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Memory in the Japanese Quail: Effects of Puromycin and Acetoxycycloheximide

Abstract. *Intracerebral injections of puromycin produced memory deficits in naive quail trained to discriminate between red and green stimuli. Puromycin aminonucleoside, acetoxycycloheximide, and saline had no such effect. After a single reversal of the visual cues, naive quail treated with puromycin performed better than control birds. Also, puromycin had no effect on performance when injected into previously trained animals. High doses both of puromycin and acetoxycycloheximide inhibited ribonucleic acid and protein synthesis to a similar extent, while low doses of puromycin inhibited only protein synthesis. Since only puromycin inhibited memory, the basis for its effect appears more likely to be mediated by the action of peptidyl-puromycin rather than by the quantitative inhibition of macromolecular synthesis or by some nonspecific toxic action.*

The prevailing theory of memory indicates that it is composed of at least two phases (*1*). The first or short-term phase is thought to be held by neuronal reverberatory electrical activity, which in time gradually gives way to a second more stable phase called the long-term memory or engram. The transition from the transient to the more permanent state is called consolidation and is thought to involve some sort of electrical interaction with the synthesis of macromolecules such as DNA, RNA, and protein. Methods which disrupt or severely reduce electrical activity—such as electroconvulsive shock, hypothermia, and deep anesthesia—have been used to interfere with consolidation and, consequently, the establishment of the engram. A more recent approach has been to inject agents which inhibit the synthesis of a particular macromolecule and, therefore, consolidation, and to observe and correlate the resulting biochemical changes with the concomitant changes in the animal's performance (which serves as an index of memory). The foregoing methods have been found to be effective in producing memory deficits only when employed shortly after (usually minutes to hours) the initial exposure to training.

Of the various inhibitors used to investigate memory only puromycin dihydrochloride has consistently produced deficits in escape behavior in mice and in avoidance responses in goldfish (*2*). Although there is still considerable question concerning the extent and nature of puromycin's action (*2*)—behavioral as well as biochemical and physiological—the focus of this report will be on the interaction of puromycin with macromolecular syntheses to test the macromolecular engram hypothesis.

Adult male Japanese quail (*Coturnix coturnix japonica*) were maintained at 85 percent of their free-feed weight. They were trained in a modified pigeon test chamber to discriminate between a red and a green light by reinforcing with food their responses (pecks) to the green light (*3*). Programming equipment successively presented, in a counterbalanced order, ten color stimuli on the translucent pecking key. Each stimulus presentation lasted for 60 seconds and was followed by an intertrial time-out period of 15 seconds during which time the key was not illuminated. All stimuli were equated for brightness. Correct responses were reinforced on a variable interval schedule of 30 seconds with 6.9 seconds access to food (Purina game bird chow). During each reinforcement, the stimulus light was turned off and the food hopper light was turned on. The total number of responses emitted during each stimulus presentation and the number of reinforcements delivered were recorded on digital counters. At the end of their first day's training (day 1)—which lasted approximately 12 minutes—the animals were placed in a stereotaxic apparatus and intracerebrally injected, with a microsyringe with a 30-gauge needle, at each of four sites with 10 μl of the appropriate solution. Two injections were made into each hemisphere to a depth of 3 mm. Unilateral sites were 3 mm from midline. The rostral sites were 2 mm anterior, while the caudal sites were 1 mm posterior to the interaural line. All injections were made slowly and were complete within 5 minutes after the last training trial. After a 3-day recovery period, training was resumed on day 4 and was continued daily until the criterion of 90 percent correct responses (100 times the responses to the reinforced stimulus divided by the number of total responses) was reached for two consecutive days. All subjects that did not respond on day 1 or day 4 or exhibited a bias by giving a correct response of greater than 90 percent on day 1 were eliminated from the experiment (*4*).

In experiment 1 (Table 1), four experimental groups (*5*) of quail were injected with either of two concentrations of puromycin (45 and 180 μg/site), puromycin aminonucleoside (PANS, 90 μg/site), or acetoxycycloheximide (AXM, 6 μg/site). All agents were prepared in 0.9 percent saline and adjusted to *p*H 7 with 1*N* NaOH. Control animals were injected with 0.9 percent saline solution. The group injected with PANS was used to determine whether

Table 1. Percentage of correct responses and days to criterion for naive control and experimental groups to a successive color discrimination. Green was the reinforced stimulus throughout experiment 1; in experiment 2, the stimulus on day 1 was green, and on day 4 and after the stimulus was red. Injections, in both experiments, were given immediately after training on day 1. Results are expressed as the mean ± standard deviation, with significant *t* scores, based on a one-tailed Dunnett's test, in parentheses. Abbreviations are: PANS, puromycin aminonucleoside; AXM, acetoxycycloheximide; and P, puromycin.

Group (μg/site)	Number	Percentage of correct responses		Days to criterion
		Day 1	Day 4	
		*Experiment 1**		
Saline	39	61 ± 16	78 ± 16	5.3 ± 1.7
PANS(90)	9	57 ± 14	77 ± 17	5.4 ± 1.6
AXM(6)	12	62 ± 15	80 ± 17	6.2 ± 2.8
P(45)	13	60 ± 14	66 ± 14 (2.30†)	6.7 ± 1.8 (2.28†)
P(180)	10	58 ± 20	60 ± 16 (3.27‡)	7.3 ± 1.0 (2.98§)
		Experiment 2 ||		
Saline	17	55.5 ± 18.9	24.9 ± 13.6	13.3 ± 2.57
P(180)	10	50.1 ± 14.8	36.8 ± 21.1	10.0 ± 2.45 (3.01‡)

* d.f. = 78. † $P < .05$. ‡ $P < .005$. § $P < .01$. \|\| d.f. = 25.

Table 2. The quail (saline controls) were trained to a red-green discrimination (with green as the stimulus) and achieved the criterion of 90 percent correct responses for two consecutive days. The animals were tested with the same discrimination task 24 hours after reaching criterion, and were then immediately injected with puromycin (180 μg/site). They were then tested 5 and 72 hours after injection. The results are expressed as the mean ± standard deviation. Nine animals were used. Abbreviations: CR, correct responses; TR, total responses; and FFW, free-feed weight.

Time (hr)	CR (%)	TR	FFW (%)
0	98 ± 3	264 ± 59	85 ± 2
5	98 ± 2	265 ± 64	84 ± 1
72	97 ± 3	288 ± 58	85 ± 2

puromycin's effect on memory was due to the inhibition of protein synthesis or to some side effect related to the molecular structure of the antibiotic. The group injected with AXM was used to determine whether another protein synthesis inhibitor would mimic the memory deficit produced by puromycin. The group injected with saline was used as a control for deficits produced by the trauma of the injection procedure.

All groups injected with puromycin on the second training session (day 4) showed significant deficits in memory when compared to groups injected with AXM, PANS, and saline, and also required significantly more time (days) to reach the criterion (Table 1). There were no differences between the AXM, PANS, and saline groups on day 4 as judged by the percentage of correct responses and by the time needed to reach the criterion. The lack of effect of PANS suggested that puromycin's effect was not solely related to its chemical structure. In addition, the lack of effect of AXM indicated that generalized inhibition of protein synthesis could not be responsible for the memory deficit. Although these results indicate that puromycin's effect is on memory, it could be argued that puromycin only affected performance by producing either difficulties in discrimination, interference with motor function, or lack of motivation for food reinforcement. The fact that all animals injected with puromycin reached criterion and that there were no significant differences between groups in total responses on days 1 and 4 or in percentage of free-feed weight may not be sufficient indicators of puromycin's toxic effects. Therefore in experiment 2 a behavioral paradigm was used to investigate this possible action of puromycin.

Experiment 2 utilized a single reversal of the visual cues on which reinforcement was contingent. With this exception, all training and injection procedures were identical to those described in experiment 1. Thus, if puromycin acts only on memory, then prior experience (day 1 conditions) should not interfere with reversal performance on day 4. The performance of the saline controls on day 4, on the other hand, would be expected to be disrupted by day 1 training to the green stimulus. However, if puromycin achieves its effect through some toxic action on performance, then both saline and puromycin groups should do poorly on day 4.

Table 3. Radioactivity in the protein and RNA precipitates and supernatants for experimental and control groups. Raw data (count/min, less background) from each portion (six portions per animal) were converted into disintegrations per minute (*14*), averaged for each animal, and normalized with respect to the saline group. Results are expressed as the mean ± standard deviation, with significant t scores, based on a one-tailed Dunnett's test (d.f. = 53), in parentheses.

Group (μg/site)	Number	Precipitate	Supernatant
		Protein	
Saline	9	1.000 ± 0.130	1.000 ± 0.194
P(45)	12	0.744 ± 0.132 (4.4*)	1.338 ± 0.197 (4.3*)
P(90)	15	0.697 ± 0.156 (6.1*)	1.452 ± 0.232 (6.0*)
P(180)	10	0.323 ± 0.078 (12.4*)	2.010 ± 0.117 (12.4*)
AXM(6)	12	0.339 ± 0.062 (12.7*)	1.986 ± 0.093 (12.5*)
		RNA	
Saline	9	1.000 ± 0.045	1.000 ± 0.035
P(45)	12	0.955 ± 0.056	1.035 ± 0.045
P(90)	15	0.890 ± 0.068 (4.9*)	1.087 ± 0.053 (5.0*)
P(180)	10	0.703 ± 0.066 (12.2*)	1.235 ± 0.052 (11.8*)
AXM(6)	12	0.837 ± 0.044 (6.7*)	1.129 ± 0.035 (7.5*)

* $P < .005$

Performance of the puromycin group on day 4 was better than that of the saline controls on that day (Table 1). Moreover, the puromycin group took fewer days to reach criterion. There were no significant differences between either group in total responses or percentage of free-feed weight on days 1 and 4. Thus, puromycin apparently does not have a toxic effect on sensory, motor, or motivational factors; it seems to act on memory. A toxic agent could not be expected to have diametrically opposite effects in the acquisition and reversal of a visual discrimination task.

It has been suggested that puromycin does not affect memory but that it affects only performance and that investigators were merely confusing the two. Evidence that puromycin may affect performance comes from experiments which found that puromycin suppresses electrical activity 5 hours after injection (*6*) and produces occult seizures (*7*). Since the two previous experiments only measured puromycin's long-term (72 hours) effects, it became necessary to measure its short-term effects on performance. Subjects for this experiment were sampled from the population of animals injected with saline that had reached the criterion of 90 percent correct responses for two consecutive days in experiment 1. Trained subjects were selected for this experiment on the assumption that long-term memory would be consolidated at this point and that, consequently, puromycin would have no effect on memory. Therefore, any behavioral deficits produced by puromycin would be attributable only to impairment of performance. On the first day after reaching the criterion (the day immediately after the second day that the animals reached 90 percent correct responses), the animals were tested on the same discrimination and then intracerebrally injected with puromycin (180 μg) and returned to their home cages. Subjects were then tested again 5 and 72 hours after the injection.

The results (Table 2) showed no significant differences in performance, total responses, or in percentage of free-feed weight during the experiment. The lack of effect of puromycin on the performance of trained subjects (but not on naive subjects) indicates that puromycin does not act on performance by the suppression of electrical activity or

by the production of occult seizures (*8*).

Puromycin's effect on memory is not confined to mice or goldfish, is reliable in naive and trained animals, and is extended to include discrimination as well as avoidance and escape behavior. Furthermore, the deficits produced by puromycin may not be attributed to sensory, motor, or motivational factors (*9*). However, puromycin's effect does not appear to be attributable to inhibition of protein synthesis, because AXM had no effect on memory.

To test the latter conclusion, the biochemical actions of these inhibitors on protein and RNA synthesis were investigated with a dual tracer method to study the incorporation of radioactive precursors into RNA and proteins precipitable by trichloroacetic acid (TCA). Five groups of adult male quail (Table 3), maintained at 85 percent of free-feed weight, were injected intracerebrally at each of four sites with either puromycin (45, 90, or 180 μg/site), AXM, or saline. One hour after intracerebral injection, each animal received an intramuscular injection of RNA precursor orotic acid-6-C^{14} (400 μc/kg; 25 mc/mmole; crystals were dissolved in 0.9 percent saline and titrated to *p*H 9 with 0.1*N* KOH to a concentration of 0.14 mc/ml) in the first quadrant of the breast. Four hours later, an intramuscular injection of protein precursor L-leucine-4,5-H^3 hydrochloride (270 μc/kg; 5 c/mmole; stock diluted in 0.9 percent saline to a concentration of 51 μc/ml) was made into the same region. One hour later, the animals were killed, and their cerebral hemispheres were removed, weighed, and homogenized by sonification in ice-cold water (1:1, volume to weight). The RNA and protein supernatants and precipitates were prepared, and the radioactivity was measured in a liquid-scintillation counter by a modification (the elimination of the heat step after the initial TCA treatment of portions of the homogenate) of the method of Brink, Davis, and Agranoff (*10*) (Table 3).

As the concentration of puromycin is increased, inhibition of RNA and protein synthesis is indicated by an increase in radioactivity in the supernatant and the concomitant decrease in the precipitate compared to control values (*11*). Quantitative inhibition of protein or RNA synthesis apparently is not responsible for the memory deficits observed with puromycin. Puromycin (45 μg) caused less inhibition of protein synthesis than AXM, but still inhibited memory; therefore puromycin apparently acts in a qualitatively different fashion from AXM in producing this memory deficit. The lack of effect of puromycin at this concentration (45 μg) on RNA synthesis, but not on protein or memory inhibition, suggests that the qualitative differences in action between puromycin and AXM on memory are related to puromycin's mechanism of action on protein synthesis.

Two possible modes of action for puromycin are either that it destroys memory by inhibiting the formation of specific proteins which make up the engram or that it blocks the expression of the engram by forming a peptidyl-puromycin-engram complex. The second alternative is supported by the report that intracerebral injections of saline restore memory previously lost after the intracerebral injection of puromycin (*12*) and also by the report that peptidyl-puromycin is stable for at least 58 days after initial injection in mouse brain (*13*).

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4. Ten uninjected subjects were eliminated on day 1 (six for lack of response and four for bias). Six subjects were eliminated from the following groups: (i) puromycin (180 μg), one eliminated (no response on day 4); (ii) AXM, three eliminated (two made no responses on day 4 and one died immediately after the intracerebral injections); and (iii) controls, two eliminated (no responses on day 4).
5. A fifth experimental group ($n = 22$) was injected with puromycin (90 μg/site). This group was not included in the analysis because their percentage of free-feed weight was held at 75 percent, 10 percent lower than that of the other groups. Performance of this group, however, was significantly lower than that of the controls on day 4 (t, 4:38; $P < .005$) and on the days to criterion (t, 4.03; $P < .005$).
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Central Neuron Initiation of Periodic Gill Movements

Abstract. *In* Aplysia *periodic spontaneous gill movements are controlled by activity endogenous to the abdominal ganglion. These movements were still observed when only the ctenidio-genital nerve was left intact between the ganglion and the gill. One kind of spontaneous gill movement (one per 5 minutes at 15°C) was correlated with the expression of activity of interneuron II; others were not. With reference to this kind of spontaneous gill movement, four types of central neurons in the ganglion send processes to the gill via the nerve. Two cell types (ii, iii) are inhibited and the other two (i, iv) are excited. Two types (i, ii) elicited gill movement—one type activating large gill areas elicited spontaneous gill movements, and the other activating specific gill regions did not participate in the spontaneous gill movements. The value of this preparation in studying the role of central neurons eliciting specific patterned movements and the temporal organization of their activity is shown.*

The neuronal organization of behavior is more amenable to detailed analysis with identification of the same neurons from animal to animal. Techniques are available to study directly neural events which initiate discrete behavior patterns especially in invertebrates. Individual neurons have been identified and their function described in several species. (*1*).

I now describe the properties of central neurons which elicit a specific