Effects of excitotoxic lesions of the central or basolateral nucleus of the amygdala on naloxone-precipitated withdrawal-induced conditioned place aversion in morphine-dependent rats

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Abstract

We examined the effects of discrete, bilateral excitotoxic lesions of the central or basolateral nucleus of the amygdala on naloxone-precipitated withdrawal-induced conditioned place aversion in morphine-dependent rats. Lesions of the central nucleus significantly attenuated the conditioned place aversion, while lesions of the basolateral nucleus had little effect. These results suggest that the central nucleus of the amygdala, rather than the basolateral nucleus, plays a crucial role in the negative affective component of morphine abstinence.

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Chronic use of opiates such as morphine is well known to lead to physical and psychological dependence, which is characterized by the expression of withdrawal symptoms including both physical and affective components, upon cessation of drug administration. In animals, morphine withdrawal produces various characteristic somatic signs, as well as disruption of schedule-controlled operant responses for food [11,16], elevation of intracranial self-stimulation thresholds [23] and aversive avoidance behavior from the environment previously associated with morphine withdrawal (conditioned place aversion) [9,20,25]. These behavioral changes are thought to reflect the negative affective component of morphine withdrawal, such as dysphoria, irritability and anxiety, which might contribute to aversively motivated drug seeking.

The amygdala is a forebrain structure composed of several distinct subnuclei including central (CeA) and basolateral (BLA) nuclei, and is thought to be a key neural substrate underlying emotional responses such as anxiety and fear in both humans and animals [4]. Several studies have investigated the involvement of the amygdala in morphine withdrawal symptoms. It has been reported that bilateral electrical lesions of the bilateral amygdala in morphine-dependent rats reduced typical somatic withdrawal signs such as jumping behavior [1], and that microinjection of opioid antagonists into the amygdala elicited some somatic withdrawal signs [1,19,28]. Furthermore, Stinus et al. reported that microinjection of methylnaloxonium, a hydrophilic opioid antagonist, into the amygdala, as well as the nucleus accumbens, produced morphine withdrawal-induced conditioned place aversion (CPA) [26]. These studies suggest that the amygdala is involved in the negative affective component of morphine abstinence, as well as somatic withdrawal signs, although they did not focus on the individual amygdaloid subnuclei.

Recent evidence has shown that individual amygdaloid subnuclei play distinct roles in several emotional behaviors such as footshock-induced freezing, fear-potentiated startle...
and the Pavlovian suppression of operant responding by a footshock-associated stimulus [10,12,14,21] and relapse to cocaine-seeking behavior [17]. However, it remains unclear which amygdaloid subnuclei are involved in the negative affective component of morphine abstinence. Therefore, in this study, we examined the effects of discrete excitotoxic lesion of CeA or BLA on the naloxone-precipitated withdrawal-induced CPA.

Morphine pellets each containing 75 mg of morphine base (Takeda Chemical Industries, Osaka, Japan) were prepared according to the method of Gibson and Tingstad [6]. A total of 111 male Sprague–Dawley rats weighing 200–300 g was used. They were kept at a constant ambient temperature of 24±1 °C under a 12-h light/dark cycle with free access to food and water. Animals were divided into eight groups, i.e., 12 CeA-sham operated and placebo pellet-implanted, 10 CeA-sham operated and morphine pellet-implanted, 14 CeA-lesioned and placebo pellet-implanted, 15 CeA-lesioned and morphine pellet-implanted, 12 BLA-sham operated and placebo pellet-implanted, eight BLA-sham operated and morphine pellet-implanted, 18 BLA-lesioned and placebo pellet-implanted, and 22 BLA-lesioned and morphine pellet-implanted animals.

Bilateral excitotoxic lesions of CeA or BLA were performed by N-methyl-D-aspartic acid (NMDA) infusion. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and placed on a standard stereotactic apparatus. The guide cannulas were held firmly in place by dental acrylic cement. For lesions of the CeA, each rat was implanted with bilateral guide cannulas (o.d., 0.5 mm; i.d., 0.22 mm) at 1.8 mm caudal to bregma, 4.0 mm lateral to the midline, 3.0 mm below the surface of the skull according to the atlas of Paxinos and Watson [22], and then 0.3 M NMDA (Sigma, St. Louis, USA) dissolved in phosphate-buffered saline was infused bilaterally at 8.0 mm below the surface of the skull in a volume of 0.3 µl/side over 2 min via the stainless steel injection cannula (33-gauge, o.d. 0.2 mm). For lesions of the BLA, each rat was bilaterally implanted with guide cannulas at 1.8 mm caudal to bregma, 4.8 mm lateral to the midline, 3.0 mm below the surface of the skull, and then 0.3 M NMDA was infused bilaterally at 8.5 mm below the surface of the skull in a volume of 0.4 µl/side over 2 min. In both cases, the injection cannulas were left in place for an additional 2 min to prevent backflow of NMDA. Sham-operated rats received identical surgical treatment, but they were not injected with anything. Then, the injection cannulas were removed, and the rats were individually returned to their cages and left to recover for at least 1 week before the experiment.

Conditioned place aversion was conducted as previously described [29]. Briefly, a place conditioning apparatus, consisting of a shuttle box (30×60×30 cm) divided into two equal-sized compartments with distinctive visual color and floor texture, was used to measure naloxone-precipitated, morphine withdrawal-induced aversion. One compartment was black with a smooth floor; the other was white with a textured floor. The time spent in each compartment during a period of 15 min (900 s) was measured automatically (KN-80; Natsume Seisakusyo, Tokyo, Japan). The apparatus was enclosed by a sound- and light-attenuated box under conditions of dim illumination (40 lux) and masking white noise. The experimental process was composed of three distinct sessions: preconditioning session, conditioning session and test session. On the first day (day 1), under light ether anesthesia, rats had either a morphine or placebo pellet implanted in the back of the neck. On days 2 and 3 (preconditioning session), the partition separating the two compartments was raised, and a neutral platform was inserted along the seam separating the compartments. The rats were individually placed on the neutral platform and allowed to freely explore the two compartments for 900 s for habituation to the apparatus (day 2) and to obtain a measure of preference (day 3). The compartment in which the rats spent greater than 50% of the total time (i.e., 450 s) was determined as the naloxone-paired compartment, and the opposite side was as the saline-paired compartment. Rats that spent more than 80% of the time (i.e., 720 s) in one side on day 3, or that spent more than 600 s in one side on day 2 and more than 600 s on the other side on day 3 were eliminated; i.e., two CeA-sham operated and placebo pellet-implanted, three CeA-sham operated and morphine pellet-implanted, one CeA-lesioned and placebo pellet-implanted, one BLA-sham operated and placebo pellet-implanted, two BLA-sham operated and morphine pellet-implanted, and three BLA-lesioned and morphine pellet-implanted animals.

There were no significant differences between time spent in the black compartment (439±15 s, n=68) and that in the white compartment (461±15 s, n=68) during the preconditioning session. This was an important step in the experimental procedure to ensure that there was no preference bias before conditioning.

On day 4, place conditioning was performed as follows: in the morning, the each rat was injected intraperitoneally with isotonic saline (1 ml/kg) and then confined to its saline-paired compartment for 1 h. After at least 3 h, in the afternoon, the rat was injected intraperitoneally with naloxone (0.3 mg/kg) and then confined to its naloxone-paired compartment for 1 h without removing the implanted pellet.

On day 5, the rat that was given no drug was individually placed on the neutral platform and allowed to freely explore the two compartments, and the time spent in each compartment for 900 s was then measured. Naloxone-precipitated morphine withdrawal-induced CPA scores represent the time spent in the naloxone-paired compartment on day 5 (test session) minus the time spent in the same compartment on day 3 (preconditioning session), and are expressed as means±S.E.M. Statistical analyses were performed using two-way (operation×pellet implantation) analysis of variance (ANOVA) and subsequent post hoc
tests were conducted using the Bonferroni tests. Differences with \( P < 0.05 \) were considered significant.

After all tests, histological analyses were performed. Rats were killed by decapitation and the brain was rapidly removed and frozen in powdered dry ice. Then, coronal sections (50 μm) including the amygdala (AP: −1.8 to −2.8 mm from bregma) were prepared on a cryostat, thaw-mounted onto gelatin-coated slides and stored at −80°C until stained. The slices were stained with cresyl violet and each section was examined by microscopy (×400).

The neuronal cells were clearly retained in the amygdala and its surroundings in the 17 CeA- and 17 BLA-sham operated rats, respectively. Photomicrographs and illustrations of NMDA-induced lesion sites in CeA and BLA are shown in Fig. 1. Bilateral neuronal cell loss in the CeA was seen in 18 of the rats that received bilateral infusion of NMDA into CeA, although the neuronal cells in the BLA were not damaged. In five of the 18 CeA-lesioned cases, neuronal damage extended into portions of the lateral globus pallidus and substantia innominata. The data from 10 rats were excluded in the final analysis, i.e., in three cases neuronal damage to the CeA extended into portions of lateral, basolateral and dorsomedial nucleus of the amygdala or Meynert basal nucleus, and in the other seven cases neuronal damage was incomplete. Bilateral neuronal cell loss in BLA was seen in 16 of the rats bilaterally infused with NMDA into BLA, although the neuronal cells in the CeA were not damaged. In seven of the 16 BLA-lesioned cases, neuronal damage extended into portions of the lateral nucleus, dorsal and ventral endopiriform nucleus. The data from 21 rats were excluded in the final analysis, i.e., in 16 cases neuronal damage extended into portions of central or basomedical and intercalated nucleus of the amygdala and lateral cortical regions, and in the other five cases neuronal damage to the BLA was incomplete.

Final group numbers subjected to statistical analysis were: 10 in CeA-sham operated and placebo pellet-implanted, seven in CeA-sham operated and morphine pellet-implanted, seven in CeA-lesioned and placebo pellet-implanted, 11 in CeA-lesioned and morphine pellet-implanted, 11 in BLA-sham operated and placebo pellet-implanted, six in BLA-sham operated and morphine pellet-implanted, eight in BLA-lesioned and placebo pellet-implanted, and eight in BLA-lesioned and morphine pellet-implanted groups.

In the preconditioning session of the present CPA paradigm, neither CeA nor BLA lesion significantly affected the times spent in the black/white compartments; they are 443 ± 31/456 ± 31 s in CeA-sham operated group \((n = 17)\), 420 ± 22/478 ± 32 s in CeA-lesioned group \((n = 18)\), 436 ± 34/463 ± 34 s in BLA-sham operated group \((n = 17)\) and 461 ± 31/438 ± 31 s in BLA-lesioned group \((n = 16)\), respectively.

Fig. 2a shows the naloxone-precipitated morphine withdrawal-induced CPA scores, i.e., the time spent in the naloxone-paired compartment in the test session minus that in preconditioning session, in the CeA-sham operated and CeA-lesioned rats. There was a significant difference in CPA scores between the placebo pellet- and morphine pellet-implantation \((F(1,31) = 10.39, P < 0.01)\), with a significant decrease seen in CeA-sham operated group \((P < 0.01)\), but not CeA-lesioned group. Furthermore, there was a significant difference in CPA scores between the sham- and lesion-operation \((F(1,31) = 6.59, P < 0.05)\), with a significant attenuation of naloxone-precipitated morphine withdrawal-induced CPA seen in morphine pellet-implanted group \((P < 0.05)\), but not placebo pellet-implanted group. On the other hand, there was no significant interaction between operation × pellet implantation \((F(1,31) = 2.59, P = 0.1177)\). The improper CeA-lesioned rats in which neuronal damage was incomplete tended to show naloxone-precipitated morphine withdrawal-induced CPA, while the rats in which neuronal damage extended into portions of BLA, lateral nucleus or caudate-putamen did not (data not shown).

Fig. 2b shows the naloxone-precipitated morphine withdrawal-induced CPA scores in the BLA-sham operated and BLA-lesioned rats. There was a significant difference in CPA scores between the placebo pellet- and morphine pellet-implantation \((F(1,29) = 16.74, P < 0.001)\), with a significant decrease seen in both BLA-sham operated group \((P < 0.001)\) and BLA-lesioned group \((P < 0.05)\). However, there were no significant operation effects \((F(1,29) = 1.96, P = 0.1724)\) nor operation × pellet implantation interaction \((F(1,29) = 0.77, P = 0.3886)\). Although the naloxone-precipitated morphine withdrawal-induced CPA of the BLA-lesioned group was slightly less than that of the BLA-sham operated group, the difference was not statistically significant. The improper BLA-lesioned rats in which neuronal damage was incomplete tended to show CPA, while the rats in which neuronal damage extended into portions of CeA and caudate-putamen did not. Furthermore, several rats in which neuronal damage widely extended into the basomedical nucleus of the amygdala and lateral cortical regions, but not CeA, tended to show CPA (data not shown). Although these improper lesioned rats were excluded from the final analysis, these results suggest that the rats in which CeA was not damaged showed CPA despite the size of the amygdaloid lesion.

In this study, we found that the naloxone-precipitated morphine withdrawal-induced CPA was significantly attenuated by bilateral excitotoxic discrete lesions of the CeA, while that of BLA had little effect, suggesting the involvement of the amygdala, particularly the CeA rather than BLA. This finding was supported by the results of several previous studies. For example, it has been reported that naloxone-precipitated withdrawal caused an increase in cerebral glucose utilization [30] and marked induction of c-fos mRNA and Fos-like immunoreactivity [7,8,27] especially in the CeA, but not BLA, of morphine-depen-
Fig. 1. Photomicrographs of cresyl violet-stained coronal sections through the amygdala (approximately −2.3 mm from bregma) from animals with sham-operation (a) and excitotoxic lesion of BLA (b) and CeA (c). Scale bar, 300 μm. BLA, basolateral nucleus of amygdala; CeA, central nucleus of amygdala; LaA, lateral nucleus of amygdala. Schematic representation of largest (pale shading) and smallest (dark shading) lesions of CeA (d) and BLA (e). Outlines represent sections ranging from 1.8 to 2.8 mm posterior to bregma.

dent animals. Stinus et al. showed that microinjection of methylaloxonium into the CeA elicited CPA in morphine-dependent rats, although they did not examine the involvement of the BLA [26]. Furthermore, we recently reported that microinjection of AMPA/kainate and NMDA glutamate receptor antagonists into the bilateral CeA attenuated naloxone-precipitated morphine withdrawal-induced CPA [29], indicating the importance of the glutamatergic system within the CeA in the negative affective components of morphine abstinence. Recently, it has been argued that the positive and negative affective components of abuse drugs are related to certain structures of the basal forebrain, termed the extended amygdala, which is a macrostructure composed of several forebrain structures
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reported that the suppression of appetitive behavioral responses conditioned by naloxone-precipitated withdrawal with a tone/light compound stimulus was abolished by BLA lesions in morphine-dependent rats [24]. Importantly, they found that the BLA-sham operated and BLA-lesioned groups showed comparable suppression of the response during conditioning trials in the presence of naloxone, suggesting that BLA is not required for the primary aversive effects of morphine withdrawal, but that it is critical for association of the negative state of withdrawal with neutral environmental stimuli. The present observation that BLA lesions tended to decrease naloxone-precipitated morphine withdrawal-induced CPA may be due to the impairment of associative learning by BLA lesions.

On the other hand, the roles of the amygdala in expression of somatic withdrawal signs are controversial. It has been reported that microinjection of opioid antagonists into the CeA elicited some somatic withdrawal signs [1,19,28], although it was also reported that there were no such effects [26]. Electrical lesion of the CeA failed to alter any somatic withdrawal signs [13] except jumping behavior [1]. We found that excitotoxic lesion of the CeA as well as BLA did not significantly affect somatic withdrawal signs, although some signs tended to increase (data not shown). The CeA and BLA seem not to play essential roles in the mediation of somatic withdrawal signs.

In summary, we have shown that discrete excitotoxic bilateral CeA lesion significantly attenuated the naloxone-precipitated morphine withdrawal-induced CPA, while that of BLA had little effect. These results suggest that CeA, rather than BLA, plays a crucial role in the negative affective component of morphine abstinence.

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