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Inhibition of glutamatergic transmission by morphine in the basolateral amygdaloid nucleus reduces pain-induced aversion

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Abstract

We examined the role of glutamatergic transmission within the basolateral amygdaloid nucleus (BLA) in pain-induced aversion using a conditioned place paradigm and an in vivo microdialysis technique in rats. Microinjection of MK-801 (1 or 10 nmol/side) into the bilateral BLA 5 min before intraplantar injection of formalin dose-dependently attenuated formalin-induced conditioned place aversion (F-CPA) without affecting nociceptive behaviors, such as lifting, licking, and biting. On the contrary, microinjection of neither CNQX (30 nmol/side) nor AP-3 (30 nmol/side) showed any significant effect on F-CPA. Microdialysis experiments revealed that intraplantar injection of formalin induced an increase in the extracellular glutamate level within the BLA. This increase in glutamate was suppressed by morphine perfusion (100 μM) via the microdialysis probe. Moreover, intra-BLA injection of morphine (10 μg/side) 5 min before formalin injection attenuated F-CPA without affecting nociceptive behaviors. These results suggest that glutamatergic transmission via NMDA receptors in the BLA plays a crucial role in the pain-induced aversion, and that in addition to the well-characterized effects on the sensory component of pain, morphine also influences the affective component of pain through an inhibitory effect on intra-BLA glutamatergic transmission.
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1. Introduction

Pain is an unpleasant sensory and emotional experience. The neural systems underlying the sensory component of pain have been studied extensively, but we are only beginning to understand those underlying its affective component. Using a conditioned place paradigm, Johansen et al. (2001) demonstrated that excitotoxic lesion of anterior cingulate cortex (ACC) suppressed conditioned place aversion (CPA) induced by the intraplantar injection of formalin without reducing formalin-induced nociceptive behaviors, suggesting the important role of this brain region in the affective component of pain. Another key neural substrate implicated in the emotional responses to noxious stimulus is the amygdaloid complex. We previously showed that the central (CeA) and basolateral (BLA) amygdaloid nuclei are differently involved in intraplantar formalin-induced CPA (F-CPA) and intraperitoneal acetic acid-induced CPA (A-CPA) (Tanimoto et al., 2003). Moreover, it has been reported that the CeA plays an important role in noxious stimulus-evoked vocalizations, which are related to the pain-induced aversive emotion (Han and Neugebauer, 2005). However, it remains to be determined which neurochemical(s) play role(s) during signal transmission in pain-induced aversive responses within these amygdaloid nuclei.

Glutamatergic transmission plays key roles in nociceptive transmission at the spinal and supraspinal levels. At the spinal level, noxious stimulus-evoked glutamate release has been demonstrated in the spinal dorsal horn (Malmberg and Yaksh, 1995; Ueda et al., 1995), and intrathecal injections of glutamate receptor antagonists have been reported to suppress thermal and mechanical hyperalgesia (Ren et al., 1992; Okano et al., 1998).

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At the supraspinal level, extracellular glutamate levels increase in the ventroposterolateral thalamic nucleus during nociceptive stimulation (Silva et al., 2001), and NMDA receptor antagonists injected into this nucleus reduce thermal and mechanical hyperalgesia (Kolhekar et al., 1997). In addition, neuronal responses in the primary somatosensory cortex evoked by noxious cutaneous stimuli are suppressed by both NMDA and AMPA/kainate receptor antagonists (Pollard, 2000). These findings suggest an important role of glutamatergic transmission in the sensory component of pain. Recently, Johansen and Fields (2004) demonstrated the suppression of F-CPA by microinjecting a glutamate receptor antagonist into the ACC, suggesting a key role of glutamatergic transmission in the affective component of pain. Thus, we first examined the involvement of intra-BLA glutamatergic transmission in pain-induced aversion using a conditioned place paradigm in rats.

Morphine produces a strong analgesic effect through the activation of opioid receptors. Supraspinal and spinal mechanisms for the pharmacological effects of this drug on the sensory component of pain have been well characterized (Dickenson, 1995; Stamford, 1995). As several lines of evidence suggest that the sensory and affective components of pain are mediated by separate neural systems (Johansen et al., 2001; Tanimoto et al., 2003; Gao et al., 2004), we sought to examine the effects of morphine on the affective component of pain. Specifically, we examined the effects of intra-BLA administration of morphine on pain-induced aversion and glutamate release. The results suggest that morphine suppresses pain-induced aversion, at least in part, through inhibition of intra-BLA glutamatergic transmission, providing the evidence regarding the effects of morphine on the neural systems mediating the affective component of pain.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) weighing 180–220 g at the start of the experiments were used. The animals were housed individually in plastic cages with woodchip bedding at a constant ambient temperature (24 ± 1 °C) under a 12/12 h light/dark cycle, with free access to food and water. All experiments were carried out with the approval of the Institutional Animal Care and Use Committee at Kyoto University and Hokkaido University.

2.2. Drugs

Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). The NMDA receptor antagonist, (5*R*, 10*S*)-(+)-5-methyl-10, 11-dihydro-5H-dibenzo [α, δ] cyclo-hepten-5,10-imine (MK-801), and the AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from RBI (Berkeley, CA, USA). Naloxone hydrochloride and the metabotropic glutamate receptor antagonist, 2-amino-3-phosphonopropionic acid (AP-3), were purchased from Sigma (St. Louis, MO, USA). These drugs were dissolved in sterile, isotonic saline.

2.3. Microinjection

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Stainless steel guide cannulae (o.d., 0.5 mm; i.d., 0.22 mm) were implanted bilaterally 5.5 mm above the BLA (coordinates for the BLA injection site from bregma: AP 1.8 mm, ML 4.8 mm, DV 8.5 mm) (Paxinos and Watson, 1998). After surgery, rats were individually returned to their cages and left to recover for at least 5 days before the experiments. For microinjection, stainless steel injection cannulae (o.d. 0.2 mm; i.d. 0.08 mm) were inserted bilaterally into the guide cannulae (injection cannulae protruded 5.5 mm from the tip of guide cannulae to reach the BLA injection site). Injection cannulae were attached to a microinfusion pump via PE 8 tubing. Microinjections of drug or vehicle were made at a rate of 1 μl/2 min (1 μl total volume/side), and the injection cannulae were left in place for 2 min after microinjection. Bilateral microinjections of MK-801 (1 or 10 nmol/side), CNQX (30 nmol/side), AP-3 (30 nmol/side) or morphine (10 μg/side) were carried out 5 min before the animals received the formalin injection.

2.4. Conditioned place aversion test

The CPA test was conducted as previously described (Tanimoto et al., 2003). A shuttle box, composed of two equal-sized compartments with distinct tactile and visual cues (one was black with a smooth floor and the other was white with a textured floor) under dim illumination (25 ± 5 lx at the center of the box), was used for a consecutive 4-day experimental procedure. On days 1 (habituation session) and 2 (preconditioning session), the rats freely explored the two compartments for 900 s, and the time spent in each compartment over 900 s was measured automatically (KN-80; Natsume Seisakusho, Tokyo, Japan). Rats that spent more than 80% of the total time (>720 s) in one side on day 2, or that spent more than 600 s in one side on day 1 and more than 600 s in the other side on day 2 were eliminated from the following experiments. No significant difference was detected between the time spent in the black (437 ± 12.2 s, $n = 116$) and white (462 ± 12.2 s, $n = 116$) compartments, indicating the absence of a significant bias in preference for a compartment before conditioning. In this study, we used a bias-like protocol (Tzschentke, 1998), and thus designated the compartment in which the rat spent longer than 50% (>450 s) of the preconditioning session time as the pain-paired compartment for each animal. On day 3 (conditioning session), place conditioning was performed as follows: in the control session, each rat was given an intraplantar injection of saline (100 μl) into the left hindpaw, and was confined in the non-pain-paired compartment for 1 h. The rat was then given an intraplantar injection of 2% formalin (100 μl) into the right hindpaw, and was confined in the pain-paired compartment for 1 h. On day 4 (test session), each rat was allowed to explore the two compartments freely, and the time spent in each compartment over 900 s was recorded. The CPA scores were calculated by subtracting the time spent in the pain-paired compartment in the test session from that in the preconditioning session.

2.5. Formalin test

For the measurement of formalin-induced nociceptive behavior, each rat was placed in a Plexiglas cylinder (30 cm diameter, 50 cm high) for 30 min to acclimatize it to the experimental environment. The rats were then given an intraplantar injection of 2% formalin (100 μl) into the right hindpaw, and immediately returned to the cylinder. The time spent lifting, licking or biting the formalin-injected paw was recorded for each 5-min (300-s) period over 50 min.

2.6. In vivo microdialysis

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and were unilaterally implanted with guide cannulae (o.d. 0.5 mm, AG-8; Eicom, Kyoto, Japan), the tips of which reached the BLA (coordinates from bregma: AP 1.8 mm, ML 4.8 mm, DV 8.0 mm). After the recovery period, microdialysis experiments were carried out with unanesthetized and freely moving animals placed in a Plexiglas chamber (30 cm wide × 30 cm long × 35 cm high). Ringer-primed microdialysis probes (dialysis membrane: length 1.0 mm, o.d. 0.22 mm, A-I-8-01; Eicom) were inserted into the guide cannulae (probes protruded 1.0 mm from the tip of guide cannulae), and were continuously perfused with Ringer's solution (Na^+ 147 mM, K^+ 4 mM, Ca^{2+} 2.3 mM, Cl^- 155.6 mM) at a constant flow rate of 2 μl/min. After a stabilization period (>1 h), nine 10-min dialysates were collected. The first three samples were taken as baseline samples. Immediately

after collection of the last baseline sample, each rat was given an intraplantar injection of 2% formalin or saline (100 μl). Effects of morphine on formalin-induced glutamate release were examined by adding this drug to Ringer's solution at a concentration of 100 μM. The glutamate concentration in each dialysate sample was measured using a high-performance liquid chromatography system equipped with an electrochemical detector. Pre-column derivatization was performed with *o*-phthaldialdehyde and 2-mercaptoethanol at 5 °C for 150 s. The derivatives were then separated in a liquid chromatography column (MA-5ODS, 4.6 mm i.d. × 150 mm; Eicom) at 30 °C with 30 % methanol in 0.1 M phosphate buffer (pH 6.0), and were measured with an electrochemical detector.

2.7. Histology

After completion of the experiments, histological analyses were performed. For the microinjection experiments, tionin (1 μl) was injected into the BLA. The brains were then removed, and coronal sections were cut in a cryostat at a thickness of 50 μm, stained with cresyl violet, and analyzed to assess the injection sites under the microscope (Fig. 1a). Localization of the microdialysis probes was examined in Nissl-stained coronal sections (50 μm) under the microscope. Data from rats with correct placements of the bilateral microinjections or microdialysis were used for the following analyses.

2.8. Statistical analyses

CPA scores were compared using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls *post hoc* test when comparing more than two groups, or Student's *t*-test when comparing two groups. The data from the formalin test were analyzed with a two-way ANOVA followed by the Bonferroni *post hoc* test. *In vivo* microdialysis data were assessed using a two-way ANOVA followed by the Bonferroni *post hoc* test, while data for the area under the curve (AUC) of extracellular glutamate levels were compared using a one-way ANOVA followed by the Newman–Keuls *post hoc* test. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of glutamate receptor antagonists on F-CPA and nociceptive behaviors

To determine the role of intra-BLA glutamatergic transmission in the affective component of pain, we examined the effects of microinjecting glutamate receptor antagonists into the bilateral BLA on F-CPA (Fig. 1b). Microinjections of MK-801 (1 or 10 nmol/side), a NMDA receptor antagonist, into the bilateral BLA dose-dependently reduced F-CPA. A one-way ANOVA performed on CPA data demonstrated a significant difference among groups ($F_{4,52} = 4.95$, $P < 0.01$). MK-801 at a dose of 10 nmol/side (1.1 ± 25.9 s; $P < 0.01$), but not 1 nmol/side (68.0 ± 33.8 s; $P > 0.05$), significantly attenuated F-CPA, compared to the vehicle-injected group (136 ± 20.1 s). In contrast, microinjections of the AMPA/kainate receptor antagonist CNQX (30 nmol/side, 86.5 ± 25.4 s) or the metabotropic glutamate receptor antagonist AP-3 (30 nmol/side, 132 ± 23.8 s) had no significant effect on F-CPA. To examine whether intra-BLA MK-801 *per se* produced conditioned place preference (CPP) or CPA, MK-801 (10 nmol/side) was microinjected into the bilateral BLA of rats conditioned with intraplantar injection of saline instead of formalin. Neither CPP nor CPA was induced by intra-BLA injection of MK-801, indicating that MK-801 alone does not have any motivational effects when injected into the BLA at this dose.

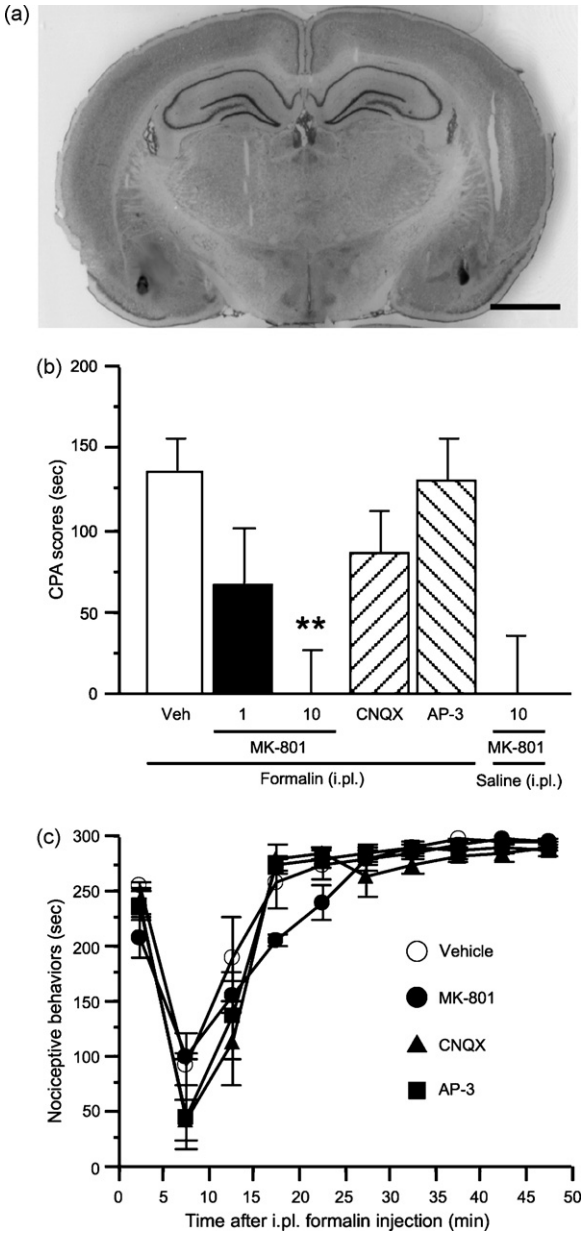

Fig. 1. Effects of intra-BLA injection of glutamate receptor antagonists on F-CPA and nociceptive behaviors. (a) Injection sites were checked by the injection of tionin (1 μl) after the behavioral experiments. (b) The effects of bilateral intra-BLA injections of vehicle ($n = 18$), MK-801 (1 nmol/side, $n = 7$; 10 nmol/side, $n = 10$), CNQX (30 nmol/side, $n = 11$), or AP-3 (30 nmol/side, $n = 11$) on F-CPA. In addition, intra-BLA injection of MK-801 (10 nmol/side, $n = 6$) without formalin injection did not induce CPP or CPA. Data are presented as means ± S.E.M. $^{**}P < 0.01$, Newman–Keuls *post hoc* test as compared to the vehicle-injected group. (c) The effects of intra-BLA injections of vehicle ($n = 5$), MK-801 (10 nmol/side, $n = 5$), CNQX (30 nmol/side, $n = 5$), or AP-3 (30 nmol/side, $n = 7$) on formalin-induced nociceptive behaviors. The time spent lifting, licking, or biting the formalin-injected paw is shown. Data are presented as means ± S.E.M.

It is well-known that intraplantar injection of formalin produces biphasic nociceptive responses in behavior such as lifting, licking and biting. Fig. 1c showed that intra-BLA injection of either MK-801, CNQX or AP-3 did not affect the first and second phases of formalin-induced nociceptive behaviors (ANOVA, $P > 0.05$).

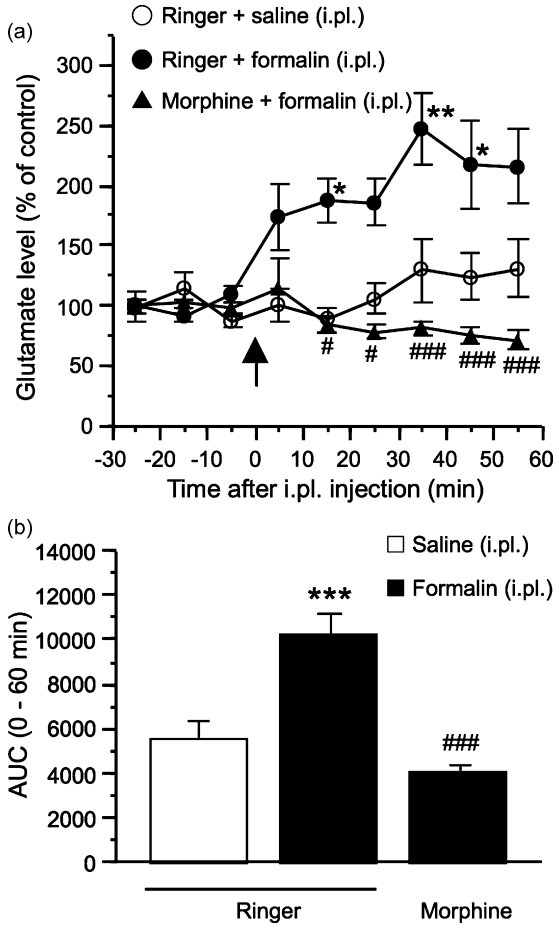

Fig. 2. Pain-induced glutamate release in the BLA. (a) Formalin ($n = 23$) or saline ($n = 9$) was injected into the hindpaw at time 0. In the group ($n = 6$) of the formalin-injected animals, morphine (100 μM) was added to the perfusing Ringer's solution. Dialysates were collected every 10 min. Data are expressed as the means ± S.E.M. of the percent basal value, calculated as an average of three consecutive dialysates before intraplantar injection. $^{*}P < 0.05$, $^{**}P < 0.01$, Bonferroni *post hoc* test as compared to the Ringer + intraplantar (i.p.l.) saline group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, Bonferroni *post hoc* test as compared to the Ringer + i.p.l. formalin group. (b) Each column represents the mean ± S.E.M. of AUC values for glutamate release during the 0–60-min period. $^{***}P < 0.001$, Newman–Keuls *post hoc* test as compared to the Ringer + i.p.l. saline group. $^{\#\#\#}P < 0.001$, Newman–Keuls *post hoc* test as compared to the Ringer + i.p.l. formalin group.

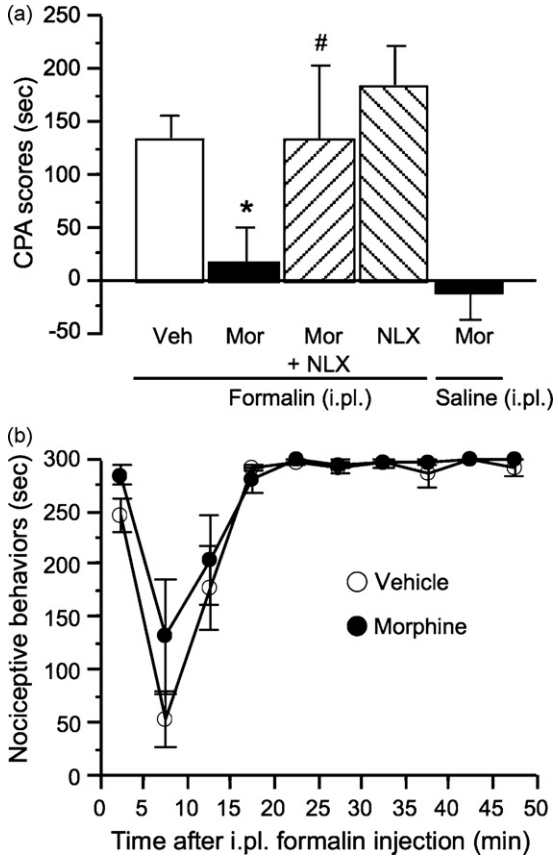

Fig. 3. Effects of intra-BLA injection of morphine on F-CPA and nociceptive behaviors. (a) The effects of bilateral intra-BLA injections of vehicle ($n = 18$), morphine (10 μg/side, $n = 12$), morphine (10 μg/side) + naloxone (30 μg/side) ($n = 9$), or naloxone (30 μg/side, $n = 9$) on F-CPA. In addition, intra-BLA injection of morphine without formalin injection did not induce CPP or CPA ($n = 7$). Data are presented as means ± S.E.M. $^{*}P < 0.05$, Newman–Keuls *post hoc* test as compared to the vehicle-injected group. $^{\#}P < 0.05$, Newman–Keuls *post hoc* test as compared to the morphine-injected group. (b) The effects of intra-BLA injections of vehicle ($n = 6$) or morphine (10 μg/side, $n = 5$) on formalin-induced nociceptive behaviors. Time spent lifting, licking, or biting the formalin-injected paw is shown. Data are presented as means ± S.E.M.

3.2. Pain-induced glutamate release in the BLA

In vivo microdialysis experiments demonstrated that intraplantar injection of formalin produced a long-lasting increase in extracellular glutamate levels in the BLA (Fig. 2a). This increase was suppressed by perfusion of morphine (100 μM) via the microdialysis probe. A two-way ANOVA showed a significant effect of treatment ($F_{2,261} = 31.63$, $P < 0.001$) and a significant interaction between treatment and time ($F_{16,261} = 2.45$, $P < 0.01$). AUC data also showed that intraplantar formalin injection significantly elevated glutamate release compared to the saline-injected group (ANOVA, $P < 0.001$), and that perfusion of morphine significantly suppressed formalin-induced glutamate release (ANOVA, $P < 0.001$; Fig. 2b).

3.3. Effects of morphine on F-CPA and nociceptive behaviors

Microinjections of morphine (10 μg/side) into the bilateral BLA produced suppression of F-CPA that was antagonized by the simultaneous injection of the opioid receptor antagonist naloxone (30 μg/side) (Fig. 3a). Morphine (19.3 ± 31.6 s; $P < 0.05$) produced a significant reduction in CPA score compared to the vehicle-injected animals (136 ± 20.1 s), and this reduction was reversed by co-administration of naloxone (136 ± 68.6 s, $P < 0.05$). Microinjections of naloxone alone tended to enhance F-CPA (184 ± 38.3 s) but it was not significant. To examine whether intra-BLA morphine *per se* produced CPP or CPA, morphine (10 μg/side) was microinjected into the bilateral BLA of rats conditioned with intraplantar injection of saline instead of formalin. Neither CPP nor CPA was induced by intra-BLA injection of morphine, indicating that morphine alone does not have any motivational effects when injected into the BLA at this dose.

Fig. 3b showed that intra-BLA injection of morphine (10 μg/side) did not affect the first and second phases of formalin-induced nociceptive behaviors (ANOVA, $P > 0.05$).

4. Discussion

We previously demonstrated that excitotoxic lesions of the BLA abolished F-CPA without affecting nociceptive behaviors (Tanimoto et al., 2003). Our current study using a conditioned place paradigm and an *in vivo* microdialysis technique extends those findings by showing that enhanced glutamatergic transmission via NMDA receptors in the BLA plays a key role in pain-induced aversion. Importantly, our results showing the suppression of both noxious stimulus-elicited glutamate release and aversive responses by intra-BLA morphine without any effect on nociceptive behaviors suggest a novel effect of morphine on the neural circuit underlying the affective component of pain, in addition to its well-characterized effects on the sensory component of pain.

There are some literatures reporting the antinociceptive effects of intra-BLA injected morphine (MacGraughty and Heinricher, 2002; Nandigama and BorszczG, 2003). They demonstrated the weak to moderate antinociceptive effects of intra-BLA morphine against radiant heat or electrical stimuli to the tail. We also observed weak but significant suppressive effect of intra-BLA morphine on nociceptive behaviors induced by intraplantar injection of the less amount (50 μl) of formalin (data not shown). In this study, for the separation of the affective component of pain from the sensory one, we used the more intense noxious stimulation (i.e., intraplantar injection of 100 μl formalin) to evade the suppressive effect of intra-BLA morphine on nociceptive behaviors (i.e., sensory component of pain).

Microinjection of MK-801, but not CNQX or AP-3, into the BLA attenuated F-CPA. The doses of CNQX and AP-3 used in this study were thought to be sufficient to block the glutamatergic transmission via intra-BLA non-NMDA and metabotropic glutamate receptors, respectively, because the lower doses of these antagonists inhibited the expression of fear-potentiated startle (Kim et al., 1993) or the consolidation of avoidance memory (Bonini et al., 2003) when injected into the BLA. Thus the present results suggest the essential role of intra-BLA NMDA receptors in F-CPA. Similar results have been obtained in the ACC. Johansen and Fields (2004) showed that microinjection of kynurenic acid, a wide-spectrum glutamate receptor antagonist, into the bilateral ACC suppressed F-CPA without reducing formalin-induced nociceptive behaviors. Furthermore, Lei et al. (2004) reported that intra-ACC injection of a NMDA receptor antagonist AP-5, but not an AMPA/kainate receptor antagonist DNQX, significantly attenuated F-CPA. These converging lines of evidence suggest the important role of glutamatergic transmission via NMDA receptors in pain-induced aversion, although further studies will be needed to elucidate how the NMDA receptors function to produce F-CPA independently of AMPA receptor activation. Anatomically, there are direct and indirect reciprocal connections between the amygdala and ACC (Ottersen, 1982; McDonald et al., 1996). It is likely that these brain regions, together with other brain areas including the mediodorsal thalamic nucleus and insular cortex, compose the neural network involved in the negative affective component of pain (Price, 2000).

Since the conditioned place paradigm used in this study is based on associative learning between noxious stimulus-induced aversive emotion and a neutral environmental context, it is difficult to determine whether the reduction in CPA is due to impairment of associative learning or reduction of the primary aversive emotion. Additionally, it is also difficult to discriminate between reduction of the primary aversive emotion and impairment of the ability to memorize the emotion. We previously demonstrated that excitotoxic lesion of BLA did not suppress the A-CPA (Tanimoto et al., 2003), suggesting that disruption of neurotransmission in the BLA is not likely to cause a general dysfunction of associative learning or emotional memory. Therefore, it is likely that the suppression of F-CPA observed in this study was due to reduction of the primary aversive emotion. However, another possibility that the BLA plays a pivotal role specifically in the associative learning or emotional memory related to F-CPA, but not to A-CPA, remains to be elucidated.

Morphine inhibits spinal pain transmission through presynaptic opioid receptors (Dickenson, 1995). Inhibitory effects of this drug on the release of pain-related neurotransmitters, such as substance P (Yaksh et al., 1980; Kuraishi et al., 1983) and glutamate (Malmberg and Yaksh, 1995; Ueda et al., 1995), have been demonstrated in the spinal dorsal horn. This presynaptic inhibition is considered to play an important role in the suppressive effects of intrathecally administered morphine on formalin-induced nociceptive behaviors (Watanabe et al., 2003). In this study, we demonstrated the inhibitory effects of morphine on noxious stimulus-induced glutamate release in the BLA. In this case, morphine suppressed F-CPA without affecting formalin-induced nociceptive behaviors. These results suggest the suppressive effect of morphine specifically on the affective component of pain, at least in part, via the presynaptic inhibition of intra-BLA glutamatergic transmission. However, further studies are needed to clarify the detailed mechanism for the suppressive effect of intra-BLA morphine on F-CPA.

As chronic pain is frequently associated with psychological and emotional dysfunction (McWilliams et al., 2003), studies on the neural circuits and molecular mechanisms for the affective component of pain may have considerable clinical importance in the treatment of chronic pain.

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