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Studies on Memory: Spontaneous Return of Memory in 6-Hydroxydopamine-Treated Mice and its Relation to Cycloheximide-Induced Transient Amnesia

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RAINBOW, T. C. AND L. B. FLEXNER. *Studies on memory: spontaneous return of memory in 6-hydroxydopamine-treated mice and its relation to cycloheximide-induced transient amnesia.* PHARMAC. BIOCHEM. BEHAV. 8(1) 1–5, 1978. – We suggested previously that cycloheximide-induced transient amnesia was due in part to side-effects of the antibiotic on the central adrenergic system at the time of testing and that spontaneous return of memory depended upon recovery of the adrenergic system. To test these possibilities, mice chronically depleted of brain catecholamines (CAs) by 6-hydroxydopamine (6–OHDA) were trained in an avoidance-discrimination task and tested for spontaneous return of memory. Contrary to our hypothesis, amnesia at 24 hr after training was followed by recovery of memory by 72 hr, which indicates that recovery of memory can occur independently of adrenergic recovery. Injection of α-methyl-para-tyrosine immediately after training prevented return of memory at 72 hr, suggesting that the residual CAs remaining after 6–OHDA are necessary for memory to spontaneously return at this time.

6–Hydroxydopamine Cycloheximide Amnesia

WE [9, 25, 26] and others [19, 20, 24, 29, 33] have found that the amnesia present 24 hr after training conducted in the presence of the inhibitors of protein synthesis, cycloheximide (CXM) and its analog, acetoxycycloheximide (AXM), is followed by the spontaneous return of memory; in contrast, other investigators [12, 13, 14, 21] have found the amnesia to be persistent. There is substantial evidence that the amnesia present at 24 hr is attenuated in rats [26], mice [4, 6, 19] and chicks [15] by adrenergic stimulants [4, 15, 19, 26] or monoamine oxidase (MAO) inhibitors [6,19] for a limited time before or after training or before retention-testing. Observations of this kind led us [26] to suggest that the amnesia at 24 hr is based at least in part on the dysfunction at this time of the catecholaminergic (CA) system caused by side-effects of the antibiotics and that the return of the memory, when it occurs, is due to recovery of the CA system. Consistent with the assumed dysfunction, tyrosine hydroxylase activity as measured in vitro [11,30] and rate of synthesis of CAs were found to be inhibited, the former for a short time and the latter up to 12 hr but not at 17 hr after treatment [10,16]. Recovery from these biochemical effects clearly precedes the disappearance of amnesia and is inconsistent with our suggestion that, in these instances, complete recovery of the CA system coincides with the appearance of memory.

To test further this part of our working hypothesis, we injected mice intraventricularly with 6-hydroxydopamine (6–OHDA) which, with minimal non-specific damage [5], severely and persistently reduces cerebral levels of CAs, and then trained the mice minimally in an aversive-discrimination task. The mice developed amnesia at 24 hr but contrary to our hypothesis, in spite of their enduring loss of CAs, spontaneously recovered memory 72 hr after training. Further depletion of CAs in 6–OHDA-treated mice with α-methyl-p-tyrosine (AMPT) shortly after training abolished the spontaneous return of memory. Furthermore, this amnesia was not relieved by treatment with an MAO inhibitor shortly before retention-testing.

METHOD

Male C57BL/6J mice (20–25 g; Jackson Laboratories) were placed in individual cages with free access to food and water. Two weeks before behavioral training they were anesthetized with nembutal (75 mg/kg IP) and either 38 μg of 6–OHDA–HBr (Regis Chemical Co.) in 5 μl of saline containing 0.1% ascorbic acid or ascorbic acid vehicle alone was injected into each lateral ventricle. Injections were made with a Hamilton syringe positioned to the stereotaxic coordinates A = 2.0 mm, L = 1.0 mm and V = 2.0 mm. Most 6–OHDA-treated mice were aphagic after injection and for 3–5 days the normal laboratory chow was supplemented with a paste made from powdered chow and warm water. By the end of the first week, cage behavior, feeding and drinking appeared normal. Postoperative mor-

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tality of mice treated with 6–OHDA was 20%.

Before training mice in the Y-maze previously described [8], they were given 4 trials without shock to determine if they had a side preference. If a preference was evident, mice were trained to the opposite side; otherwise the choice of side was arbitrary. Mice were trained to a criterion of 3 out of 4 correct consecutive trials. Intermittent foot-shock manually applied (0.2–0.4 mA from a d.c. source; 2 sec on, 2 sec off) was given for a 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 5 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. Mice that took fewer than 4 or greater than 30 errors to reach criterion were discarded.

Mice were tested for retention by retraining them and comparing the percentage savings in total errors between training and testing. Total errors were the sum of all mistakes (latencies greater than 5 sec and incorrect choices) made to achieve a criterion of 3 correct out of 4 successive trials. The percentage savings in errors was calculated by subtracting the number of errors to criterion in the retention tests from the number to criterion in training, dividing by the errors made in training and multiplying by 100. A mouse that made the same number of errors at both training and testing would have a retention of 0% while a mouse that made no errors during testing would have a retention of 100%.

Immediately after training, some 6–OHDA-treated mice received either 50 mg/kg AMPT-methyl ester (Sigma Chemical Co.) or 75 mg/kg pargyline-HCl (Regis), a monoamine oxidase inhibitor. Both drugs were dissolved in saline and injected intraperitoneally in a volume of 0.2 ml Pargyline was also given 1 hr before testing in a separate experiment.

Catecholamines were extracted and norepinephrine (NE) assayed according to the method of Anton and Sayre [2] as modified by Moore and Smith [18]. Dopamine (DA) was determined 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. Values expressed are uncorrected for 70% recovery. For the CA assays on brain areas, tissues from 3 mice were pooled to obtain sufficient material. To determine the effects of AMPT on brain CAs remaining after 6–OHDA, 6–OHDA-treated mice were injected IP with 50 mg/kg AMPT and sacrificed 1 hr later. All animals used for CA determinations had been used 1 or 2 days previously in behavioral studies. Statistical significance for all studies were calculated by the 2-tailed Mann Whitney U test [27], chosen because of its utility in analyzing small samples.

RESULTS

Biochemical

Figure 1 shows the effect of 6–OHDA on CA levels in the cerebral hemispheres at 2–3 weeks after injection. Median NE and DA after 75 μg 6–OHDA-HBr were reduced, respectively, by 84 and 63% of control values. In 4 6–OHDA-treated mice given 50 mg/kg AMPT and sacrificed after 1 hr, there was a further significant reduction

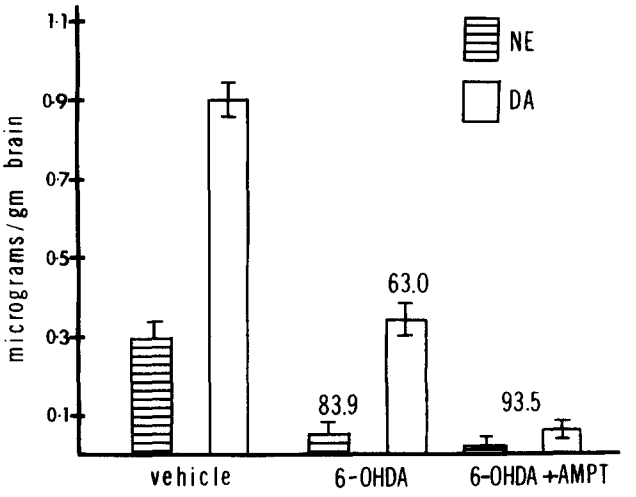

FIG. 1. Effects of 75 μg 6–OHDA·HBr alone or supplemented by α-methyl para tyrosine (AMPT) on NE and DA levels in the cerebral hemispheres of mice. Each value is the median ± SEM of 4–7 separate determinations, uncorrected for 70% recovery. Numbers indicate % of control NE and DA lost after treatment. CA levels were measured 2–3 weeks after intraventricular injection of 6–OHDA or ascorbic acid vehicle. 6–OHDA + AMPT mice were given 50 mg/kg AMPT IP 1 hr before sacrifice. NE and DA levels in 6–OHDA-treated mice were significantly lower than vehicle-treated mice ($p = 0.002$, U = 0) and CA levels in 6–OHDA + AMPT mice were significantly lower than CAs of 6–OHDA mice ($p = 0.006$, U = 0).

of CAs ($p = 0.006$) amounting to a 94% depletion of vehicle-treated values. Table 1 shows that severe depletion of amine was present throughout the brain with cortical NE showing the greatest depletion to 4% of control values.

Behavioral

The median number of acquisition errors to criterion in 6–OHDA-treated mice (N = 52) was 16.0 ± 1.6 errors; in vehicle-treated mice (N = 15), 14.5 ± 1.7 errors; these values are not significantly different. Nor did significant differences exist in errors to criterion among individual groups used in the experiments. Four 6–OHDA-treated mice and 1 vehicle control were discarded for inability to achieve criterion at training. No differences were noticed in the sensitivity of 6–OHDA or vehicle-treated mice to footshock.

As seen in Table 2, 6–OHDA-treated mice showed significant amnesia when tested 24 hr after training ($p<0.001$, U = 1), compatible with our observations on passive avoidance training [22]. Injection of pargyline immediately after training resulted in significant improvement of savings when compared to 6–OHDA mice that received only saline ($p = 0.028$, U = 0). When tested at 72 hr after training, however, 6–OHDA mice showed nearly uniform 100% savings, significantly better than at 24 hr ($p<0.001$, U = 0) and better than vehicle injected mice tested at 72 hr ($p = 0.006$, U = 2). To test the possibility that the improvement in savings at 72 hr was dependent on the CAs remaining after 6–OHDA, 6–OHDA mice were injected with AMPT immediately after training. This resulted in a significant amnesia at 72 hr compared to 6–OHDA mice given only saline ($p = 0.028$, U = 0). This dose of AMPT reduces CA synthesis for only a few hours [10] so it is unlikely that any enduring effects of the drug could inhibit the ability of 6–OHDA mice to perform the

TABLE 1

EFFECT OF 6-OHDA ON CATECHOLAMINES (CAs) IN VARIOUS REGIONS OF MOUSE BRAIN

Region	CA	Median ± SEM Vehicle μg/g	Median ± SEM 6-OHDA μg/g	% depletion after 6-OHDA %
Hippocampus + Entorhinal cortex	NE	0.19 ± 0.02	0.02 ± 0.01	89.5
Diencephalon	NE	0.40 ± 0.08	0.12 ± 0.03	70.0
Striatum	DA	4.40 ± 0.08	0.98 ± 0.02	77.7
Cortex	NE	0.23 ± 0.06	0.01 ± 0.00	95.5

CA concentrations in various regions of mouse brain after intraventricular injection of 75 μg 6-OHDA·HBr 2-3 weeks before sacrifice. Each value is the median ± SEM of 4 determinations made on tissue pooled from 3 mice. 6-OHDA treated regions were significantly different from their respective controls (p = 0.028, U = 0).

TABLE 2

EFFECT OF 6-OHDA ON RETENTION AT 24 HR AND 72 HR AFTER TRAINING

Time after training and procedures	% savings errors Median ± SEM
1. 24 hr	
a. Vehicle (N = 9)	85.5 ± 8.9
b. 6-OHDA (N = 10)	28.8 ± 10.9
c. 6-OHDA-saline (N = 4)	46.0 ± 8.3
d. 6-OHDA-pargyline (N = 5)	80.8 ± 8.9
2. 72 hr	
a. Vehicle (N = 6)	73.5 ± 18.3
b. 6-OHDA (N = 9)	100 ± 3.5
c. 6-OHDA-saline (N = 5)	100 ± 0.0
d. 6-OHDA-AMPT (N = 5)	30.0 ± 10.3
e. 6-OHDA-AMPT → pargyline $\xrightarrow{1\ hr}$ Test (N = 4)	44.5 ± 17.3

Retention of 6-OHDA-treated mice at 24 and 72 hr after avoidance-discrimination training expressed as % savings errors. AMPT and saline (0.2 ml) were injected IP immediately after training; in Group (1)d pargyline was given IP immediately after training and in Group (2)e 1 hr before retention-testing. All experimental groups were significantly different from their respective controls (p<0.03). 6-OHDA and 6-OHDA-saline at 24 hr are not significantly different. Exact statistical comparisons given in text.

task at 72 hr. Amnesia after 6–OHDA + AMPT treatment could not be reversed by injection of pargyline 1 hr before testing, a procedure that attenuates amnesia following treatment with cycloheximide or inhibitors of CA synthesis [6,19].

Finally, we considered the possibility that the improvement in performance of 6–OHDA mice between 24 hr and 72 hr was due to a recovery of adrenergic function. Although no recovery of CA levels has been observed after 6–OHDA [7,32], we thought it possible that adrenergic receptor sensitivity increased sufficiently between 14 and 17 days after injection to permit memory expression. To examine this, we retrained 6–OHDA mice in a passive avoidance task after memory had recovered for the avoidance-discrimination learning. 6–Hydroxydopamine (N = 5) or vehicle mice (N = 4) were trained as previously described [22] one day after avoidance-discrimination testing, and tested for retention 24 hr later. Median entry latency at retention testing for 6–OHDA mice was 33.6 ± 40 sec, significantly shorter than the entry latency of vehicle-treated mice (180 ± 00 sec, p = 0.028, U = 0). There was no significant difference in entry latencies at training. This suggests that a behaviorally active deficit was still present in the adrenergic system 72 hr after the 6–OHDA mice were trained.

DISCUSSION

CXM and AXM, 6–OHDA and inhibitors of the synthetic enzymes of CAs, all of which may produce transient amnesia, share the property of reducing during consolidation the newly synthesized, functional pools of CAs [10, 16, 23]. Inhibition of synthesis by amnestic doses of CXM and AXM was observed, as has been mentioned, up to 12 hr after treatment; with AMPT, an inhibitor of tyrosine

hydroxylase, rate of synthesis had recovered at 7 hr and with diethyldithiocarbamate, an inhibitor of DA β-hydroxylase, substantial recovery was observed at 8.5 hr [10,23]. By contrast, the severe destruction of CA terminals by 6–OHDA is permanent [7,32]. Thus it appears that the amnesia at 24 hr may occur both in the absence of any known abnormality of the CA system at that time or in the presence of a severe lesion of the system. Similarly, recovery of memory may occur both in the absence and presence of an abnormality. The possibility that a fully effective compensatory supersensitivity had developed in the 6–OHDA-treated mice during the interval between training and testing at 72 hr when memory spontaneously returned is not supported by the finding that these mice were unable to retain a passive avoidance task at 72 hr.

These observations suggest that the CA system is essential for effecting changes in one or more other systems responsible for retrieval. For these changes to occur normally, a sufficient quantity of newly synthesized CAs must be present during consolidation. In the presence of an insufficient quantity during consolidation, amnesia occurs at 24 hr in spite of full recovery of the CA system at this time to be followed by return of memory when, with added time, the necessary changes have occurred in the relevant retrieval systems.

The changes in the retrieval systems that we assume to be essential can be produced by agents that increase sufficiently the synaptic concentration of NE and DA or NE alone [6, 19, 31] either during consolidation or shortly before training. For treatment with the MAO inhibitor, pargyline, to be effective, however, a minimal level of CAs must be present. In our 6–OHDA-treated mice the NE and DA of the cerebral hemispheres were reduced by 84 and 63%, respectively, the greatest depletion of NE occuring in the cerebral cortex and amounting to 96% as compared to 90% for the hippocampus plus entorhinal cortex and 70% for the diencephalon. By comparison, supplementary treatment of these mice with AMPT immediately after training reduced both NE and DA levels in the cerebral hemispheres by 94%. In this instance, treatment with pargyline before testing at 72 hr was ineffective. We take this as evidence that reduction of CA levels (a reflection of decreased rate of synthesis) to a sufficiently great extent may lead to an irreversible amnesia at 72 hr after training. Among the amnestic agents we have considered, 6–OHDA is unique in causing an adrenergic supersensitivity [28]. We would expect reversibility in the absence of supersensitivity to require a higher level of CAs than in its presence.

As has been mentioned, some investigators unlike ourselves and others, have failed to find spontaneous return of memory following CXM and AXM-induced amnesia and there is no satisfactory explanation for this difference in experience. The problem is complicated by the neurochemical differences found among different strains of mice [34]. The most insightful observation that has been made relates to strength of training. In a passive avoidance paradigm using C57BL/6J mice, Quartermain and McEwen [20] reported that low shock led to a persistent amnesia while high shock resulted in transient amnesia. Based on the experiments presented here we suggest that the degree of depression of synthesis of CAs may also influence spontaneous recovery from CXM and AXM-induced amnesia and that the critical level of depression may vary among different strains of mice.

Two differences have been noted between mice treated with CXM and those treated with 6–OHDA. In contrast to the loss of short-term memory in a passive avoidance task caused by CXM, short-term memory is present in 6–OHDA treated mice [22], as it is in mice treated with an inhibitor of NE synthesis [23,35]. These observations led to the suggestion that absence of short-term memory in this task might result from CXM's side effects on transmitter or other systems [3,22] in addition to the CA system. Secondly, 6–OHDA-treated mice, after spontaneous return of memory, perform unlike CXM-treated mice, at a significantly higher level than their controls. This improvement in behavior may be related to the decreased rate of extinction found after treatment with 6–OHDA [17].

In spite of these differences there are striking similarities between mice treated with CXM and 6–OHDA. In both instances amnesia is present 24 hr after training with subsequent spontaneous return of memory. This adds to the evidence that the effects of the antibiotics on CAs may be important in understanding their effects on memory and that it may be mistaken to consider these effects solely due to inhibition of protein synthesis.

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