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Puromycin's Suppression of Memory in Mice as Affected by Caffeine[1]

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FLEXNER, J. B. AND L. B. FLEXNER. *Puromycin's suppression of memory in mice as affected by caffeine.* PHARMAC. BIOCHEM. BEHAV. 3(1) 13–17, 1975. – It has previously been shown that expression of maze-learning in mice is blocked for long periods of time by puromycin injected intracerebrally one or more days after the training experience. Treatment with caffeine after training has now been found to reduce greatly the amnestic effects of puromycin. With a high dose of caffeine (200 mg/kg) this reduction is evident 6 days after treatment with puromycin. With a lower dose of caffeine (25 mg/kg) the effect becomes evident only after a more extended period of time. In view of control experiments, we suggest that caffeine modifies factors necessary for the expression of memory and that this alteration makes puromycin relatively ineffective in blocking memory.

Memory Puromycin Caffeine

SEVERAL biochemical actions of caffeine make it potentially interesting in a study of memory. It inhibits cyclic 3′, 5′-nucleotide phosphodiesterase [5] and thus may lead to an increase in cyclic adenosine-3′-5′ monophosphate which, among other effects, may be concerned in synaptic transmission [17,20]. Caffeine increases the rate of synthesis of norepinephrine from tyrosine in brain, one of several observations consistent with the conclusion that it increases the turnover and release of this transmitter [2, 7, 31] which has been implicated in memory processes [24, 26, 28]. Finally, caffeine causes a marked release of Ca^{++} from intracellular pools [3] with the possibility that in this way it indirectly alters the physical properties of membranes, among them those of the synapse [6,25].

Numerous studies have been made of the effects of caffeine on locomotor activity, on maze learning and on the acquisition and expression of conditioned reflexes (see review [8]). The results of these studies are difficult to interpret since they are frequently complicated by the opposite effects of high and low doses, by the nature of the learning procedure and by variations in dose-response intervals. Coffee has been reported to increase the persistence of memory in man. Except for one study, however, the effect of caffeine on a conditioned avoidance response does not appear to have been investigated. Paré [23] found in rats trained in an avoidance-discrimination task that caffeine (30 mg/kg) injected 5 sec after massed training trials improved retention tested 48 h later but that injections 2 or more minutes after training were ineffective.

Our approach with caffeine depends upon the finding that expression of maze-learning in mice is blocked for long periods of time by the intracerebral injection of puromycin one or more days after the training experience [11]. We have found that the analog of lysine vasopressin, deglycinamide^{9}-lysine vasopressin, which facilitates acquisition of a conditioned-avoidance response in hypophysectomized rats and inhibits extinction [19], has the additional property of protecting memory against puromycin's suppression [18]. We interpret this result to mean that this vasopressin analog modifies memory consolidation in such a way that the expresssion of memory becomes insensitive to puromycin. We have used this same approach with caffeine. Caffeine also reduces puromycin-induced amnesia although, unlike vasopressin, this effect may not be maximally evident until several weeks after treatment.

METHOD

Animals and Procedure

Male and female Swiss-Webster mice (6 to 7 months old, 30–35 g) from our inbred colony were housed four to a cage at room temperature and were placed in individual cages the day before use. They were trained in a single

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session in a Y-maze to a criterion of 9 out of 10 correct responses. 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 consistent with the desired behavioral response. After entering the correct side of the maze and remaining there for 10 sec, the mouse was allowed to climb up a ladder and was rested for about 30 sec before starting the next trial. The same procedure was used in tests for retention of memory of the training experience. These tests were made 7 or 31 days after learning, depending upon the purpose of the experiment. A final test of retention of relearning was made 1 to 2 weeks after the first retention test.

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 of trials and errors. These percentages were calculated by subtracting the number of trials and errors to criteron in the retention tests from the number to criterion in training, dividing by the number in training and multiplying by 100. Savings of 100% indicate perfect memory; zero savings, complete loss of memory.

The intracerebral injection technique has been fully described [11]. Mice were lightly anesthetized with evipal (150 mg/kg). When treatment with puromycin was limited to bitemporal injections, each injection contained 90 μg of the dihydrochloride (neutralized with NaOH) dissolved in 12 μl of water. As judged by their inhibition of protein synthesis, these bitemporal injections primarily affect the hippocampi and entorhinal cortices. Bitemporal plus biventricular plus bifrontal injections contained 30 μg of puromycin in 12 μl of water per injection site. Again as judged from their inhibition of protein synthesis, these multiple injections lead to high concentrations of the antibiotic throughout the neocortices, corpora striata and thalami in addition to the hippocampi and entorhinal cortices [13]. All injections of puromycin were made 1 or 2 days after training. Each injection required about 2 sec for delivery; multiple injections were made with an interval of less than one minute between injections.

Caffeine hydrate was dissolved in water and injected subcutaneously after training in a volume of 0.2 ml per site. One subcutaneous site was used with caffeine at a concentration of 25 mg/kg; two sites were used with a concentration of 200 mg/kg.

Experimental Plan

After finding that treatment with caffeine 1 to 24 hr after training does not impair memory, we planned experiments to answer the following questions:

(1) Does a relatively low dose of caffeine (25 mg/kg) protect memory against puromycin? Mice were treated with caffeine within an hour after training, one day later injected bitemporally with puromycin and then retention-tested either 6 or 30 days later. Choice of the 30 day interval was made after noting in preliminary experiments that savings improved with increase in time between treatment with puromycin and testing of retention.

(2) Does a high dose of caffeine (200 mg/kg) protect memory against puromycin? The same procedure with bitemporal injections of puromycin was used as with the low dose except that an estimate was made of the duration of the interval after training during which the high dose was protective.

(3) In the presence of caffeine, does memory develop resistance to bitemporal injections of puromycin more rapidly than in normal mice? In a normal mouse, bitemporal or bitemporal plus biventricular plus bifrontal injections are consistently effective in suppressing memory for 3 days after training, whereas at later times bitemporal injections are ineffective and the 6 combined injections are necessary [11]. These observations raise the possibility that in the presence of caffeine memory develops resistance to bitemporal injections of puromycin more rapidly than in untreated mice. Accordingly, one group of mice treated with the high dose of caffeine received one day later the 6 combined injections of puromycin.

RESULTS

Mice treated with caffeine were normal in appearance and cage behavior. Treatment with puromycin gave the same symptoms as in normal mice – about two days of lethargy, reduced intake of food and water and occasional convulsions.

The effects on memory of the basic control procedures are given in Table 1. In Table 1, arrows separate sequential procedures with the time between two procedures given over the arrows. Thus, in Group 1a, mice were trained, 24 hr later injected bitemporally with puromycin and 30 days later retention-tested. These experiments resulted in the consistent amnesia previously observed [14]. Treatment with 200 mg/kg of caffeine from 1 to 24 hr after training, followed one day later by bitemporal injections of saline gave a normally high level of savings 6 and 30 days later (Groups 1b, c and d).

The results of the experimental procedures are also given in Table 1. Statistical significance was calculated by the *t*-test, 2-tailed. Treatment with 25 mg/kg of caffeine up to 1 hr after training gave insignificant protection against the effects of puromycin when retention was tested at 6 days (Group 2a). At 30 days (Group 2b), however, savings of both trials and errors were significantly higher ($p<0.001$) than in puromycin controls and only marginally significantly lower ($p<0.05$) than in the controls treated with caffeine and injected intracerebrally with saline (Group 1c).

In contrast to 25 mg/kg of caffeine, treatment after training with 200 mg/kg of caffeine up to 6 hr (immediately, n = 8; 1 hr, n = 10; 6 hr, n = 10) or at 16 hr gave a highly significant ($p<0.001$) degree of protection against the effects of bitemporal injections of puromycin when retention was tested at 6 days (Groups 3a and b). Savings of mice in these two groups as well as those treated 1 hr after training with the 6 combined injections of puromycin (Group 3c) were essentially equivalent. At 30 days (Group 3d) savings of errors were only marginally higher ($p<0.05$) than at 6 days, there was no significant differences ($p>0.1$) in savings of trials, and there was no significant difference ($p>0.1$) from the controls treated with caffeine and injected bitemporally with saline (Group 1c). All protection against the effects of puromycin was lost when treatment with caffeine was delayed until 24 hr after training (Group 3e).

An effort was made to improve memory by making

TABLE 1

EFFECT OF CAFFEINE ON SUPPRESSION OF MEMORY BY PUROMYCIN

Groups and Procedures	Group % Savings Means ± S.E. Trials	Errors
1. Controls		
a. Train —(24 hr)→ Puro (T) —(30 days)→ Test (n = 10)	0.0 ± 0.0	0.0 ± 0.0
b. Train —(1-24 hr)→ Caffeine 200 mg/kg —(24 hr)→ Saline (T) —(6 days)→ Test (n = 14)	86.4 ± 4.15	92.4 ± 2.25
c. Train —(1 hr)→ Caffeine 200 mg/kg —(24 hr)→ Saline (T) —(30 days)→ Test (n = 8)	82.0 ± 6.05	87.5 ± 4.42
d. Train —(24 hr)→ Saline (T) —(6 & 30 days)→ Test (n = 8)	87.5 ± 4.77	89.0 ± 3.60
2. Caffeine 25 mg/kg		
a. Train —(0-1 hr)→ Caffeine —(24 hr)→ Puro (T) —(6 days)→ Test (n = 10)	2.5 ± 2.25	7.9 ± 5.15
b. Train —(0-1 hr)→ Caffeine —(24 hr)→ Puro (T) —(30 days)→ Test (n = 10)	46.7 ± 11.30	56.6 ± 12.80
3. Caffeine 200 mg/kg		
a. Train —(0-6 hr)→ Caffeine —(24 hr)→ Puro (T) —(6 days)→ Test (n = 28)	48.9 ± 9.04	54.0 ± 7.84
b. Train —(16 hr)→ Caffeine —(24 hr)→ Puro (T) —(6 days)→ Test (n = 7)	49.7 ± 18.10	51.0 ± 18.30
c. Train —(1 hr)→ Caffeine —(24 hr)→ Puro (T+V+F) —(6 days)→ Test (n = 6)	51.6 ± 17.40	53.8 ± 18.10
d. Train —(0-1 hr)→ Caffeine —(24 hr)→ Puro (T) —(30 days)→ Test (n = 13)	64.8 ± 9.00	76.2 ± 6.80
e. Train —(24 hr)→ Caffeine —(24 hr)→ Puro (T) —(6 days)→ Test (n = 12)	0.0 ± 0.0	0.0 ± 0.0

Time between procedures indicated over arrows. Puro = puromycin. 2HCl neutralized with NaOH. T = bitemporal, and T+V+F = bitemporal + biventricular + bifrontal injections. In Group 1d savings at 6(n = 4) and 30 days (n = 4) were practically equivalent.

multiple injections of caffeine after training. Three injections of 25 mg/kg each of caffeine given immediately and then at 3 hr intervals after training failed to reduce the amnesia caused by puromycin when memory was tested at 6 days after training (n = 4) or at 30 days (n = 4). A second injection of 200 mg/kg of caffeine was lethal.

Twelve of the mice distributed among the different groups had impaired memory on their final retention tests; the remainder showed retention of relearning at a high level.

DISCUSSION

Numerous observations, not all of them consistent, have been made on the effects of central nervous system stimulants other than caffeine on learning, memory and the attenuation of amnesia caused by drugs. Evidence has been reported that post-training injections of strychnine, pentylenetetrazol, amphetamine and nicotine facilitate one or another of a variety of tasks and these facilitative effects have suggested that these drugs enhance storage processes (see review [21]). The adrenergic stimulants, amphetamine and metaraminol, reduce the amnesia which follows training in the presence of cycloheximide and acetoxycycloheximide [1,29]. Adrenergic stimulants and the monoamine oxidase inhibitor, tranylcypromine, have been shown to remove the block to memory caused by puromycin [26].

The blood-brain barrier is highly permeable to caffeine. Olendorf [22] found that 85% of the caffeine injected rapidly into a carotid artery of a rat was taken up by the brain in what was estimated to be a single microcirculatory passage. Burg and Werner [4] reported that 5 min after an oral dose in mice, caffeine reached its maximal concentration in the brain and that this concentration was maintained for at least an hour. They also found that 1,7-dimethylxanthine is the major tissue metabolite of caffeine. It reached its highest concentration (30% that of caffeine) 30 min after administration of caffeine, decreasing to half this value at one hour.

We have previously considered several possibilities to

explain results of the kind we have obtained with caffeine [10]. Three of these deserve comment here. There is the possibility that caffeine alters the cellular interactions of puromycin rather than processes concerned with memory. This hypothesis appears untenable in view of the drastic loss of memory observed in mice treated with caffeine 24 hr after training and with puromycin one day later, the same interval of time between the two injections that was used in the experiments in which puromycin failed to block memory after treatment with caffeine. Secondly, there is the possibility that motivational or other factors present at the time of retention testing and not a part of the memory process itself might account for the results. This explanation has been ruled out in view of the finding that treatment with caffeine up to 16 hr after training protects memory, whereas caffeine is ineffective when its injection is delayed until 24 hr after training. Finally, we found no support for the possibility that caffeine's protection is limited to bitemporal injections of puromycin since treatment with the 6 combined injections also failed to suppress memory.

Our results suggest that caffeine causes a change during consolidation of memory that leads with a high dose to an early and sustained reduction of puromycin-induced amnesia similar to that resulting from over-training [14], and vasopressin [18], and with a low dose to partial recovery of memory after an initial period of amnesia. Several findings are consistent with the view that the amnesia following puromycin is dependent upon the persistence of puromycin above a critical level at critical sites. Thus puromycin's block of memory is dose dependent [15] and restoration of memory by intracerebral injection of saline [9] or peripheral injection of adrenergic stimulants [26] is accompanied by selective loss of puromycin from synaptosomes [16]. Furthermore, puromycin persists for at least two months in brain, decreasing from its initial high concentration to a value at 31 days after injection that is little different from that one month later [12] when amensia is still present. We suppose that a high dose of caffeine makes puromycin ineffective either by increasing the number of critical sites formed during consolidation or by modifying sites so that binding of puromycin is reduced. The low dose of caffeine acts similarly but with the important difference that its effect is smaller and is not clearly evident until, with time, sufficient puromycin is lost to fall below the level necessary for amnesia.

The mechanism of action of caffeine which accounts for its reduction of puromycin-induced amnesia is unknown. Its interaction with the general arousal produced by training cannot be ruled out nor can minor effects which might become important with the larger of the 2 doses we have used. It appears improbable that cyclic adenosine monophosphate is involved since Sattin [27] found no increase in its concentration in mouse brain following treatment with 100 mg/kg of caffeine and Vernikos-Daniellis and Harris [30] reported that pharmacologically active doses of caffeine (20 mg/kg) failed to change the phosphodiesterase activity of rat cerebral cortex. There is no direct evidence available to evaluate the possibility that the increase in intracellular Ca^{++} caused by caffeine might provide protection by modifying the properties of central synaptic membranes. Caffeine appears to decrease the turnover of serotonin and dopamine [7] while increasing the turnover and release of norepinephrine [2, 7, 31]. At present the most attractive possibility is based upon this increase in turnover and release of norepinephrine. Principal support for this possibility comes from observations that suggest norepinephrine is involved in the memory process [24, 26, 28]. Reduction of the availability of norepinephrine during training and/or consolidation leads to amnesia; perhaps an increase in its release during consolidation leads to the formation of a more stable memory trace.

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