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Endorphin Analogs with Potent and Long-Lasting Analgesic Effects

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WALKER, J. M., C. A. SANDMAN, G. G. BERNTSON, R. MCGIVERN, D. H. COY AND A. J. KASTIN. *Endorphin analogs with potent and long-lasting analgesic effects.* PHARMAC. BIOCHEM. BEHAV. 7(6) 543–548, 1977. – The analgesic effects of intracerebroventricular administration of α, γ, and β-endorphin and their D-Ala²-analogs were examined in the rat using the tail-flick test. Analgesia was produced by all substances. The actions of D-Ala²-α and -β-endorphin were considerably greater than the parent forms, whereas D-Ala²-γ-endorphin was approximately equivalent to the parent compound. The marked analgesia was dose dependent and prolonged for all analogs. Since these effects were reversed by the opiate antagonist naloxone, it was concluded that opiate receptors mediate the action of these analogs. It is suggested that these analogs may be useful in behavioral tests when a longer duration of action is desirable.

Endorphin Analgesia Pain Naloxone Opiate Peptide Enkephalin

SHORTLY after its identification in brain tissue, the opiate pentapeptide methionine enkephalin [17] was shown to have analgesic effects in vivo [1]. This analgesia, however, was weak and transient and it became of increasing interest to develop more potent and longer lasting analogs of enkephalin. Our previous work [9,31], together with an independent report [26], demonstrated that substitution of D-alanine in the Gly² position of the molecule results in an enkephalin analog with greatly increased potency both in vivo and in vitro.

The amino acid sequence of methionine-enkephalin is identical to residues 61–65 of the β-lipotropin molecule (β-LPH). Other opiate-like peptides, α, γ, and β-endorphin, were isolated from pituitary extracts and also characterized as fragments of the putative prohormone β-LPH [6, 11, 14, 20, 21] corresponding to β-LPH_{61-76}, β-LPH_{61-77}, and β-LPH_{61-91}, respectively. Since the β-LPH_{61-65} sequence is shared by Met-enkephalin and the pituitary endorphins, it seemed likely that the D-Ala²-analogs of α, γ, and β-endorphin would also show greater potency than their naturally occurring parent compounds. Britton *et al.* [7], and Coy *et al.* [8] described enhanced effects of these and other synthetic analogs in the mouse vas deferens assay and in stimulating the release of growth hormone and prolactin. We now report enhanced effects of analogs of α, γ, and β-endorphin, having D-alanine substituted in the second amino acid position, in tests of analgesia in the rat.

METHOD

Animals

Male albino (Holtzman) rats, 90–120 days old were used in the present study. Rats were housed individually and fed ad lib throughout the experiments.

Drugs

Peptides (α, γ, β-endorphin and D-Ala²-α, D-Ala²-γ, and D-Ala²-β-endorphin), synthesized by solid phase methods [8], were dissolved in Ringer's solution no more than three hours before injections. Since opioid peptides are essentially ineffective in producing analgesia after peripheral administration, all peptides were injected intracerebroventricularly (ICV) in 10 μl volumes over a period of one minute. Naloxone [4], supplied by Endo Laboratories, was administered subcutaneously in physiological saline (2 mg/cc).

Surgery

At least one week before testing, ventricular cannulae were surgically implanted, under sodium pentobarbital anesthesia, into the left lateral ventricle of each animal. Stereotaxic coordinates (with lambda and bregma at the same dorso-ventral level) were 1 mm posterior to bregma, 1.5 mm lateral to the midline, and 4.1 mm below the skull

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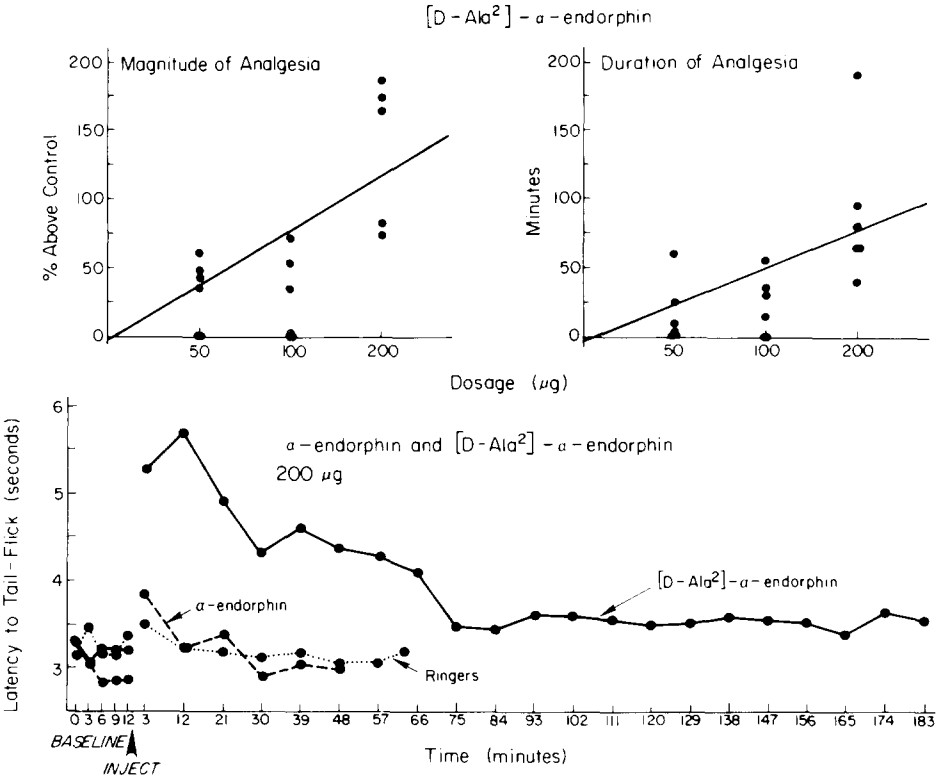

FIG. 1. Upper: Regression analysis as a function of Ringer's solution and 3 doses of D-Ala²-α-endorphin. ($\bar{X}$ magnitude ± SEM = 31.2 ± 10.4, 50.3 ± 11.7, 135.6 ± 23.8; $\bar{X}$ duration ± SEM = 17.5 ± 9.3, 31.7 ± 8.9, 135.6 ± 23.8, for 50, 100 and 200 μg respectively.) Lower: Mean tail flick latency before and after Ringer's solution, 200 μg-α-endorphin and 200 μg D-Ala²-α-endorphin.

surface. Dental acrylic and stainless steel hooks were used to secure the cannula to the skull. Cannulae were constructed from 22 ga stainless steel hypodermic tubing sharpened and beveled at the tips. The 26 ga needle of a microsyringe used for injections extended 1 mm beyond the tip of the cannula. Cannulae placements were verified histologically by examination of the ventricles for the presence of a marker dye injected before sacrifice.

Apparatus

Analgesia was measured by the tail-flick test of D'Amour and Smith [10]. This widely used test has the advantages of being relatively selective for narcotics [12], highly quantitative, and insensitive to experimenter bias. The apparatus consisted of an adjustable heat source which can be directed onto the rat's tail. The application of power to the heat source starts a solid state latency timer which in turn is stopped by withdrawal of the tail allowing the light from the heat source to activate a photocell. A digital readout, to tenths of a second, is provided for the latency to tail withdrawal, which is the index of pain sensitivity.

Procedure

Preliminary experiments were first conducted to determine the appropriate dosage levels of the peptides to be used in studies comparing potencies of the D-Ala²-analogs and the parent compounds. These experiments examined the dose-related potencies of the endorphin analogs (D-Ala²-α, -γ and -β) in producing analgesia as measured by the tail-flick test. Three doses of each compound were selected based on pilot tests. These doses were 50, 100 and 200 μg for D-Ala²-α and -γ, and 0.1, 2 and 40 μg for D-Ala²-β-endorphin. Separate groups of 6 rats each were used to test the three analogs. Each rat received three doses of a given analog and two Ringer's control tests on separate days. Tests were separated by 24 hours and the order of dose level was counterbalanced across animals. In each test the output of the heat source was adjusted to obtain tail-withdrawal latencies between 2.5 and 4 sec. Tail-flick latencies were then measured every 3 minutes for a baseline period of 15 min. The peptide or control solution was then administered, and testing continued, at 3 minute intervals (5 min for β) for a minimum of one hr or until tail-flick latencies had returned to within 30% of their baseline values on three consecutive trials.

Based on the results of the dose-response tests described above, a dosage level of each endorphin producing reliable analgesia was selected for further testing (α and γ-endorphin = 200 μg; β-endorphin = 10 μg). Separate groups of 10 rats each were used to compared endorphin analogs to their parent compounds. In order to insure appropriate cannula placements, each animal was given a preliminary test with 20 μg morphine ICV. Only those animals who showed an analgesic response to this treatment were used for endorphin tests. At least 4 days after screening with morphine, each animal of a given group received five tests, one with the parent endorphin, one with the D-Ala²-analog, and three with control administration of the vehicle alone. All tests were separated by 24 hours; the order of drug administration was counterbalanced with vehicle injections occurring on the first, third, and fifth tests, and endorphins

on the second and fourth. Individual testing sessions for analgesia were conducted as described above.

After completion of dose-response studies, two animals were given additional tests in order to assess the role of opiate receptors in analgesia produced by the analogs. The opiate antagonist naloxone (2 mg/kg) was administered, subcutaneously, after an analgesic response to the peptide treatment (200 μg of D-Ala^2-α and -γ, 40 μg of D-Ala^2-β) was observed. Tail-flick tests were then repeated until responses had returned to normal.

Data Analysis

As in a previous report [31], dose-response data were reduced to give an estimate of the magnitude and duration of analgesia. For each animal, the duration was computed as the number of minutes between tests showing a 30% increase in latency over baseline mean. The magnitude of effect was taken as the mean latency during this interval. Linear regression was performed by the method of least squares on each measure to give an estimate of dose-related changes in the effect.

Experiments designed to compare analogs with parent compounds were subjected to repeated measures analysis of variance, the effectiveness of each compound tested by separate analyses over blocks of trials with the first block consisting of baseline measures. Since these analyses did not include data over the entire period of the effect, a separate *t*-test was used to compare the duration of action of each analog to that of its parent.

RESULTS

D-Ala^2-α-endorphin

Figure 1 (upper panel) shows the increasing analgesic effect with increasing doses of D-Ala^2-α-endorphin. As indicated, both the magnitude and duration of the effect increased in a dose-related fashion; regression analysis showed a significant linear component ($r = .77$, $p<0.001$, for magnitude and $r = .45$, $p<0.025$ for duration). The results of the comparison of α-endorphin with D-Ala^2-α-endorphin are shown in the lower panel of Fig. 1. Both substances produced statistically reliable increases in tail-flick latencies (α: 3,24, $F = 3.05$, $p<0.05$; D-Ala^2-α: 3,27, $F = 3.78$, $p = 0.02$) whereas the Ringer's vehicle had no significant effect. The apparent greater potency of the analog was highly significant (1,9,$F = 14.95$, $p<0.005$) as was its duration of action ($df = 9$, $t = 1.86$, $p<0.05$). It thus appears that substitution of D-alanine in the Gly^2 position of α-endorphin dramatically enhances its potency in vivo. Moreover the effect of the analog was blocked by the opiate antagonist naloxone. Two animals who showed large increases in tail-flick latency after 200 μg of D-Ala^2-α-endorphin returned to baseline response levels shortly after injection of the naloxone ($\bar{X}$ latency after D-Ala^2-α = 8.1 sec, 10 min after naloxone = 2.6 sec).

D-Ala^2-γ-endorphin

D-Ala^2-γ-endorphin produced dose dependent analgesic effects as shown in Fig. 2 (upper panel). Both the duration and magnitude increased linearly to a significant degree with increasing doses (magnitude: $r = .70$, $p<0.001$; duration: $r = .82$, $p<0.001$). Furthermore, repeated measures analysis of variance revealed that although Ringer's solution had no effect, both γ and D-Ala^2-γ-endorphin produced reliable increases in tail-flick latency (γ: 4,36, $F = 4.11$, $p<0.01$; D-Ala^2-γ: 4,36, $F = 4.23$, $p<0.01$). As shown in the lower panel of Fig. 2, the analog was very similar to the parent compound in its effects. Although analysis of variance suggested that the two compounds did not differ significantly, it appears that the analog may be longer lasting since the mean duration of its effect (86 min) was considerably greater than that of the naturally occurring parent (58.2 min).

As with D-Ala^2-α-endorphin, naloxone completely reversed the effects of D-Ala^2-γ-endorphin. Tail-flick latencies of two rats, elevated by injection of 200 μg of the analog, returned to normal within 10 minutes after injection of naloxone ($\bar{X}$ latency after D-Ala^2 = 8.5 sec; 10 min after naloxone = 2.76 sec).

D-Ala^2-β-endorphin

As with the other endorphin analogs, D-Ala^2-β-endorphin produced a marked analgesia which was an increasing function of its dose. A significant linear effect of dose was indicated by regression analysis for both magnitude ($r = .69$, $p<0.001$) and duration ($r = .52$, $p<0.005$) of effect. The 40 μg dose was particularly powerful, in some cases producing analgesia that lasted over 9 hours. (See Fig. 3, upper panel.)

In view of the extreme effects of high doses of D-Ala^2-β-endorphin, a more moderate dose of 10 μg was chosen for comparison with its naturally occurring parent compound. Again, the control injections had no effect on tail-flick latency; however, as shown in Fig. 3 (lower panel), both β-endorphin and its D-Ala^2-analog produced significant increases over baseline (ANOVA; β: 6,54, $F = 2.41$, $p<0.05$; D-Ala^2-β: 6,54, $F = 7.76$, $p<0.001$). Moreover, the overall effect of D-Ala^2-β-endorphin was found to be significantly greater than that of the parent compound (ANOVA; 1,9, $F = 7.19$, $p<0.025$). The greater potency of the D-Ala^2-analog was especially apparent in its greater duration of action. The mean duration of analgesia was 45.1 min for β-endorphin and 176.5 min for the analog ($t = 2.67$, $p = 0.012$).

The results of naloxone administration again indicated that the actions of D-Ala^2-β-endorphin are mediated through opiate receptors. The opiate-antagonist naloxone completely reversed the analgesia induced by 40 μg of this potent analog. The mean latency of tail withdrawal after D-Ala^2-β-endorphin was 10.0 sec which returned to 3.2 sec 10 min after naloxone injection, a value approximately that of the pre-drug baseline.

Behavioral Observations

The analgesia produced as a result of the endorphins and their analogs in the present study was generally accompanied by other behavioral changes as well. As we previously observed with D-Ala^2-Met-enkephalin [31], short bursts of course tremors often occurred shortly after injections. With α- and γ-endorphin and their analogs, tremors were often observed within 30 sec after injection injection, whereas with β and D-Ala^2-β-endorphin, 5–15 minutes usually elapsed before such tremors were observed. In addition, animals often showed signs of hyperactivity and hypersensitivity to touch for transient periods occurring irregularly throughout the testing session. At higher doses of β-endorphin and the analogs, seminal emission and the straub tail phenomenon were occasionally observed.

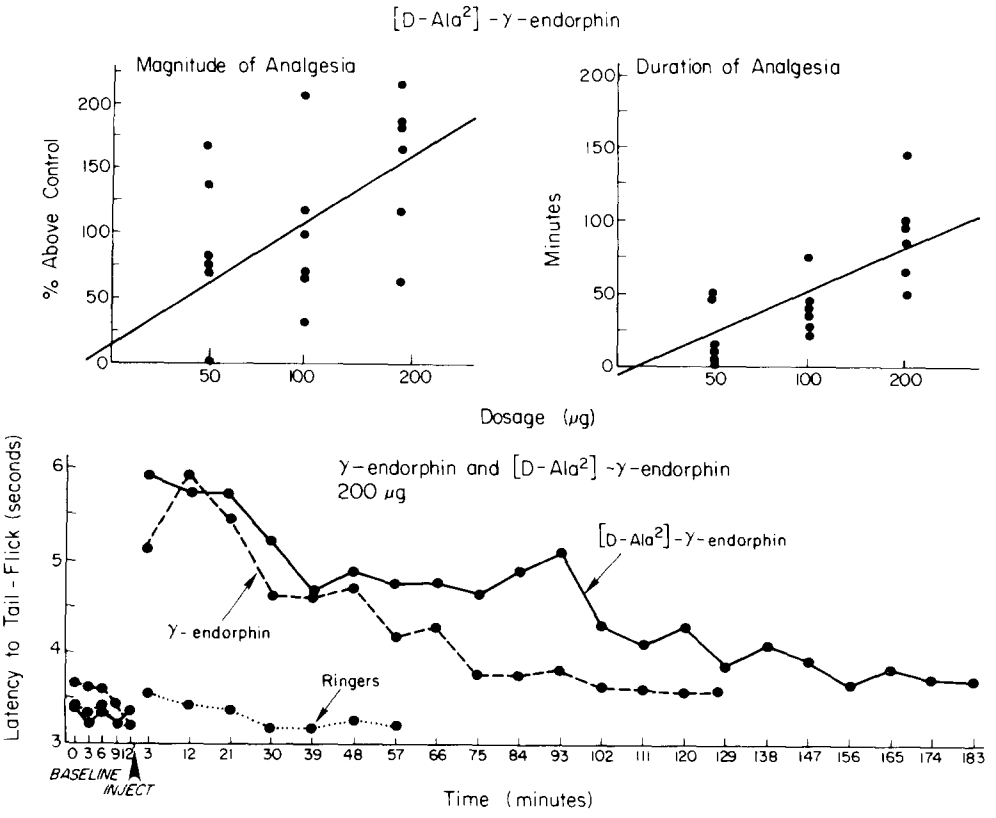

FIG. 2. Upper: Least squares linear regression of effects of Ringer's solution and 3 doses of D-Ala²-γ-endorphin on pain sensitivity. ($\bar{X}$ magnitude ± SEM = 88.8 ± 23.7, 98.8 ± 24.7, 154.7 ± 22.6; $\bar{X}$ duration ± SEM = 2.08 ± 8.7, 41 ± 7.75, 90 ± 13.4, for 50, 100 and 200 μg respectively). Lower: Mean tail flick latency before and after Ringer's solution, 200 μg γ-endorphin, and 200 μg D-Ala²-γ-endorphin.

DISCUSSION

The results of the present experiments document the analgesic properties of the naturally occurring pituitary endorphins, α, γ, and β-endorphin [18]. Although some other investigators have failed to find analgesia after central administration of α and γ-endorphin [3,18], our results clearly indicate that they do have reliable effects in the tail-flick test. The present experiments were not designed to directly compare potency among these naturally occurring substances; however, it was obvious that β-endorphin is the most potent followed by γ and then α.

These experiments further demonstrate the increased potency of synthetic endorphin analogs having D-alanine substituted in the Gly² position, extending and confirming recent reports showing increased potency of D-Ala²-endorphin analogs in other systems [7, 8, 30, 31]. All three analogs produced dramatic and prolonged analgesic effects. Moreover, the analgesia we observed did not appear to be a secondary consequence of the other effects of these compounds since analgesia could be observed in the absence of other behavioral effects. Since the first five residues of the endorphin sequences are identical to those of Met-enkephalin, this work stands as a logical extension of research showing enhanced activity of D-Ala²-Met-enkephalin [9, 26, 31]. As in the case of methionine-enkephalin, it is possible that a β-type II bend [5, 19, 27] is stabilized by the D-amino acid. Studies of endorphin analogs in vitro prompted Britton *et al.* [7] to argue for greater resistance to enzymatic destruction as well as greater binding affinity as the cause of the increased activity of these analogs in the vas deferens assay. Our results support the view that these factors may account for the enhanced potency of the D-Ala²-endorphin analogs.

Lord *et al.* [23] have suggested that the binding characteristics of D-Ala²-Met-enkephalin to various classes of opiate receptors are essentially indistinguishable from those of the naturally occurring parent. It is, therefore, becoming increasingly reasonable to use this analog in behavioral experiments where a longer duration of action is necessary. It seems likely that a similar relationship will hold for the analogs in the present study. If so, the application of the D-Ala²-analogs as a research tool is particularly suitable for α-endorphin whose actions are extremely weak in tests of analgesia.

While there is some evidence suggesting a great deal of similarity between the D-Ala²-analogs and parent compounds, other observations from this study suggest some differences in action between the enkephalins and endorphins. For example, in experiments with β-endorphin and its D-Ala²-analog, low doses often produced powerful analgesia with few other pronounced behavioral changes. In contrast, animals treated with D-Ala²-Met-enkephalin rarely showed analgesia without an accompanying state of nearly total immobility; lower doses often produced varying degrees of hyperactivity and hypersensitivity but with little

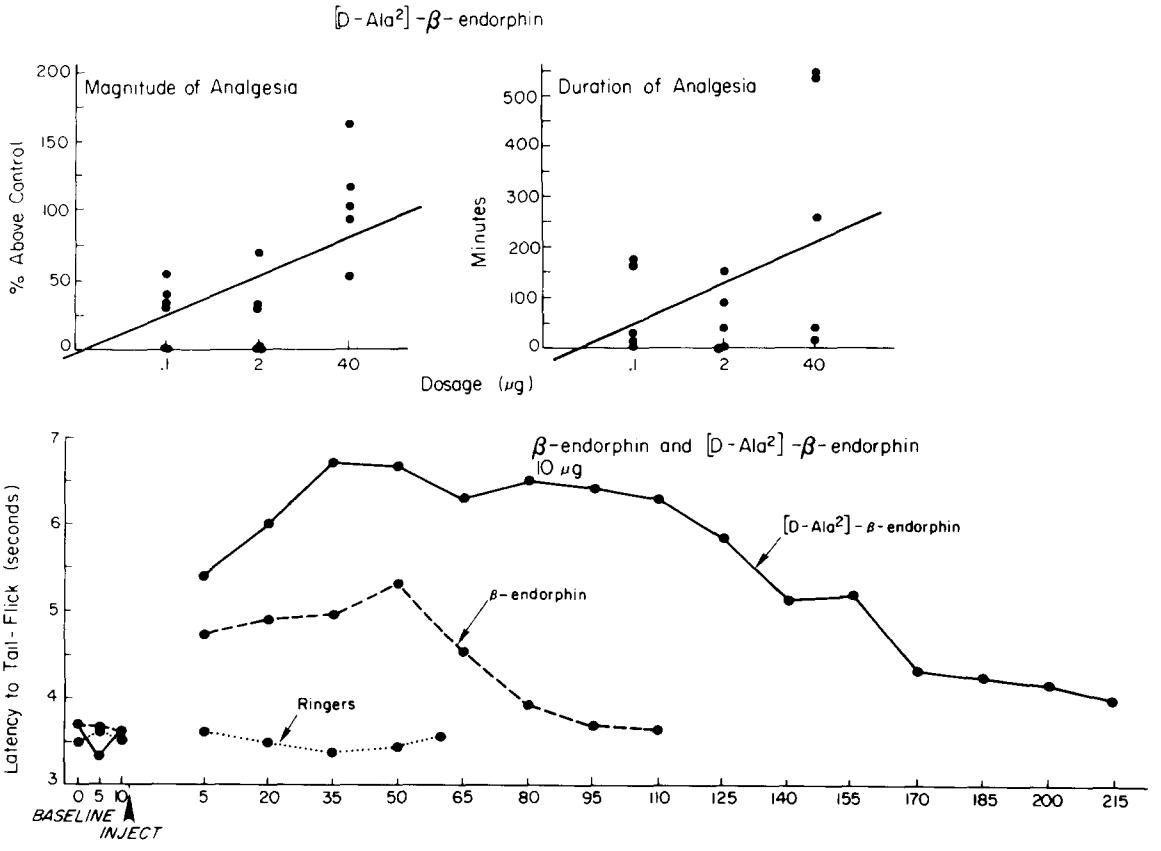

Fig. 3. Upper: Regression analysis of effects of Ringer's solution and three doses of D-Ala²-β-endorphin on tail-flick latency. ($\bar{X}$ magnitude ± SEM = 25.8 ± 8.87, 22.3 ± 11.6, 106 ± 17.8; $\bar{X}$ duration ± SEM = 65 ± 35.1, 46.7 ± 25.3, 275 ± 17.8, for 0.1, 10, and 40 μg respectively.) Lower: Mean tail flick latency before and after injections of Ringer's solution, β-endorphin and D-Ala²-β-endorphin.

analgesia [31]. It is clear, therefore, that intraventricular injection of these various opiate-like peptides produce the same classes of behavioral effects but possibly in different proportions. The neurological basis for this observation is unclear since dose level, rate of degradation [13, 15, 16], access to receptor populations, and varying affinity to different classes of opiate receptors [24,25] may interact in producing the final behavioral outcome. The observations of different effects of the opiate-like peptides lend support to the idea that these substances may have different functional roles. Not only have previous observations led to this conclusion [29] but the distinct anatomical differences in localization between the enkephalins and endorphins [2,28] provide a neurological basis for different actions of these substances.

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