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FURTHER OBSERVATIONS ON RESTORATION OF MEMORY LOST AFTER TREATMENT WITH PUROMYCIN†

Intracerebral injections of the antibiotic, puromycin dihydrochloride, neutralized with NaOH, cause loss of memory of maze learning in mice.[1,2] This loss persists for at least three months, the longest interval over which we have made retention tests. During the first three days after training in a maze (recent memory), bitemporal injections of the antibiotic, which primarily involve the hippocampal area (hippocampus plus entorhinal cortex), lead to loss of memory. Combined, bilateral temporal plus ventricular plus frontal injections, which involve all of the neocortex in addition to the hippocampal area, are necessary to cause loss of memory six or more days after training (longer-term memory).

These findings were first interpreted to mean that puromycin destroys the basic memory trace, the effective locus of which is restricted to the hippocampal area for three days after training; it then spreads to include large parts of the neocortex. In more recent experiments we have found that memory lost after treatment with puromycin is recovered following intracerebral injections of a small volume of a solution of NaCl.[3] Thus, it now seems clear that puromycin blocks expression of memory in mice without substantially altering the process that maintains the basic memory trace.

The mechanism by which intracerebrally injected NaCl removes puromycin's block of expression of memory is entirely obscure. The present experiments were made to test other agents for their effectiveness in restoring memory in the hope that this approach would contribute to our understanding of the mode of action of NaCl. We have used intracerebral injections of the chlorides of several other cations and, in addition, distilled water and an ultrafiltrate of pooled mouse blood serum. All of these substances duplicate the effects of NaCl.

MATERIALS AND METHODS

The behavioral procedures have been described.[1] Male and female Swiss Webster mice from 5-10 months old and from our closed colony were used. The mice were

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trained in a Y-maze with a grid floor through which intermittent shock, usually of 40 volts, could be applied. The animal was placed in the stem of the Y. To avoid shock the mouse had to move into the correct arm within five seconds. If the mouse entered the incorrect arm, it received shock until it moved to the correct arm. Mice with position preferences were trained to the opposite arm. Training was continued in one session of 10-20 minutes (usually about 15 trials) with an intertrial interval of a minute to a criterion of 9 out of 10 correct responses. The same procedure was used in tests for retention of memory of the training experience. These tests were given at 9 or 16 days after intracerebral injection of the salt solutions or water, the longer interval having been used for mice that recovered slowly from the effects of the treatment. A final test of retention of re-learning was given two weeks after the first retention test. Memory is evaluated in the retention tests in terms of the percentage savings of trials and total errors. These percentages are calculated by subtracting the number of trials or errors to criterion in the retention tests from the number to criterion in training, dividing by the number in training, and multiplying by 100. Savings of 100 percent indicate perfect memory; zero savings, complete loss of memory. We have noted in these and other studies that the results are not significantly altered if avoidance errors (failure to move from the stem of the Y) are ignored and savings based only upon errors of discrimination (failure to move into the correct arm of the Y).

The injection technique has also been described.[1] All injections were bilateral and each had a volume of 12 μl. Puromycin dihydrochloride (Nutritional Biochemicals Corp.) was neutralized to pH6 with NaOH. Bitemporal injections of the neutralized puromycin were used to produce loss of memory in mice treated one day after first learning (recent memory) and contained 90 μg of puromycin per injection in mice weighing 28-32 g.; 120 μg in those weighing 34-42 g.; and 150 μg in those weighing 43-48 g. Bilateral temporal plus ventricular plus frontal injections of puromycin were used to cause loss of memory in the mice treated 14-19 days after first learning (longer-term memory), each of the injection sites having received 30 μg of puromycin irrespective of the weight of the mouse.

The mice treated with puromycin were subsequently injected bilaterally with 12 μl per injection of glass distilled water, an ultrafiltrate of pooled mouse blood serum, or a solution of either KCl, LiCl, $CaCl_2$ or $MgCl_2$ to test for the effectiveness of these substances in restoring memory. The substances were injected bitemporally four days after puromycin had been injected bitemporally. Bilateral temporal plus ventricular plus frontal injections were used eight days after corresponding injections of puromycin. In some experiments, however, mice treated with puromycin to block longer-term memory subsequently received only two injections, i.e., either bifrontal, bitemporal or biventricular. These last experiments were made to test the possibility that longer-term memory might be restored by exposure of a limited portion of the brain to the agent that was used.

Because of their toxicity, it was not always possible to inject isotonic salt solutions. Isotonic KCl, LiCl and $MgCl_2$ were used in the bitemporal injections while $CaCl_2$ was hypotonic, one-fifth of the concentration required for isotonicity having been used. All solutions were hypotonic in the six combined injections. For these injections the concentrations of KCl, LiCl and $MgCl_2$ were half that required for isotonicity; that of $CaCl_2$, one-sixteenth.

The ultrafiltrate of pooled mouse blood serum was prepared by repeatedly passing the serum through a "Centriflo" ultrafiltration membrane cone (Amicon Corp.) until the ultrafiltrate was free of precipitate on addition of trichloracetic acid.

Three series of control experiments were made. In one, we examined the effect on the memory of otherwise untreated mice of bitemporal injections of the substances that we have used in an effort to restore memory after puromycin. These experiments were made in part because of observations that show deterioration of conditioned avoidance responses in the cat after ventricular injections of cations.[4] Mice were trained to criterion and one day later injected bitemporally with the same quantities of salt, water or serum ultrafiltrate as were used for treatment after bitemporal injections of puromycin. In a second control series, we have tested the possibility that sham injections might restore memory. Four days after treatment with puromycin, a needle was inserted into the brain as in our routine bitemporal injections; nothing was injected through the needle. A final series of controls, essential because of the nature of the results in the present studies, consisted of frequent testing of the effectiveness of our puromycin solutions in blocking memory. All of these mice, treated only with puromycin, also served as controls for other experiments[6] which were performed concurrently with those reported here.

RESULTS

There was substantial variation in the recovery time of the mice after injections of the salt solutions, water and the ultrafiltrate of serum. When these substances were injected into mice not treated with puromycin, only $CaCl_2$ caused notably prolonged excitability. The whole group was retention tested nine days after the injections. Half of the mice that received $CaCl_2$ were too excitable at this time for reliable testing and were discarded. Those mice injected bitemporally after corresponding injections of puromycin were also retention tested nine days later. At this time about half of those that received KCl or $CaCl_2$ were hyperexcitable and were discarded; all others gave reliable maze performance. Hyperexcitability was particularly severe in the series of mice treated after puromycin with the six combined injections so that retention testing was delayed for 16 days. Only those mice injected with water failed to show this effect. It was particularly interesting to note that those mice injected with the ultrafiltrate of serum after both bitemporal and the six combined injections of puromycin were hyperexcitable in contrast to the calmness of the mice similarly injected in the control series without puromycin. Delay of retention testing for 16 days in the group with the six injections resulted in satisfactory recovery of all but a few mice treated with the chlorides of the four cations.

The effects of the various procedures on memory are shown in Tables 1, 2, and 3. In mice that had not been treated with puromycin (Table 1) bitemporal injections of the chlorides of potassium, lithium, calcium and magnesium as well as of the ultrafiltrate of serum and of water were without deleterious effect on memory. Table 2 shows that our bitemporal injections of puromycin were consistently effective in blocking recent memory and that sham injections after puromycin were ineffective in restoring expression of memory. The Table also shows the striking improvement in memory

TABLE 1. EFFECT ON MEMORY OF MICE NOT TREATED WITH PUROMYCIN OF BITEMPORAL INJECTIONS OF INDICATED SUBSTANCES

Substance injected	*No. mice with memory:*		
	Retained	*Impaired*	*Lost*
KCl	5	1	0
LiCl	3	0	0
$CaCl_2$	3	0	0
$MgCl_2$	3	0	0
U.F.	3	0	0
H_2O	4	0	0

Note: U.F. = ultrafiltrate of pooled mouse blood serum. All salts were isotonic except for $CaCl_2$ which was hypotonic. For the mice with retention of memory, the means ± S.D. for percentage of savings of trials and errors were, respectively, 93 ± 9 and 95 ± 6; for the mouse with impaired memory 14 and 60.

TABLE 2. RESTORATION OF RECENT MEMORY*

Substance injected after puromycin	*No. mice with memory:*		
	Restored	*Impaired*	*Lost*
None	0	1	33
Sham	0	0	7
KCl	5	1	1
LiCl	5	3	1
$CaCl_2$	7	2	1
$MgCl_2$	4	2	4
U.F.	11	2	3
H_2O	8	0	0

* Effect on memory of mice treated with bitemporal injections of indicated substances 4 days after 1-day-old memory was blocked by bitemporal injections of puromycin. The mice of the group designated "None" received no treatment after puromycin and served as controls. The group designated "Sham" had a needle inserted into the brain as for bitemporal injections; nothing was injected. Salt concentrations were as in Table 1. For the mice with restored memory, the means ± S.D. for percentage of savings of trials and errors were, respectively, 90 ± 12 and 93 ± 9; for those with impaired memory, 36 ± 17 and 67 ± 13; and for those with lost memory 0 ± 2 and 4 ± 7.

in the majority of the mice consequent upon the six different bitemporal treatments we have used after blocking recent memory with puromycin. Table 3 shows the recovery of longer-term memory, initially blocked by bitemporal plus ventricular plus frontal injections of puromycin, which followed corresponding injections of the six substances. Not included in the table are eight mice treated with puromycin for suppression of longer-term memory and subsequently injected either bitemporally, biventricularly or bifrontally with LiCl, $MgCl_2$, water or serum ultrafiltrate. The two injections were equally as effective as the six injections in the remainder of the

TABLE 3. RESTORATION OF LONGER-TERM MEMORY*

Substance injected after puromycin	*No. mice with memory:* *Restored*	*Impaired*	*Lost*
KCl	4	1	0
LiCl	2	0	0
$CaCl_2$	4	0	0
$MgCl_2$	4	0	0
U.F.	4	0	0
H_2O	4	0	0

* Effect on memory of mice treated with bitemporal plus biventricular plus bifrontal injections of indicated substances 8 days after 14-19-day-old memory was blocked by corresponding injections of puromycin. All salts were hypotonic. For the mice with restored memory, the means ± S.D. for the percentage of savings of trials and errors were, respectively, 92 ± 11 and 94 ± 10; for the mouse with impaired memory, 33 and 75.

series, all of these mice having recovered memory.

The mice of all experiments were given a final test for retention of re-learning two weeks after the first retention test. All had memory of this re-learning at a high level.

DISCUSSION

We assume as a working hypothesis that puromycin's effect on memory is due to puromycin peptides (peptidyl-puromycin) formed in its presence[6] and that these peptides alter the characteristics of neuronal synapses. This hypothesis is supported by several observations: 1) Puromycin does not affect memory in the presence of acetoxycycloheximide or cycloheximide,[7] both of which inhibit the formation of peptides.[8] 2) After treatment with puromycin, established memory disappears only after 10-20 hours,[3] possibly because some substance must accumulate with time to sufficient concentration to produce its effect. 3) This interval of 10-20 hours corresponds with the time at which puromycin peptides are at their maximum concentration.[9] 4) Peptidyl-puromycin has been shown to persist in the brain for at least two months after intracerebral injection of the tritiated antibiotic.[10] 5) It has been shown by Murphy and Miller[11] and by Bohus and deWied[12] that different peptides can delay or accelerate the rate of extinction of a conditioned avoidance response.

As has been said, the mechanism by which intracerebrally injected substances remove puromycin's block of memory is entirely obscure. The present experiments indicate that, as shown previously with NaCl, the block can be removed by the chlorides of one or another of several other cations, by an ultrafiltrate of serum and by water. Also, as shown earlier with NaCl, memory returns after two injections (bilateral temporal or

ventricular or frontal) of several of these latter substances in mice previously treated with the six injections of puromycin to produce loss of longer-term memory. Thus, it again seems only necessary to free a relatively small area of the brain from the block produced by puromycin for longer-term memory to reappear.[3] In terms of our working hypothesis, recovery of memory after any one of these treatments should be accompanied by a modification of peptidyl-puromycin in neuronal synapses. We are presently testing these assumptions by studying the concentration of peptidyl-puromycin in subcellular fractions of the brain[13] prepared from two groups of mice, one with memory blocked by puromycin and a second with memory restored as a result of our intracerebral treatment.

SUMMARY

It has previously been shown in mice that expression of maze learning, lost after treatment with puromycin, can be restored by intracerebral injections of a solution of NaCl. The present experiments demonstrate that memory is recovered after intracerebral injections of the chlorides of several other cations, of an ultrafiltrate of mouse blood serum and of water, whereas sham injections are ineffective.

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