Attenuation of morphine withdrawal signs by a GABA_B receptor agonist in the locus coeruleus of rats

Esmail Riahia, Iraj Mirzai-Dizgah, Seyed Morteza Karimian, Hamid Reza Sadeghipour Roodsaria, Ahmad Reza Dehpour

Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Clinical Research Center, Qom University of Medical Sciences, Qom, Iran

Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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Abstract
In the present study, the effects of intra-locus coeruleus (LC) injection of GABA_B receptor-interacting agents on naloxone-induced withdrawal signs of morphine-dependent rats were examined. The GABA_B receptor agonist and antagonists were injected 5 min prior to naloxone injection. Baclofen, a GABA_B receptor agonist, decreased the TWS in a dose-dependent manner but CGP35348, a GABA_B receptor antagonist, alone had no effect. On the other hand, baclofen effects were reversed by CGP35348. It may be concluded that activation of GABA_B receptor mechanisms in the LC reduces precipitated withdrawal symptoms from chronic morphine treatment.

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1. Introduction
It is well established that chronic administration of μ-opioid receptor agonists like morphine results in the development of tolerance and physical dependence. Although the exact mechanisms that mediate these effects have not yet been firmly identified, certain changes in catecholaminergic, serotonergic, cholinergic, GABAergic or peptidergic transmission have been reported during chronic opiate administration. It was shown that both GABA_A and GABA_B receptor subtypes may have an inhibitory influence on naloxone induced withdrawal signs. For example i.p. or i.c.v. injection of muscimol, a GABA_A receptor agonist, and baclofen, a GABA_B receptor agonist, reduced naloxone-induced jumping in morphine-dependent mice [1–3]. In addition, chronic morphine and naloxone-precipitated withdrawal treatments exhibited changes in GABA receptor expression in the LC [4] and produced an increase in GABA binding in the pons and medulla [5].

The LC area was found to be the most sensitive site for the elicitation of motor aspects of opiate withdrawal [6]. It is a bilateral nucleus in the brain stem consisting mostly of noradrenergic neurons. During withdrawal of the exogenous opiates, LC neurons exhibit an augmented activation of their discharge activity [7–9]. The noradrenergic neurons of the LC are under the inhibitory control of GABA [10], the major inhibitory transmitter in the mammalian CNS. Direct iontophoretic application of GABA has an inhibitory effect on the LC neurons [11–13]. Furthermore, LC receives GABAergic innervations terminating on postsynaptic and presynaptic sites [10,14,15]. In addition, our previous study indicated that intra-LC injection of muscimol, a GABA_A receptor agonist, attenuated withdrawal signs in morphine-dependent rats. However, the effect of muscimol on withdrawal signs was reversed by a GABA_B receptor antagonist, but not by a GABA_A receptor antagonist [16]. To understand whether a GABA_B receptor agonist might attenuate opioid withdrawal symptoms, we examined the effects of GABA_B receptor-interacting agents that microinjected in the LC of morphine-dependent rats.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 220–320 g were housed in a group of four animals in a cage. They were kept in an animal room maintained at 23±2 °C and a 12-h light/dark cycle with free access to food and tap water. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Ani-
2.2. Experimental groups

Rats were randomly divided into 13 groups: 2 saline treated groups and 11 morphine treated groups. One of saline and one of the morphine treated groups was not cannulated but the other groups underwent stereotaxic surgery and received intra-LC or i.c.v. microinjection of saline, baclofen or CGP35348 as follow:

- One of saline treated group (n = 5) received intra-LC injection of baclofen (200 pmol/site) and locomotor activity test was done before and after injection of baclofen.
- The other saline treated group (n = 8) and uncanulated morphine treated group (n = 8) received only naloxone (3 mg/kg s.c.).
- Seven morphine treated groups (n = 8) received intra-LC microinjections of saline (0.2 μl/site), baclofen (40, 100, 200 or 400 pmol/site), CGP35348 (200 pmol/site) or a combination of baclofen (200 pmol/site) and CGP35348 (200 pmol/site) before naloxone injection (3 mg/kg).
- Two morphine treated groups (n = 5) received intra-LC microinjections of saline (0.2 μl/site) or CGP35348 (200 pmol/site) before naloxone injection (0.5 mg/kg).
- One morphine treated group (n = 5) received bilateral i.c.v. injection of baclofen (200 pmol/site) before naloxone injection (3 mg/kg).

2.3. Surgery and induction of morphine dependence

Rats were deeply anesthetized with i.p. injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) and then placed on a stereotaxic device. Two 23-gauge stainless steel thin wall cannulae (12 mm long) were implanted bilaterally into the LC with the cannula tip being aimed 1 mm above it at the following coordinates: anteroposterior (AP), −0.8 mm from interaural line; mediolateral (ML), ±1.3 mm from midline and dorsolateral (DV), −6.3 mm below the skull surface; the tooth bar was at −3.3 mm [17]. The guide cannulae were fixed to the skull using two stainless screws and dental acrylic cement. For insertion of cannulae into the lateral ventricles the same method was used except for the cannulae were 9.5 mm long and their tips were located at following coordinates from the bregma as the landmark: AP = −0.7 mm, ML = ± 1.6 mm and DV = −3.8 mm.

Daily ones subcutaneous injection of morphine (2 ml/kg) was started on the third day after surgery and raised incrementally for 1 week (6, 16, 26, 36, 46, 56 and 66 mg/kg, from day 1 through day 7, respectively) as described previously [18]. Non-dependent animals received same volume saline only. Morphine injections were done at the same time of days in whole period of study. Opioid withdrawal was precipitated 1 day after the last dose administration of morphine by naloxone hydrochloride (0.5 or 3 mg/kg, s.c.).

2.4. Microinjection of drugs

Animals received bilateral intra-LC injections of saline, baclofen, CGP35348, or a combination of baclofen and CGP35348 and bilateral i.c.v. injections of saline or baclofen according to their groups. Saline and the drugs were injected 5 min prior to naloxone injection. Our preliminary results had shown that the above doses and intervals were adequate to elicit a response. Drug doses had also been shown in a previous study to be adequate [19]. The drugs were injected into the LC through a 30-gauge stainless steel injecting cannula and the injecting needle tip protruded 1 mm beyond the guide cannula tip. The volume of 0.2 μl/site was injected using a 2-μl Hamilton microsyringe connected to a length of PE-10 polyethylene tubing. The efficacy of injection was monitored by observing the movement of a small air bubble through the tube. Injection period on each side was 1 min, and the injecting cannula was then left in place for an additional 30 s to minimize the drug back flow into the injection track. The injection volume was 0.2 μl/site and the doses were as following: saline, baclofen, 40–100–200–400 pmol/site and CGP 200 pmol/site.

2.5. Measurement of behavioral signs during morphine withdrawal

For behavioral assessment of opioid withdrawal, animals were studied individually in a clear Plexiglas chamber (50 cm × 25 cm × 15 cm) that was placed in other dark chamber to avoid environmental perturbations. A digital camera connected to a recording computer was placed on the inner chamber to simultaneously show the rat behaviors. The reactions of each animal were evaluated by an observer who was not aware of the nature of the treatment received by that animal. The behaviors of all animals were evaluated by the same observer. Whenever necessary, the records were replayed for meticulous analysis.

Twenty distinct behaviors (16 scale behaviors: jumping, rearing, walking sniffing, sniffling, wet dog shakes, head shakes, body grooming, face wiping, penis licking, chewing, teeth chattering, swallowing, writhing, fore paw tremor, percent of body weight loss and percent of dysphoria time; 2 ordinal behaviors: ptosis and diarrhea; and 2 checked behaviors: irritability and eye twitching) were scored during a 30-min period following the naloxone injection as behavioral signs of withdrawal. Body weight was measured before and 30 min after the administration of naloxone. Percent of dysphoria time was determined as: (dysphoria duration time/30 min) × 100. The score of each behavior was divided by a weighing factor (Table 1) as described previously [18] and the results were added and came to a total withdrawal score (TWS) for each animal [20].

2.6. Locomotor activity test

Locomotor activity was assessed by direct observation using interval scale that measures the frequency of crossing the lines painted on the underside of floor of the Plexiglas behavioral arena by each animal [20].

2.7. Histological verification

On completion of each experiment, the precise injecting site was evaluated by intra-LC injection of methylene blue. The animals were anesthetized and their brains removed. Paraffin sections (40 μm thick) were prepared through the LC for histological examinations. Animals were accepted for data analysis only when both injection needle placements were located within the LC (Fig. 1).

2.8. Drugs

The following drugs were used: morphine sulfate (Temad, Tehran, Iran), naloxone hydrochloride and CGP35348 (Tocris, Bristol, UK), baclofen (Sigma, Germany), ketamine (Trillat, Germany) and xylazine (Alfasan, Netherlands). All the drugs were dissolved in saline. The injection volume of morphine and naloxone were 2 ml/kg.

2.9. Statistical data analysis

Data were analyzed by ANOVA followed by the Tukey post hoc test. Paired and unpaired t-test was also used for comparison of two means. Differences amongst

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Table 1

<table>
<thead>
<tr>
<th>Behavior</th>
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<td>Jumping</td>
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<td>Whritting</td>
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<td>Wet-dog-shakes</td>
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<td>Head shakes</td>
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<td>Paw tremor</td>
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<td>Penis licking</td>
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<td>Walk sniffing</td>
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<td>Sniffing</td>
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<td>Body grooming</td>
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<td>Face wiping</td>
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<td>Teeth chattering</td>
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<td>Rearing</td>
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Fig. 1. Representative locations of intra-LC injections (black dots) used in this study. Shown is brain section (−0.8 mm from interaural line) modified from [17] indicating the regions where the bilateral injection tips were determined to be within the intended site.
3. Results

Chronic administration of morphine sulfate caused weight loss (5.75%) and death (29%).

A one-way ANOVA indicated that there was a significant difference in total withdrawal score (TWS) among saline and morphine treated groups ($F(2, 21) = 49.8, P < 0.05$) (Fig. 2). Post hoc analysis showed that the TWS was not significantly different between cannulated and uncannulated morphine-dependent rats, but the scores in both groups were significantly higher than uncannulated saline treated group.

The TWS induced by naloxone (3 mg/kg) was altered by intra-LC microinjection of baclofen ($F(3, 28) = 13.6, P < 0.05$) (Fig. 3). Post hoc analysis showed that the TWS was significantly decreased by baclofen in a dose-dependent manner (Fig. 3). Doses of 40 pmol/site did not have significant effect on TWS, and dose of 400 pmol/site resulted in ataxia (limiting dose). About 21% of rats that received baclofen 200 pmol/site exhibited ataxia, so their data were excluded from the statistical analysis.

Unpaired t-test analysis showed that the TWS was not significantly different between i.c.v. injection of baclofen (200 pmol/site) (37.7 ± 2.4) and intra-LC injection of saline (36.1 ± 4.2, $P = 0.76$) in morphine-dependent rats.

Intra-LC microinjection of CGP35348 (200 pmol/site) did not affect on TWS in morphine-dependent rats neither with 3 mg/kg of naloxone (40.53 ± 1.63) as compared to the control group (36.02 ± 2.38, $P = 0.13$) (Fig. 4) nor with 0.5 mg/kg of naloxone (47.4 ± 6.1) as compared to the control group (42.1 ± 2.1, $P = 0.46$).

Co-microinjection of baclofen (200 pmol/site) and CGP35348 into the LC resulted in an increase of TWS (30.17 ± 3.66) compared to the baclofen (200 pmol/site) treated group (19.35 ± 2.36, $P < 0.05$) but not a significant decrease in comparison with control group (36.02 ± 2.38, $P > 0.05$) (Fig. 4). In other word, the CGP35348 almost completely reversed the alleviative effect of baclofen on opioid withdrawal syndrome.

The data showed that intra-LC injection of 200 pmol/site baclofen (23.6 ± 3.8) as compared to before injection of it (24.6 ± 9.6) had no significant effect on rats' motor performance in saline treated rats ($P = 0.6$).

4. Discussion

The results of this study show that stereotaxic surgery and microinjection of saline into the LC did not affect naloxone-induced opioid withdrawal symptoms in morphine-dependent rats. The results also demonstrate that the intra-LC injection of baclofen, a GABA$_B$ receptor agonist, can alleviate opioid withdrawal symptoms in morphine-dependent rats in a dose-dependent manner. It is in agreement with the results of other studies, in which, it has been shown that i.p. or i.c.v. administration of baclofen attenuate the naloxone-precipitated withdrawal symptoms in mice [1–3,21].

Since the LC is a structure that lies on the floor of fourth ventricle; bilateral i.c.v. injection of baclofen in a set of morphine-dependent rats were also made to fully prove that the suppressive effect of baclofen on opioid withdrawal is directly related to LC but not to the diffusion of the drug into the cerebral ventricles following intra-LC injection. The results showed that i.c.v. injection of baclofen had no effect on TWS, supporting the role of LC in mediating of baclofen response.

Locomotor activity test of rats with LC microinjected by baclofen was done to rule out the occurrence of motor performance-hampering effect of baclofen. The data showed that intra-LC injection of baclofen had no significant effect on rats' motor performance. Thus, the alleviative effect of baclofen on opioid withdrawal is not ensued from suppression of motor activity.

Our data also showed that the intra-LC administration of CGP35348, a GABA$_B$ receptor antagonist, alone had no effect on morphine withdrawal severity. We have previously shown that bicuculline, a GABA$_A$ receptor antagonist, had no effect on withdrawal symptoms per se [16]. These findings may indicate that the
GABA tone during influence of naltrexone in morphine-dependent rats may be very low or nonexistent within the LC. Owing to the repressive effect of GABA on electrical features of LC neurons, the lack of GABA tone in this brain region may be consistent with the fact that LC neurons have an increased electrical activity during opioid withdrawal.

The effect of baclofen on withdrawal signs was reversed by CGP35348, supporting the role of LC GABA receptors in mediation of baclofen response. We have previously shown that the intra-LC injection of muscimol attenuated the withdrawal symptoms, an effect which did not reversed by bicuculline but interestingly blocked by CGP35348 [16], leading us to assume that muscimol effect in LC is accomplished by way of GABA receptors. The results of this study further emphasized on the capacity of these receptors in abatement of morphine withdrawal syndrome. It seems that the GABA receptors mechanism in the LC is involved in naltrexone-induced withdrawal signs.

It has been previously shown that after withdrawal of the chronic treatment with exogenous opiates, LC neurons exhibit an augmented activation of their discharge activity [7–9,22], and that opiate withdrawal behaviors are associated temporally with LC neuronal hyperactivity [9]. Moreover, blockade of LC neuronal activity of glutamate-mediated synaptic neurotransmission and the lack of GABA tonus in this brain region may be consistent with the fact that LC neurons have an increased electrical activity during opioid withdrawal.

Intra-LC injection of baclofen prior to naltrexone injection attenuated the expression of withdrawal signs dose dependently in morphine-dependent rats. This effect was antagonized by a GABA receptor antagonist. Further studies may elucidate the likely role of these receptors in clinical management of opioid withdrawal syndrome.

References


