

that very well may be related to intra-neuronal communication. Once located and tentatively identified these structures presumably can be isolated in ultrasections and examined with a transmission electron microscope. The scanning electron microscope in conjunction with the transmission microscope thus may become an extremely efficient tool in mapping the nervous system (9). This seems significant to us, since the lack of efficient mapping techniques is undoubtedly one of the greatest obstacles in neuroscience today.

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References and Notes

1. W. T. Frazier, E. R. Kandel, I. Kupfermann, R. Waziri, R. E. Coggeshall, *J. Neurophysiol.* **30**, 1288 (1967).
2. R. E. Coggeshall, *ibid.*, p. 1263.
3. E. R. Lewis, Y. Y. Zeevi, F. S. Werblin, *Brain Res.*, in press.
4. Examples of other applications of scanning electron microscopy studies of biological preparations are found in: V. C. Barber and A. Boyde, *Z. Zellforsch.* **84**, 269 (1968); A. Boyde and V. C. Barber, *J. Cell Sci.* **4**, 223 (1969); G. A. Horridge and S. L. Tamm, *Science* **163**, 817 (1969); W. E. Jaques, J. Coalson, A. Zervins, *Exp. Mol. Pathol.* **4**, 576 (1965).
5. "Stereoscan" scanning electron microscope, Cambridge Instrument Company Limited, England.
6. Employing transmission electron microscopy, Frazier *et al.* (*1*, pp. 1330-1331) observed vesicle-filled profiles contacting each other in addition to contacting the giant axon of cell R-2. They too were uncertain as to whether these contacts represent functional connections. They point out that if they are functional, they might account for the presynaptic interactions which were postulated by E. R. Kandel and L. Tauc [*J. Physiol.* **181**, 1 (1965)] for heterosynaptic facilitation in R-2. The connected knobs observed with the scanning electron microscope definitely are not attached to the giant axon of R-2, so they are not the same structures as those reported by Frazier *et al.* Furthermore, if all the fibers of the clusters are at once postsynaptic and presynaptic the consequences of the connections might be considerably more complicated than heterosynaptic facilitation.
7. E. R. Lewis and Y. Y. Zeevi, in preparation.
8. T. L. Hayes and R. F. W. Pease, in *Advances in Biological and Medical Physics*, J. H. Lawrence and J. W. Gofman, Eds. (Academic Press, New York, 1968), vol. 12.
9. We could find only two previous papers reporting studies of nervous tissue with the scanning electron microscope: A. Boyde, D. W. James, R. L. Tresman, R. A. Willis, *Z. Zellforsch.* **90**, 1 (1968); L. W. McDonald, R. F. W. Pease, T. L. Hayes, *Lab. Invest.* **16**, 532 (1967).
10. Stereoscan microscope purchased under NSF grant GB-6428 and maintained under PHS grant GM-15536-02, which also supported the research of T.E.E. The work of E.R.L. was supported in part by the Joint Services Electronics Program under grant AF-AFOSR-68-1488 and in part by NSF grant GK-3845. Y.Y.Z. was supported under PHS training grant GM 01418-05. We thank M. K. Nemanik for his invaluable assistance in microscope operation and G. O. Kreutziger for his many helpful suggestions about tissue preparation.

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Puromycin: Effect on Memory of Mice When Injected with Various Cations

Abstract. Puromycin dihydrochloride neutralized with bases of potassium, lithium, calcium, or magnesium fails to block expression of memory of maze learning in mice, unlike puromycin neutralized with NaOH. This failure may be due to cationic binding at anionic membrane sites with a resultant exclusion of sufficient peptidyl-puromycin to make it ineffective in blocking memory.

Memory of maze learning in mice is blocked by puromycin dihydrochloride neutralized with NaOH and injected intracerebrally one or more days after the training experience (1); small intracerebral injections of saline made at least up to 2 months later remove the block and restore memory (2). Thus it is apparent that these injections of puromycin interfere with retrieval without substantially altering the process which maintains the basic memory trace. Several observations support our working hypothesis that this reversible suppression of the expression of memory is due to the interaction of peptidyl-puromycin with neuronal membranes, particularly synaptic membranes (3). Our report is a first step in testing the possibility that certain cations, through

binding at anionic membrane sites, might interfere with the postulated interaction of peptidyl-puromycin and thus modify the effects of puromycin on memory. We have been concerned with expression of memory as affected by intracerebral injections of puromycin neutralized with a base of potassium, lithium, calcium, or magnesium and a comparison of the effects of these injections with those obtained with puromycin neutralized with NaOH.

The behavioral procedures have been described (1). Male and female Swiss Webster mice (5 to 10 months old) from our closed colony were used. The mice were trained in a Y maze with a grid floor through which intermittent shock, usually 40 volts, could be applied. The animal was placed in the stem of the

Y maze. To avoid shock the mouse had to move into the correct arm within 5 seconds. If it 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 to 20 minutes (usually about 15 trials) with an intertrial interval of a minute to a criterion of nine out of ten correct responses. Total errors were the sum of incorrect choices and of latencies greater than 5 seconds. The same procedure was used in tests for retention of memory of the training experience. These retention tests were given 5 to 10 days after intracerebral injection of the puromycin solutions, the longer intervals having been used for mice that recovered slowly from the effects of the injections. A final test of retention of relearning was given 2 weeks after the first retention test. Memory is evaluated in the retention tests in terms of the percentage savings of trials and 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.

In previous experiments with puromycin (Nutritional Biochemicals) we have neutralized the solution of the dihydrochloride to pH 6 with 1 equivalent of NaOH. In the present experiments, in addition to NaOH, we have used KOH, Li_2CO_3 , $\text{Ca}(\text{OH})_2$, or MgO for neutralization.

The injection technique has been described (1). All injections were bilateral and each had a volume of 12 μl . Bitemporal injections were used in mice treated 1 day after first learning (recent memory). For mice weighing 28 to 32 g each injection site received 90 μg of puromycin; for mice weighing 34 to 42 g and 43 to 48 g, each injection site received 120 and 150 μg , respectively. Combined bitemporal, biventricular, and bifrontal injections were used in the series of mice that were treated 9 days after first learning (longer-term memory). Each injection site received 30 μg of puromycin regardless of animal weight. The bitemporal injections with 120 μg of puromycin per injection contained a total of approximately 380 nmole of univalent cation or 190 nmole of divalent cation; those with 90 or 150 μg of puromycin per injection had proportionate amounts of cation. The combined bitemporal, biventricular, and

bifrontal injections contained a total of 290 nmole of K^+ .

Except for Na^+ , the amounts of cations injected intracerebrally were substantial compared to those calculated to be present in the extracellular fluid of the cerebral hemispheres. In these calculations we have used the average weight of 300 mg for the cerebral hemispheres and 10 percent of the volume of the cerebral hemispheres as the extracellular fluid volume; for the concentrations of cations we used those in cerebrospinal fluid (4). On this basis, in the bitemporal series the ratios of the amounts of K^+ , Ca^{2+} , or Mg^{2+} injected to the amount present in cerebral extracellular fluid was between 4 and 5. The ratio of Na^+ was 0.09, and the ratio for K^+ in the six combined injections was 3.

We have consistently found that bitemporal injection of puromycin dihydrochloride neutralized with NaOH in the amounts used here and the injection at six sites of 30 μ g in each site cause loss of recent and longer-term memory, respectively. Nevertheless, because of the nature of our results, the effectiveness in blocking expression of memory of our puromycin dihydrochloride neutralized with NaOH has been checked throughout the experiments. Some of the mice treated in this way also served as controls for other experiments (5) that were performed concurrently with ours.

There was substantial variation in the reactions of the mice to injections of puromycin containing the different cations. Mice which receive Na^+ usually lose their excitability and give reliable maze performance 5 days after treatment; this interval between treatment and retention testing was used in the Na^+ series. The group of mice which received Mg^{2+} approached most closely those with Na^+ ; they were in satisfactory condition for retention testing 7 days after treatment. The effects of injections of puromycin together with K^+ , Li^+ , or Ca^{2+} were more drastic. Mice which had these injections were highly excitable 1 day after treatment, usually required 10 days to recover sufficiently for reliable testing, and in some cases showed further improvement 14 days later when tested for retention of relearning. The numbers of mice which failed to recover sufficiently for reliable retention testing are given in Table 1.

Table 1 also shows the unique effectiveness of puromycin neutralized with

Table 1. Effect on memory of intracerebral injections of puromycin dihydrochloride neutralized with the indicated bases. All injections were bitemporal and were made 1 day after training except for "KOH" in which combined bitemporal, biventricular, and bifrontal injections were made 9 days after training. Mice too excitable for testing are indicated by not testable. For the mice with retention of memory, the percentage of savings of trials and errors (mean \pm S.D.) were, respectively: 89 ± 13 and 91 ± 12 ; for those with impaired memory, 46 ± 14 and 57 ± 17 ; and for those with lost memory, 0 ± 2 and 3 ± 6 .

Base	Mice with memory (No.)			Mice not testable (No.)
	Retained	Impaired	Lost	
NaOH	0	1	33	3
KOH	9	4	1	5
"KOH"	5	0	0	4
Li_2CO_3	6	3	0	1
$Ca(OH)_2$	10	4	0	1
MgO	13	3	0	2

NaOH in blocking memory. Consistent with our previous experience it caused loss of memory, with savings of trials and errors closely approaching zero with a single exception. The number of mice which were tested on their retention in the other 5 groups totaled 58. Of these, 43 retained memory with means savings of trials and errors of about 90 percent, and all but one of the remaining 15 had savings of trials and errors at about 50 percent. There was no significant difference (*t*-test) in savings among the K^+ , Li^+ , Ca^{2+} , and Mg^{2+} groups in which bitemporal injections were made 1 day after training. The savings for trials and errors (mean \pm S.E.) were, respectively: for the K^+ group, 75 ± 5.9 and 79 ± 5.8 ; for Li^+ , 78 ± 7.0 and 74 ± 7.3 ; for Ca^{2+} , 75 ± 6.5 and 78 ± 5.3 ; and for Mg^{2+} , 79 ± 6.0 and 86 ± 4.6 . Much the same results were obtained 9 days after training with combined bitemporal, biventricular, and

bifrontal injections containing these ions. As an example, Table 1 shows the results of such injections of puromycin neutralized with KOH. All but two of the testable mice of Table 1 on second retention testing had high savings of trials and errors consistent with retention of memory; the two exceptions showed moderate impairment.

It is premature to attempt an explanation of these results. They appear consistent with the possibility that K^+ , Li^+ , Ca^{2+} , and Mg^{2+} protect memory by binding to the anionic sites of neuronal membranes with the resultant exclusion of sufficient peptidyl-puromycin to make it ineffective in blocking memory. If this mechanism is in fact involved, it must be supposed, because of the high concentration of intracellular K^+ , that relevant anionic sites are unavailable to intracellular K^+ . The experimental situation is, however, complex and demands more direct evidence of exclusion of peptidyl-puromycin from neuronal membranes by the ions we have used.

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References and Notes

1. J. B. Flexner, L. B. Flexner, E. Stellar, *Science* **141**, 57 (1963).
2. ———, *Proc. Nat. Acad. Sci. U.S.* **57**, 1651 (1967).
3. J. V. Murphy and R. E. Miller, *J. Comp. Physiol. Psychol.* **48**, 47 (1955); B. Bohus and D. deWied, *Science* **153**, 318 (1966); L. B. Flexner and J. B. Flexner, *Proc. Nat. Acad. Sci. U.S.* **55**, 369 (1966); *ibid.* **60**, 923 (1968).
4. A. White, P. Handler, E. L. Smith, *Principles of Biochemistry* (McGraw-Hill, New York, 1968).
5. J. B. Flexner and L. B. Flexner, *Proc. Nat. Acad. Sci. U.S.*, in press.
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Visual Reinforcement of Nonnutritive Sucking in Human Infants

Abstract. *High-amplitude sucking was studied as a conditioned operant response reinforced by visual feedback in 4- and 12-month infants. Typical response acquisition and extinction effects were obtained. With the 12-month infants the conditioned sucking rates were influenced by amounts of redundancy in the visual reinforcers.*

The development of learning tasks for the human infant, in which his behavior is made experimentally effective for producing changes in the exteroceptive environment, may provide important tools for studying the ontogeny of exploratory behavior over the first

weeks and months of human life. In experiments with infants from 3 weeks to 1 year of age we began to explore the feasibility of using the sucking response as a conditioned operant to assess the infant's response to visual feedback as a reinforcing event. Sucking is a re-