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Research Report

Peripheral electrical stimulation reversed the cell size reduction and increased BDNF level in the ventral tegmental area in chronic morphine-treated rats

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ABSTRACT

Chronic morphine administration induces functional and morphological alterations in the mesolimbic dopamine system (MLDS), which is believed to be the neurobiological substrate of opiate addiction. Our previous studies have demonstrated that peripheral electrical stimulation (PES) can suppress morphine withdrawal syndrome and morphine-induced conditioned place preference (CPP) in rats. The present study was designed to investigate if PES could reverse the cell size reduction induced by chronic morphine treatment in the ventral tegmental area (VTA), which is an important area of the MLDS. Immunohistochemical observations showed that the cell size of dopaminergic neurons in the VTA reduced significantly in the chronic morphine-treated rats with a concomitant decrease in the number of BDNF-positive cells compared to the saline-treated rats. A much milder morphological change, accompanying with an increased number of BDNF-positive cells, was observed in dopaminergic neurons in the rats that received repeated 100 Hz PES after morphine withdrawal. In another experiment, enzyme-linked immunosorbent assay (ELISA) reconfirmed a significant up-regulation of BDNF protein level in the VTA in the rats received 100 Hz PES after morphine abstinence. These results indicate that PES could facilitate the morphological recovery of the VTA dopaminergic cells damaged by chronic morphine treatment and up-regulate the BDNF protein level in the VTA. Activation of endogenous BDNF by PES may play a role in the recovery of the injured dopaminergic neurons in the morphine addictive rats.

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1. Introduction

The mesolimbic dopamine system (MLDS) has been known as the neural substrate involved in opiate reinforcement and

addiction (Koob et al., 1992; Shippenberg et al., 1993). It includes the dopaminergic neurons located in the VTA and their anterior projections to the limbic forebrain, for example, the nucleus accumbens (NAc) and the frontal cortex (Volkow

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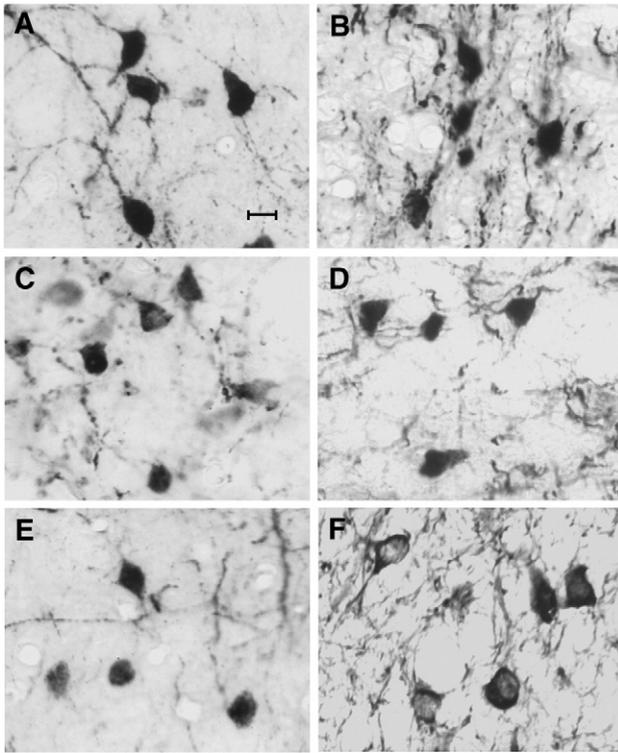


Fig. 1 – 100 Hz PES treatment accelerated the recovery of VTA dopaminergic neurons shrinkage induced by chronic morphine administration. The figure shows dopamine neurons taken from: (A) NS control rats; (B) M-3 h group; (C) M-24 h group; (D) M-14 d group; (E) restraint control group; (F) 100 Hz PES group. $n=4$. Scale bar = 10 μm .

and Li, 2005). Chronic administration of morphine produces a number of adaptive changes in the MLDS: a hypofunction of the dopaminergic neurons of the VTA (Diana et al., 1995; Bonci and Williams, 1997; Manzoni and Williams, 1999; Diana et al., 1999), a decrease of the content of neurofilament proteins (intermediate filament proteins specific to neurons) (Beitner-Johnson et al., 1992; Garcia-Sevilla et al., 2004), an impairment of the axonal transport in the VTA-NAc pathway (Beitner-Johnson and Nestler, 1993) and a reduction of the dopaminergic cell size in the VTA (Spiga et al., 2003; Sklair-Tavron et al., 1996). These modifications represent the opiate-induced neuronal plastic changes, which are believed to contribute to opiate addiction (Nestler, 2004, 1997).

Previous results have shown that PES can suppress both morphine withdrawal syndrome (Liu et al., 2005; Han and Zhang, 1993) and morphine-induced CPP expression in rats (Shi et al., 2003, 2004; Chen et al., 2005), as well as heroin craving in the addicts (Zhang et al., 2000; Zhong et al., 2006). It seemed important to study whether PES can reverse chronic morphine-induced neuronal plasticity changes in the MLDS as above mentions. Thus, the first aim of the present study was to investigate if PES can accelerate the recovery of dopaminergic cell size reduction in the VTA induced by chronic morphine administration.

The morphological changes in the VTA dopaminergic neurons induced by chronic morphine exposure may indicate neural injury. In this context, neurotrophic factors could play a

role in the prevention or protection of long-term changes in responses to the drug exposure. Indeed, direct infusion of brain-derived neurotrophic factor (BDNF) or related neurotrophins into the VTA can block morphine-induced biochemical and morphological changes in this brain region (Berhow et al., 1995; Sklair-Tavron et al., 1996). Moreover, *in vivo* study revealed that BDNF protected dopaminergic neurons in the substantia nigra against toxic effects of 6-hydroxydopamine ions (Baquet et al., 2005; Zheng et al., 2005) and stimulated their neuronal activity (Shen et al., 1994). Taken together, these results suggest that BDNF may implicate in regulating neuronal plasticity in the CNS (Shen et al., 1994; Gaiddon et al., 1996). Liang et al. (2002) has reported that 100 Hz PES increased the abundance of BDNF mRNA in the VTA and substantia nigra in a rat model of Parkinson's disease. And our second aim in the present study was to determine whether PES can increase the endogenous BDNF pathway along with the recovery of damaged dopaminergic neurons in the VTA of chronic morphine-treated rats. Nerve Growth Factor (NGF) was used as a control growth factor, since NGF was known not to be related with morphine-induced neuronal plasticity (Berhow et al., 1995).

2. Results

2.1. Repeated 100 Hz PES accelerated the morphological recovery of the VTA dopaminergic neurons damaged by chronic morphine treatment

TH immunohistochemical staining was performed and the morphological changes of dopaminergic neurons in the VTA were measured as described in Experimental procedures. As shown in Fig. 1, chronic morphine treatment decreased the size of dopaminergic neurons in the VTA. The neuronal areas decreased by 34.5%, 17.8% and 44.0% respectively, 3 h, 24 h and 14 days after the last morphine injection compared to NS control groups (see Experimental procedures for the animal grouping). A two-way ANOVA for treatment (saline-morphine) \times time showed significant effects for drug ($F_{1,18}=31.78$; $P<0.01$), but not for time, with no interaction. Reduction on the mean calculated area and perimeter of the neurons is shown in

Table 1 – Effects of PES on the morphometrical changes of dopaminergic neurons induced by chronic morphine administration

Groups	Area (μm^2)	Perimeter (μm)
NS-3 h	115.2 \pm 7.11	56.65 \pm 2.53
M-3 h	75.43 \pm 5.11**	43.04 \pm 2.25*
M-24 h	94.67 \pm 3.29*	47.08 \pm 2.45*
NS-14 d	119.20 \pm 11.71	55.72 \pm 3.80
M-14 d	64.50 \pm 4.28##	39.23 \pm 1.07##
Restraint	61.21 \pm 3.92##	38.64 \pm 2.21##
100 Hz PES	102.0 \pm 7.95 $\Delta\Delta$	50.10 \pm 3.03 Δ

Data are expressed as means \pm SEM. * $P<0.05$, ** $P<0.01$: compared with NS-3h group. # $P<0.05$, ## $P<0.01$: compared with NS-14d group. $\Delta P<0.05$, $\Delta\Delta P<0.01$: compared with M-14 d group. Comparisons between means of groups were analyzed with ANOVA followed by Newman-Keuls post-test.

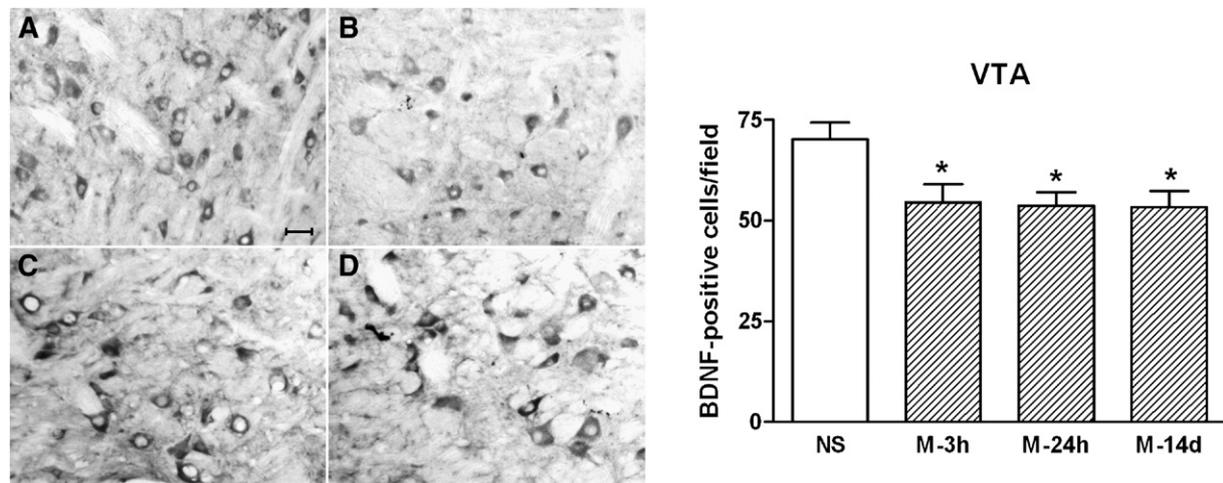


Fig. 2 – Representative photomicrographs of BDNF-positive cells in the VTA after chronic morphine administration. The figure shows BDNF-positive cells taken from: (A) NS control group; (B) M-3 h group; (C) M-24 h group; (D) M-14 d group. The column figure shows quantitative analysis of the number of BDNF-positive cells in the VTA. * $P < 0.05$, compared with NS control groups. One-way ANOVA followed by Newman-Keuls post-test, $n = 4$ in each group. Scale bar = 40 μm .

Table 1. In the rats that received 100 Hz PES after chronic morphine administration for 13 sessions (10 days), the cell area of dopaminergic neurons in the VTA was significantly recovered than morphine withdrawal 14-day rats without PES treatment ($F_{3,12} = 9.72$, $P < 0.01$), as shown in Fig. 1 and Table 1. However, in the adjacent area of substantia nigra, dopaminergic neurons showed normal appearance without obvious morphological abnormalities (data not shown).

2.2. 100 Hz PES increased the number of BDNF-positive cells in the VTA of the morphine withdrawal rats

To determine the quantitative changes of BDNF-positive cells after chronic morphine administration, we examined the

number of BDNF-positive cells in the VTA using immunohistochemistry. Our preliminary study showed that there were no significant differences of the BDNF- and NGF-positive cells in NS group at different times after the last injection of drugs, so we combined N-3 h and N-24 h groups as one for the convenience of description. As shown in Fig. 2, the BDNF-positive neurons in the VTA were decreased significantly in the rats of M-3 h, M-24 h and M-14 d groups ($F_{3,15} = 4.167$, $P < 0.05$), and the BDNF-positive cells decreased by 22.4%, 23.5% and 24.2%, 3 h, 24 h and 14 days after the last morphine injection ($P < 0.05$), respectively. There was, however, no change of the number of NGF-positive cells in the three groups (data not shown).

As shown in Fig. 3, 100 Hz PES increased (+37.6%, against M-14 d group) significantly the number of BDNF-positive cells in

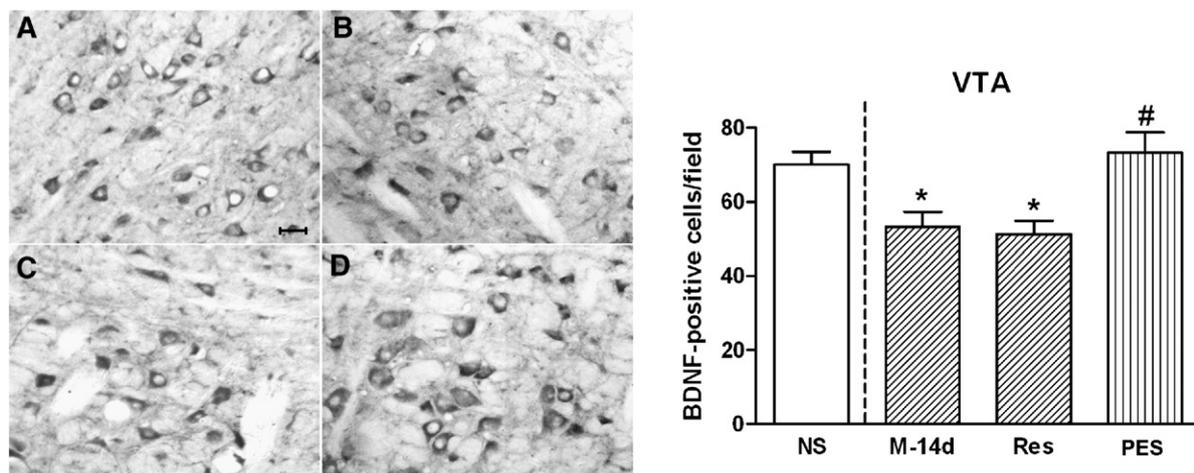


Fig. 3 – Influence of PES on BDNF-positive cells in the VTA on the 14th day of morphine withdrawal. The figure shows BDNF-positive cells taken from: (A) NS control group; (B) M-14 d group; (C) restraint control group; (D) 100 Hz PES group. The column figure shows quantitative analysis of BDNF-positive cells in the VTA. * $P < 0.05$, compared with NS control groups. # $P < 0.05$, compared with M-14 d group. One-way ANOVA followed by Newman-Keuls post-test, $n = 4$ in each group. Scale bar = 40 μm .

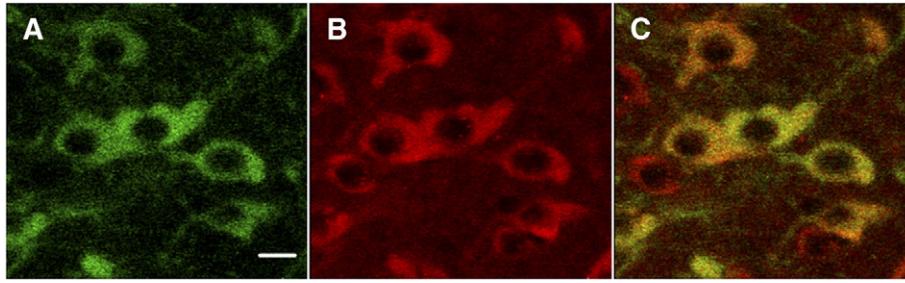


Fig. 4 – Co-immunofluorescence of TH-positive and BDNF-positive cells in the VTA. (A) TH-positive cells (green). (B) BDNF-positive cells (red). (C) Merged staining profile. Scale bar = 10 μ m.

the VTA in the morphine withdrawal rats ($F_{2,11}=7.32$, $P<0.05$). No significant differences of the number NGF-positive cells in the VTA were found between the two groups (data not shown). Co-immunofluorescence was executed to identify the locational relationship between BDNF-positive cells and TH-positive cells in the VTA in NS group. It was found that most of the BDNF-positive cells and TH-positive cells were co-localized or localized closely (Fig. 4).

2.3. 100 Hz PES up-regulated BDNF protein levels in the VTA in the morphine withdrawal rats

Influence of chronic morphine administration on the levels of two neurotrophic factors was measured with enzyme-linked immunosorbent assay (ELISA), and the results are shown in Fig. 5. No significant changes in the level of the BDNF were found in the VTA 3 h and 24 h after drug abstinence. The content of BDNF decreased significantly 14 days after the last injection of morphine. For NGF, the content was rather stable, except for a significant increase in the VTA at 24 h of morphine withdrawal ($F_{2,6}=14.56$, $P<0.01$).

Effect of the PES on the BDNF and NGF protein levels is shown in Fig. 6. Two weeks after the last administration of morphine, the BDNF content in the VTA was significantly lower than that of the NS group (79.37 ± 9.526 vs. 139.6 ± 16.07 pg/mg protein). One-way ANOVA showed a significant difference among the groups ($F_{4,18}=8.49$, $P<0.01$). 100 Hz PES up-regulated BDNF protein levels

to approach the normal level in the VTA ($P<0.05$ compared to M-14 d group; $P>0.05$ compared to the NS control group) on 14-day morphine withdrawal.

No such changes were observed in the content of NGF in the VTA (Fig. 6B) in morphine withdrawal rats.

3. Discussion

In the present study, we found for the first time that multiple 100 Hz PES could accelerate the recovery of morphine-induced morphological changes of dopaminergic neurons in the VTA. Our results showed that the cell size reduction induced by chronic morphine administration lasted for at least 14 days after morphine abstinence, which is consistent with the earlier studies (Sklair-Tavron et al., 1996; Spiga et al., 2003; Kalivas and Nakamura, 1999). The cell size of VTA dopaminergic neuron in M-24 h group was increased compared to that of M-3 h group in our study, which maybe related to withdrawal-induced adaptive reaction of the organism and up-regulated TH in the early stage of morphine withdrawal (Beitner-Johnson and Nestler, 1991). Our results differed from Spiga et al., who found that VTA dopaminergic neurons did not change after 1 h morphine abstinence and reduced greatly at 24 h of morphine withdrawal. A plausible reason for this discrepancy could be the differences in the way and time of the animal sacrifices. In the study of Spiga et al., rats were

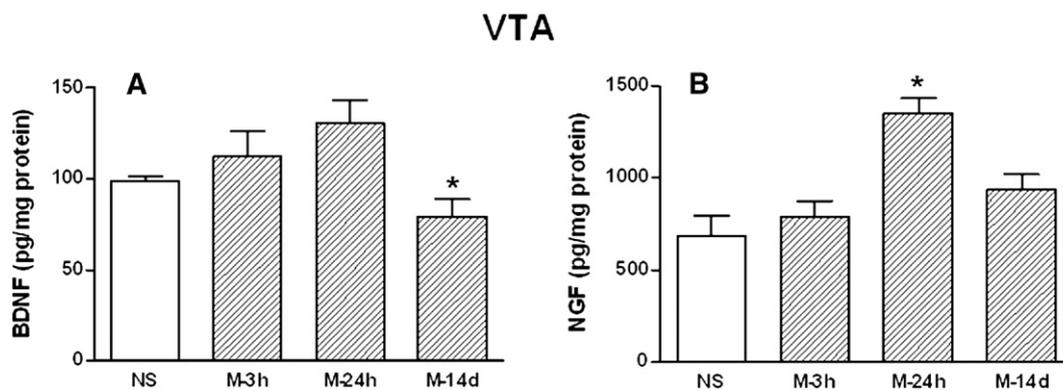


Fig. 5 – ELISA analysis of immunoreactivity changes of BDNF (A) and NGF (B) in the VTA of rats 3 h, 24 h and 14 days after morphine abstinence (shaded column). Equal numbers of rats were given normal saline (empty column) as control. * $P<0.05$, compared with NS control groups. One-way ANOVA followed by Newman–Keuls post-test, $n=3-4$ in each group.

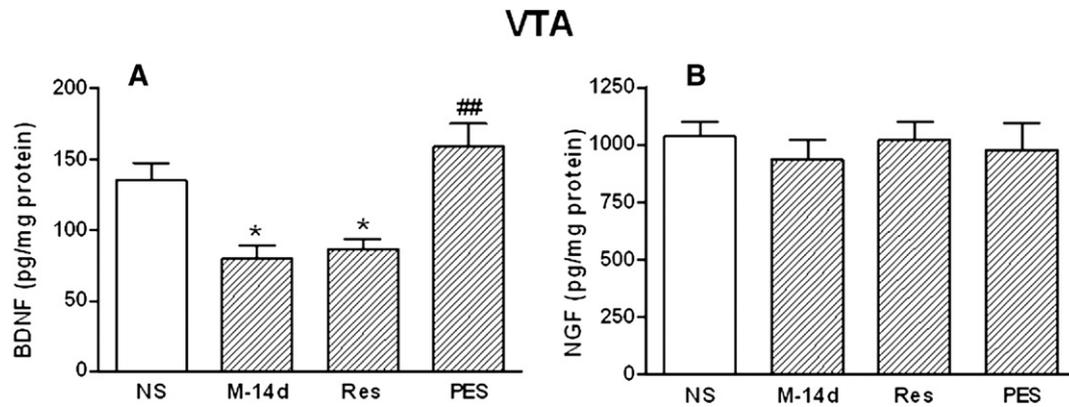


Fig. 6 – Effects of 100 Hz PES on the levels of BDNF, and NGF in the VTA of rats on the 14th day of morphine withdrawal. * $P < 0.05$; compared with NS control groups. ## $P < 0.01$, compared with M-14 d group. One-way ANOVA followed by Newman–Keuls post-test, $n = 4$ in each group (morphine withdrawal groups, shaded column; saline control groups, NS, empty column).

killed by decapitation. Whereas in our treatment, the rats were anesthetized with sodium pentobarbital and perfused intracardially with saline followed by 4% paraformaldehyde, with the whole procedure took about 2 h.

Interestingly, the cell size reduction occurred in the VTA in our study, but not in the substantia nigra, an adjacent area that is also rich of dopaminergic neurons. The substantia nigra dopaminergic neurons, referred to as A9, innervate the dorsal striatum and serve a motor control function. The VTA dopaminergic neurons, referred to as A10, mainly innervate the NAc, frontal cortex, the bed nucleus of the stria terminalis and amygdala complex, and constitute the MDLS, which is known to be involved in motivation, reward and sustained attention processes (Kalivas and Nakamura, 1999). Since VTA is one of the key neural substrates for the rewarding effects of drugs (Bozarth and Wise, 1983; Wise, 1989; Koob and Le, 2005), the chronic morphine-induced cell size reduction in the VTA contributes significantly to the neurobiology of addiction. It is reported that the decreased size of VTA dopaminergic neurons was associated with reward tolerance during early withdrawal, which may result in escalation of drug taking (Russo et al., 2007). The effect of PES reversing morphine-induced VTA cell size reduction may underlie one of the mechanisms of PES in treating morphine addiction.

Acupuncture has become one of the standard procedures in many detoxification programs worldwide (Whitehead, 1978; Ulett et al., 1998; Montazeri et al., 2002; D'Albarto, 2004; Liu et al., 2005). Unlike the drug approach, which usually has aversive side effects, there is little if any adverse effect with the acupuncture therapy (Lu et al., 2004). As a modified form of acupuncture, PES has been proven to be efficacious in treating morphine addiction by our previous study (Wu et al., 1999, 2000; Cui et al., 2004; Zhong et al., 2006). Han et al. have summarized that 100 Hz (high frequency) PES was effective in ameliorating the morphine withdrawal syndrome in chronic morphine-treated rats (Han and Zhang, 1993; Han et al., 2005), so in this study we used the frequency of 100 Hz. It is known that brain opioid-receptors are involved in mediating PES-induced inhibition of morphine CPP in rats (Shi et al., 2003). Compared to the research on opioid mechanisms, there has been much less investigation on dopamine mechanisms

underlying the PES effect. Besides, a possible role played by neurotrophic factors deserves serious consideration.

It is well known that neurotrophic factors, such as BDNF, NGF, neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5), support and maintain normal neuronal function, including their survival, differentiation and plasticity. BDNF has been shown to have trophic effects on noradrenergic neurons (Chao, 2003). And BDNF signaling pathway is essential for neural plasticity in response to drugs of abuse. Behavioral responses to cocaine were reduced in the BDNF knockouts (Hall et al., 2003). Intra-VTA infusion of BDNF prevented the characteristic biochemical (Berhow et al., 1995) and morphological (Sklair-Tavron et al., 1996) changes after chronic morphine exposure. Moreover, IRS2-Akt pathway may play an important role in the effect of BDNF for the revitalization of dopaminergic neurons shrinkage in the VTA (Russo et al., 2007). In the present study, we found that the number of BDNF-positive cells decreased after morphine abstinence, accompanying with the cell size reduction in the VTA. This is a very interesting phenomenon, which may partly explain the mechanisms of morphine-induced morphological changes of dopaminergic neurons in the VTA. 100 Hz PES increased the number of BDNF-positive cells in the VTA on 14 days of morphine withdrawal. Meanwhile, there was an accelerated recovery of the shrinkage of dopaminergic neurons in PES group. Our co-immunofluorescence result showed that BDNF-positive cells and TH-positive cells were co-localized or sitting closely in the VTA. Both BDNF and its receptor TrkB can be found in the VTA (Numan et al., 1998). BDNF released from single cells acted directly on nearby recipient neurons on an exquisitely local scale (Horch and Katz, 2002). So it may be concluded that increased BDNF protein induced by PES acts on the VTA dopaminergic neurons in an autocrine and/or paracrine manner.

To reconfirm the changes of BDNF and NGF observed in the immunohistochemistry study, we used ELISA, which is a method commonly used to detect neurotrophic factors (Sei et al., 2000; Grimm et al., 2003; Yan et al., 2005). Our results of ELISA showed that no significant changes of BDNF protein level were found 3 h and 24 h of morphine withdrawal in the VTA. A significant decrease was found only 14 days after morphine withdrawal. This is in contrast with the immunohistochemical

data, which showed a decrease of the number of BDNF-positive cells in the VTA 3 h after the last injection of morphine and remained as such through the whole course of withdrawal for 14 days. The variance may result from the different experimental methods. In the immunohistochemistry study, the number of BDNF-positive cells was counted; whereas in the ELISA study, the content of BDNF in the tissue of VTA was measured, including the peptide located within the cells as well as in the extracellular space. In other words, disintegrated neurons disappeared morphologically but may still exist neurochemically in the tissue for a short period of time. A significant decrease of both the content and the number of BDNF-positive cells in the VTA seemed to remain for as long as 14 days. It was interesting to find that these dramatic and long-lasting changes were rescued effectively by the application of 100 Hz PES after the discontinuation of the drug. The possibility that the changes of the content of neurotrophic factors in the VTA may be accounted for by the change in body weight or the ages of the rats has been excluded by the setting of parallel saline control groups, and the data obtained in other laboratories seem to substantiate this finding (Miller, 2004).

A concomitant finding was that morphine-treated rats subjected to 24 h withdrawal expressed a significant increase in NGF level in the VTA (Fig. 5). The mechanisms and the impacts of this phenomenon remain obscure. It is reported that NGF was constitutively secreted whereas BDNF remains in the cytoplasm under resting conditions (Miller, 2004). Depolarization triggered the release of BDNF but not NGF, suggesting that while the secretion of NGF is constitutive, that of BDNF is activity-dependent (Schinder and Poo, 2000). Patterned electrical field stimulation of cultured primary sensory neurons was shown to increase extracellular BDNF levels (Balkowiec and Katz, 2000), so it is rational to suggest that the facilitated release or synthesis of BDNF induced by PES could be achieved by means of increasing the neuronal activity in the VTA. Further studies are needed to substantiate this speculation. The current literature does not support a close relationship between NGF and dopaminergic neurons (Berhow et al., 1995). Our results showed that PES treatment had no significant influence on the content of NGF. Therefore NGF may not be involved in the effect of PES for the treatment of opiate addiction. In contrast, the marked increase of the content of BDNF in the VTA in PES group may explain the mechanism of PES in rescuing dopaminergic neurons in morphine withdrawal rats, although direct evidence is still lacking to support that the effect of PES on dopaminergic neurons is mediated by BDNF.

In summary, (1) escalating doses of morphine treatment twice a day for 14 days resulted in a cell size reduction of dopaminergic cells and reduced the number of BDNF cells in the VTA of the rats, with the effects lasting for at least 14 days; (2) these changes were site-specific, occurring in the VTA, but not in the adjacent area of substantia nigra; (3) 100 Hz PES treatment for 13 sessions was capable of rescuing the dopaminergic cells and BDNF cells in the VTA from the damage produced by chronic morphine administration and (4) an up-regulation of BDNF protein level in this area by PES may play a role in the repairing of morphological damage of the dopaminergic cells.

4. Experimental procedures

4.1. Subjects

Male Sprague–Dawley rats, weighing 180–220 g at the beginning of the experiment, were obtained from the Institute of Animal Research, Chinese Academy of Sciences, Beijing. They were housed four per chamber, in a standard 12:12-h light/dark cycle (light on at 07:00) with food and water *ad libitum*.

The room temperature was maintained at 22 ± 1 °C. The rats were habituated to the environment and handled daily for 5 days before the experiment. The experimental procedures were approved by the Committee on Animal Care and Use of Peking University.

4.2. Morphine and PES administration

Morphine hydrochloride, purchased from the First Pharmaceutical Factory of Shenyang, China, was dissolved in sterile saline and administered twice daily (at 08:00 and 20:00 h) for 14 days as described (Diana et al., 1995). Briefly, the initial dose administered was 20 mg/kg and was increased by 20 mg/kg every other day until the 14th day of treatment, reaching a dose of 140 mg/kg for the last injection. Morphine doses up to 100 mg/kg were administered *s.c.* in a volume of 1 ml/kg, whereas higher doses were administered *i.p.* in a volume of 1 ml/0.1 kg. Normal saline (NS) control rats received an equal volume of saline.

Rats chronically treated with morphine for 14 days were randomly assigned to the following groups: (1) morphine withdrawal 3 h group (M-3 h): rats were decapitated 3 h after the last morphine injection; (2) morphine withdrawal 24 h group (M-24 h): morphine spontaneous withdrawal for 24 h before sacrifice, without any further treatment; (3) morphine withdrawal 14-day group (M-14 d): morphine withdrawal for 14 days before sacrifice, without any further treatment; (4) 100 Hz PES group: after chronic morphine administration, rats were given 100 Hz PES for 13 sessions; 100 Hz PES group was decapitated 14 days after the last morphine administration; (5) restraint control (Res) group: rats were restrained in the holder 30 min per session for 13 sessions in 10 days without receiving PES.

The PES treatment was executed as follows: the rats were given 12 h after the last injection of morphine, two stainless steel needles of 0.3 mm diameter were inserted into each hind leg, one in the acupoint ST36 (5 mm lateral to the anterior tubercle of the tibia) and the other in the acupoint SP-6 (at the level of the upper border of the medial malleolus, posterior border of the tibia). In traditional Chinese medical practice, ST36 (Zusanli) and SP6 (Sanyinjiao) are two most commonly used points in the lower extremity. They have been proved to be effective in producing analgesic effect in humans as well as for the treatment of heroin withdrawal syndrome in the addicts. Therefore these points are also used in the present study. Constant current square-wave electric stimulation generated by a programmed pulse generator HANS LH-800 (produced by Peking University of Astronautics and Aeronautics Aviation) was given via the two needles for a total of 30 min. The frequency of stimulation used was 100 Hz (0.2 ms pulse width). The intensity of the stimulation was increased stepwise from

0.5 mA to 1 mA and then 1.5 mA, with each step lasting for 10 min. To control for the unavoidable effects of restraint stress from PES treatment, the subjects of the restraint group were simply restrained in the holder for 30 min. On the 14th day after the last injection of morphine, the rats were sacrificed for immunohistochemical study and ELISA.

4.3. Experiment 1: immunohistochemistry

4.3.1. Observation of dopaminergic neuron morphology in the VTA

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused intracardially with saline (0.9% NaCl, 150 ml), followed by 300 ml of 4% ice-cold paraformaldehyde prepared in 0.1 mol/l phosphate buffer solution (pH 7.4). Brains were dissected out and placed in the same fixative solution at 4 °C overnight. After the postfixation, brains were soaked in 30% sucrose (prepared in 0.1 mol/l phosphate buffer, pH 7.4) at 4 °C overnight. They were then frozen and coronal slices (20 μm thick) were obtained with a cryostat, beginning at –5.80 mm and ending at –6.30 mm from bregma (according to Paxinos and Watson, 1997) (Fig. 7), and immediately mounted onto 3-aminopropyltriethoxysilane (APES)-coated glass slides. The slides were immunostained for detection of the tyrosine hydroxylase (TH, Sigma, St. Louis, USA), which is used as the marker of dopaminergic neurons. First, slides were air dried for 30 min, rehydrated with phosphate-buffered saline (PBS) for 15 min and treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. Slides were washed thrice in PBS for 5 min each, followed by 30 min of incubation with 10% normal goat serum (NGS) in PBS plus 0.3% Triton X-100 and kept at room temperature (RT) according to the protocol of the manufacturer. After excess fluid was drained, slices were then incubated overnight at 4 °C with anti-TH antiserum (diluted 1:8000). Slices were then washed 3 × 5 min in PBS, and immunostained with avidin–biotin–peroxidase method (Elite ABC, Vector Labs, CA, USA). The bound complex was visualized by incubating sections in a solution containing 0.1% 3,3'-diaminobenzidine (Sigma), 1% H₂O₂ and 8% ammonium nickel sulfate (Fluka Chemie GmbH, Switzerland). Images of TH-positive cells were collected from the VTA marked off ventrally by the interpeduncular nucleus (IPDL) and laterally by the medial lemniscus (ml) and parabrachial pigmented nucleus (PBP). The dorsal boundary was defined by the red nucleus magnocellularis (RMC), whereas the medial mark was given by the caudal linear nucleus of the raphe (Cli) (Paxinos and Watson, 1997; Spiga et al., 2003). Images were generated by using a 40× objective under normal bright-field illumination (Olympus, Japan). The morphological parameters of the TH-positive cells were analyzed by an investigator blind to the animal treatment using an advanced image-analysis system (Metmorph). The area of the cell's body was obtained by marking its profile, excluding all dendritic trunks. This yielded the bounded area in calibrated square units (μm²). Perimeter values were evaluated similarly and the total boundary length was expressed in μm.

4.3.2. Observation of BDNF and NGF-positive cells in the VTA
The procedures of immunostaining BDNF and NGF were just like that of TH. The sections were from the same rats that were used

for detecting TH. The BDNF and NGF (Chemicon) antibody was diluted at 1:200 and 1:1000, respectively. No staining was observed when NGS was applied in place of the antibodies. Images of BDNF-positive and NGF-positive cells were generated by using a 10× objective under normal bright-field illumination from the square area depicted in Fig. 7, and the number BDNF-positive cells and NGF-positive cells in the VTA was counted.

4.3.3. Co-immunofluorescence for TH and BDNF in the VTA
Each primary antiserum was reacted for 2 h at room temperature. Unspecific antibody binding was abrogated by preincubation in 10% NGS. Tissue permeability was facilitated by addition of 0.3% Triton X-100 to all solutions. Appropriate secondary antisera were reacted over 1 h at room temperature. Specificity of the signals was verified by omitting primary antisera. Confocal images were generated using SP2 Leica, 40× oil.

4.4. Experiment 2: ELISA analysis of BDNF and NGF protein levels

Three to 5 rats were used for each group in each experiment. To minimize possible pharmacological interactions between anesthetic and various drug treatments, rats were sacrificed by decapitation. The ventral midbrain (consisting mainly of VTA) was dissected out on ice under a surgical magnifier. Tissues were sonicated in lysis buffer consisting of 137 mM NaCl, 20 mM Tris (pH 8.0), 1% Nonidet P (NP)-40, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin, 1 μg/ml leupeptin and 0.5 mM sodium vanadate. Lysates were clarified by centrifugation at 50,000×g for 20 min, and the resulting supernatants were diluted 5-fold with DPBS. Samples were then acidified to pH 3.0 by adding 1 N HCl, vortexed briefly and kept at room temperature for 10 min, then neutralized with 1 N NaOH to pH 7.5. Aliquots of each sample were used for measurement of BDNF or NGF levels with the ELISA procedure using BDNF and NGF Emax ImmunoAssay System kits (Promega, Madison, WI) according to the protocol of the manufacturer. The Promega Emax Immuno Assay System kits have typically less than 3% cross-reactivity with other neurotrophic factors and can detect a minimum of 15.6 pg/ml BDNF and

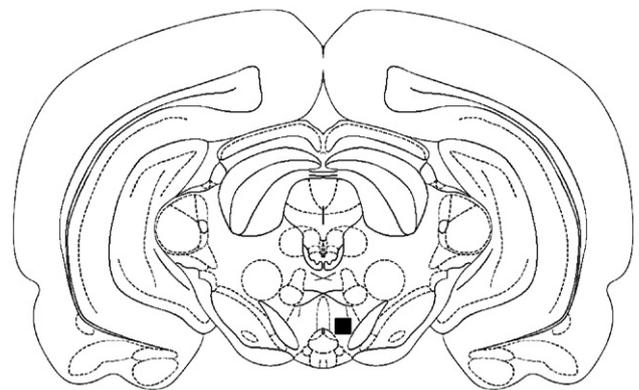


Fig. 7 – Schematic illustration of the location of VTA neurons sampled in the present study (inserted box). They were sampled from –5.6 to –6.3 mm of Bregma, according to Paxinos and Watson (1997).

7.8 pg/ml NGF, respectively. Total protein content in the tissue extracts was determined by the bicinchoninic acid (BCA) method using BCA Protein Assay kits (Pierce, Rockford, IL). The proportion of BDNF or NGF/mg total protein for each sample was then calculated.

4.5. Statistical analysis

Data were processed using commercially available software GraphPad PRISM 4.0 and Microsoft EXCEL 2000. Results were presented as mean \pm S.E.M. and were analyzed with one-way analysis of variance (ANOVA) followed by Newman–Keuls post-test or with two-way ANOVA followed by Bonferroni post-test. Statistical significance was set at $P < 0.05$.

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