotine doors. The floor consisted of stainless steel rods set 1.3 cm apart and 3.2 cm off the ground. All arms were wired to a standard constant current shock source. The arms of the maze contained two lights set 10.8 cm above the grid floor.

Design and Procedure

On Day 1, mice were placed in the start alley of the Y-maze facing away from the arms and 5 sec later a constant current footshock (0.2 mA) was automatically delivered to the grids in the start alley. Mice were required to choose the dark-arm, the position of which was randomly varied, in order to avoid shock. An animal choosing incorrectly was allowed to correct and permitted to remain in the safe arm for 15 sec. Guillotine doors prevented retracing. Thereafter the animal was removed from the maze and placed in a holding cage for 45 sec after which he was returned to the start alley for the next trial. Training was terminated when the animal made 9 correct responses. Extensive pilot data obtained in our laboratory indicate that for the shock-motivated brightness discrimination task the criterion of 9 correct responses produces a robust amnesia on the 5-day retention test in ANI-injected mice, whereas saline controls still demonstrate adequate retention. Animals reaching this criterion in less than 17 or more than 30 trials were discarded. Immediately after training mice were injected with either ANI (150 mg/kg SC) or SAL. One hour prior to the 5 day retention test different groups of mice were injected with either SAL, clonidine, or with clonidine 30 min following treatment with an alpha

TABLE 3

MEAN PERCENTAGE SAVINGS FOR THE GROUPS IN EXPERIMENT 4a

Group	N	Drug After Training	Drug Before Testing	Retention Test	Percentage Savings
1	11	ANI	SAL	Standard	−18.4
2	13	ANI	SAL	Standard	24.4
3	13	ANI	SAL	Reversal	15.9
4	10	SAL	SAL	Reversal	−32.8
5	10	ANI	Clon	Standard	35.2
6	10	SAL	Clon	Standard	37.9
7	10	ANI	Clon	Reversal	−24.0
8	10	SAL	Clon	Reversal	−10.6
9	10	ANI	phentol-clon	Standard	−17.9
10	10	SAL	phentol-clon	Standard	27.4

Anisomycin (ANI) 150 mg/kg, injected immediately after training; clonidine (clon) 0.5 mg/kg, injected 1 hr before testing; phentolamine (phentol) 10 mg/kg, injected 30 min before clon; Standard retention refers to mice retrained to the original S^D; Reversal refers to mice retrained to the original S^Δ.

adrenergic antagonist phentolamine. The design of this experiment is shown in Table 3. Two measures of retention were employed. Mice were retained to either the dark arm or reversed to the lighted arm to a criterion of 9 correct responses.

RESULTS AND DISCUSSION

Mean percentage savings for all groups in Experiment 4a is shown in Table 3. The basic effect of ANI was assessed by carrying out a 2 × 2 ANOVA on Groups 1, 2, 3 and 4. The results indicated a highly significant interaction between drug group and type of retention test, $F(1,40) = 16.04$, $p<0.001$. This interaction shows that ANI–SAL mice had significantly lower mean percentage savings than SAL–SAL animals when tested to the same side, but a significantly higher mean percentage savings score when the reversal measure was employed. This finding shows that ANI is creating an amnesia which is specific for the discrimination. A further 2 × 2 ANOVA assessed the effect of clonidine on both ANI and SAL treated mice tested on the two retention measures. The results of this analysis revealed a highly significant interaction between drug group (ANI–SAL vs. ANI-clon) and type of retention test, $F(1,37) = 11.89$, $p<0.002$. This finding indicates that clonidine induces a specific recovery of the discriminated avoidance habit. This parallels the findings for the food motivated position discrimination response described in Experiment 1. A one way ANOVA was carried out on Groups 1, 2, 5, 6, 9 and 10 in order to determine whether phentolamine would block clonidine-induced recovery of the avoidance response. The results indicate a significant difference among the 6 groups, $F(5,58) = 4.88$, $p<0.001$. To assess the effect of phentolamine, the retention score of teh ANI-clon (Group 5) and the ANI-phentolamine group (Group 9) were compared by means of Duncan's Range Test. The difference between these groups was significant, $F(1,58) = 5.42$, $p<0.01$. Neither phentolamine nor clonidine significantly influenced the retention scores of SAL-treated mice.

The results of this experiment indicate that amnesia induced by immediate posttraining injections of ANI in a shock-motivated brightness discrimination task can be reversed by NE receptor stimulation with clonidine 5 days after learning, and suggests that the enhancing effect of clonidine on memory retrieval is not specific to the class of protein synthesis inhibitor used to induce amnesia, the learning task employed, the training retest interval, or the motivational variable.

EXPERIMENT 4b

A possible explanation for the failure of CXM and ANI to induce a permanent amnesia is that a single dose of these compounds may not induce inhibition of protein synthesis for a sufficient period of time to prevent storage of information. That duration of inhibition may be an important determinant of the strength and durability of amnesia is suggested by results of a study by Flood *et al.* [11]. They showed that prolonging protein synthesis inhibition for several hours beyond time of training could overcome the effects of increased habit strength which typically blocks the amnesic effect of protein synthesis inhibition. The aim of Experiment 4b was to determine whether clonidine administered before a 5 day retention test would induce a reversal of amnesia when protein synthesis was inhibited for a duration of 6–8 hr following training.

Design and Procedure

Apparatus and procedure were the same as for Experiment 4a. Twenty-four mice were injected with 2.5 mg of ANI, and 12 mice with SAL 30 min prior to training. The 24 ANI treated mice were injected with an additional 0.5 mg of ANI, at 1.5, 3.5 and 5.5 hr after training. The 12 mice treated with SAL prior to reversal were given a second injection of saline at these times. Thirty min before the retention test on Day 5, 12 of the mice which had received the multiple ANI treatment were injected with 0.5 mg/kg of clonidine, and the remaining 12 were injected with SAL.

The effect of single and multiple injections of ANI on protein synthesis was determined using the method previously described. Thirty min after the last injection, 20 μC

of ^{3}H-tyrosine was injected intraperitoneally. Animals were sacrificed at 30 min and brains processed as described in Experiment 2 and level of protein synthesis estimated by calculating ratio of ^{3}H-tyrosine in PCA insoluble and PCA soluble fractions.

RESULTS AND DISCUSSION

Both the single and multiple ANI injection schedules inhibited mouse brain protein synthesis to a similar degree. The PCA insoluble/soluble ratios were reduced from 0.351 in the control group (N = 6) to 0.073 in the single ANI group(N = 5) and to 0.067 in the multiple ANI group (N = 6); a decline of 79.2 and 81.0%, respectively. Flood *et al.* [11] have previously shown that multiple injections of ANI at 2 hr intervals can maintain a constant level of protein synthesis inhibition. Our data are consistent with the findings of Flood *et al.* and indicate that protein synthesis was inhibited for a 6 hr period in this experiment.

Mean percent savings for the SAL treated mice was 20.4 and −4.7% for the multiple ANI group treated with SAL before testing, $t = 2.86$, df 22, $p<0.02$. That this amnesia could be reversed by adrenergic stimulation is indicated by the results of the ANI-clonidine group. Mean percent savings for this group was 21.06, which was significantly different from the ANI–SAL group, $t = 2.15$, df 22, $p<0.025$, and not significantly different from the SAL–SAL control group, $t = 0.08$. These data indicate that stimulation of adrenergic receptors can induce recovery from an amnesia which has been produced by prolonged inhibition of protein synthesis.

EXPERIMENT 5

The experiments reported in this study indicate that pharmacological manipulation of the adrenergic system can reverse amnesias induced by protein synthesis inhibitors. As an initial attempt to evaluate the role of other neurotransmitter systems we have tested the effectiveness of two agents that induce cholinergic activation; physostigmine an anticholinestrase inhibitor, and pilocarpine a cholinergic receptor stimulator.

Design and Procedure

The procedure for this experiment was the same as in Experiment 1. On Day 2 mice were injected with either CXM (N = 50) or SAL (N = 30) 30 min before reversal training. The following day all CXM treated mice were injected with either SAL (N = 10) pilocarpine, 0.5 mg/kg (N = 8), 5.0 mg/kg (N = 8) or physostigmine 0.5 mg/kg (N = 8), 1.0 mg/kg (N = 8) 5.0 mg/kg (N = 8). Pilocarpine was injected 30 min and physostigmine 15 min before the retention test. Animals treated with SAL were divided into 3 groups: Group 1 (N = 10) were injected with SAL; Group 2 (N = 10) with 5.0 mg/kg of physostigmine and Group 3 (N = 10) injected with 5.0 mg/kg pilocarpine before the retention test.

RESULTS AND DISCUSSION

Results are shown in Table 4. Physostigmine, a drug which potentiates the effect of acetylcholine does not reverse CXM-induced amnesia over a 10 fold dose range. Direct cholinergic receptor stimulation by pilocarpine is also ineffective over a similar dose range. These data suggest that activation of the cholinergic system prior to the retention test cannot overcome CXM-induced amnesias in this task.

TABLE 4

EFFECT OF ACTIVATION OF THE CHOLINERGIC SYSTEM ON REVERSAL OF CXM-INDUCED AMNESIA

Group*	Dose mg/kg	% Savings ± SEM
SAL-SAL	—	20.4 ± 0.51
SAL-PHYSO	5.0	19.1 ± 0.036
SAL-PILO	5.0	20.0 ± 0.60
CXM-SAL	—	−4.5 ± 0.73
CXM-PILO	0.5	−8.7 ± 0.14
	5.0	−8.9 ± 0.19
CXM-PHYSO	0.5	−1.8 ± 0.29
	1.0	−8.7 ± 0.21
	5.0	−5.2 ± 0.56

*N = 8 per group, Saline (SAL), Cycloheximide (CXM), Physostigmine (PHYSO) and Pilocarpine (PILO).

GENERAL DISCUSSION

The results reported in these experiments provide clear evidence that stimulation of adrenergic receptors can reverse the amnestic effects of protein synthesis inhibition. The present findings taken together, with our previous results [17] which demonstrated memory restoration with d- (but not l-) amphetamine indicate that activation of both pre- and postsynaptic adrenergic mechanisms can facilitate recovery from amnesia.

It should be emphasized that recovery of memory following receptor stimulation is not merely the result of increased general arousal. Clonidine, a drug which has potent antihypertensive effects [14] facilitates retention despite the fact that at the effective doses used in these studies it produces mild sedation and depresses activity and exploratory behavior [24]. More direct evidence that clonidine is facilitating retrieval of a specific memory rather than non-specifically enhancing all behavior, comes from the results of the reversal tests in Experiment 1. When clonidine treated mice are tested on a reversal measure the number of trials required to reach criterion is significantly increased relative to similarly treated animals tested in the conventional manner. Since slower learning of a reversal indicates the existence of a source of proactive interference, these findings strongly suggest that clonidine is restoring memory of the specific spatial response rather than nonspecifically enhancing learning.

The data from Experiment 3 suggests that clonidine and isoproterenol are acting through alpha and beta receptor mechanisms. Clonidine-induced recovery was reversed by alpha and not by beta receptor blockade whereas isoproterenol-induced recovery was reversed by beta and not by alpha receptor blockade. Recently, Gibbs [12] has reported data which confirms the present findings that alpha and beta receptors are involved in memory retrieval. This study showed that both NE and amphetamine can overcome amnesias in chicks induced by CXM, and that the effect of

these agents can be blocked by both alpha and beta adrenergic antagonists but not by dopamine or histamine receptor antagonists.

The results of Experiment 1 do not indicate a specific role for dopamine in the recovery from amnesia. The potent DA agonist piribedil failed to reverse the amnestic effects of CXM despite the wide dose range which was employed. It would however, be premature to conclude that dopamine does not play a role in memory retrieval processes. There is recent evidence which suggests the existence of both an excitatory and an inhibitory dopamine receptor [6] and that these two populations of DA receptors may respond differently to DA agonists. Excitatory receptors are stimulated by apomorphine and are relatively unaffected by piribedil; inhibitory mediating receptors are maximally stimulated by piribedil and are relatively unaffected by apomorphine. This hypothesis would suggest that apomorphine would be a more effective DA agonist than piribedil in these experiments.

We have not been able to test the effects of apomorphine administered prior to the retention test. At effective doses, apomorphine induced some anorexia in mice so that they lose motivation to run the maze. We did however inject apomorphine (0.5 and 1.0 mg/kg) immediately after reversal training (a time at which clonidine is maximally effective in reversing amnesia) and were unable to demonstrate recovery when retention was tested 24 hr later. It cannot, of course, be concluded that NE is playing an exclusive role in retrieval of memories learned under appetitive motivation. While our preliminary data reported in Experiment 5 does not indicate an important role for acetycholine, other putative neurotransmitters cannot be ruled out.

The results reported in these experiments are consistent with Flexners' contention that protein synthesis inhibition may be producing amnesia by virtue of its disruptive action on adrenergic neurotransmission [9,21]. The demonstration in Experiment 2 that amnesia is prevented if adrenergic receptors are stimulated before CXM is administered, clearly supports this hypothesis. CXM has been shown to result in decreased rate of synthesis of both NE and DA 30 min after training [9] and it is not unreasonable to suspect that some dynamic aspect of the CA system, such as release, reuptake, or receptor sensitivity is malfunctioning at time of testing when animals are amnestic. Transient amnesias, such as those studied in this laboratory in which spontaneous recovery takes place 48 hr after CXM may ultimately be explained in terms of the effects of CXM on catecholamine systems. The major obstacle to the acceptance of the catecholamine hypothesis as a general explanation for all amnesias induced by protein synthesis inhibition is the existence of amnesias which last for days or weeks and do not show spontaneous recovery. It is possible that some persistent defect in CA metabolism could account for these amnesias but this remains to be demonstrated.

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