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Effect of Two Inhibitors of Dopamine β-Hydroxylase on Maturation of Memory in Mice

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FLEXNER, J. B. AND L. B. FLEXNER. *Effect of two inhibitors of dopamine β-hydroxylase on maturation of memory in mice.* PHARMAC. BIOCHEM. BEHAV. 5(2) 117–121, 1976. – Bitemporal injections of puromycin that primarily affect the hippocampal-entorhinal cortical areas suppress memory of maze-learning in mice for 3 days after training but are ineffective 6 or more days after training. At these later times, injections affecting widespread areas of the brain in addition to the hippocampal-entorhinal area are necessary for amnesia. These observations are interpreted to indicate that the locus of the memory trace has enlarged at 6 days to include other parts of the central nervous system in addition to the hippocampal-entorhinal area. To produce an imbalance of neurotransmitters and so to test their importance in enlargement of the memory trace's locus, we treated mice for 7 days after training with inhibitors of dopamine β-hydroxylase. These mice, unlike untreated controls, developed amnesia after bitemporal injections of puromycin. In view of additional control experiments, we interpret these results to suggest that an imbalance of transmitters suppresses the normal enlargement of the locus of the memory trace.

Puromycin Hippocampus and recent memory Neurotransmitter imbalance and longer-term memory Memory Longer-term memory Recent memory

WE REPORTED [8,9] that puromycin injected bitemporally up to 3 days after training (90 μg/injection; total, 180 μg) consistently caused a persistent amnesia of avoidance discrimination learning (Y-maze) in our inbred Swiss-Webster mice. These injections, as judged by the distribution of a fluorescent dye and by the degree and duration of inhibition of protein synthesis [9,11], primarily affected the hippocampus and entorhinal cortex. Injections of the antibiotic that primarily affected the neocortex, thalamus and corpus striatum but largely spared the hippocampus or entorhinal cortex were not amnestic [8]. At 4 and 5 days after training the bitemporal injections were inconsistently amnestic and at 6 or more days they were consistently without effect on retention. At these later times, amnesia was obtained only by making 6 injections of puromycin (30 μg/injection; total, 180 μg) designated as bitemporal, biventricular and bifrontal injections [8]. These 6 injections primarily affected, in addition to the hippocampus and entorhinal cortex, all of the neocortex and to a substantial but lesser degree the thalamus and corpus straitum [10]. Results consistent with these came from a reversal experiment [8] in which mice were initially trained to one arm of a Y-maze, 3 weeks later retrained to the opposite arm, and one day later injected bitemporally with puromycin. These mice, unlike their controls, forgot their most recent training and reverted with a high level of performance to their first behavior pattern. These several observations led to the suggestion that in our mice and in our training situation, recent memory (up to 3 days after training) is supported by the hippocampus and entorhinal cortex and that the locus of the effective memory trace of longer-term memory (6 days or more after training) is enlarged to include wide areas of the central nervous system in addition to the hippocampus and entorhinal cortex.

This view is supported by the findings of Uretsky and McCleary [19] on cats trained in a one-way avoidance task. At 3 hr after training, the cats received a combined entorhinal-fornix lesion (hippocampal isolation) or one of these 2 lesions alone. Only the combined lesion led to a retention deficit. At 8 days after training the combined lesion had no effect on retention, consistent with the puromycin results.

The present experiments were made to test the possibility that the normal enlargement of the locus of the memory trace might be suppressed by creating an imbalance of neurotransmitters. To this end, the synthesis of norepinephrine was inhibited by daily injections for 7 days of one or another inhibitor of dopamine β-hydroxylase. Our observations indicate that this treatment restricts the memory trace to the hippocampal-entorhinal area, preventing the normal enlargement of its locus.

METHOD

Animals and Procedure

Male and female Swiss-Webster mice (30–35 g) from our

inbred colony were housed 4 to a cage at room temperature and were placed in individual cages the day before use. They were trained in a single session in a Y-maze to a criterion of 9 out of 10 correct responses. The maze was constructed of wood painted dull gray and consisted of 3 equally sized arms, 20 cm long, 11 cm wide and 15 cm deep, joined to an equilateral triangularly shaped center compartment with sides of 11 cm. The floor of the maze was composed of brass rods, 3 mm in dia., spaced 1 cm apart. Each compartment could be separated from the center by a guillotine door. Each of the arms had a hinged lid of clear Plexiglas. Intermittent foot-shock manually applied (0.2 0.4 mA from a d.c. source; 2 sec on, 2 sec off) was given for failure to move from the stem of the Y within 5 sec and for errors of left-right discrimination. Shock was adjusted with individual mice to the minimal level (not less than 0.2 mA) that produced the desired behavioral response. After entering the correct arm of the maze and remaining there for 10 sec, the mouse was allowed to climb up a ladder and was returned to its home cage for 30 sec before starting the next trial. The same procedure was used in tests for retention of memory of the training experience. A final test of retention of relearning was made 1 2 weeks after the first retention test. The large majority of mice had good retention on this final test; the 8 exceptions were considered unreliable and were discarded although they did not differ from their respective norm in earlier tests.

Total errors were the sum of latencies greater than 5 sec and of incorrect choices, i.e., all mistakes were added until, in 10 consecutive runs in the maze, the mouse had performed correctly in 9 of them. Memory was evaluated in the retention tests in terms of the percentage savings in errors. This percentage was calculated by subtracting the number of errors to criterion in the retention tests from the number to criterion in training, dividing by the number in training and multiplying by 100. Negative savings were scored as zero. Savings of 100% indicate perfect memory; zero savings, complete loss of memory.

The intracerebral injection technique has been described [8]. Mice were lightly anesthetized with evipal (150 mg/kg). Treatment with puromycin was limited, per mouse, to a total of 180 μg of the dihydrochloride neutralized with NaOH. Each of the bitemporal injections contained 90 μg dissolved in 12 μl of water. Bitemporal plus biventricular plus bifrontal injections contained, per injection site, 30 μg of puromycin in 12 μl of water. Each injection required about 2 sec for delivery; injections were made with an interval of less than one minute between them.

FLA-63 [5] and U14624 [14] were used to inhibit dopamine β-hydroxylase. A minimal amount of IN HCl was used to dissolve FLA-63 (bis (4-methyl-l-homopiperazinl-thiocarbonyl) disulphide; Regis Chemical Co.), the pH adjusted to 5 with NaOH and water added to the desired volume. U14,624 (1-phenyl-3(2-thiazolyl)-2-thiourea; Aldrich Chemical Co.) was prepared as a suspension in 12% gelatin. Both inhibitors were injected intraperitoneally in a volume of 0.2 ml. FLA-63 in a dosage of 13 mg/kg was given daily at 9 a.m.; all mice survived in excellent condition. This dose was chosen after doses of 13 and 25 mg/kg given on alternate days caused an unacceptably high rate of mortality (31%). U14,624 in a dosage of 200 mg/kg was given twice daily at 9 a.m. and 5 p.m.; the mortality rate was 10%. Survivors were in excellent condition.

Norepinephrine of the cerebral hemispheres was determined according to Anton and Sayre [2]; dopamine, according to Adler's unpublished method [1], which employs ethanolic iodine for oxidation, a basic solution of sodium sulfite for reduction, and acetic acid for pH adjustment.

Experimental Plan

We first confirmed our earlier findings [8] that, at 8 days after maze-learning, memory is insensitive to bitemporal injections of puromycin whereas bitemporal plus biventricular plus bifrontal injections of the antibiotic produce profound amnesia, previously found to persist for at least 3 months [9]. We next observed that FLA-63 and U14,624 given one day after training and continued for 7 consecutive days was without effect on retention of memory. With this background, we planned experiments to answer the following questions: (1) Do bitemporal injections of puromycin cause amnesia in mice treated for 7 days after training with the inhibitiors of dopamine β-hydroxylase? These treated mice, unlike their controls, in fact developed amnesia following bitemporal injections; (2) Might the inhibitors enhance the amnestic action of puromycin and so account for the abnormal effectiveness of bitemporal injections noted above? To test this possibility the beginning of the 7 days of drug treatment was delayed until the eighth day after training. Puromycin was given one day later. Delay of drug treatment according to this schedule permitted development of the enlarged locus of the memory trace. If the action of puromycin was unaffected by the inhibitors, it would be expected that the 6 combined injections, but not bitemporal injections, would be amnestic; (3) Since the enlarged locus of the memory trace apparently failed to develop during 7 consecutive days of treatment, would it appear within an extended period of time after termination of treatment? To test this possibility, mice were injected bitemporally with puromycin on the 14th day (twice the normal time for development of the enlarged locus) after treatment was terminated; (4) What was the magnitude and duration of the effect of the inhibitors on the concentrations of norepinephrine and dopamine and did tolerance to the inhibitors develop during treatment? These questions were answered by assaying cerebral catecholamines during the first and seventh days of treatment. Additional assays were made 14 days after training, corresponding to the time of retention-testing.

RESULTS

Behavioral

Mice treated with FLA-63 and U14624 were, with few exceptions, normal in appearance and cage behavior. Treatment with puromycin gave the same symptoms as in otherwise untreated mice -- about 2 days of lethargy, reduced intake of food and water and occasional convulsions.

The effects on retention of memory of the basic control procedures are given in Table 1. In Table 1, arrows separate sequential procedures with the time between 2 procedures given over the arrows. Thus, in Group 1c, mice were trained and 1 day later, intraperitoneal injections of saline were started and were continued for 7 consecutive days. On the day after this treatment, the mice were injected bitemporally with puromycin. Six days later they were tested for retention. The series of controls substantiated previous

TABLE 1

EFFECT OF FLA-63 AND U14,624 ON PUROMYCIN-INDUCED AMNESIA

Groups and Procedures	Medians ± SEM Errors to Criterion	Medians ± SEM % Savings Errors
1. Saline and puromycin controls		
a. Train 1 day Saline i.p. for 7 days 7 & 20 days Test (n=8)	11.5 ± 2.16	100.0 ± 19.7
b. Train 8 days Saline i.p. for 7 days 7 days Test (n=8)	8.0 ± 0.74	82.5 ± 6.35
c. Train 1 day Saline i.p. for 7 days 1 day Puro (T) 6 days Test (n=13)	11.0 ± 0.98	91.0 ± 2.68
d. Train 1 day Saline i.p. for 7 days 1 day Puro (T+V+F) 6 days Test (n=6)	11.0 ± 1.71	0.0 ± 0.0
2. FLA-63		
a. Train 1 day FLA for 7 days 1 day water (T&T+V+F) 6 days Test (n=8)	10.0 ± 0.83	95.0 ± 5.12
b. Train 1 day FLA for 7 days 1 day Puro (T) 6 days Test (n=16)	9.0 ± 0.69	0.0 ± 6.60
c. Train 8 days FLA for 7 days 1 day Puro (T) 6 days Test (n=6)	10.0 ± 0.31	95.0 ± 8.82
d. Train 8 days FLA for 7 days 1 day Puro (T+V+F) 6 days Test (n=6)	8.5 ± 0.99	0.0 ± 0.0
e. Train 1 day FLA for 7 days 14 days Puro (T) 6 days Test (n=7)	8.5 ± 1.19	0.0 ± 11.7
3. U14,624		
a. Train 1 day U14,624 for 7 days 1 day water (T&T+V+F) 6 days Test (n=8)	9.0 ± 1.28	100.0 ± 14.3
b. Train 1 day U14,624 for 7 days 1 day Puro (T) 6 days Test (n=12)	9.0 ± 1.09	0.0 ± 11.2
c. Train 8 days U14,624 for 7 days 1 day Puro (T) 6 days Test (n=6)	10.0 ± 2.67	93.0 ± 14.4
d. Train 8 days U14,624 for 7 days 1 day Puro (T+V+F) 6 days Test (n=6)	11.0 ± 2.08	0.0 ± 6.15

Time between procedures indicated over arrows. Puro = puromycin 2 HCl neutralized with NaOH. T = bitemporal, T + V + F = bitemporal + biventricular + bifrontal injections, IP = intraperitoneal. Savings were not significantly different in Group 1a at 7 (n = 4) and 20 days (n = 4) after IP saline nor were they significantly different in Groups 2a and 3a, respectively, after water (T; n = 4) and water (T + V + F; n = 4). Negative savings scored as zero.

experience [7]. Mice retained a high level of memory of maze-training up to 28 days after training (Group 1a and b). Bitemporal injections of puromycin 9 days after training had no apparent effect on memory (Group 1c) whereas combined bitemporal plus biventricular plus bifrontal injections of the antibiotic at this time were severely amnestic (Group 1d).

The results of the experimental procedures are also given in Table 1. Statistical significance was calculated by Mann-Whitney U test, 2-tailed. Treatment with FLA-63 started 1 day after training and continued for 7 consecutive days (Group 2a) had no significant effect on the normally high level of savings (Group 1a). Treatment with FLA-63 for 7 days followed 1 day later by bitemporal injections of puromycin (Group 2b) resulted in severe amnesia ($p<0.002$), suggesting that the effective memory trace in these mice was restricted, like recent memory, to the hippocampal-entorhinal area. To test the possibility that FLA-63 might enhance the amnestic effects of bitemporal injections of puromycin, treatment with FLA-63 was delayed until the eighth day after training (Groups 2c and d) when longer-term memory associated with a widespread memory trace could be assumed to be present. As in the control experiments (Group 1c) bitemporal injections of puromycin were not amnestic whereas the 6 combined injections (Group 1d) caused profound amnesia ($p<0.002$). It consequently appeared that FLA-63 did not modify the amnestic effects of bitemporal injections of puromycin.

Treatment with U14624 gave results like those with FLA-63. Retention of memory was unaffected by 7 days treatment with U14624 (Group 3a). Bitemporal injections of puromycin one day after the end of the period of treatment led to amnesia (Group 3b). There was no evidence that U14624 enhanced the amnestic effects of bitemporal injections of puromycin. As with FLA-63 bitemporal injections were ineffective when treatment with U14624 was delayed until the eighth day after training (Group 3c); production of amnesia required the 6 combined injections (Group 3d).

Finally, mice were treated for 7 days with FLA-63 and after an additional 14 days were injected bitemporally with puromycin (Group 2e) to test whether the enlarged locus of the memory trace would develop under these circumstances. The small savings following puromycin were not significantly different ($p>0.1$) from those mice that had complete amnesia (Group 1d). It therefore appeared that within the time limits of the experiment longer-term memory with its enlarged locus failed to develop.

Biochemical

A single injection of FLA-63 (13 mg/kg) on the first day of treatment depressed the level of norepinephrine to a statistically significant degree during the interval from at least 3 to 8 hr after the injection with a maximum depression of 50% at 3 hr (Fig. 1). Much the same pattern was found on the seventh day of treatment. Concentrations of dopamine were not significantly changed during either of these days. Both norepinephrine and dopamine were at control concentrations 7 days after termination of treatment when retention tests of memory were usually made.

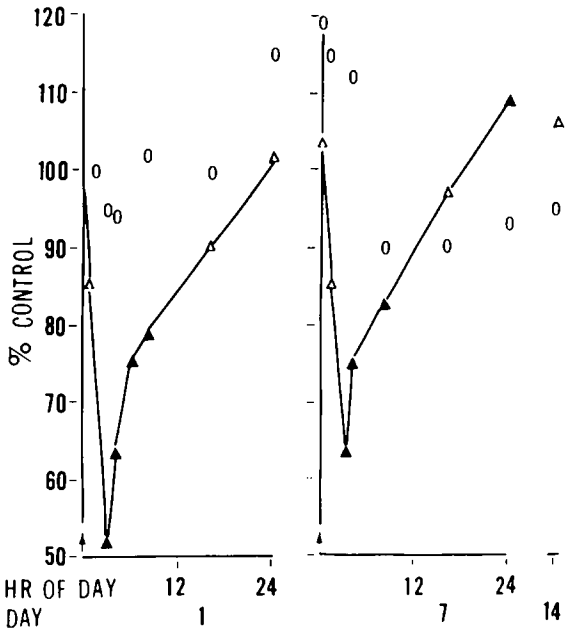

FIG. 1. Effect of FLA-63 (13 mg/kg daily for 7 days) on cerebral levels of norepinephrine (^) and dopamine (0) during Days 1 and 7 of treatment. Injection time indicated by arrows. Values are averages of 2–4 mice. Solid symbols indicate statistically significant ($p<0.05$) differences from controls; open symbols, insignificant differences (Mann-Whitney U test, 2-tailed). Control means ± SEM n = 9) for norepinephrine and dopamine = 0.39 ± 0.01 and 1.35 ± 0.11 μg/g, respectively (both corrected for 75% recovery).

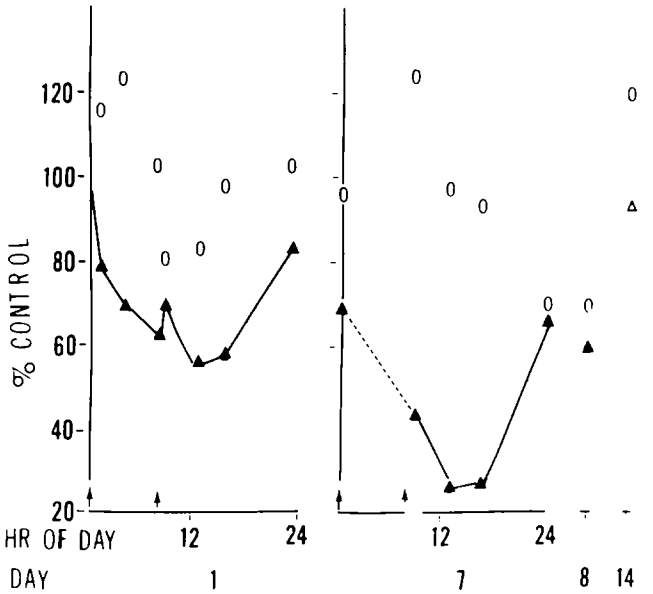

FIG. 2. Effect of U14,624 (200 mg/kg twice daily for 7 days) on cerebral levels of norepinephrine (^) and dopamine (0) during Days 1 and 7 of treatment. Other details as in Fig. 1.

The twice daily injections of U14624 (200 mg/kg per injection) depressed norepinephrine levels to a significant degree throughout the 24 hr of the first day with a maximum depression of 45% (Fig. 2). The effects of the drug were more pronounced on the seventh day with a maximum depression of 75%. Norepinephrine levels had returned to normal 7 days after treatment at the time of retention tests for memory (Day 14 in Fig. 2). The variations in concentrations of dopamine were not statistically significant at any time.

DISCUSSION

It appears well established in man that the hippocampal formation is critically concerned in recent memory. Severe impairment of recent memory has been found after bilateral surgical hippocampectomy [16, 18, 20], in cases of senile dementia with prominent lesions in the hippocampus [12], and in instances of inclusion-cell encephalitis with predominant lesions of the hippocampus [4]. Distant memory is not impaired in these instances nor by a variety of other cerebral lesions indicating that with time there is a delocalization of the mechanisms that support memory and that they become more widely distributed in the central nervous system.

The numerous observations in laboratory animals on the role of the hippocampus in recent memory have not led to the same general agreement as in man. The difference in viewpoints is exemplified in two recent reviews. Kesner and Wilburn [15] support the hypothesis that the hippocampus is important in memory consolidation. They point out that in the majority of studies stimulation of the hippocampus at intensity levels below initiation of seizure after-discharge has a disruptive effect on consolidation. Izquierdo [13], however, notes that hippocampal injury in laboratory animals has not been shown to cause deterioration of all types of learning but rather to show diverse degrees of impairment depending upon the type of learning. Our results [8,9] and those of Uretsky and McCleary [19], presented above, suggest that in certain but not all types of learning [3], more consistent findings with respect to recent memory might result from extending lesions of the hippocampus to include the entorhinal cortex. The results of these investigators also provide evidence, paralleling that in man, that with time the effective memory trace spreads from the hippocampal-entorhinal area to include other parts of the central nervous system.

Our experiments with inhibitors of dopamine β-hydroxylase are the first effort known to us to gain insight into neurochemical mechanisms that may underlie the delocalization of mechanisms that support memory. The evidence suggests that delocalization can be suppressed by an imbalance of neurotransmitters and that, consequently, an appropriate balance of transmitters is essential for the normal development of the process. There is, however, the possibility that treatment with the inhibitors for 7 days produced unknown biochemical side-effects, not related to transmitters, that contributed to the suppression.

The nature of the imbalance of transmitters cannot be adequately defined. Our analyses indicate that levels of cerebral norepinephrine were reduced by FLA-63 for at least 8 hr a day for 7 days and by U14624 for the whole of the 7 days of treatment without significant effects on dopamine. Corrodi *et al.* [5] have also shown in the rat that FLA-63 does not affect the stores of serotonin. Important indirect effects of the treatment, however, may well have

occurred. There is, for example, evidence that norephinephrine from adrenergic terminals modulates the release of acetylcholine from cholinergic terminals [17] suggesting that our treatment may have affected the release of acetylcholine in addition to norepinephrine. It has also been found that depletion of the stores of norepinephrine in reserpine-treated rats leads to a central receptor supersensitivity to norepinephrine that persists for at least 7 days after treatment [6], suggesting that a similar change might have occurred in our experiments. Such a change, if it occurred, was not accompanied by a change in footshock sensitivity of individual mice.

The failure of delocalization of the memory trace to occur 14 days after cessation of treatment may have been due to a persistent indirect effect of the treatment; alternatively, perhaps delocalization is restricted to a critical period after learning. Fortunately, methods are available to test some of the possibilities and to study the effects of transmitter imbalance produced in other ways.

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