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Increased c-Fos expression in the medial part of the lateral habenula during cue-evoked heroin-seeking in rats

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Abstract

Conditioned environmental stimuli are known to be important determinants of drug seeking behavior. c-Fos, the protein product of the protooncogene c-Fos, is expressed in neurons when there are drug-associated cue-induced drug-seeking behaviour. Therefore, its expression could serve as a marker of regional neuronal activation. Using an extinction/reinstatement paradigm of relapse animal model, we trained Sprague-Dawley rats to nose-poke for i.v. heroin (0.05 mg/kg/infusion) either daily for 4 h or 25 infusions for 14 consecutive days. We then tested these animals for cue-evoked heroin-seeking behavior after abstinence from self-administration of heroin for 14 days. Expression of c-Fos was examined in the lateral habenula (LHb), a region important for conveying information between the limbic forebrain and midbrain. Findings showed that heroin-associated conditioned stimuli could induce robust heroin-seeking behavior that was associated with increased c-Fos immunoreactivity in the medial part of the LHb. This observation suggests the involvement of the LHb in mediating drug cue-induced heroin-seeking behavior after abstinence from self-administration of heroin.

Keywords: c-Fos; Lateral habenula; Heroin; Cue; Drug seeking

Relapse is common to drug addiction. The extinction/reinstatement paradigm has been widely used as one of the animal models for investigating the neuronal mechanisms underlying relapse [15]. In this animal model, drug-priming, stress, as well as re-exposure to the environmental stimuli that are previously paired with heroin infusions can reliably reinstate drug-seeking behavior even after prolonged abstinence from self-administration [15,23].

The neuronal mechanisms that environmental stimuli could reinstate heroin-seeking behavior are largely unknown. Both human imaging and animal studies have provided evidences suggesting that the limbic brain regions, especially nucleus accumbens [10,20], ventral tegmental area [1], amygdala complex [20] and prefrontal cortex are critical structures for conditioned-cued relapse [14,16,19]. Exploring new brain regions that participate in mediating the conditioned-cued relapse to drug-seeking is essential to an understanding of the neurobiological mechanisms of drug addiction. Among the brain regions relating to drug addiction, the lateral habenula (LHb) is of special importance for its anatomical structure. There is increasing evidence that the habenula appears to be vulnerable to damage following chronic administration of most of the abused drugs [6,12,13]. Furthermore, the LHb functions as a major station conveying information between the limbic forebrain and midbrain. It also mediates the negative feedback from forebrain dopamine-rich and limbic structures onto midbrain dopamine-secreting cells.

c-Fos is one of the most commonly used markers of neuronal activation resulting from physiological, pharmacological, and environmental manipulations. Previous studies have reported activated c-Fos expression in the LHb relating to conditioned hyperactivity when tested in cocaine-paired environment [2,8]. In this study, c-Fos immunostaining was used to identify neurons in the habenula activated by

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heroin-associated conditioned stimuli (CS) during a test for reinstatement of heroin seeking behavior.

Sixteen male Sprague-Dawley rats (280–300 g, Experimental Animal Center of Zhejiang Province, China) were catheterized in the right jugular veins under sodium pentobarbital (50 mg/kg, i.p.) anesthesia. All experimental procedures were approved by the Experimental Animal Care and Use Committee of Zhejiang Province, China. Seven days after surgery, behavioral training started as previously described [22]. In brief, the rats were trained to self-administer heroin (obtained from the National Institute of Forensic Science, China) in operant chambers equipped with two nose-pokes (ENV-114 M, Med Associates, Lafayette, IN) under an escalating fixed ratio schedule for 4 h daily. Each trial began with illumination of a green light inside the active nose-poke hole. Responding in the active hole resulted in an infusion of heroin (0.05 mg/kg) delivered by an infusion pump (PHM-100, Med Associates, Lafayette, IN). The green nose-poke light was turned off during heroin infusions. A 30 s inter-trial interval (time out) followed and then another trial began. Responding in the inactive hole had no consequences. The response requirements started with one and increased one after each five-heroin infusion. Each earned heroin infusion was also paired with a 5 s cue-light (located in the middle of the same panel with the nose pokes) that served as the CS. The apparatus was controlled by an IBM-compatible PC running a program written in Pascal (Borland Delphi 6.0).

The rats were transferred to the operant cages and returned to their individual home cages after each session every day. Twelve rats were trained with heroin self-administration. Training sessions were conducted daily for 14 consecutive days, and sessions ended after 4 h or 25 heroin infusions, whichever occurred first. Then the rats were abstinent from heroin for two weeks, during which they lived in their individual home cages. The same experimental procedures were used for the control (SAL, $n = 4$) rats except the heroin was substituted with the same volume of saline. Water was provided ad libitum in both training and home cages, and food was provided after each training session in the home cages.

During testing, the rats were reintroduced into the operant cages. The heroin-trained rats were divided into two groups: the extinction only (EXT, $n = 6$) and the extinction/reinstatement (CUE, $n = 6$). The EXT rats were allowed to nose-poke for 2 h with all the light signals off. The SAL and the CUE rats were allowed to nose-poke for two consecutive 1 h testing phases. During the first phase, all the light signals were turned off, and this phase is generally regarded as an extinction phase. The second phase was signaled by a 5 s presentation of the CS that had previously accompanied with pump injections and after which each nose-poke resulted in another 5 s presentation of the CS.

Immediately after behavioral testing, the rats were deeply anaesthetized using sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with approximately 200 ml of saline containing 200 units of heparin, followed by 200 ml of 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS; pH 7.4). The brains were then removed, post-fixed overnight in 4% paraformaldehyde in PBS, and finally placed in 30% sucrose/PBS. Coronal sections were cut at 35 μm on a freezing microtome (Leica, Germany). Alternate sections through the extent of the habenula were saved and processed for c-Fos immunoreactivity (IR). Sections were rinsed with PBS and incubated with a Fos-specific rabbit polyclonal antibody (1:1000, Santa Cruz Biotechnology, USA) for 24 h at 4 °C in PBS containing 1.5% goat serum and 0.3% triton X-100, and then processed with an ABC kit (Vector Laboratories) using diaminobenzadine as the chromagen. Finally, the sections were mounted onto gelatin-coated slides, dehydrated through a graded series of ethanol, cleared in xylene and coverslipped. The sections were analyzed under a microscope (Olympus BX51, Olympus Optical Co.), c-Fos positive neurons were

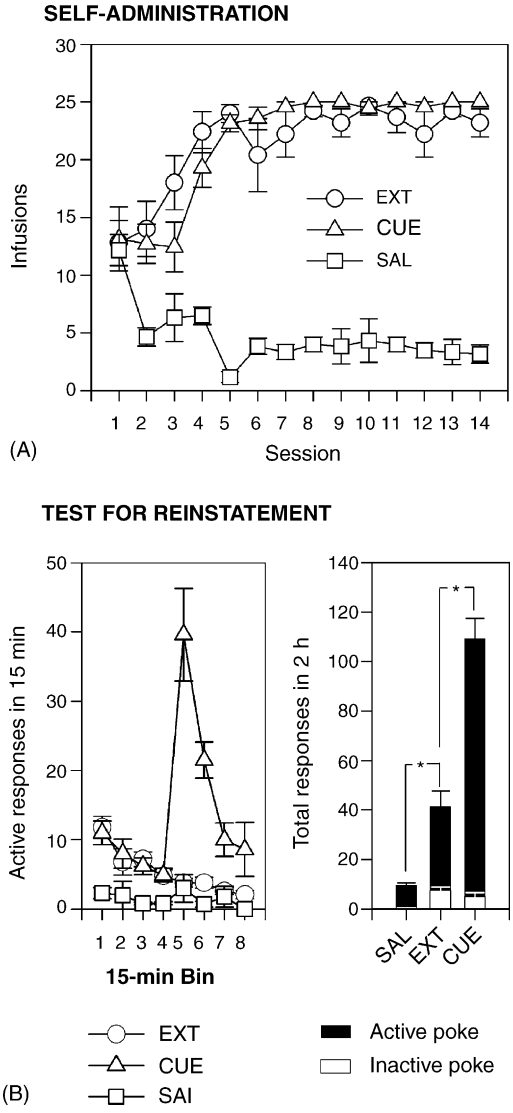

Fig. 1. (A) Mean ± S.E.M. number of heroin (0.05 mg/kg per infusion) or saline infusions at each day of self-administration training. (B) Cue-evoked heroin-seeking after two weeks of abstinence from self-administration. Data were expressed with mean ± S.E.M. number of responses in 15 min blocks (left panel) or total responses in 2 h (right panel). $^{*}p < 0.05$.

counted and collected using a computer-assisted image analysis system (Microimage, Olympus Optical Co.).

Statistical analysis was done using one-way ANOVA. Post hoc comparisons were conducted using Newman–Keuls tests. p values less than 0.05 were considered significant.

Similar to previous findings [22], both the EXT and the CUE rats reached a stable level of heroin infusions within 14 days of heroin self-administration. However, the SAL rats trained with saline were unable to establish stable self-administration. Only a few infusions were made within each session (Fig. 1A).

After 14 days of abstinence from heroin self-administration, when the rats were returned to the operant chambers, contingent presentation of the discrete CS previously associated with heroin infusions did induce robust heroin-seeking behavior. Heroin cue-induced reinstatement of active responding occurred mainly in the first 15 min blocks, which decreased gradually across blocks (Fig. 1B, left). The EXT rats also showed significant amount of active responding in the first 15-min block during testing (Fig. 1B, left). One-way ANOVA revealed that the total number of active nose-poke responses was significantly higher in the CUE than the EXT rats ($p < 0.05$), which was significantly higher than that of the SAL rats ($p < 0.05$) (Fig. 1B, right).

In the SAL rats, re-exposure to the operant chambers resulted in c-Fos expression in a few immunoreactive cells in the LHb. In contrast, in the heroin-trained rats, when the rats were re-exposed to the contingent presentation of the heroin-associated CS (CUE), a large number of darkly stained cells were observed in the LHb, almost all of which were located in the medial part of the LHb. Substantial amount of c-Fos immunoreactive cells were seen in the medial part of the LHb in the EXT rats, but the number of c-Fos immunoreactive cells and the staining in these rats were significantly lower than that in the CUE rats (Fig. 2). Quantitative results are also shown in Fig. 2. The one-way ANOVA indicated that significant between-group differences. Post hoc comparisons indicated that the total number c-Fos positive neurons was significantly higher in the CUE than the EXT rats, and that of both groups was higher than that observed in the SAL rats (all ps < 0.05).

Consistent with the mounting evidences suggesting the role of environmental cues in precipitating relapse [4,5,11,14,16], our results further confirmed that discrete CS previously associated with the availability and subjective effects of heroin could elicit robust heroin-seeking behavior after prolonged abstinence. Furthermore, selective induction of c-Fos IR in the medial part of the LHb during a test of reinstatement of heroin-seeking behavior was observed. The association between instrumental and genomic responses during reinstatement test indicates that cue-induced heroin-seeking responses could relate to functional changes in the medial part of the LHb. In this study, the EXT rats also showed a significantly amount of c-Fos IR in the medial part of the LHb. Since the extinction training was conducted in the same self-administration chamber, self-administration chamber alone appeared to be sufficient for inducing c-Fos IR in the LHb. This observation is consistent with some previous reports that cocaine-paired context could also induce c-Fos IR in the LHb [2,8].

c-Fos IR appears to be related to the amount of nose-poke responding. However, it has been demonstrated that nose-poke responding alone could not increase c-Fos IR in the LHb in food trained rats [18]. Nonetheless, the medial portion of

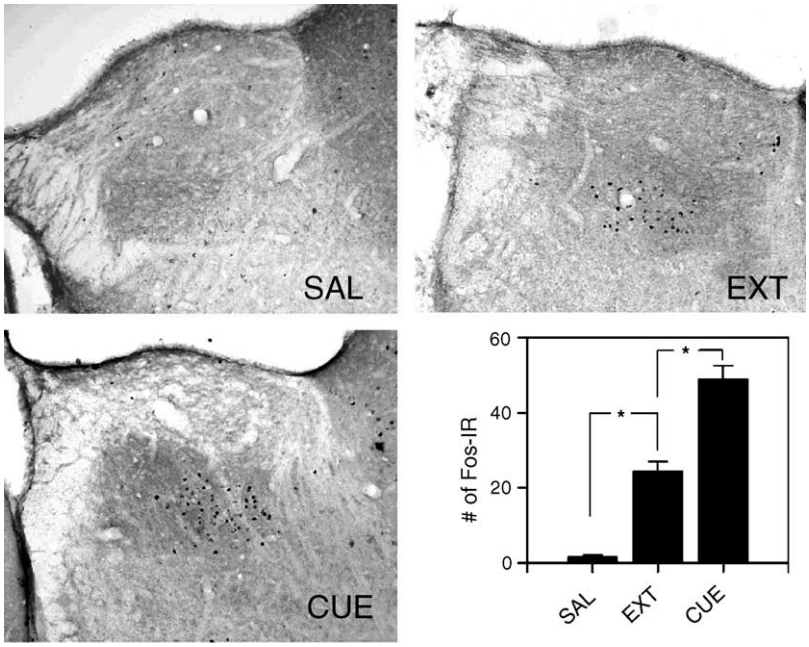

Fig. 2. c-Fos IR labeling in the habenula of representative rats from each of the experimental conditions and mean ± S.E.M. counts of c-Fos IR cells. $^{*}p < 0.05$.

the LHb did exhibit increased c-Fos IR in response to restraint stress [3]. There has been growing evidence suggesting that drug cue and stress manipulations induce a similar pattern of craving and cue reactivity, which implies an overlap between neural substrates controlling craving evoked by drug cues and stress [19]. These data further suggest increases in neural activation in the habenula following exposure to heroin-paired cues are not causally related to conditioned increases in nose-poke responding. Alternative explanation of the c-Fos activation in the medial LHb to both restraint stress and cue-evoke heroin seeking could be that both behaviors are associated with an increased state of wakefulness. The increased c-Fos IR in the medial LHb might reflect the arousal-related aspect of the behavioral of nose-poke responding to heroin-related cues.

Anatomically, the LHb complex composes of two divisions: a medial division and a lateral division. Each of which has distinct biological functions. The LHb contains a moderate density of dopamine binding sites. Most of the dopaminergic markers concentrate within the medial portion of the LHb [9]. The distribution of c-Fos positive nuclei observed in the present study (Fig. 2) corresponds to the dopaminergic innervations of the LHb [21]. The mesocorticolimbic dopamine system is important for conditioned-cued relapse to drug-seeking behavior [14,19]. Anatomically the LHb receives first order afferent projections from both the nucleus accumbens and the ventral tegmental area. It also receives second-order afferent projection from the amygdala [7,9]. Besides, the LHb functions as a major station conveying information between the limbic forebrain and midbrain. Thus, our findings may help clarify the role of the LHb in drug-associated conditioning and relapse to heroin seeking.

In conclusion, our data indicate that drug cue-induced heroin-seeking was associated with increased expression of c-Fos IR in the medial part of the LHb. This suggests that the LHb is a critical site for cue-evoked reinstatement of heroin-seeking. Given the fact that the proposed role of the LHb in a feedback loop from frontal cortex to the midbrain, as well as its significance for motivated behavior [17], further examination of the role of LHb in cue-induced heroin-seeking behavior is recommended.

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References

[1] J.M. Bossert, S.Y. Liu, L. Lu, Y. Shaham, A role of ventral tegmental area glutamate in contextual cue-induced relapse to heroin seeking, J. Neurosci. 24 (2004) 10726–10730.

[2] E.E. Brown, G.S. Robertson, H.C. Fibiger, Evidence for conditional neuronal activation following exposure to a cocaine-paired environment: role of forebrain limbic structures, J. Neurosci. 12 (1992) 4112–4121.

[3] N. Chastrette, D.W. Pfaff, R.B. Gibbs, Effects of daytime and nighttime stress on Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus, the habenula, and the posterior paraventricular nucleus of the thalamus, Brain Res. 563 (1991) 339–344.

[4] H.S. Crombag, Y. Shaham, Renewal of drug seeking by contextual cues after prolonged extinction in rats, Behav. Neurosci. 116 (2002) 169–173.

[5] P. Di Ciano, B.J. Everitt, Reinstatement and spontaneous recovery of cocaine seeking following extinction and different durations of withdrawal, Behav. Pharmacol. 13 (2002) 397–405.

[6] G. Ellison, Neural degeneration following chronic stimulant abuse reveals a weak link in brain, fasciculus retroflexus, implying the loss of forebrain control circuitry, Eur. Neuropsychopharmacology 12 (2002) 287–297.

[7] T.M. Felton, L. Linton, J.S. Rosenblatt, J.I. Morell, First and second order maternal behavior related afferents of the lateral habenula, NeuroReport 10 (1999) 883–887.

[8] T.R. Franklin, J.P. Druhan, Expression of Fos-related antigens in the nucleus accumbens and associated regions following exposure to a cocaine-paired environment, Eur. J. Neurosci. 12 (2000) 2097–2106.

[9] S. Geisler, K.H. Andres, R.W. Veh, Morphologic and cytochemical criteria for the identification and delineation of individual subnuclei within the lateral habenular complex of the rat, J. Comp. Neurol. 458 (2003) 78–97.

[10] U.E. Ghitza, A.T. Fabbricatore, V. Prokopenko, A.P. Pawlak, M.O. West, Persistent cue-evoked activity of accumbens neurons after prolonged abstinence from self-administered cocaine, J. Neurosci. 23 (2003) 7239–7245.

[11] K.N. Gracy, L.A. Dankiewicz, F. Weiss, G.F. Koob, Heroin-specific stimuli reinstate operant heroin-seeking behavior in rats after prolonged extinction, Pharmacol. Biochem. Behav. 65 (2000) 489–494.

[12] C.K. Meshul, K. Noguchi, N. Emre, G. Ellison, Cocaine-induced changes in glutamate and GABA immunolabeling within rat habenula and nucleus accumbens, Synapse 30 (1998) 211–220.

[13] C.A. Murphy, L. Ghazi, A. Kokabi, G. Ellison, Prenatal cocaine produces signs of neurodegeneration in the lateral habenula, Brain Res. 851 (1999) 175–182.

[14] R.E. See, Neural substrates of conditioned-cued relapse to drug-seeking behavior, Pharmacol. Biochem. Behav. 71 (2002) 517–529.

[15] Y. Shaham, U. Shalev, L. Lu, H. de Wit, J. Stewart, The reinstatement model of drug relapse: history, methodology and major findings, Psychopharmacology 168 (2003) 3–20.

[16] U. Shalev, W. Jeffrey, J.W. Grimm, Y. Shaham, Neurobiology of relapse to heroin and cocaine seeking: a review, Pharmacol. Rev. 54 (2002) 1–42.

[17] R.J. Sutherland, The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex, Neurosci. Biobehav. Rev. 6 (1982) 1–13.

[18] S. Tronel, S.J. Sara, Mapping of olfactory memory circuits: region-specific c-fos activation after odor-reward associative learning or after its retrieval, Learn Mem. 9 (2002) 105–111.

[19] F. Weiss, Neurobiology of craving, conditioned reward and relapse, Curr. Opin. Pharmacol. 5 (2005) 9–19.

[20] F. Weiss, C.S. Maldonado-Vlaar, L.H. Parsons, T.M. Kerr, D.L. Smith, O. Ben-Shahar, Control of cocaine-seeking behavior by drug-associated stimuli in rats: effects of recovery of extinguished operant-responding and extracellular dopamine levels in amygdala and nucleus accumbens, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 4321–4326.

[21] D. Wirtshafter, K. Asin, M.R. Pitzer, Dopamine agonists and stress produce different patterns of Fos-like immunoreactivity in the lateral habenula, Brain Res. 633 (1994) 21–26.
[22] F. Zhang, W. Zhou, S. Tang, M. Lai, H. Liu, G. Yang, Motivation of heroin-seeking elicited by drug-associated cues is related to total amount of heroin exposure during self-administration in rats, Pharmacol. Biochem. Behav. 79 (2004) 291–298.
[23] W. Zhou, F. Zhang, S. Tang, H. Liu, M. Lai, G. Yang, Low dose of heroin inhibits drug-seeking elicited by cues after prolonged withdrawal from heroin self-administration in rats, NeuroReport 15 (2004) 727–730.