Involvement of Glutamate Receptors Within the Central Nucleus of the Amygdala in Naloxone-Precipitated Morphine Withdrawal-Induced Conditioned Place Aversion in Rats

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ABSTRACT—Chronic use of morphine leads to physical and psychological dependence. The amygdala is known to be involved in the expression of emotion such as anxiety and fear, and several studies have shown that the central nucleus of the amygdala (CeA) is involved in morphine dependence. In the present study, we investigated the role of glutamate receptors within the CeA in the negative affective component of morphine abstinence by evaluating naloxone-precipitated withdrawal-induced conditioned place aversion (CPA) in morphine-dependent rats. We found that microinjection of the AMPA/kainate-glutamate-receptor antagonist CNQX (30 nmol/side) into the bilateral CeA significantly attenuated the naloxone-precipitated withdrawal-induced CPA, as well as several somatic signs, in morphine-dependent rats, without preference or aversive effects by itself in non-dependent rats. Furthermore, microinjection of the non-competitive NMDA-receptor antagonist MK-801 (30 nmol/side) or competitive NMDA-receptor antagonist D-CPPene (0.01 and 0.1 nmol/side) into the CeA significantly attenuated the naloxone-precipitated morphine withdrawal-induced CPA, but not somatic withdrawal signs. These results suggest that the activation of AMPA/kainate and NMDA receptors within the CeA play a crucial role in the negative affective component of morphine abstinence.

Keywords: Morphine withdrawal, Conditioned place aversion, Central nucleus of amygdala, Glutamate, Morphine dependence

Chronic use of opiates such as morphine leads to physical and psychological dependence, characterized by the expression of withdrawal symptoms upon cessation of drug administration. The withdrawal symptoms include both physical and affective components. In animals, morphine withdrawal produces various characteristic somatic signs, as well as disruption of schedule-controlled operant responses for food (1, 2), elevation of intracranial self-stimulation thresholds (3), and aversive avoidance behavior from the environment previously associated with morphine abstinence (conditioned place aversion (CPA)) (4). These behavioral changes are thought to reflect the negative affective components of morphine abstinence, such as dysphoria, irritability and anxiety, which might contribute to aver-sively motivated drug seeking.

The neuroanatomical substrates involved in morphine withdrawal symptoms have been investigated extensively. There is a body of evidence implying the involvement of multiple brain regions such as the locus coeruleus, nucleus paragigantocellularis of the rostral ventrolateral medulla, periaqueductal gray matter, several thalamic or hypothalamic nuclei, and amygdala in morphine somatic withdrawal signs (5 – 8). On the other hand, morphine withdrawal-induced CPA has been shown to involve the amygdala, as well as the nucleus accumbens, in experiments using microinjection of methylmaloxonium, a hydrophilic opioid antagonist (9). The amygdala is a forebrain structure composed of several distinct subnuclei including the central (CeA), basolateral and medial nuclei; and it is thought to be a key neural substrate underlying emotional responses such as anxiety and fear in both humans and animals (10). Naloxone-precipitated morphine withdrawal has been reported to cause an increase of cerebral glucose utilization (11) and a marked induction of c-fos mRNA and Fos-like immunoreactivity (12, 13) in the CeA of mor-
phine-dependent animals. Furthermore, studies by Kelsey and Arnold (14) showed that electrical lesions of the CeA reduced morphine withdrawal-induced CPA. Recently, it was suggested that the negative affective component of morphine withdrawal was related to certain structures of the basal forebrain, termed the extended amygdala, which is a macrostructure composed of several forebrain structures including the central and medial nucleus of the amygdala, the bed nucleus of the stria terminalis, the nucleus accumbens shell, and the area termed the sublenticular substantia innominata (15, 16). These findings suggest that activation of the CeA neurons contributes to naloxone-precipitated morphine withdrawal-induced CPA.

On the other hand, there is evidence that excitatory amino acids, particularly glutamatergic, systems participate in physical and psychological morphine dependence (17–22). Furthermore, it has been reported that systemic administration of NMDA-receptor antagonists such as MK-801, d-CPPene and memantine attenuate naloxone-precipitated morphine withdrawal-induced CPA, as well as morphine-induced conditioned place preference (CPP) (23–25). These findings suggest that glutamate receptors could be involved in the negative affective component of morphine abstinence, as well as the rewarding effects of morphine.

To investigate the involvement of glutamate receptors within the CeA, we examined the effects of microinjection of several glutamate-receptor antagonists, i.e., the AMPA/kainate-glutamate-receptor antagonist CNQX, the non-competitive NMDA-receptor antagonist MK-801 and the competitive NMDA-receptor antagonist d-CPPene, into the bilateral CeA on naloxone-precipitated withdrawal-induced CPA in morphine-dependent rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180–250 g were 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. After arrival, rats were individually housed in plastic cages with woodchip bedding for at least 1 day until surgery.

Materials

Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka). Morphine pellets each containing 75 mg of morphine base were prepared according to the method of Gibson and Tingstad (26). 6-Cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX): 2-hydroxypropyl-β-cyclodextrin complex, which is water-soluble complex of CNQX, and (5R,10S)−(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cycloheptan-5,10-imine (MK-801) were purchased from RBI (Berkeley, CA, USA). R[−]-3-[2-Carboxy-piperazin-4-yl]-propyl-1-phosphonic acid (d-CPPene) and naloxone hydrochloride were purchased from Sigma (St. Louis, MO, USA). Naloxone hydrochloride was dissolved in saline and other drugs were dissolved in phosphate-buffered saline (PBS). These drug solutions were made fresh each day.

Surgery

Under sodium pentobarbital anesthesia, each rat was implanted with bilateral guide cannulas (o.d., 0.5 mm; i.d., 0.22 mm) above the CeA at coordinates of 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 (27). After surgery, the rats were individually returned to their cages and left to recover for at least 1 week before the experiments.

CPA paradigm

Apparatus: CPA was conducted as previously described (15, 19) with slight modifications. A place conditioning apparatus, consisting of a shuttle box (30 × 60 × 30 cm: width × length × height) divided into two equal-sized compartments was used. 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 using a computer system (KN-80; Natsume Seisakusyo, Tokyo).

Preconditioning session: 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. The implanted pellet remained until test session. On day 2, the rats were individually placed on the platform and climbed down to the horizontal floor. The rats were allowed to freely explore the two compartments for 900 s and habituated to the apparatus. On day 3 (preconditioning session), the same trial was performed. The preferred compartment was determined by establishing the compartment in which the rats spent greater than 50% of the total time (i.e., 450 s). 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. There were no significant differences between time spent in the black compartment with a smooth floor (445 ± 14 s, n = 104) and the white compartment with a textured floor (453 ± 14 s, n = 104) during the preconditioning phase. This was an important step in the experimental procedure to ensure that there was no preference bias before conditioning.

Conditioning session: On day 4, place conditioning was performed as follows: in the morning, the rats were intra-peritoneally injected with saline (1 ml/kg) and confined to
the non-preferred compartment for 1 h. After at least 3 h, in the afternoon, they were intraperitoneally injected with naloxone (0.3 mg/kg) without removing the implanted pellet and confined to the preferred compartment for 1 h. Drugs were microinjected into the CeA 10 min before i.p. injection of naloxone. Injection cannulas (33 gauge) were inserted just 8.0 mm below the surface of the skull when attached to the guide cannula. Drugs or vehicle (PBS) were bilaterally administered in a volume of 1 μl/side over 2 min by a microinfusion pump. The injection cannula was left in place for an additional 2 min to prevent backflow of drugs.

Test session: On day 5, the rats that were given no drug were allowed to freely explore the two compartments. The time spent in each compartment for 900 s was then measured.

Measurement of naloxone-precipitated morphine withdrawal signs

Measurement of naloxone-precipitated morphine withdrawal signs was performed as previously described (28) with slight modifications. On the first day (day 1), under light ether anesthesia, rats had a morphine pellet implanted in the back of the neck. After 72 h (day 4), each rat was placed in a Plexiglass cylinder to acclimatize it to the experimental environment. After the 30 min habituation period, drugs were microinjected into the CeA as described above. Ten minutes after microinjection, naloxone (0.3 mg/kg) was administered without removing the implanted pellet. Then, the rats were immediately returned to the cylinder and behavior was observed for 1 h. The numbers of occurrences of rearing, stretching, wet dog shake, teeth chattering, jumping, paw shake, head shake and backwards walk were counted; and the occurrence of diarrhea, salivation, ptosis, lacrimation and rhinorrhea was checked. Body weight was measured just before and 1 h after administration of naloxone.

Histology

After all tests, histological analyses were performed. Rats were killed by decapitation and the brain was rapidly removed and frozen in powdered dry ice. To verify the placement of the injection cannulas, 1 μl of a solution of cresyl violet including thionine in saline was infused through a similar injection cannula before rats were killed. Then, coronal sections (50 μm) including the amygdala (anteriorposterior −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 use. The slices were stained with cresyl violet and each section was examined by microscopy (× 400).

Statistical analyses

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 they are expressed as means ± S.E.M. Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. Counts of each naloxone-precipitated morphine withdrawal signs are presented as means ± S.E.M. of total numbers during a period of 1 h, and the data were analyzed by the Mann-Whitney U-test. The occurrence of a behavior is presented as the number of rats showing positive signs over the total number of rats tested, and the data were compared by the Fisher’s Exact Probability test. Differences with P<0.05 were considered significant.

RESULTS

Histology

The microinjection sites of glutamate-receptor antagonists into the CeA are illustrated in Fig. 1. The microinjection sites were found to be correctly placed within the CeA in 128 rats from which experimental data were finally analyzed. The data of 27 rats were excluded in the final analysis because of wrong placement of the microinjection sites.

Effects of microinjection of glutamate-receptor antagonists into the CeA on the naloxone-precipitated morphine withdrawal-induced CPA

In the rats microinjected with vehicle into the bilateral CeA, the time that placebo pellet-treated rats spent in the naloxone (0.3 mg/kg, i.p.)-paired compartment in the test session was 612 ± 42 s (n = 11), which was not significantly different from that in the preconditioning session (606 ± 24 s, n = 11). The CPA score (i.e., the time spent in the naloxone-paired compartment in the test session minus that in the preconditioning session) of the placebo pellet-treated group was 6 ± 32 s (n = 11). On the other hand, the time that morphine pellet-treated rats spent in the naloxone-paired compartment in the test session was 379 ± 15 s (n = 17), which was significantly shorter than that in the preconditioning session (571 ± 17 s, n = 17, P<0.01). The CPA score in this group was −191 ± 22 s (n = 17), which was statistically significant compared with that of placebo pellet-treated rats. These results indicate that naloxone-precipitated morphine withdrawal significantly induced CPA.

Microinjection of CNXQ, an AMPA/kainate-glutamate-receptor antagonist (10 and 30 nmol/side) into the CeA of morphine pellet-treated rats attenuated the naloxone-precipitated morphine withdrawal-induced CPA. A signifi-
cant inhibitory effect was observed at a dose of 30 nmol /side ($P<0.01$), but not 10 nmol /side, as compared with the morphine pellet-treated and vehicle-microinjected groups. On the other hand, CNQX (30 nmol /side) into the CeA of placebo pellet-treated rats had no effect on the CPA score. Microinjection of MK-801, a non-competitive NMDA-receptor antagonist (10 and 30 nmol /side) into the CeA of morphine pellet-treated rats attenuated the naloxone-precipitated morphine withdrawal-induced CPA. A significant inhibitory effect was observed at a dose of 30 nmol /side ($P<0.01$), but not 10 nmol /side. However, microinjection of MK-801 (30 nmol /side) into the CeA of placebo pellet-treated rats produced a slight place preference, although it was not significant compared with the placebo pellet-treated and vehicle-microinjected group. Similarly, the competitive NMDA-receptor antagonist D-CPPene significantly attenuated the CPA at doses of 0.01 and 0.1 nmol /side ($P<0.01$ and $P<0.05$, respectively), but not 0.001 nmol /side. Microinjection of D-CPPene (0.1 nmol /side) into the CeA of placebo pellet-treated rats produced a slight CPA, although it was not significant compared with the placebo pellet-treated and vehicle-microinjected group (Fig. 2).

**Effects of microinjection of glutamate-receptor antagonists into the CeA on the naloxone-precipitated morphine withdrawal signs**

In rats implanted with a morphine pellet and microinjected with vehicle into the bilateral CeA, i.p. injection of naloxone (0.3 mg/kg) elicited characteristic withdrawal signs such as weight loss, rearing, stretching, wet dog shake, teeth chattering, paw shake, head shake, backwards walk, diarrhea, ptosis and rhinorrhea, but not jumping, salivation and lacrimation in this paradigm. Microinjection of CNQX (30 nmol /side) into the CeA significantly attenuated several withdrawal signs such as teeth chattering, diarrhea, rhinorrhea and weight loss; and it tended to attenuate other signs such as wet dog shake, paw shake, head shake, backwards walk and ptosis. On the other hand, microinjection of MK-801 (30 nmol /side) and D-CPPene (0.1 nmol /side) into the CeA, which showed significant attenuation of naloxone-precipitated morphine withdrawal-induced CPA, showed no inhibitory effects on naloxone-precipitated morphine withdrawal signs (Table 1).
DISCUSSION

Recent evidence supports the involvement of glutamate in physical and psychological morphine dependence (17, 19, 21, 22). It has been reported that several non-competitive and competitive NMDA-receptor antagonists such as MK-801, ketamine and LY274614 (18, 20, 29, 30), and AMPA-receptor antagonists (31, 32) attenuate the development of morphine dependence and the expression of morphine withdrawal. Furthermore, electrophysiological studies indicated that NMDA and AMPA/kainate receptors play an important role in the morphine withdrawal-induced activation of locus coeruleus neurons (30, 31, 33, 34). With regard to the rewarding effects of morphine, systemic
administration of the non-selective glutamate-receptor anwstagonist kynurenic acid (17) or several NMDA-receptor antagonists (19, 21) was reported to attenuate the CPP induced by morphine. In this study, we found that microinjection of an AMPA/kainate-glutamate-receptor antagonist, CNQX, into the CeA attenuated naloxone-precipitated withdrawal-induced CPA in morphine-dependent rats. In non-dependent rats, CNQX itself had no significant effect on the CPA score, indicating that CNQX microinjected into the CeA produced neither preference nor aversive effects. Similarly, microinjection of the non-competitive NMDA-receptor antagonist MK-801 or competitive NMDA-receptor antagonist D-CPPene into the CeA significantly attenuated naloxone-precipitated morphine withdrawal-induced CPA without significant effects on the CPA score by themselves in non-dependent rats. In support of our findings, it has been reported that systemic administration of MK-801, D-CPPene or memantine significantly attenuated naloxone-precipitated morphine withdrawal-induced CPA, as well as morphine-induced CPP (23 – 25). These results suggest that both AMPA/kainate and NMDA glutamate receptors within the CeA are involved in naloxone-precipitated morphine withdrawal-induced CPA.

The roles of the CeA in expression of somatic withdrawal signs are controversial. It has been reported that microinjection of opioid antagonists into the CeA elicited several somatic withdrawal signs (5, 8) or showed no such effects (9). Electrical lesions of the CeA failed to alter somatic withdrawal signs (14) except for jumping behavior (5). In this study, we found that microinjection of CNQX into the CeA significantly attenuated several somatic withdrawal signs, consistent with a previous report (35), while MK-801 and D-CPPene had no effect. These observations suggest that AMPA/kainate receptors, rather than NMDA receptors, within the CeA are involved in the expression of naloxone-precipitated somatic withdrawal signs.

Recent neurochemical studies using in vivo microdialysis have directly demonstrated elevation of extracellular glutamate levels within the locus coeruleus (36), striatum and nucleus accumbens (ref. 37 and our unpublished data) during morphine withdrawal. Furthermore, it has been reported that direct injection of glutamate into the lateral ventricle or locus coeruleus dose-dependently precipitates opioid withdrawal-like signs in opioid-dependent, but not non-dependent rats (38, 39). Indeed, we found that the extracellular glutamate level within the CeA was transiently elevated during morphine withdrawal (unpublished data). These findings suggest that the increased glutamate release within the CeA is associated with naloxone-precipitated morphine withdrawal-induced CPA.

It is debatable whether the inhibitory effects of these glutamate-receptor antagonists on the naloxone-precipitated withdrawal-induced CPA are due to attenuation of the negative affective component of morphine abstinence. It has been reported that MK-801 produced a potent CPP by repeated systemic administration (40, 41), suggesting that MK-801 has reinforcing properties. On the other hand, systemic administration of the non-selective glutamate receptor antagonist kynurenic acid or several NMDA antagonists such as D-CPPene, ifenprodil, and memantine failed to produce either CPP or CPA (17, 19, 23, 25). These findings suggest that the mechanism underlying CPP by MK-801 is distinct from the antagonism of NMDA receptors. In this study, although microinjection of MK-801 into the CeA tended to produce a slight CPP in non-dependent rats, it was not significant. D-CPPene, which tended to produce a slight CPA, had a pronounced inhibitory effect on naloxone-precipitated morphine withdrawal-induced CPA. Furthermore, it was reported that CNQX itself also failed to produce either CPP or CPA (42). Taken together, we consider that the attenuation of naloxone-precipitated withdrawal-induced CPA was not due to a simple additive effect between morphine withdrawal and glutamate-receptor antagonists.

However, it is difficult to determine whether the inhibitory effects of glutamate-receptor antagonists microinjected into the CeA are due to impairment of associative learning during conditioning or due to attenuation of the negative affective component of morphine abstinence. To date, many studies have shown that NMDA- or AMPA/kainate-glutamate-receptor antagonists microinjected into the amygdala impaired memory of fear-motivated conditioning (43 – 45). It has been proposed that the impairing effects are due to the blockade of glutamate-receptor-dependent neural plasticity mechanisms such as long-term potentiation in the amygdala, which could underlie the formation of fear memory (44, 45). Nevertheless, glutamate-receptor antagonists have been shown to attenuate the development of physical morphine dependence and the expression of withdrawal signs, as well as tolerance, in which the influence of memory and learning processes is minimal. Furthermore, it has been reported that ifenprodil, which selectively inhibits NR2B-containing NMDA receptors and has little effect on learning, inhibited morphine-induced CPP without preference or aversion effects by ifenprodil alone (19). Similarly, Popik and Danylsz (25) demonstrated that a non-competitive NMDA-receptor antagonist, memantine, inhibited the morphine-induced CPP and naloxone-precipitated morphine withdrawal-induced CPA, but not food-induced CPP, without affecting memory retrieval in the Morris water maze spatial task. These findings suggest that microinjection of glutamate-receptor antagonists into the CeA at least partly attenuated the negative affective component of morphine abstinence.

In summary, we found that microinjection of AMPA
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