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Studies on Memory: The Cerebral Spread of An Engram in Mice as Affected by Inhibitors of Dopamine β-Hydroxylase

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FLEXNER, J. B., L. B. FLEXNER AND A. C. CHURCH. *Studies on memory: The cerebral spread of an engram in mice as affected by inhibitors of dopamine β-hydroxylase.* PHARMACOL BIOCHEM BEHAV **18**(4) 519–523, 1983.— Bitemporal injections of puromycin that primarily affect the hippocampal-entorhinal areas consistently cause amnesia of maze-learning in mice for 3 days after training but become consistently ineffective if given 6 or more days after training. At these later times, additional puromycin injection sites covering widespread areas of the forebrain are necessary to induce amnesia. These observations are interpreted to indicate that the locus of the engram has become more widespread within the 6-day period. Treatment with inhibitors of dopamine β-hydroxylase for 3 days following training, retarded the spread of memory from a matter of days to a period of weeks. Repeated treatment with the inhibitors restricted engram spread for about 3 months; again spread was evident about a month after the last treatment. These observations imply that the mechanisms responsible for engram spread are capable of surviving for extraordinarily long periods of time.

Recent memory Remote memory Memory spread Dopamine β-hydroxylase Hippocampus Puromycin

WE have previously observed that recall of shock-motivated Y-maze learning in mice appears to be initially dependent on the integrity of neural systems located within the temporal lobe. With time, this memory trace spreads to wider cerebral areas [6,8]. The forebrain distribution of the engram can be followed by exploiting the amnesic effects of intracerebral injections of puromycin. If bitemporal injections of puromycin (90 μg/injection; total 180 μg) that primarily reach the hippocampus and entorhinal cortex [8,9] are administered within 3 days after learning, a persistent amnesia of the maze learning occurs in our inbred Swiss-Webster mice. If puromycin administration is delayed until 6 or more days after training, the bitemporal injections are without effect on retention. At these later times, amnesia is obtained only by making 6 injections (30 μg/injection; total 180 μg) that affect, in addition to the temporal lobe areas, all of the neocortex and to a lesser degree the thalamus and corpus striatum [7]. These observations with puromycin are supported by the findings in cats trained in a one-way avoidance task [21]. At 3 hours after training, the cats received a bilateral combined entorhinal-fornix lesion (hippocampal isolation) that caused a large retention deficit. However, similar to our results with bitemporal injections of puromycin, the lesion did not produce significant retention deficits if performed 8 days after training. We have pointed out [19] that the development with time of multiple or redundant neural pathways, widely distributed, would be sufficient to explain these and related behavioral phenomena.

In other experiments [5] we have found that treatment of mice with inhibitors of dopamine β-hydroxylase for 7 consecutive days after training suppressed the spread of the memory engram from its initial temporal lobe locus; namely, bitemporal injections of puromycin produced amnesia for at least 22 days after training. The present experiments were designed: (1) to evaluate the effects of inhibitor dosage on the spread of memory; (2) to test the possibility that, by waiting for longer periods of time after the inhibition of norepineprhine (NE) synthesis, engram spread might be observed; (3) to determine whether administration of inhibitors prior to training would retard the spread of memory; and (4) to test if the effectiveness of a drug in retarding spread was related to its degree of inhibition of dopamine β-hydroxylase. The results indicate that relatively low doses of the inhibitors restrict the engram to the hippocampal-

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entorhinal area but that with sufficient time (a matter of weeks in contrast to 6 days) the enlargement of its locus does occur.

METHOD

Animals and Behavioral Procedures

Males and female Swiss-Webster mice from our closed colony were housed four to a cage and, after ramdom selection, were placed in individual cages the day before use. The mice were trained in a single session in a Y-maze, previously described [5], to a criterion of 9 out of 10 correct responses. Details of the training experience have been given [5]. In brief, intermittent foot shock, manually applied (0.2–0.4 mA from a DC source), was given both for failure to move from the stem of the Y within 5 sec and for errors of left-right discrimination. The same procedure was used 6–8 days after treatment with puromycin in tests for retention of memory of the training experience. An error was assigned for a failure to make a choice within 5 sec and for an error in choice. These mistakes were added until in 10 consecutive runs in the maze the mouse performed correctly in nine out of them. Memory was evaluated in the retention tests in terms of the percentage of savings or errors [5]. Negative savings were scored as zero.

The intracerebral injection technique has also been described [6]. Mice were lightly anesthetized with evipal (150 mg/kg). Treatment with puromycin was limited to bitemporal injections each of which contained 90 μg of the dihydrochloride neutralized with NaOH and dissolved in 12 μl of water. Each injection required about 2 sec for delivery; the interval between injections was less than a minute.

Fla-63 (Fla; Regis Chemical Co.) and U14624 (Aldrich Chemical Co.), inhibitors of dopamine β-hydroxylase [2,14], were prepared for injection as previously noted [5]. Both inhibitors were injected intraperitoneally in a volume of 0.2 ml. Fla was given daily in single doses of 13, 6 or 3 mg/kg, while the daily single dose for U14624 was 200 mg/kg. With four exceptions, all mice survived these treatments in excellent condition.

Biochemical Procedure

Mice were sacrificed by cervical dislocation. The cerebral hemispheres were rapidly removed and the catecholamines then extracted by a modification of the butanol extraction method of Shore and Olin [20]. Tissue was homogenized in 20 volumes of acid n-butanol (0.085% HCl) and centrifuged at 10,000 $\times$g for 10 minutes. To 1.0 ml of the supernatant, there was added 250 μl of a solution containing 0.1 N HCl, 100 μM EDTA and 250 pmol of 3,4-dehydroxybenzylamine (an internal standard; Aldrich Chemical Co.) followed by 2 ml of cold heptane. The mixture was briefly vortexed and again centrifuged at 10,000 $\times$g for 10 minutes. The organic phase (upper layer) was discarded and heptane was again added to the aqueous phase. Following centrifugation, an aliquot of the aqueous phase was injected into a high performance liquid chromatography system. The amines were separated on a 25 cm C-8 Ultrasphere reverse phase column (Altex) at 30° using a mobile phase consisting of 1% acetic acid, 6% methanol, 5 mM heptane sulfonic acid and 100 μM EDTA (pH 2.8). The effluent was passed through a thin layer detector cell (TL-3; Bioanalytical Systems) containing a graphite paste electrode, the potential of which was maintained at 720 mV versus a Ag/AgCl reference electrode. Changes in current were measured on a LC-4 electronic controller (Bioanalytical Systems). The chromatogram was recorded on a Houston Instruments strip chart recorder. Catecholamines were quantified by peak height comparison to the internal standard [3].

Other Procedures

Statistical analyses were made with the Kruskal-Wallis one-way analysis of variance and post hoc comparisons with the Mann-Whitney U test.

The appearance of amnesia following bitemporal injections of puromycin was taken to mean that the memory trace was confined to the temporal lobe; the absence of amnesia following this treatment was taken to mean that the widespread engram was present.

RESULTS

Behavioral

After treatment with Fla and U14624, mice were normal in appearance and in cage and maze behavior. Treatment of these mice with puromycin gave the typical symptoms of otherwise untreated mice—about 2 days of lethargy, reduced intake of food and water and occasional convulsions.

Fla and U14624 affected engram spread as given in Table 1. Variance of the training scores between mice with high (>75%) savings and with low relearning scores was insignificant, $H(1)=2.9$, $p>0.05$; variance of the relearning savings of the 2 groups was significant, $H(1)=10.6$, $p<0.01$.

In Table 1, arrows separate sequential procedures with the time between procedures noted above the arrows. For example, in Group 1d the mice were trained, then after a day injected intraperitoneally with Fla (13 mg/kg per day) for 3 days and then after 10 days injected bitemporally with puromycin. The mice were retrained 6–8 days later.

Group 1 of Table 1 shows the effectiveness of post-training Fla in suppressing memory spread as a function of dose, duration of treatment and delay of treatment after training. As noted above, to suppress spread of the engram, we previously used a daily dose of 13 mg/kg of Fla for 7 consecutive days [5]. Treatment with the same dose for 3 days following training (Group 1d) was equally effective in blocking memory spread (i.e., bitemporal injections of puromycin 14 days after training were fully amnesic). Treatment for 2 days (Group 1c) was significantly ($p=0.029$) but incompletely effective and for one day (Group 1b) was ineffective (i.e., bitemporal injections of puromycin were not amnesic). Treatment with a daily dose of 6 mg/kg of Fla (Group 1e) for 3 days following training again led to complete suppression of spread whereas 3 doses of 3 mg/kg of the inhibitor (Group 1g) were ineffective. Fla in 3 daily doses of 6 mg/kg became ineffective when the beginning of treatment was delayed from day 2 (Group 1e) after training to day 4 (Group 1f).

Group 2 of Table 1 shows that after suppressive Fla treatment the engram eventually spread beyond the temporal region. With daily doses of 13 mg/kg of Fla started one day after training and continued for 7 consecutive days, statistically significant engram spread was absent 24 days after treatment (Group 2a) but was present 40 days after treatment (Group 2b). Treatment with a smaller daily dose of Fla, 6 mg/kg, for days 2 through 4 after training led to much the same results. There was no evidence of the spread of memory 15 days after treatment (Group 2c); the savings on re-

TABLE 1

EFFECT OF Fla-63 AND U14624 ON SPREAD OF ENGRAM

Groups	Procedures	Initial Training Errors to Criterion	Relearning* % Savings Errors
1. Fla-63 Dose responses			
a.	Train —1 day—> saline days 2–4 —10 days—> Puro (4)	9.8 ± 1.0	90.3 ± 6.4
b.	Train —2 days—> Fla 13 mg/kg day 3 —10 days—> Puro (4)	8.3 ± 0.9	77.0 ± 3.1
c.	Train —1 day—> Fla 13 mg/kg days 2,3 —10 days—> Puro (4)	9.0 ± 1.1	31.3 ± 18.8
d.	Train —1 day—> Fla 13 mg/kg days 2–4 —10 days—> Puro (4)	8.5 ± 0.6	0.0
e.	Train —1 day—> Fla 6 mg/kg days 2–4 —10 days—> Puro (5)	10.0 ± 1.4	0.0
f.	Train —1 day—> Fla 6 mg/kg days 4–6 —10 days—> Puro (4)	8.5 ± 0.6	91.0 ± 4.3
g.	Train —1 day—> Fla 3 mg/kg days 2–4 —10 days—> Puro (4)	9.3 ± 0.9	84.0 ± 3.1
2. Fla-63 Appearance of engram spread following its suppression			
a.	Train —1 day—> Fla 13 mg/kg days 2–8 —24 days—> Puro (6)	7.3 ± 1.2	11.7 ± 11.7
b.	Train —1 day—> Fla 13 mg/kg days 2–8 —40 days—> Puro (4)	10.0 ± 1.6	81.3 ± 5.2
c.	Train —1 day—> Fla 6 mg/kg days 2–4 —15 days—> Puro (4)	8.8 ± 1.4	8.3 ± 8.3
d.	Train —1 day—> Fla 6 mg/kg days 2–4 —26 days—> Puro (5)	8.0 ± 1.1	34.8 ± 25.8
e.	Train —1 day—> Fla 6 mg/kg days 2–4 —40 days—> Puro (7)	9.6 ± 0.7	82.3 ± 3.8
f.	Train —1 day—> Saline days 2–4; 19–21; 36–38; 53–55; 70–72 —10 days—> Puro (7)	7.4 ± 0.7	81.2 ± 2.6
g.	Train —1 day—> Fla 6 mg/kg as in 2f —10 days—> Puro (5)	9.0 ± 0.5	0.0
h.	Train —1 day—> Fla 6 mg/kg as in 2f —39 days—> Puro (5)	9.2 ± 0.6	90.8 ± 5.0
3. Fla 63 Effect of 6 mg/kg daily given before training			
a.	Fla days 1–3 —9 days—> Train —10 days—> Test (4)	8.0 ± 0.7	97.0 ± 3.0
b.	Fla days 1–3 —9 & 15 days—> Train —10 days—> Puro (8)	8.0 ± 0.9	3.6 ± 3.6
c.	Fla days 1–3 —21 days—> Train —10 days—> Puro (5)	9.0 ± 1.4	94.4 ± 3.4
4. U14624. 200 mg/kg daily in all experiments			
a.	Train —1 day—> U14624 days 2–4 —10 days—> Puro (4)	10.5 ± 1.0	0.0
b.	Train —1 day—> U14624 days 2–4 —24 days—> Puro (5)	8.2 ± 0.6	88.6 ± 3.2
c.	U14624 days 1–3 —9 days—> Train —10 days—> Puro (5)	8.4 ± 0.7	0.0
d.	U14624 days 1–3 —15 days—> Train —10 days—> Puro (6)	7.0 ± 0.4	90.7 ± 4.4

Time between procedures indicated over arrows. Puro=puromycin 2 HCl neutralized with NaOH and in all instances injected bitemporally. Relearning tests 6–8 days after treatment with puromycin. Fla, U14624 and saline (0.2 ml) injected intraperitoneally. Savings in groups 3b were not significantly different at the 2 times indicated. Figures in parentheses=number of mice per group. Negative savings scored as zero. Training and testing results expressed as means ± SEM.

*In mice with puro, high scores (savings > 75%) = full and low scores = incomplete or no engram spread. See text for additional details.

learning 26 days after treatment were statistically insignificant (Group 2d) while at 40 days after treatment the enlarged engram had appeared (Group 2e).

Group 2 also shows that the widespread engram developed after a more prolonged period of suppression. In these experiments, mice received on days 2 through 4 after training daily doses of 6 mg/kg of Fla; then, with intervals of 14 days, this treatment was repeated 4 times to end 72 days after training. Consistent with previous expereince [6], mice similarly treated with saline and injected bitemporally 10 days later with puromycin had high retraining scores (Group 2f). Bitemporal injections of puromycin 10 days after the 5 groups of treatment with Fla caused complete amnesia; there was no evidence of the spread of memory (Group 2g). Extension of the interval between termination of treatment with Fla and treatment with puromycin to 39 days, however, was followed by the full development of the widespread engram (Group 2h).

Our next experiments tested the effect of pre-training injections of Fla on the spread of memory (Group 3). In these experiments, Fla was injected in a daily dosage of 6 mg/kg for 3 consecutive days ending 9, 15 or 21 days before training. Memory of maze-learning was unaffected by this treatment (Group 3a). To test for the appearance of the enlarged engram in subsequent experiments, mice received bitemporal injections of puromycin 10 days after training. In mice trained 9 or 15 days after termination of Fla injections there was no evidence of spread (Group 3b). Full development of the wider engram was, however, observed when the interval between treatment with Fla and training was extended to 21 days (Group 3c). Thus it appears that a 3-day treatment before training with 6 mg/kg of Fla renders the brain incapable of transferring mnemenic information for at least 25 days.

Experiments with U14624 were limited to a repetition of those that we considered basic in our studies with Fla. In our previous studies with U14624 [5], mice that failed to develop the widespread engram 9 days after training received 400 mg/kg of the drug daily for 7 consecutive days. In the present experiments, daily dosage was reduced to 200 mg/kg during days 2 through 4 after training with the results that engram spread was completely suppressed 14 days after training (Group 4a) but had occurred 28 days after training (Group 4b). The same treatment with U14624 given 9 days before training which was followed after 10 days with bitemporal injections of puromycin failed to lead to engram spread (Group 4c) while extension of the interval between the termination of drug treatment and training to 15 days resulted in its appearance (Group 4d).

Biochemical

The biochemical experiments were made to test the relationship between the degree and duration of inhibition of dopamine β-hydroxylase (as judged by levels of NE) and the suppression of spread of the engram. One group of mice was injected with the suppressive dose of 6 mg/kg of Fla and a second group with the ineffective dose of 3 mg/kg. As shown in Fig. 1, measurements were made 1, 3, 6 and 17 hr after a single treatment. Variance of the concentrations of NE of these 2 groups and of the controls was significant, $H(2)=10.50, p<0.01$; that of dopamine was again [5] found to be insignificant, $H(2)=4.02$, $p>0.20$. NE levels following treatment with 6 mg/kg of Fla were significantly reduced ($p=0.022$) at all times that were tested. Maximum reduction to 60% of the control value was observed 6 hr after injection

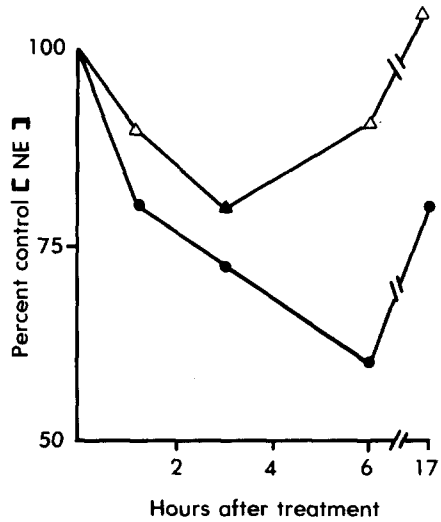

FIG. 1. Effect of a single subcutaneous dose of Fla-63 (△=3 mg/kg; 0=6 mg/kg) on cerebral levels of NE. Values are averages of 2 (6 mg/kg) or 4 (3 mg/kg) mice. Solid symbols indicate statistically significant differences from controls; open symbols, insignificant differences. Control mean±SEM (n=8)=0.77±0.03 μg/g. See text for additional details.

with recovery to 80% of the control value at 17 hr. With 3 mg/kg of Fla, the single significant ($p=0.008$) reduction of NE values amounting to 80% of the control value occurred 3 hr after treatment. In previous studies with U14624 [5], significant reductions in NE levels were found 1, 4, and 8 hr after the injection of 200 mg/kg with a maximum reduction to 60% of the control value 8 hr after injection. It is evident that the effectiveness of a treatment in suppressing engram spread is correlated with its effectiveness in inhibiting dopamine β-hydroxylase.

DISCUSSION

As noted previously [5], severe impariment of recent memory in man has been found after bilateral surgical hippocampectomy [16, 18, 22] as well as in cases of senile dementia [11], inclusion-cell encephalitis [1], and vascular accidents [24] when there are prominent lesions in the hippocampus. Remote memory persists in spite of such gross hippocampal damage. Apparently, with time, the mechanisms that support memory become widely distributed in the brain.

There are also numerous observations in laboratory animals that support the role of the hippocampal-entorhinal area in recent memory [12, 13, 15, 17]. Relatively little has been done, however, with respect to studying the means by which recent memory in this area is encoded in other cerebral regions. Our interest in this problem came from the observations noted above that bitemporal injections of puromycin block recently acquired memory whereas 6 widespread intracerebral injections of the antibiotic are required to block more remote memory. Results consistent with these came from a reversal experiment [6] in which mice were initially trained to one arm of a Y-maze and 3 weeks later retrained to the opposite arm. One day later, they were 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

behavioral pattern; i.e., memory of recent training was blocked by bitemporal injections of puromycin whereas more remote memory was spared. A further test of the concept of wide spread multiple or redundant neural pathways was provided by the finding that puromycin's block of memory could be removed by intracerebral injections of isotonic saline [4]. Although 6 widespread intracerebral injections (12 μl/injection) of puromycin are required to block remote memory, one injection of saline (12 μl/injection) at only 2 of the 6 sites is needed to restore the memory. Similarly, the results in cats [21] clearly illustrate that as in man, the engram spreads from the hippocampal-entorhinal areas to include other parts of the brain.

Our finding of chief interest in the present studies is that the spread of memory did occur after surprisingly long periods of its suppression. When a single period of treatment with Fla or with U14624 was started one day after training and continued for 3 or 7 days, suppression lasted for about a month. When multiple periods of treatment with the inhibitors were employed, suppression lasted for about 3 months. These observations imply that the underlying mechanisms responsible for spreading memory (1) continue to operate on the "unmoved engram" until memory redundancy is achieved; (2) are capable of surviving for extraordinarily long periods of time.

In our earlier experiments, following treatment with Fla or U14624, we failed to find evidence of a widespread engram up to 22 days after training [5]. We concluded that this failure might have been due to persistent side effects of the treatment, or, alternatively, that the transfer of memory is restricted to a critical period after learning. This last possibility is made untenable by the results presented here whereas the first possibility is strengthened. The assays made here indicate that effective doses of Fla inhibit the synthesis of NE for at least 6 hr and that normal levels of NE are present about 24 hr after treatment. These results leave us with the problem of the nature of the changes in the brain that follow either pre- or post-training treatment with the inhibitors of dopamine β-hydroxylase and that account for the month-long period necessary for the brain to recover its ability to initiate the spread of memory. As shown in these and the earlier experiments, the inhibitors, as we have used them, had no effect on learning or, in the absence of puromycin, had no effect on memory. The spread of the engram is evidently a more vulnerable process than its initial formation.

It will be of interest to supplement our studies of the actions of the inhibitors of dopamine β-hydroxylase by blocking cerebral noradrenergic receptors. Because of observations suggesting that adrenergic processes outside the brain may influence memory [10,23], it is also desirable to block peripheral noradrenergic systems selectively in order to assess the possible interference of autonomic changes produced by our drug treatments.

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