Brain Struct Funct
DOI 10.1007/s00429-013-0599-2

ORIGINAL ARTICLE

# Mesoaccumbens dopamine signaling alteration underlies behavioral transition from tolerance to sensitization to morphine rewarding properties during morphine withdrawal

**Linlin Sun · Ling Hu · Yijing Li · Cailian Cui**

Received: 18 March 2013 / Accepted: 7 June 2013


**Abstract** Although the firing activity of dopamine (DA) neurons in the ventral tegmental area (VTA) and the behavioral response to morphine rewarding properties alter as opiate withdrawal, little is known about the dynamic changes in DA signal pathway from the VTA to the nucleus accumbens (NAc) during prolonged withdrawal, and whether the changes are indicative of vulnerability to relapse of drug abuse. Here we report that morphine spontaneously withdrawn (SW) rats are incapable of responding to small dose of morphine-induced conditioned place preference (CPP) from 24h-SW to 30d-SW, but recover response at 45d-SW. Interestingly, mesoaccumbens DA signaling, including the firing of DA neurons in the VTA, contents of DA and its metabolic ratio, and the membrane level of dopamine $D_1$ receptor in the NAc elicited by morphine challenge, display a similar pattern of time-dependent changes during morphine withdrawal. Moreover, blockade of $D_1$ receptor abolishes this behavioral transition. In addition, a strong correlation was found between % change in CPP score and membrane $D_1$ receptor level induced by morphine challenge. These results indicate a time-dependent behavioral switch from tolerance to sensitization during the prolonged withdrawal, which could offer a window for therapeutic intervention via manipulations of $D_1$ receptors.

**Keywords** Dopamine (DA) neuronal firing · Mesoaccumbens DA signaling · Morphine withdrawal · Nucleus accumbens (NAc) · Ventral tegmental area (VTA)

L. Sun, L. Hu and Y. Li contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00429-013-0599-2) contains supplementary material, which is available to authorized users.

L. Sun · L. Hu · Y. Li · C. Cui (✉)
Neuroscience Research Institute, Peking University,
38 Xueyuan Road, Beijing 100191, China
e-mail: clcui@bjmu.edu.cn; sarah_lyn@pku.edu.cn

L. Sun · L. Hu · Y. Li · C. Cui
Department of Neurobiology, School of Basic Medical Sciences,
Peking University, Beijing 100191, China

L. Sun · L. Hu · Y. Li · C. Cui
Key Laboratory for Neuroscience of the Ministry of Education
and the Ministry of Public Health, Beijing 100191, China

## Introduction

Behavioral responses in rats re-exposed to drugs during withdrawal have been extensively studied (Ahmed et al. 2000; Kenny et al. 2006; Koob and Le Moal 2005; Shippenberg et al. 1988). However, some researchers found that morphine pretreatment led to sensitization to the reinforcing effects of morphine using the conditioned place preference (CPP) paradigm (Shippenberg et al. 1996), whereas other studies reported tolerance to reward in morphine-induced CPP (Russo et al. 2007) and heroin self-administration in rats (Kenny et al. 2006). These discrepant findings might be attributable to differences in opiate pretreatment paradigms, the duration of withdrawal, the pretreatment dose of the drug, and the challenge dose of the drug after withdrawal. We sought to resolve this issue by administering the same dose of morphine (0.5 mg/kg) during conditioning following a similar pretreatment regimen as previously reported (Hu et al. 2009) with a sufficiently long withdrawal period (i.e., 45 days). The present study was designed to determine whether morphine challenge elicits a behavioral transition from reward tolerant to sensitive state in the CPP paradigm in rats.

Published online: 18 June 2013

Adaptive changes in the mesocorticolimbic dopaminergic system are considered crucial for morphine dependence and withdrawal (Di Chiara and North 1992; Laviolette et al. 2002). The mesocorticolimbic dopamine (DA) system originates in the ventral tegmental area (VTA) and drives reward-related behavior (Sun 2011; Fields et al. 2007). The VTA contains a majority (~65 %) of DA neurons (Dobi et al. 2010). Acute morphine has been shown to increase the firing of VTA DA neurons by inhibiting γ-aminobutyric acid neurons (Gysling and Wang 1983; Kalivas and Stewart 1991). Chronic morphine administration in rats decreased the size of VTA DA neurons (Sklair-Tavron et al. 1996), and this reduction persisted up to 2 weeks after the cessation of morphine exposure (Chu et al. 2007). In vivo electrophysiological recording showed that VTA DA neurons exhibited tolerance to firing in response to acutely administered morphine as long as 14 days after withdrawal (Georges et al. 2006). Opiate addicts relapse even after years of withdrawal when re-exposed to drugs, which means there must be a withdrawal time point when this tolerant response of the DA neurons returns to normal state and the neurons can react to the drug rewarding properties again. Despite previous research that demonstrated a prolonged opiate withdrawal lasting for 14 days at most, still unclear are how long this tolerance endures, whether a switch to sensitization subsequently occurs, and, importantly, whether DA neurons eventually recover their response to morphine reward.

Morphine exerts stimulation in the firing activity of DA neurons that project from the VTA to the NAc (i.e., the mesoaccumbens DA pathway) (Manzanedo et al. 2005; Di Chiara 1999) and increased DA release from DA terminals in the NAc (Herz 1998; Willins and Meltzer 1998). Moreover, previous studies revealed a role for NAc $D_1$ receptors in the mediation of morphine-induced motivational effects (Shippenberg et al. 1993; Manzanedo et al. 2005). Therefore, second aim in the present study is to explore the dynamic alterations in the mesoaccumbens DA pathway and whether these adaptations underlie the behavioral response to a subsequent challenging dose of morphine.

## Materials and methods

Male Sprague-Dawley rats, weighing 200–220 g, were obtained from the Peking Center of Experimental Animals. The animals were housed in groups of four under a normal 12 h/12 h light/dark cycle with food and water available ad libitum. Room temperature was maintained at 24 ± 1 °C and 50 % relative humidity. All of the experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the Animal Use Committee of Peking University Health Science Center.

### Chronic morphine pretreatment

Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Shenyang (Shenyang, China). It was dissolved in saline and administered twice daily (at 8:00 am and 8:00 pm) for 14 days. Briefly, the initial dose injected was 20 mg/kg, and the dose increased by 20 mg/kg every other day until reaching a dose of 140 mg/kg for the last injection (Figure S1). Saline control rats received the same volume of saline twice daily for 14 days. Rats treated with morphine were randomly divided into the following spontaneous withdrawal (SW) groups: 24h-SW, 7d-SW, 14d-SW, 21d-SW, 30d-SW, and 45d-SW.

### Drug injection

The jugular vein was cannulated for intravenous administration of the pharmacological agents. Morphine hydrochloride (1 mg/kg) was prepared in isotonic saline. The DA agonist apomorphine (APO; 0.1 mg/kg; Sigma) and DA $D_2$ receptor antagonist eticlopride (ETI; 0.1 mg/kg; Sigma) were prepared in isotonic saline and used to pharmacologically identify VTA DA neurons (Figure S2).

D1 antagonist SCH23390 (0.2 mg/kg; Sigma) was intraperitoneally injected 25 min prior to each CPP conditioning session.

### Conditioned place preference

Place conditioning was conducted in a three-compartment apparatus using an unbiased design. The apparatus was described in a previous study (Ma et al. 2007). The CPP experiment was conducted using procedures described previously (Shi et al. 2004), with minor modifications as described below. The CPP procedure consisted of three phases: pretest, conditioning, and test. Overall, the conditioning phase commenced 24 h, 14, 30, and 45 days after the last morphine treatment. During pretest, rats were placed in the central compartment and allowed to freely explore the entire apparatus for 15 min. Rats that spent more time (>100 s) in one of the outer compartments than in the other were excluded from the study. Conditioning then occurred over a 2-day period after the pretest. The rats were injected with morphine (0.5 mg/kg, i.p.) and confined to one outer compartment for 1 h in the morning and then injected with saline and confined to the alternate for 1 h in the afternoon. On the following day (test), the rats were returned to the central compartment and allowed to freely explore the entire apparatus for 15 min. Their preference for one of the outer compartments was then determined.

### Surgery

On the day that VTA DA neuron firing was electrophysiologically recorded, all of the groups of rats were anesthetized with 20 % urethane (1.3 mg/kg, i.p.), cannulated in the jugular vein for intravenous drug administration, and 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.

### Immunofluorescence

Immunofluorescence detection was performed as we previously described (Chu et al. 2007). Coronal slices (30 μm thick) were obtained with a cryostat, beginning at −5.80 mm and ending at −6.30 mm from bregma. Collected sections were rinsed in 0.1 M PBS, followed by preincubation in 10 % normal goat serum (NGS) in PBS plus 0.3 % Triton X-100 and kept at room temperature for 2 h and then incubated overnight with rabbit anti-TH antiserum (diluted 1:8,000, Sigma, St. Louis, USA), which is used as the marker of DA neurons. The sections were then rinsed again in 0.1 M PBS and incubated with a secondary antibody mixture of goat anti-rabbit IgG conjugated with Alexa fluor 488 for 2 h at room temperature. After rinsing with PBS, sections were mounted onto slides and dried before coverslipping. Fluorescence immunolabeling was detected using confocal laser scanning microscope, under 20× and 40× objective, FV1000 Olympus. The morphological parameters of the TH-positive cells were analyzed by an investigator blind to the animal treatment using an advanced image-analysis system (Image-J). 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 ($\mu m^2$). For each rat, we used 3–4 brain slices, which generated bilateral 6–8 immunofluorescent images. The body areas of an average of 10 DA neurons were measured in one image under 40× object condition.

### Extracellular single-unit recording

A parylene-coated tungsten microelectrode (impedance 1–3 MΩ, Far East Harris Communications Bowdoinham, ME, USA) driven by a micro-step 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 midline; 7.3–8.5 mm below the cortical surface) according to the brain atlas of Paxinos and Watson (1998). Dopamine neurons in the VTA were identified by anatomical location and according to established physiological criteria (Guyenet and Aghajanian 1978; Grace and Bunney 1983; Tepper et al. 1984; Chiodo 1988; Ungless et al. 2004; Georges et al. 2006). These neurons had (1) action potentials (APs) with biphasic or triphasic waveforms and a half AP width >1.1 ms 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 in response to DA receptor agonists and subsequent reversal by DA receptor antagonists. The electrical signal was amplified and filtered (0.3–5 kHz band pass) by a bioelectric amplifier (AVB-10, Nihon Kohden, Japan). Simultaneously, the signal was visually monitored on an oscilloscope (VC-10, Nihon Kohden, Japan), auditorily monitored using a bioelectric amplifier (SZF-1, Shanghai, China), and fed to a Pentium computer via a Cambridge Electronic Design 1401 interface for off-line analysis using Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

### HPLC analysis of dopamine and its metabolites

The rats were decapitated 80 min after an intraperitoneal morphine challenge (5 mg/kg). The brains were removed and placed on an ice-cold plate to dissect the NAc according to the stereotaxic atlas of Paxinos and Watson (1998). Immediately afterward, the tissue samples were weighed and placed in 1.5 ml plastic tubes that contained ice-cold perchloric acid (200 μl, 0.4 M), ultrasonically (0.5 Hz) homogenized for 10 s, and centrifuged at 15,000×*g* for 20 min at 4 °C. The supernatant was passed through a 0.2-μm filter and kept at 4 °C until high-performance liquid chromatography (HPLC) analysis.

DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were analyzed using reversed-phase ion-pair chromatography combined with electrochemical detection under isocratic conditions (Teismann and Ferger 2001). The six-channel detector potentials were set at +50, 100, 200, 300, 400, and 500 mV using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase (0.6 mM 1-octanesulfonic acid, 0.27 mM Na2EDTA, 0.043 M triethylamine, and 50 ml acetonitrile/L, adjusted to pH 2.95 with $H_3PO_4$) was delivered at a flow rate of 0.5 ml/min at 22 °C onto the reversed-phase column (125 mm × 3 mm with 5 mm × 3 mm precolumn filled with nucleosil 120-3 C18; Knauer, Berlin, Germany). Ten microliter aliquots were injected with an autoinjector with a cooling module set at 4 °C. The data were calculated using external standard calibration.

### Western blot for $D_1$ receptor protein expression

For the Western blotting experiments, the rats were given an overdose of chloral hydrate (250 mg/kg, i.p.), and their brains were quickly removed and frozen in *N*-hexane

(−70 °C) for approximately 40 s. The brains were then stored at −80 °C until further use. Bilateral tissue punches (16 gauge) of the NAc were obtained from 60-μm-thick sections obtained using a sliding freezing microtome. The punches were sonicated in 300 μl ice-cold cytosol extraction reagent. The homogenate was then centrifuged at 800×*g* for 5 min, half of the supernatant was reserved for total $D_1$ receptor analysis, and the other half was mixed with membrane extraction reagent at a 10:1 volume ratio. The mixture was left on ice for 5 min and further centrifuged at 25,000×*g* for 30 min. The supernatant was discarded, and 60 μl suspension buffer was added to the deposition (Applygen, Nucl-Cyto-Mem Preparation Kit, #P1201). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA). Sample buffer was immediately added to the homogenates, and the samples were boiled for 5 min. Protein extracts (60 μg) were then electrophoresed in 10 % sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05 % Tween 20) with 5 % dry milk and incubated with an anti-DRD1 antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were then incubated with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; 1:2,000; Zhongshan Biotechnology, Beijing, China) and developed using West Dura chemiluminescent substrate (Pierce Laboratories). Densitometry was determined based on band intensity, and relative protein expression was quantified densitometrically using the Total Lab 2.01 analysis system (Phoretix, UK).

## Data analyses

### *Conditioned place preference*

The 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). Two-way analysis of variance (ANOVA) was used, followed by the Bonferroni post hoc test.

### *Immunofluorescence*

The cell body area represents the size of VTA DA neurons. ANOVA was used, followed by the Bonferroni post hoc test.

### *Extracellular single-unit recording*

The basal firing activity of VTA DA neurons and their response to morphine challenge were analyzed for 200 s before and after drug administration. The following parameters were evaluated: (1) average firing rate, (2) bursting activity (i.e., the percentage of spikes that occurred in bursts [% spikes in bursts (SIB)]; %SIB was calculated by dividing the number of spikes that occurred in bursts by the total number of spikes that occurred during the same period of time), (3) burst size (i.e., the number of spikes within each burst), and (4) regularity of DA neuron firing, measured with the coefficient of variation (i.e., the standard deviation and mean value of instantaneous interspike intervals (Georges and Aston-Jones 2002; Georges et al. 2006). Changes in burst firing are expressed as %SIB after the drug minus %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 was defined as the occurrence of two spikes with an interspike interval >160 ms (Grace and Bunney 1983). Values were analyzed using one-way ANOVA followed by the Dunnett or Newman-Keuls post hoc test.

### *HPLC*

The content of DA and its metabolites was calculated as ng/mg tissue, and the data are expressed as mean ± SEM. DA ratio was calculated as [DOPAC + HVA]/DA. Changes in content were analyzed using two-way ANOVA followed by the Bonferroni post hoc test, and DA ratio were using unpaired *t* test.

### *Western blot*

The net intensities of the bands for membrane $D_1$ receptors are expressed as the percentage of the net intensities in the control group. The intensities of total $D_1$ receptors were divided by the intensities of actin, and that of the membrane $D_1$ receptors were normalized to the average value of the naive controls. Differences between groups were analyzed using ANOVA followed by the Bonferroni post hoc test. For all of the analyses, statistical significance was set at $p < 0.05$.

## Results

### Morphine SW rats displayed a time-dependent behavioral transition in response to the rewarding properties of morphine

To investigate the behavioral response to the rewarding properties of morphine in SW rats, 0.5 mg/kg morphine conditioning was performed over a 2-day period before the CPP test at different withdrawal time points, including 24 h, 14, 30, and 45 days after the last morphine injection

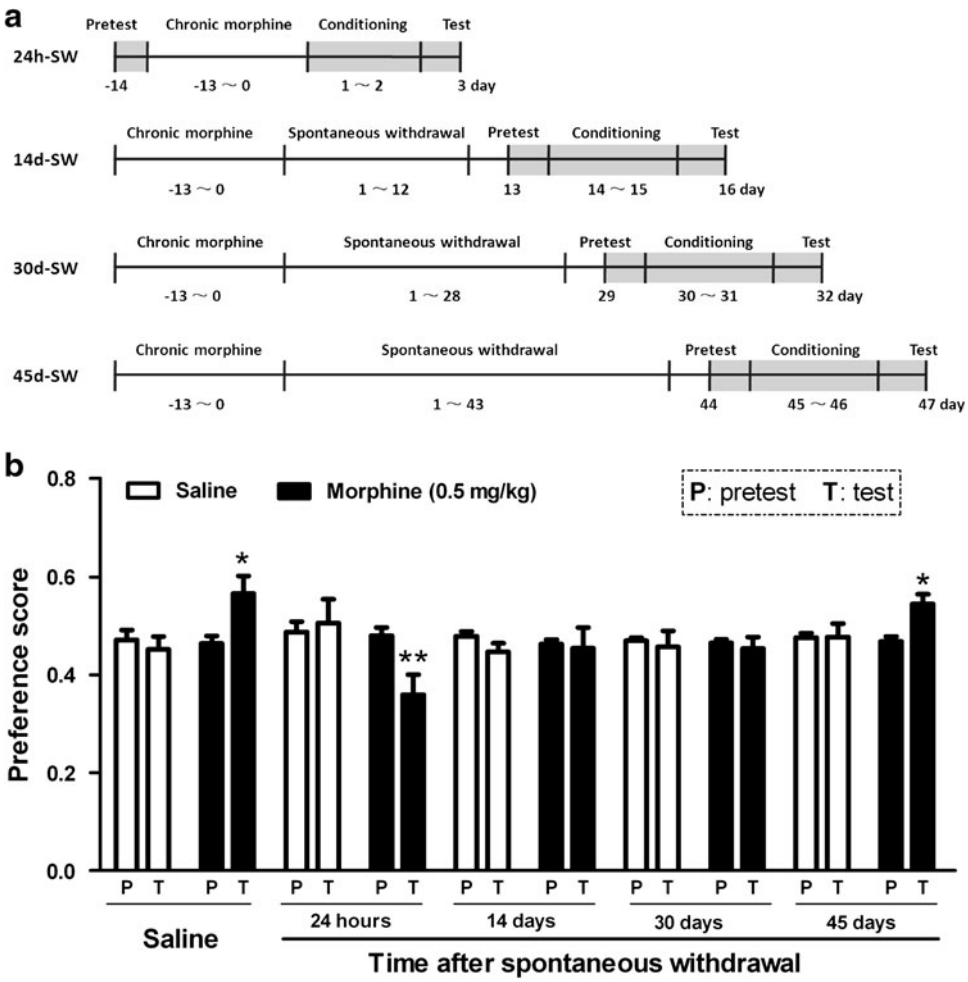



**Fig. 1** Morphine-induced conditioned place preference (CPP) at different time points during spontaneous withdrawal (SW). **a** Experimental procedure. **b** The 0.5 mg/kg dose of morphine induced significant CPP only in the 45d-SW and control groups (two-way repeated-measures ANOVA, $F_{9,\ 79} = 1.971$; $^{*}p < 0.05$, $^{**}p < 0.01$, compared with pretest; $n = 9$–13). The data are expressed as mean ± SEM

(Fig. 1a). The results showed that 0.5 mg/kg morphine induced significant place preference in saline-pretreated rats, considered the normal sensitization state. In contrast, rats pretreated with chronic morphine did not display a preference for the morphine-paired compartment at 24 h of withdrawal and exhibited an aversive response to the drug-paired compartment. This anhedonia-like effect remained until 30 days of withdrawal. Remarkably, 45d-SW rats recovered place preference for the 0.5 mg/kg morphine-paired compartment, similar to the control group (Fig. 1b). Altogether, these data demonstrate that SW rats displayed tolerance to CPP induced by 0.5 mg/kg morphine from 24 h to 30 days of withdrawal, but eventually recovered to a reward sensitive state at 45 days of withdrawal as control rats.

## Basal firing properties of DA neurons in the VTA during morphine withdrawal

Chronic morphine treatment decreased the size of DA neurons in the VTA 24 h after the last morphine injection compared to saline-pretreated group. The reduction in size began to recover after 7 days of withdrawal, and resumed upon 21 days post-withdrawal (saline, 184.4 ± 8.54; 24h-SW, 92.99 ± 3.118; 27d-SW, 123.6 ± 1.734; 14d-SW, 160.4 ± 1.219; 21d-SW, 172.8 ± 6.192; 30d-SW, 176.4 ± 6.841; 45d-SW, 175.3 ± 5.849; Fig. 2). This result was in consistent with our previous finding that the cell size reduction induced by chronic morphine administration lasted for at least 14 days after morphine abstinence (Chu et al. 2007). These morphological changes in VTA DA neurons may imply alterations in the function of VTA DA neurons during morphine withdrawal. Therefore, we performed in vivo single-unit recording of VTA DA neuronal firing. Electrophysiological data were obtained from VTA DA neurons identified by their electrophysiological properties (see "Materials and Methods"). The identification of VTA DA neurons used APO and ETI. 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.), shown in Figure S2, which have been previously reported as the identification of DA neurons (Hu et al. 2009). Figure 3a illustrates the protocol used for the electrophysiological experiments. The gray area indicates

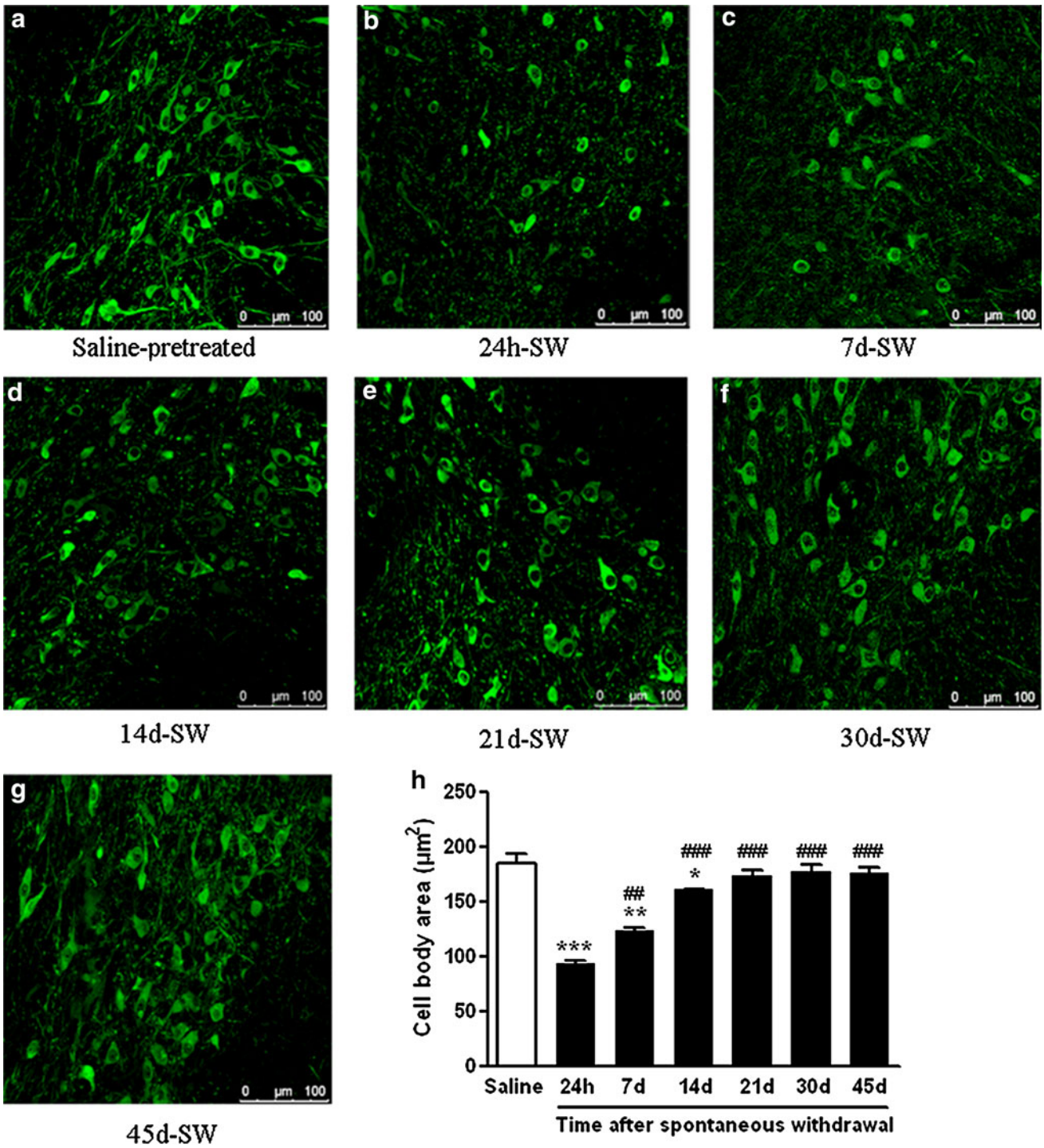



**Fig. 2** The morphological changes in the VTA DA neurons induced by chronic morphine exposure at different time points during spontaneous withdrawal. **a**–**g** Immunofluorescence of TH-positive neurons in the VTA. **h** The cell body area reduced in 1d-, 7d- and 14d-SW group compared with the saline group (one-way ANOVA, $F_{6,\ 2,617} = 25.36$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with saline group), and the reduction was higher in 24h-SW than that of the 7d-SW and 14d-SW groups (one-way ANOVA, $F_{6,\ 2,617} = 35.36$; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with 24h-SW group). $n = 4$ rats per group, and the numbers of neurons measured are 399, 346, 363, 381, 386, 361, 388 in the saline, 24h-SW, 7d-SW, 14d-SW, 21d-SW, 30d-SW, and 45d-SW groups, respectively

the time of data collection used to analyze changes in DA neuron activity induced by morphine withdrawal, including firing rate, firing regularity, bursting activity and size, and firing rate distribution. The firing rates of VTA DA neurons in the seven groups of rats were normally distributed (Fig. 4a–g; range 0.2–9.0 Hz), indicating similar neuronal

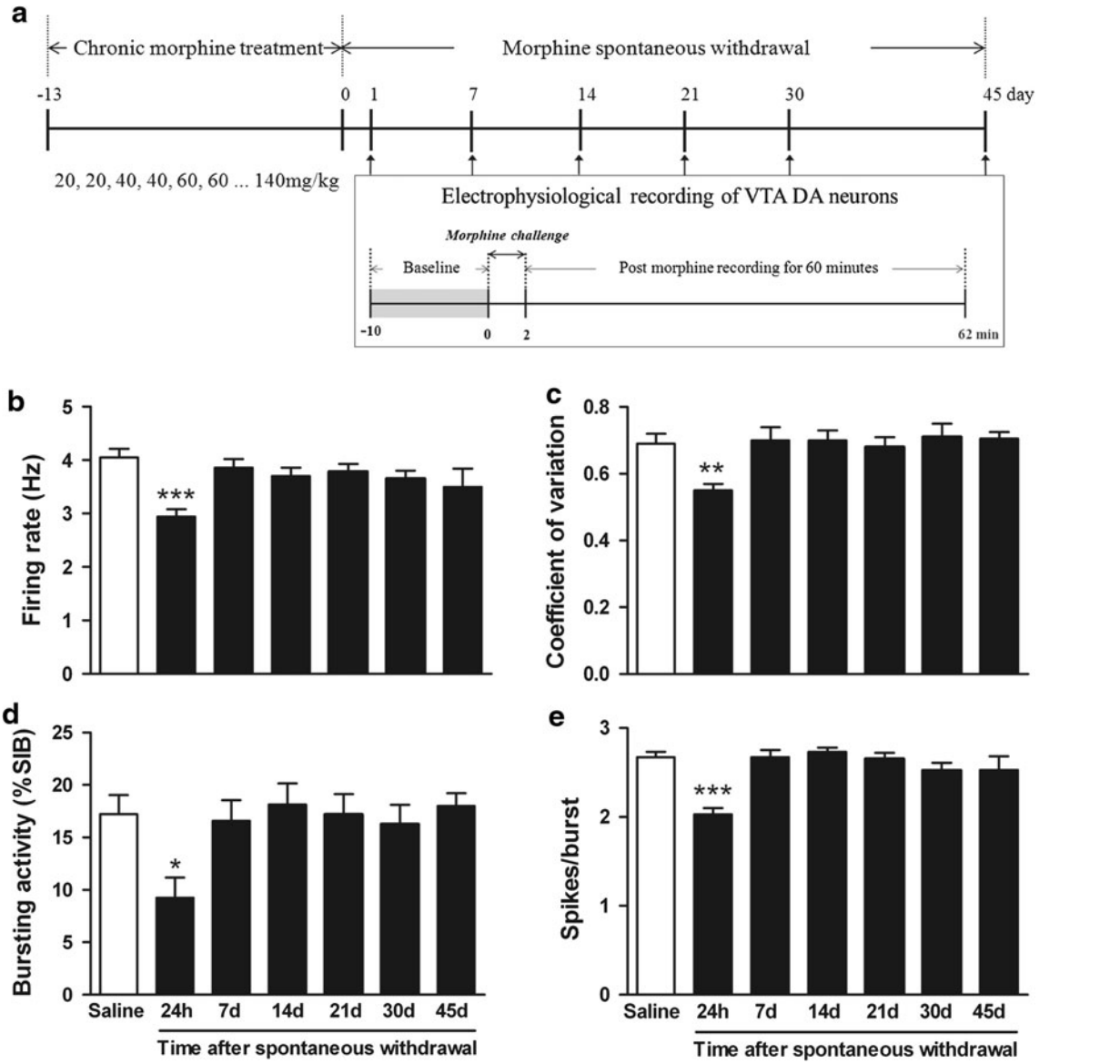



**Fig. 3** Analysis of basal firing properties of VTA DA neurons in saline control rats ($n = 8$ rats, 105 cells), 24h-SW rats ($n = 8$ rats, 96 cells), 7d-SW rats ($n = 6$ rats, 92 cells), 14d-SW rats ($n = 7$ rats, 93 cells), 21d-SW rats ($n = 6$ rats, 90 cells), 30d-SW rats ($n = 8$ rats, 91 cells), and 45d-SW rats ($n = 8$ rats, 27 cells). **a** Experimental procedure. *Upper arrows* indicate the time points of electrophysiological recordings. *Lower* baseline recording of DA neuronal firing lasted 10 min. A morphine (1 mg/kg, i.v.) challenge injection was administered over 2 min, and the DA neuron firing response to the morphine challenge was monitored up to 60 min. **b** Basal firing rate (one-way ANOVA, $F_{6,\ 652} = 3.520$; $^{***}p < 0.001$, compared with saline group). **c** Regularity of DA neuron firing (one-way ANOVA, $F_{6,\ 652} = 3.425$; $^{**}p < 0.01$, compared with saline group). **d** Burst-event frequency (one-way ANOVA, $F_{6,\ 652} = 2.832$; $^{*}p < 0.05$, compared with saline group). **e** Mean burst size (one-way ANOVA, $F_{6,\ 652} = 7.866$; $^{***}p < 0.001$, compared with saline group). The data are expressed as mean ± SEM

populations in all of the groups. However, the Gaussian curve was shifted ~1.0 Hz to the left in the 24h-SW group compared with saline-pretreated and other withdrawn rats (Fig. 4h), indicating that hypoactivity occurred in the VTA DA neuronal population in the 24h-SW group. Further analysis revealed that the 24h-SW group exhibited a significant decrease (~27 %) in basal firing rate compared with the saline-pretreated group. The basal firing rate resumed upon 7 days post-withdrawal (saline, 4.05 ± 0.16; 24h-SW, 2.94 ± 0.14; 7d-SW, 3.86 ± 0.16; 14d-SW, 3.70 ± 0.16; 21d-SW, 3.78 ± 0.15; 30d-SW, 3.65 ± 0.15; 45d-SW, 3.50 ± 0.34; Fig. 3b). The regularity of firing of VTA DA neurons was lower in the 24h-SW group, reflected by the coefficient of variation (see "Materials and Methods"; saline, 0.69 ± 0.03; 24h-SW, 0.55 ± 0.02; 7d-SW, 0.70 ± 0.04; 14d-SW, 0.70 ± 0.03; 21d-SW, 0.68 ± 0.03; 30d-SW, 0.71 ± 0.04; 45d-SW, 0.71 ± 0.02; Fig. 3c). The basal burst activity and burst size decreased at 24 h of

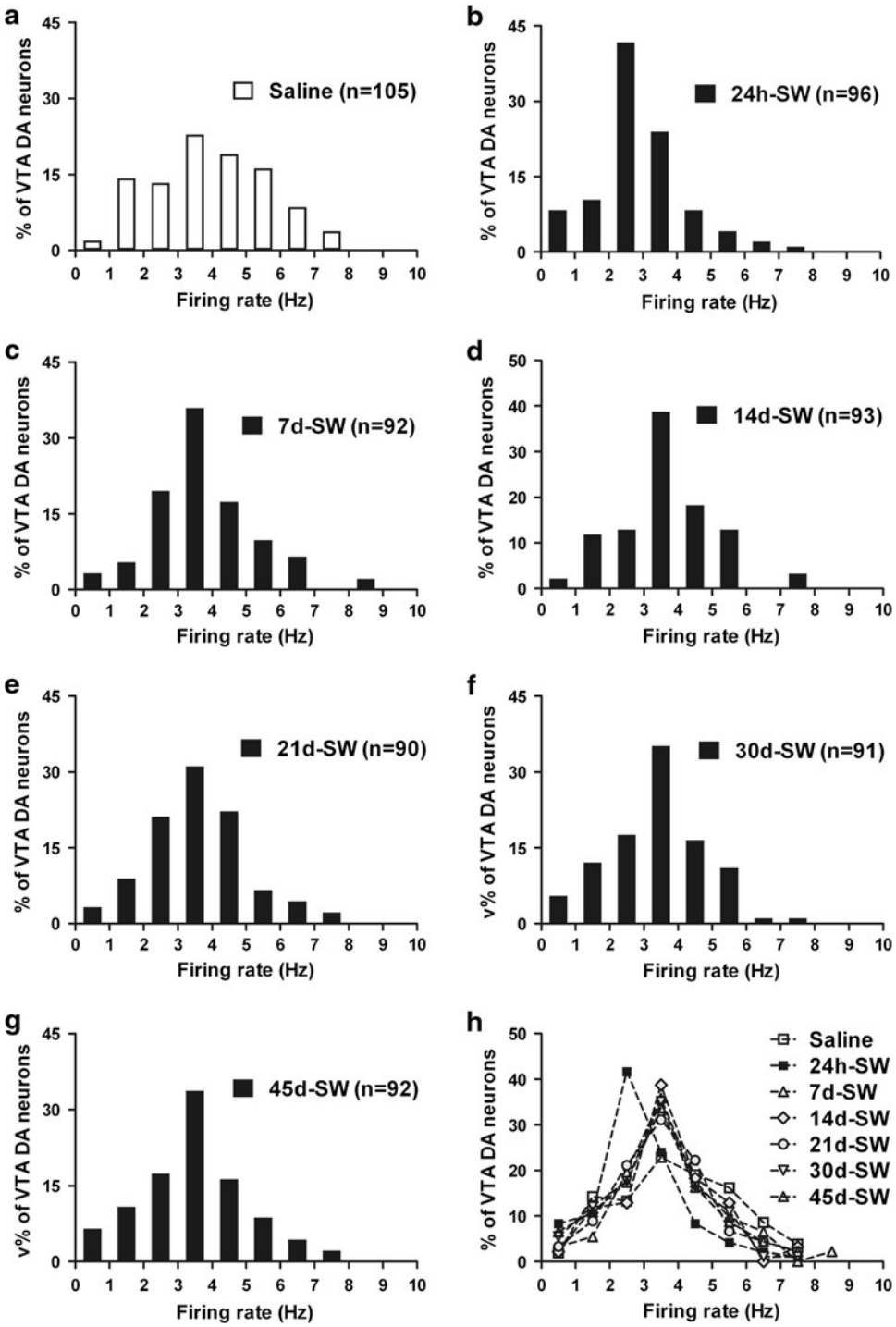



**Fig. 4** Analysis of firing rate distributions of VTA DA neurons at different time points during morphine withdrawal. The figure shows the firing rate distributions of VTA DA neurons in saline rats ($n = 32$ rats, 105 cells) and withdrawn rats (24h-SW rats, $n = 30$ rats, 96 cells; 7d-SW rats, $n = 20$ rats, 92 cells; 14d-SW rats, $n = 22$ rats, 93 cells; 21d-SW rats, $n = 23$ rats, 90 cells; 30d-SW rats, $n = 20$ rats, 91 cells; 45d-SW rats, $n = 57$ rats, 92 cells). **a**–**g** Firing rates for the seven groups of cells are normally distributed (range 0.2–9 Hz). 24h-SW induced a shift to the left of the Gaussian curve of $\sim$1.0 spikes per second, indicating hypoactivity of the VTA DA neuron population

withdrawal and returned to saline-pretreated levels upon 7 days post-withdrawal (saline, 17.21 ± 1.83 %; 24h-SW, 9.26 ± 1.92 %; 7d-SW, 16.58 ± 1.98 %; 14d-SW, 18.13 ± 2.03 %; 21d-SW, 17.18 ± 1.96 %; 30d-SW, 16.25 ± 1.86 %; 45d-SW, 18.00 ± 1.21 %; Fig. 3d; Saline, 2.67 ± 0.06; 24h-SW, 2.03 ± 0.07; 7d-SW, 2.67 ± 0.08; 14d-SW, 2.73 ± 0.05; 21d-SW, 2.65 ± 0.07; 30d-SW, 2.52 ± 0.09; 45d-SW, 2.53 ± 0.15; Fig. 3e).

## Firing response of VTA DA neurons to morphine challenge during withdrawal

We then monitored the response of VTA DA neurons to morphine challenge (1 mg/kg, i.v.) in saline-pretreated rats and all six SW groups. Changes in DA neuron firing were first analyzed from the data recorded for 200 s before and after intravenous morphine administration (Fig. 5). Time-

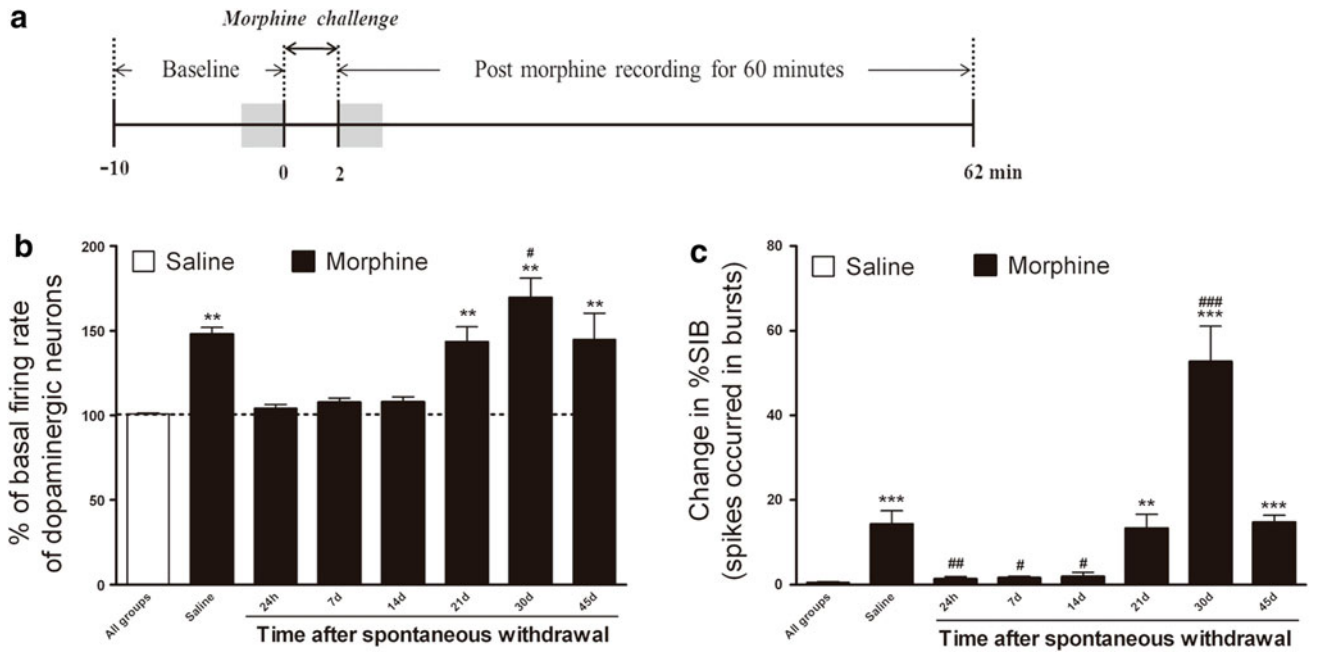
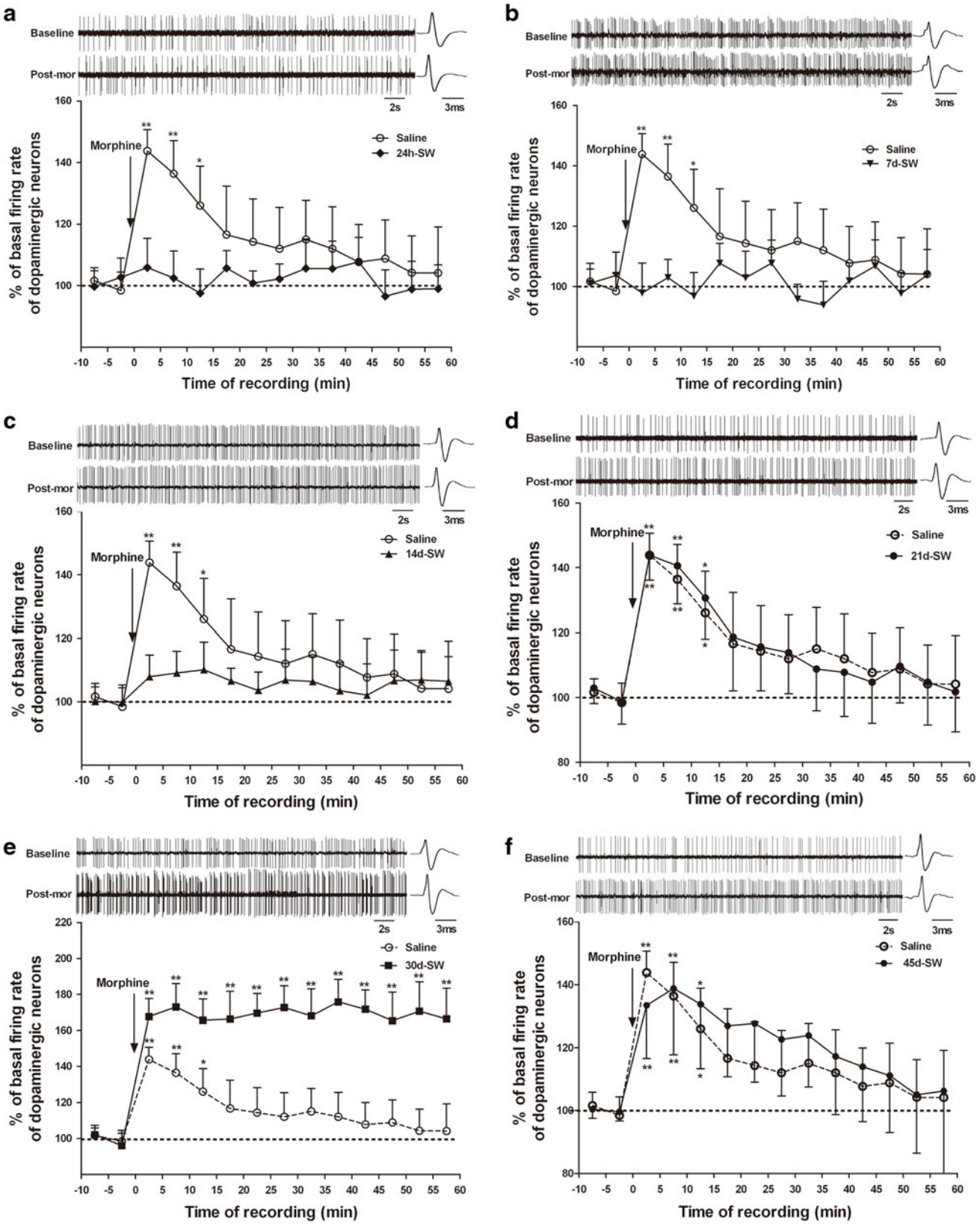



**Fig. 5** Overall effects of morphine challenge on VTA DA neuron activity in saline rats ($n = 8$ rats, 8 cells), 24h-SW rats ($n = 8$ rats, 8 cells), 7d-SW rats ($n = 6$ rats, 6 cells), 14d-SW rats ($n = 7$ rats, 7 cells), 21d-SW rats ($n = 6$ rats, 6 cells), 30d-SW rats ($n = 8$ rats, 8 cells), and 45d-SW rats ($n = 8$ rats, 8 cells). **a** The *gray area* represents the data analyzed in **b**, **c**. Morphine challenge (1 mg/kg, i.v.) induced changes in (**b**) firing rate (one-way ANOVA, $F_{7,\ 86} = 25.68$; $^{**}p < 0.01$, compared with baseline in all groups; $^{\#}p < 0.05$, compared with saline + 1 mg/kg morphine) and **c** burst size of VTA DA neurons (one-way ANOVA, $F_{7,\ 86} = 49.25$; $^{**}p < 0.01$, $^{***}p < 0.001$, compared with baseline in all groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with saline + 1 mg/kg morphine). The data are expressed as mean ± SEM

course changes in firing activity were then analyzed from the data recorded for 10 min before and 60 min after intravenous morphine administration (Fig. 6). The intravenous injection of 1 mg/kg morphine had no effects on the firing rate of VTA DA neurons or burst size (change in %SIB) within 200 s (Fig. 5b, c) and 60 min (Fig. 6a–c) compared with the pre-morphine baseline in the 24h-SW, 7d-SW, and 14d-SW groups. An intravenous injection of 1 mg/kg morphine increased the firing rate of VTA DA neurons to ~45 % of pre-morphine baseline (all groups, 100.9 ± 0.5087 %; saline, 147.9 ± 3.951 %; 24h-SW, 104.0 ± 2.489 %; 7d-SW, 107.6 ± 2.541 %; 14d-SW, 107.8 ± 3.072 %; 21d-SW, 143.4 ± 8.852 %; $N = 8$ 30d-SW, 169.5 ± 11.58 %; 45d-SW, 144.7 ± 15.49 %; Fig. 5b) and significantly increased the burst size (~16 %) in the 21d-SW group within 200 s (all groups, 0.5555 ± 0.1440 %; saline, 14.32 ± 3.148 %; 24h-SW, 1.350 ± 0.5612 %; 7d-SW, 1.603 ± 0.4139 %; 14d-SW, 1.897 ± 1.015 %; 21d-SW, 13.31 ± 3.318 %; 30d-SW, 52.72 ± 8.306 %; 45d-SW, 14.71 ± 1.665 %; Fig. 5c). Neuronal activation was maximal 3 min after the injection, and all of the neurons returned to baseline levels 12 min after the injection, which was almost equivalent to the saline-pretreated group (Fig. 6d). In the 30d-SW group, an intravenous injection of 1 mg/kg morphine dramatically increased the firing rate of VTA DA neurons by ~70 % (Fig. 5b) and elicited a greater increase in burst size (~53 %) compared with the pre-drug baseline within 200 s (Fig. 5c). This neuronal activation reached a maximum 3 min after the injection and was maintained at a high level for at least 60 min (Fig. 6e). The morphine challenge-induced increases in firing rate and burst size (Fig. 5b, c) and the time-course of neuronal activation (Fig. 6f) of VTA DA neurons in the 45d-SW group were similar to the 21d-SW and saline-pretreated groups. The top panels in Fig. 6a–f present typical examples of oscilloscope traces of VTA DA neuron firing before and after intravenous morphine injection at different withdrawal time points.

## Dynamic changes in mesoaccumbens DA signaling during morphine withdrawal

As shown in Table 1, morphine challenge (5 mg/kg, i.p.) increased DA content in the NAc by ~56 % in saline-pretreated rats. Importantly, in morphine-withdrawn rats, the content of DA, DOPAC, and HVA in the NAc also exhibited an early decrease and late increase with withdrawal time in response to morphine challenge. The DA ratio ([DOPAC + HVA]/DA) associated with 5 mg/kg morphine challenge significantly decreased in the 24h-SW group (Fig. 7b), but 30d-SW and 45d-SW group returned to the control group level (Fig. 7a, f, g), which is similar to


a
Baseline
Post-mor
2s
3ms
Morphine
Saline
24h-SW
% of basal firing rate of dopaminergic neurons
Time of recording (min)
b
7d-SW
c
14d-SW
d
21d-SW
e
30d-SW
f
45d-SW

◀ **Fig. 6** Time-course of changes in the firing rate of VTA DA neurons in morphine-withdrawn rats at different time points after morphine challenge. **a–f** *Upper left* sample spikes before and after morphine challenge for 20 s. *Upper right* single sample waveforms before and after morphine challenge. *Lower* 60-min recording of changes in the firing rate of VTA DA neurons after morphine challenge. The morphine injection is indicated by the *arrow* above the *curve*. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared with pre-morphine baseline (one-way ANOVA; $n = 6$–9 rats, 6–9 cells). The data are expressed as mean ± SEM

the change of DA neuron activity in the VTA during withdrawal.

Different withdrawal time-induced changes in membrane $D_1$ receptor level are represented as white columns in Fig. 8, which increased after 24 h of withdrawal (Fig. 8a) and returned to saline-pretreated levels after 7 days of withdrawal (Fig. 8b–f). 5 mg/kg morphine-induced changes in membrane $D_1$ receptor expression in each SW group are represented as black columns. The SW groups has no remarked alterations in total $D_1$ receptor level, and only the 45d-SW group responded to the morphine challenge with a ~76 % increase in membrane $D_1$ receptor protein (Fig. 8b–f). The gray density data of membrane $D_1$ receptors was normalized to the average value of saline-pretreated controls.

We further assessed the effect of $D_1$ receptor blockade on the acquisition of morphine-induced CPP on day 45 of withdrawal. The $D_1$ receptor antagonist SCH23390 or its vehicle was injected 25 min before each conditioning session. The results showed that 0.5 mg/kg morphine was sufficient to induce a significant preference for the drug-paired compartment (Figs. 1b, 8g), and SCH23390 completely prevented the acquisition of morphine-induced CPP, in contrast to the vehicle-treated group (Fig. 8g).

## Discussion

### Behavioral transition from early tolerance to late sensitization in response to the rewarding properties of morphine during withdrawal

In the present study, we found that 0.5 mg/kg morphine, an effective dose to induce CPP in saline-pretreated rats (Bardo et al. 1995; Ma et al. 2009), was unable to induce CPP in SW rats from 24 h to 30 days of withdrawal, and the response recovered on 45 days of withdrawal, indicating a dynamic change with withdrawal time from early tolerant to late sensitive to the reward. Our results are consistent with previous findings. Russo et al. (2007) found

**Table 1** Effects of morphine challenge on DA, its metabolites (DOPAC and HVA) and DA ratio (DOPAC + HVA/DA) in the NAc

| Treatment | DA (ng/mg of tissue) | DOPAC (ng/mg of tissue) | HVA (ng/mg of tissue) | DA ratio |
|---|---|---|---|---|
| Saline | | | | |
| Vehicle | 2.932 ± 0.2149 | 1.488 ± 0.01001 | 0.4534 ± 0.02713 | 0.6730 ± 0.05052 |
| Morphine challenge | 4.654 ± 0.2380* | 3.010 ± 0.2010*** | 1.053 ± 0.05351*** | 0.8723 ± 0.02205* |
| 24h-SW | | | | |
| Vehicle | 7.519 ± 0.1871 | 3.189 ± 0.1446 | 0.9835 ± 0.02990 | 0.5573 ± 0.03340 |
| Morphine challenge | 5.284 ± 0.4537** | 1.241 ± 0.1432$^{***\#\#\#}$ | 0.3433 ± 0.06990$^{***\#\#\#}$ | 0.3157 ± 0.07112* |
| 7d-SW | | | | |
| Vehicle | 1.972 ± 0.1438 | 1.102 ± 0.04719 | 0.4199 ± 0.01502 | 0.7881 ± 0.07933 |
| Morphine challenge | 0.9352 ± 0.07753$^{*\#\#\#}$ | 0.2995 ± 0.02857$^{*\#\#\#}$ | 0.2109 ± 0.08184$^{\#\#\#}$ | 0.5643 ± 0.09434 |
| 14d-SW | | | | |
| Vehicle | 4.344 ± 1.157 | 1.187 ± 0.2212 | 0.4359 ± 0.05394 | 0.4583 ± 0.2346 |
| Morphine challenge | 3.957 ± 0.7656 | 0.9494 ± 0.04926$^{\#\#\#}$ | 0.2819 ± 0.05922$^{\#\#\#}$ | 0.3251 ± 0.1411 |
| 21d-SW | | | | |
| Vehicle | 4.166 ± 0.2902 | 2.153 ± 0.1454 | 0.3866 ± 0.03072 | 0.5063 ± 0.1480 |
| Morphine challenge | 5.884 ± 0.3320 | 2.529 ± 0.1587 | 0.6703 ± 0.04577$^{\#\#}$ | 0.4313 ± 0.1015 |
| 30d-SW | | | | |
| Vehicle | 4.278 ± 0.1924 | 1.735 ± 0.07889 | 0.5284 ± 0.03116 | 0.5320 ± 0.02774 |
| Morphine challenge | 6.315 ± 0.2962$^{***\#}$ | 3.530 ± 0.2039*** | 0.9981 ± 0.1088*** | 0.7216 ± 0.04167** |
| 45d-SW | | | | |
| Vehicle | 3.176 ± 0.2021 | 1.510 ± 0.08071 | 0.4668 ± 0.04253 | 0.6244 ± 0.01158 |
| Morphine challenge | 5.695 ± 0.1417** | 3.268 ± 0.1340*** | 1.073 ± 0.03706*** | 0.7648 ± 0.04156* |

Two-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with vehicle challenge; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, compared with saline group + morphine challenge; $n = 4$. The data are expressed as mean ± SEM

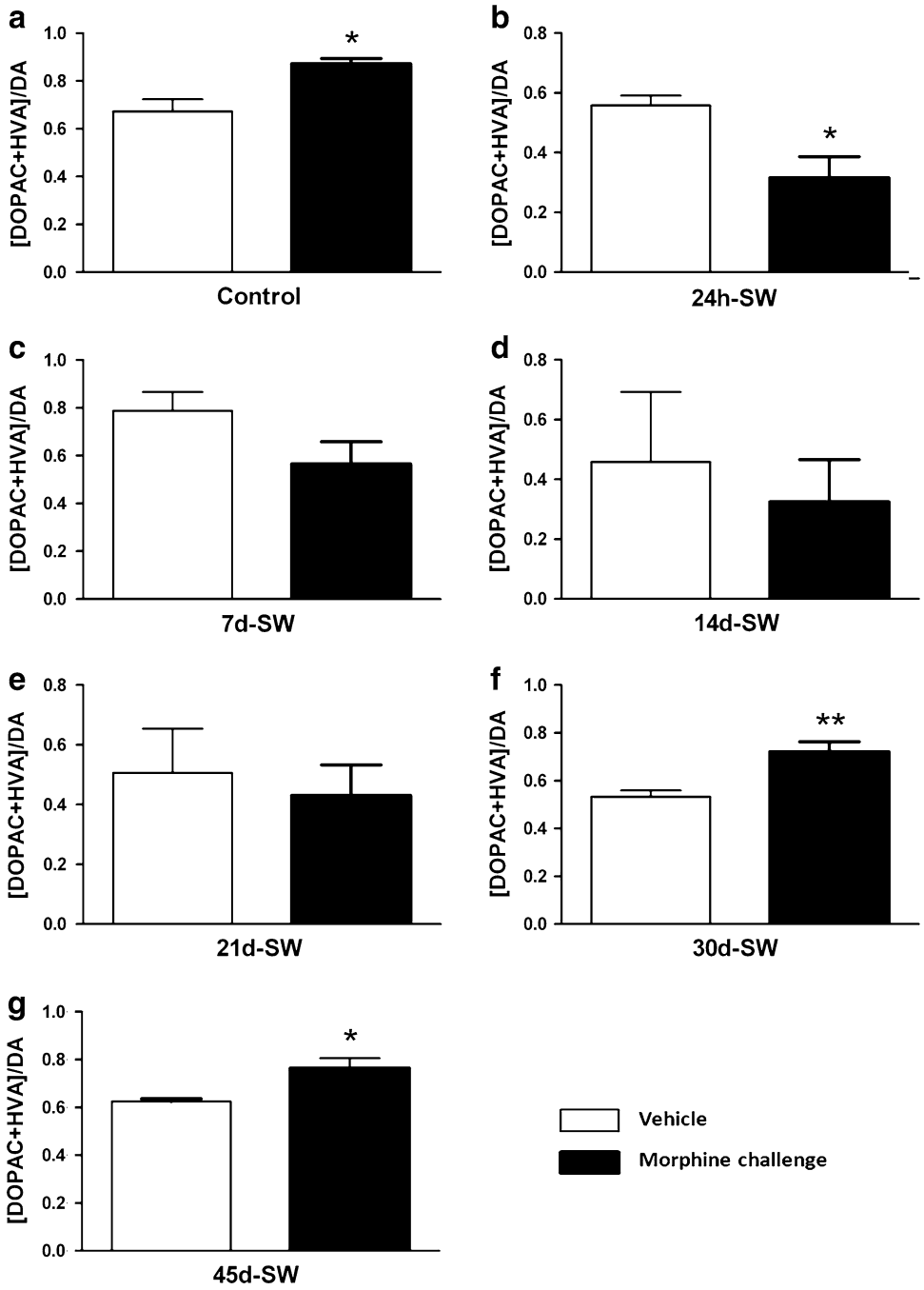



**Fig. 7** Effects of morphine challenge on the DA ratio in rats of different withdrawn groups in the NAc. Morphine challenge (5 mg/kg, i.p.) increased DA ratio in the **a** control group ($t$ test, $F_{3,\ 3} = 5.249$; $^{*}p < 0.0111$, $n = 4$), **f** 30d-SW group ($t$ test, $F_{3,\ 3} = 2.256$; $^{**}p < 0.0091$, $n = 4$), **g** 45d-SW group ($t$ test, $F_{3,\ 3} = 12.89$; $^{*}p < 0.0174$, $n = 4$) but decreased the DA ratio in the **b** 24h-SW group ($t$ test, $F_{3,\ 3} = 4.535$; $^{*}p < 0.0218$, $n = 4$). The data are expressed as mean ± SEM

that chronic morphine-pretreated rats did not exhibit CPP in response to 0.5 mg/kg morphine until 14 days of withdrawal. Mazei-Robison et al. (2011) also reported that chronic morphine-pretreated rats were tolerant to morphine's rewarding effects in the CPP paradigm. In contrast, Shippenberg et al. (1996, 1998) found that pre-exposure to morphine in rats led to sensitization to 5 mg/kg morphine CPP from 3 to 21 days of withdrawal. The apparent contradiction may be attributable to differences in the dose and temporal pattern of drug pretreatment and subsequent drug challenge. We employed an increasing morphine dose regimen for 14 days, prolonged withdrawal for 45 days, and both a lower and higher dose of morphine for CPP induction during withdrawal, which closely resemble opiate addiction in humans. The high dose of morphine (10 mg/kg) effectively induced CPP during the entire withdrawal period and eliminated potential learning deficits in withdrawn rats (Figure S3). The lack of response in SW rats to the low dose of morphine (0.5 mg/kg) during early withdrawal may indicate tolerance to the drug.

The 24h-SW rats displayed an aversive response to the 0.5 mg/kg morphine-paired compartment, this might be

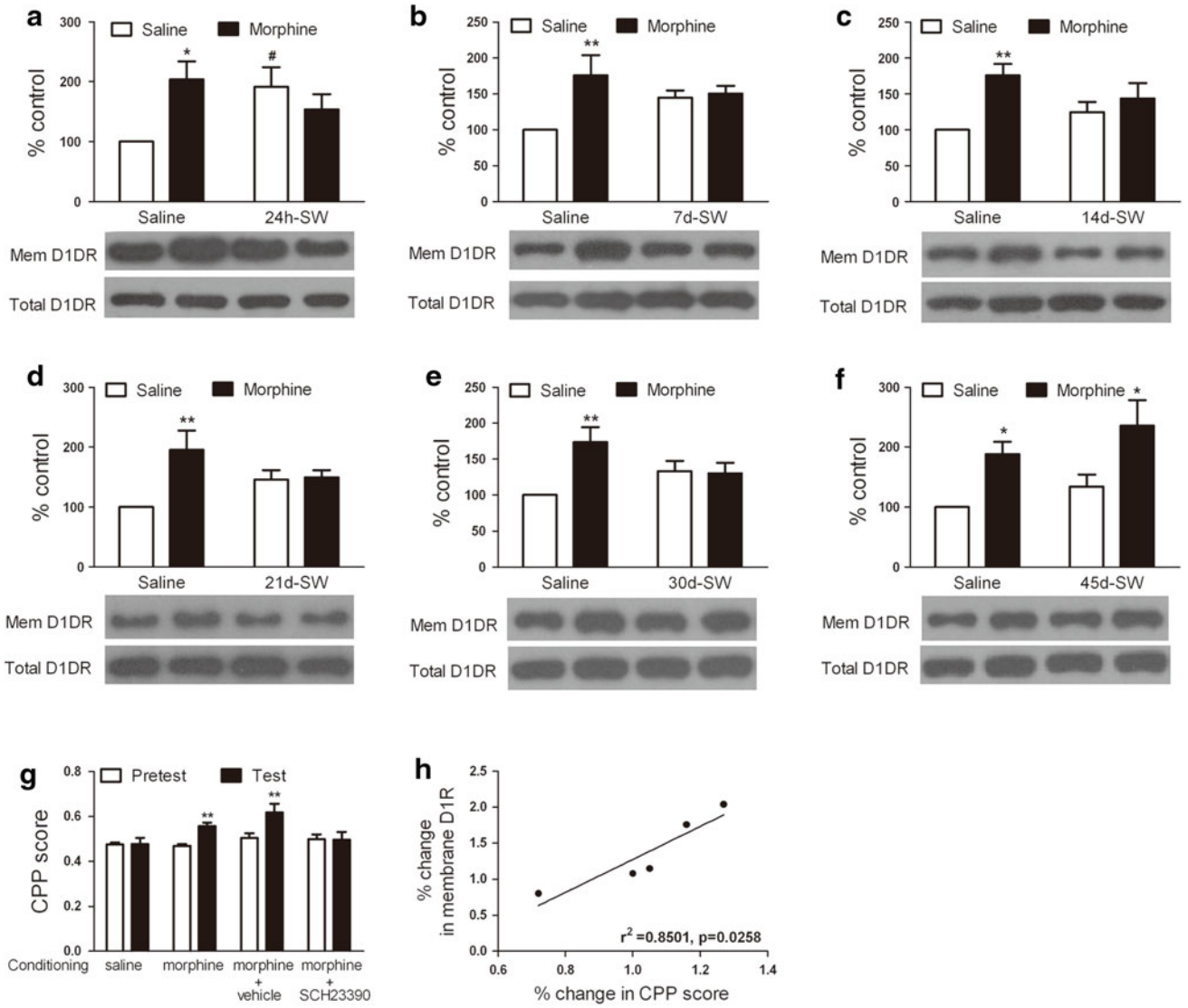



**Fig. 8** The membrane and total protein levels of $D_1$ receptors in the NAc with or without morphine challenge shown in **a–f**. **a** 24h-SW induced a significant increase in the membrane protein level of $D_1$ receptors, and 5 mg/kg morphine challenge significantly decreased the membrane protein level of $D_1$ receptors in 24h-SW rats (two-way ANOVA, $F_{1,\ 20} = 1.729$; $^{*}p < 0.05$, compared with the saline challenge; $^{\#}p < 0.05$, 24h-SW vs. saline-pretreated group; $n = 6$). **b–e** Morphine challenge had no effects on the total or membrane protein level of $D_1$ receptors in the 7d-SW, 14d-SW, 21d-SW, or 30d-SW groups (two-way ANOVA; $^{**}p < 0.01$, compared with saline challenge; $n = 4$–6). **f** Morphine challenge produced a marked increase in membrane $D_1$ receptor protein level in the 45d-SW group but no changes in total $D_1$ receptor protein level (two-way ANOVA, $F_{1,\ 18} = 3.505$; $^{*}p < 0.05$, compared with saline challenge; $n = 4$–6). **g** The 0.5 mg/kg dose of morphine induced significant CPP at 45 days of withdrawal, and pretreatment with SCH23390 blocked the reactivation of CPP (two-way ANOVA, $F_{3,\ 27} = 3.041$; $^{**}p < 0.01$, compared with pretest; $n = 6$–9). **h** A positive linear correlation was found between morphine-induced changes in CPP scores and changes in membrane $D_1$ receptor expression. The data are expressed as mean ± SEM

related to impairment in the morphology and excitability of VTA DA neurons induced by chronic morphine (Chu et al. 2007, 2008. Our result was in line with the previous findings. Russo et al. (2007) found that both chronic morphine administration and heroin self-administration in rats decreases the soma size of VTA DA neurons 24 h after last morphine treatment, and the reductions in cell size were associated with a diminished response to the rewarding effects of morphine. Of note, the author also used 0.5–10 mg/kg morphine conditioning and found that rats previously exposed to chronic morphine require much higher doses of morphine to produce a CPP and 0.5 mg/kg morphine conditioning also caused a significant place aversion in animals previously exposed to chronic morphine (Russo et al. 2007). Mazei-Robison et al. (2011) also reported that chronic morphine decreased VTA DA soma

size and decreased morphine CPP reward 24 h after the last morphine pellet s.c. implantation. Therefore, it is probable that the aversive CPP response in the 24h-SW rats might be due to the morphological or excitability changes in VTA neurons. To eliminate the possible contribution of a physical withdrawal syndrome to the behavioral transition, we measured four withdrawal symptoms for 1 h in all of the rats at all of the withdrawal time points. The results showed that withdrawal symptoms were not detectable on 7 days of withdrawal (Figure S4). Thus, withdrawal syndrome cannot explain the time-dependent behavioral transition from early to prolonged withdrawal.

## Temporally parallel changes in mesoaccumbens DA signaling with the behavior transition

Mesoaccumbens DA signaling has been considered as basis for the rewarding effects of opiates (Shippenberg et al. 1993; Willins and Meltzer 1998). We analyzed both the basal and burst firing activity of VTA DA neurons. Burst firing is believed to specifically convey reward-related information (Schultz 2002), and dopamine release during burst firing is substantially greater than during regular spiking (Chergui et al. 1994; Gonon 1988). The basal and burst firing activity of VTA DA neurons in chronic morphine-pretreated rats were reported to be unaltered when challenged with 1 mg/kg morphine (i.v.) during 2 weeks of spontaneous withdrawal (Georges et al. 2006). Our results confirmed this finding and further demonstrated that this tolerance switched to sensitization after 21 days of withdrawal. This sensitization then fluctuated and was most prominent on 30 days of withdrawal and returned to normal sensitization level after 45 days of withdrawal. We verified that the lack of response to 1 mg/kg morphine challenge during early withdrawal was the result of tolerance, as the two higher doses of morphine (3 and 5 mg/kg, i.v.) both successfully drove significant increases in VTA DA neuron firing activity in the 24h-SW group (Figure S5). The seemingly norm-sensitive DA response to the morphine challenge on 45 days of withdrawal may reflect the manifestation of equilibrium around a new set point in the brain (e.g., resetting the system parameters at a new set point) rather than total recovery (Sterling and Eyer 1988). This is consistent with allostasis theory, which is a physiological concept, in which an organism must vary all of the parameters and match them appropriately to maintain stability (Koob and Le Moal 2001). Notably, the observed tonic hypersensitivity of DA neuron firing in the 30d-SW group might reflect electrophysiological deviation toward the new equilibrium manifested on 45 days of withdrawal.

We then monitored ensuing changes in the NAc, specifically DA neurotransmitter and $D_1$ receptor protein levels in the NAc, both of which underwent a transition from an early decrease to a late increase in response to morphine challenge during withdrawal, with only minor changes in the switch time points. The motivational effects of opiates, both rewarding and aversive, are well known to be paralleled by an increase and decrease, respectively, in DA release in the NAc (Herz 1998). In line with this, we found that with morphine challenge, increased DA ratio was associated with increased DA neuron firing and enhanced reward-related behavior, but decreased DA ratio was associated with tolerant DA neuron firing and aversive behavior. It has been known for over 20 years now that microdialysate DA is reduced during opiate withdrawal (Acquas and Di Chiara 1992; Pothos et al. 1991; Rossetti et al. 1992). Electrophysiological investigations further suggested that reduction in DA release during morphine withdrawal was due to marked reductions in VTA DA neuron firing rates and pattern (Diana et al. 1995) probably owing to an increased GABA tone (Bonci and Williams 1997) and/or a decreased glutametergic input onto VTA neurons (Manzoni and Williams 1999). However, in our results we found decreased DA, DOPAC, and HVA contents upon acute 24 h of withdrawal, but increased DA, DOPAC, and HVA contents in the NAc after 7 days of withdrawal. This inconsistency may be because of the different experimental methods used, that the microdialysis could directly reflect the DA release while the HPLC method could only detect the intraneuronal contents of DA and its metabolites in the local. In order to better understand the firing activity of DA neurons in the VTA and the DA transmitter in the NAc, we calculated the DA metabolism as [DOPAC + HVA]/DA, which is also an indicator of presynaptic DA neuronal activity (Thiblin et al. 1999) and the results were highly in line with the electrophysiological finding. Taken together, increased VTA-NAc DA neuronal activity may contribute to the morphine challenge-induced CPP behavior during withdrawal. Increased stimulation of $D_1$ receptors in the NAc contributes to the enhanced strength in response to morphine reward (Chartoff et al. 2009) and counteracts negative affective states during morphine withdrawal (Chartoff et al. 2003), moreover $D_1$ receptor antagonism blocked the potentiation of morphine reward (Koo et al. 2012). The behavioral transition pattern observed in the present study was paralleled by a switch in mesoaccumbens DA signaling, indicating that mesoaccumbens DA signaling may underlie the behavioral transition in the rewarding properties of morphine from early to prolonged withdrawal. Supporting this possibility, blockade of $D_1$ receptor activation abolished morphine-induced CPP in the 45d-SW group (Fig. 8g).

Because of method limitation, we employed different doses of morphine in CPP, electrophysiological, and DA content and $D_1$R studies. In morphine-induced CPP model, we used 0.5 mg/kg i.p. morphine over a 2-day conditioning

period, we took this as an equal dose with the 1 mg/kg intravenous morphine in the in vivo electrophysiology experiment. For the HPLC and WB experiments in the NAc, it is not possible to perform the intravenous morphine challenge in every rat and just to get the brain tissue within the finite time. Therefore, we employed a single i.p. injection of 5 mg/kg morphine challenge and detect the morphine effect on the targeted molecules at its most effective time point, 80 min post injection for analysis of DA and its metabolites, 30 min post injection for analysis of $D_1R$ in the NAc.

### Incubation of motivational value of morphine challenge as a function of withdrawal time

The incubation theory of craving means that cocaine-seeking induced by reexposure to cocaine-associated cues progressively increased over 2 months of withdrawal from cocaine self-administration, suggesting that drug-craving behavior incubates over the withdrawal period (Grimm et al. 2003; Lu et al. 2004). The present results revealed that the motivational value in withdrawn rats increased with prolonged withdrawal in response to 0.5 mg/kg morphine (i.p.) reinforcement. The 0.5 mg/kg dose of morphine was insufficient to induce CPP for as long as 30 days of withdrawal. In fact, the 24h-SW group exhibited aversion while the 45d-SW group displayed preference to the same dose. This behavioral transition may reflect the incubation of drug-seeking behavior in SW rats. An analogous pattern was also observed in the mesoaccumbens DA signaling, which became more sensitive to morphine challenge as the withdrawal time extended. This increase in sensitization over time could be viewed as incubation of the motivational value of the drug.

### Mesoaccumbens DA signaling underlies the time-dependent behavioral transition during withdrawal

Membrane $D_1$ receptors in the NAc were the response-limiting factor in mesoaccumbens DA signaling. This is better understood when analyzing the results presented in Table 2. The 0.5 mg/kg dose of morphine (i.p.) could only induce CPP when membrane $D_1$ receptors recovered their response to the morphine challenge. In addition, when a decrease in membrane $D_1$ receptor expression occurred, an aversive behavioral response ensued, as observed in the 24h-SW group. Moreover, the VTA DA neuron firing response was the first to exhibit a trend toward recovery, with a positive response to morphine challenge on 21 days of withdrawal, which is reasonable because the firing of DA neurons in the VTA is the initial step in mesoaccumbens DA signaling.

**Table 2** Morphine challenge-induced changes in DA neuronal firing, DA metabolic rate, membrane D1-type receptor and CPP score

| % Control | Neuronal firing | DA ratio | D1R expression | CPP test |
|---|---|---|---|---|
| Control | + | + | + | √ |
| 24h-SW | 0 | − | − | × |
| 7d-SW | 0 | 0 | 0 | |
| 14d-SW | 0 | 0 | 0 | × |
| 21d-SW | + | 0 | 0 | |
| 30d-SW | + | + | 0 | × |
| 45d-SW | + | + | + | √ |

"0" represents no alteration; "−" represents decreased response; "+" represents increased response. "√" stands for successful CPP, and "×" stands for no CPP

To clarify whether a correlation exists between the behavioral transition and changes in mesoaccumbens DA signaling in morphine-withdrawn rats in response to a morphine challenge, we assessed the effect of $D_1$ receptor blockade on the acquisition of morphine-induced CPP on day 45 of withdrawal, and SCH23390 completely prevented the acquisition of morphine-induced CPP. Linear regression analysis revealed a positive relationship between % change in CPP score and membrane $D_1$ receptor expression induced by morphine challenge ($r^2 = 0.8501$, $p = 0.0258$; Fig. 8h).

In conclusion, chronic morphine-pretreated rats displayed a behavioral transition from early to prolonged withdrawal in response to the rewarding properties of morphine that paralleled the mesoaccumbens DA pathway response to morphine challenge. Morphine chronic exposure triggered adaptive changes in mesoaccumbens DA signaling during withdrawal, which increased the DA-encoding value of the same reward properties of morphine as a function of withdrawal time. The present findings may explain why even years after withdrawal, a single drug exposure may induce relapse in addicts.

**Acknowledgments** This project was supported by grants from the National Basic Research Program (2009CB522003) and the National Natural Science Foundation (31271163) of China to Cailian Cui. We thank Dr. Lu Lin of the National Institute on Drug Dependence, Peking University, Beijing 100191, China, for his helpful comments on the manuscript.

**Conflict of interest** The authors declare that they have no conflict of interest.

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