EFFECTS OF LEUPEPTIN ON AVERSIVELY MOTIVATED SPATIAL MEMORY

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EFFECTS OF LEUPEPTIN ON AVERSIVELY MOTIVATED SPATIAL MEMORY

BY

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B.A. Bowling Green State University, 1981
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DISSERTATION

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Recent evidence indicates that the activation of a calcium-dependent proteinase, calpain, results in the formation of new glutamate receptors in the hippocampus (Lynch, Halpain, & Baudry, 1982), a brain area commonly thought to play a crucial role in mammalian memory formation. In vitro, this effect is correlated with a long-lasting enhancement of efficacy in synaptic transmission (long-term potentiation, LTP) following brief pulses of electrical stimulation in the hippocampus (Dunwiddie & Lynch, 1979). Because the induction properties of LTP are similar to the behavioral properties of memory, it has been proposed to be the physiological basis of memory (Lynch & Baudry, 1984). If the calcium-dependent activation of calpain is responsible for the induction of LTP and ultimately memory formation, then pharmacologically blocking the calcium-calpain interaction should block those forms of memory dependent on this mechanism.

Three experiments were conducted to assess the effects of leupeptin, a proteinase (calpain) inhibitor on aversively motivated spatial memory in rats. In Experiment One, either leupeptin (8 mg /
ml isotonic saline) or isotonic saline was infused continuously into the cerebrospinal fluid, and rats were trained in a one-way avoidance task with one-hour intertrial intervals, three trials per day, for 11 days. Leupeptin significantly impaired rats' acquisition of this task. Experiment Two assessed rats' performance in a win-stay or a win-shift avoidance task, and it was found that rats in the win-shift group learned the task more efficiently. This win-shift avoidance task was then used in Experiment Three to assess the temporal dependence of leupeptin's behavioral effects by introducing a delay of 1 min, 15 min, or 30 min between the information and choice trials. Leupeptin impaired choice performance at the 30 min delay only. In addition, leupeptin significantly impaired previously-acquired avoidance responding. Because a time-dependent memory deficit was observed, these data are considered to be consistent with the biological model of memory formation advanced by Lynch & Baudry (1984), however no adequate explanation of leupeptin's deleterious effects on previously acquired responding presently exists.
The neural basis of learning and memory has proven to be among the most profoundly interesting and intrinsically problematic areas in behavioral neuroscience. Since the time of Descartes, physiologists have offered a plethora of theoretical neural models of the acquisition and storage of information. Most notably, the Russian physiologist Sechenov (1863/1965) offered a theoretical account of neural "changeability" (p. 72) by which memories and sensations were "...preserved in a latent state by the nervous apparatus" (p. 71). A similar concept of selective strengthening of synaptic transmission was advanced by Hebb (1949) in his connectionistic theory of learning and memory. Although the cellular machinery of more recent theoretical accounts of the neural basis of learning and memory has changed significantly, this notion of neural changeability, or plasticity, has endured.

Before any mechanistic, neurobiological account of memory formation can be advanced, the critical brain structures or systems involved must be identified. An exhaustive search for a cortical locus of such a mechanism led Lashley (1950/1960) to conclude that his experiments "...yielded a good bit of information about what and where the memory trace is not" (p. 500). Indeed, until recently, even the anatomical locus of a potential memory mechanism has remained elusive. The hippocampal formation first became a candidate anatomical locus important to memory processes following documentation of the amnesic effects of medial temporal lobe resection in the epileptic HM (Scoville & Milner, 1957).
Although the resection included structures in addition to the hippocampus, including the amygdala, uncus, and hippocampal gyrus, Scoville & Milner (1957) and Milner (1974) have produced several lines of evidence indicating that the hippocampectomy is largely responsible for HM's memory impairment. Indeed, there appears to be mounting, converging evidence implicating the hippocampus in memory processes. In humans, the hippocampus appears to be most active when subjects answer questions requiring recent memory (Halgren, Engel, Wilson, Walter, Squires, & Crandall, 1983). Further, the extent of hippocampal damage incurred during surgery is correlated with the severity of induced memory deficits (Milner, 1974). In monkeys, conjoint lesions to the amygdala and hippocampus have been shown to produce amnesic effects (Mahut & Cordeau, 1963; Mishkin, 1978). An examination of the relative effects of amygdalo-hippocampal lesions and lesions to the hippocampus alone suggest further a critical role of the hippocampus in primate memory formation (Mahut, 1971). The observed memory deficits following hippocampal lesions in monkeys are most pronounced in, but not limited to, tasks requiring spatial memory (Mahut, 1971). Although hippocampal lesions have no effect on monkeys' pattern discrimination ability, they do significantly impair delayed nonmatching-to-sample performance (Zola-Morgan & Squire, 1986).

The hippocampus is also strongly implicated in nonprimate memory. Electrical stimulation of the hippocampus has been shown to disrupt learning and memory in rats (Bierly, Kesner, & Novak, 1983; Knowlton, McGowan, Olton, & Gamzu, 1985; Maki, 1985; McNaughton, Barnes, Rauch, Baldwin, & Rasmussen, 1986). Single and multiple unit recordings of
hippocampal cells during classical conditioning (Berger & Thompson, 1978; Hoehler & Thompson, 1980) further implicate this structure in memory. By far, the bulk of evidence implicating the hippocampus in nonprimate memory comes from studies assessing behavioral impairments in memory-related tasks following hippocampal lesions in the rat. Although there is considerable controversy concerning the 'types' of memory in which the hippocampus is implicated (Mishkin, Malamut, & Bechevalier, 1984; Oscar-Berman, 1979), hippocampal lesions clearly have a profound, deleterious effect on spatial memory in rats (Becker, Walker, & Olton, 1980; Jackson & Strong, 1969; Jarrard, 1980; Jarrard & Elmes, 1982; Nadel & MacDonald, 1980; Olton, Becker, & Handelmann, 1979; Thomas, Brito, Stein, & Berko, 1982).

Through a series of radial arm maze studies, Olton et al. (1979) demonstrated convincingly that hippocampal lesions disrupt rats' memory for places visited. In a typical experiment, rats are placed individually in a central compartment, radiating from which are eight arms with a food pellet at the end of each. The optimal strategy in this task is to visit each arm only once and consume the reinforcer. An error is scored if the subject enters a previously entered arm within a session. Rats learn rapidly not to enter previously visited arms, usually in 20-30 sessions. Following acquisition of this task, a delay is typically imposed between the fourth and fifth arm choices. After this delay, the subject is again placed in the central compartment, and allowed to choose as many arms as needed to retrieve the remaining food pellets. Unoperated rats perform well in this task, making few errors after delays as long as four hours (Mizumori, Rosenzweig, & Benet,
1985), or in conditions in which they are forced to solve a second radial arm maze during the delay between the fourth and fifth arm choices (Maki, Brokofsky, & Berg, 1979). Hippocampally lesioned rats, on the other hand, perform poorly in this task, reentering arms that were chosen prior to the imposed delay (Kesner, 1985). The results of a number of control experiments indicate that rats solve this maze using extramaze spatial cues (Olton et al., 1979). Thus it appears that lesions to the hippocampus disrupt spatial memory. The poor performance of hippocampally lesioned rats in spatial alternation tasks supports this conclusion (Douglas, 1975; Johnson, Olton, Gage, & Jenko, 1977).

Disruptive effects of hippocampal lesions are also observed in one-way active and passive avoidance tasks. Avoidance tasks typically take advantage of rats' tendency to prefer dark places to lighted ones. The apparatus normally used in one-way avoidance tasks consists of two chambers, one lighted and the other dark, separated by a guillotine door. In active avoidance, the rat is placed in the dark chamber, and after a short delay, a tone is sounded (CS) and the guillotine door is raised. After a specified length of time, usually 10 sec, the floor of the dark chamber is electrified (US), and the latency to enter the lighted chamber is recorded. Normal rats learn this task rapidly, avoiding the shock by entering the lighted compartment within the 10 sec CS-US interval. Animals with hippocampal lesions are impaired in both the acquisition and retention of this task (Liss, 1968; McNew & Thompson, 1966; Olton & Isaacson, 1968a, 1968b; Rich & Thompson, 1965).
Passive avoidance tasks employ a similar apparatus, but the rats are initially placed in the lighted compartment. This task is referred to as 'passive' because, to avoid shock, the rat must remain in the lighted compartment. Again, unoperated controls learn this task rapidly, while hippocampally lesioned rats require many more trials to learn to avoid the shock (McNew & Thompson, 1966; Olton & Isaacson, 1968b).

While lesions to the hippocampus disrupt passive, and one-way active avoidance, they facilitate the learning of two-way avoidance tasks (Isaacson, Douglas, & Moore, 1961; Olton & Isaacson, 1968a, 1968b). Two-way avoidance tasks employ a two compartment apparatus, but the two compartments are similarly lit. In a task of this type, the rat is placed in one of the compartments, a tone is sounded, and the guillotine door is raised. After a short delay, the floor of the compartment in which the rat was placed is electrified. The latency to enter the other compartment is recorded, and the rat is confined in the 'safe' compartment. After a specified intertrial interval, the tone is again sounded and the door raised. The rat must now enter the previously shocked compartment within the CS-US interval to avoid the shock. Hippocampally lesioned rats require fewer trials to learn this task than unoperated controls (Olton & Isaacson, 1968a, 1968b).

It can be argued that rats solve one-way active and passive and two-way avoidance tasks in much the same way as they do radial maze tasks, that is, by utilizing spatial cues (Black, Nadel, & O'Keefe, 1977; Olton, 1973; Olton & Isaacson, 1968a). In the one-way tasks, normal rats would have the advantage of using spatial cues to determine
which compartment to avoid. Hippocampally lesioned rats, on the other hand, may be impaired in associating shock with the location of that shock. Because of their inability to associate shock with its location, hippocampal rats may then more readily enter the dark, shocked compartment in passive avoidance tasks, and similarly would be more apt to remain in the dark, shocked compartment in active avoidance tasks. With respect to two-way avoidance, unoperated controls presumably remember on each trial that the compartment into which they must escape was, on the previous trial, the compartment in which they were shocked. This spatial memory competes with conditions of the current trial. Thus, by virtue of being impaired in spatial memory, hippocampally lesioned rats perform better in two-way avoidance.

The hypothesis that hippocampal damage disrupts spatial memory, and that these avoidance tasks are essentially spatial tasks, predicts rats' performance in the three types of avoidance tasks discussed. Hippocampal lesions impair one-way passive and active avoidance acquisition, and enhance two-way avoidance acquisition (Olton, 1973; Olton & Isaacson, 1968a). Interestingly, hippocampal lesions facilitate the acquisition of free-operant avoidance performance, presumably because of a reduced tendency to freeze in the presence of shock (Duncan & Duncan, 1970).

Based on the evidence outlined above, it appears that the hippocampus is in some way involved in some memory-related behavior in both appetitively and aversively motivated tasks. Although studies examining the effect of telencephalic damage on memory have been useful in identifying the anatomical locus of a possible memory mechanism, they
tell us little about the physiological mechanism which may be responsible for the formation of memory. Furthermore, while lesions remove the anatomical structure of interest, they also disrupt countless interconnections passing through the lesioned area. To identify the biological mechanism responsible for memory formation, one needs a paradigm by which this mechanism can be manipulated without destroying or disrupting existing neuronal pathways.

Lynch & Baudry (1984) outline several conditions which must be satisfied by any plausible, testable biological memory mechanism. To be considered 'plausible', this mechanism must meet the conditions imposed by the behavioral properties of memory. First, this mechanism must be activated by brief physiological events. Second, this mechanism must produce changes in the operating characteristics of neuronal circuitry. Third, these induced changes must last for substantial lengths of time.

Lynch & Baudry suggest that long-term-potentiation (LTP), meets the conditions described above. LTP refers to the phenomenon of long-lasting enhancement of efficacy in synaptic transmission following brief periods of intense firing. LTP has been demonstrated in vivo (Bliss & Lomo, 1973; Bliss & Garner-Medwin, 1971, 1973) and in vitro in hippocampal slices (Schwartzkroin & Wester, 1971; Alger & Teyler, 1976). LTP is typically measured by recording hippocampal population excitatory post-synaptic potentials following electrical stimulation of the perforant pathway, a major source of afferents to the hippocampus. Although observations of LTP have previously been limited to the hippocampal pathways (McNaughton, 1984) using the neurotransmitter glutamate (Storm-Mathieson, 1977), recent evidence suggests that
cortical neurons may also exhibit LTP (Roman, Staubli, & Lynch, in press).

Two forms of hippocampal LTP, differentiated by their half-life, have been found to exist. One type, LTP1, decays with a half-life of several hours, and a second type, LTP2, decays with a half-life of several days (Racine & Milgram, 1983; Racine, Milgram, & Hafner, 1983).

Lynch & Baudry (1984) and others (McNaughton, 1983) argue that, because the properties of LTP2 so closely resemble the behavioral properties of memory, it may be the physiological substrate of memory. Indeed, LTP2 is induced by brief physiological events (Dunwiddie & Lynch, 1978), is by definition a change in the operating characteristics of neuronal circuitry, and lasts for substantial lengths of time. LTP is also strengthened by repetition (Barnes, 1979), and with repetition can last indefinitely (Bliss & Lomo, 1973), two characteristics which further resemble the behavioral properties of memory. The behavioral consequences of disrupting LTP induction should then be predictable: an impairment of memory.

But what is the locus of the physiological mechanism which accounts for LTP? Lynch & Baudry argue that, since a large scale modification of the nucleus or axon of a neuron would result in a change of countless neural interconnections, the mechanism must be localized synaptically. It is unclear, however, whether the biochemical mechanism resulting in LTP is localized pre- or post-synaptically (Bliss & Dolphin, 1982, 1984). A presynaptic mechanism refers to one which results in an afferent fiber releasing more neurotransmitter per action potential. A
postsynaptic mechanism refers to one in which the same amount of neurotransmitter results in a significantly potentiated post-synaptic response. Determining whether LTP is the result of pre- or post-synaptic changes is important for behavioral tests of the relationship between LTP and memory because pharmacological disruption of LTP induction depends upon this knowledge.

According to Farley & Alkon (1985), there are four lines of evidence indicating that LTP arises from postsynaptic changes. First, as shown by Dunwiddie, Madison, & Lynch (1978), LTP is prevented by blocking glutamate binding with AFB, a glutamate receptor antagonist. Second, morphological changes in postsynaptic spine density (Desmond & Levy, 1983), shape (Fifkova & Van Harreveld, 1975), and number (Lee, Schottler, Oliver, & Lynch, 1980) occur following LTP induction. Third, Baudry, Oliver, Creager, Wieraszko, & Lynch (1980) found increased glutamate binding following LTP induction; statistical (Scatchard) analyses indicated that this effect was due to an increase in the number of receptors, not in the affinity of those receptors. Finally, intracellular injection of the calcium chelator EGTA prevented LTP in the injected postsynaptic neuron, but not in surrounding neurons (Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983). Based on this evidence, one should observe behavioral consequences of interfering with post-synaptic biochemical events. Specifically, by negating the induction of LTP through biochemical manipulations designed to alter post-synaptic events, one should observe memory impairments.
In order to determine the trigger for LTP and the associated increase in glutamate receptor number and glutamate binding, neurons were bathed in various calcium concentrations. In a low calcium medium, the potentiation effect (Dunwiddie & Lynch, 1979) and the increased number of receptors (Lynch, Halpain, & Baudry, 1982) were greatly reduced, suggesting that these effects are calcium dependent. The calcium-dependent effects on glutamate binding are most pronounced in the hippocampus, and are greatly reduced or absent in the brainstem (Baudry & Lynch, 1980). Further, this calcium-dependent increase in glutamate binding appears to be temporally dependent, with a half maximal effect between 1-2 min following exposure to calcium, and maximal effects between 10-15 min following exposure (Baudry, Kramer, & Lynch, 1983). Because these calcium-dependent effects were temperature-sensitive, and because enzymes cause the irreversible, long-lasting cleavage of peptide bonds, Lynch & Baudry hypothesized that an enzyme was involved in the formation of new receptors. They tested the effects of proteinase inhibitors, among them leupeptin, and found that inhibition of intracellular enzymes blocked the increased binding of glutamate (Baudry, Bundman, Smith, & Lynch, 1981).

Fodrin is a protein known to line the inner face of neuronal membranes and it is concentrated postsynaptically (Carlin, Bartelt, & Siekevitz, 1983). Fodrin is degraded by the calcium-dependent proteinase calpain (Murachi, 1983), which has a high affinity for fodrin (Zimmerman & Schlaepfer, 1982). Further, fodrin has been implicated in the regulation of glutamate receptor number (Baudry & Lynch, 1984). In support of this regulatory role of fodrin, Siman, Baudry, & Lynch (1985)
found that blocking the calpain-induced fodrin degradation with fodrin antibodies also blocks calcium-dependent increases in glutamate receptor binding.

Based on the physiological findings described above, Lynch & Baudry (1984) put forth a specific theory regarding the neurobiology of memory. They suggest that, during periods of high frequency activity, intracellular calcium concentrations increase. This increase in intracellular calcium activates the degrading enzyme calpain, which degrades a localized portion of the fodrin network. This degradation results in a structural change in the postsynaptic membrane: the uncovering of previously latent glutamate receptors. An increase in the number of receptors then increases the size of the postsynaptic response to this neurotransmitter, which in turn results in LTP. LTP, then, is postulated to be the physiological correlate of memory.

This theory generates several testable hypotheses. First, LTP induction should result in an increase in glutamate binding and be accompanied by dendritic morphological changes. Second, memory formation should be accompanied by both an increase in glutamate binding and an induction of LTP. Third, pharmacological manipulations inhibiting the calcium-calpain interaction should block LTP induction, increased glutamate binding, and those forms of memory dependent on brain regions utilizing this mechanism (i.e., the hippocampus). Finally, the behavioral effect of calpain inhibitors should be temporally dependent, with a time course reflecting the temporal course of calcium's effect on increased glutamate receptors.
With respect to the first of these predictions, induction of LTP was shown to produce an increase in glutamate binding due to an increase in the number of receptors (Baudry et al., 1980; Lee et al., 1980), as well as a change in both the shape (Fifkova & Harreveld, 1975) and density (Desmond & Levy, 1983) of dendritic spines.

Support for the second of these predictions came from Mamounas, Thompson, Lynch, & Baudry (1984), who found an increase in hippocampal glutamate receptor binding after classically conditioning the eyeblink response in rabbits. Further, the amplitude of induced LTP has been shown to correlate strongly with the speed of complex maze learning (Barnes, 1979) and with memory formation in an olfactory learning set paradigm (Roman, Staubli, & Lynch, in press) in rats.

Finally, pharmacological manipulations inhibiting the calcium-calpain interaction should block LTP induction, increased glutamate binding, and those forms of memory dependent on brain regions utilizing this mechanism. In support of the first of these, intracellular injection of the calcium chelator EGTA has been shown to block LTP induction (Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983). Further, intracerebroventricular infusion of leupeptin, a proteinase (calpain) inhibitor, has been shown to impair memory related behavior in an olfactory learning set task (Staubli, Baudry, & Lynch, 1985), and perhaps more germane to the present studies, in a radial arm maze task (Staubli, Baudry, & Lynch, 1984).
After acquisition of the radial arm maze task, Staubli et al. (1984) implanted subcutaneous osmotic minipumps which, through a cannula implanted into the lateral ventricle, infused leupeptin continuously for 14 days. They found that leupeptin severely impaired radial arm maze performance. That is, their experimental subjects made many more errors than did controls after delays were imposed between the fourth and fifth arm choices, effects consistent with those found after lesions to the hippocampus.

In an attempt to investigate the role of this mechanism in memory for avoidance contingencies, Staubli et al. (1984) implanted one group of rats with pumps containing leupeptin, and another group with pumps containing isotonic saline. Following two days' post-operative recovery, the rats were given eight training trials (30 sec intertrial interval) on a one-way active avoidance task. The next day, eight trials of the same task were conducted. Staubli et al. found that leupeptin did not affect rats' within-day acquisition of this task. Furthermore, except for the first three trials, latencies to avoid the shock on day two testing trials were essentially the same for both groups. Staubli et al. concluded that the putative calcium-calpain mechanism may be responsible for long-lasting spatial memory, but is uninvolved in avoidance memory. This is because, presumably, the hippocampus is not involved in memory for avoidance contingencies. Given the findings of Olton & Isaacson (1968a, 1968b) and others (Liss, 1968; McNew & Thompson, 1966; Rich & Thompson, 1965) regarding the deleterious effect of hippocampal lesions on active and passive avoidance performance in rats, however, this seems unlikely. Further,
if indeed these avoidance tasks are essentially aversively motivated spatial tasks, as suggested above (Black et al., 1977; Olton, 1973), avoidance memory and spatial memory are not dichotomous, and the distinction is without meaning.

Several lines of evidence suggest that the conclusion of Staubli et al. regarding the noninvolvement of the calcium-calpain interaction in aversively motivated memory tasks is premature. First, their results are inconsistent with the effects of hippocampal lesions. Second, the calcium-induced increase in glutamate binding occurs with a half-maximal effect of 1-2 min, and reaches asymptote between 10-15 min (Baudry et al., 1983). Given this time course, one would not expect proteinase inhibitors to impair acquisition of any task across 30 sec delays. If indeed this calpain mechanism is a memory mechanism, it can be invoked only in situations in which the organism is required to 'remember' for periods as long as, or longer than, the minimum time required for the mechanism to be active. Thus, one would expect leupeptin to impair memory-related behavior given retention intervals of longer than a minimum of perhaps 10 min. Indeed, a close examination of Staubli et al.'s one-way avoidance data indicate that although leupeptin-treated subjects were not impaired in within day acquisition, their performance in the first three trials of day two was significantly impaired. Across these first three trials, an average of 33.3% of the leupeptin-treated rats avoided the shock, while 60% of the controls made avoidance responses. Thus, although leupeptin may not have impaired the within-day acquisition of the task across 30 sec intertrial intervals, it may have impaired between-day retention. This finding would be
consistent with the logic regarding the temporal relationship between calcium-dependent increases in glutamate receptor number and behavior. Using a 24 hr intertrial interval, Davis & Pico (in press) found that intracerebroventricular injection of leupeptin impaired avoidance behavior in chicks. Given long intertrial intervals, similar leupeptin induced deficits were obtained using rats in a swimming task requiring them to remember from trial to trial the location of a platform hidden just beneath the surface of opaque water (Morris & Baker, 1984). Administration of the glutamate receptor antagonist d,L-AP5 (Morris, Anderson, Lynch, & Baudry, 1986) and hippocampal lesions (Morris, Garrud, Rawlins & O'Keefe, 1982) also impair performance in the Morris water tank.

It appears, then, that the conclusion that leupeptin does not impair, and by inference that the putative calcium-calpain mechanism is not involved in, memory for avoidance contingencies may be erroneous. The present studies were conducted to investigate further the effect of leupeptin on avoidance performance, and to provide information pertinent to the third prediction discussed above regarding the time course of leupeptin-induced behavioral impairments.
I. EXPERIMENT ONE

The Effect of Leupeptin on One-Way Avoidance Acquisition

As stated previously, the failure of Staubli et al. (1984) to find a leupeptin-induced deficit in one-way avoidance acquisition is inconsistent with several lines of evidence. Given the time course of calpain-induced increase in glutamate receptor number, one would not expect leupeptin to impair acquisition of any task with 30 sec intertrial intervals. The present experiment was conducted to determine the effects of leupeptin on acquisition of one-way avoidance performance with long (1 hr) intertrial intervals.

Method

Subjects

Nine male Sprague-Dawley rats (Charles Rivers Breeding Laboratories), aged five months at the beginning of testing, served as subjects. Subjects were housed individually in rack mounted wire mesh cages (24 cm x 20 cm x 18 cm) from weaning to age five months, during which time they were handled and weighed twice weekly to render them docile. Starting at age five months, they were housed individually in rack mounted clear plastic tubs (45 cm x 24 cm x 20 cm), which served as their home cage for the remainder of the experiment. Subjects were maintained in an environmentally controlled animal vivarium in the Department of Psychology, University of New Hampshire, on a 12:12 light:dark cycle, lights on at 0700. Food and water were available ad
libitum. Four randomly assigned rats served as the control (saline) group, five randomly assigned rats served as the experimental (leupeptin) group.

Apparatus
A 52 cm long x 13 cm wide x 30 cm high plexiglas chamber was used. This chamber was divided into two equal-sized compartments (26 cm x 13 cm x 30 cm) by a sliding guillotine door which, when lowered by means of a cord threaded through a spar above the apparatus, formed a barrier 10 cm high. One compartment was lined with white cardboard, the other compartment was lined with black cardboard. One wall of the entire chamber was left uncovered to permit viewing. The floor of the apparatus consisted of 0.3 cm stainless steel rods spaced 1.4 cm center to center. The grid floor of the black compartment was connected to a Grason-Stadler scrambler and shock generator (E1064GS).

Cannulae were constructed from 22g stainless steel tubing (21022, Hamilton Co.). This tubing has an O.D. of 0.7 mm and an I.D. of 0.4 mm. Pieces of 28g stainless steel tubing (21028, Hamilton), cut to length and crimped at the ends, served as plugs for the cannulae during implantation and prior to attaching the pumps. These cannulae were held in place with four skull screws (obtained from a local optician) and Kerr Fastcure dental cement. A Kopf stereotaxic instrument was used to implant the cannulae.

Osmotic minipumps (2002, Alza Corp.) were used. These pumps have a 0.2 ml capacity, and deliver 0.5 ul solution per hour for 14 consecutive days. These pumps were filled with either isotonic saline (0.9%) or with 8 mg leupeptin (L2884, Sigma) / ml isotonic saline. Given a
ventricular volume of 250 ul (Cserr, 1965), and a cerebrospinal fluid exchange rate of 2.2 ul/min (Bass & Lundborg, 1973), leupeptin reached a steady-state concentration of between 20-40 uM. The pumps were attached to the cannulae with polyethylene tubing (Intramedic PE-60, Clay Adams).

Procedure

All behavioral training and testing was conducted during the light cycle, and took place in a room located approximately 20 m from the housing vivarium. Animals were wheeled to the testing room in their home cages in squads of four or five. After all four or five rats in a squad were run, they were returned to the vivarium.

Surgery. Rats were anesthetized with Nembutal (50mg/kg) prior to surgery. A cannula with plug was placed stereotaxically into the left lateral ventricle at the level of the anterior hippocampal commissure (exact coordinates, measured from Bregma, flat skull position: anterior-posterior, -1.0 mm, lateral, +1.5 mm, depth, -4.0 mm). Following placement and anchoring of the cannula, the 28g plug was removed and the osmotic minipumps were attached. These pumps were implanted interscapularly and subcutaneously, and were attached to the cannula with 5 cm polyethylene tubing.

Avoidance training. Avoidance training commenced following two days post-surgical recovery. Rats were given three trials per day, with a 1 hr intertrial interval, for 11 days.

Before a trial, a rat was placed in the black compartment facing the guillotine door. A trial began 10 sec later with the lowering of the door separating the two compartments. A trial was terminated when the subject traversed the barrier and placed all four feet onto the
floor of the white, 'safe' compartment. An avoidance response was recorded if the rat traversed the barrier and entered the safe compartment within 10 sec of trial onset. If no avoidance response occurred, a 0.5 mA shock, 0.5 sec every 1.0 sec, was delivered to the floor of the black compartment. Latency to enter the white compartment was recorded. Failure to avoid or escape from the shock within 30 sec of trial onset resulted in termination of the trial, and the rat was placed manually into the safe arm. In either case, the rat was retained in the safe arm for 30 sec, after which it was returned to its home cage. After all four or five rats in a group were run, they were returned to the vivarium.

Results

Results are plotted in Figures la-c. As illustrated in Figure la, leupeptin-treated rats failed to avoid the shock significantly more often than their saline-treated counterparts across all 33 trials (Total, mean leupeptin = 16, mean saline = 9.25, Mann-Whitney U = 2.0, p < .05, one tailed). Similarly, rats receiving leupeptin failed to avoid the shock prior to attaining a criterion of eight avoidance responses in 10 consecutive trials significantly more often than rats receiving saline (mean leupeptin = 11.8, mean saline = 5, Mann-Whitney U = 1.5, p < .05, one tailed).

Figure lb illustrates trial-by-trial performance averaged across subjects and days. Rats treated with leupeptin made significantly fewer avoidance responses on the second daily trial, on the average, than rats treated with saline (mean percent avoid leupeptin = 45.45, mean percent avoid saline = 81.82, Mann-Whitney U = 16, p < .01). Figure lc, which
Figure 1A. Leupeptin's effects on avoidance performance across all 33 trials and prior to attaining a criterion of 8 avoidance responses out of 10 consecutive trials (+ Is). Both differences are significant (Mann-Whitney U, p < 0.05).

Figure 1B. Leupeptin's effects on avoidance performance by trial (+ Is).
Figure 1C. Leupeptin's effects on trial by trial avoidance performance.
documents trial by trial avoidance performance averaged across individual rats within a group, further illustrates this point. Unlike the curves describing both trial one and trial three performance of the two groups, the curves representing trial two performance of the two groups are clearly divergent, with the exception of days six and eight.

Discussion

These data suggest that, given long intertrial intervals, leupeptin impairs one-way avoidance performance. This finding is consistent with the time course governing calpain's effect on glutamate binding, the effects of hippocampal lesions on one-way avoidance acquisition, and the effects of leupeptin on avoidance behavior of chicks (Davis & Pico, in press) and rats in a water maze (Morris et al., 1984). Leupeptin appeared to manifest its effects differentially across trials within a day, significantly impairing performance on the second daily trial. It is unclear at present why during the third daily trial leupeptin-treated subjects were responding similar to saline-treated subjects. It is possible that, while between-day retention was impaired, within-day retention was not impaired as severely by leupeptin, as was suggested earlier with reference to the study of Staubli et al. (1984).

Although these data are suggestive of a time dependent memory deficit, they are not conclusive. It is possible that leupeptin's disruptive effects on avoidance acquisition were due to another, nonspecific, unknown effect of the drug. For example, leupeptin could potentially impair attentional or motor processes, disrupting avoidance acquisition through a nonmemorial mechanism.
Thus, the present experiment raises two questions: first, whether leupeptin's impairment of avoidance acquisition is a manifestation of its effects on some nonmemorial mechanism and second, whether the temporal course of leupeptin's behavioral effects resembles closely the known time course of the calcium-calpain mechanism. These questions can be answered by choosing a paradigm with which one can assess leupeptin's effects on previously learned avoidance responding and on delayed choice performance. For example, a delayed choice task similar to the radial arm maze using aversive motivation would be ideal; however, rats' choice performance has not been assessed using such a paradigm. The experiment which follows was conducted to determine the viability of such a paradigm by assessing rats' choice performance in a plus-shaped maze.
II. EXPERIMENT TWO

Win-Stay vs. Win-Shift Preference and Discrimination Learning in an Avoidance Task

Olton (1982) found that rats learn win-shift appetitive tasks more quickly than win-stay appetitive tasks. Win-stay and win-shift tasks are typically comprised of a series of two trial sessions. In the first trial of a win-shift task, food is placed in one of two possible locations, and a food deprived the rat is allowed (or forced) to find and consume the food. In trial two of a session, food is placed in the other of the two possible locations, and the rat must choose between the two. A correct response consists in choosing the food location not chosen in trial one. Thus, the rat must 'shift' between the two potential food locations within a session. Win-stay tasks employ a similar design, except that food is placed in the same location in trials one and two, and a correct response consists in choosing the same food location in trial two as in trial one. Olton (1982) suggests that rats perform better in win-shift tasks because they are evolutionarily predisposed to seek food in previously unvisited locations.

Win-stay vs win-shift performance has not been assessed using an avoidance paradigm. From an evolutionary perspective, animals might be predisposed to follow a win-stay strategy in the avoidance of noxious stimuli because, unlike food sources, safe places are not subject to
depletion and renewal.

The purpose of the present experiment was threefold. First, win-stay vs. win-shift preference was assessed in an aversively motivated task. Second, win-stay and win-stay learning was assessed. Third, and perhaps most important, the findings regarding win-stay and win-shift learning served as the basis of the paradigm used in Experiment Three to assess the temporal dependence of leupeptin's effects on aversively motivated spatial memory.

Method

Subjects

Twelve Harvard Brown rats, bred in the laboratories of the Department of Psychology, University of New Hampshire, served as subjects. These animals were housed individually in rack-mounted clear plastic tubs (45 cm x 24 cm x 20 cm), which served as their home cage throughout the experiment. Subjects were maintained in an environmentally controlled animal vivarium in the Department of Biochemistry, University of New Hampshire, on a 12:12 light:dark cycle, lights on at 0700. Food and water were available ad libitum.

Apparatus

A four-arm radial maze adapted for avoidance training was used. The sides of the maze were constructed of plexiglas and the floor of 0.3 cm stainless steel rods spaced 1.4 cm center to center. Four arms (25 cm long x 13 cm wide x 15 cm high) with removable tops radiated from an octagonal central compartment measuring 25 cm across and 25 cm high. Each of the arms was separated from the central compartment by a
guillotine door, which could be raised by means of a cord attached to
the top of the door and threaded through a spar above the central
compartment.

The grid floor of the central compartment and of each of the arms
could be connected to a Grason-Stadler scrambler and shock generator
(E1064GS). By making the appropriate connections, the experimenter
could control which areas of the maze floor delivered shock.

Procedure

All behavioral training was conducted during the light cycle, and
took place in a corner of the vivarium.

Stay vs. Shift Preference. Training sessions assessing rats' preference for stay or shift strategies were comprised of two trials: a
forced-choice trial and a free-choice trial. In the first,
forced-choice trial, a rat was placed in the central compartment and 10
sec later the guillotine door leading to one randomly chosen arm was
raised. An avoidance response was recorded if the rat placed three feet
into the arm within 10 sec after the door was raised. If no avoidance
response occurred, a 0.5 mA shock, 0.5 sec every 1.0 sec, was delivered
to the floor of the central compartment. Latency to enter the arm was
recorded, and the rat was confined in the arm for 30 sec, after which
the rat was placed immediately into the central compartment. The
second, free-choice trial commenced 10 sec later.

Free-choice trials were similar to forced-choice trials except that
two doors were raised: one leading to the arm entered in the
forced-choice trial, the other leading to the arm 180 degrees opposite
the arm entered in the forced-choice trial. Neither arm delivered shock. As in the forced-choice trial, an avoidance response was recorded if the subject placed three feet onto the floor of either arm within 10 sec of the raising of the two doors. If no avoidance response occurred, a 0.5 mA shock, 0.5 sec every 1.0 sec, was delivered to the floor of the central compartment. Both the arm entered and the entrance latency were recorded. The same pair of randomly chosen arms was used for each of the rats during a given session. Rats received two such sessions daily, 1 hr intersession interval, for 14 days.

*Stay vs. Shift Learning.* Following assessment of strategy preference, each of the 12 rats was randomly assigned to one of two conditions. In the 'stay' condition, a win-stay strategy was enforced; similarly, in the 'shift' condition, a win-shift strategy was enforced. Training sessions in this phase of the experiment were identical to those used in determining strategy preference except that in the second, free-choice trial, only one of the two arms was free from shock. For the 'stay' group, only the arm entered in the forced-choice trial was safe; for the 'shift' group, only the arm 180 degrees opposite the arm entered in the forced-choice trial was safe. In both tasks, the floor of the other, incorrect arm delivered shock at the same time and rate as the floor of the central compartment. In both the 'stay' and 'shift' conditions, a correction procedure was used. If an animal entered an incorrect arm and remained there for 30 sec after the onset of shock, it was pushed out of the arm into the central compartment and then into the correct arm. An error was scored only if the subject entered the incorrect arm, regardless of whether it was an escape or an avoidance response. Two such sessions were run daily for 30 days.
Results

*Stay vs. Shift Preference.* Performance and preference data are illustrated in Figures 2a-c. In Figures 2a and 2b, the solid line represents trial one (forced-choice) performance, while the dashed line represents trial two (free-choice) performance. As can be seen in these two figures, animals learned quickly to avoid the shock by entering either the one available arm (trial 1) or one of the two available arms (trial 2). Figure 2c illustrates average percent 'stay' responses across the 12 rats as a function of sessions. There was no consistent preference either to stay or to shift (mean percent stay = 48.81, s.e.m. = 2.77, t = -0.43, p = 0.67).

*Stay vs. Shift Learning.* Figure 3 illustrates average errors for each group of rats across the 60 training sessions, grouped in blocks of 10 sessions. An 'error' for animals in the 'stay' group consisted of entering the arm in the free-choice trial which was opposite to that entered on the forced-choice trial; similarly, an error was recorded for animals in the 'shift' condition if on the free-choice trial they entered the same arm as was entered on the forced-choice trial. The solid, horizontal line in Figure 3 represents chance performance.

Overall, the performance of both groups appears to be well below chance. An analysis of total errors across the final 30 training sessions revealed a significant difference between 'shift' and 'stay' groups (mean errors 'stay' = 11.7, mean errors 'shift' = 5.33, Mann-Whitney U = 4.5, p<.05).
Figures 2A-C. Response latency, percent avoidance responses, and percent 'stay' responses across all 28 stay vs. shift preference assessment sessions.
Figure 3. Average errors per animal in each group across six blocks of 10 sessions.
Discussion

These data indicate that, unexpectedly, rats show no preference for either a win-stay or a win-shift strategy when faced with the task of avoiding shock. Further, and perhaps more unexpectedly, rats are more efficient in learning a win-shift avoidance strategy. It is unclear why this might be the case; however, among the advantages of recourse to evolutionary theory in searching for 'explanations' of behavior is the freedom to speculate. From an evolutionary perspective, it may be adaptive for organisms to seek safety in novel locations. Imagine a rat fleeing from a pursuing predator. Returning consistently to the same refuge could conceivably be a maladaptive escape strategy. All things being equal (such as distance from the predator), unpredictability in escape locations across escape episodes may be a more adaptive escape strategy.

Regardless of the potential evolutionary determiners of win-shift avoidance behavior, it is clear that, indeed, rats do learn a shift strategy more efficiently. More importantly, this paradigm can be used to investigate the temporal dependence of leupeptin's effects on aversively motivated spatial memory by introducing a variable delay between the forced-choice and free-choice trials. Further, leupeptin's effects on previously learned avoidance responding can be tested by training animals on this task prior to the infusion of the drug.
III. EXPERIMENT THREE

The Temporally Dependent Effects of Leupeptin on Aversively Motivated Spatial Memory

Pharmacological manipulations inhibiting calpain's action are predicted to impair memory in a time-dependent manner. Given that the half-maximal effect of calcium-activated calpain on glutamate receptors occurs between 1-2 min, and reaches asymptote between 10-15 min (Baudry et al., 1983), one would expect proteinase inhibitors to impair memory in a similarly time-dependent fashion. If this calpain mechanism is a memory mechanism it would be invoked only in situations in which an organism was required to 'remember' information over a critically long time period, perhaps 10-15 min. Further, leupeptin would be expected to leave shorter-term memories intact, impairing memory only during longer retention intervals. Finally, inhibition of the calpain mechanism should impair the acquisition of new information only; previously well-learned responses have presumably already resulted in the induction of LTP, and, theoretically, inhibiting the calcium-calpain interaction should leave existing LTP and existing memory undisrupted.

The purpose of the present experiment was twofold: first, to provide concrete evidence of a temporally dependent effect of leupeptin on memory, and second, to determine leupeptin's effect on previously well learned avoidance responses. The win-shift avoidance task
described in Experiment Two was used, but a delay of variable length was imposed between the forced- and free-choice trials. The win-shift strategy was chosen because of the proficiency with which rats learned this task in Experiment Two.

Method

Subjects

Twenty-five male Sprague Dawley rats (Charles Rivers Breeding Laboratories), purchased at weaning, served as subjects. Subjects were raised in rack mounted wire mesh cages (24 cm x 20 cm x 18 cm) until six months of age. They were handled and weighed twice weekly during this period to render them docile. At age six months, subjects were transferred to rack mounted clear plastic tubs (45 cm x 24 cm x 20 cm), which served as their home cage throughout the experiment. Subjects were maintained in an environmentally controlled vivarium in the Department of Psychology, University of New Hampshire, on a 12:12 light:dark cycle, lights on at 0700. Food and water were available ad libitum.

Apparatus

The four arm radial maze adapted for avoidance conditioning, described in detail in Experiment Two, was used. Cannulae, and osmotic minipumps, described in Experiment One, were also used.

Procedure

All behavioral training and testing was conducted during the light cycle, and took place in a room located approximately 20 m from the vivarium. Subjects were wheeled to the training room in their home.
cages in squads of eight or nine. After all subjects in a squad were run, they were returned to the vivarium. The walls and floor of the entire apparatus were wiped with a damp sponge and dried with a paper towel between trials to minimize olfactory cues.

**Acquisition.** On the first day of training, animals were placed individually into the central compartment of the apparatus and allowed to explore the four arms freely for five minutes. Shift training followed this acquaintance procedure.

All subjects were given 35 'forced-shift' sessions, each of which was comprised of two forced-choice avoidance trials. In trial one, the subject was placed into the central compartment, and a trial began 10 sec later when a randomly chosen door was raised. An avoidance response was recorded if the subject placed three feet onto the floor of the accessible arm within 10 sec after trial onset. If no avoidance response occurred, a 0.5 mA shock, 0.5 sec every 1.0 sec, was delivered to the floor of the central compartment. Latency to enter the arm was recorded. The subject was retained in the arm for 30 sec, after which it was placed immediately into the central compartment.

Trial two began 10 sec later with raising the door leading to the arm 180 degrees opposite the arm entered on trial one. Contingencies of avoidance identical to those in trial one prevailed in trial two. After a 30 sec confinement period following trial two, the animal was returned to its home cage. Subjects received three to five daily sessions, with a one hour inter-session interval, seven days a week.
Following forced-shift training, subjects received 70 shift sessions. These sessions were identical to the shift sessions described in Experiment Two. Briefly, sessions were composed of a forced-choice trial, in which only one door was raised and the corresponding arm was free from shock, and a free-choice trial, in which two doors were raised but only one of the corresponding arms was free from shock. A correct response consisted of entering on the free-choice trial the arm opposite the arm entered on the forced-choice trial. A correction procedure was used. An error was scored only if the subject entered the incorrect arm, regardless of whether it was an escape or an avoidance response. Failure to avoid the shock but entering the correct arm was not scored as an error.

Acquisition of the shift strategy at no delay was followed by a series of sessions in which increasingly longer delays were imposed between the forced- and free-choice trials. Following a forced-choice trial and the subsequent 30 sec confinement period, the subject was returned to its home cage for a variable delay. Delays were measured from the time of entry into the arm on the forced-choice trial to the start of the free-choice trial; thus, the 30 sec confinement period was included in the delay. Length of the imposed delay increased gradually from 1 min to 30 min. Subjects were run in 30 sessions with 1 min delay, followed by 10 sessions each with delays of 3 min, 6 min, 9 min, 15 min, and finally 30 min. Three to five such sessions were run daily, seven days a week. The final 30 sessions consisted of delays of 1 min, 15 min, and 30 min, counterbalanced across days. These final 30 sessions were conducted at the rate of five per day for six days.
Individual percent correct responses for each of the 15 min and 30 min delays in these final 30 counterbalanced sessions were combined with similar data from the previous 10 noncounterbalanced sessions at those delays, yielding a total of 20 sessions at each delay. Similarly, the data from the 1 min delay counterbalanced sessions were combined with the data from the final 10 sessions of the 30 total sessions run previously at 1 min delay. Based on these combined choice data, subjects were assigned to two groups so as to be about equal in percent correct responses across the three delays. Twelve subjects comprised the saline (control) group, thirteen comprised the leupeptin (experimental) group.

Surgery. Surgical procedures commenced following the final day of acquisition training. Animals were anesthetized and cannulated, and the osmotic minipumps containing either saline (0.9%) or leupeptin (8 mg/ml) were implanted as described in Experiment One.

Testing. Following two days' post-surgical recovery, subjects were run for 12 consecutive days, five sessions per day, yielding 20 sessions at each of the delays of 1 min, 15 min, and 30 min. Delays varied across days but were kept constant on a given day. The order of delay presentation was as follows: 1 min, 1 min, 15 min, 15 min, 30 min, 30 min, 1 min, 15 min, 30 min, 30 min, 15 min, 1 min. Forced-choice trial response latency was recorded, as were free-choice trial response latency to the first entry, latency to the correct entry if the first entry comprised an error, and errors.

Results

Acquisition. Avoidance performance during the initial 35 forced-shift sessions and during the shift sessions at zero and
increasing delays is shown in Figure 4. Average percent avoidance responses for the forced-shift phase of the experiment are grouped in blocks of five sessions. Average percent avoidance responses for the shift sessions are grouped in blocks of 10 sessions. Underlined delays represent blocks of 10 sessions in which delay length was counterbalanced across days. The solid line represents trial one performance, the dashed line trial two performance. As shown, subjects learned quickly to avoid the shock during the forced-shift sessions. Avoidance performance declined slightly during the shift sessions at zero delay, and appear to decline further during delay trials. For the final 30 sessions counterbalanced for delay across days, 60.33% of the subjects were avoiding the shock on the forced-choice trial (trial one) and 63.6% on the free-choice trial (trial two).

Shift acquisition at zero and increasing delays is illustrated in Figure 5, which shows the average number of errors per animal within blocks of 10 sessions. An error consisted of entering the arm on the free-choice trial which was entered on the forced-choice trial of a given session. These data do not differentiate between avoidance and escape responses. The solid, horizontal line represents chance performance (5 errors in 10 sessions). Underlined delays represent blocks of 10 sessions in which delay length was counterbalanced across days.

As can be seen in Figure 5, animals improved rapidly over the 70 sessions at zero delay, with error rates well below chance at delays as long as 30 min.

Post-surgical Performance. Figure 6 illustrates presurgical and
Figure 4. Average percent avoidance responses during 35 forced-shift sessions, 70 shift-acquisition sessions (0 delay) and 110 delay sessions. Data from all 25 subjects are grouped in blocks of 5 sessions for the forced-shift phase. The remainder are grouped in blocks of 10 sessions.
Figure 5. Mean errors per animal across 70 Shift-acquisition sessions (0 delay) and 110 delay sessions. Data from all 25 subjects are grouped in blocks of 10 sessions.
Figure 6. Average percent correct as a function of delay and condition (+ 1s).
Figure 7. Average presurgery - postsurgery avoidance difference scores (± 1 s.e.m.).
### Table 1

Table 1. Analysis of variance for presurgical - postsurgical avoidance difference scores.

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<th>p</th>
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<td>.020**</td>
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<td>.151</td>
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postsurgical choice performance for the two groups of subjects, plotted as average percent correct across the twenty sessions at each delay. Open bars represent saline group performance, filled bars represent leupeptin group performance.

Postsurgical percent correct responses were normalized by the probit transformation (log ratio correct/incorrect). A repeated measures analysis of variance on these transformed scores revealed a significant effect of delay only (Delay: $F = 22.195$, $df = 2.46$, $p < .001$). Neither the condition nor the condition by delay interaction were significant (Condition: $F = 1.53$, $df = 1.23$, $p = .229$; Condition by Delay: $F = 0.447$, $df = 2.46$, $p = .642$). On the other hand, pairwise comparisons of the percent correct data using the Mann-Whitney test of significance revealed a significant difference between saline and leupeptin treated rats at the 30 min delay only (mean percent correct leupeptin $= 66.9$, mean percent correct saline $= 77.5$, Mann-Whitney $U = 32$, $p < .01$, one tailed).

Leupeptin's effects on avoidance performance are illustrated in Figure 7, which shows the mean difference between the number of presurgical avoidance responses and postsurgical avoidance responses for the two groups as a function of delay length. Open bars represent the average difference scores for rats receiving saline, filled bars represent the average difference scores for rats receiving leupeptin. Because there was no significant difference in avoidance difference scores between forced- and free-choice trials (Trial, $F=2.206$, $df= 1.23$, $p=.151$), the data depicted are average difference scores across animals and forced and free-choice trials.
The results of an analysis of variance on presurgical-postsurgical avoidance scores (see Table 1) indicate that leupeptin treated rats made significantly fewer avoidance responses after surgery than rats treated with saline (Condition, F=6.262, p<.02). None of the other main effects or interactions was significant.

Discussion

These data are significant for two reasons. First, it is clear that indeed leupeptin will impair memory in an aversively motivated task; this conclusion is significant in light of Staubli et al.'s (1984) conclusion to the contrary. Second, and perhaps more important, these are the first data chronicling the time dependence of a leupeptin-induced behavioral deficit in any task. A time dependent behavioral effect of leupeptin, as illustrated by the results of pairwise comparisons using the Mann-Whitney test of significance, is consistent with what might be predicted from Lynch & Baudry's biochemical theory of memory given the existent data regarding the time course of calcium-stimulated glutamate binding.

The present experiment raises two questions. First, why did leupeptin impair postsurgical avoidance performance? Second, why did leupeptin's effects on choice performance not reflect exactly the time course of the calcium-calpain mechanism? Specifically, why was no deficit observed at the 15 min delay?
Leupeptin's effects on previously acquired avoidance responses is indicative of one of two things. One, leupeptin may be disrupting previously acquired memories, inducing retrograde amnesia. This possibility can almost certainly be ruled out given that saline and leupeptin treated rats behaved so similarly in their choice performance in postsurgical trials with short delays. Further, Lynch & Baudry's theory would predict that leupeptin should not impair previously acquired behavior because presumably the calcium-calcium mechanism has already resulted in an induction of LTP associated with that acquisition. A second possibility is that, among its unknown effects, leupeptin impairs sensory, motivational, or motor processes, all of which could account for the observed impairment of previously learned avoidance responding. In a series of control studies, Staubli et al. (1984) found that leupeptin delivered in concentrations identical to those used in the present experiments did not affect spontaneous activity, rearing, exploration, or food or water intake.

One possible explanation for the failure of leupeptin's temporally dependent effects to reflect exactly the time course of the calcium-calcium mechanism is that the in vivo time course of this mechanism may not be the same as that in vitro. Alternatively, although the putative calcium-calcium memory mechanism may be functionally able to account for (store) memory within 15 min, it cannot logically be the only memory mechanism available to an organism. That is, some other mechanism must account for the fact that organisms are able to remember for periods shorter than 15 min. Thus, although the calcium-calcium mechanism may be functional at shorter times, its use may not be
necessary until some critically long time.
IV. GENERAL DISCUSSION

Three experiments designed to assess leupeptin's effects on averishly motivated spatial memory have been reported. It was found in Experiment One that, given long (one hour) intertrial intervals, leupeptin impairs acquisition of one-way avoidance performance. Experiment Two was conducted to provide basic behavioral data regarding rats' performance in an averishly motivated spatial choice task, and it was found that, although they had no preference with respect to win-stay or win-shift strategies, rats made significantly fewer errors when required to adopt a win-shift strategy. This paradigm was then used in Experiment Three to assess the temporal dependence of leupeptin's effects on memory and the effects of leupeptin on previously acquired avoidance responding. Leupeptin impaired choice performance at the 30 min delay only, while leaving choice performance at shorter delays intact. This finding leads directly to the conclusion that leupeptin produced an impairment of memory. Because subjects were able to respond appropriately at shorter delays, one can argue against a leupeptin-induced impairment of spatial discrimination ability, or a global debilitation produced by some as yet unknown property of the substance.

Leupeptin also impaired postsurgical avoidance responding. It is at the present time unclear why leupeptin disrupted this previously learned response; however, leupeptin's effects on postsurgical avoidance performance has obvious implications for the interpretation of the
results of Experiment One. Because leupeptin impaired this previously
acquired response, and because theoretically it should not impair
previously acquired memory, one must consider the possibility that
leupeptin's effects on one-way avoidance acquisition are due to an
impairment of some aspect of avoidance acquisition other than
between-trial memory. Indeed, one need not, and in fact cannot,
conclude that leupeptin impaired memory for the avoidance contingency in
Experiment One (although a memorial explanation is certainly not ruled
out).

The results of Experiment Three demonstrate convincingly that,
under appropriate conditions, leupeptin will impair averesively motivated
spatial memory in a time dependent fashion. Although the behavioral
effects do not reflect precisely the in vitro time course of the
calcium-calpain mechanism, leupeptin clearly manifests its effects
differentially over time. These results are consistent with Lynch &
Baudry's (1984) biochemical theory of memory formation, and the
suggestion offered by Olton (1973) and others (Black et al., 1977) that
avoidance tasks are a type of spatial task. Given the plethora of data
implicating the hippocampus in spatial memory and in one-way avoidance
performance, and the present data implicating the calcium-calpain
mechanism in averesively motivated spatial memory, one must consider the
possibility that these 'types' of memory share a common chemistry.

Using a double dissociation technique, Staubli, Faraday, & Lynch
(1985) found that leupeptin impaired 'spatial' and 'olfactory' memory
while leaving 'avoidance' memory intact, while anisomycin, a protein
synthesis blocker, impaired 'avoidance' memory but not olfactory memory.
They went on to conclude that different 'types' of memory are regulated by different chemistries. It was subsequently shown that anisomycin, like leupeptin, impairs appetitively motivated memory in a radial arm maze (Mizumori, Rosenzweig, & Bennett, 1985), and that leupeptin, like anisomycin, impairs avoidance memory (Davis & Pico, in press; Morris & Baker, 1984). Thus it appears that, although Staubli et al.'s suggestion regarding the differential involvement of various chemistries in different 'types' of memory may be true, it is not true of the putative calcium-calpain mechanism and its involvement in 'avoidance' versus 'spatial' memory.

While disruption of hippocampal function produces deleterious behavioral effects in spatial tasks, manipulation of other brain areas has been shown to produce similar deficits. For example, lesions to the mammilary bodies impair spatial alternation learning (Greene & Naranjo, 1986; Rosenstock, Field, & Greene, 1977). Similar results are found following lesions to the septum (Herrmann, Poucet, & Ellen, 1985). Chemical lesions to the noradrenergic bundle, a major afferent to the hippocampus, have been shown to produce spatial learning deficits (Mason & Fibinger, 1978), although contrary results have also been found (Pisa & Fibinger, 1983). One must consider the possibility that leupeptin manifests its effects through a disruption of the functioning of one or more of these alternate brain areas, and not through the proposed disruption of the hippocampal calcium-calpain mechanism.
The present experiments raise many questions. Among the most pressing issues for future research is determining leupeptin's effects on previously acquired behavior. If it is found that leupeptin produces a retrograde amnesia, Lynch & Baudry's theory must undergo substantial revision. The nonmemorial effects of leupeptin, such as its possible effect on attentional processes, must be investigated. Also, other pharmacological manipulations designed to interfere with the calcium-calpain mechanism at different points, such as glutamate receptor blockers and other proteinase inhibitors, will have to be tested in both the behavioral and biochemical domains. Interestingly, leupeptin's effects on LTP induction have not yet been determined. This is one of many studies which needs to be done before any conclusions can be reached regarding the role of the calcium-calpain mechanism, leupeptin, and LTP in memory.

Recent evidence indicates that a subacute bout of thiamine deficiency results in an impairment of both appetitively (Mair, Anderson, Langlais, & McEntee, 1985) and aversively (Mair, Otto, Knoth, & Goodness, in preparation) motivated spatial memory. Further, this treatment regimen produces lesions to the intralaminar nuclei of the thalamus (Mair & Langlais, unpublished observations), an area rich in NMDA receptors. These induced lesions are similar to those found in Alcoholic Korsakoff's Syndrome, a degenerative brain disorder induced by thiamine deficiency and characterized by a profound anterograde amnesia. Future studies aimed at uncovering the possible role of the calcium-calpain mechanism in post-thiamine deficiency amnesia would be useful in bringing together these literatures. For example, assaying
brain calpain concentration following thiamine deficiency would indicate whether this chemical system is involved in the observed memory deficits. Further, the effects of thiamine deficiency on the induction of hippocampal LTP would indicate whether hippocampal LTP is involved in the observed memory deficits following such treatment. A failure of thiamine deficiency to disrupt in vivo LTP induction while producing a memory deficit in the same organism would present a problem for many modern theorists. Conversely, a thiamine deficiency-induced blockade of LTP induction would fit nicely into the rapidly emerging overall scheme of the physiology of memory.

A final set of studies which need to be conducted follow from the finding that both hippocampal lesions (Staubli, Ivy, & Lynch, 1984) and intracerebroventricular infusions of leupeptin (Staubli, Baudry, & Lynch, 1985) block olfactory learning set formation, which in rats resembles the acquisition of learning sets for various sensory cues by primates (Nigrosh, Slotnick, & Nevin, 1975). Rats can discriminate electrical stimulation of discrete patches of olfactory bulb and lateral olfactory tract, and these stimuli can be used effectively as discriminative stimuli in a learning set paradigm (Roman, Staubli, & Lynch, 1986). Further, these learned discriminations are strongly correlated with the induction of LTP in the pyriform cortex. A more detailed evaluation of the role of the calcium-calpain mechanism in memory could be pursued by assessing the effect of various pharmacological manipulations, such as infusions of leupeptin or glutamate receptor antagonists, on both learning set formation and LTP induction in the same organism.
The results of the present studies, in particular Experiment Three, are consistent with the calcium-calpain memory mechanism proposed by Lynch & Baudry (1984). Leupeptin, a proteinase (calpain) inhibitor, impaired aversively-motivated spatial memory in a time dependent fashion. These findings are significant because they suggest that 'spatial' and 'avoidance' memory are not truly dichotomous, and may be subserved by the same biochemical mechanism. Further, and perhaps more importantly, the temporal dependence of leupeptin's behavioral effects are strongly suggestive of a memory impairment. While it is clear that more work needs to be done before firm conclusions can be drawn, the present studies support the theory of calpain-induced neural 'changeability' by which memories are 'preserved in a latent state by the nervous apparatus.'
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