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Electroacupuncture treatment reverses morphine-induced physiological changes in dopaminergic neurons within the ventral tegmental area

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ABSTRACT

Chronic morphine administration decreases the size of dopamine (DA) neurons in the ventral tegmental area (VTA). These transient morphological changes are accompanied by a reduced sensitivity of morphine-induced conditioned place preference (CPP) after chronic exposure to the drug. In this study we examined alterations in the firing rate of DAergic neurons by means of extracellular recording following chronic morphine exposure and applied 100 Hz electroacupuncture (EA) treatment to reverse the reduced firing rate of these neurons. In the first set of experiments we show that in rats, which received chronic morphine treatment for 14 days, a small dose of morphine was not able to induce a CPP response anymore. However, the sensitivity to morphine was reinstated by consecutive EA treatment for 10 days. The electrophysiological response of VTA DA neurons to morphine was markedly reduced in chronic morphine-treated rats compared to saline-treated controls. A substantial recovery of the reactivity of VTA DA neurons to morphine was observed in rats that received 100 Hz EA for 10 days. Our findings suggest that 100 Hz EA is a potential therapy for the treatment of opiate addiction by normalizing the activity of VTA DA neurons.

Keywords Conditioned place preference (CPP), dopamine neurons, electroacupuncture (EA), extracellular recording morphine, ventral tegmental area.

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INTRODUCTION

The mesolimbic dopamine (DA) system, which arises in the ventral tegmental area (VTA), is an important neural substrate for opiate reinforcement and addiction (Spanagel & Weiss 1999). Chronic morphine administration decreases the size of DA neurons in the VTA (Sklair-Tavron *et al.* 1996). After cessation of morphine exposure the reduction in size of VTA DA neurons persists up to 2 weeks (Russo *et al.* 2007). These transient morphological changes are accompanied by a diminished morphine-induced conditioned place preference (CPP) (Russo *et al.* 2007; Tzschentke 2007).

A recent morphological study has demonstrated that the reduction of the size of VTA DA neurons in chronic morphine-treated rats could be reversed by 100 Hz electroacupuncture (EA) (Chu *et al.* 2007, 2008). We were interested to see whether the 100 Hz EA was also capable of reversing the diminished sensitivity of morphine-induced CPP. Furthermore, in the chronic morphine-treated rats, the firing response of VTA DA neurons to acute morphine was modified (Diana *et al.* 1999; Georges, Moine & Aston-Jones 2006). Therefore, we also studied the effects of 100 Hz EA on the recovery of the function of VTA DA neurons using extracellular recording technique.

MATERIALS AND METHODS

All experiments were performed on Male Sprague–Dawley rats obtained from the Peking Center of Experimental Animals, weighing 200–220 g at the beginning of the experiment. Animals were housed four per cage in a 12 hours light/12 hours dark normal cycle with food and water available at all times. The room temperature

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was maintained at 24 ± 1°C and relative humidity at 50%. All experimental procedures were approved by the Animal use Committee of Peking University Health Science Center and carefully designed to minimize the number of animals used and their suffering.

Chronic morphine administration and EA treatment: Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Shenyang (Shenyang, China). It was dissolved in saline and administered twice daily (at 8 AM and 8 PM) for 14 days as described (Diana *et al*. 1995). Briefly, the initial dose injected 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/100 g. Saline control rats received an equal volume of saline.

Rats treated with morphine for 14 days were randomly divided into the following groups: (1) spontaneous withdrawal group (14d-SW): morphine withdrawal for 14 days, without any further treatment; (2) 100 Hz EA group: after chronic morphine administration, rats were gently restrained in specially designed plastic holders, given 100 Hz EA (30 minutes per session) twice a day for 3 days, followed by once a day for 7 days, totaling 13 sessions in 10 days; and (3) restraint control group: rats were restrained in the holder 30 minutes per session for 13 sessions in 10 days without receiving EA.

The EA treatment was executed as follows: 12 hours after the last morphine injection, 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). Constant current square-wave electric stimulation generated by a programmed pulse generator, HANS LH-800 (Peking University of Astronautics and Aeronautics Aviation, Beijing, China), was given via the two needles for a total of 30 minutes. The intensity of the stimulation was increased stepwise from 0.5 to 1 mA, and then to 1.5 mA, with each step lasting for 10 minutes.

Experiment 1: the effects of EA on morphine-induced CPP

Place conditioning was conducted in a three-compartment apparatus with an unbiased design (Morales *et al*. 2007). The apparatus has been described in our previous study (Ma *et al*. 2007). The CPP experiment was conducted using procedures described previously (Shi *et al*. 2004), with minor changes as described below. The CPP procedure consisted of three phases including pre-test, conditioning and test. Briefly, on the 14th day after the last morphine or saline injection (pre-test), all groups of rats were placed in the central compartment and allowed to freely explore the entire apparatus for 15 minutes. Rats that spent more time (over 100 seconds) in one of the outer chambers than the other were excluded from the study. On the 15th and 16th day, after the last morphine or saline injection (conditioning), rats were injected with morphine (0.5 mg/kg, i.p.) and confined to one outer chamber for 60 minutes in the morning and then injected with saline and confined to the alternate outer chamber for the same time period in the afternoon. On the subsequent day (test), rats were placed back into the central compartment, allowed to freely explore the entire apparatus for 15 minutes and tested which outer chamber they preferred to stay in.

Data analysis

CPP score represents the index of place preference for each rat, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments (Shi *et al*. 2004). Values are expressed as mean ± SEM of CPP score. CPP results were analyzed with two-way analysis of variance (ANOVA) followed by Bonferroni post-test. The accepted level of statistical significance was $P < 0.05$.

Experiment 2: the effects of EA on electrophysiological properties of VTA DA neurons

Surgery and intravenous drug injection

On the 15th day after the last morphine or saline injection, all groups of rats were respectively anesthetized with 20% urethane (1.3 mg/kg, i.p.), cannulated in jugular vein for intravenous administration of drugs and then mounted on a stereotaxic apparatus (SN-3N, Narishige, Japan). A cranial window was opened on top of the VTA. Body temperature was maintained at 36.5–37.5°C via a feedback-controlled under-body heating pad. Morphine hydrochloride (1 mg/kg) was prepared in isotonic saline. The DA receptor agonist apomorphine (APO, 0.1 mg/kg; Sigma) and the D_2 DA receptor antagonist eticlopride (ETI, 0.1 mg/kg; Sigma) were dissolved in isotonic saline and used for pharmacological identification of VTA DA neurons. When investigating the response of VTA DA neurons to acute morphine (1 mg/kg), only one DA neuron was recorded from each rat.

Extracelluar single-unit recording

A parylene-coated tungsten microelectrode (impedance 1–3 MΩ, FHC, USA) driven by a micro-stepping motor (PC-5N, Narishige, Japan) was lowered into the VTA (5.3–5.8 mm posterior to bregma; 0.8–1.0 mm lateral to the midline; 7.3–8.5 mm below the cortical surface)

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according to the brain atlas of Paxinos & Watson (1998). VTA DA neurons were identified by anatomical location of the VTA and according to established physiological criteria (Guyenet & Aghajanian 1978; Grace & Bunney 1983; Tepper, Young & Groves 1984; Chiodo 1988; Ungless, Magill & Bolam 2004; Georges, Moine & Aston-Jones 2006). These neurons had (1) action potential (AP) with biphasic or triphasic waveform and a half AP width > 1.1 ms in duration, (2) spontaneous firing with either a slow irregular firing pattern or a slow bursting pattern (decreasing spike amplitude and increasing interspike interval) and (3) inhibition of spontaneous firing by DA receptor agonists and its subsequent reversal by DA receptor antagonists. The bioelectric signal was amplified and filtered (0.3–5 kHz bandpass) by a bioelectric amplifier (AVB-10, Nihon Kohden, Japan). Simultaneously, the signal was visually monitored on the oscilloscope (VC-10, Nihon Kohden, Japan) and auditorily monitored by a bioelectric amplifier (SZF-1, Shanghai, China), and fed to a Pentium computer via a CED (Cambridge Electronic Design) 1401 interface for off-line analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

Histological identification

At the end of each recording, the site was marked by electrolytic lesion (20 μA positive depolarizating DC current for 20 seconds). The animal was then euthanized by an overdose of pentobarbital sodium and perfused intracardially with saline (0.9% Nacl, 150 ml), followed by 300 ml of 4% paraformaldehde. The brains were removed and fixed at 4°C for at least 24 hours. The tissue was then sectioned into 20 μm-thick coronal slices on a cryostat and stained with cresyl violet.

Data analysis

Basal firing activity of VTA DA neurons and their response to acute morphine were analyzed over 200 seconds epochs before and after the administration. The following analyses of parameters were included: (1) the average firing rate and (2) the percentage of spikes that occurred in bursts (% spilces in bursts (SIB)). %SIB was calculated by dividing the number of spikes occurring in bursts by the total number of spikes occurring in the same period of time. Changes in burst firing are expressed as the %SIB after the drug minus the %SIB before the drug. The onset of a burst was defined as the occurrence of two spikes with an interspike interval < 80 ms, and the termination of the burst defined as the occurrence of two spikes with an interspike interval > 160 ms (Grace & Bunney 1983). When two means were compared, statistical significance of their difference was assessed using two-tailed paired Student's *t*-tests. For multiple comparisons, values were subjected to a one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett or Newman-Keuls tests.

RESULTS

Repeated 100 Hz EA recovered the sensitivity of morphine-induced CPP in morphine-withdrawn rats

According to the experimental procedure shown in Fig. 1a, we observed the effect of 100 Hz EA on the sensitivity of morphine-induced CPP in morphine-withdrawn rats. As shown in Fig. 1b, morphine (0.5 mg/kg) conditioning could induce a significant place preference in saline-treated rats. In rats received chronic morphine treatment followed by spontaneous withdrawal for 14 days, or in chronic morphine-treated rats given physical restraint for 10 days during the 14-d withdrawal period, 0.5 mg/kg of morphine failed to induce CPP, suggesting a diminished sensitivity in response to morphine-induced reinforcing behavior. In contrast, rats received 100 Hz EA for 10 days during the spontaneous morphine withdrawal showed a significant preference for morphine at 0.5 mg/kg (ANOVA, $F_{3,66} = 9.699$; $P < 0.0001$). No preference was observed in rats received saline conditioning (data not shown).

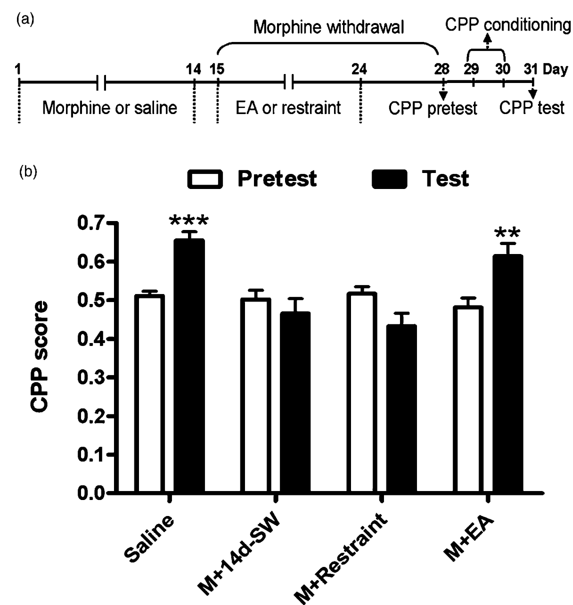

Figure 1 Effect of 100 Hz EA on place preference induced by 0.5 mg/kg morphine in 14d-SW rats. (a) Procedures of the behavioral experiment. (b) Unlike the significant place preference shown in saline-treated rats, 14d-SW or restraint rats failed to show a significant place preference for morphine, but the rats that received 100 Hz EA treatment displayed a place preference for the drug. Data are expressed as mean ± SEM, 9–10 rats per group. $**P < 0.01$, $***P < 0.001$ compared with the pre-test value of the same group. EA = electroacupuncture

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Identification of VTA DA neurons

Electrophysiological data were obtained from 36 histologically verified VTA neurons that were identified as DA neurons according to the established electrophysiological characteristics (see Materials and Methods). Figure 2a displays a representative example showing the inhibition of firing activity of a VTA DA neuron after injection of DA receptor agonist APO, and its reversal by subsequent injection of D_2 DA receptor antagonist ETI. Figure 2b shows the firing rate histogram of the data from the same experiment.

Repeated 100 Hz EA resumed the response of VTA DA neurons to acute morphine in morphine-withdrawn rats

Figure 3a represents a schedule of the electrophysiological experiment. In saline-treated rats, intravenous injection of 1 mg/kg morphine increased the firing rate of VTA DA neurons to ~45% of the pre-drug baseline within 200 seconds (Fig. 3b) (ANOVA: $F_{4,67} = 112.4$; $P < 0.0001$; $n = 10$) and elicited a significant increase of their burst firing (~16%) (Fig. 3c) (ANOVA: $F_{4,67} = 312.6$; $P < 0.0001$; typical example in Fig. 3e). The neuronal activation was maximal 3 minutes after the injection, and all cells returned to baseline level 12 minutes after the injection (Fig. 3d).

Comparing with the activation in saline-treated rats, intravenous injection of 1 mg/kg morphine to rats received chronic morphine treatment followed by spontaneous withdrawal for 14 days, or to chronic morphine-treated rats given physical restraint for 10 days during the 14-day withdrawal period, showed no significant changes on firing rate and burst firing of VTA DA neurons. However, in rats that received 100 Hz EA for 10 days during morphine withdrawal, morphine (1 mg/kg, i.v.) induced a marked increase of firing rate (~40%) (Fig. 3b) (ANOVA: $F_{4,67} = 112.4$; $P < 0.0001$; $n = 10$) and burst firing (~15%) (Fig. 3c) (ANOVA: $F_{4,67} = 312.6$; $P < 0.0001$; typical example in Fig. 3e). The neuronal activation was maximal 3 minutes after the injection, and all cells returned to the baseline 12 minutes after the injection (Fig. 3d). Administration of saline had no effect on the firing rate and the burst firing of VTA DA neurons in saline-treated, 14d-SW, restraint or EA-treated rats (all groups pooled together; ANOVA: $F_{3,32} = 2.25$; $P = 0.10$).

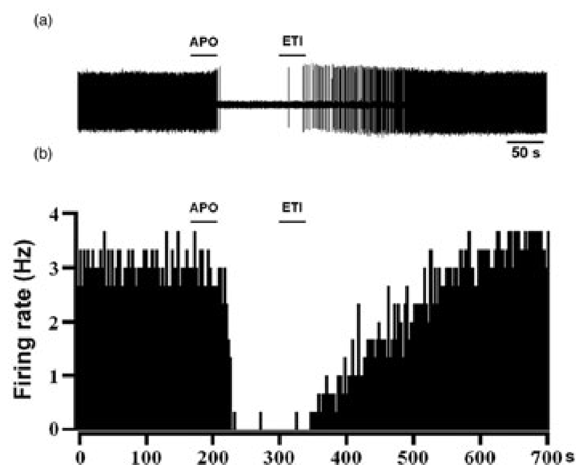

Figure 2 Electrophysiological identification of VTA DA neurons. (a) The firing activity of a typical VTA DA neuron was inhibited by APO (0.1 mg/kg, i.v.), and the effect of APO was reversed by ETI (0.1 mg/kg, i.v.). (b) The firing rate histogram of the typical VTA DA neuron. The drug injections are marked by the short lines above the figure. APO = apomorphine, DA = dopamine, ETI = eticlopride, VTA = ventral tegmental area

DISCUSSION

DA neurons originating in the VTA and primarily projecting to the nucleus accumbens are one of the key neuronal substrates implicated in the reinforcing properties of drugs of abuse (Spanagel & Weiss 1999). Long-term opiate exposure produced some adaptive changes in VTA DA neurons: a reduction of VTA DA cell size (Sklair-Tavron *et al*. 1996; Spiga *et al*. 2003), a hypofunction of VTA DA neurons (Diana *et al*. 1995, 1999; Bonci & Williams 1997; Manzoni & Williams 1999) and a decrease of DA-mediated neurotransmission (Diana *et al*. 1999). This hypofunction of VTA DA neurons is thought to contribute to the negative consequences of acute and protracted opiate withdrawal (Nestler 1997, 2004) and to the decreased motivation for natural reinforcers (Zhang *et al*. 2007).

Figure 3 Overall effects of intravenous injections of MOR (1 mg/kg) on VTA DA neuronal firing activity in saline-treated rats ($n = 10$ rats, 10 cells) and morphine-treated rats (M + 14d-SW: $n = 8$ rats, 8 cells; M + Restraint: $n = 8$ rats, 8 cells; M + EA: $n = 10$ rats, 10 cells). For firing rate, data are expressed as a percentage of the mean basal level (mean ± SEM). For burst firing analysis, data are expressed as percentage of SIB postdrug minus the percentage of SIB predrug. (a) Procedures. (b, c) Effect of morphine (1 mg/kg, i.v.) on the firing rate and burst firing of VTA DA neurons ($^{###}P < 0.001$ compared to all groups; ANOVA followed by Newman-Keuls tests). (d) Time-course of changes in the firing activity of VTA DA neurons after MOR injection ($^{*}P < 0.05$, $^{**}P < 0.01$; ANOVA followed by Dunnett's test). The MOR injection is designated by the arrow above the curve. (e) Oscilloscope traces of impulses from four VTA DA neurons showing the typical changes of firing activity after the MOR injection. ANOVA, analysis of variance, DA = dopamine, EA = electroacupuncture, MOR = morphine, SIB = spikes in bursts, VTA = ventral tegmental area

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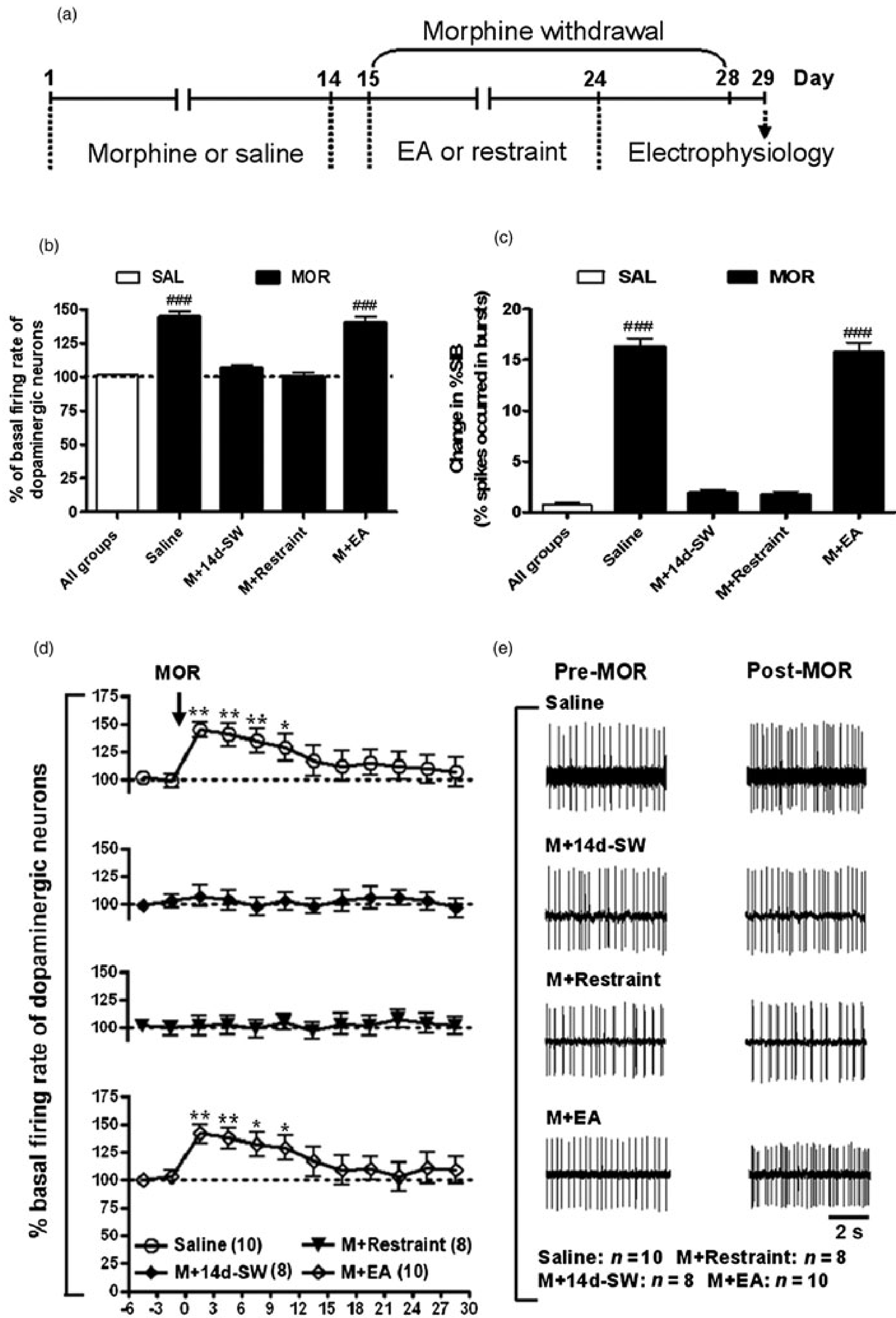
(a)
Morphine withdrawal
1 14 15 24 28 29 Day
Morphine or saline
EA or restraint
Electrophysiology
(b)
SAL MOR
% of basal firing rate of dopaminergic neurons
###
###
All groups
Saline
M+14d-SW
M+Restraint
M+EA
(c)
SAL MOR
Change in %SIB (% spikes occurred in bursts)
###
###
All groups
Saline
M+14d-SW
M+Restraint
M+EA
(d)
MOR
% basal firing rate of dopaminergic neurons
Saline (10)
M+Restraint (8)
M+14d-SW (8)
M+EA (10)
(e)
Pre-MOR
Post-MOR
Saline
M+14d-SW
M+Restraint
M+EA
2 s
Saline: n = 10 M+Restraint: n = 8
M+14d-SW: n = 8 M+EA: n = 10

Recently, Russo *et al*. (2007) reported that the decreased size of VTA DA neurons was associated with a profound reduction in the sensitivity of morphine-induced CPP at 1 or 14 days of morphine withdrawal, and both effects dissipated at least 1 month after morphine withdrawal in rats. In a previous study, we have demonstrated that the reduction of the size of VTA DA neurons after repeated morphine administration could be reversed by 100 Hz EA for 10 days (Chu *et al*. 2008). It would thus be interesting to examine whether the 100 Hz stimulation was capable of reversing the decrease of sensitivity in response to morphine-induced behavioral reinforcement.

The behavioral results from this study revealed that morphine induced a significant place preference in saline-pretreated rat, but in chronic morphine exposed rats, the same dose of this drug was not able to induce a CPP response anymore. This result is consistent with the report from Russo *et al*. (2007). Interestingly, in the rats that received 100 Hz EA treatment, the same dose of morphine could induce a CPP response. Simultaneously, we observed that an acute morphine administration to saline-pretreated rats significantly increased the firing activity of VTA DA neurons, but administration of the same dose of the drug to chronic morphine exposed rats failed to increase the activity of VTA DA neurons. In line with our finding a lasting reduction in mesolimbic DAergic activity following cessation of chronic morphine exposure has been described previously (Diana *et al*. 1999; Georges, Moine & Aston-Jones 2006). Furthermore, we found that the decreased response of VTA DA neurons to acute morphine in chronic morphine-treated rats was reversed by 100 Hz EA treatment for 10 days during morphine withdrawal period.

A major question which arises from the present findings is: what is the molecular mechanism underlying the effectiveness of 100 Hz EA for the recovery of neuronal atrophy induced by chronic morphine treatment? It is well known that brain-derived neurotrophic factor (BDNF) signaling pathway is essential for neural plasticity in response to drugs of abuse. Behavioral responses to cocaine were reduced in the BDNF knockout mice (Hall *et al*. 2003), and intra-VTA infusion of BDNF prevented the characteristic biochemical (Berhow *et al*. 1995) and morphological (Sklair-Tavron *et al*. 1996) changes occurred after chronic morphine exposure. We also reported that chronic morphine exposure resulted in a decrease in BDNF level in the VTA, and this alteration could be reversed by 100 Hz EA treatment (Chu *et al*. 2007). Therefore, we speculate that an accelerated expression of endogenous BDNF after 100 Hz EA treatment may play a role in the reversal of electrophysiological and behavioral alterations induced by chronic morphine exposure. Further studies are necessary to investigate the effect of 100 Hz EA on chronic morphine-induced electrophysiological and behavioral changes after intra-VTA infusion of BDNF antibody or silencing of the local BDNF gene expression.

In conclusion, the results of the present study show that chronic morphine exposure diminishes the sensitivity of a small dose of morphine to induce CPP, and decreases the response of VTA DA neurons to acute morphine as well. These reductions could be reversed by 100 Hz EA treatment. Our findings suggest that 100 Hz EA might be used as a potential therapy for the treatment of opiate addiction by normalizing the activity of VTA DA neurons.

Acknowledgements

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