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RAPID COMMUNICATION

[Leu]Enkephalin and Its Metabolite, Tyr-Gly-Gly, Impair Active Avoidance Retention

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SCHULTEIS, G. AND J. L. MARTINEZ, JR. *[Leu]Enkephalin and its metabolite, Tyr-Gly-Gly, impair active avoidance retention.* PHARMACOL BIOCHEM BEHAV **42**(3) 523–527, 1992.—The current study examined the effects of [leu]enkephalin and its metabolite, Tyr-Gly-Gly, given immediately posttraining on active avoidance performance measured 24 h later. Initial experiments revealed that, in comparison to zero or one training trials, providing mice with two training trials significantly increased active avoidance performance measured 24 h later; this enabled us to examine the effects on retention of peptides administered immediately after the two training trials. It was found that Tyr-Gly-Gly (16 and 53 μg/kg) and [leu]enkephalin (30 and 100 μg/kg) administered in this fashion both significantly impaired retention; the dose–response functions for both peptides were U-shaped. Since the effects of enkephalins are most likely mediated by opioid δ-receptors, and Tyr-Gly-Gly has little or no activity at opioid receptors, the effects of the parent peptide(s) and metabolite are presumably pharmacologically distinct.

[Leu]enkephalin Opioid peptides Tyr-Gly-Gly Enkephalin fragments Posttraining administration Active avoidance conditioning Retention Mice

THE enkephalins are known to affect the acquisition and retention of a variety of conditioned responses in a number of species, including rats, mice, monkeys, and chicks (3,6,7,8, 12,13,17–23,26,28,35,36). As suggested by de Wied and colleagues (1,2), it is possible that peptides may act as precursors to metabolic fragments that have behavioral activity of their own. Izquierdo and Dias (7) reported that des-tyr-[met]enkephalin impaired retention of two-way shuttle avoidance conditioning. Thus, this fragment of [met]enkephalin, which is produced by aminopeptidase M activity in the plasma of mice and rats (30–32), produces an effect on memory that is identical to that produced by the parent peptide. Izquierdo and Dias (7) suggested that this effect of des-tyr-[met]enkephalin was mediated by opioid receptors. While substantial evidence now suggests that des-tyr-[met]enkephalin and other enkephalin metabolites do not possess opioid activity as measured in standard guinea pig ileum or mouse vas deferens bioassays (4), and therefore probably do not modulate learning and memory through activation of opioid receptors, the study of Izquierdo and Dias (7) is important because it provided the first direct evidence that a major enkephalin metabolite could affect conditioning.

More recently, a number of metabolic fragments of [met]- and [leu]enkephalin have been examined for their ability to modulate active avoidance conditioning. In vitro studies of the hydrolysis of enkephalins suggests that tyrosine (Tyr), tyrosylglycine (Tyr-Gly), tyrosylglycylglycine (Tyr-Gly-Gly), des-tyr-[leu]- and des-tyr-[met]enkephalin, glycylphenylalanine, and glycylphenylalanyl-leucine (or methionine) are all products of enkephalin metabolism in mouse and rat plasma in vitro (30–32,37). Furthermore, membrane bound aminopeptidases and dipeptidylcarboxypeptidases also contribute to the formation of these products in vivo from circulating enkephalins (9,29). Of these enkephalin fragments, des-tyr-[leu]enkephalin and Tyr do not influence one-way active avoidance acquisition in mice (8,17,36), and des-tyr-[leu]enkephalin is also without effect in an appetitive task (12). However, both Tyr-Gly and Tyr-Gly-Gly impair acquisition of an active

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avoidance response when systemically administered to mice prior to training (8,17,36). Neither of these peptides alter shock-induced locomotor activity as measured in an open field, suggesting that their effects on avoidance learning are not due to indirect performance effects. While Tyr-Gly-Gly, along with Tyr, is one of two primary metabolic products of [leu]- and [met]enkephalin hydrolysis in mouse plasma, Tyr-Gly is produced in only minute amounts (31,32).

To summarize, several studies reported that pretraining administration of Tyr-Gly-Gly, a major product of enkephalin hydrolysis in mouse plasma, impairs avoidance acquisition following its intraperitoneal administration to mice (8,17,36). This effect mimics that of the parent peptides, [met]- and [leu]enkephalin, in this same training paradigm. The current study sought to determine whether posttraining administration of Tyr-Gly-Gly and one of its parent peptides, [leu]enkephalin, could influence retention of active avoidance performance tested 24 h after training.

METHOD

All procedures were approved in advance by the Animal Care and Use Committee of the University of California at Berkeley, in accordance with NIH guidelines.

Subjects

Male Swiss Webster mice (Harlan-Sprague-Dawley, Indianapolis, IN) weighing between 24–32 g served as subjects (n = 152). Upon arrival from Harlan, animals were housed three to a standard laboratory cage; food and water were available at all times. Mice were acclimated to laboratory conditions, including a standard light/dark cycle (12 L:12 D, lights on at 0800), for 3–6 days before treatment began. Twenty-four hours prior to the start of an experiment, mice were weighed and their tails marked with indelible red ink for identification. At least 2 h before training, mice were placed in a darkened, sound-attenuated testing room with an electric fan to provide background masking noise; after training, mice remained in the testing room until retention testing the following day. All testing occurred between 1100 and 1730.

Drugs

All drugs were dissolved in saline in polyethylene vials 30–45 min prior to use and blind coded. IP injections were made with disposable plastic syringes in a volume of 1.0 ml/100 g body weight. Mice were injected immediately after training and returned to their home cages until retention testing on the following day. [Leu]enkephalin was obtained from Bachem (Torrance, CA), and tyrosylglycylglycine (Tyr-Gly-Gly) was purchased from Sigma (St. Louis, MO).

Active Avoidance Paradigm

The apparatus and procedure are described in detail elsewhere (14,26). Briefly, the one-way active avoidance apparatus consisted of a trough-shaped alley with two chambers separated by a sliding door. The larger, darkened shock chamber had metal floor plates through which foot-shock could be delivered; the smaller, white compartment had Plexiglas sides and floor and was illuminated by a Dazor lamp (14 W) set at high intensity. To start each trial, mice were placed in the dark chamber facing away from the door. In Experiment 1, training consisted of one, two, or three trials, separated by an intertrial interval of 20 s. A control group that received no training on day 1 was included in this experiment. In Experiment 2, all mice received two training trials on day 1. On day 1, mice were placed in the shock compartment while the door to the safe compartment remained closed; after 10 s, the door was opened and a foot-shock (Lafayette Instrument Co. shocker, Model 5226) of 280-μA rms intensity was simultaneously delivered. On day 2, mice in Experiments 1 and 2 received 10 additional trials that differed from those on day 1 in that the door was opened as soon as subjects were placed in the shock compartment and animals were given 10 s to move into the safe compartment. Failure to cross within the 10-s period resulted in the administration of a foot-shock that could be terminated by escape into the safe compartment. If a mouse failed to escape after 20 s of foot-shock delivery, then the shock was terminated and the subject gently moved into the safe compartment by the experimenter. The latency to avoid or escape on each trial was recorded, and the total number of avoidances made during retention testing (maximum possible score of 10) served as the measure of retention.

RESULTS

Experiment 1: Effects of 0,1,2, or 3 Training Trials on Day 1 on Day 2 Avoidance Performance

Data analysis for this experiment consisted of an overall one-way analysis of variance (ANOVA) followed by all possible simple comparisons between means using the Bonferroni correction for multiple planned comparisons (10). The overall ANOVA revealed a significant effect of number of day 1 training trials on day 2 avoidance performance, $F(3, 51) = 5.54$, $p < 0.005$. Individual comparisons revealed that animals receiving two ($\bar{X} = 3.86 \pm 0.42$) or three ($\bar{X} = 3.92 \pm 0.54$) training trials made significantly more avoidances on day 2 than mice receiving either zero ($\bar{X} = 2.00 \pm 0.43$) or one ($\bar{X} = 2.00 \pm 0.48$) training trial (all ps < 0.05). Mice receiving zero training trials did not differ in their performance on day 2 from mice receiving one training trial; mice receiving two training trials also did not differ from mice receiving three pretraining trials. These results indicate that two training trials leads to significant learning that can be measured as enhanced avoidance performance on day 2 as compared to administration of zero or one training trials. Therefore, it was decided that posttraining drug effects would be examined with a paradigm using two day 1 training trials.

Experiment 2: Effects of [Leu]Enkephalin and Tyr-Gly-Gly Given Posttraining on One-Way Active Avoidance Retention

Data analysis for this experiment consisted of overall one-way ANOVAs followed by planned comparisons of each peptide-treated group to its appropriate saline-treated control group using the Student's t-test (all p values reported as two tailed). Keppel (10) maintains that if the number of planned comparisons to be made does not exceed the degrees of freedom associated with the between-groups mean square of an overall ANOVA (three in this case) then no correction for familywise error need be made. The results of this experiment are presented graphically in Figs. 1 and 2.

The overall ANOVA performed on the [leu]enkephalin data (see Fig. 1) revealed a significant effect of this peptide on day 2 avoidance performance, $F(3, 46) = 5.90$, $p < 0.002$. When compared to saline treatment ($\bar{X} = 3.67 \pm 0.37$), [leu]enkephalin given immediately after the day 1 training trials at doses of 30 μg/kg ($\bar{X} = 1.50 \pm 0.76$) and 100 μg/kg ($\bar{X} = 1.17 \pm 0.35$) significantly impaired avoidance performance on day 2 [$t(24) = 2.90$, $p < 0.01$, and $t(28) = 4.67$,

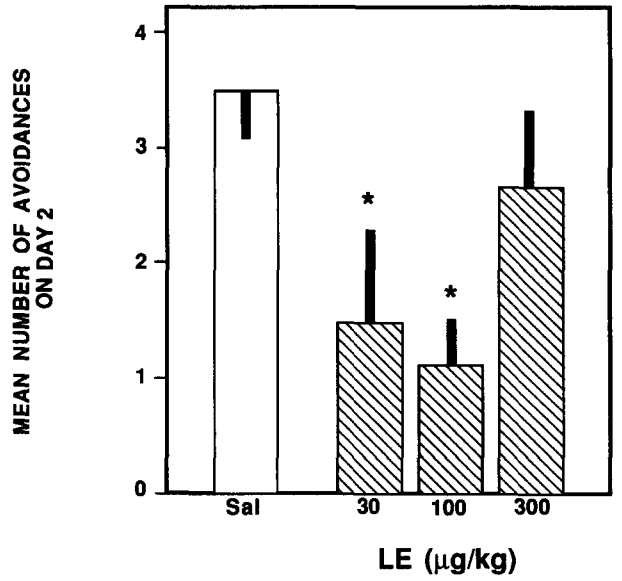

FIG. 1. U-shaped dose-response function for the impairment of one-way active avoidance retention produced by [leu]enkephalin (LE). n = 18 for the saline (Sal) group and n = 8-12 for all LE groups. *$p < 0.05$ vs. Sal.

$p < 0.0001$, respectively]. The effect of [leu]enkephalin was U-shaped, as animals treated with 300 μg/kg [leu]enkephalin ($\bar{X}$ = 2.75 ± 0.63) were not significantly different from animals receiving saline treatment, $t(28) = 1.34, p > 0.19$.

As shown in Fig. 2, Tyr-Gly-Gly also had a significant effect on day 2 avoidance performance, $F(3, 44) = 3.73$, $p < 0.02$. Individual comparisons revealed that animals treated with Tyr-Gly-Gly at doses of 16 μg/kg ($\bar{X}$ = 1.39 ± 0.39) or 53 μg/kg ($\bar{X}$ = 1.50 ± 0.42) differed significantly in their avoidance performance on day 2 from saline-treated mice ($\bar{X}$ = 2.75 ± 0.25)[$t(23) = 2.92$, $p < 0.01$, and $t(22) = 2.57$, $p < 0.02$, respectively]. The dose-response function was again U-shaped, as mice injected with a 160-μg/kg dose ($\bar{X}$ = 2.64 ± 0.43) of Tyr-Gly-Gly did not differ significantly from saline controls, $t(21) = 0.23$, $p > 0.80$.

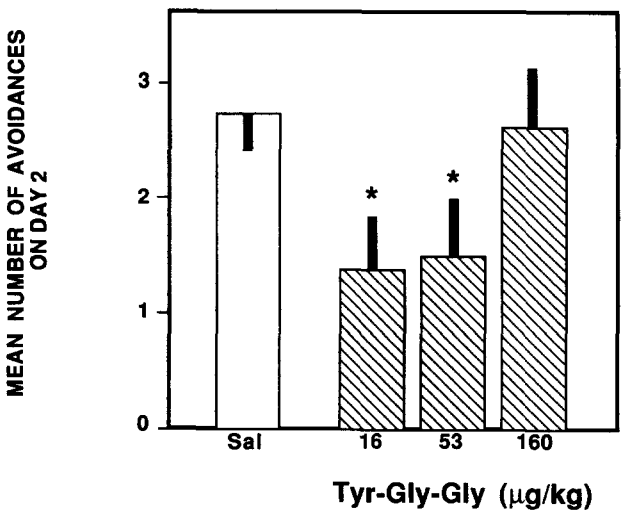

FIG. 2. U-shaped dose-response function for the impairment of one-way active avoidance retention produced by Tyr-Gly-Gly. n = 12 for the saline (Sal) group and n = 11-13 for all Tyr-Gly-Gly groups. *$p < 0.05$ vs. Sal.

DISCUSSION

It was reported previously that the enkephalin metabolite Try-Gly-Gly, like its parent peptides [met]- and [leu]enkephalin, impaired active avoidance acquisition following pretraining, systemic administration (8,17,36). The present study extended these findings in demonstrating that [leu]enkephalin and Tyr-Gly-Gly also can impair one-way active avoidance performance when given immediately after training. The doses of Tyr-Gly-Gly given posttraining on day 1 that impaired day 2 avoidance performance are equimolar to the doses of [leu]enkephalin that produced the same effect. Furthermore, the doses of both peptides are equal to those previously reported to impair one-way active avoidance acquisition in mice after pretraining administration (8,26,28,36).

The dose-response functions for both Tyr-Gly-Gly and [leu]enkephalin in the present study were U-shaped; this is in agreement with the effects of these peptides on avoidance acquisition (8,26,28,36). As we argued previously [(16,25); Schulteis and Martinez, submitted], a U-shaped dose-response function suggests that a drug effect on learning and memory processes is mediated by an interaction with modulatory systems and not a direct interaction with the memory trace per se. Modulatory systems provide inputs to the memory trace that are capable of either increasing or decreasing the strength of the trace. As formulated by Martinez et al. (15), the operational definition of learning and memory modulation states that whether enhancement or impairment of learning and memory is observed following an experimental treatment (such as administration of a peptide) is dependent upon the strength of the experimental treatment (i.e., dose administered), the strength of training, and their interaction [see also (25)]. U-shaped dose-response functions provide one piece of experimental evidence for a modulatory effect as defined in this fashion. Thus, we maintain that the effects of both [leu]enkephalin and Tyr-Gly-Gly reported herein involve modulation of memory storage processes.

What contribution, if any, do the effects of enkephalin fragments make to the effects of the parent peptides on avoidance conditioning? Considerable evidence supports the notion that intact enkephalins modulate learning and memory. For example, the effects of [leu]enkephalin on active avoidance conditioning in mice (26) and on peck avoidance conditioning in chicks (21) are reversed by the δ opioid receptor antagonist ICI 174,864, suggesting the effects of [leu]enkephalin are mediated through δ-receptors. Since enkephalin metabolites have little or no opioid receptor affinity (4), the reversal of [leu]enkephalin's effect by ICI 174,864 argues against the possibility that the conditioning effects of the parent peptide are mediated solely through production of behaviorally active metabolites.

Further evidence for the importance of the intact parent compound comes from the finding that metabolically more stable analogs of enkephalins such as D-Pen^2-[D-Pen^5]enkephalin (DPDPE) and D-Pen^2-[L-Pen^5]enkephalin (DPLPE) (21,26,35) and DADLE (22,23), which share opioid agonist properties and affinity for the δ-receptor with the enkephalins, also share the enkephalins' modulatory actions on avoidance conditioning.

Taken together, these results suggest that intact enkepha-

lins are capable of modulating learning and memory processes through an action on opioid δ-receptors, and that enkephalin fragments are likely exerting their effects on conditioning through some mechanism other than direct binding and activation of opioid receptors. An understanding of the nature of this mechanism awaits further research, although, as discussed elsewhere (8), it is possible the enkephalin fragments may act on opioid systems, even if they do not act directly at opioid receptors. For example, the peptides kentsin (Thr-Pro-Arg-Lys) and kyotorphin (Tyr-Arg) produce naloxone-reversible analgesia, apparently without binding to opioid receptors directly, and it is hypothesized that this is due to their ability to release endogenous enkephalins (5,24,33). A second possible indirect mechanism of enkephalin fragment actions on opioid systems is through competition with the parent compound for binding to degradative enzymes or enkephalin-binding plasma proteins (11,34). As suggested by Janak and Martinez (8), the net effect of this competition would be to increase the concentration of intact endogenous enkephalins (either in plasma or more locally at the site of release), which may then bind to δ-receptors and produce behavioral effects.

In conclusion, while effects of [leu]enkephalin given posttraining were reported previously (3), we report here for the first time that Tyr-Gly-Gly given posttraining impairs one-way active avoidance retention. In combination with earlier studies describing the effects of Tyr-Gly-Gly and [leu]enkephalin on avoidance acquisition (8,17,26,28,36), the current findings demonstrate that the enkephalin metabolite Tyr-Gly-Gly shares the effects of its parent peptides on both active avoidance acquisition and retention. However, Tyr-Gly-Gly most likely produces these effects through some mechanism distinct from the direct activation of opioid δ-receptors and therefore can be thought of as an agent that is pharmacologically distinct from its parent peptides.

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