[Leu]Enkephalin Enhances Active Avoidance Conditioning in Rats and Mice

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The effects of intraperitoneal (IP) administration of the endogenous opioid peptide, [Leu]enkephalin (LE), on avoidance conditioning in rodents were investigated. At a dose of 30 µg/kg (IP), LE enhanced acquisition of a one-way step-through active avoidance response when administered 2 minutes before training to Swiss Webster mice. [Leu]enkephalin produced a U-shaped dose–response function because both lower and higher doses of LE did not affect avoidance responding. [Leu]enkephalin-induced enhancement of avoidance acquisition was also observed in Sprague-Dawley rats; the intraperitoneal injection of 10 µg/kg LE, administered 5 minutes before training, enhanced acquisition of a

jump-up one-way active avoidance response. When administered to Sprague-Dawley rats immediately after training, LE (30 µg/kg IP) enhanced jump-up avoidance responding at test 24 hours after peptide injection. Previously, we found LE to impair acquisition in the same tasks in both rats and mice, also at microgram doses, and also in a U-shaped manner. Thus, LE can either enhance or impair learning within the same species and the same task; these findings are in agreement with recent theoretical proposals regarding the nature of compounds, such as LE, that modulate learning and memory. [Neuropsychopharmacology 10:53-60, 1994]

In 1981, McGaugh and Martinez designated neurohormones that affect learning and memory as learning modulatory hormones. Since that time, empirical research has determined the defining characteristics of endogenous substances that modulate learning (Martinez et al. 1991a; Koob 1987; Gold 1989; McGaugh 1989). Two of these defining characteristics are that 1) the dose–response functions produced by modulatory substances are U-shaped, rather than hyperbolic, and 2) each modulatory substance has the ability to either

improve or worsen learning and memory (Martinez et al. 1991a; Schulteis et al. 1990; Schulteis and Martinez 1992a; Gold 1989). These characteristics are derived from discovering commonalities among the considerable list of endogenous hormones that influence learned performance in experimental animals.

Opioid peptides, such as β-endorphin, dynorphin, [Met]enkephalin (ME), and [Leu]enkephalin (LE), are endogenous neuropeptides that appear to be learning and memory modulatory substances. For each of these opioid peptides, the characteristic U-shaped dose–effect function has been demonstrated (for examples, see Martinez et al. 1981, 1991a; Schulteis et al. 1988; Colombo et al. 1992; Martinez and Rigter 1980), thus fulfilling the first of the defining characteristics for a modulatory substance.

The second defining characteristic of bidirectional memory effects of modulatory substances is less clear for these opioid peptides. For example, the endogenous opioid peptide LE, when administered to a variety of species in a multitude of tasks, most frequently impairs learning (Rigter et al. 1980a, 1980b, 1981; Izquierdo et

al. 1980; Izquierdo and Dias 1981; Martinez et al. 1981, 1984, 1985a, 1985b, 1988, 1990; Martinez and Rigter 1982; Introini et al. 1985; Dana and Martinez 1986; Linden and Martinez 1986; Schulteis et al. 1988, 1990; Patterson et al. 1989; Janak and Martinez 1990; Sandi et al. 1990; Schulteis and Martinez 1992b). [Leu]enkephalin impairs active avoidance acquisition in mice (Martinez et al. 1985b; Dana and Martinez 1986; Schulteis et al. 1988; Janak and Martinez 1990) and in rats (Rigter et al. 1980a, 1980b, 1981; Martinez and Rigter 1982; Martinez et al. 1985a). [Leu]enkephalin also impairs retention of active avoidance conditioning in mice (Dana and Martinez 1986; Schulteis and Martinez 1992b) and rats (Izquierdo et al. 1980; Izquierdo and Dias 1981).

In contrast, some studies report that LE enhances learning and memory. [Leu]enkephalin attenuates CO_2-induced amnesia for a passive avoidance response in rats (Rigter 1978) and enhances retention of a passive avoidance response in rats (Martinez et al. 1981). Thus, a small number of studies suggest that LE enhances learning and memory in animals.

As with LE, the most frequently observed effect of exogenous administration of other opioid peptides is learning and memory impairment (see Schulteis et al. 1990; Martinez et al. 1991a for review), although a few cases of enhancement were observed. For example, although ME is reported to impair acquisition (Rigter et al. 1980a) and retention (Introini et al. 1985; Izquierdo and Dias 1981) of avoidance conditioning in rats and mice, other reports indicate that ME enhances acquisition of Y-maze (Martinez et al. 1984) and complex maze (Kastin et al. 1976) learning and delays extinction of a pole-jump response (de Wied et al. 1978) in rats. [Met]enkephalin also enhances retention of and attenuates CO_2-induced amnesia for passive avoidance conditioning in rats (Martinez et al. 1981; Rigter 1978).

Because the methodologies (task and injection route; strain and species used) varied between these studies of the learning and memory effects of enkephalins, it is difficult to directly compare the results. Thus, the evidence indicates that some enkephalins either enhance or impair learned behavior; however, it has yet to be demonstrated that enkephalins are capable of producing enhancement and impairment under the same conditioning parameters. The following studies demonstrate that the pre- or posttraining intraperitoneal injection of LE enhances active avoidance conditioning in two separate species; in each case, the task parameters were identical to those in which we have previously demonstrated LE-induced learning impairment (Janak and Martinez 1990; Weinberger et al. 1989). This demonstration supports current views that the behavioral effect of an exogenously administered opioid may depend upon the basal state of the subject, rather than task parameters alone (Martinez et al. 1991a; Schulteis and Martinez 1992a).

GENERAL METHODS

Subjects

Food and water were available ad lib; the animals were maintained on a standard 12-hour light/12-hour dark schedule with lights on at 7 A.M. Newly arrived animals were allowed at least 4 days to acclimate before any experimental procedures were conducted. Housing conditions were in accord with National Institutes of Health (NIH) guidelines, and all experimental procedures were approved in advance by the Animal Care and Use Committee at the University of California, Berkeley.

Drugs

[Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu), obtained from Bachem (Torrance, CA), was dissolved in 0.9% saline and administered via intraperitoneal injection in a volume of 1 ml/100 g for mice and 1 ml/kg for rats. The experimenter was unaware of the specific treatment a subject received until testing was concluded.

Procedures

The subjects were brought into the testing room a minimum of 3 hours before conditioning began. All experimental procedures occurred between the hours of 11 AM and 4 PM. A tensor lamp provided dim lighting, and a fan provided background noise.

Data Analysis

Planned single degree-of-freedom comparisons between the treatment (LE-injected) and control (vehicle-injected) groups were conducted. To control for Type I error, the Bonferroni correction method was applied to adjust the alpha level for each experiment (Keppel 1991); family-wise error was set to .10. Because a limited number of animals may be used in each day's study, completion of a full dose-response curve required combining data across consecutive experimental days. Because a saline-injected control group was included in each experimental session, pooling of the saline-treated subjects created a larger sample size in the saline-treated groups than in the drug-treated groups.
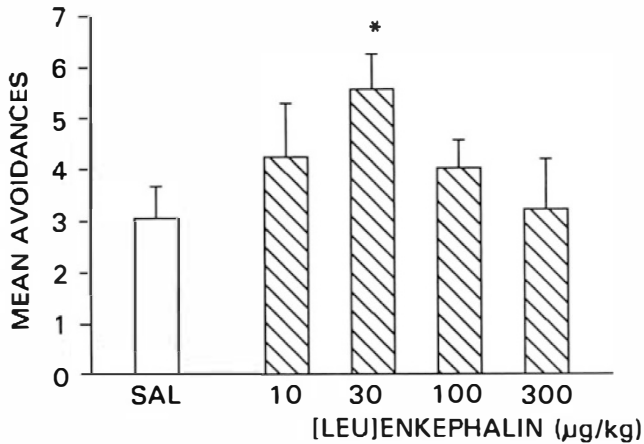
Figure 1. Effect of pretraining LE administration on active avoidance acquisition in Swiss Webster mice. [Leu]enkephalin (30 µg/kg) enhanced the mean number of avoidance responses made in 14 trials. Sample sizes are as follows: saline, $n = 19$; 10-µg/kg LE, $n = 9$; 30-µg/kg LE, $n = 13$; 100-µg/kg LE, $n = 19$; 300-µg/kg, $n = 5$. *$p < .05$.

EXPERIMENT 1

This experiment investigated the effect of pretraining LE administration on one-way step-through active avoidance conditioning in mice.

Methods

Subjects. Male Swiss Webster mice, weighing between 29 and 36 g, were obtained from Benton-Kingman and were housed three to a cage.

Apparatus and Procedure. The avoidance chamber consisted of two trough-shaped compartments: a smaller well-lit compartment ($10 \times 10 \times 10$ cm), made of white acrylic, separated by a black acrylic sliding door from a metal-floored shock compartment. The shock compartment ($23 \times 10 \times 10$ cm) was constructed of black acrylic, except for the metal floor. Peptide (10, 30, 100, or 300 µg/kg) or saline was administered 2 minutes prior to avoidance training. On the first trial, animals were placed in the shock compartment facing away from the closed entrance to the lit compartment. After 10 seconds, the doorway separating the two compartments was opened and the animals received a mild footshock (390 µA) and were allowed to escape into the lit compartment. On all subsequent trials, the animals were placed facing away from the lit compartment and allowed 10 seconds within which they could successfully avoid footshock by crossing through the open doorway into the smaller lit compartment. Failure to avoid resulted in application of footshock. All mice that did

not escape the footshock within 20 seconds were placed into the smaller lit compartment by the experimenter. Mice received a total of 14 trials with 20-second intertrial intervals. The number of avoidances made by each animal was recorded and used to measure performance. All subjects completed training and were included in the final analysis of the data.

Results

Pretraining LE enhanced acquisition of one-way active avoidance responding in mice. As seen in Figure 1, a 30-µg/kg dose of LE significantly enhanced acquisition of the active avoidance task in mice as compared with saline-treated control animals ($F[1,30] = 6.94$, $p < .02$), whereas mice receiving doses of 10 ($F[1,26] = 1.01$, $p = .33$), 100 ($F[1,36] = 1.30$, $p = .26$), and 300 µg/kg LE ($F[1,22] = .01$, $p = .91$) did not differ from saline control animals. The dose–response function produced is an inverted U shape, as indicated by a significant quadratic trend component: ($F[1,60] = 6.28$, $p < .02$) (Keppel 1991).

EXPERIMENT 2

This experiment investigated the effects of pretraining LE administration on acquisition of active avoidance responding in rats. The task used differs from the step-through active avoidance task in that a distinctive conditioned stimulus (CS) (houselight) signals the unconditioned stimulus (US) (footshock), and jumping, rather than forward locomotion, is required for shock escape or avoidance. In addition, the jump-up avoidance apparatus is automated, thus eliminating the need for intertrial experimenter handling, as required by the step-through one-way avoidance apparatus.

Methods

Subjects. Male Sprague-Dawley rats, weighing between 260 and 300 g, were obtained from Simonsen and were singly housed.

Apparatus and Procedure. The shelf-jump conditioning chamber (Lafayette Instruments, Lafayette, IN) was a 23- × 21-cm rectangle with two opposing lucite walls and a third metal wall (all 21 cm tall). The fourth wall contained a rectangular metal compartment, that, when open, was recessed 12.5 cm from the chamber proper, 21 cm in length, and elevated 9 cm from the floor of the chamber. When the shelf was in the closed position, the back wall of the platform was brought flush with the two side lucite walls, appearing then as a con-
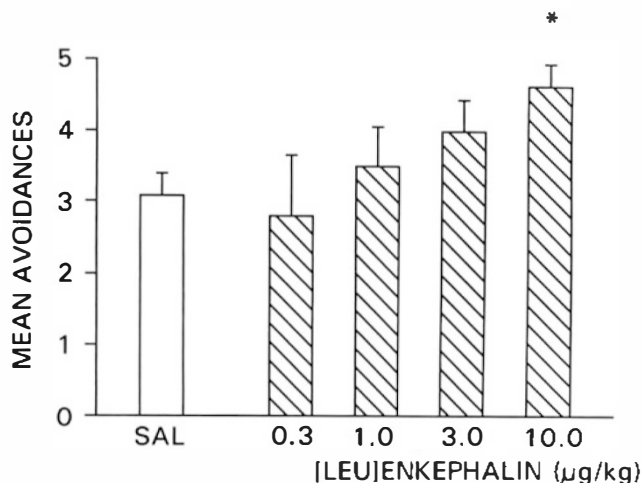
Figure 2. Effect of pretraining LE administration on active avoidance acquisition in Sprague-Dawley rats. [Leu]enkephalin (10 µg/kg) enhanced the mean number of avoidance responses made in eight trials. Sample sizes are as follows: saline, $n = 13$; 0.3-µg/kg LE, $n = 5$; 1.0-µg/kg LE, $n = 6$; 3.0-µg/kg LE, $n = 7$; 10-µg/kg, $n = 8$. *$p < .01$.

tinuous metal wall. The floor of the chamber consisted of a steel-rod grid floor, through which a constant current footshock was delivered. A houselight was mounted 15 cm from the floor on the metal wall opposite the shelf. A pressure-sensitive switch under the platform registered jump-up responses. All equipment was controlled by an Apple IIe computer (Apple Computers, Cupertino, CA) interfaced with solid state control equipment (Lafayette Instruments).

Peptide (.3, 1, 3, or 10 µg/kg) or saline was administered 5 minutes before the first acquisition trial. On trial one, each rat was placed on the floor of the chamber, with the shelf in the closed position. Ten seconds after the initiation of the trial, three events occurred simultaneously: 1) the back wall of the platform withdrew, exposing the platform; 2) the CS (houselight) came on; 3) and a footshock of 290 µA was delivered to the floor of the chamber. The footshock (US) and CS remained on for 30 seconds or until the subject escaped by jumping up onto the exposed platform. Following a 30-second intertrial interval, the back wall of the platform moved forward into the "closed" position, gently depositing the rat onto the steel-rod floor and initiating the next trial. For the following 7 trials, the platform was exposed and the CS came on 10 seconds prior to footshock (US) onset; therefore, the subject could avoid footshock by jumping onto the exposed platform prior to shock initiation. Maximum trial length was 40 seconds. Subjects that did not escape the footshock for two or more trials were excluded from the study (5%); exclusion was uniform across treatment groups.

Results

[Leu]enkephalin administration enhanced acquisition of jump-up avoidance conditioning. As illustrated in Figure 2, the pretraining administration of 10 µg/kg LE to rats significantly enhanced acquisition of jump-up avoidance responding, as indicated by a higher mean number of avoidance responses relative to saline-treated control rats ($F[1, 19] = 10.84$, $p < .004$). Pretraining administration of 0.3, 1.0, and 3.0 µg/kg LE did not affect acquisition (0.3 µg/kg LE: $F[1,16] = .15$, $p = .71$; 1.0 µg/kg LE: $F[1,17] = .51$, $p = .49$; 3.0 µg/kg LE: $F[1,18] = 3.04$, $p = .10$).

EXPERIMENT 3

The previous two experiments demonstrated that the pretraining injection of LE enhances avoidance response acquisition. This experiment investigated the effects of posttraining LE injection on jump-up avoidance responding in a 3-day paradigm in rats.

Methods

Subjects. Male Sprague-Dawley rats, weighing between 230 and 280 g, were obtained from Harlan Sprague-Dawley and were singly housed.

Apparatus and Procedure. The apparatus was the same as that used in Experiment 2. Conditioning consisted of three consecutive daily sessions. On day 1, each rat was given one escape-only trial in which the CS (houselight) onset and footshock (US) (290 µA) onset were paired for 30 seconds or until the subject jumped up onto the platform, thus terminating the CS and the shock. Each subject was then removed from the apparatus, injected with LE (3 or 30 µg/kg) or vehicle, and replaced into its home cage. The procedure on day 2 was exactly the same as on day 1. On day 3, each rat received 12 trials in which the shelf was exposed 10 seconds prior to footshock onset, allowing the subject to either avoid or escape the shock, as described in Experiment 2. Subjects that did not learn the escape response on day 1 or 2 were excluded from the study (10%); exclusion was uniform across treatment groups.

Results

The results indicated that 2 days of posttraining LE administration enhanced acquisition of the jump-up avoidance response, as measured by avoidance performance on day 3. Analysis of the data revealed that 30 µg/kg LE ($F[1,22] = 8.01$, $p < .01$), but not 3 µg/kg LE ($F[1,21] = .32$, $p = .58$) enhanced performance on day 3, as evi-
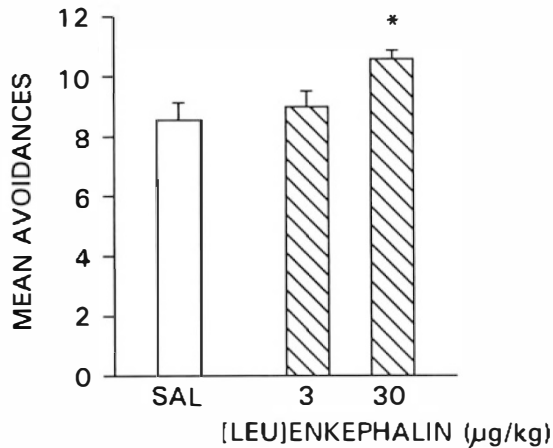
Figure 3. Effect of posttraining LE administration on active avoidance retention in Sprague-Dawley rats. [Leu]enkephalin (30 µg/kg) enhanced the mean number of avoidance responses made in the 12-trial retention test. Samples sizes are as follows: saline, n = 13; 3.0-µg/kg LE, n = 10; 30-µg/kg, n = 11. $*p < .01$.

denced by a higher mean number of avoidance responses when compared to saline-treated control animals. These data are illustrated in Figure 3.

DISCUSSION

In the present study, LE dose dependently enhanced one-way active avoidance acquisition in Swiss Webster mice when administered prior to training (Experiment 1). These results were extended to rats. In Experiment 2, we found that the pretraining administration of LE to Sprague-Dawley rats enhanced acquisition of jump-up active avoidance conditioning. In addition, the posttraining administration of LE to Sprague-Dawley rats enhanced retention of jump-up active avoidance conditioning (Experiment 3). The only previously reported instances of learning enhancement produced by LE were found with passive avoidance conditioning; the present report represents three instances of LE-induced learning enhancement in active avoidance tasks. Because extensive previous study demonstrated that LE most frequently impairs learning and memory when administered either pretraining or posttraining to both rats (Rigter et al. 1980a, 1980b, 1981; Martinez and Rigter 1982; Martinez et al. 1985a) and mice (Martinez et al. 1985b; Dana and Martinez 1986; Schulteis et al. 1988; Janak and Martinez 1990), these results show that LE can both enhance and impair acquisition of active avoidance conditioning. Importantly, the enhancement of avoidance responding produced by LE in this study was

observed under task conditions in which LE usually produces impaired performance. These results therefore demonstrate that it is not the nature of the behavioral task that determines the direction of the effect of this peptide, but perhaps other variables such as the basal stress levels of subjects play a role in determining the behavioral effect of LE.

The learning and memory enhancement produced by LE in the present study is similar in two respects to the LE-induced learning impairment that we have observed previously in these same tasks. First, the dose range for both enhancement and impairment is similar. For example, in the mouse, a 30-µg/kg dose of LE enhanced acquisition (Experiment 1), whereas this same dose impaired acquisition in previous studies (Schulteis et al. 1988; Schulteis and Martinez 1990). Likewise, in the rat, we found that pretraining administration of a 10-µg/kg dose of LE enhanced acquisition of a jump-up avoidance response (Experiment 2), whereas in a previous study, pretraining administration of 1 or 3 µg/kg LE (Weinberger et al. 1989), or 10 µg/kg LE (Martinez and Rigter 1982; Rigter et al. 1981), impaired jump-up avoidance learning. The dose of LE (30 µg/kg) that enhanced jump-up avoidance learning following its posttraining administration to rats in the present study is also within the 1- to 100-µg/kg dose range. These results reveal that the direction of LE's avoidance conditioning effects cannot be accounted for simply on the basis of differing doses.

The second similarity between LE-produced learning enhancement and impairment is that the dose-response functions produced in both cases are U shaped. Thus, we found that a dose of 30 µg/kg LE enhanced acquisition in mice, whereas doses both lower and higher had no effect. This quadratic-shaped dose-response function, although opposite in direction, also is produced in experiments in which LE impairs avoidance conditioning (Martinez et al. 1988; Schulteis et al. 1988). U-shaped, dose-response curves commonly are produced by treatments that modulate learning and memory, whether they are enhancing or impairing (see Martinez et al. 1991a; Schulteis and Martinez 1992a for discussion of U-shaped curves and memory). Therefore, at least in mice, the conditioning enhancement and impairment produced by LE are similar to one another. The LE dose-response functions obtained in rats in the present study were not extended beyond the doses found to significantly affect avoidance responding and therefore cannot lend support to these results obtained in mice.

Our results are similar to the effects of the endogenous opioid peptides ME and β-endorphin on learning. Although some studies report impairment of aversive conditioning (Martinez and Rigter 1980, 1982; Izquierdo et al. 1980; Zhang et al. 1987), others report

enhancement (de Wied et al. 1978; Kovacs et al. 1981; Kovacs and de Wied 1981; Martinez et al. 1984). None of these studies in which β-endorphin or ME enhanced learning and memory are easily compared to those studies in which the peptides produced impairment, as the studies did not use identical training and testing procedures. For example, β-endorphin impaired retention of an inhibitory avoidance response when administered intraperitoneally to rats in a study by Martinez and Rigter (1980), whereas subcutaneous administration of the same peptide enhanced rats' retention for an inhibitory avoidance response in a study by Kovacs and de Wied (1981). It is notable that the studies reported here were conducted identically to previous studies in which we found LE to impair learning and memory.

What factors might have contributed to the results obtained in the present study? These results are not due to circadian or seasonal effects, because the time of day and year that testing occurred is similar to that of studies in which LE-induced impairment was observed previously by us in both rats and mice. In addition, the results of additional studies conducted by the experimenters indicate that the present results cannot be attributed to differences in experimenter handling. Indeed, the automated shelf-jump avoidance apparatus used for Experiments 1 and 2 minimized experimenter handling.

However, although the avoidance acquisition enhancement observed in Experiment 1 was conducted in Swiss Webster mice, the strain of mice that we commonly use, the subjects came from a different commercial breeder than used by us in previous studies. In fact, we observed that the saline-treated mice included in Experiment 1 performed more poorly than saline-treated mice from some other breeders. Factors that lead to opposite effects in the same strain, but different suppliers, are not known at this time. The albino rat subjects used for Experiments 2 and 3 were obtained from the same breeders we commonly use; however, the subjects were housed and the experiments were conducted in a different building than used by us in previous studies. How different buildings contribute to opposite pharmacologic effects is unknown. However, when compared to saline-treated subjects in our previous study of opioid effects on jump-up avoidance acquisition (Weinberger et al. 1989), the saline-treated rats from Experiment 2 performed more poorly, suggesting that basal arousal levels of rats in the present studies may have been affected by the housing environment.

The present study thus suggests that determination of the overall direction of effect of a given pharmacologic treatment is complex. Previous research with the classic learning and memory modulatory treatments, adrenocorticotrophic hormone (ACTH) and epinephrine, specified variables that may impact the direction of the effect of pharmacologic treatments on learning

and memory. In the studies of Gold and van Buskirk (1975, 1976), either enhancement or impairment of retention of an inhibitory avoidance response could be obtained with the same hormone by manipulating the dose and keeping the footshock level constant or by manipulating the footshock level and keeping the dose constant. Similar results were obtained following the posttraining administration of dynorphin$_{1-17}$ to rats: dynorphin enhanced retention of passive avoidance conditioning when subjects were trained with weak footshock, and dynorphin impaired retention of passive avoidance conditioning when subjects were trained with strong footshock (del Cerro and Berrell 1990). These studies indicate that a hormone usually released as a consequence of arousal or stress also may modulate learning. In addition, these studies indicate that factors that influence arousal levels in animals may have an impact on the conditioning effects of exogenously administered stress hormones, such that the influence of a particular hormone on memory formation could vary depending on experimental conditions that alter the concentration of the hormone available for receptor action (Schulteis and Martinez 1992a; Martinez et al. 1991a, 1991b; McCarty and Gold 1981; Gold 1989; McGaugh 1989).

[Leu]enkephalin is similar to ACTH and epinephrine in that it is a hormonelike substance that is released into the circulation in response to arousal or stress (DiGiulio et al. 1978; Hanbauer et al. 1982; Hexum et al. 1980; Schultzberg et al. 1978), and it alters learned performance. Thus, as with ACTH and epinephrine, factors that influence the arousal of experimental subjects may alter the effects of exogenous administration of LE. Factors that were not explicitly manipulated in our studies, such as the commercial supplier and the housing environment, could have been important determinants of stress levels in our subjects. Thus, one possibility is that the basal arousal state (and, thus, the basal levels of endogenous circulating LE) of our subjects helped determine the direction of effect of LE on avoidance conditioning. Interestingly, direct evidence that stress affects memory via an opioid-dependent mechanism is provided by the finding that posttraining restraint stress strain-specifically alters retention of passive avoidance conditioning in mice, and these effects are reversed by naloxone (Castellano and Puglisi-Allegra 1983).

The present results in which the endogenous opioid peptide LE enhances learning, together with the more typical previous results in which LE impairs learning, provide evidence that fulfills both defining characteristics of a learning modulatory substance, as first described by McGaugh and Martinez (1981) and later extended (Martinez et al. 1991a; Schulteis and Martinez 1992a). Thus, LE can either enhance or impair learning and memory, independent of task specifics such as

the response requirement or the route of peptide administration, and the function produced by LE administration is U shaped, regardless of whether the peptide is enhancing or impairing behavior. Therefore, LE provides a good example of learning and memory modulation by endogenous neuropeptides.

ACKNOWLEDGMENT

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