

Expression of brain-derived neurotrophic factors, neurotrophin-3, and neurotrophin-4 in the nucleus accumbens during heroin dependency and withdrawal

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Neurotrophins, brain-derived neurotrophic factors (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), have been implicated in the modulation of heroin dependency. This study was designed to explore the expression alterations of BDNF, NT-3, and NT-4 in the context of heroin dependence and withdrawal in the rat nucleus accumbens (NAc). Heroin dependence was induced by a progressive intraperitoneal treatment of heroin. The results showed that the expression levels of BDNF and NT-4 were significantly decreased in the NAc of rats with heroin addiction in comparison with the control group, whereas there was a significant increase in BDNF and NT-4 expressions in the groups of rats with both naloxone-induced and spontaneous withdrawal. Moreover, NT-3 expression was markedly increased in the NAc of rats with heroin addiction and spontaneous withdrawal in comparison with the control group, but decreased in the NAc of rats with naloxone-induced withdrawal. These

results indicated that chronic administration of heroin results in the alterations of BDNF, NT-3, and NT-4 expressions in the rat NAc. BDNF, NT-3, and NT-4 may play a critical role in the development of heroin dependency and withdrawal. *NeuroReport* 28:654–660 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.

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Keywords: brain-derived neurotrophic factor, heroin dependence, heroin withdrawal, neurotrophin-3, neurotrophin-4, nucleus accumbens, rat

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Introduction

Heroin dependence is a chronically relapsing brain disease characterized by a compulsion to take heroin with an inability to limit the drug intake, continuous demand for heroin, tolerance (i.e. craving for larger doses), and painful withdrawal [1,2]. This disorder is a global healthcare burden and contributes toward serious behavioral, medical, psychiatric, and social consequences [3]. In response to continued opiate administration, several long-term structural and biochemical alterations occur in the brain that can be explained collectively as neuroplasticity or neuroadaptations [4]. The functional brain impairment caused by long-term opioid use might intensify dependence and contribute toward a relapse [5]. Although the underlying pathogenesis of heroin addiction remains poorly understood, recent studies have shown that neurotrophins play an important role in the development of heroin dependency and withdrawal.

The family of neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are important regulators of neural survival, development,

function, and plasticity [6,7]. BDNF is a critical neurotrophic factor involved in cell growth, survival, and differentiation by regulating synaptic plasticity and neurogenesis [8]. Studies of acute exposure to cocaine have shown an increased expression of BDNF in the prefrontal cortex, ventral tegmental area (VTA), striatum, and nucleus accumbens (NAc) [4,9]. Grimm *et al.* [10] reported that a time-dependent increase in BDNF expression was observed within the mesolimbic dopamine system up to 90 days after withdrawal from cocaine. However, chronic morphine administered to mice decreased BDNF expression in the VTA [11]. In addition, clinical studies have shown contradictory results of BDNF, with the serum levels being increased and decreased in heroin addicts [12–14]. Also, it is documented that NT-3 contributes toward the initiation of behavioral sensitization to cocaine and modulates noradrenergic neuron function and opiate withdrawal [15,16]. NT-4 has been shown to be required for tolerance to morphine in the mouse [17]. Overall, these studies indicate that changes in neurotrophic factors BDNF, NT-3, and NT-4 may be involved in the pathophysiology of opiate addiction.

The NAc, a basal forebrain structure receiving glutamatergic inputs from the prefrontal cortex, the amygdala, and VTA, is a critical component of the mesocortico-limbic system, a brain circuitry involved in reward and

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motivation and it has been the target of addictive drugs [18]. Considerable evidence supports the involvement of dopaminergic neurons in the VTA and their projections to the VTA or NAc in drug-induced rewarding properties [18,19]. Besides glutamate and dopamine, recent studies have shown that administration of exogenous neurotrophic factors in the NAc increased the frequency of behaviors related to addiction and relapse; conversely, a decrease in neurotrophic factors is linked to opposite behaviors [9,20]. However, the response of endogenous neurotrophins in the NAc to heroin treatment remains elusive. In the present study, we sought to investigate the expression of BDNF, NT-3, and NT-4 in the rat NAc during heroin dependency and withdrawal to further understand the roles of neurotrophins in the development of heroin dependence and withdrawal and explore the potential treatment for the prevention and management of heroin addiction.

Materials and methods

Animals

In all, 32 male Sprague–Dawley rats weighing 180–220 g (Experimental Animal Center of Guizhou Medical University) were divided randomly into four groups: heroin-dependent group, spontaneous withdrawal group, naloxone-induced withdrawal group, and saline control group. Rats were housed three per cage under a 12 h light/dark cycle (light: 8:00–20:00) in a room with controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$), and free access to water and food. Rats were individually tested in the procedure room adjacent to the animal room. All animal protocols and experimental procedures were approved by the Ethics Committee of Guizhou Medical University Drug Administration. The study was carried out in the Animal Laboratory of Guizhou Medical University, Guiyang, China, between May 2015 and February 2016.

Heroin dependence and withdrawal

For repeated heroin or saline treatment, Sprague–Dawley rats were administered intraperitoneal injections of either saline (0.9% NaCl, 1 ml/kg) or heroin (provided by Police Bureau of Guizhou Province, China, containing 92.09% of diacetylmorphine, 7.91% of acetylmorphine) for 9 days. Heroin was dissolved in a 0.9% NaCl solution immediately before injection. In the heroin-dependent group, the spontaneous withdrawal group, and the naloxone-induced withdrawal group, rats were subcutaneously injected with heroin twice a day for 9 days. The dose for each injection was 3 mg/kg on day 1, and was increased by 3 mg/kg each day. On day 9, the dose was 27 mg/kg. This pattern of heroin administration has been shown to induce physiological dependence in rats [21]. Rats in the control group were administered saline treatment with the same procedure.

After the last injection of heroin on day 9, rats in the spontaneous withdrawal group were observed for

withdrawal symptoms on day 10. In the naloxone-induced withdrawal group, heroin withdrawal symptoms were precipitated by the administration of naloxone (5 mg/kg, intraperitoneally) on day 10. Symptoms of withdrawal were scored for 30 min.

Behavioral observations

Behavioral observations were carried out as described previously [21]. In rats with heroin administration, behaviors were recorded during visual checks for 2 h, starting after the first daily injection. Typical heroin-induced behaviors including explore, rear walk, hyperactivity, straggling, erecting, licking the hair, eat/drink, and biting paws were recorded for each rat. Five behavioral characteristics of the rat heroin abstinence syndrome including writhing, jumping, wet-dog shakes, teeth chatting, and rearing were recorded and scored after an intraperitoneal injection of naloxone and lasted for 30 min. At the end of the rating period, the amount of body weight loss was measured and scored as described previously [22]. The individual who measured behavior was blinded to the experimental groups.

Sample preparation

In the heroin-dependent group and the control group, brains were extracted after the last injection of heroin or saline. In the spontaneous withdrawal group, rats received the same chronic treatment as the heroin-dependent group, and brains were extracted 7 days after the last injection of heroin. In the naloxone-induced withdrawal group, brains were extracted 30 min after the injection of naloxone. Rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneally) and perfused with PBS and a 4% paraformaldehyde solution sequentially from the left ventricle. The paraffin-embedded brain tissue was horizontally sliced at a thickness of 10 μm sections and then detected with immunohistochemical staining. The NAc was punched with a 14 G needle, and then stored in liquid nitrogen until they were determined by western blotting.

Immunohistochemical staining

To localize the expression of BDNF, NT-3, and NT-4, immunohistochemistry staining was performed. First, sections were rinsed with PBS (3×5 min), treated with 0.5% H_2O_2 in PBS for 10 min, and then rinsed again in PBS (3×10 min). Then, sections were placed in a blocking solution containing 3% normal goat serum for 1 h, and then incubated with the primary antibodies to BDNF, NT-3, and NT-4 (diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, Texas, USA) overnight at 4°C . A negative control without a primary antibody was performed. On the second day, tissue sections were rinsed in PBS and then incubated for 1 h at room temperature with the secondary antibodies (diluted 1:1000; Jackson ImmunoResearch Inc., West Grove, Pennsylvania, USA). The optical densities of positive staining were determined using Image Pro-Plus software

(version 3.0.1; Media Cybernetics, Guiyang, Guizhou, China). Paxinos and Watson [23] were used to identify the different brain regions.

Western blot assay

NAC samples were homogenized in lysis buffer (Heart Biological Technology Co. Ltd., Xi'an, China) and incubated on ice for 30 min. The homogenates were centrifuged at 14 000g for 20 min at 4°C. The protein concentration was determined using a bicinchoninic acid assay. The proteins were separated and transferred to a polyvinylidene difluoride membrane. After blocking in 3% bovine serum albumin/tris-buffered saline with Tween 20 at room temperature for 1 h, the membranes were incubated with the appropriate primary antibodies overnight at 4°C (anti-BDNF 1:500, anti-NT-3 1:1000, anti-NT-4 1:1000, anti-β-actin 1:2000; Santa Cruz Biotechnology). After washing three times, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (1:2000). The relative intensities of bands were quantified using the enhanced chemiluminescence method.

Statistical analysis

Data are shown as means ± SD. The SPSS11.5 statistical package was applied to analyze experimental data with a one-way analysis of variance and *P* less than 0.05 was considered statistically significant for all tests.

Results

Test of dependence on heroin

Both heroin-treated rats in the spontaneous withdrawal group and the naloxone-induced withdrawal group had higher numbers of wet-dog shakes, writhing, and rearing compared with the vehicle-treated controls (*P* < 0.05) (Table 1). In addition, naloxone precipitated a significant increase in jumping and teeth chatter (*P* < 0.05) (Table 1). Heroin caused a decrease in body weight. Rats were checked daily for body weight for 10 days. Both heroin-treated rats in the spontaneous withdrawal group (209 ± 3 g) and the naloxone-induced withdrawal group (183 ± 3 g) had significant less weight than the vehicle-treated controls (217 ± 1 g) (*P* < 0.05) (Fig. 1).

Alterations of BDNF expression in the rat NAC during heroin dependence and withdrawal

As shown in Fig. 2a–d, BDNF expression was found to be mainly located in the cytoplasm in the rat NAC. The BDNF

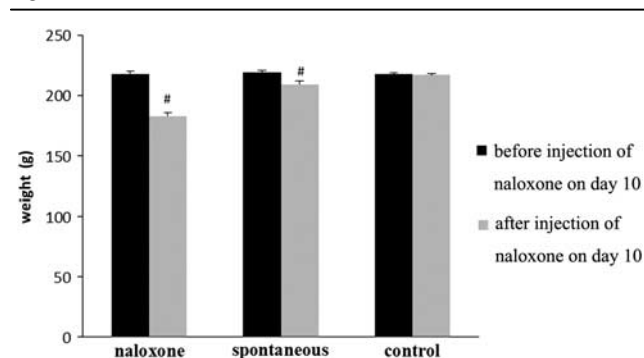
Table 1 Withdrawal symptoms (mean ± SD)

Withdrawal symptoms	Naloxone	Spontaneous	Control
Jumping	10 ± 2* [#]	0	0
Teeth chatting	12 ± 1* [#]	0	0
Wet-dog shakes	23 ± 3* [#]	12 ± 2*	0
Writhing	18 ± 2* [#]	11 ± 3*	0
Rearing	15 ± 1* [#]	7 ± 2*	2 ± 1*

**P* < 0.05 compared with the control group.

[#]*P* < 0.05 compared with the spontaneous withdrawal group.

Fig. 1



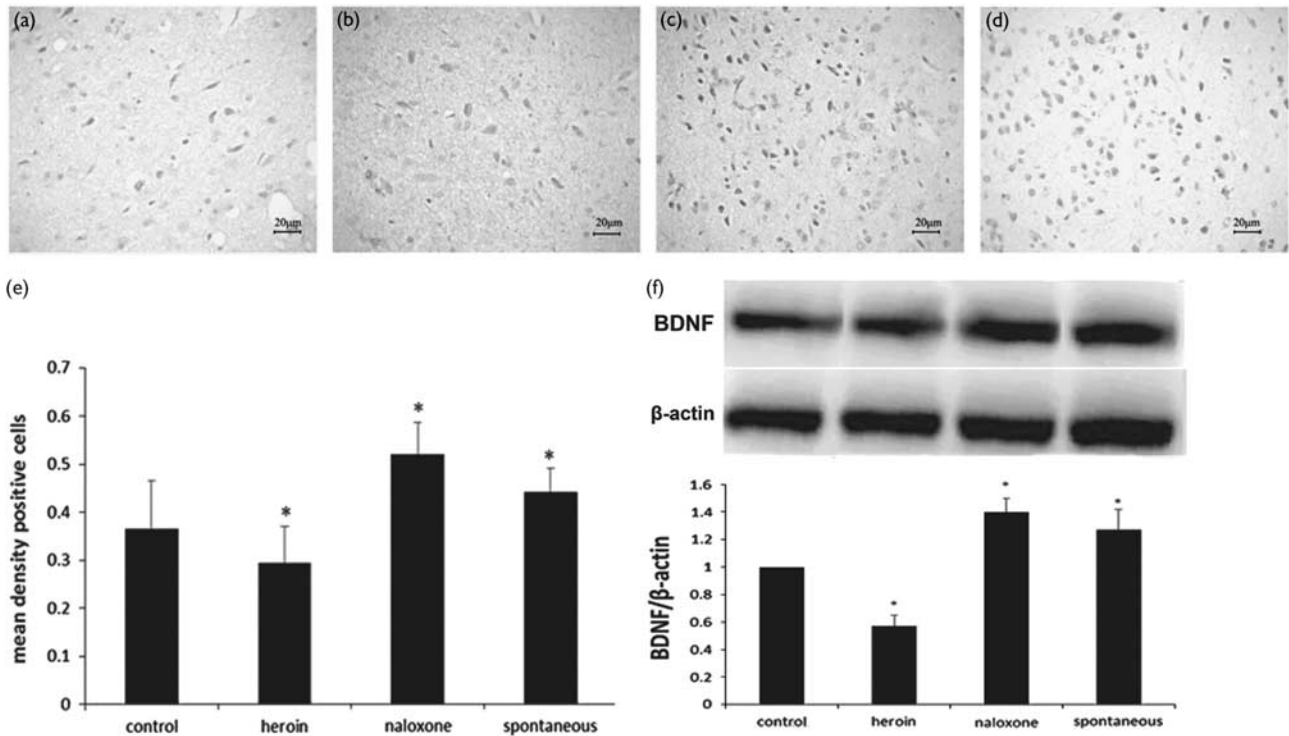
Withdrawal syndrome test. Heroin caused a decrease in body weight. Rats were checked daily for body weight for 10 days. Both heroin-treated rats in the spontaneous withdrawal group (209 ± 3 g) and the naloxone-induced withdrawal group (183 ± 3 g) weighed significantly less than the vehicle-treated controls (217 ± 1 g) ([#]*P* < 0.05).

expression levels changed in the rat NAC in response to chronic heroin treatment. After injection of heroin for 9 days, the expression levels of BDNF were markedly decreased in the NAC of rats with heroin addiction in comparison with control rats (mean optical density: 0.295 ± 0.075 vs. 0.366 ± 0.099, *P* < 0.05) (Fig. 2c), whereas there were a significant increase in BDNF expressions in the groups of rats with naloxone-induced and spontaneous withdrawal (mean optical density: 0.521 ± 0.066 vs. 0.442 ± 0.049 vs. 0.366 ± 0.099, *P* < 0.05) (Fig. 2e). The heroin-induced changes in BDNF expression were represented by changes in the mean optical density in the NAC investigated. Western blotting was performed to further confirm the similar changes in BDNF expression in the rat NAC during heroin dependence and withdrawal (BDNF/β-actin: 0.57 ± 0.08 vs. 1.4 ± 0.1 vs. 1.27 ± 0.15 vs. 1.0 ± 0.0, *P* < 0.05) (Fig. 2f).

Alterations of NT-3 expression in the rat NAC during heroin dependence and withdrawal

Figure 3a–d shows that NT-3 expression was found to be mainly located in the cytoplasm in the rat NAC. After an injection of heroin for 9 days, the NT-3 expression levels changed in the rat NAC in response to chronic heroin treatment. The expression levels of NT-3 were markedly increased in the NAC of rats with heroin addiction and spontaneous withdrawal in comparison with the control group (mean optical density: 0.498 ± 0.076 vs. 0.435 ± 0.037 vs. 0.372 ± 0.066, *P* < 0.05) (Fig. 3e), but significantly decreased in the groups of rats with naloxone-induced withdrawal (mean optical density: 0.322 ± 0.033 vs. 0.372 ± 0.066, *P* < 0.05) (Fig. 3e). The heroin-induced changes in BDNF expression were represented by the changes in the mean optical density in the NAC investigated. Western blotting was performed to further confirm the similar changes of NT-3 expression in the rat NAC during heroin dependence and withdrawal (NT-3/β-actin: 1.42 ± 0.1 vs. 0.62 ± 0.07 vs. 1.38 ± 0.21 vs. 1.0 ± 0.0, *P* < 0.05) (Fig. 3f).

Fig. 2



Alterations of brain-derived neurotrophic factor (BDNF) expression in the rat nucleus accumbens (NAc) during heroin dependence and withdrawal. (a–d) BDNF expression was found to be mainly located in the cytoplasm in the rat NAc. (e) After injection of heroin for 9 days, the expression levels of BDNF were markedly decreased in the NAc of rats with heroin addiction in comparison with the control rats (mean optical density: 0.295 ± 0.075 vs. 0.366 ± 0.099 , $P < 0.05$), whereas there was a significant increase in BDNF expressions in the groups of rats with naloxone-induced and spontaneous withdrawal (mean optical density: 0.521 ± 0.066 vs. 0.442 ± 0.049 vs. 0.366 ± 0.099 , $P < 0.05$). (f) Western blotting further confirmed the similar changes in BDNF expression in the rat NAc during heroin dependence and withdrawal (BDNF/β-actin: 0.57 ± 0.08 vs. 1.4 ± 0.1 vs. 1.27 ± 0.15 vs. 1.0 ± 0.0 , $P < 0.05$).

Alterations of NT-4 expression in the rat NAc during heroin dependence and withdrawal

As shown in Fig. 4a–d, NT-4 expression was found to be mainly located in the cytoplasm in the rat NAc. Chronic heroin treatment altered the expression of NT-4 in the rat NAc. The levels of NT-4 expression were markedly decreased in the NAc of rats with heroin addiction compared with the control group (mean optical density: 0.312 ± 0.035 vs. 0.401 ± 0.059 , $P < 0.05$) (Fig. 4e), but significantly increased in the groups of rats with spontaneous and naloxone-induced withdrawal (mean optical density: 0.485 ± 0.102 vs. 0.479 ± 0.039 vs. 0.401 ± 0.059 , $P < 0.05$) (Fig. 4e). The heroin-induced changes in NT-4 expression were represented by the changes in the mean optical density in the NAc investigated. Western blotting was performed to further confirm the similar changes in NT-4 expressions in the rat NAc during heroin dependence and withdrawal (NT-4/β-actin: 0.58 ± 0.09 vs. 1.37 ± 0.13 vs. 1.24 ± 0.03 vs. 1.0 ± 0.0 , $P < 0.05$) (Fig. 4f).

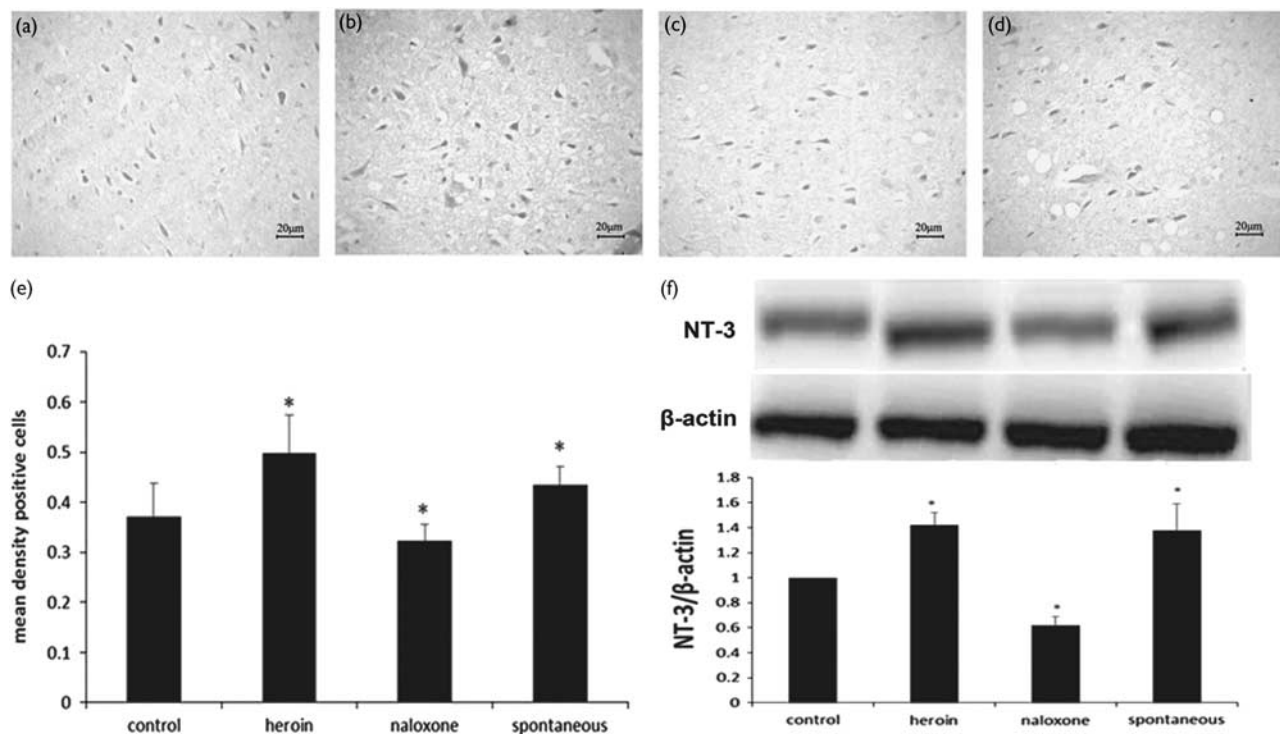
Discussions

The results of the present study show that chronic heroin administration and withdrawal differentially regulate the

expression of neurotrophins BDNF, NT-3, and NT-4 in the rat NAc. Chronic heroin treatment led to a significant decrease in the expression levels of BDNF and NT-4 in the NAc of rats in comparison with the control group, whereas there was a significant increase in BDNF and NT-4 expression in the groups of rats during heroin withdrawal. In contrast to BDNF and NT-4, chronic heroin administration significantly increased NT-3 expression in the NAc of rats with heroin addiction and spontaneous withdrawal, but decreased in the NAc of rats with naloxone-induced withdrawal. These results support the notion that neurotrophins might play a critical role in opiate addiction and withdrawal.

The neurotrophins, a family of structurally and functionally related polypeptides, have been implicated in the maintenance of neuronal excitability and synaptic transmission, synaptic plasticity, neuroprotection, and drug addiction [24,25]. The four neurotrophins produce their biological effects by activating three paralogous receptor tyrosine kinases (TrkA, TrkB, and TrkC) and the 75 kDa a neurotrophin receptor (p75^{NTR}) [26]. BDNF and NT-4 are selective for TrkB, whereas NT-3

Fig. 3



Alterations of NT-3 expression in the rat nucleus accumbens (NAc) during heroin dependence and withdrawal. (a–d) NT-3 expression was found to be mainly located in the cytoplasm in the rat NAc. (e) After injection of heroin for 9 days, expression levels of NT-3 were markedly increased in the NAc of rats with heroin addiction and spontaneous withdrawal in comparison with the control group (mean optical density: 0.498 ± 0.076 vs. 0.435 ± 0.037 vs. 0.372 ± 0.066 , $*P < 0.05$), but significantly decreased in the groups of rats with naloxone-induced withdrawal (mean optical density: 0.322 ± 0.033 vs. 0.372 ± 0.066 , $*P < 0.05$). (f) Western blotting further confirmed similar changes of NT-3 expression in the rat NAc during heroin dependence and withdrawal (NT-3/β-actin: 1.42 ± 0.1 vs. 0.62 ± 0.07 vs. 1.38 ± 0.21 vs. 1.0 ± 0.0 , $*P < 0.05$).

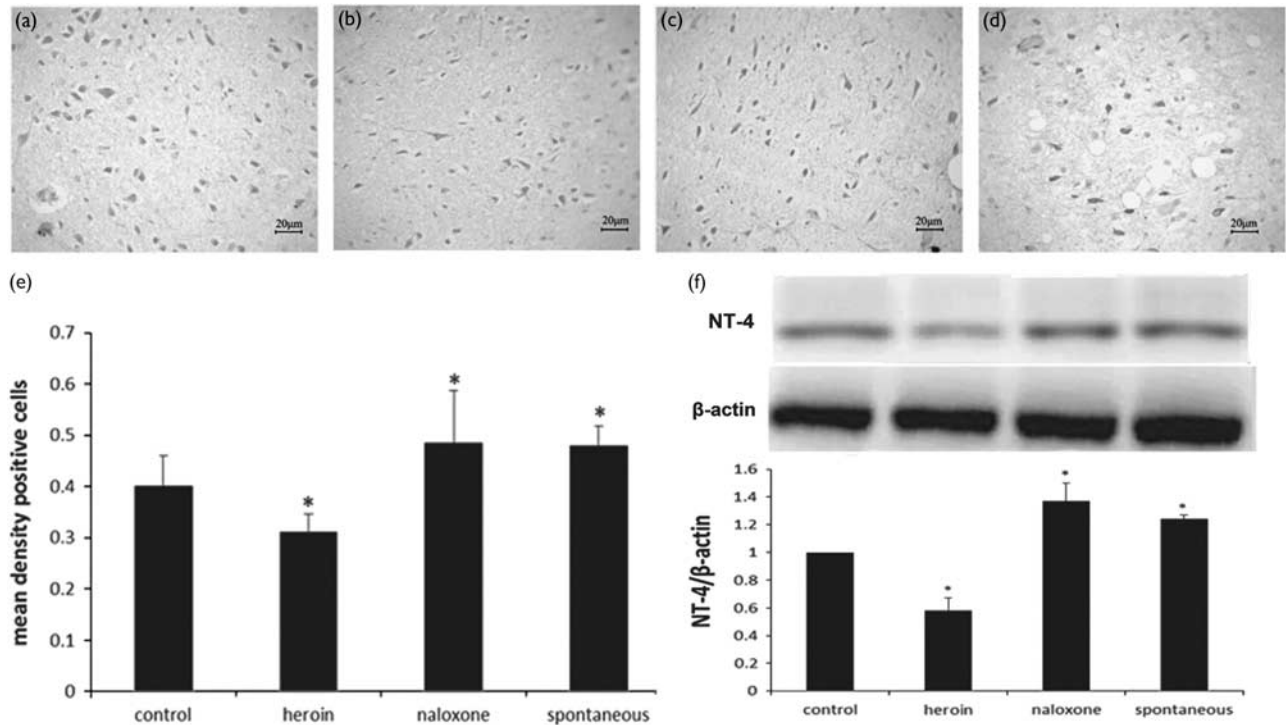
preferentially binds TrkA and TrkC receptors [22]. Neurotrophins have been associated with the addictive process, mostly regulating the dopaminergic transmission in the NAc, which receives projections from the VTA [19]. Intra-infusion of exogenous neurotrophic factors directly into the VTA or NAc has been shown to prevent some of the biochemical and structural adaptations to chronic drug exposure [6,27,28]. However, the effect of heroin on the expression of endogenous BDNF, NT-3, and NT-4 in the rat NAc has not yet been well elucidated.

BDNF is best characterized for its role in promoting the neural and behavioral plasticity induced by cocaine or other stimulants through a key reward circuit where BDNF is engaged in a feed-forward loop that promotes further actions of stimulant drugs [29,30]. It has been shown previously that exogenously added BDNF and NT-4 can produce similar effects in the NAc [6]. In the present study, the expression levels of BDNF and NT-4 were significantly decreased in the NAc of rats with heroin addiction in comparison with the control group. Our result is consistent with previous studies that showed that chronic morphine administration decreases BDNF

expression in the NAc [31,32]. BDNF in the NAc is predominantly supplied by anterograde axonal transport from cortical pyramidal neurons in PFA, with a minor contribution from dopamine neurons in VTA [33]. Therefore, a possible speculation of the decrease in BDNF expression observed was because of the decrease in protein synthesis, anterograde axonal transport from PFA and VTA, where decreased BDNF expression was induced by chronic cocaine and morphine exposure [11,34]. BDNF expression in the NAc in response to different stimulants, however, is not uniform. Several studies have found increased BDNF expression in the NAc [9,35]. In addition, Smith *et al.* [17] reported mice lacking NT-4 reduces tolerance to morphine. Thus, the endogenous expression of BDNF and NT-4 to opiates may depend not only on the particular drug type or drug regimen used but also on the presence of other factors that are not yet defined.

In our results, increases in BDNF and NT-4 expressions were found in the groups of rats with both naloxone-induced and spontaneous withdrawal compared with the control group. These results are in agreement with previous observations showing a time-dependent increase in

Fig. 4



Alterations of NT-4 expression in the rat nucleus accumbens (NAc) during heroin dependence and withdrawal. (a–d) NT-4 expression was found to be mainly located in the cytoplasm in the rat NAc. (e) The levels of NT-4 expression were markedly decreased in the NAc of rats with heroin addiction compared with the control group (mean optical density: 0.312 ± 0.035 vs. 0.401 ± 0.059 , $*P < 0.05$), but significantly increased in the groups of rats with naloxone-induced and spontaneous withdrawal (mean optical density: 0.485 ± 0.102 vs. 0.479 ± 0.039 vs. 0.401 ± 0.059 , $*P < 0.05$). (f) Western blotting further confirmed similar changes of NT-4 expressions in the rat NAc during heroin dependence and withdrawal (NT-4/β-actin: 0.58 ± 0.09 vs. 1.37 ± 0.13 vs. 1.24 ± 0.03 vs. 1.0 ± 0.0 , $*P < 0.05$).

BDNF expression in the NAc after withdrawal from prolonged cocaine self-administration [10,35]. In addition, increased serum/plasma BDNF levels were found in heroin-dependent patients during withdrawal [14]. It has been hypothesized that BDNF and NT-4 may be responsible for maintenance of the neuronal phenotype [6]. The increases in BDNF and NT-4 expressions could serve to counter the neurotoxicity caused by chronic heroin withdrawal on the biochemistry of NAc neurons. Future studies with antagonists to BDNF and NT-4 will be needed to examine the exact effects of chronic heroin withdrawal.

In contrast to the alterations in BDNF and NT-4 following heroin dependence and withdrawal, there was a significant increase in NT-3 expression in the NAc of rats with heroin addiction and spontaneous withdrawal, but decrease in the NAc of rats with naloxone-induced withdrawal. Our results are similar to earlier studies in which the effects of different stimulants on NT-3 in the different brain regions were assessed. For example, Pierce *et al.* [15] reported that an acute injection of cocaine resulted in a marked increase in NT-3 expression in the VTA, which would be expected to increase BDNF

signaling from the VTA to the NAc. Hatami *et al.* [22] reported that chronic morphine treatment and withdrawal induced a significant upregulation of NT-3 expression in paraventricular nucleus to cellularis. In addition, a significant increase in NT-3 expression in the hippocampus was found following chronic exposure to ethanol [36]. Increased NT-3 may function as a trophic factor for many neurotrophin-responsive neuronal populations. Our results indicate that increased expression of NT-3 in the NAc during heroin treatment and spontaneous withdrawal could then contribute toward adaptations and homeostatic mechanisms that occur in the NAc as well.

Conclusion

Our investigation showed the differential regulation of BDNF, NT-3, and NT-4 in the rat NAc following heroin dependency and withdrawal. These findings provide further evidence for the involvement of neurotrophins particularly in the NAc in the development of heroin dependence and withdrawal. Although the exact physiological role played by these neurotrophin alterations during the course of heroin treatment and withdrawal will require further investigation, these results may highlight

a potential influence of neurotrophic factors in heroin addiction.

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Conflicts interest

There are no conflicts of interest.

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