Pharmacology Biochemistry & Behavior, Vol. 37, pp. 655–659. © Pergamon Press plc, 1990. Printed in the U.S.A. 0091-3057/90 $3.00 + .00

Only Tyrosine-Containing Metabolites of [Leu]Enkephalin Impair Active Avoidance Conditioning in Mice

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Received 26 March 1990

JANAK, P. H. AND J. L. MARTINEZ, JR. *Only tyrosine-containing metabolites of [Leu]enkephalin impair active avoidance conditioning in mice.* PHARMACOL BIOCHEM BEHAV 37(4) 655–659, 1990.—The effects of the enkephalin metabolites, Tyr, des-Tyr-[Leu]enkephalin (GGFL), and Tyr-Gly-Gly (YGG), on acquisition of an active avoidance task following their IP administration to mice were determined. Neither free Tyr (3.9–390.0 μg/kg) nor GGFL (7.1–710.0 μg/kg) altered acquisition of the avoidance response. In contrast, 53, but not 16 μg/kg, of YGG significantly impaired response acquisition. A 390.0, but not 39.0 μg/kg, dose of Tyr decreased locomotor activity levels measured in an open field. Together with previous findings that the enkephalin metabolites Tyr-Gly and Tyr-Gly-Gly-Phe also impair avoidance acquisition, these data indicate that the dipeptide Tyr-Gly is the minimum sequence needed to intefere with acquisition of an active avoidance response. Because the various enkephalin metabolites do not bind to opioid receptors, it is likely that their effects on avoidance acquisition represent a separate class of pharmacological agents whose effects are mediated by a nonopioid receptor mechanism. These results are important to the interpretation of behavioral studies involving peripheral administration of the opioid peptide, [Leu]enkephalin (LE).

[Leu]Enkephalin Enkephalin metabolites Avoidance acquisition Memory modulation Mouse

THE endogenous opioid peptide, [Leu]enkephalin (LE), influences acquisition of a number of conditioned behaviors in a number of species (7, 8, 13, 14, 17–19, 22–25, 29). For example, peripherally administered LE impairs acquisition of active avoidance conditioning in both mice and rats (14, 16, 18, 19, 29). LE undergoes rapid enzymatic degradation in vitro in mouse and rat plasma (30,36). This degradation rate is even faster in vivo in the mouse and rat (9,30). For example, following IP LE administration in both mice and rats, 97–98% of the peptide is in the form of metabolites 2 minutes after the injection (9,30). Since conditioning training typically does not commence until at least 2 minutes after drug injection, the rapid hydrolysis of LE degradation following its IP administration raises the question of whether LE degradation products themselves influence conditioning.

Previously we reported that the enkephalin metabolites Tyr-Gly (YG), Tyr-Gly-Gly (YGG), and Tyr-Gly-Gly-Phe (YGGF) all impair one-way active avoidance acquisition in mice (16,37), while des-Tyr-LE (GGFL) is without effect in either an appetitively motivated Y-maze discrimination task (13) or an active avoidance task (37). These conditioning effects of the Tyr-containing metabolites are similar to the impairment produced by LE itself. Thus, in accord with previous suggestions for other behaviorally active peptides (1, 3, 11), it is possible that behaviorally active metabolites of LE could account for some or all of the behavioral effects seen after administration of the parent peptide.

The present study was designed to determine whether the most abundant metabolite of LE, Tyr, influences acquisition of an avoidance response in mice. In addition, we examined whether Tyr affects open-field locomotor activity in the mouse. A further examination of the effects of GGFL and YGG on avoidance conditioning was also conducted.

METHOD

Subjects

Male Swiss Webster mice (24–31 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed three per cage under standard laboratory conditions. Food and water were available ad lib. The animals were maintained on a standard 12:12-h light:dark cycle, with lights commencing at 7 a.m. Newly arrived animals were allowed at least 4 days to acclimate to their housing conditions before experimentation began. Each animal was only used once. All housing conditions and experimental procedures are in accord with NIH guidelines. All experimental procedures were approved in advance by the Animal Care and Use Committee at the University of California, Berkeley.

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Behavioral Testing

Active avoidance task. The apparatus is a two-chamber alley with a floor consisting of two metal plates. The chamber design requires an animal to make contact with both plates at all times. Testing begins by placing an animal in the darkened start chamber. After 10 s the door connecting the two chambers is opened and a constant current footshock (280 μA) is delivered across the metal floor plates. The shock is terminated either when an animal escapes to the safe white compartment or after 20 s has elapsed. On subsequent trials the door is opened and the animal is given 10 s to move into the safe compartment before the shock is activated and subsequently terminated by escape or the pasage of 20 s. The intertrial interval is 20 s. Training begins two min after drug injection, and fourteen training trials are completed. The number of successful shock avoidances made is used to measure performance.

Locomotor activity. The apparatus is a 30 cm square, 20 cm high, open-field chamber, the floor of which is marked into 16 squares of equal area. An animal is placed in the center two minutes after drug injection, immediately following a 2-s, 280 μA footshock administered in the avoidance chamber, and is videotaped for later behavioral analysis. All crossovers, defined as the passage of all four feet across any line, are counted. The counts are tabulated in one-min intervals. Each animal is observed in the open-field chamber for a total of 10 min, the approximate time needed to train an animal in the active avoidance task.

Drugs

YGG, GGFL, Tyr and naloxone were obtained from Sigma (St. Louis, MO). LE was obtained from Bachem (Torrance, CA). All drugs were dissolved in 0.9% saline and blind-coded. Intraperitoneal injections were administered in a volume of 1 ml/100 g. A range of doses, centered around a dose equimolar to the 100 μg/kg LE dose known to impair avoidance acquisition (21), was tested for each metabolite. For Tyr, a 39.0 μg/kg dose is equimolar to the 100 μg/kg LE dose, and Tyr doses over a 3.9–39.0 μg/kg range were tested. For GGFL, a 71.0 μg/kg dose is equimolar to the 100 μg/kg LE dose, and GGFL doses over a 7.1–710.0 μg/kg range were tested. For YGG, a dose of 53.0 μg/kg is equimolar to 100 μg/kg LE; this dose, as well as the lower dose of 16.0 μg/kg YGG, was tested. LE (100 μg/kg) provided a positive control in all active avoidance experiments, since it reliably impairs avoidance acquisition, while naloxone (1.0 mg/kg) was included as a positive control in locomotor activity testing, since it is known to decrease open-field locomotor activity.

Data Analysis

The data from each individual study were analyzed by conducting an analysis of variance, followed by Dunnett's test for comparing a treatment mean to a control mean (10). The effect of the positive control treatment of 100 μg/kg LE or 1 mg/kg naloxone was verified for each experiment by comparing this group to saline-treated animals using the Student's *t*-test (10). Since a limited number of animals can be trained each day, completion of dose-response functions necessitated combining data across consecutive experimental days. The necessity for including positive control animals one each day of experimentation resulted in large numbers of animals (up to 25) in some treatment groups.

RESULTS

Effects of TYR, GGFL, and YGG on Avoidance Acquisition

As can be seen in Fig. 1, Tyr (3.9, 11.8, 39.0, 118.0, 390.0 μg/kg) failed to influence acquisition of the active avoidance response, as indicated by the absence of significant differences between the number of avoidances made by Tyr-treated animals and the number made by saline control animals [one-way ANOVA: $F(5,87)=0.877$, $p=0.5$]. The positive control group, administered a dose of 100 μg/kg LE, made significantly fewer avoidances than the saline-treated animals, $t(42)=-2.90$, $p<0.006$.

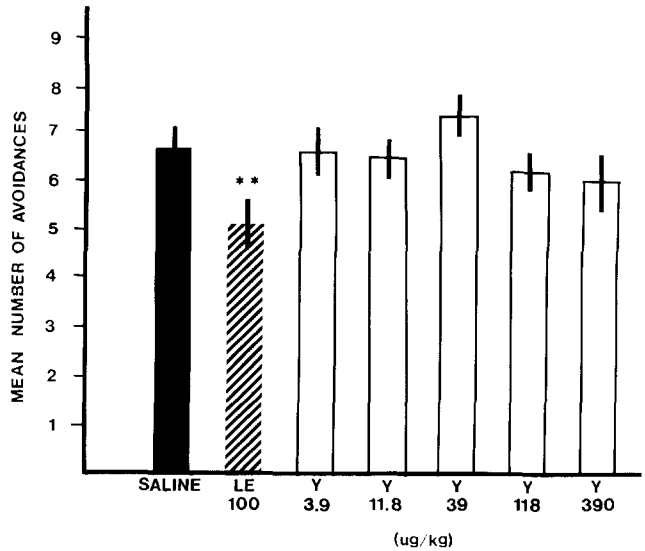

FIG. 1. Effect of pretraining Tyr administration on acquisition of active avoidance responding. None of the Tyr doses altered avoidance acquisition, as compared to saline-treated animals, $F(5,87)=0.877$, $p=0.5$. In contrast, LE (100 μg/kg) impaired avoidance acquisition, $t(38)=-2.92$, $p<0.006$. The n's for each group are as follows: saline, 23; LE 100 μg/kg, 17; Tyr 3.9 μg/kg, 11, Tyr 11.8 μg/kg, 12; Tyr 39.0 μg/kg, 11; Tyr 118.0 μg/kg, 12; Tyr 390 μg/kg, 11.

GGFL (7.1, 21.0, 71.0, 210.0, 710.0 μg/kg) did not affect the average number of avoidances made, as compared to saline-treated control animals [one-way ANOVA; $F(5,76)=0.838$, $p=0.527$]. LE (100 μg/kg) again impaired avoidance acquisition, as indicated by fewer avoidances in LE-treated animals as compared to saline controls, $t(45)=-2.75$, $p<0.01$.

An examination of the acquisition effects of YGG found a significant effect of treatment [one-way ANOVA: $F(2,28)=4.228$, $p<0.03$]. Further analysis revealed that a dose of 53 μg/kg of YGG, equimolar to the behaviorally effective dose of 100 μg/kg of LE, significantly impaired acquisition of the avoidance response [Dunnett's test against saline mean: $t(28)=2.64$, $p<0.05$; see Fig. 3], while 16 μg/kg YGG was without effect [Dunnett's test: $t(28)=-0.03$, $p>0.05$]. The positive control animals receiving 100 μg/kg of LE also were impaired when compared to saline-treated control animals [Student's $t(14)=2.49$, $p<0.025$].

No difference between the individual saline control groups was found when the three experiments were compared [one-way ANOVA: $F(2,56)=2.19$, $p=0.12$; saline means: Tyr Expt.: 6.76; GGPL Expt.: 7.42, YGG Expt.: 8.20].

Effects of Tyr on Locomotor Activity in an Open Field

Analysis of the effect of Tyr administration on locomotor activity revealed a marginally significant effect of treatment on total number of lines crossed in ten minutes [one-way ANOVA: $F(2,75)=3.15$, $p<0.05$]. As can be seen in Table 1, this treat-

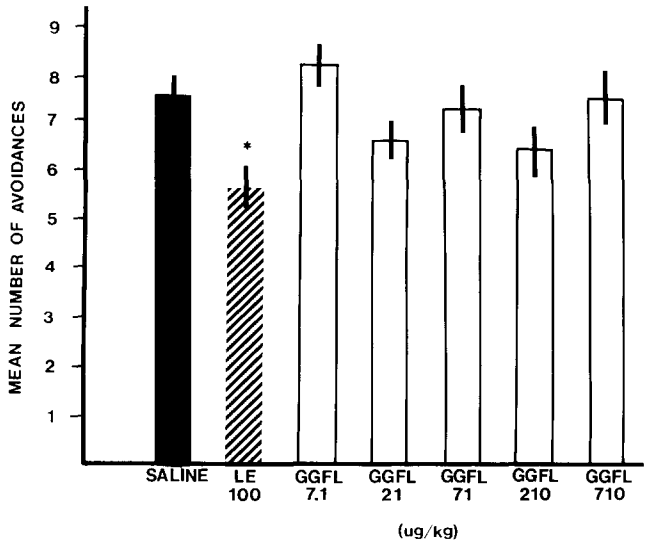

FIG. 2. Effect of pretraining des-Tyr-[Leu]enkephalin (GGFL) administration on acquisition of active avoidance responding. None of the GGFL doses altered avoidance acquisition, $F(5,76)=0.838$, $p=0.527$. LE (100 μg/kg) impaired avoidance acquisition, $t(41)=-3.17$, $p<0.01$. The n's for each group are as follows: saline, 22; LE 100 μg/kg, 21; GGFL 7.1 μg/kg, 6, GGFL 21.0 μg/kg, 10; GGFL 71.0, 10; GGFL 210.0 μg/kg, 11; GGFL 710.0, 11.

ment effect is reflected by a decrease in the average number of lines crossed by mice receiving the 390.0 μg/kg Tyr dose as compared to saline-treated control animals [Dunnett's test against saline mean: $t(75)=2.46$, $p<0.05$]. However, a dose of 39.0 μg/kg Tyr, equimolar to the 100 μg/kg dose of LE that impairs avoidance acquisition, did not affect locomotor activity [Dunnett's test: $t(75)=0.73$, $p>0.05$]. The positive control group that received a 1.0 mg/kg dose of naloxone also had significantly decreased locomotor activity [Student's $t(29)=-2.31$, $p<0.05$].

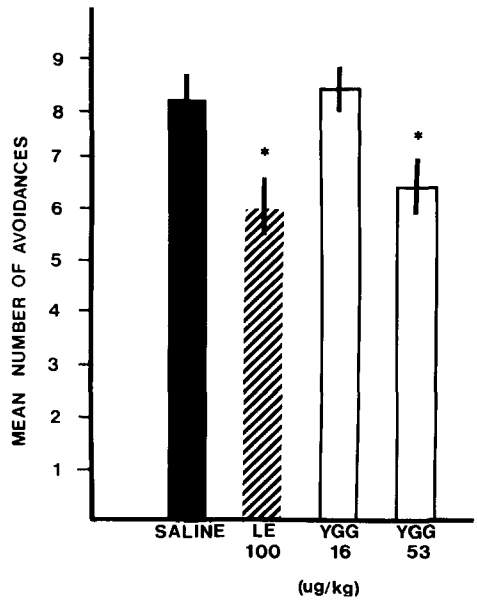

FIG. 3. Effect of pretraining Tyr-Gly-Gly (YGG) administration on acquisition of active avoidance responding. YGG (53.0 μg/kg), as well as LE (100 μg/kg, impaired avoidance acquisition as compared to saline-treated control animals (saline vs. YGG 53.0 μg/kg: Dunnett's $t(28)=2.64$, $p<0.05$; saline vs. LE 100 μg/kg: Student's $t(14)=2.49$, $p<0.025$. YGG (16.0 μg/kg) was without effect [Dunnett's $t(28)=-0.03$, $p>0.05$]. The n's for each group are as follows: saline, 8; LE 100 μg/kg, 8; YGG 16.0 μg/kg, 7; YGG 53.0 μg/kg, 10.

TABLE 1

EFFECTS OF TYROSINE ON OPEN-FIELD LOCOMOTOR ACTIVITY

Treatment	Dose	Mean Crossovers ± SEM	n
Saline	—	346.64 ± 17.59	25
Tyr	39.0 μg/kg	329.78 ± 16.46	27
	390.0 μg/kg	289.62 ± 15.16*	26
Saline	—	390.00 ± 28.08	14
Naloxone	1.0 mg/kg	300.82 ± 26.22*	17

*$p<0.05$ compared to saline-treated animals.

DISCUSSION

We recently determined that Tyr and the complementary fragment des-Tyr-LE (GGFL) are the two most numerous LE degradation products formed in vitro in mice (31). Since 97–98% of IP-administered LE is in the form of metabolites by 2 minutes after injection (9), we sought to determine whether exogenously administered LE metabolites influence avoidance conditioning. In the present study we found that free Tyr, when tested across a wide range of doses, was without effect on avoidance aquisition. A previous study in chicks found that Tyr produces amnesia for a one-trial taste avoidance task (6). However, the chick study used intracranial, rather than peripheral, injection of relatively high doses, administered posttraining. Pretraining effects of Tyr in tests measuring acquisition may differ from the effects of Tyr in paradigms examining retention following posttraining injections.

We previously found that a 71 μg/kg GGFL dose, which is equimolar to the behaviorally effective 100 μg/kg LE dose, failed to influence avoidance acquisition in mice (37). The lack of effect of GGFL on active avoidance acquisition was confirmed and extended in the present study by examining its effects over a wider dose range. Our failure to find an effect of GGFL on avoidance acquisition contrasts with the previous reports that des-Tyr-[Met]enkephalin (GGFM) impairs retention of a shuttle avoidance task in rats (8) and that GGFM enhances retrieval of a passive avoidance response (4). However, the conditioning effects of GGFL may differ from the effects of GGFM, since each peptide has a different COOH-terminal amino acid.

The present results partially replicate our previous finding of avoidance impairment produced by the enkephalin metabolite, YGG. In our previous study, 16 μg/kg of YGG was found to impair avoidance acquisition in mice, but a higher dose (160 μg/kg) was without effect (37). In the present study, a 53 μg/kg YGG dose impaired acquisition, while the lower 16 μg/kg dose was without effect. Although both studies observed a YGG-induced avoidance impairment, the discrepancy in effective dose of YGG may be accounted for by procedural differences. For example, both the footshock level and the animal breeder differed in the two studies. The original study was conducted using a 330 μA footshock in Swiss Webster mice obtained from Simonsen (CA); the present study was conducted using a 280 μA footshock in Swiss Webster mice obtained from Harlan Sprague-Dawley

(IN). Obvious behavioral differences between these two groups of animals are seen in their saline means. Animals received from Harlan Sprague-Dawley consistently perform better, even at a reduced shock level, as indicated by a higher saline mean than animals obtained from Simonsen. This difference in baseline performance between saline-treated animals from the two breeders likely shifted the dose-reponse function such that different optimal doses were observed in the two studies, although the nature of the impairing effect remains the same.

We previously reported that two other enkephalin metabolites, YG and YGGF, also impair acquisition of active avoidance conditioning in mice (16). The combined evidence suggests that Tyr-containing enkephalin metabolites can impair avoidance conditioning. This suggestion is further supported by the absence of an effect of GGFL, and the fact that Tyr itself is not sufficient to affect acquisition of the avoidance response. Therefore, the Tyr-Gly sequence appears to be the minimum fragment needed for altering avoidance acquisition.

The conditioning effects of all acquisition-impairing enkephalin fragments tested to date are similar to the conditioning effects produced by LE in that both the parent peptide and the active metabolites impair acquisition at microgram doses and their dose-response functions for this effect are U-shaped. Many studies suggest that peripherally administered LE affects avoidance conditioning through a site of action located outside the blood-brain barrier [(14, 15, 18, 19); see (21) for review]. While no direct evidence is yet available concerning a peripheral or central site of action for behaviorally active LE metabolites, the fact that the metabolites are effective at similar or lower dose than those needed to produce an acquisition impairment by LE suggests the metabolites also may act outside the blood-brain barrier (37).

Since our avoidance acquisition studies used pretraining injections of metabolites, we investigated the effects of these same metabolites on locomotor activity. The avoidance impairments produced by two enkephalin metabolites, YG and YGG, are not attributable to drug-induced locomotor activity changes, since these peptide fragments do not affect horizontal activity measured in an open field (16,37). By contrast, both Tyr (this study) and GGFL (37) decrease open-field horizontal activity in mice, but do not alter avoidance conditioning. An exception to the rule that metabolite effects on acquisition and locomotor activity appear to be mutually exclusive is seen with the enkephalin metabolite, YGGF, which both impairs avoidance acquisition and decreases locomotor activity, suggesting that, for this metabolite, conditioning effects may be secondary to locomotor activity effects (16).

How relevant are the behavioral effects of exogenously administered enkephalin metabolites to behavioral effects observed following exogenous administration of LE? Studies using HPLC determination of metabolites formed in vitro 10 minutes following addition of LE to mouse plasma reveal that, in addition to Tyr, small amounts of both YGG and YG are produced, accounting for approximately 18% and 2% of the total LE metabolized, respectively (31). Accumulation of YGGF after LE addition to plasma was not detectable in the mouse (31). Our finding that two natural enkephalin metabolites, YG and YGG, are behaviorally active following their exogenous administration, together with our finding that 97–98% of peripherally administered LE is metabolized within two minutes (9), might suggest that one or more enkephalin metabolites could account for the acquisition impairments observed over the approximately ten-minute active avoidance training session. However, other pharmacological evidence suggests to us that LE affects conditioning as an intact peptide.

For example, several lines of evidence lend substantial support for involvement of the opioid delta receptor type in the effects of IP-administered LE. The avoidance impairment produced by LE is attenuated by simultaneous administration of the delta receptor selective antagonist, ICI 174,864 (28). Administration of ICI 154,129, a different delta receptor selective antagonist, by itself enhances avoidance conditioning (29). Additionally, d-Pen^2-d-Pen^5-enkephalin, a delta selective agonist that may be more enzymatically stable than is LE, has effects on avoidance conditioning similar to those produced by LE (29,35). Together, this evidence suggests that the avoidance conditioning effects of LE are mediated by delta opioid receptors (29).

In contrast, the mechanism through which the behaviorally active YG-containing enkephalin metabolites produce their avoidance conditioning effects is not known. Results from bioassays and receptor binding assays indicate that none of the LE metabolites is capable of stimulating delta opioid receptors (2), while YGGF may have minor biologic activity at mu opioid receptors (26). However, neither YG nor YGG have detectable mu receptor activity (2). Therefore, the behaviorally active enkephalin metabolites, especially the di- and tripeptide, must exert their effects through a mechanism that does not directly involve opioid receptors.

It is possible that LE metabolites may exert an effect on opioid systems, even though they are not direct opioid receptor agonists. For example, both kentsin (Thr-Pro-Arg-Lys) and kyotorphin (Tyr-Arg) produce naloxone-reversible analgesia, yet neither peptide appears to bind opioid receptors (5, 27, 32, 33). These compounds are thought to produce their analgesic effects through induced release of endogenous enkephalin stores (5, 27, 32). Another possible mechanism for nonopioid receptor-mediated effects of LE fragments is through metabolite competition with the parent compound for binding to degradative enzymes (12) or to LE-binding plasma proteins (34). This would effectively increase the concentration of intact circulating endogenous LE available for binding to opioid receptors in order to produce its behavioral effect.

Thus, while some enkephalin metabolites share conditioning effects with intact LE, they most likely represent a separate class of pharmacological agents, with behavioral effects produced by a mechanism distinct from the direct activation of delta opioid receptors that characterizes the behavioral effects of peripherally administered LE.

ACKNOWLEDGEMENTS

We thank Dr. Susan Weinberger for her editorial assistance. Supported by PHS National Research Service Award DA-5375-01 to P.H.J. from the National Institute on Drug Abuse, and PHS grant DA 04195 to J.L.M.Jr. also from the National Institute on Drug Abuse.

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