Brain Research 1652 (2016) 151–157

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Research report

Effects of histone deacetylase inhibitor sodium butyrate on heroin seeking behavior in the nucleus accumbens in rats

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ARTICLE INFO

Keywords:
Heroin
Self-administration
Addiction
Epigenetic
Histone acetylation

ABSTRACT

Histone acetylation and other modifications of the chromatin are important regulators of gene expression and may contribute to drug-induced behaviors and neuroplasticity. Inhibition of histone deacetylases (HDAC) activity results in the change of some drug-induced behaviors , however, relatively little is known about the effects of HDAC inhibitors on heroin-seeking behavior. In the present study, male rats were trained to self-administer heroin under a FR1 schedule for consecutive 14 days, followed by 14 daily 2 h extinction session in the operant chamber. After training, the heroin priming (250 μg/kg) was introduced for the reinstatement of heroin-seeking behavior. Pretreatment with sodium butyrate (NaB) (200 or 400 mg/kg, i.p.), an inhibitor of HDAC, failed to affect heroin self-administration. Additionally , systemic administration of NaB (400 mg/kg, i.p.)increased significantly the reinstatement of heroin-seeking induced by heroin priming when NaB administered 12 h, but not 6 h before the reinstatement test. The same effect was observed after the intracerebroventricular injection of NaB (5 μL, 100 μg/μL). Moreover, the levels of histone H3 acetylation at lysine 18 (H3K18) and H4 acetylation at lysine 5 or lysine 8 (H4K5 or H4K8) in the accumbens nucleus core and shell were remarkably increased during the reinstatement and were further strengthened after intracerebroventricular injection of NaB. These results demonstrated that activation of histone acetylation may be involved in the heroin-seeking behavior, and identifying these epigenetic changes will be critical in proposing a novel pharmacological strategy for treating heroin addiction.

1. Introduction

Heroin addiction is a chronic, relapsing brain disease characterized by persistent and uncontrolled drug seeking behavior. After abstinence, simple exposure to heroin or heroin-associated cues can lead to potent cravings and ultimately a resumption of heroin-seeking behavior. There is converging evidence showing that chromatin remodeling and epigenetic changes occur during both development and maintenance of drug addiction (Renthal et al., 2009). Epigenetic modifications such as histone acetylation may play a key modulator for drug-induced gene expression and long-lasting aspects of addiction (Nestler, 2014). Epigenetic alterations can register and maintain durable structural chromatin adaptations (Bird, 2007). Specific changes of the histone acetylation in the key brain reward regions (e.g., accumbens nucleus, prefrontal cortex) seem to contribute to drug-induced behaviors and neuroplasticity (McQuown and Wood, 2010; Renthal et al., 2009; Simon-O'Brien et al., 2015).

The histone cores are composed of 8 subunits, two each of the histones H2A, H2B, H3 and H4. Now over 100 acetylations of histone have been identified with most having an unclear function (Tan et al., 2011; Finegersh et al., 2015). While four unique histone modifications such as H3 acetylated at lysine 14 (H3K14) or at lysine 18 (H3K18), and H4 acetylated at lysine 5 (H4K5) or lysine 8 (H4K8) are of interest because these acetylations of histone may be belong to the class of so-called common modification module that is present on active and poised promoters (Martin et al., 2012). Histone acetylation is a highly dynamic process regulated by two families of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs), which promote gene activation and gene repression, respectively. Indeed, inhibitors of HDAC promote the synaptic plasticity and the long-term memory (Barrett and Wood, 2008), and also alter rewarding responses to psychostimulants(e.g., cocaine, amphetamine), ethanol and nicotine (Kumar et al., 2005; Alaux-Cantin et al., 2013; Sakharkar et al., 2014; You et al., 2014; Pandey et al., 2008; Legastelois et al., 2013; Castino

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http://dx.doi.org/10.1016/j.brainres.2016.10.007
Received 28 May 2016; Received in revised form 6 October 2016; Accepted 7 October 2016
Available online 11 October 2016

et al., 2015). Inhibitors of HDAC also significantly enhance heroin place preference (Sheng et al., 2011) and potentiate morphine-induced behavioral sensitization (Wei et al., 2016). However, the role of epigenetic regulatory events in mediating the lasting effects of heroin seeking behavior is little known. In the present study, we investigated the effects of systemic and intracerebroventricular (i.c.v.) injection of NaB, an inhibitor of HDAC on the heroin self-administration and the reinstatement of heroin-seeking behavior. Next, we also examined the effect of i.c.v. injection of NaB on expression of acetylated histone H3 (H3K14 and H3K18) and H4 (H4K5 and H4K8) in the NAc core and shell immediately after reinstatement test.

2. Results

2.1. Effect of Systemic injection of NaB on heroin self-administration

A total of 100 rats were used to the heroin self-administration, but six of them were excluded because they did not meet the training standard for heroin self-administration or extinction. Twenty rats were tested for the effect of NaB on the heroin reinforcement. After heroin self-administration training for 12 d under the FR1 schedule, the rats were randomly divided into three groups, which were treated intraperitoneally (i.p.) with NaB at doses of 200 mg/kg (n=7), 400 mg/kg (n=7) or vehicle (n=6) at 12hr prior to the training on day 13. After test of NaB treatment, the rats were also trained to heroin self-administration without NaB treatment on day 14. As shown in Fig. 1, the two-way repeated-measures ANOVA revealed no significant effect of NaB treatment on the accumulated responses of active nose-pokes (NaB treatment ($F(2,17)=0.25$, $P=0.78$), time ($F(2,34)=0.44$,$P=0.65$), and interaction between treatment and time ($F(4,34)=0.81$, $P=0.53$)), and no significant effect of NaB treatment on the infusions (NaB treatment ($F(2,17)=0.15$,$P=0.86$), time ($F(2,34)=1.33$, $P=0.28$), and interaction between treatment and time ($F(4,34)=2.32$,$P=0.077$). The results showed that NaB at doses of 200 and 400 mg/kg failed to affect the heroin reinforcement at 12 h or 24 h after its systemic injection.

2.2. Effect of Systemic injection of NaB on the reinstatement of heroin-seeking induced by heroin priming

After the last session of self-administration, the rats underwent 2 h daily extinction training for 14 days. After the last session of extinction training, twenty-six rats were allocated randomly to four groups. Two groups of rats received an injection of NaB (400 mg/kg, i,p.) at 6 (NaB/6 h, n=7) or 12 h (NaB /12 h, n=7) prior to the reinstatement test, respectively. Another two groups of rats were injected with the same volume of saline at 6 or 12 h prior to reinstatement as Veh/6 h (n=6) and Veh /12 h (n =6). There were no group differences in active ($F(3, 22)=0.10$, $P=0.96$) and inactive ($F(3, 22)=0.03$, $P=1.0$) responding on the last day of extinction training prior to the reinstatement tests. The multiple comparisons showed that active nose-pokes were no significantly different between NaB and vehicle group (NaB/6 h group (4.3 ± 2.0) compared with Veh/6 h group(4.8 ± 2.4, $P > 0.05$; NaB /12 h group(4.6 ± 2.6) compared with Veh /12 h(5.0 ± 2.8, $P > 0.05$) as well as inactive nose-poke responses (NaB/6 h group(3.3 ± 2.7) compared with Veh/6 h group(3.3 ± 2.7, $P > 0.05$; NaB /12 h group (3.3 ± 2.1) compared with Veh /12 h (3.0 ± 1.7, $P > 0.05$). The active or inactive nose-pokes during the reinstatement were recorded and accumulated over 120 min (Fig. 2). The two-way ANOVA analysis revealed a significant main effect of NaB treatment ($F(1,22)=5.36$, $P=0.03$), time ($F(1,22)=3.99$, $P=0.05$) on the active nose-pokes, and interaction between treatment and time ($F(1,22)=11.00$, $P=0.003$). The multiple comparisons showed that active nose-poke responses were significantly enhanced by treatment with NaB at 12 h ($F(1,11)=12.95$, $P=0.004$), but not at 6 h ($F(1,11)=0.65$, $P=0.44$) (Fig. 2a). In contrast, there was no change in inactive-pokes (NaB treatment ($F(1,22)=1.70$, $P=0.21$), time ($F(1,22)=2.09$, $P=0.16$), and interaction between treatment and time $F(1,22)=0.67$, $P=0.42$) (Fig. 2b). Systemic injection of NaB increased the reinstatement of heroin-seeking induced by heroin priming after 12 h of treatment.

2.3. Effect of the i.c.v. injection of NaB on the heroin-seeking induced by heroin priming

To confirm that our observations were due to a central effect of NaB, but not to the peripheral side effects, the rats were administered NaB via the i.c.v. route (5 μL, 100 μg/μL). After extinction training from heroin self-administration, sixteen rats divided randomly into two groups, which implanted with the stainless steel guide cannula in the left lateral cerebral ventricle, were microinjected NaB at a total dose of 500 μg (100 μg/μL, n =8) or vehicle (n =8) into the left lateral cerebral ventricle of each rat at 12 h prior to reinstatement. There were no group differences in active ($F(1, 15)=0.01$, $P=0.92$) and inactive ($F(1, 15)=0.02$, $P=0.90$) responding on the last day of extinction training prior to the reinstatement tests. Fig. 3 illustrated that active nose-poke responses were significantly increased by treatment with NaB ($F(1, 15)=11.29$, $P=0.005$). There is not significant difference in the inactive responses between NaB treated and vehicle group ($F(1, 14)=3.30$, $P=0.09$).

2.4. Effect of NaB treatment on locomotion activity

As shown in Fig. 4, The statistic analysis showed no significant

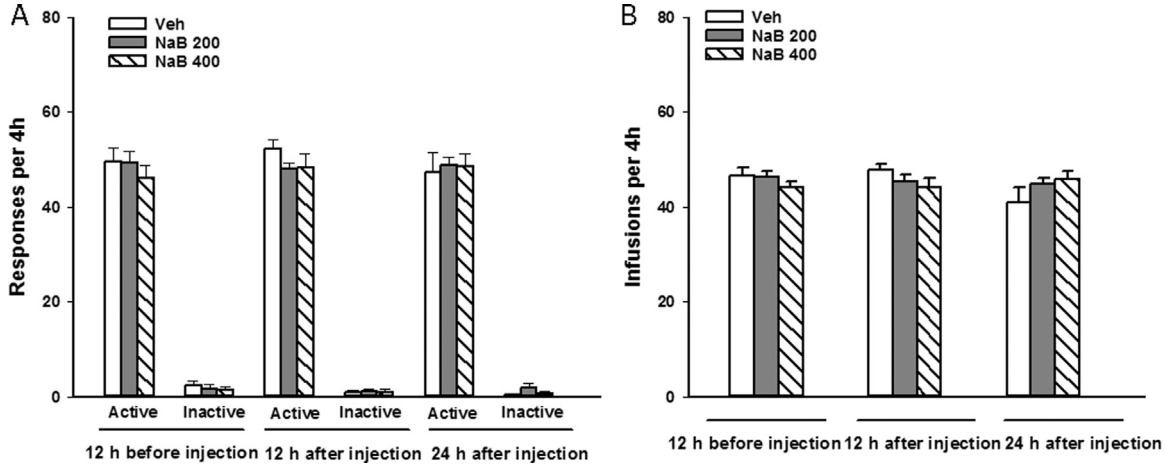

Fig. 1. Effect of Systemic injection of NaB (vehicle, n=6; NaB at 200 mg/kg, n=7 or NaB at 400 mg/kg, n=7) on the reinforcement of heroin self-administration. Nose-poke responses (A) and heroin infusions (B) of heroin self-administration in rats treated with NaB at 12 h before injection, 12 h and 24 h after injection. Data were expressed with mean ± S.E.M.

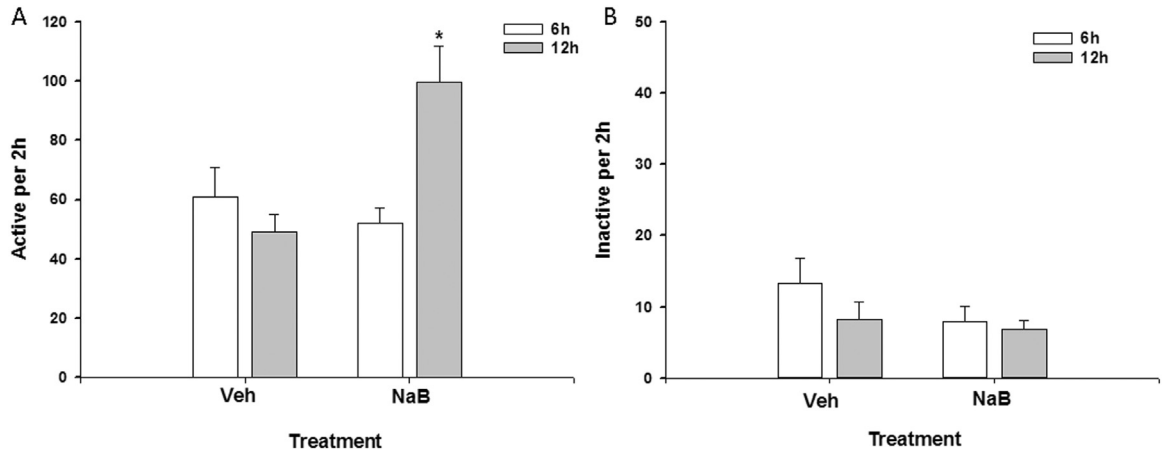

Fig. 2. Effect of Systemic injection of NaB (Veh/6 h and Veh /12 h, n=6; NaB/6 h and NaB/12 h, n=7) on the reinstatement of heroin-seeking induced by heroin priming following extinction from heroin self-administration. Active (A) and inactive (B) nose-poke responses induced by heroin priming in the rats injected with NaB (i.p., 400 mg/kg) at 6 h or 12 h prior to reinstatement test. Data were expressed with mean ± S.E.M. *P < 0.05 vs. vehicle group on the same time point test.

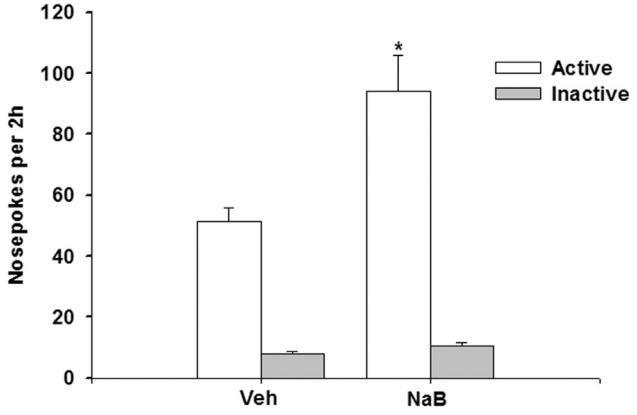

Fig. 3. Effect of i.c.v. injection of NaB on the reinstatement of heroin-seeking induced by heroin priming. Nose-poke responses induced by heroin priming in the rats injected with NaB (500 μg) at 12 h prior to reinstatement test. Data were expressed with mean ± S.E.M. *P < 0.05 vs. vehicle group.

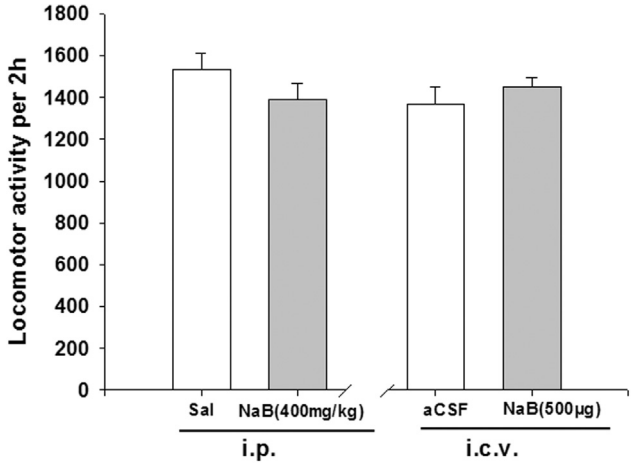

Fig. 4. Effect of NaB treatment on locomotion activity. The rats were allowed to adjust to their new environment. One hour later, the rats were treated with NaB at the dose of 400 mg/kg (i.p.) or 500 μg (i.c.v) and immediately placed back and observed for two hours. Data were expressed with mean ± S.E.M.

effect of either systemic injection with NaB (400 mg/kg) or i.c.v injection with NaB (500 μg) on the horizontal locomotion activity compared with the corresponding vehicle control ($F=(1,10)=1.75$, $P=0.22$; $F=(1,10)=0.33$, $P=0.58$). Since pretreatment with NaB failed to alter the locomotor activity in a separate group of heroin extinguished rats, the enhancement of heroin induced reinstatement by central or systemic treatment with NaB may not be caused by the locomotor hypersensitization.

2.5. Effect of i.c.v. infusions of NaB on histone acetylation in the NAc core and shell

A total of 16 rats were used in the experiment and were randomly divided into four groups (n=4 per group). The rats received an i.c.v injection of NaB (5 μL, 100 μg/μL) or aCSF 12 h before the reinstatement test as NaB/Her or Veh/Her group respectively. The rats received the same above treatment except saline instead of heroin in self-administration as NaB/Sal or Veh/Sal control group. As shown in Figs. 5 and 6, the statistics analysis revealed the significant effect of treatment with NaB on the levels of H3 acetylation on the Lys K18 residue (H3K18) in the NAc core ($F(3,12)=27.40$, $P < 0.01$) and in the NAc shell ($F(3,12)=28.87$, $P < 0.01$), and H4 acetylation on the Lys K5 or K8 residue (H4K5 or H4K8) in the NAc core ($F(3,12)=107.05$, $P < 0.01$; $F(3,12)=68.05$, $P < 0.01$) and in the NAc shell ($F(3,12)=63.73$, $P < 0.01$; $F(3,12)=169.59$, $P < 0.01$). The multiple comparison of Veh/Her group with Veh/Sal group showed a significant elevation of levels of H3K18 and H4K5 or H4K8 were elevated specifically in the NAc core and shell ($P < 0.05$). Meanwhile, the levels of H3K18 and H4K5 or H4K8 in the NAc core and shell remarkably enhanced in the rats treated with NaB (NaB/Her group compared with Veh/Her group, $p < 0.05$; NaB/Sal group compared with Veh/Sal group $P < 0.05$). In contrast, the NaB (5 μL, 100 μg/μL) infusions 12 h before the heroin reinstatement were not significantly changed the levels of H3 acetylation on the Lys K14 residue (H3K14) in the NAc core ($F(3,12)=0.22$, $P > 0.05$) and shell ($F(3,12)=3.12$, $P > 0.05$).

3. Discussion

The major findings of the present study were that both systemic and i.c.v. administration with NaB enhanced the reinstatement of heroin-seeking induced by heroin priming when NaB administered at 12 h, but not at 6 h prior to the reinstatement test. Meanwhile, the levels of acetylated H3K18 and H4K5 and H4K8 in the NAc core and shell were increased remarkably in heroin exposed rats and were augmented after i.c.v. injection of NaB. In contrast, NaB at doses of 200 or 400 mg/kg did not affect heroin self-administration at 12 h or 24 h after its systemic injection. These results demonstrated that enhancement of specific lysine acetylation of histone H3 and H4 in the NAc may be involved in the heroin-seeking behavior.

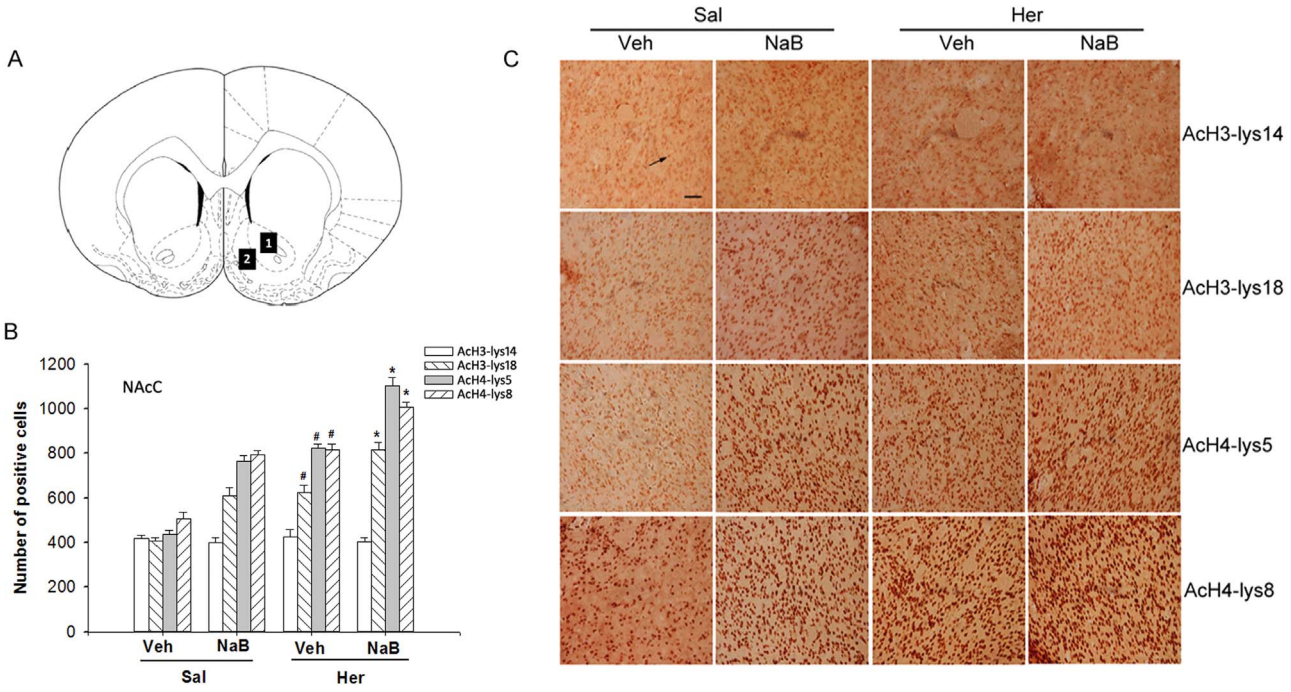

Fig. 5. Effect of i.c.v. infusions of NaB on histone acetylation in the NAc core. (A) Schematic representation of coronal sections of the rat brain taken at +1.6 mm from Bregma illustrating the regions in this study as follows: (1) nucleus accumbens core (NAcC); (2) nucleus accumbens shell (NAcS). Two sample areas were 0.155 mm^2. (B) Number of AcH3 (K14 or K18) and AcH4 (K5 or K8) immunoreactive cells in the NAcC. Data were expressed with mean ± S.E.M; n=4 per group. * P < 0.05 vs. Veh/Her group. # P < 0.05 vs. Veh/Sal group. (C) Representative photomicrographs of histone acetylation in the NAcC of rats from all experimental groups at 40× magnification, where histone acetylation was visible as dark ovals (highlighted by arrows). Scale bar is equal to 50 μm.

Inhibition of histone deacetylation by systemic and i.c.v. injection of NaB increased the heroin seeking behavior induced by heroin priming, which is consistent with the effects of NaB on the addictive behaviors of other abused drug. For example, NaB significantly potentiates the amphetamine-induced behavioral sensitization in mice and produces an increase of histone H4 acetylation in the striatum (Kalda et al., 2007), as well as enhances the cocaine-maintained responding in rats during cocaine self-administration (Sun et al., 2008). Additionally, co-administrations of NaB with ethanol prolonge the extinction of conditioned place aversion and increase the reinstatement effects of ethanol (Pascual et al., 2012). The effects of HDAC inhibitors on the cocaine- or alcohol-seeking behavior probably were observed by repeated uses of the inhibitors more than single injection (Jeanblanc et al., 2015; Romieu et al., 2011). Systemic injection of NaB enhances morphine-, cocaine- and alcohol-induced locomotor sensitization or morphine-induced conditioned place preference in mice (Sanchis-Segura et al., 2009). Furthermore, trichostatin A, another HDAC inhibitor, also significantly augments the heroin-induced histone H3

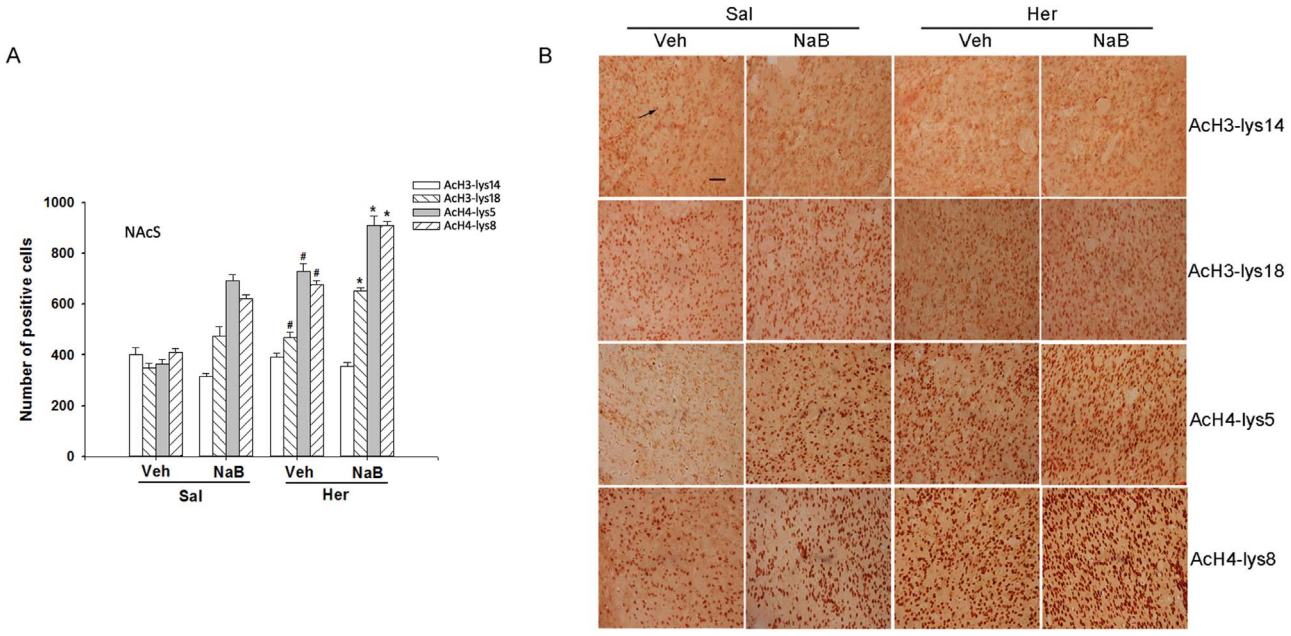

Fig. 6. Effect of i.c.v. infusions of NaB on histone acetylation in the NAc Shell. (A) Number of AcH3 (K14 or K18) and AcH4 (K5 or K8) immunoreactive cells in the NAcS. Data were expressed with mean ± S.E.M; n=4 per group. * P < 0.05 vs. Veh/Her group. # P < 0.05 vs. Veh/Sal group. (B) Representative photomicrographs of histone acetylation in the NAcS of rats from all experimental groups at 40× magnification, where histone acetylation was visible as dark ovals (highlighted by arrows). Scale bar is equal to 50 μm.

phosphoacetylation and enhances the heroin place preference (Sheng et al., 2011). Daily infusion of trichostatin A into the NAc shell enhances the motivation for cocaine self-administration (Wang et al., 2010), and sufficiently induces an enhancement of the ethanol sensitization (Sprow et al., 2014). HDAC inhibitors also enhance the morphine-induced CPP or morphine behavioral sensitization (Wang et al., 2015; Wei et al., 2016). Thus, the present results provided further evidence that the enhancement of the histone acetylation may play an important role in heroin-seeking behavior. However, a few studies show the opposite effects of HDAC inhibitors, such as blockade of the ethanol sensitization (Legastelois et al., 2013), decrease of excessive ethanol intake and the ethanol relapse (Simon-O'Brien et al., 2015), reduce of nicotine-seeking behavior (Castino et al., 2015), inhibition of morphine-induced hyperactivity and sensitization to a single morphine exposure in mice (Jing et al., 2011). This discrepancy could be attributed to many factors, such as experimental paradigm, dosage and time point of HDAC inhibitors, brain region-specificity tested.

Since both systemic and central administration of NaB enhanced the reinstatement of heroin-seeking induced by heroin priming when NaB administered 12 h, but not 6 h before the reinstatement test, indicating that at least 12 h could remodel the chromatin sufficiently to modify the drug-induced behavioral. Given that NaB is rapidly eliminated from the body (Egorin et al., 1999), its behavioral effect may be due to the neurochemical changes induced by its administration rather than its direct pharmacological effect. Previous studies showed that the effects of NaB on cocaine-induced locomotor activity are apparent on the second day but not the first day of cocaine exposure (Kumar et al., 2005; Kalda et al., 2007), and its effects are transient and only apparent within 2 days after the last treatment of NaB (Schroeder et al., 2008). Interestingly, the effects of NaB on cocaine-maintained responding are significant across two time points (12 h and 36 h) after NaB administration in reinforcement schedule of cocaine self-administration (Sun et al., 2008). Finally, the effective doses of NaB failed to alter the locomotion activity and sucrose self-administration (Sun et al., 2008; Simon-O'Brien et al., 2015), indicating that its potentiating of the heroin-seeking behavioral seems to be a specific response for the histone modifications.

The previous evidence showed that the single injection of methamphetamine leads to substantial time-dependent increases in acetylated H4K5 and H4K8 but decreases in the acetylation of histone H3K9, H3K18, and H4K16 (Martin et al., 2012). Additionally, some studies showed that chronic cocaine or ethanol exposure results in increasing acetylation of histone subunits H3 and H4 in the NAc and ventral tegmental area (Wang et al., 2010; Shibasaki et al., 2011). The ethanol-induced histone modifications observed are transient and varied significantly between brain regions, although the acetylated histone H3K9 but not H3K14 or H3K27 in the NAc and prefrontal cortex is remarkably increased by chronic intermittent ethanol exposure (Finegersh et al., 2015). Notably, the acetylation of histone H3K18 is found almost exclusively at the transcriptional start site of actively transcribed genes (Wang et al., 2008). Acetylated histone H3K9 in the ventrolateral orbital cortex is further strengthened by inhibitor of HDAC in morphine-treated rats (Wei et al., 2016). In present study, the levels of H3 acetylation on the Lys K14 or K18 residue and H4 acetylation on the Lys K5 or K8 residue in the NAc were quantified, by treated or not with NaB during the reinstatement of heroin-seeking. There were H3K18, H4K5 and H4K8 hyperacetylation specifically in the NAc core and shell in heroin addicted rats. This observation indicates the implication of the histone acetylation in the NAc in heroin-related behaviors. Moreover, chronic heroin exposure increases in histone acetylation in the NAc , which were notably augmented by pretreatment with an inhibitor of HDAC, which are correlated to enhancement of heroin seeking behavior.

Histone acetylation is associated with the activation of chromatin structure, which promotes transcriptional activity (McQuown and Wood, 2010). Altered histone acetylation has been demonstrated to regulate several candidate genes in the NAc in response to addictive drugs. For example, H4 acetylation increases at the c-Fos promoter acutely, with no changes seen chronically, which is consistent with desensitization of c-Fos expression after chronic drug exposure (Kumar et al., 2005; Renthal et al., 2008). In contrast, BDNF and Cdk5 genes are induced by chronic treatment with cocaine, while H3 hyperacetylation only is observed at the BDNF and Cdk5 promoters after chronic cocaine (Kumar et al., 2005; Nestler, 2014). Induction of CaMKIIα in the NAc by cocaine is associated with histone H3 acetylation at the CaMKIIα gene (Wang et al., 2010; Robison et al., 2013). Expression of postsynaptic density protein 95 (PSD-95) mRNA and protein encoded by the disks large homolog 4 (Dlg4) gene increases in the VTA, histone H3 hyperacetylation occurs in the promoter region of Dlg4 in rats exposed to chronic morphine (Wang et al., 2014). Furthermore, the previous evidence showed the methamphetamine-induced alters the expression of the global genes (such as c-fos, fosB, corticotropin-releasing factor, cholecystokinin, and neuronal PAS domain protein 4 transcripts) in the NAc, which is related, in part, to the methamphetamine-induced changes in histone H3K9, H3K18, H4K5 and H4K8 acetylation (Martin et al., 2012). HDAC inhibitor induces a general increase in histone H3K14 or H3K9 acetylation together with upregulation of BDNF, ΔFosB, p-ERK and CREB activation in the morphine exposed rats (Wang et al.,2015; Wei et al., 2016). Thus, it may be useful to employ additional studies of gene expression or ChIP-seq to reveal the roles of histone acetylation in the development and maintenance of heroin addiction.

In conclusion, the present results demonstrated that the pharmacological inhibition of HDAC activity not only boosted the heroin-seeking induced by heroin priming, but also strengthened the histone acetylation in the NAc, suggesting that histone acetylation may be one of the epigenetic mechanisms underlying heroin addiction.

4. Methods and materials

4.1. Subjects

Male Sprague-Dawley rats (250–300 g) purchased from the Experimental Animal Center of Zhejiang Province (Hangzhou, China) were housed in a temperature- and humidity-controlled room with a reversed 12-h light/dark cycle (lights onset 19:00 h, offset 07:00 h). Food and water were freely available except when specified. The rats were weighed and handled daily for one week prior to surgery.

4.2. Drugs

Heroin (diacetylmorphine HCl) was obtained from National Institute of Forensic Science (Beijing, China). The heroin dose used for the self-administration experiment was chosen on the basis of previous reports (Zhou et al., 2004; Lai et al., 2014a, 2014b). NaB was purchased from Sigma-Aldrich (St. Louis, MO, USA). For intraperitoneal (i.p.) injections, NaB was dissolved in 0.9% sterile physiological saline and administered with an injection volume of 1 ml/250 g body weight. For intracerebroventricular (i.c.v.) injections, NaB was dissolved in artificial cerebrospinal fluid solution (aCSF, CMA Microdialysis, Solna, Sweden).

4.3. Surgery

The rats were catheterized in the right jugular vein according to the method described previously (Zhou and Kalivas, 2008). Briefly, the rats were anesthetized with sodium pentobarbital anesthesia (50 mg/kg, i.p.; Serva) and atropine sulfate (0.3 mg/kg, s.c.) was given at the time of surgery. A silicon catheter (Silastic; length 3.5 cm, 0.5 mm inner diameter, 0.94 mm outer diameter) was inserted into the right external jugular, and the other end of the catheter (10 cm, PE20) was passed

subcutaneously to an incision on the back of the body where it exited into the custom-made fluid connector fixed to a jacket. The catheter was flushed daily with heparinized saline (0.2 ml of 100 IU) and cefazolin antibiotic (0.2 ml of 0.1 g/ml). For i.c.v. treatment, the rats were placed in a stoelting stereotaxic instrument. A stainless steel guide cannula was implanted into the left lateral cerebral ventricle of each animal, according to coordinates from Paxinos & Watson (1998) rat brain atlas: anteroposterior (AP) relative to bregma, −0.8 mm; lateral (L) to midline, +1.4 mm; ventral (V) from the skull surface, −3.0 mm. Obturators were extended 0.5 mm beyond the tip of each cannula to prevent the obstruction of debris. After surgery, the rats were flushed with heparinized cefazolin via the catheter for 7 days and then heparin alone throughout the remaining self-administration. All animal treatments were performed strictly according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23 revised 1996).

4.4. Self-administration procedure

Seven days after surgery, behavioral training started as previously described (Liu et al., 2011). In brief, The rats were first trained to self-administer heroin in operant chambers equipped with two nose pokes (ENV-114 M, Med Associates, St. Albans, VT) under fixed ratio reinforcement schedule (FR1) for 4 h daily. Each response in the active hole was immediately reinforced with an infusion of heroin (0.1 mg/kg for days 1–3, 0.05 mg/kg for days 4–10, 0.025 mg/kg for days 11–14) prepared in 0.9% sterile saline via the pump over 4 s as previously described (Lou et al., 2014). The delivery of each heroin infusion was accompanied with the 20-s illumination of the stimulus light located above the active nose poke. Responses made during this 20-s period were counted but resulted in no heroin delivery. Training sessions were conducted daily for 14consecutive days. The criterion used to determine stable heroin self-administration was when the rats touched the active poke for less than 10% variability in the active nose poking on the last three days (Lai et al., 2014a, 2014b).

4.5. Extinction and reinstatement procedures

After the last session of self-administration, rats underwent 2 h daily extinction session for 14 days. The rats were replaced to the operant chambers for 2 h without conditioned cues and heroin, and pressing the active or inactive pokes had no programmed consequences. The criterion used to determine extinction was when the rats pressing the active nose-poke for less than 10% of the average response on the active nose-poke during maintenance. Reinstatement tests were conducted next day after the last extinction training. The rats were injected with NaB (i.p. and i.c.v.) at 6 or 12 h prior to reinstatement. During the heroin priming-induced reinstatement, the rats first received a single injection of uncontingent heroin (0.25 mg/kg, s.c.) at 30 min before reinstatement testing and then were exposed to the conditioned cues such as light and tone previously associated with heroin infusion for 5 s. The subsequent active nose-poke response resulted in the presentation of the conditioned cue light and tone. Nose-pokes during the reinstatement were accumulated over 120 min

4.6. Injection procedure and histology

For i.p. treatments, NaB (200 and 400 mg/kg) or saline was tested. This dose was chosen based on the previous study (Legastelois et al., 2013). For i.c.v. treatments, aCSF or NaB (5 μL, 100 μg/μL) was i.c.v. infused using an injection cannula projecting 1 mm beyond the tip of the guide cannula. The doses of NaB were based on the previous study (Engelhard et al., 2001). All injections into the left lateral cerebral ventricle were delivered by a microinjection pump (MD-1001, Bioanalytical System Inc., IN) in a volume of 5 μL over 2 min, and the injector needles were left in place for an additional 2 min to allow for diffusion from the site of the injection. Once all testing were completed, the rats were anesthetized and transcardially perfused with phosphate-buffered saline, followed by 4% polyformaldehyde solution. Their brains were sectioned on a Cryostat Microtome (Leica CM1850, Germany) in the coronal plane at a thickness of 50 μm and stained with cresyl violet. These slices were examined for correct location of the internal cannulae within the lateral ventricles. No animals were excluded for misplacements of the cannula. The infusion-cannula tip placement was located using light microscopy and was mapped onto a schematic diagram of the rat brain.

4.7. Locomotor activity assessment

After extinction training from heroin self-administration, the locomotor activities were measured in acrylic locomotor monitoring cages (AccuScan Instruments, Inc., Columbus, OH). Each cage contained 16 photocell beams measuring horizontal distance traveled. Beam breaks were continuously counted and recorded once every 10 min by a PC running VersaMax/Digiscan System Software(AccuScan Instruments, Inc.). After one hour habituation in the chamber, the rats (n=6 in each group) were injected with NaB (400 mg/kg, i.p. and 500 μg, i.c.v) or vehicle (i.p., saline; i.c.v, aCSF) respectively and placed back in the AccuScan chamber after 12 h. Horizontal locomotor activities traveling were recorded for 2 h.

4.8. Immunohistochemistry

Rats were killed immediately after the completion of reinstatement for immunohistochemistry analysis as described previously (Liu et al., 2012). The rats were deeply anesthetized with pentobarbital (80 mg/ml) and decapitated. Coronal sections (20 μm) were cut from blocks containing the NAc and collected in ice-cold PBS for free-floating immunohistochemistry. Sections were blocked in 0.1 M sodium phosphate (pH 7.4) containing 0.3% Triton X-100(Sigma, USA) and 5% normal goat serum (Vector Laboratories, USA). Sections were next incubated overnight at 4 °C with AcH3 (rabbit polyclonal, Lys-14 or Lys-18) or AcH4 (rabbit polyclonal, Lys-5 or Lys-8) antibodies (1:500, Abcam, Cambridge, MA). Slices were then incubated for 1 h at room temperature with biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories) and processed with avidin-biotinylated horseradish peroxidase complex (Elite ABC kit, Vector Laboratories) for 45 min at room temperature. The reaction was visualized using diaminobenzidine (DAB, Sigma). Pictures of the regions of interest (the NAcC or NAcS) were taken with a microscopic CDD camera in x40 object lens (0.155 mm^2/graph). Quantification of immunoreactive cells was performed with an Olympus Opticals (Olympus BX51, Olympus Optical Co., Japan) microscope attached to an image analysis system (Mcroimage, Olympus Optical Co.). In brief, taking into account that the positive cells were defined with nuclear staining above basal background, counts above threshold were taken in a standard frame sample area from the two consecutive sections across both hemispheres per rat, with four rats per group, and these counts were averaged to produce a mean.

4.9. Statistical analyses

All data were expressed as mean ± SEM. Effects of systemic injection of NaB on the nose-poke responses and infusions during heroin self-administration or reinstatement were analyzed by two-way ANOVA with treatment group and time as factors. Effects of the microinjection of NaB into the i.c.v. on the nose-poke responses during extinction, reinstatement, the locomotor activity, and the histone acetylation were analyzed by using one-way ANOVA, followed by Student–Newman–Keuls *post hoc* comparisons. A value of P less than 0.05 was considered to be statistically significant.

Acknowledgments

This work was supported by Natural Science Foundation of Zhejiang (LY14H310002) and China (81471350, 81671321), the National Basic Research Program of China (2015CB553504), and Science and Technology Program of Ningbo (2013C50033, 2015C110026).

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