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Pharmacological Dissociation of Memory: Anisomycin, a Protein Synthesis Inhibitor, and Leupeptin, a Protease Inhibitor, Block Different Learning Tasks

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Inhibition of protein synthesis by anisomycin for a short duration impairs memory of a one-trial inhibitory avoidance task in rats. Memory of escape conditioning involving eight trials is disrupted only if the duration of protein synthesis is prolonged by repeated injections. In marked contrast, olfactory memory of rats trained on two odor discriminations is not affected by anisomycin even if the duration of inhibition is prolonged and the number of trials is reduced to a minimum. In previous work, leupeptin, a thiol proteinase inhibitor, was shown to impair olfactory discrimination learning, but left inhibitory and avoidance conditioning intact. Together, these results provide a pharmacological double dissociation of memory, and suggest that the same chemistries, or mixtures of chemistries, may not be involved in all types of memory.

One of the fundamental questions in learning research concerns the extent to which various forms of memory involve different chemistries. Recent biochemical studies from this laboratory have demonstrated that low micromolar levels of calcium irreversibly uncover what may be synaptic receptors for the putative transmitter glutamic acid (Baudry & Lynch, 1979, 1980) and have linked this effect to the activation of a specific proteinase (Baudry, Bundman, Smith, & Lynch, 1981) found associated with synaptic membranes (Siman, Baudry, & Lynch, 1983). Recently, we reported that intraventricular infusions of the calcium proteinase inhibitor leupeptin produced an impairment of tasks requiring either spatial (Stäubli, Baudry, & Lynch, 1984a) or olfactory (Stäubli, Baudry, & Lynch, 1985) memory; these treatments did not influence spontaneous activity, habituation to novel environments, escape conditioning, or the learning of avoidance responses (Stäubli et al., 1984a). The nature and selectivity of leupeptin's effects led us to suggest that

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the proteinase–receptor interaction may be involved in those forms of memory that require the lasting modification of telencephalic circuitries while some other process subserves the storage of escape and avoidance conditioning.

An alternative explanation is that olfactory and spatial memory are both more readily disrupted than aversive conditioning and that leupeptin produced generalized effects that were above threshold for impairment in the one case but not the other. One approach to this problem is to determine if experimental manipulations exist that produce effects opposite to those of the treatment of interest, or, by analogy to the lesion literature, a double dissociation.

Protein synthesis inhibitors have been reported to produce pronounced impairments in avoidance conditioning. More specifically, these drugs are known to block memory tests given a considerable time (e.g., 24 h) after the training episode and to have little effect on tests given within minutes of initial learning (e.g., Bennett, Rosenzweig, & Flood, 1977). This led to the idea that protein synthesis is required for memory "consolidation." Among a variety of protein synthesis inhibitors used in memory research anisomycin was found to have the fewest side effects (Bennett et al., 1977). Most studies on the effects of protein synthesis inhibitors on memory have been performed on conceptually rather simple tasks such as avoidance conditioning, and there appears to have been very little work done using spatial or olfactory memory.

In the studies described in the present paper, we established anisomycin treatment regimens that interfered with two forms of avoidance conditioning that are not affected by leupeptin, and then measured the effects of the same treatments on a leupeptin sensitive test of olfactory memory.

EXPERIMENT 1A: INHIBITORY AVOIDANCE

Animals. Twelve young adult male Sprague–Dawley rats (280 g), which were housed individually and kept in a reversed light–dark cycle, were used.

Apparatus. The avoidance conditioning apparatus was a trough-shaped alleyway (90 cm long, 15 cm deep, 20 and 6 cm wide at top and bottom, respectively) separated by a sliding door into a lighted small compartment (30 cm long) and a long dark compartment (60 cm long). The floor of the dark compartment was covered with metal plates through which a footshock could be delivered.

Procedure. Two groups of six animals each were tested. One group received a single subcutaneous injection of anisomycin (25 mg/kg body wt) prepared in 1 ml of 0.9% NaCl. To dissolve the drug HCl was added and the pH was adjusted with NaOH to 7.4. The control group received 1 ml of saline. For mice it has been demonstrated (Bennett et al., 1977) that protein synthesis inhibition has to be fully established at the time

of training in order to affect memory. The best results have been achieved with injections done 15 min (Bennett et al., 1977) or 30 min (Squire & Barondes, 1974) before training, and this procedure was used in the present study. Thirty minutes after the injection each rat was placed in the well-lit small compartment, facing away from the door; when the animal turned around, the door was opened and the rat was allowed to step through. The door was then closed and a footshock 650 μA for 1 s) was delivered. Entrance latency from the time the door was opened was recorded. After receiving the footshock the rat was removed from the apparatus.

Twenty four hours later, each rat was placed again in the lighted compartment and the step-through latency to the dark compartment was recorded. If the rat failed to cross within 300 s, the testing trial was terminated.

Results. Figure 1 summarizes the effects of 25 mg/kg of anisomycin on inhibitory avoidance learning. Subcutaneous injections in rats treated 30 min before training did not affect step-through latencies compared to rats injected with saline, but had a severe amnestic effect on retention of the task 24 h later ($p < 0.002$; U Test) compared to controls. Entrance latencies on training and testing day were virtually identical in the anisomycin group. In marked contrast, saline treated rats waited approximately 10 times longer on the testing day before entering the dark compartment (see Fig. 1). This is in accord with previous work with rats trained on a step-down avoidance task (Bennett, Orme, & Hebert, 1972), where an even lower dose of anisomycin (5 mg sc compared to 7 mg in this study) injected before training caused amnesia. The authors established that 5

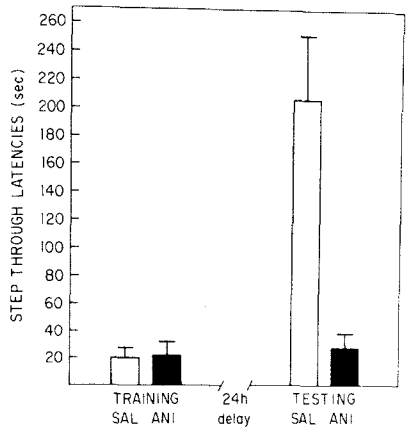

FIG. 1. Mean latencies of two groups of rats (n = 6 each) during training and testing (24 h later) in a inhibitory avoidance task: the effect on long-term memory (retention) of a single 30-min pretraining sc injection of anisomycin (25 mg/kg) given to one group was compared to a saline injection given to the control group.

mg anisomycin produces a 90% inhibition of protein synthesis for 1 h in rats.

EXPERIMENT 1B: OLFACTORY DISCRIMINATION

Animals. Nine adult male Sprague–Dawley rats (280 g), which were housed individually and kept in a reversed light–dark cycle, were used.

Apparatus and procedure. The animals were water deprived and trained on an olfactory discrimination task using a water reward. An eight-arm radial maze was used with one arm always serving as the starting position. Two distinct odors were ejected by air pressure from tubes in different arms of the maze (i.e., one odor per arm). The two arms adjacent to the starting alley were not used and remained permanently blocked. The location of a given odor was randomized across the 5 remaining arms in different trials; the three arms containing no odor tubes were blocked on each trial. One (correct) odor led to a water dish placed at the end of the arm, the other to an empty dish. When the rat selected the incorrect odor a flashing light was turned on for 10 s when it reached the end of the arm. After a trial the animal was removed to its home cage for 1 to 10 min and then returned to the maze for a second trial, with the odors now being in two different arms. Twenty trials were run per day. The same odor pair was used until the animal reached a criterion of 80% correct responses (usually in 3 to 5 days) after which a second pair of odors was introduced. After three to four such pairs the animals acquired the correct response in three to five trials for all subsequent pairs, independent of whether a short or long delay separated the trials.

The nine animals were trained on 10 pairs, at which point the anisomycin study was initiated: 30 min before the training each rat was subcutaneously injected with anisomycin (25 mg/kg body wt, dissolved in 1 ml saline, pH 7.4). Twenty trials with a 3- to 4-min delay were given, and 24 h later the rats were tested again on the same odor pair but with the significance of the odors reversed. It is difficult to test for long-term olfactory memory of the type employed in our experiments using savings (i.e., more rapid aquisition on a retest than on initial learning) since the rats acquire the discrimination quickly. Therefore, we reversed the significance of the cues given 24 h later, reasoning that if the rats remembered the previously correct smell then they would commit a measurable number of errors before switching responses. This has been proven to be true (Stäubli, Ivy, & Lynch, 1984b) as shown by the fact that normal rats make many more errors in the first 20 trials using reversed cues than they did during the initial acquisiton 24 h earlier.

Results. Figure 2 summarizes the effect of a single injection of anisomycin (25 mg/kg), given 30 min before training, on acquisition and on retention of olfactory information. Anisomycin had no detectable effects on acquisition. In the reversal 25 h later, all the rats clearly took much longer

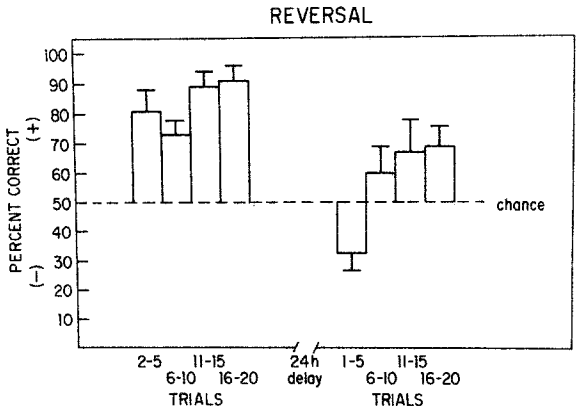

FIG. 2. Mean number (in percent) of correct choices in an olfactory discrimination reversal problem by a group of nine animals injected with a single injection of anisomycin (25 mg/kg) 30 min before training. The original training on the problem and its reversal 24 h later consisted of 20 trials separated by 1 min and are illustrated as four blocks of five trials. The first training trial was discarded since its outcome is necessarily random with regard to correctness.

than normally to acquire the discrimination. If the rats had forgotten what they learned 24 h ago they would not be expected to recognize a reversal of the cues and thus their performance should be identical to the original acquisition (i.e., scores of 70–80% correct responses in the first five reversal trials). Instead, the rats persisted in choosing the wrong, previously correct, odor. This effect was, as expected, more pronounced on the first few reversal trials (their scores were well below chance level) but was still significant for Trials 6–10 ($p < .025$ for Trial 6–10 vs Trial 6–10 on the previous day; U test). These data indicate that the memory of the specific olfactory cues, acquired under the influence of anisomycin, is intact 24 h later.

It has been noted that single injections of protein synthesis inhibitors are not effective in well-trained subjects but even these animals will become amnestic if the period of inhibition is long enough. Since we used two different behavioral tasks (electrical footshock vs water reward; 1 trial vs 20 trials) to test the effect of anisomycin on memory it could be argued that the training strength of olfactory discrimination and thus its impact on memory consolidation was too strong to be overcome by protein synthesis inhibition. Therefore, we repeated the experiment by reducing the number of trials from 20 to 8 and at the same time increasing the duration of protein synthesis inhibition by giving repeated anisomycin injections. In addition, this prolonged time course of protein synthesis inhibition was also tested on a different multiple-trial avoidance-conditioning task.

EXPERIMENT 2A: ESCAPE CONDITIONING

Apparatus. The avoidance conditioning apparatus as described above was used.

Procedure. The naive rats were assigned to an experimental ($n = 5$) and a control ($n = 6$) group. Each rat received four subcutaneous injections in the following time course: 30 min before training, immediately after training, 2 h after training, and 4 h after training. In the experimental group, each injection consisted of 10 mg anisomycin dissolved in 1 ml 0.9% NaCl (ph 7.4). The control group received four injections of 1 ml saline. It has been shown that the duration of inhibition by anisomycin can be controlled and extended by administering doses at 2-hr intervals (Bennett et al., 1977). Escape conditioning consisted of eight training trials on Day 1 and eight testing trials on Day 2. At the start of the trial the rat was placed in the larger, dark compartment facing the door to the smaller, lighted compartment. The door was opened and 10 s later a 400-μA footshock was administered for 30 sec, and the latency to enter the lighted compartment was recorded. If the rat did not escape to the lighted compartment within 30 s after onset of the shock (i.e., 40 s from the beginning of the trial) the trial was terminated and the rat was placed in the smaller compartment and retained there during the 30-s intertrial interval. The procedure of the testing trial was identical to that of the training trial.

Results. Both groups acquired the avoidance response in a comparable fashion and had virtually identical mean escape latencies after shock onset at the end of the training session. In the first two trials, two experimental animals entered the "safe" compartment spontaneously within 10 s, i.e., without experiencing the shock. Therefore, for each individual rat the first six trials after the first trial on which shock was administered were used for data analysis. Figure 3 (left side) shows the group medians of these six training trials. Evidently, anisomycin did not affect acquisition of the task. However, 24 h later, the anisomycin group, in clear contrast with the control animals, did not show any savings. Their mean escape latency in the first testing trial was slightly higher compared to their first training trial. There was a strong tendency for reacquisition of the avoidance response in the anisomycin group in the first three testing trials but only slight improvement could be detected in the following five trials. This suggests that multiple injections of anisomycin have prolonged effects on aspects of behavior (e.g., motivation, fear) in addition to memory. The bars on the right side of Fig. 3 represent the mean retention latencies for each group across the first three testing trials. Compared to anisomycin-treated animals performance of the controls was significantly better in the first three testing trial ($p < .015$, U Test).

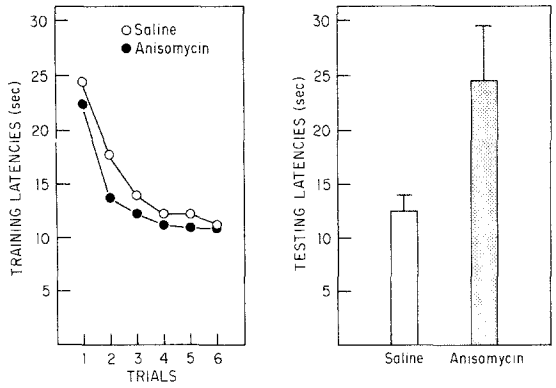

FIG. 3. Left: median escape latencies after experiencing a mild footshock of two groups of rats (n = 6 each) during six consecutive training trials in an escape conditioning task. The two groups received four injections of 10 mg anisomycin or saline, respectively, 30 min before training, immediately after, 2 h after, and 4 h after training. Right: The two bars represent mean retention latencies (+SE) of the anisomycin and the control group 24 h after training averaged across the first three testing trials.

EXPERIMENT 2B: OLFACTORY DISCRIMINATION

Apparatus and procedure. The apparatus and procedure were the same as in Experiment 1b, except for the number of trials and the drug regimen. Training consisted of eight consecutive trials with a 1-min delay; 24 h later the animals (n = 7) were again tested for 8 trials but with the significance of the odors reversed. As in Experiment 2A (escape conditioning) anisomycin was injected 30 min before training, immediately after the training session, 2 and 4 h after training.

Results. Figure 4 summarizes the effects of four consecutive injections of 10 mg anisomycin each on learning and retention of olfactory information. Similar to Experiment 1B, anisomycin had no obvious effect on acquisition, and 24 h later all rats continued responding to the previously correct odor; ($p < .003$ for Trial 1–4 vs Trial 2–4 on the previous day; U test). Evidently, the rats still remembered the specific olfactory information acquired 24 h earlier despite prolonged protein synthesis inhibition.

DISCUSSION

The above results address two important and related problems concerning the pharmacology of memory: (1) the possibility that drug-induced impairments are due to generalized disturbances of the nervous system or unsuspected side effects and (2) the unitary nature of the biochemical processes that underly storage.

Memory storage represents one case of a "higher order" operation of brain circuitries since we can assume that many if not most physiological and psychological functions can occur without it; yet it requires the

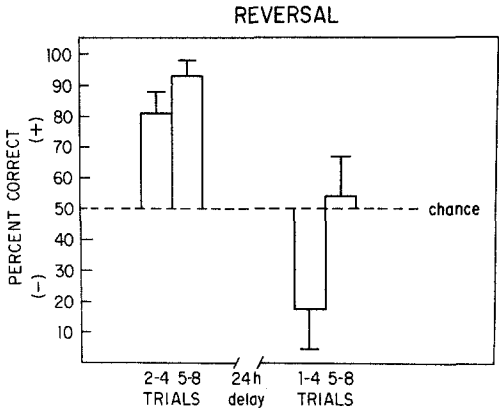

FIG. 4. Effect of four injections of 10 mg anisomycin administered to a group of seven rats (30 min pretraining, immediately after training and 2 h and 4 hr post-training) on acquisition and reversal of an olfactory discrimination problem: the eight trials (which were separated by a 1-min delay) of the original problem and its reversal 24 h later are illustrated as two blocks of four trials. The first training (but not testing) trial was not taken into account since the choice is random.

coordinated contributions of sensory, motor, and motivational systems. From this it follows that any treatment which produces a significant effect on the primary operations of neurons will produce memory disturbances. Similar problems are encountered in lesion studies and, as a response to them, the double-dissociation tactic (Teuber, 1955) has gained increasingly widespread usage. In this two lesions and two behaviors are used and an attempt is made to show that each lesion selectively interrupts one behavior—thus each lesion–behavior pair serves as a control for nonspecific effects of the other lesion. This approach has not been widely used in pharmacological studies of memory, perhaps because of a belief that a single chemical process subserves all forms of memory.

In previous work we found that the protease inhibitor leupeptin blocked the daily learning of a complex spatial problem, and it did so at concentrations that had no detectable effects on activity, ingestive behavior, and body weight (Stäubli et al., 1984a). In recent studies we have found that intraventricular infusions of the drug do not produce detectable changes in baseline physiology of the hippocampus or in size of the potentials evoked by stimulation of the perforant path (Wilson & Lynch, unpublished data). Moreover, leupeptin had little or no effect on the acquisition or retention measured in the shock-conditioning problems described above (Stäubli et al., 1984a). This provides a single dissociation of the effects of leupeptin on memory.

However, it is still possible that this dissociation was along a dimension of sensitivity to disruption. The radial maze problem might well be more

readily affected by nonspecific disturbances since it requires the rat to remember several complex, distant cues while the avoidance tasks involve only two (dark, light) adjacent and salient cues. But leupeptin also blocked the learning of a simple two-odor discrimination problem (Stäubli et al., 1985), a form of learning rats acquire about as quickly as active avoidance (i.e., three to five trials).

The present experiments provide a further dissociation of memory in that they demonstrate that anisomycin dissociates avoidance and olfactory memory in a direction opposite to that produced by leupeptin. It was clear that animals treated with dosages of this drug that profoundly impair active and passive avoidance both acquired the olfactory discrimination and remembered it the following day. At a minimum these findings demonstrate that acquisition and retention of olfactory information is not easily disrupted. Anisomycin causes a profound suppression of protein synthesis (Bennett et al., 1977; Squire & Barondes, 1974) and has a number of side effects as well. (It has been argued that the amnestic effects of anisomycin are due to actions other than inhibition of protein synthesis: see Gold & Sternberg, 1978.) Apparently these effects of the drug, as well as the handling, injections, and other manipulations involved in the study, were not sufficient to interfere with the storage of information about specific odors. This adds support to the conclusion that the effects of leupeptin on this task are not due to generalized or nonspecific actions of the drug. In a more general sense the double dissociation points to the possibility that the cellular mechanisms underlying avoidance conditioning are in some important way different from those responsible for spatial and olfactory memory.

Before considering what these differences might be, we need to add a caveat to the above interpretations. The negative aspects of the dissociations (leupeptin doesn't impair avoidance conditioning and anisomycin does not block olfactory memory) do not rule out the possibility that low levels of protein synthesis are involved in spatial/olfactory memory or that some minimal proteolytic activity is part of the conditioning chemistry. These drugs are graded in their effects and almost certainly do not totally suppress their target processes. The difficulty with testing this possibility lies in the greater likelihood of producing side effects with much higher concentrations of drugs.

As described in the introduction, leupeptin blocks thiol proteases including calpain. This enzyme is found in synaptic membrane fractions (Siman et al., 1983) and selectively degrades the submembraneous cross-linking protein fodrin (Baudry et al., 1981; Siman, Baudry, & Lynch, 1984a); one consequence of this is the uncovering of glutamate binding sites (Siman, Baudry, & Lynch, 1984b). It has also been suggested that the activation of calpain produces the structural changes in spines and synapses found after high-frequency stimulation of the type that elicits

long-term potentiation of excitatory post-synaptic potentials. (See Lynch and Baudry, 1984, for a review.) This is evidence that some aspect of the calpain mechanism is absent from certain brain regions or circuitries. Thus, calcium induces glutamate binding sites in crude synaptic membranes prepared from all regions of telencephalon but has little effect on membranes from cerebellum and brain stem. While it remains possible that particular circuitries in these latter regions utilize the calpain mechanism to affect synapses, the regional distribution studies indicate that significant differences exist in the mechanism or its consequences between forebrain and hindbrain. The olfactory task can be assumed to involve processing by and modification of telencephalic circuitries. The primary, secondary, and tertiary connections of the olfactory bulb are localized to forebrain and recent studies have shown that lesions of entorhinal cortex (the primary link between bulb and hippocampus) produce a "rapid forgetting" syndrome for the olfactory cues in the experimental situation used in the present experiments (Staubli, Ivy, & Lynch, 1984b). The acquisition of spatial memory, which like the learning of specific olfactory information is interrupted by leupeptin, is also severely and irreversibly disrupted by lesions to hippocampus and its connections (O'Keefe and Nadel, 1978).

Conditioning can be obtained in decerebrate animals (Whitfield, 1979) and it is possible that some versions of this type of memory are found at all levels of brain. Protein synthesis is a fundamental activity of all cells and it does not seem likely that the differential effects of anisomycin on memory reflect unequal effects of the drug on synthesis across the brain. However, it can be assumed that selected groups of cells or circuitries are involved in the types of conditioning used in our experiments, and it is possible that these utilize protein synthesis (or some other cellular chemistry affected by anisomycin) as part of the storage process.

REFERENCES

Baudry, M., Bundman, M., Smith, E., & Lynch, G. (1981). Micromolar levels of calcium stimulated proteolytic activity and glutamate receptor binding in rat brain synaptic membranes. *Science (Washington, D.C.),* **212,** 937–938.

Baudry, M., & Lynch, G. (1979). Regulation of glutamate receptors by cations. *Nature (London),* **282,** 748–750.

Baudry, M., & Lynch, G. (1980). Regulation of hippocampal glutamate receptors: Evidence for the involvement of a calcium-activated protease. *Proceedings of the National Academy of Sciences,* **77,** 2298–2302.

Bennett, E. L., Orme, A., & Hebert, M. (1972). Cerebral protein synthesis inhibition and amnesia produced by scopolamine, cycloheximide, streptovitacin A, anisomycin, and emetine in rat. *Federation Proceedings,* **31,** 838.

Bennett, E. L., Rosenzweig, M. R., & Flood, F. J. (1977). Protein synthesis and memory studied with anisomycin, In S. Roberts, A. Lajtha, & W. Gispen (Eds.), Mechanism, regulation and special function of protein synthesis in the brain. Amsterdam: Elsevier/ North Holland Biomedical Press, 1977.

Gold, P. E., & Sternberg, D. B. (1978). Retrograde amnesia produced by several treatments: Evidence for a common biological mechanism. *Science* (Washington, D.C.), **201,** 367–369.

Lynch, G., & Baudry, M. (1984). The biochemistry of memory: a new and specific hypothesis. *Science* (Washington, D.C.), **224,** 1057–1063.

O'Keefe, J., and Nadel, L. (1978). The hippocampus as a cognitive map. London/New York: Oxford Univ. Press (Clarendon).

Siman, R., Baudry, M., & Lynch, G. (1983). Purifications from synaptosomal plasma membranes of calpain I, a thiol-protease activated by micromolar calcium concentrations. *Journal of Neurochemistry,* **41,** 950–956.

Siman, R., Baudry, M., & Lynch, G. (1984a). Brain fodrin: Substrate for the endogenous calcium-activated protease calpain I. *Proceedings of the National Academy of Sciences USA,* **81,** 3572–3576.

Siman, R., Baudry, M., & Lynch (1984b). Regulation of glutamate receptor binding by the cytoskeletal protein fodrin. *Nature (London),* **313,** 225–228.

Squire, L. R., & Barondes, S. H. (1974). Anisomycin, like other inhibitors of cerebral protein synthesis, impairs long-term memory of a discrimination task. *Brain Research,* **66,** 301–308.

Stäubli, U., Baudry, M., & Lynch, G. (1984a). Leupeptin, a thiol proteinase inhibitor, causes a selective impairment of spatial maze performance in rats. *Behavioral and Neural Biology,* **40,** 58–69.

Stäubli, U., Baudry, M., & Lynch, G. (1985). Olfactory discrimination learning is blocked by leupeptin, a thiol protease inhibitor. *Brain Research,* in press.

Stäubli, U., Ivy, G., & Lynch, G. (1984b). Hippocampal denervation causes rapid forgetting of olfactory information in rats. *Proceedings of the National Academy of Sciences USA,* **81,** 5885–5887.

Teuber, H.-L. (1955). Physiological psychology. *Annual Review of Psychology,* **34,** 267–296.

Whitfield, I. C. (1979). The object of the sensory cortex. *Brain, Behavior and Evolution,* **16,** 129–154.