Antinociception following application of DAMGO to the basolateral amygdala results from a direct interaction of DAMGO with Mu opioid receptors in the amygdala

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Accepted 30 September 2005

Abstract

Previous studies from our laboratory have shown that application of the mu opioid agonist DAMGO into the basolateral region of the amygdala (BLA) suppresses the radiant heat tail flick (TF) reflex in anesthetized rats. This antinociceptive effect can be blocked by lesions of brainstem regions such as the periaqueductal gray (PAG) or the rostral ventromedial medulla (RVM) or by functional inactivation of neurons in these regions, suggesting the activation of brainstem-descending antinociceptive systems from the amygdala. However, little is known about the direct interaction of DAMGO with mu receptors in the amygdala. In the present series of experiments, the BLA was pretreated with opioid receptor antagonists and a G protein inhibitor prior to TF testing with application of DAMGO into the same site. Rats pretreated with the non-selective opioid antagonist naltrexone (1.25–3.75 μg/0.25 μl per side) or the G protein inhibitor pertussis toxin (0.25 μg) failed to show inhibition of TF reflexes following infusion of DAMGO (0.168–0.50 μg), indicating that DAMGO works through G-protein-coupled opioid receptors in the BLA. Furthermore, pretreatment with the mu antagonist β-FNA (1.00–2.00 μg) attenuated antinociception induced by DAMGO injection, suggesting DAMGO’s action on mu receptors in the BLA. Accordingly, we confirm a direct interaction of DAMGO with G-protein-coupled mu receptors in the BLA contributing to induction of opioid antinociception in the amygdala.

1. Introduction

Brainstem regions such as the periaqueductal gray matter (PAG) and the rostral ventromedial medulla (RVM) are known to be critical for descending pain modulation. Cells in the PAG innervate neurons in the RVM that in turn send their axons to the dorsal horn of the spinal cord and activation of these PAG or RVM cells suppress activity of ascending nociceptive projection neurons in the dorsal horn [3,4,10]. In accordance, opioid, electrical, or excitatory chemical stimulation of cells in the PAG leads to activation of cells in the RVM, and stimulation of neurons in either of these brainstem regions can abolish responsiveness to peripheral noxious stimulation in rats and humans [5,6,22,24,36,46].

The amygdala is a forebrain site that provides important inputs to the descending antinociceptive systems of the brainstem. Anatomical and neuropharmacological data support the amygdala’s important neural and functional connections to the brainstem. The central nucleus of the amygdala (CeA), which receives inputs from the basolateral region of the amygdala (BLA) including the basolateral and lateral nuclei [9], sends dense neural projections to the PAG [1,15,35,40]. Electrical, opioid, or non-opioid stimulation of cells in the amygdala suppresses tail flick (TF) reflexes evoked by radiant heat or paw-licking responses induced by...
formalin in rats [12–14,23,26,38]. This antinociceptive
effect can be blocked by electrolytic lesions of the PAG or
the RVM or by functional inactivation of cells in these
brainstem regions [14,23,26].

A considerable amount of evidence suggests that mu
opioid synapses in the BLA subserve antinociceptive
actions of the amygdala. Autoradiographic studies have
shown that mu opioid receptors are mostly located in the
BLA rather than in other amygdalar nuclei and are more
densely localized than delta or kappa receptors in the BLA
[2,19]. Converging lines of pharmacological and behavioral
data indicate that the non-selective agonist morphine or the
mu-selective agonist [d-Ala², N-MePhe⁴, Gly-ol⁵]-enkephal-
alin (DAMGO) is more effective in producing antinocicep-
tion in rats as measured in the radiant heat-evoked TF test or
in the tail shock-induced vocalization test, when applied into
BLA rather than into any other amygdalar region
[12,23,25]. Thus, mu opioid receptors in the BLA are believed to be critical for amygdala contributions to
descending pain control.

However, we have been unable to make firm conclusions
regarding direct interactions of DAMGO with mu receptors
in the BLA due to little existing evidence for local
mechanisms of mu opioid actions on amygdalar neurons.
Accordingly, in the present study, we tested the ability of
several mu receptor antagonists and related compounds to
block antinociception produced by intra-BLA injections of
DAMGO.

2. Results

2.1. Experiment 1: BLA pretreatment with the non-selective
opioid antagonist naltrexone

Sixteen rats with bilateral injection sites located inside
the BLA were accepted for statistical analyses. One rat was
excluded due to missing the target site. Location of
injections for the accepted animals is depicted in Fig. 1.

Fig. 2 illustrates dose response curves for the non-
selective opioid antagonist naltrexone against DAMGO. This figure was constructed with mean TF latency data for
baseline and naltrexone pre-DAMGO trials and the data
obtained at 0, 10, 20 and 30 min after DAMGO injection.

Intra-BLA infusion of DAMGO elevated TF latencies
over time. An omnibus test of the analysis of variance
(ANOVA) on TF latency data for post-DAMGO injection
period yielded a significant main effect for time ($F_{3,36} = 37.09, P < 0.001$).

Naltrexone was effective in blocking DAMGO-induced
antinociception. The overall data analysis for the post-
DAMGO injection period produced a significant main effect
for group ($F_{3,12} = 6.35, P < 0.01$). Subsequent post hoc
comparisons on the group revealed that rats pretreated with
intermediate (2.50 µg) or high (3.75 µg) dose of naltrexone
were capable of attenuating the DAMGO-induced TF

inhibition, relative to animals pretreated with saline.
Furthermore, intermediate and high doses of naltrexone
significantly prevented elevation of the TF latencies at 20
and 30 min. The post hoc comparisons over each time point
showed that at these time points of the post-DAMGO
period, mean TF latencies for animals pretreated with
intermediate or high dose of naltrexone were significantly
lower than those for saline-pretreated subjects.

The ability of naltrexone to prevent DAMGO effects
became more evident over time, as indicated by a significant
group by time interaction ($F_{9,36} = 4.64, P < 0.001$).

2.2. Experiment 2: BLA pretreatment with the mu-selective
antagonist β-FNA

BLA placement of injections for fifteen accepted rats is
depicted in Fig. 3. Four animals were excluded due to
injection sites outside the BLA or reduced body weight on the
testing day (less than 90% relative to their presurgery weight).

Fig. 4 displays mean TF latencies for pre- and post-
DAMGO injection trials. Intra-BLA application of
DAMGO elevated TF latencies over time. An overall test
on TF latency data for post-DAMGO injection trials showed
a significant main effect for time ($F_{3,36} = 52.15, P < 0.001$).

Notably, this DAMGO-induced antinociception was
prevented by intra-BLA infusion of the mu-selective
antagonist beta-funaltrexamine (β-FNA). A significant
main effect for group was seen in the overall test
($F_{2,12} = 6.49, P < 0.05$). Subsequent post hoc comparisons on the
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Fig. 2. Dose response function for rats injected with naltrexone prior to DAMGO. Pretreatment with the intermediate (grey square) and high (black square) dose of NTX prevented inhibition of the TF reflex by DAMGO when applied to the amygdala. Each number in the legend indicates the dose of NTX or DAMGO contained in an injection volume of 0.25 µl per side. This way of designations is also applied to Figs. 4, 6 and 8. *Indicates a statistical significance (α = 0.05) resulting from Tukey comparisons of NTX versus saline-pretreated animals in TF latencies, at a given time point. NTX, naltrexone; SAL, saline. BL, baseline trials; Pre-DAMGO, time period prior to application of DAMGO.

2.3. Experiment 3: BLA pretreatment with the mu-selective antagonist CTAP

Twenty-nine rats were accepted for data analyses (Fig. 5), excluding three animals due to their injection sites outside the BLA.

Fig. 6 shows mean TF latencies for baseline, the mu antagonist d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr (CTAP) pre-DAMGO and post-DAMGO injection trials. The TF latency data are displayed with three separate subfigures to improve clarity. Intra-BLA application of DAMGO elevated TF latencies over time. Each overall F test on post-DAMGO TF latency data for each subfigure A, B or C representing respective low (0.168 µg) (F3,21 = 5.74, P < 0.01), intermediate (0.25 µg) (F3,18 = 14.63, P < 0.001) or high (0.50 µg) (F3,27 = 18.57, P < 0.001) dose of DAMGO resulted in a significant main effect for time.

However, in no case observed were there significant differences between groups as a function of CTAP (1.25 or 62.5 ng) versus DAMGO (0.168, 0.25 or 0.50 µg) treatment. The omnibus F tests on TF latency data for post-DAMGO injection period as reflected in the subfigures A, B and C produced no significant main effects for group (F < 1.71, P > 0.23). In addition, group by time interaction was not seen in any case (F < 1.0).

2.4. Experiment 4: BLA pretreatment with the G protein inhibitor PTX

Fig. 7 depicts injection sites for ten accepted rats. Three other animals were not included due to loss of body weight prior to the TF test day.
Fig. 8 illustrates mean TF latencies for pre- and post-DAMGO injection trials. Infusion of DAMGO into the BLA was effective over time in inhibiting TF reflexes. An omnibus test on TF latency data for post-DAMGO injection period showed a significant main effect for time ($F_{3,24} = 18.24, P < 0.001$).

This DAMGO effect was blocked by pretreatment of BLA with the guanine nucleotide binding protein (G protein) inhibitor pertussis toxin (PTX). The overall test resulted in a significant main effect for group ($F_{1,8} = 5.28, P < 0.05$). Subsequent pair-wise $t$ tests further indicated that at 30 min of the post-DAMGO period, PTX in the BLA significantly prevented the DAMGO-induced suppression of TF reflexes ($t = 8.06, P < 0.05$).

During the post-DAMGO injection period, differences in TF latencies between saline and PTX-pretreated rats became more evident over time, as implied by a significant group by time interaction ($F_{3,24} = 3.60, P < 0.05$).

2.5. Experiment 5: determination of the spread of DAMGO

A set of autoradiograms for one rat was excluded due to an error in film processing. Fig. 9 illustrates data for the other two rats. Representative registered images of the spread of $[^3H]$-DAMGO (for the left hemisphere) are seen in Fig. 9A. In this figure, the upper and lower serial panels represent small and large diffusions, respectively. For clarity, estimated coronal and sagittal views were reconstructed based on ranges of the two series of visualized images (Figs. 9B and C).

Most of the injected $[^3H]$-DAMGO labeled brain tissues in the BLA. Estimation of three-dimensional volume of the radiolabeled brain tissues revealed that 100% (0.892 mm$^3$; for the small diffusion) and 82.2% (1.562 mm$^3$; for the large diffusion) of labeled tissue was restricted to the BLA. Approximately 2.9% (0.055 mm$^3$) and 14.7% (0.279 mm$^3$) for the large diffusion covered respective CeA and caudate putamen (adjacent brain regions).

3. Discussion

In the present series of experiments, intra-BLA application of a range of doses (0.168–0.50 μg) of DAMGO, a potent $\mu$ opioid receptor agonist, suppressed TF reflexes in anesthetized rats, replicating previous work from our laboratory [13,14,42]. This opioid antinociception was blocked by pretreatment of the BLA with the non-selective opioid antagonist naltrexone. This observation supports the expected interaction of DAMGO with opioid receptors. Furthermore, the $\mu$-selective effects are supported by previous findings from our laboratory showing that DAMGO in concentrations similar to those used here is capable of elevating TF latencies while the $\kappa$ agonist (trans-3, 4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)-cyclohexyl)-benzene acetamide methanesulfonate-hydrate, or U50, 488H) or the $\delta$ agonist ([D-Pen$_2$, D-Pen$_5$]-enkephalin, or DPDPE) is not in the BLA [13].

However, there may still be reason to doubt that the DAMGO’s antinociceptive effect as shown in the present
along with previous observations by our group [13,14,42] has resulted from selective interactions of DAMGO with mu receptors in the BLA, since higher concentrations of this compound have been required in all these studies for producing a significant level of antinociception. Indeed, we have needed concentrations of DAMGO in the BLA that are 10–100 times higher than effective doses of the same peptide applied to the PAG or cerebral ventricles [28,43].

When one considers DAMGO’s impressive affinity and selectivity for mu receptors (i.e., several hundred or thousand times higher for mu than other types of opioid receptors) [47], the current range of doses of DAMGO may have acted primarily on mu receptors. However, the general observation that high concentrations of opioid ligands can reduce receptor subtype specificity [11] still may suggest that the present (or our previous) use of higher concentrations of DAMGO may have worked through other populations of receptors in the amygdala. In the present study, we thus subsequently introduced pharmacological antagonistic probes with several mu opioid inhibitors to investigate whether the DAMGO’s antinociceptive effect resulted from its direct actions on mu receptors in the BLA.

Pretreatment of BLA neurons with h-FNA known as a mu receptor antagonist blocked the increase in TF latencies following an infusion of DAMGO into the same site. Indeed, this h-FNA’s antagonism of the DAMGO-induced antinociception was effective at 14 min and 24 min or more of the post-DAMGO time period (data not shown). This h-FNA’s antagonistic effect against mu opioids is consistent with existing data that subcutaneous (s.c.) or intra-PAG application of beta-FNA 24 h prior to TF testing blocks antinociception following intracerebroventricular (i.c.v.) or intra-amygdala infusion of the mu agonist DAMGO or morphine [30,31].

Some existing evidence states that beta-FNA may have kappa agonist [34,44] and delta antagonist properties
but other accounts of its pharmacological properties still support an antagonist action on mu receptors [8,18,20,41]. First, β-FNA may work as a short-lasting kappa agonist. For example, pharmacological and behavioral work has demonstrated that s.c. infusion of β-FNA inhibits writhing in mice induced by acetic acid, and this antinociceptive effect is maximal 25 min after the β-FNA application [44]. This study has further documented that at this time interval, the β-FNA maximally enhances the antinociception produced by the s.c. injected kappa agonist nalorphine. In contrast, the present study used a long (24 h) time interval between β-FNA injection and the nociception test. This minimized the possibility that kappa receptors may be implicated in β-FNA’s action. Furthermore, when noting that β-FNA binds irreversibly to mu rather than kappa receptors at a low concentration (less than 2 μM) [41], our present effective blockade of DAMGO by a low concentration (16 nM equivalent to the present dose 2.0 μg/0.25 μl) of β-FNA can also exclude another possibility that the current β-FNA may act on kappa receptors. Thus, the present antagonistic effect of β-FNA against DAMGO is attributable to β-FNA’s direct interactions with mu rather than kappa receptors. Second, at high concentrations (78 nM or more), β-FNA has been reported to irreversibly bind to delta receptors as well as to mu receptors [41]. However, several other studies have demonstrated that at a low concentration, β-FNA has only mu antagonist features. For example, i.c.v. application of 20 nM (10 μg) of β-FNA 24 h prior to ligand-receptor binding assay significantly decreases the amount of DAMGO binding due to a desensitization of mu receptors, while the same manipulation fails to prevent the delta ligand DPDPE’s binding to delta receptors [8,17,18,20]. In agreement, i.c.v. pretreatment (24 h) of 20 nM (10 μg) of β-FNA attenuates the mu

Fig. 9. The estimated spread of [3H]-DAMGO injected in the amygdala. (A) Two series of autoradiograms overlayed on corresponding Nissl stained sections representing small (upper panels) and large (lower panels) diffusions. (B) Serial reconstruction of coronal sections for small (red) and large (blue) diffusions. (C) Reconstruction of sagittal sections (at 5 mm lateral to the midline) for the diffusions. The numbers seen in panels (A and B) indicate distances (mm) posterior to bregma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
agonist morphine-induced, but not the delta agonist DPDPE-produced, analgesia [18]. In light of these observations, in the present study, beta-FNA is likely to have acted on mu rather than delta receptors.

Unexpectedly, the current intra-BLA infusion of the potent mu receptor antagonist CTAP failed to prevent the antinociception induced by DAMGO into the BLA. Previous data from our laboratory indicate that rats injected into the brainstem with a dose of CTAP (1.25 ng, identical to the present low dose) blocked the increase in TF latencies following intra-BLA infusion of DAMGO [33,42]. The same experimental protocols including time interval (10 min) between CTAP and DAMGO injections as utilized in these studies were applied to the present CTAP versus DAMGO experiment. However, in the current study, neither this (1.25 ng) nor a higher (62.50 ng) dose of CTAP injected into the BLA was able to prevent antinociception induced by DAMGO applied into this same site. The present range of doses of CTAP should be sufficient based on prior work. That is, the current high dose of CTAP was fifty times higher than the previous effective dose in the brainstem which reduced 70–80% of antinociception following intra-BLA injection of DAMGO [33,42] but failed to affect this DAMGO-induced antinociception. In contrast, the present effective dose (2.00 μg) of beta-FNA was only twice the effective dose (equivalent to the present dose of 1.00 μg) in the brainstem which decreased 68% (approximately equipotent to the effect by the dose of CTAP in the brainstem) of antinociception seen after intra-amygdala application of morphine [31].

As stated above, in the present study, CTAP’s failure to block the DAMGO effect is obviously discrepant to the beta-FNA’s effective blockade of the DAMGO effect. Although it is not clear why this differential effect was observed after CTAP versus beta-FNA pretreatment of the BLA, a possible explanation may be made in light of existing pharmacological evidence. The third extracellular loop (EL3) of the mu receptor has been shown to be critical for the selectivity of several mu ligands. Notably, whereas the EL3 of the mu receptor is necessary for binding of mu ligands including DAMGO and beta-FNA [8,45], this loop is not critical for binding of CTAP [45]. Thus, the present failure of CTAP to prevent the DAMGO-induced antinociception may have resulted from CTAP versus DAMGO’s incongruence in effective binding sites on the mu receptor. In contrast, beta-FNA’s effective blockade of the DAMGO’s effect is thought to have arisen from beta-FNA’s prevention of DAMGO’s action on the EL3 of the mu receptor.

The idea that DAMGO interacts with mu receptors in the BLA can be further supported by other data from our recent work with antisense oligodeoxynucleotides (ODN) targeting opioid receptors. Inhibition of the synthesis of mu receptor subunits in the BLA via intra-BLA application of antisense ODN can prevent antinociception induced by intra-BLA injection of DAMGO ([39]; manuscript in progress). This same manipulation also reduces the amount of [3H]-DAMGO’s specific binding within the BLA.

It has been well documented that DAMGO interacts with G-protein-coupled mu receptors in brain regions including the amygdala. Opioid receptors are known to couple to PTX-sensitive Gα and/or Gα subtypes of G proteins [16], which inhibit accumulation of adenylate cyclase [7] resulting in hyperpolarization by modulating potassium and/or calcium channels [16]. DAMGO has been found to stimulate 5’-[y-S thio]-triphosphate (GTPyS) binding to G proteins in brain regions including the BLA, and this effect can be blocked by beta-FNA [21]. Behaviorally, i.e. v. [43] or intra-PAG [29] pretreatment with PTX has been observed to inhibit antinociception produced by morphine or DAMGO infusion into the same regions. A similar result was obtained in the present study in which BLA treatment with PTX prior to TF testing prevented antinociception produced by DAMGO into the BLA. Indeed, this PTX’s blockade of DAMGO effect was seen at 12 min and 22 min or more of the time period after DAMGO injection (data not shown). Thus, the current finding suggests that the DAMGO-induced antinociception has arisen from an interaction of DAMGO with G-protein-coupled mu receptors in the BLA.

The primary site of action of DAMGO in the present study is the BLA. In the current autoradiographic study, rats given intra-BLA injections of [3H]-DAMGO revealed limited spread (less than 10% of the [3H]-DAMGO, at average) to structures outside the target region the BLA, indicating that intra-BLA applied DAMGO acts directly on cells in the BLA. Furthermore, the present study (data not shown) along with previous work by our group [13,42] has observed that TF latencies during post-DAMGO injection period for rats with injection sites outside the BLA are comparable to the baseline level. Thus, converging lines of these observations suggest that the present antinociception following intra-BLA injection of DAMGO has arisen from DAMGO’s direct action on neurons in the BLA.

Taken together, intra-BLA administration of DAMGO suppresses radiant heat TF reflexes in anesthetized rats. This antinociceptive effect is blocked by intra-BLA application of a mu opioid antagonist and a G protein inhibitor, suggesting that the opioid-related antinociception is achieved through a direct interaction of the mu ligand DAMGO with G-protein-coupled mu receptors in the BLA. Accordingly, the findings from our present study firmly support the idea that mu opioid receptors in the BLA contribute to the descending pain modulation.

4. Experimental procedure

4.1. Experiment 1: pretreatment of the BLA with naltrexone

4.1.1. Subjects

Seventeen male Long Evans rats (350–500 g) obtained from Harlan Sprague–Dawley (Madison, WI) served as
subjects. Each animal was housed in a hanging stainless steel cage and allowed free access to rat chow and water. A 14:10 h light/dark cycle and a temperature of 22 °C were maintained in the vivarium. All experimental manipulations were conducted during the light portion of the cycle. All of the present procedures employing animals were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin–Milwaukee.

4.1.2. Preparations and procedures

Our present preparation and procedures were based on previous standard methods utilized by our group [13,14,42]. A custom-made radiant heat source was employed that contained a 3-mm diameter aperture beneath which a 500-W projection bulb was mounted in a forced air-cooled, stainless steel enclosure. A thermocouple located within the apparatus detected the stimulus intensity on each trial. Temperature of the surface of the apparatus was maintained between 28 °C and 32 °C by the cooling fan. Lateral deflection of the tail resulted in triggering of a photocell and digital timing circuit that deactivated the heat source and subsequently recorded the TF latency to the nearest 10 ms. The heat stimulus intensity was adjusted to produce baseline TF latencies of 4–6 s. The same stimulus temperature was used throughout the test session. If a response did not occur within 20 s, the testing was automatically terminated to prevent tissue damage, and the TF latency was recorded as 20 s. Presentation of the radiant heat stimulus was computer controlled.

Prior to the TF testing session, each animal was anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/ml/kg). A polyethylene catheter (PE-20) was inserted into the jugular vein 1.0 cm deep and secured with silk sutures. Each subject as mounted on a Kopf stereotaxic frame was maintained in a slight recovery from surgery, the animals were administered with anesthetizet with stainless steel screws and cement. After 7 days of surgery) were used for Experiment 2. Prior to surgery each rat was injected i.p. with ketamine HCl (100 mg/ml/kg) and sodium pentobarbital (50 mg/ml; 0.15 ml/rat) and mounted in the stereotaxic frame. Bilateral 26-gauge chronic guide cannulae were implanted targeting the BLA and secured immediately after the catheterized animal was mounted on the stereotaxic frame.

When baseline TF latencies reliably measured 4–6 s, a standard testing protocol was initiated, in approximately 1 h after the initiation of MH infusion. TF latency was recorded every 2 min throughout the testing session. Immediately after the 5th baseline trial, naltrexone (1.25, 2.50 or 3.75 µg/0.25 µl per side) or the sterile isotonic saline vehicle was bilaterally administered into the BLA via 33-gauge stainless steel injection cannulae that extended 1 mm beyond the guide cannula tips, followed by infusion of DAMGO (0.25 µg/0.25 µl per side) into the same site 10 min later. Each set of an injection cannula and 1.0 µl Hamilton syringe was connected to tips of a PE-20 tubing. Each drug was infused over 45-s period. The dose of DAMGO utilized in this experiment was chosen from a previous range of doses effective in inhibiting TF reflexes of rats [13].

4.1.3. Histology

After testing, each animal was injected with sodium pentobarbital (100 mg/ml/kg) and perfused with 0.9% saline followed by a buffered 10% formalin solution. The head was removed with cannulae in place and soaked overnight in buffered formalin. Brains were extracted and put in 20% sucrose formalin for 1 day. Frozen 40 µm brain sections were obtained and Nissl stained. The location of injection sites was determined with the aid of a rat brain atlas [32].

4.1.4. Data analyses

TF latency data for baseline, pre-DAMGO and post-DAMGO trials were analyzed with a repeated measures ANOVA. Following the overall analysis, post hoc comparisons on group were conducted via Tukey HSD test (α = 0.05). This way of data analyses was also applied to TF latency data obtained from Experiments 2 and 3 in which more than two groups of subjects were employed. However, TF data from Experiment 4 using two groups of animals were analyzed with the repeated measures ANOVA, followed by pair-wise t tests to examine whether the two groups differed in TF latencies over each time point.

Mean TF latencies for five baseline and five pre-DAMGO injection trials were averaged, since the TF latencies did not differ between groups or change over time.

4.2. Experiment 2: pretreatment of the BLA with β-FNA

A total of nineteen rats (350–500 g on the day of the surgery) were used for Experiment 2. Prior to surgery each rat was injected i.p. with ketamine HCl (100 mg/ml/kg) and sodium pentobarbital (50 mg/ml; 0.15 ml/rat) and mounted in the stereotaxic frame. Bilateral 26-gauge chronic guide cannulae were implanted targeting the BLA and secured with stainless steel screws and cement. After 7 days of recovery from surgery, the animals were administered with β-FNA (1.00 or 2.00 µg/0.25 µl) [31] or sterile isotonic saline into the BLA. After 24 h [18,31], each animal was TF-tested with DAMGO (0.25 µg/0.25 µl) applied bilaterally to the BLA. Only animals that had recovered 90% or more of their presurgical body weight were used for the TF testing. The other aspects of procedures and methods were same as described for Experiment 1.

4.3. Experiment 3: pretreatment of the BLA with CTAP

In Experiment 3, thirty-two rats (350–500 g) were employed. The same procedures and methods were conducted as in Experiment 1 except that CTAP (1.25 or 62.50 ng/0.25 µl) [33,42] instead of naltrexone was injected into
the BLA prior to application of several doses of DAMGO (0.168, 0.25 or 0.50 µg/0.25 µl) [13] into the same site.

4.4. Experiment 4: pretreatment of the BLA with PTX

As with Experiment 2, thirteen rats were implanted with bilateral chronic guide cannulae aimed at the BLA. After 7 days of recovery from surgery, each animal was given a bilateral injection of PTX (0.25 µg/0.25 µl) or saline into the BLA. Three days after the PTX injection, each animal was TF tested with bilateral administration of DAMGO (0.25 µg/0.25 µl) into the BLA. All the other procedures and methods were same as in Experiment 2.

The choice of PTX dose and the pretreatment period of this chemical were based on previous studies [29,43].

4.5. Experiment 5: autoradiography

The purpose of this experiment was to determine the spread of DAMGO injected in the amygdala. A set of three male Long Evans rats (350—500 g) was implanted with bilateral 26-gauge guide cannulae aimed at the BLA. Each rat was given a bilateral intra-BLA injection of [3H]-DAMGO (0.125 µCi/0.25 µl per side). Twenty minutes after the injection [27], each brain was extracted and immediately frozen. The frozen brains were coronally sectioned (40 µm) and then exposed to tritium-sensitive film (Hyperfilm MP; Amersham Pharmacia Biotech) under darkroom safelight. The exposed brain sections were contained in X-ray film cassettes and stored at 4 °C for 6 weeks. Latent images were developed and fixed with X-ray developer and fixer (Milwaukee X-ray Technics, Milwaukee, WI) for 12 weeks. Latent images were then Nissl stained. The autoradiograms were registered on the stained sections using a computerized densitometry and image analysis system (MCID, Imaging Research Inc).

Acknowledgments

We thank Dr. Elliot Stein at the Medical College of Wisconsin for his generous help with the autoradiography. This research was supported by NIDA Grant DA09429 to F.J. Helmstetter.

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