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Authors

Stäubli, U
Baudry, M
Lynch, G

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Leupeptin, a Thiol Proteinase Inhibitor, Causes a Selective Impairment of Spatial Maze Performance in Rats

URSULA STÄUBLI, MICHEL BAUDRY, AND GARY LYNCH[1]

Center for the Neurobiology of Learning and Memory, University of California, Irvine

The effects of chronic intraventricular infusion of leupeptin, a potent inhibitor of thiol proteinases, were tested on ingestive behaviors, escape and avoidance conditioning, and spatial memory in rats. The drug did not detectably influence feeding, drinking, body temperature, or the latency to escape from a mild footshock or inhibitory avoidance behavior. However, rats treated with leupeptin made numerous errors (reentries) in an eight-arm spatial maze. These results are interpreted as supporting the hypothesis that calcium-activated thiol proteinases are involved in the formation of certain types of memory.

Recent experiments from this laboratory have shown that activation of a membrane associated calcium sensitive proteinase leads to the rapid and irreversible uncovering of a class of binding sites for glutamic acid in the telencephalon but not the brain stem of rats (Baudry, Bundman, Smith, & Lynch, 1981; Baudry & Lynch, 1980). The subcellular localization, developmental pattern, pharmacological properties, and ionic sensitivities of these sites suggest that they are associated with the postsynaptic transmitter receptor, at least in the rat hippocampus (Baudry & Lynch, 1981). For several reasons, the proteinase-induced increase in binding sites seems a plausible candidate for the substrate(s) underlying some types of memory (Lynch & Baudry, submitted): (a) it is activated by concentrations of calcium (10 μM) that can reasonably be expected to occur intracellularly during intense physiological activity, (b) its consequences (added receptors) develop within minutes and are irreversible (Baudry, Kramer, & Lynch, 1983), and (c) added receptors should significantly influence the strength of synaptic transmission and thus be of functional importance. Two clear predictions follow from the hypothesis that a proteinase-receptor interaction is involved in memory storage: (1)

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learning should produce a change in receptor numbers and (2) inhibition of the enzyme should block memory. Recent experiments have shown that a learning paradigm that causes long-lasting changes in hippocampal physiology also produces an increase in the number of hippocampal glutamate binding sites (Mamounas, Thompson, Lynch, & Baudry, in press). It may also be feasible to test the second prediction of the hypothesis. That is, the membrane-associated proteinase is activated by calcium concentrations (5–10 μM; Siman, Baudry, & Lynch, 1983) in excess of the levels of the cation thought to mediate most calcium related cellular processes. It follows then that the enzyme can be blocked without interrupting ongoing physiological activity. Leupeptin is a small peptide that exerts a profound and reversible inhibition of several thiol proteinases including the calcium activated enzymes (Aoyagi & Umezawa, 1975). In the present paper, we describe the first attempts to measure the effects of this drug on behavior; the results indicate that it causes a pronounced and selective impairment of spatial memory tasks.

GENERAL METHOD

Animals. Male hooded rats of the Sprague–Dawley strain, approximately 60 days of age at the beginning of each of the several experiments, were used. They were housed individually and kept in a reversed light–dark cycle. Animals trained in appetitive tasks were reduced to 85% of free feeding weight and were maintained on this food deprivation schedule throughout the experiment.

Apparatus. The eight-arm radial maze was raised 1 m above floor level and consisted of a central platform (60 cm in diameter) that gave access to eight arms (45-cm long) separated from each other by a wall (18-cm high). A hidden well (2-cm deep, 7-cm in diameter) containing a small chocolate chip was located at the end of each arm.

The left–right discrimination maze was of a trough-shaped elevated Y with three identical arms (45-cm long, 18-cm deep, 19- and 3.5-cm wide at top and bottom, respectively). The three arms were covered with opaque Plexiglas, and one arm always served as start arm. A small food cup containing a chocolate chip was placed at the far end of each of the two remaining arms.

The apparatus for avoidance conditioning 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 longer dark compartment (60-cm long). The floor of the dark compartment was covered with metal plates through which a footshock could be delivered.

Surgery. Osmotic minipumps (Alzet model 2002, Alza Corp., Palo Alto, CA) were implanted under the skin and connected via fine tubing to a cannula implanted into the lateral ventricle at the level of the anterior

hippocampal commissure. The pumps (volume 0.2 ml) were loaded with one of three dosages of leupeptin (low: 4 mg/ml; intermediate: 8 mg/ml; or high: 20 mg/ml in sterile saline) or with aprotinin (10 mg/ml of saline) or with saline alone. The pumps deliver 0.5 μl per hour; given a ventricular volume of 250 μl (Cserr, 1965), and a cerebrospinal fluid exchange rate of 2.2 μl/min (Bass & Lundborg, 1973), the infused drugs should have reached a steady-state concentration between 10–20, 20–40, and 50–100 μM for the low, moderate, and high dosages of leupeptin, respectively, and 25–50 μM for aprotinin, a potent inhibitor of serine proteinases (Barrett, 1980). The pumps operate continuously for 14 days.

Procedure

Eight-arm radial maze. The naive rats were assigned to two experimental groups (leupeptin, high dosage: $n = 8$, low dosage: $n = 5$) and to two control groups (aprotinin: $n = 5$ and saline: $n = 12$). Before the start of the drug phase of the experiment the rats were trained for 25 days on the radial maze, with one or two daily trials. The animal was placed in the center of the maze surrounded by a cylinder. After a few seconds the cylinder was raised and the animal was allowed to explore the arms and retrieve the chocolate rewards. The sequence of choices and the number of errors (reentering an already visited arm) were noted. The animals were trained to a criterion of entering seven or eight different arms in the first eight choices on 3 consecutive days. On the following trials a delay, varying between 10 min and 2 hr, was interposed between the fourth and fifth choice of the trial. The animal was removed from the maze and returned to its home cage after four choices, the maze was rotated 90°, and the arms in the four unentered positions baited. The training period was terminated when all animals fulfilled a criterion of making no more than two errors for 5 consecutive days in the delay trials.

Following this training period the osmotic minipumps were implanted, and 2 days later the rats were reintroduced to the maze.

The animals treated with the high dose of leupeptin were given 3 to 5 days of testing with no delay, followed by 7 to 9 days of testing with delays interposed between the fourth and fifth choice. Delays ranged between 1 min to 1 hr. The low dosage leupeptin group, the aprotinin group, and the saline group were tested for 12 days with delays ranging from 1 min to 3 hr.

The maze was moved to a new location every 3 to 4 days.

Y maze. The animals were trained in the Y maze for 16 days (one to two daily trials) before the start of the drug phase of the experiment. On each trial the rat was placed in the start alley (at the far end from the choice point) and allowed to enter one of the two remaining arms. After consuming the reward the animal was returned to its home cage.

After a delay ranging between 10 min and 3 hr the rat was returned to the Y maze and allowed to make a second choice. Only the previously unentered arm was baited. An error was scored if the rat entered the same arm as on the first choice, i.e., did not alternate arms.

Following this training period the osmotic minipumps were implanted (leupeptin, high dosage: $n = 5$ and saline: $n = 5$). Two days later, the rats were reintroduced to the maze and tested for 9 days with one daily trial. The delays ranged between 1 min and 2 hr.

Escape conditioning. Rats implanted with osmotic minipumps (leupeptin, high dosage: $n = 5$ and saline: $n = 5$) received eight training trials on Day 1 and eight testing trials on Day 2. At the start of the trial the rat was placed into the larger, dark compartment facing the door to the smaller, lighted compartment. The door was opened and the trial was terminated when the rat entered the smaller compartment. An avoidance response was recorded if the rat entered the smaller compartment within 10 sec. If no avoidance response was made, a 640-μA/30-sec footshock was administered. If the rat did not escape to the smaller compartment within 30 sec after the onset of the shock (i.e., 40 sec 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-sec intertrial interval. Latency to enter the smaller compartment was recorded for each trial.

Inhibitory avoidance. Two groups of five animals each (leupeptin, intermediate dosage, and saline) were tested. The rat was placed in the lighted small compartment. When the rat turned around, the door was opened and the rat was allowed to step through. The door was then closed and a footshock (650 μA/1 sec) 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 again placed in the lighted small compartment and the step through latency to the dark compartment was recorded. If the rat failed to cross within 300 sec, the testing trial was terminated.

Activity and rearing measurements. Naive rats were implanted with osmotic minipumps and starting 3 days later were tested once per day for spontaneous activity and rearing. The apparatus was a 76 $\times$ 32 $\times$ 36-cm box with the floor divided into equal squares by a series of lines. Two experiments were conducted. In the first, line crossings were recorded for a total of 15 min in five animals with leupeptin (high dosage) and five with saline minipumps. The experiment was then repeated using additional groups of five animals with intermediate dosages of leupeptin or saline, measuring line crossing every 2 min for 14 min. In the second study a novel object (a small box 21 $\times$ 21 $\times$ 6 cm) was placed in one corner of the activity box on the 4th day of testing.

Rearing was measured for the leupeptin (intermediate dosage) and the saline control groups using 6-min periods in a separate enclosure of 32 $\times$ 32 $\times$ 36 cm.

Histology. At the end of the experiments the rats were sacrificed and their brains quickly removed. The half-brain ipsilateral to the cannula implantation site was fixed in 10% formaldehyde and subsequently sectioned and then processed using the Nissl staining technique. The sections were analyzed in the light microscope to verify the location of the cannula tip within the lateral ventricle.

RESULTS

Table 1 shows the amount of daily food and water intake as well as body weight gain and body temperature for both intermediate and high dosage leupeptin and saline treated animals over a period of 11 days. These four measurements were comparable for the groups.

The high dose of leupeptin (20 mg/ml) produced inconsistent effects on spontaneous activity; two and possibly three of the rats appeared less active than controls, particularly on the 2nd day of testing (Table 2). (One of the rats with saline pumps was extremely active and did not appear healthy; its data are not included). The difference between means for the high dosage and saline groups are not statistically significant. Spontaneous activity scores over a 4-day period (Days 4–7 after implantation of the pumps) for the second activity study using intermediate dosages of leupeptin are summarized in Fig. 1. The total scores for the leupeptin and saline groups were virtually identical for all 4 days of testing. Moreover, as shown in the figure, habituation both within and between days was not detectably different for the two groups. On the 4th day of testing, a novel object (a small cardboard box) was introduced into the testing box. This caused a slight and equivalent increase in activity in both leupeptin and saline treated rats. The right hand panel of Fig. 1 summarizes

TABLE 1
Body Weight Changes, Food and Water Intake, and Body Temperature of Four Groups (n = 5) of Rats[a]

	% weight gain[b]	Food intake (g)[c]	Water intake (ml)[c]	Temp (°C)[d]
Control	16.2 ± 2.4	26.5 ± 1.0	38.2 ± 1.5	38.3 ± .3
	19.8 ± 6.7	25.3 ± 1.7	35.9 ± 2.3	
Leupeptin				
20 mg/ml	21.2 ± 2.2	25.0 ± 4.2	39.7 ± 2.7	38.6 ± 0.1
8 mg/ml	16.2 ± 1.8	25.3 ± 2.6	35.9 ± 2.3	

[a] Two infused with saline alone, one with saline plus an intermediate dosage of leupeptin, and one with a high dosage (mean ± SE).
[b] Measured over 11 days.
[c] Average per day for 11 days.
[d] Mean of two measurements made on successive days.

TABLE 2
Spontaneous Activity Measured as Number of Line Crossings over a 15-min Period of Rats Infused with Saline (n = 4) or Saline plus Leupeptin (20 mg/ml: n = 5)

	Day		
	1	2	3
Control	62.3 ± 19.8	49.5 ± 25.7	27.8 ± 10
Leupeptin	57.4 ± 13.7	25.6 ± 15.7	25.6 ± 24

the results of the rearing tests; as is evident, there were no differences between the two groups.

Figure 2 illustrates the effects of leupeptin (4 and 20 mg/ml) and aprotinin (10 mg/ml) on eight-arm radial maze performance compared to control (saline) animals. Only trials with a delay interposed are depicted. Means and medians are shown for all four groups. The rats were unaffected by infusion of saline and performed both continuous and delayed tasks with no evident change from before surgery. As shown, the average number of reentries in the delay tasks was about one, many of which occurred on the first trial after the rat was returned to the maze. The rats treated with leupeptin entered arms and consumed the rewards as freely as controls but had considerable difficulty with the radial maze. In the high dose group only two out of the eight rats performed at control level in the no-delay tests while the remaining six made an average of 4.1 ± 0.6 (mean ± SD) reentries per trial. In the low dose group (which was given only delay tests) only one of the five animals performed at control level.

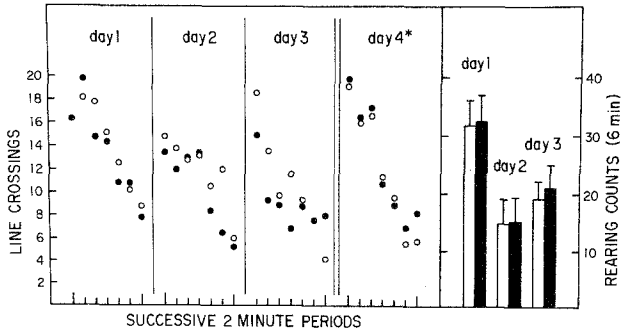

FIG. 1. Left hand side: activity scores expressed as numbers of line crossings in successive 2-min periods for rats infused with saline (n = 5; open circles) or with saline plus leupeptin (8 mg/ml: n = 5; closed circles). On Day 4 a novel object was added to the activity box. Right hand side: Rearing counts during a 6-min period on 3 successive days for control rats (n = 5; white bars) and rats infused with leupeptin (20 mg/ml; n = 5).

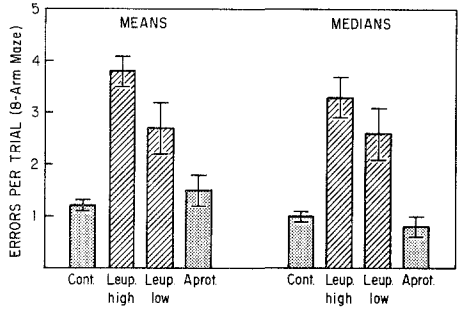

FIG. 2. Number of reentries per trial by rats infused with saline (n = 12), saline plus leupeptin (high dose 20 mg/ml: n = 8; low dose 4 mg/ml: n = 5), or saline plus aprotinin (10 mg/ml: n = 5). The group means are indicated by the height of the bars. The data used for the right hand bars were the median score for each animal over the several days of testing. The value indicated is thus the mean of the medians. The left hand bars were constructed from the simple means (total number of errors/number of days of testing) for each animal. Only trials with a delay interposed between the fourth and fifth choice are taken into account.

The remaining four animals in this group as well as all eight animals infused with the high dose of leupeptin made a number of errors in the trials with delays interposed between the fourth and fifth choices: 3.8 ± 0.8 for the high dose; 2.7 ± 1.2 for the low dose (see Fig. 2). The errors did not fall into any detectable pattern and in particular were not due to response perseveration. Animals infused with aprotinin exhibited no impairment in running the maze. They were identical to control animals with an average number of reentries of one (see Fig. 2).

Figure 3 illustrates the effect of leupeptin (high dosage) on eight consecutive trials to escape (or avoid) a footshock (Fig. 3, top) and on eight consecutive testing trials 24 hr later (Fig. 3, bottom). The escape latency after shock onset in the first training trial was virtually identical for the two groups. Except for trial 2 and 3 on which the leupeptin rats had slightly longer latencies to escape the shock, the decrease in latency that occurred over trials was comparable for both groups (Fig. 3, top). The leupeptin rats may have been slightly slower in learning to avoid the shock since four of five controls avoided the shock at the end of the 1st day of eight trials compared to two of five experimental rats. All animals in both groups successfully avoided the shock after a 2nd day of testing (Fig. 3, bottom).

Leupeptin (intermediate dosage) had no apparent effect on the learning of a standard inhibitory avoidance task; both saline and drug groups showed perfect retention 24 hr after exposure to the footshock, as is summarized in Table 3.

As Figure 4 shows, delayed alternation in the Y maze was markedly disrupted by infusion of leupeptin (high dosage). The mean number of

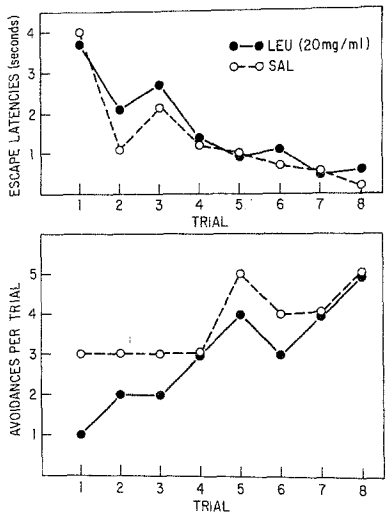

FIG. 3. Latency to escape from a mild shock (650 μA for 30 sec) by running to an adjacent "safe" compartment (leupeptin 20 mg/ml: $n = 5$; saline: $n = 5$). Eight training trials were given in succession with a 30-sec interval between them and 24 hr later eight testing trials were given.

correct responses over a period of nine trials was 34.4% for 10 leupeptin treated rats versus 84.4% correct responses for the five control animals.

Histological analyses of the brain sites that had contained the cannula yielded 85% exact locations of the cannula tip in the lateral ventricle whereas the remaining 15% were too dorsal. No correlation between quality of performance and location of cannula tip was obvious.

DISCUSSION

Leupeptin produced reasonably selective behavioral effects. Intermediate and high concentrations of the drug did not affect food and water intake

TABLE 3
Latency to Step from a Small Lighted Compartment into a Dark Compartment of Rats ($n = 5$ per group) Infused with Saline or Saline plus Leupeptin (8 mg/ml) before (= training) Receiving a Mild Footshock (650 μA for 1 sec) and 24 hr after (= testing) the Footshock

	Training (sec)	Testing (sec)
Control	25 ± 4	148 ± 41
Leupeptin	20 ± 5	189 ± 47

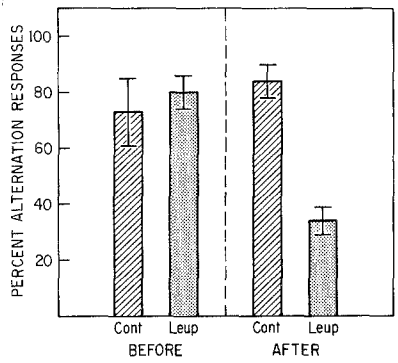

FIG. 4. Mean number (in percent) of correct responses over nine trials by a group of 5 rats (controls) versus 10 animals infused with leupeptin (20 mg/ml). Left hand side: performance of the delayed alternation task before surgery (five trials). Right hand side: performance after implantation of pumps.

or body temperature. Moreover, the latency to escape from a mild footshock in naive animals (i.e., on the first trial of conditioning) treated with high concentrations of the drug were comparable to those found in controls. In contrast, inhibitors of amino peptidases and enkephalinases are reported to cause a marked increase in escape latency (de la Baume et al., 1983). Certain forms of learning also remain intact in the presence of leupeptin. Escape conditioning clearly occurred over eight trials and savings were evident a day later. Active avoidance (i.e., leaving the shock compartment in the 10-sec period before shock onset) may have been slightly slower in the leupeptin group but acquisition clearly occurred in a relatively small number of trials. Inhibitory avoidance, tested 24 hr after a single footshock, was not affected by infusions of intermediate (8 mg/ml) concentrations of leupeptin. It is worth noting that escape learning and both forms of avoidance have been shown to be sensitive to a variety of pharmacological and peripheral manipulations (see McGaugh, 1983).

The high dosage leupeptin may have reduced spontaneous activity in some rats. However, rats treated with the intermediate dosage of the drug were virtually identical to controls in every aspect of spontaneous activity tested. They habituated both within and between the 15-min trial period; moreover, their response to a novel element in the activity box appeared completely normal. Rearing scores for these animals were also indistinguishable from those of controls.

Leupeptin did produce a substantial impairment in both radial maze performance and delayed alternation learning. The maze study was particularly informative. The animals clearly retained their predrug learning about the task since they rapidly ran from arm to arm and consumed the rewards, behaviors that are seen only in well-trained animals. The errors they committed did not fall into any obvious patterns, and in

particular were not due to response perserveration or the formation of stereotyped response patterns (e.g., always turning left upon leaving an arm). It seems reasonable then to assume that leupeptin interfered with some aspect of the memory needed to avoid already-entered maze arms. These effects were found in every rat tested with the high drug dosage and were clearly present in four out of five animals infused with the lowest concentration tested. This latter dosage was one-half of that which had no effect on any aspect of spontaneous activity, rearing, or feeding and drinking. Finally, aprotonin, a powerful inhibitor of serine proteinases, had no measureable effects on radial maze performance.

Leupeptin then does not produce a generalized block of short-term memory (as measured by within trials habituation) nor does it interfere with all types of long-lasting memory (e.g., savings in escape conditioning or inhibitory avoidance). The question of why its actions should be restricted to radial maze and Y maze memory tests is better discussed after a consideration of its possible biochemical effects.

The pharmacological actions of leupeptin have been intensively studied in muscle cells where it has been shown to inhibit two types of thiol proteinases: (1) a calcium-activated enzyme that is maximally effective at neutral pH and (2) a small group of lysosomal enzymes (e.g., cathepsin B) which are only active at acidic pH. Given that proteinase inhibitors as a group are not particularly selective, it is possible that the drug has smaller effects on other proteolytic enzymes. As mentioned, aprotonin does not affect the radial maze test and leupeptin does not cause changes in escape latencies of the type reported to occur after administration of aminopeptidase or enkephalinase inhibitors. This, combined with the very selective nature of the drug's effects, leads us to conclude that under the conditions used in our experiments leupeptin does not produce generalized disturbances in proteoloytic activity in brain.

Given the paucity of information concerning the functions of lysosomes (Katunuma & Kominami, 1983), it is difficult to predict the effects of inhibition of the acidic thiol proteinase on cell physiology and behavior. Using light and electron miscroscopy, it has been found that high concentrations of leupeptin rapidly induce an accumulation of lipofuscin-like material in neurons and glia cells throughout the neuroaxis (Ivy, Schottler, Baudry, & Lynch, 1983). This is almost certainly due to an inhibition of lysosomal enzymes since the same result is obtained with chloroquine, a drug that elevates lysosomal pH above the range in which the acidic proteinases are effective. Lipofuscin accumulation did not occur with infusion of leupeptin in the dosages equal to the intermediate levels used in the present experiments (Ivy, Baudry, & Lynch, in preparation) and thus cannot explain the impairments in spatial maze performance found in rats treated with low and intermediate doses of the drug.

While the effects of inhibition or partial inhibition of lysosomes are not easily predicted, there is no reason to suspect that they would be selective since these organelles are found throughout the brain in every type of cell. This is not true for the calcium-activated proteinase. This enzyme is located in both the soluble (Murachi, Tanaka, Hatanaka, & Murakami, 1981) and membrane (Siman, Baudry, & Lynch, 1983) fractions of brain homogenates but apparently in different proportions in different parts of the brain. High levels of activity are found in the soluble fractions from brain stem and cerebellum but very little can be detected in equivalent samples from the telencephalon (Simonson, Baudry, Simon, & Lynch, submitted for publication). The distribution of the membrane-associated variant of the enzyme had not been studied in equivalent detail but there is reason to suspect that it is the reverse of that of the soluble form (Baudry et al., 1981). Recent work in this laboratory supports the earlier conclusion (Baudry & Lynch, 1980) that the calcium-induced, leupeptin sensitive increase in glutamate binding sites is a telencephalic phenomenon and is greatly reduced or altogether absent from the lower brain. Since this effect is very likely to be mediated by the calcium sensitive proteinase (Baudry et al., 1981; Lynch & Baudry, 1983) it appears that the enzyme is either absent in the lower brain or lacks access to its substrates.

From this it follows that any involvement of the proteinase–receptor interaction in memory would necessarily be restricted to those forms that require modifications of telencephalic circuitries. This in turn could explain why leupeptin blocks only certain memory related behaviors. The spatial memory tasks disrupted by the drug are exquisitely sensitive to lesions of the hippocampus and its connections (see Olton, Becker, & Handelmann, 1979, for a review). Escape and avoidance conditioning as well as ingestive behaviors are much less vulnerable to such damage. It is of course possible that spatial learning is peculiarly sensitive to leupeptin for reasons other than its dependency on telencephalic brain structures. However, we have recently found that leupeptin also blocks the learning of successive olfactory discriminations in tasks in which spatial cues are irrelevant (Staubli et al., unpublished data). Since the primary and secondary olfactory pathways are almost exclusively telencephalic, it is very likely that even simple forms of smell memory involve modification at that end of the neuroaxis. In any event, these results strengthen the hypothesis that different forms of learning exist which could involve different biochemical mechanisms.

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