RAPID COMMUNICATION

Inhibition of the Acquisition of Conditioned Place Aversion by Dopaminergic Lesions of the Central Nucleus of the Amygdala in Morphine-Treated Rats

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Summary

The negative affective state of opiate abstinence plays an important role in craving and relapse to compulsive drug use. The dopamine system participates in the reward effects of opiate use and the aversive effect of opiate abstinence. The amygdala is an essential neural substrate for associative learning of emotion. To establish a model of conditioned place aversion (CPA) in morphine-treated rats, we used different visual and tactual cues as conditioned stimuli (CS) within a conditioning apparatus. An injection of naloxone served as the unconditioned stimulus (US). The 6-hydroxydopamine (6-OHDA) lesion technique was used to investigate the effects of the dopaminergic system of the central nucleus of the amygdala (CeA) on naloxone-induced CPA. Rats were rendered physically dependent *via* administration of increasing doses of morphine delivered *via* intraperitoneal injection. Doses increased by 20 % each day for 14 days, starting from an initial dose of 6 mg/kg. All rats also received a low dose of naloxone (0.1 mg/kg) by injection 4 hours after morphine treatment on days 11 and 13 to induce CPA in a biased two-compartment conditioned place apparatus. Morphine-dependent rats with sham lesions were found to develop significant CPA after naloxone treatment. Bilateral 6-OHDA lesions of the CeA impaired the acquisition of CPA but had no effect on locomotor activity. These results suggest that the dopaminergic system of the CeA plays an important role in the negative affective state of opiate abstinence.

Key words

Conditioned place aversion • Central nucleus of amygdala • Dopamine • 6-OHDA

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Introduction

Drug addiction is a chronic, debilitating brain disorder characterized by compulsive drug seeking behavior. The high relapse rate that occur during abstinence is a pervasive problem in drug addiction treatment (Leshner 1997, Polunina and Brium 1997, O'Brien 2011). The negative emotions induced by withdrawal and drug abstinence are major contributors to the high relapse rate. The central nucleus of the amygdala (CeA) sits at a crucial position in the neuronal circuits that mediate negative emotion. It plays an important role in several processes, such as the acquisition and expression of associative learning of emotion and regulation of attention to the conditional stimulation (Duvarci *et al.* 2011, Pessoa 2011). The neurotransmission that takes place in the amygdala is involved in the process of negative emotion related to learning and memory, but this has yet to be thoroughly investigated. The mesolimbic dopamine system from the ventral tegmental area (VTA) projects heavily to the amygdala, possibly suggesting that it is involved in mediating the negative affective state in opiate abstinence (Koob *et al.* 1992, Maldonado *et al.* 1992, Harris and

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Aston-Jones 1994).

Conditioned place aversion (CPA) can be used to measure the negative motivational aspect of opiate withdrawal, serving as an index of conditioned affective withdrawal (Stinus *et al.* 2000). The acquisition phase of conditioning has been widely used to explore the neurobiological mechanisms underlying withdrawal aversion in chronic-dependent animals. Previous studies have shown significant increases in Fos expression in the shell of the nucleus accumbens (NAc), bed nucleus of the stria terminal (BNST), and central amygdala (CeA) in rats that had developed significant CPA (Skoubis and Maidment 2003). Electric lesioning of CeA reduced the aversion response of morphine withdrawal in rats without affecting somatic symptoms, but lesions of NAc had no visible influence on CPA (Li *et al.* 2007, Li *et al.* 2011).

In the present study, we carefully established a steady CPA model and examined the effects of 6-OHDA lesions of the CeA on the acquisition naloxone-precipitated CPA induced by morphine withdrawal in rats.

Materials and Methods

Animals

Forty-five adult male Sprague-Dawley rats (Charles River in Beijing), weighing 220±20 g at the beginning of the experiments, were housed individually in stainless steel mesh cages in a temperature-controlled (22±2 °C) colony room with a 12/12 h day/night cycle. Food and water were available ad libitum. Every effort was made to minimize both the suffering of the animals and the number of animals used. All animals were allowed to adapt to the laboratory conditions for at least 1 week before surgery. Behavioral tests were conducted during the dark phase of the daily cycle. The experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the local animal ethics committee.

Drugs

Morphine (Qinghai Pharmaceutical, China) and naloxone hydrochloride (Sigma Chemical CO, U.S.) were dissolved and diluted with 0.9 % saline and injected intraperitoneally at 1 ml/kg according to experimental sessions. Pargyline hydrochloride, desipramine hydrochloride, ascorbic acid, and 6-OHDA were purchased from Sigma Chemical Co (St Louis, MO, U.S.). Neurotoxins, 8 mg of 6-OHDA were dissolved in normal saline containing 0.1 % of ascorbic acid to avoid oxidation. Pargyline hydrochloride (inhibitor of metabolism of 6-OHDA by monoamine oxidase) and desipramine hydrochloride (an inhibitor of norepinephrine (NE) uptake) were mixed into 50 mg/kg/ml and 25 mg/kg/ml with physiological saline to protect against noradrenergic transmission damage.

Apparatus

The conditioned place apparatus consisted of an enclosed rectangle Plexiglas chamber (60 cm long × 30 cm wide × 33 cm high) with a small front door to allow the rats to be placed in the chamber. The chamber was divided into two compartments of identical size and material, one with black walls and black textured floor, and the other with white walls and white textured floor. A removable partition with a hole (10 cm × 10 cm) between the compartments allowed rats to cross freely between the two rooms. A weight sensor positioned below the teeterboard floor was used to detect the position of the rats. The signals detected by the sensor were transferred to an interface box, which was connected to a computer. Commercially available software was used to convert the signal from the weight sensor into the time spent in each compartment. The time that each rat spent in each compartment was recorded and the data were converted according to the following formula: Relative amount of time spent in naloxone-paired chamber (time %) = time spent in naloxone-paired chamber (time spent in naloxone-paired chamber + time spent in saline-paired chamber).

Surgery

As 6-OHDA may destroy both dopaminergic and noradrenergic terminals in the target areas, the rats received 25 mg/kg desipramine (i.p.) and 50 mg/kg pargyline 30 minutes before the surgery to protect against damage to the noradrenergic terminals (Louis and Clarke 1998). Then the rats were anesthetized with 10 % chloral hydrate (4 ml/kg, i.p.) and placed in a stereotaxic apparatus. Eight micrograms of 6-OHDA in 2 µl of saline containing 0.1 % of ascorbic acid was injected bilaterally into the CeA (bregma –2.3 mm, lateral ±4.4 mm; dorsoventral –7.4 mm, according to the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson 1986). The injection needle was kept in for 5 min to prevent flush back. All rats were treated with penicillin to prevent infection (80,000 units) and were allowed 7 days to

recover. The rats were handled every other day to reduce stress at the time of testing. There are four groups: (1) 6-OHDA-lesioned rats given naloxone (6-OHDA lesion+NAL), (2) 6-OHDA-lesioned rats given saline (6-OHDA lesion+SAL), (3) sham-lesioned rats given naloxone (sham+NAL), and (4) sham-lesioned rats given saline (sham+SAL).

Induction of physical dependence

Seven days after the surgery, rats were subjected to an opiate physical dependence procedure involving increasing doses of morphine delivered *via* intraperitoneal injection (one injection per day). Doses increased by 20 % every day for 14 days. The initial morphine dose was 6 mg/kg and the dose reached 31 mg/kg on day 10, and that dose of 31 mg/kg was continued during days 11-14. On days 8-10, morphine was administered to rats in the home cage at least 6 hours after the natural chamber preference test. The dose regime was chosen based on our preliminary data showing that 14-day morphine regimen with doses that increased by 20 % every day developed physical dependence by showing wet dog shaking, writhing, and diarrhea.

Induction of CPA

Compared to conditioned place preference, the conditioned place aversion (CPA) is much easier to develop and maintain longer, some lab showed that even one trial training can develop robust CPA (White *et al*. 2005). In the present study, the CPA procedures were based on our previous studies (Wu *et al.* 2012) with two days of conditioning. Briefly, it consisted of four different phases: adaptation (day 8), pretesting (days 9-10), conditioning (days 11-14), and post-conditioning testing (day 15). On the adaptation day, animals were placed into the apparatus and allowed to explore freely for 30 minutes to reduce stress and novelty. During the next two days, the animals' initial preferences were assessed by placing each rat in the chamber for 20 minutes a trial, one trial a day. The average time spent in the same compartment over these two days was used as the natural preference score (pretest). The pretest result showed that most of the rats preferred the black chamber. Accordingly, the 5 rats that spent less than 70 % of their time in the black chamber were not used in further experiments; and in conditioning, a biased conditional place preference (CPP) design was used, in which each animal received a naloxone injection in the black chamber and a saline injection in the white chamber.

Conditioning started on day 11. On days 11 and 13, the rats received naloxone by injection (0.1 mg/kg) 4 hours after the morphine injection. They were placed into the black chamber immediately after the naloxone injection. On days 12 and 14, the rats received saline injection and were placed in the white chamber. The control animals received saline injections paired with both the black and white chambers. Each conditioning session lasted 40 minutes. Post-testing was conducted on day 15. The animals were placed in the compartment through the front door and allowed free access to the entire compartment for 20 min. The amount of time spent in each of the chambers and the number of times each rat crossed between the two chambers were recorded.

Histology

The rats were sacrificed 24-36 hours after the behavioral testing, and the brains were removed and fixed in 10 % formaldehyde for 7 days. Then, 20 μm coronal sections were frozen and cut. Every fifth section of the lesioned area was stained with thionin. The sections were observed under an optical microscope (Leica DMR). Photos were taken with a digital camera (Diagnostic Spot RT). Six rats in which the needle tip had been deflected from the target structure were excluded (6-OHDA+NAL, n=10; 6-OHDA+SAL, n=8; Sham+NAL, n=8; Sham+ SAL, n=8).

Statistics

CPA scores were converted to relative amount of time spent in the naloxone-paired chamber and analyzed using two-way ANOVAs with "treatment" serving as the between-subject factor (lesion-naloxone, lesion-saline, sham-naloxone, and sham-saline) and "test" serving as the within-subject factor (pretest, posttest). Post-hoc testing (LSD) was used whenever indicated by ANOVA results. Results with $P<0.05$ were accepted as statistically significant. All data were analyzed using SPSS 13.0 software for Windows.

Results

Histological results

The key to accurate analysis is here accurate identification of the lesion effect. To examine the effects of 6-OHDA-induced lesions on the CeA, we used histochemical dyeing technology to detect the morphological changes in the nissl bodies. Our results

showed that 6-OHDA was capable of causing a robust lesion effect in the CeA (Fig. 1A). The areas targeted by 6-OHDA were specifically located on the CeA, and the CPU and BLA remained uninjured (Fig. 1B). Thionin staining showed that the nissl bodies of rats subjected to 6-OHDA lesioning were very different from those of rats injected with ascorbic acid (Fig. 1C). The nissl bodies were totally destroyed by 6-OHDA treatment, indicating that 6-OHDA exerted a lesion effect on dopaminergic (DA) neurons.

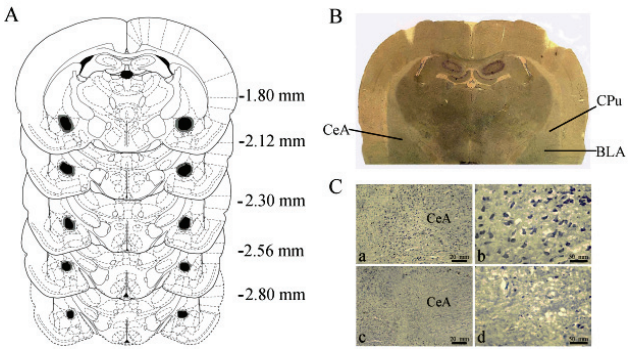

Fig. 1. Illustration of the location and effects of 6-OHDA lesion in the nucleus of the rat amygdala. **(A)** Effects of 6-OHDA lesions. The deep color shows the minimum range of lesion, and the light color shows the maximum range. Representative sections of the amygdala (−1.80 mm, −2.12 mm, −2.30 mm, −2.56 mm, −2.80 mm, and −3.14 mm from bregma) were taken from the rat brain atlases of Paxinos and Watson. **(B)** Typical thionin staining coronal section of 6-OHDA lesion of CeA under the microscope (about 2.30 mm after bregma). CeA, central nucleus of the amygdala; BLA, basolateral amygdala; CPu, caudate nucleus. **(C)** The thionin staining coronal section pictures of 6-OHDA damages CeA. (**a**: 6-OHDA lesion group, 100×; **b**: 6-OHDA lesion group, 400×; **c**: sham-operated control group, 100×; **d**: sham-operated control group, 400×).

6-OHDA lesions of the CeA impaired the acquisition of CPA

In order to investigate the ability of 0.1 mg/kg naloxone (NAL) to induce CPA in opiate abstinence, we established a model of CPA in morphine-dependent rats by using naloxone as a US and the chamber with the black wall and textured floor as the CS. Two-way ANOVA revealed a significant interaction effect between "treatment" and "test" ($F_{3,30}$=33.25, P<0.01). Before the conditioning, there was no significant difference across groups in the amount of time spent in one chamber or another ($F_{3,30}$=0.09, P>0.05). However, after conditioning, there were significant differences ($F_{3,30}$=31.34, P<0.01; Fig. 2A). Post-hoc analysis showed that the relative amount of time spent in the naloxone-associated chamber by rats in the lesion-naloxone group was significantly shorter than that of rats in the sham-naloxone group (P<0.01); there were no significant differences among the three control groups: the lesion-saline, sham-saline, and sham-naloxone groups. This indicated that 6-OHDA lesions could impair the acquisition of CPA.

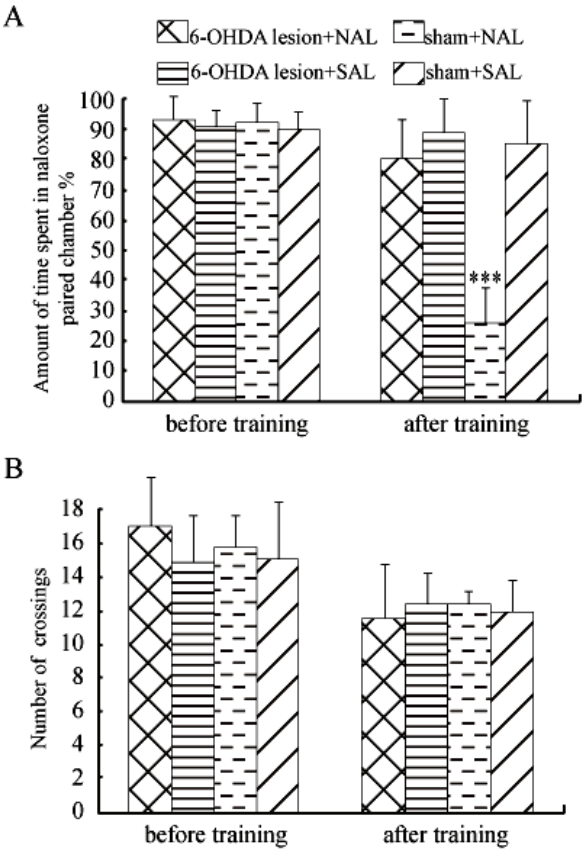

Fig. 2. Effects of 6-OHDA lesion of CeA on the development of conditioned place aversion (CPA) in morphine-dependent rats. **(A)** Effects of 6-OHDA lesions on CPA induced by 0.1 mg/kg naloxone (6-OHDA+NAL, n=10; 6-OHDA+SAL, n=8; Sham+NAL, n=8; Sham+SAL, n=8). **(B)** Effects of 6-OHDA lesion on locomotor activity. ***P<0.01, relative to sham+NAL. NAL, naloxone; SAL, saline.

It is possible that the 6-OHDA lesions may change the animal's locomotor activity and thus influence its place preference, the times that each rat crossed between the white and black chambers both before and after conditioning were counted and compared. No significant difference was observed on the main effect of "treatment" ($F_{3,30}$=0.02, P>0.05; Fig. 2B), the main effect of "test" ($F_{3,30}$=2.49, P>0.05), or the interaction between "treatment" and "test" ($F_{3,30}$=0.08, P>0.05), suggesting that in our model 6-OHDA lesions did not affect the rats' physical ability to move. Therefore, our results show that the CeA DA system induced by 6-OHDA indeed

suppresses the acquisition of CPA in opiate abstinence.

Discussion

The main finding of the present study is that 6-OHDA lesions of the DA systems of the CeA inhibited the acquisition of CPA in the context of opiate abstinence, but they have no effect on the activity levels of the animal. The CPA paradigm has long been known to be a highly sensitive index of the aversive motivational consequences of withdrawal from acute and chronic opioid dependence. Withdrawal-induced CPA in rats results from learning based on the Pavlovian contingency between CS (context cues in CPA apparatus) and US (aversive state induced by withdrawal from morphine dependence), and is positively related to number of trials, magnitude of the US (i.e. doses of morphine and naloxone), and the interval between the opioid agonist and opioid antagonist. In accordance with a Pavlovian analysis, there are three requirements for the acquisition of CPA: First, withdrawal must induce an unconditioned aversive state; second, there must be distinct context cues; third, there must be an association between the aversive state and context cues. Previous studies have shown that c-*fos* mRNA responses occur uniquely in the extended amygdala, including the CeA, when opiate withdrawal is precipitated by low doses of naloxone that induce only an aversive state without inducing somatic signs (Frenois *et al.* 2002). This suggests that CeA can specifically mediate the affective value of withdrawal-paired stimuli. In this way, it is most likely that 6-OHDA lesions of CeA can attenuate aversive emotional state induced by naloxone-induced withdrawal.

The behavior of rats subjected to chemical lesions in specific parts of the brain indicates that the DA system of the CeA plays an important role in the withdrawal-induced negative affective state. There are two possible functional mechanisms by which the DA system affects aversion state. First, the effect may be associated with the function of the CeA in the conditional learning of emotion. The D1 receptor in the amygdala participates in the expression of LTP by changing the excitability of synapse activity and so affects the acquisition of emotional learning (Darvas *et al.* 2011). Dopamine reuptake mechanisms within the amygdala are considerably less efficient than those in the cortical and striatal regions, suggesting that dopamine has a much longer extracellular lifetime within the amygdala. Prolonged elevation of extracellular transmitter levels may be part of the normal functioning of the mesoamygdaloid dopamine system. They may therefore be related to the involvement of this system in modulating the formation of associations. Second, the DA system of CeA may be involved in the regulation of the other neurotransmitters or neuropeptide systems. The DA neurons in this part of the brain region can activate and inhibit brain stress systems by regulating corticotrophin releasing factor (CRF). The CRF neurons in different parts of the brain may be involved in the behavioral and physiological responses to addictive drugs during different phases of the addiction process (Wise and Morales 2010). The CRF receptors of CeA are critically involved in the anxiogenic and aversive effects of acute withdrawal (Wise and Morales 2010). The immunohistochemical method shows that the number of CRF immune response neurons in the central amygdala decreases after 6-OHDA damage to the substantia nigra and VTA or the midbrain DA neuron of the bunch of central forebrain (Day *et al.* 2002). However, the mechanism underlying this process requires further study.

In summary, our results indicate that 6-OHDA lesions of the DA system of the CeA may inhibit the acquisition of CPA in the context of opiate abstinence, suggesting that the DA system plays a crucial role in mediating the negative emotions associated with opiate abstinence.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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