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The role of the nucleus accumbens OXR1 in cocaine-induced locomotor sensitization

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ABSTRACT

Re-exposure to drug or drug-associated cues after withdrawal can induce behavioral sensitization expression in animals or increase in the expected effect to drug in humans, which mean an enhanced drug seeking/taking motivation to trigger relapse after abstinence. The Nucleus accumbens (NAc) is known to play a key role in mediating this motivation. Recently, it has been shown that systemic administration of orexin receptor 1 (OXR1) antagonist attenuates animals' motivation behavior to take drug by self-administration paradigm, which is more effectively than orexin receptor 2 (OXR2) antagonist. However, the effect of OXR1 in the NAc on drug-induced locomotor sensitization remains elusive. The present study was designed to investigate the effect of OXR1 in the NAc on chronic cocaine-induced locomotor sensitization. Rats were given 10 mg/kg cocaine intraperitoneal injection (i.p.) for five consecutive days, followed by 10 mg/kg cocaine re-exposure (challenge) on the 14th day of withdrawal. Results showed that re-exposure to cocaine after withdrawal could induce locomotor sensitization expression in cocaine-sensitized rats. Simultaneously, the number of OXR1 positive neurons and OXR1 membrane protein level in the NAc core but not the shell were significantly increased following the cocaine re-exposure. Further, micro-infusion of SB-334867, an OXR1 selective antagonist, into the NAc core but not the shell before cocaine re-exposure, significantly attenuated the expression of locomotor sensitization in rats. The findings demonstrate that OXR1 in the NAc core partially mediates the expression of chronic cocaine-induced locomotor sensitization.

1. Introduction

Drug addiction is a healthy and socio-economic problem, which is a chronic and recrudescent brain disease caused by substance abuse [1]. The main difficulty confronted with treating drug addiction is to prevent relapse after detoxification [2]. Behavioral sensitization, including locomotor sensitization and motivation sensitization, refers to enhanced response to equal dose of addictive drug after constant same dose stimulation in animals or humans [3]. It reflects a gradual increase in drug craving that is related to relapse after withdrawal [4]. Locomotor sensitization induced by addictive drugs, has been widely used as one of the animal models to investigate the mechanism of relapse induced by drug re-exposure after withdrawal [5]. Research have shown that neuron activity in the Nucleus accumbens (NAc) is increased upon a cocaine challenge in cocaine-treated rats with functional MRI [6], followed with an increase of △FosB [7], as well as increases of dopamine (DA) release and DA receptors (DAR) D1R, D2R, D3R uprate in the NAc [8]. These studies indicate that the NAc is a key structure in mediating drug-induced locomotor sensitization.

Additionally, Quarta et al. found that systemic administration of orexin receptor 1 (OXR1) selective antagonist, SB-334867, can reduce amphetamine-evoked DA outflow into the NAc shell and decrease the expression of amphetamine sensitization [9]. This enlightened that orexin (OX) may be involved in behavioral sensitization. OX, as a classical neuropeptide, is initially found in intestinal epithelial cells. In 1998, Sakurai and de Lecea reported that OX is also expressed in brain, which is mainly synthetized and secreted in the Lateral hypothalamic area (LHA) [10]. OX is divided into OXA and OXB, which are synthetized from same precursor. Accordingly, OX receptors (OXR), divided into OXR1 and OXR2, belong to G protein-coupled receptors. OXA has been shown to combine with both OXR1 and OXR2, and OXB can only combine with OXR1 [11]. Besides the original function of modulating feeding behavior [12,13], OX also participates in regulating sleep-awake cycle [14–16], spontaneous physical activity [17,18] and analgesia [19,20]. Recently, Haghparast et al. and Schmeichel et al. found that orexinergic neurons can project to multiple brain regions,

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including the NAc and the Ventral tegmental area (VTA), which are considered to be crucial regions involved in drug addiction [21,22], and OXR are expressed at high level at these regions [23]. Additionally, Baimel et al, Lei et al. and Schmeichel et al. reported one after another that systemic blockade of OXR1 but not OXR2 can reduce cocaine-induced DA uptake in the NAc and inhibit cocaine self-administration behavior [24–26]. Obviously, these studies indicate that central OX system participates in drug-enhanced motivation, in which OXR1 might play a more important role than OXR2.

However, whether OXR1 in the NAc is involved in locomotor sensitization induced by addictive drugs remains elusive. The present study was designed to investigate the effect of OXR1 in the NAc core and the shell on chronic cocaine-induced locomotor sensitization in rats.

2. Materials and methods

2.1. Animals

Male Sprague Dawley (total 180) rats weighing 200 − 220 g on arrival (purchased from Laboratory Animal Center of Peking University Health Science Center) were housed in pairs in a light controlled room (12 h/12 h dark/light cycle: lights off at 7:00 a.m.). The room temperature was maintained at 23 ± 2 ℃, with relative humidity at 50 ± 5 %. Food and water were available ad libitum. All saline or cocaine injection and behavioral recording were conducted during the dark phase between 9:00 a.m. and 4:00 p.m. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

2.2. Chemical drugs

Cocaine hydrochloride was purchased from the First Pharmaceutical Factory of Qinghai, China and dissolved in sterile saline (at a concentration of 10 mg/ml) just before use. A specific non-peptide OXR1 antagonist N-(2-methyl-6-benzoxazolyl)-N'-1, 5-naphthyridin-4-yl (SB-334867 hydrochloride, Tocris) was dissolved in DMSO as a stock solution of 60 nmol/μl and was further diluted with DMSO to the final concentration of 20 nmol/μl before use.

2.3. Development of cocaine locomotor sensitization

After one week of acclimation to the home cage and daily handling, the animals were habituated to the intraperitoneal injection (normal saline, 1 ml/kg, i.p.), and the behavioral test was conducted by placement in the recording box (40 × 40 × 40 cm^3, installed with infrared camera on the ceiling) for 30 min during three consecutive days. For chronic cocaine treatment, rats were habituated to the testing box for 15 min before cocaine injection. Subsequently, cocaine (10 mg/kg, i.p.) was administered intraperitoneally once daily for five consecutive days. Immediately after cocaine injection, the rats were placed into the recording box under dim light illumination. Rats were allowed to move freely in the chamber for 30 min because locomotor activity induced by a variety of cocaine dose is apparent within 30 min after injection [27]. Since not all rats developed locomotor sensitization, they were allocated into sensitized and non-sensitized groups. Criterion for sensitization was based on the ratio of horizontal travel distance on cocaine 5th day vs. cocaine 1st day. For this analysis, horizontal travel distance was calculated based on the total locomotor activity in 30 min after cocaine injection. The criterion used for sensitization was that at least a 20 % increase in horizontal travel distance over 5-day injection period (Day 5-to-Day 1 locomotor activity ratio ≥ 1.2) [28]. Rats get normal saline (1 ml/kg, i.p.) administration intraperitoneally, once per day, for five consecutive days were used as control group.

2.4. Expression of cocaine locomotor sensitization

Before the cocaine challenge, rats were placed into the recording box for 15 min and locomotor activity was recorded as the baseline locomotor activity. Subsequently, rats were taken out and received a challenge injection of cocaine (10 mg/kg, i.p.). Rats get the injection of normal saline (1 ml/kg, i.p.) were used as control group.

2.5. Immunofluorescence staining

Waiting for 90 min after the cocaine challenge, the rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused with 200 ml of 0.9 % saline, followed by 400 ml of 4 % PFA in 0.1 M PBS (pH 7.4). Brains were post-fixed in 4 % PFA for 24 h before transferred to 30 % sucrose in PBS aiming for dehydration. Six coronal brain sections (30 mm thick, including AP + 1.7 mm, +1.6 mm, +1.5 mm, +1.4 mm, +1.3 mm, +1.2 mm) were cut in each rat using a freezing microtome. The brain slices were kept in cryoprotectant (20 % glycerol, 30 % ethylene glycol and 2 % DMSO in 0.1 M PBS, pH = 7.4) and stored at -30 ℃ for further study.

The frozen brain slices were rinsed with PBS three times in a twelve-well plate by shaking the slices 5 min for each time, after put at room temperature for 30 min. Then, the slices were rinsed with PBS with 0.3 % Triton X-100 (PBS-Tx) for 30 min, followed by the incubation with PBS-Tx containing 5 % normal donkey serum for 1 h. Next, the brain slices were incubated with mouse anti-c-Fos antibody at a dilution of 1:500 (Santa Cruz Biotechnology) monoclonal antibody and rabbit anti-NeuN at a dilution of 1:800 (Millipore) monoclonal antibody or rabbit anti-OXR1 antibody at a dilution of 1:300 (Rockland) overnight at 4 ℃. The sections were then rinsed with PBS-Tx and incubated for 1 h with the following secondary antibodies: Alexa Fluor 488 donkey anti-mouse IgG (c-Fos, 1:500, Invitrogen) and Alexa Fluor 555 donkey anti-rabbit IgG (NeuN, OXR1, 1:500, Invitrogen). After rinsed with PBS, sections were mounted onto slides and cover slipped with anti-fade solution (Applygen Technologies Inc). The stained sections were examined under an Olympus FV1000 confocal microscope (Olympus Corporation). Image quantifications of every total brain area were conducted by manual counting that finished by two people in a blind manner. The number of c-Fos/NeuN immunoreactive cells and c-Fos/OXR1 immunoreactive cells in each brain region were determined by averaging the results from two hemisections per rat.

2.6. Western blot

Protein extracts (total protein: 30 μg; membrane protein: 20 μg) were electrophoresed in 10 % sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked in TBST buffer (Tris-buffered saline with 0.1 % Tween 20) containing 5 % nonfat milk and then incubated with primary antibody diluted in TBST overnight at 4 ˚C (rabbit anti-OXR1, 1:1000, Rockland; mouse anti-β-actin, 1:3000, Sigma-Aldrich; mouse anti-Na-K-ATPase, 1:3000, Abcam). The blots were then washed in TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG for 1 h at room temperature (1:2000, ZB-2305 or ZB-2301, Zhongshan Biotechnology), followed by the development with a chemiluminescence detection kit (WBKLS0500, Millipore). The blots were washed with stripping buffer (P1650, Applygen Technologies Inc) to incubate with other antibodies. The immunoreactive bands were analyzed quantitatively by densitometry with the Quantity One® 1-D analysis software. The optical density of each band was standardized as a percentage of the total before statistical treatment.

2.7. Cannulation and micro-infusion

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and permanent guide cannulas were implanted at 2 mm above the

target regions bilaterally for the study of expression of chronic cocaine-induced locomotor sensitization. Coordinates for the NAc core were AP, +1.2 mm; ML, ± 3.7 mm (15° inclination); DV, +7.0 mm, and for the NAc shell were AP, +1.6 mm; ML, ± 0.9 mm; DV, +7.0 from the skull [29]. After the surgery, the rats were raised in pairs to avoid the stress of social isolation and were allowed to recover for one week. To eliminate the impact of the cannula detachment, the rats were excluded from the study when the cannula was detached from their skull. In the micro-infusion study (n = 9–10), time and dosage of drugs were based on previous reports and pre-experiment: SB-334867 hydrochloride, 20 nmol/μl, 0.5 μl per side [3031,32,33]. Drugs were microinjected into the target regions 15−20 min prior to cocaine challenge. To minimize the restraint stress, all micro-infusions were administered in freely moving rats. Infusions were made with a syringe pump at a rate of 0.5 μl/min connected to Hamilton syringes attached via polyethylene tubing to injectors. The injectors were left in place for an additional 1 min to allow for drug diffusion. At the end of the behavioral tests, cannula placements were estimated by Nissl staining.

2.8. Statistical analysis

Data were expressed as mean ± SEM and analyzed with one-way ANOVA or two-way ANOVA, followed by a Tukey post hoc test. When two groups' data were analyzed, the data were treated using a two-tailed unpaired t-test. The software used for the statistical analysis was Graph Pad Prism 6.0. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Cocaine challenge after withdrawal can induced locomotor sensitization expression in chronically cocaine-treated rats

As shown in Fig. 1A, rats were randomly divided into two groups, and were given cocaine (10 mg/kg) or normal saline (1 ml/kg) as the control, by intraperitoneal injection (i.p.) once per day for five consecutive days, respectively. Locomotor activity (horizontal travel distance) of the rats in the two groups were immediately tested and recorded in 5 min intervals for 30 min right after every injection. On the 14th day of withdrawal after the last injection, half of the rats in each group were given a 10 mg/kg cocaine injection again (challenge), and the other half were given normal saline (1 ml/kg) as control. Locomotor activity of the rats were immediately tested and recorded for 30 min. In other words, the data were generated for following four groups: 1) cocaine-treat + cocaine- challenge (coc + coc), 2) cocaine-treat + saline-challenge (coc + sal), 3) saline-treat + cocaine-challenge (sal + coc), and 4) saline-treat + saline-challenge (sal + sal).

Results showed that most (72.2 %) of the rats get the cocaine injection for five consecutive days displayed locomotor sensitization, which were defined as cocaine-sensitized rats [34]. The cocaine-sensitized rats (n = 65) had a significant increase on horizontal travel distance in all observed time intervals after cocaine injected for five consecutive days compared to the distance after the 1st day's injection (Fig. 1B1; a significant main effect of time F (8, 496) = 50.25, a significant main effect of day F (1, 62) = 114.0, a significant interaction of day * time F (8, 496) = 69.78, repeated measures two-way Anova with Tukey post hoc test). The horizontal travel distance of the rats on the 5th day after cocaine injection was significantly increased than that on the 1st day (Fig. 1B2; df = 64, t = 11.18, $p < 0.0001$, n = 65; paired t-test, two-tailed); The other 27.8 % rats (n = 25) were defined as cocaine-unsensitized rats, which showed an opposite trend compared to the sensitized rats, i.e., the horizontal travel distance on the 5th day were significantly decreased than that on the 1st day, for all observed time intervals after cocaine injection (Fig. 1C1; a significant main effect of time F (8, 192) = 18.92, a significant main effect of day F (1, 24) = 31.90, a significant interaction of day * time F (8, 192) = 5.130, repeated measures two-way Anova with Tukey post hoc test). The horizontal travel distance of the cocaine-unsensitized rats on the 5th day after cocaine injection was significantly decreased than that on the 1st day (Fig. 1C2; df = 24, t = 5.075, $p < 0.0001$, n = 25; paired t-test, two-tailed).

All experiments discussed below were conducted in the cocaine-sensitized rats. During the five consecutive days' cocaine or normal saline injection, an increase trend of the 30 min horizontal travel distance after injection day by day in cocaine-treated rats was observed, but there was no significant change of which in saline-treated rats (Fig. 1D1). Meanwhile, a significant increase of horizontal travel distance after cocaine injection between the 1st day and the 5th day was observed, and the horizontal travel distance after cocaine injection was significantly more than that after saline injection on the 1st day and the 5th day, respectively (Fig. 1D2; a significant main effect of drug formation F (1, 160) = 228.9, a significant main effect of day F (1, 160) = 174.3, a significant interaction of drug formation * day F (1, 160) = 187.2, repeated measures two-way Anova with Tukey post hoc test).

Fig. 1E1 shows the horizontal travel distance after injection from the 1 st day of injection to the challenge on the 14th day after withdrawal (a significant main effect of group F (3, 40) = 17.43, a significant main effect of day F (5, 200) = 9.922, a significant interaction of group * day F (15, 200) = 8.192, repeated measures two-way Anova with Tukey post hoc test). In addition, results showed that the horizontal travel distance after injection of the coc + coc group was significantly increased compared to the coc + sal group, for all observed time intervals after injected (Fig. 1E2; a significant main effect of group F (3, 40) = 25.19, a significant main effect of time F (8, 320) = 12.34, a significant interaction of group * time F (24, 320) = 13.23, repeated measures two-way Anova with Tukey post hoc test). The 30 min horizontal travel distance after injection of the coc + coc group was significantly increased than that of the sal + coc group and the coc + sal group (Fig. 1E3; a significant main effect of drug formation F (1, 40) = 12.21, a significant main effect of drug challenge F (1, 40) = 58.07, a significant interaction of drug formation * drug challenge F (1, 40) = 5.777, two-way Anova with Tukey post hoc test), indicating that the expression of locomotor sensitization was induced by cocaine re-exposure successfully. Meanwhile, results also showed that the 30 min horizontal distance after injection of the sal + coc group was significantly increased than that of the sal + sal group (Fig. 1E3), indicating that a single exposure to cocaine could also increase the locomotor activity of the rats.

3.2. The number of c-Fos$^+$/NeuN$^+$ cells in the NAc core was specifically increased following expression of locomotor sensitization

It has been confirmed that the NAc is activated by cocaine-induced locomotor sensitization in rats. To investigate the role of the NAc subregions in the locomotor sensitization, we tested the number of c-Fos (a kind of immediate early gene regarded as biomarker of cell activation) and NeuN (biomarker of mature neuron) double-positive cells in the NAc core and the shell of the rats after challenge. As shown in Fig. 2A1, the number of c-Fos$^+$/NeuN$^+$ cells in the NAc core of the coc + coc group was significantly increased compared to the sal + coc group and the coc + sal group (Fig. 2A2; a significant main effect of drug formation F (1, 19) = 11.24, a significant main effect of drug challenge F (1, 19) = 5.155, a significant interaction of drug formation * drug challenge F (1, 19) = 2.087, two-way Anova with Tukey post hoc test); As shown in Fig. 2 B1, no signifant change of the number of c-Fos$^+$/NeuN$^+$ cells in the NAc shell between every group was observed (Fig. 2B2; main effect of drug formation F (1, 18) = 0.1695, main effect of drug challenge F (1, 18) = 0.007281, interaction of drug formation * drug challenge F (1, 18) = 0.1315, two-way Anova with Tukey post hoc test). Results also showed that the number of c-Fos$^+$/NeuN$^+$ cells in the NAc core of coc + coc group was significantly more than that in the shell (Fig. 2D; a significant main effect of group F (3, 37) = 3.104, a significant main effect of brain region F (1, 37) = 5.837, interaction of

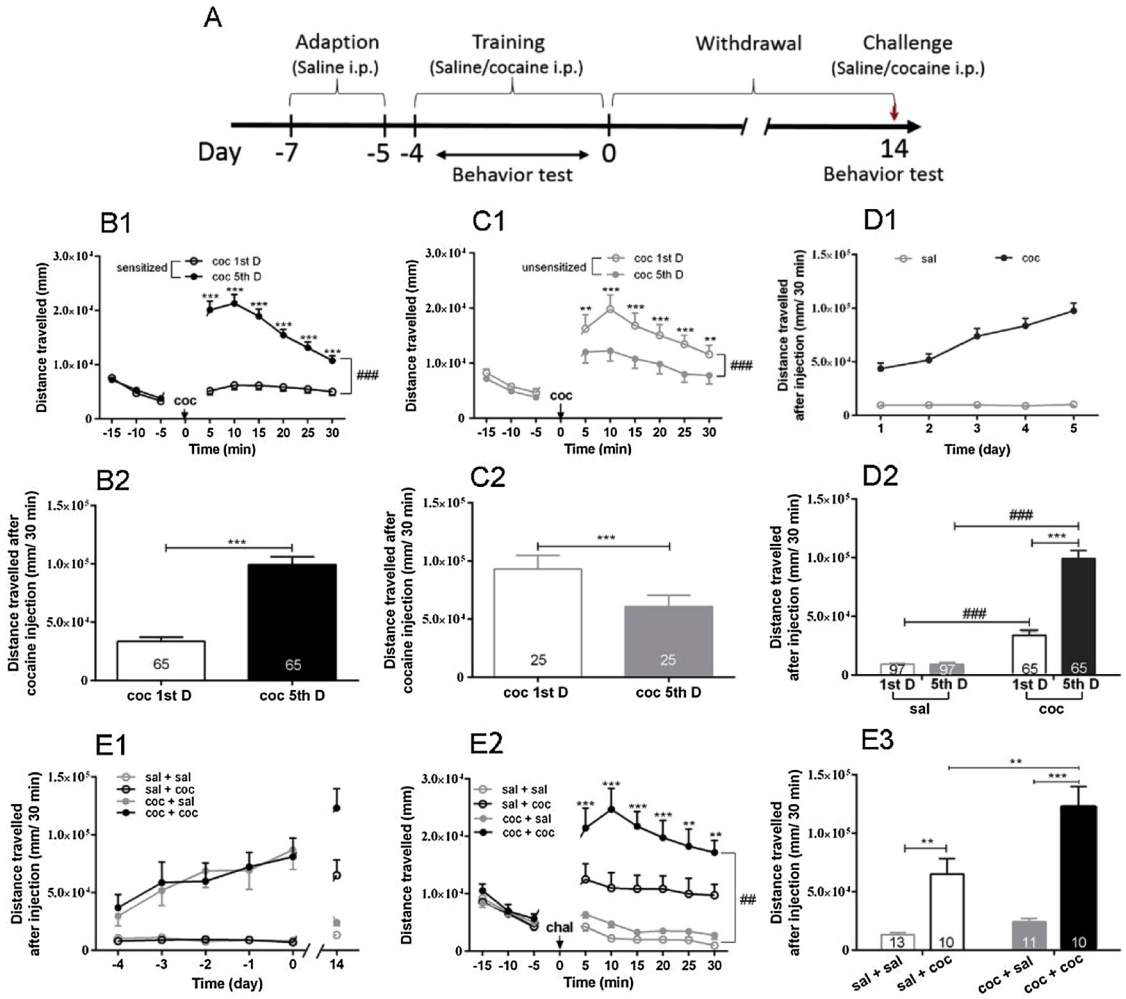

Fig. 1. Cocaine challenge after withdrawal can induced locomotor sensitization expression in chronically cocaine-treated rats. A. The experimental procedure. B1. Horizontal travel distance of every 5 min before and after cocaine injection on the 1st and the 5th day in cocaine-sensitized rats (### $p < 0.001$, *** $p < 0.001$, repeated measures two-way ANOVA with Tukey post hoc test). B2. Horizontal travel distance of 30 min after cocaine injection on the 1st and the 5th day in cocaine-sensitized rats (*** $p < 0.001$, paired t-test, two-tailed). C1. Horizontal travel distance of every 5 min before and after cocaine injection on the 1st and the 5th day in cocaine-unsensitized rats (### $p < 0.001$, ** $p < 0.01$, *** $p < 0.001$, repeated measures two-way ANOVA with Tukey post hoc test). C2. Horizontal travel distance of 30 min after cocaine injection on the 1st and the 5th day in cocaine-unsensitized rats (*** p < 0.001, paired t-test, two-tailed). D1. 30 min horizontal travel distance after cocaine or saline injection in five consecutive days. D2. Horizontal travel distance 30 min after cocaine or saline injection on the 1st and the 5th day (### $p < 0.001$, *** $p < 0.001$, repeated measures two-way ANOVA with Tukey post hoc test). E1. Horizontal travel distance after injection from the 1st day of injection to the challenge on the 14th day after withdrawal of the four groups. E2. Horizontal travel distance in every 5 min before and after challenge of the four groups (### $p < 0.001$, ** $p < 0.01$, *** $p < 0.001$, repeated measures two-way ANOVA with Tukey post hoc test). E3. Horizontal travel distance 30 min after challenge of the four groups (** $p < 0.01$, *** $p < 0.001$, two-way ANOVA with Tukey post hoc test). sal: saline; coc: cocaine; chal: challenge.

group * brain region F (3, 37) = 4.240, two-way Anova with Tukey post hoc test), indicating that the NAc core plays a more important role in chronic cocaine-induced locomotor sensitization expression than the shell. Besides, we found that c-Fos$^+$ cells were almost NeuN$^+$ cells, indicating that almost all activated cells were neurons. Thus, in the following research, we used c-Fos$^+$ signals to represent activated neurons. The staining position of the NAc core and the shell is shown in Fig. 2C.

3.3. The number of c-Fos$^+$/OXR1$^+$ cells in the NAc core was specifically increased following expression of locomotor sensitization

We have certificated that the NAc core plays an important role in mediating chronic cocaine- induced locomotor sensitization expression. To further authenticate whether the activated neurons in the NAc core following locomotor sensitization expression are OXR1$^+$ neurons, we tested the number of c-Fos and OXR1 double-positive cells in the NAc core and the shell of the rats after challenge. As shown in Fig. 3A1, no significant change of the number of OXR1$^+$ cells in the NAc core between every group was observed (Statistical graph is not shown; main effect of drug formation F (1, 17) = 2.077, main effect of drug challenge F (1, 17) = 0.07296, interaction of drug formation * drug challenge F (1, 17) = 0.008690, two-way Anova with Tukey post hoc test); The number of c-Fos$^+$/OXR1$^+$ cells in the NAc core of the coc + coc group was significantly increased compared to the sal + coc group and the coc + sal group (Fig. 3A2; a significant main effect of drug formation F (1, 17) = 9.003, a significant main effect of drug challenge F (1, 17) = 10.01, interaction of drug formation * drug challenge F (1, 17) = 4.447, two-way Anova with Tukey post hoc test); There was no significant change between every group in the percentage of c-Fos$^+$/OXR1$^+$ cells among c-Fos$^+$ cells (Statistical graph is not shown; main effect of drug formation F (1, 17) = 0.5725, main effect of drug challenge F (1, 17) = 0.0002434, interaction of drug formation * drug challenge F (1, 17) = 0.05908, two-way Anova with Tukey post hoc test). As shown in Fig. 3B1, for the cells in the shell, no significant changes of the number of OXR1$^+$ cells (Statistical graph is not shown;

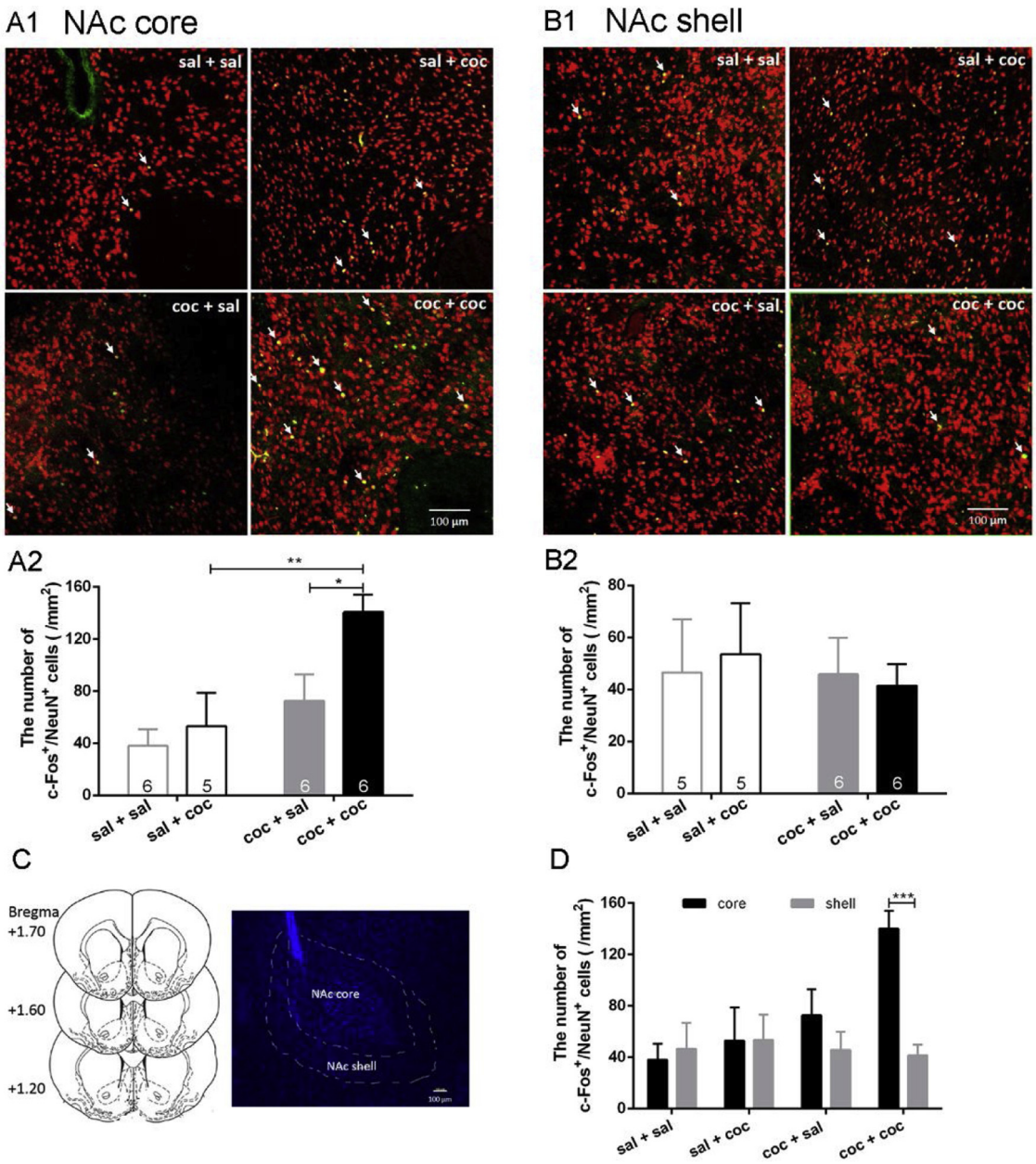

Fig. 2. The number of c-Fos$^+$/NeuN$^+$ cells in the NAc core was specifically increased following expression of locomotor sensitization. A1. c-Fos and NeuN staining in the NAc core (green: c-Fos; red: NeuN). A2. The number of c-Fos$^+$/NeuN$^+$ cells in the NAc core of the four groups (* $p < 0.05$, ** $p < 0.01$, two-way ANOVA with Tukey post hoc test). B1. c-Fos and NeuN staining in the NAc shell (green: c-Fos; red: NeuN). B2. The number of c-Fos$^+$/NeuN$^+$ cells in the NAc shell of the four groups. C. The staining position of the NAc core and the shell. D. The number of c-Fos$^+$/NeuN$^+$ cells in the NAc core and the shell (*** $p < 0.001$, two-way ANOVA with Tukey post hoc test). sal: saline; coc: cocaine.

main effect of drug formation F (1, 16) = 0.5859, main effect of drug challenge F (1, 16) = 0.5141, interaction of drug formation * drug challenge F (1, 16) = 0.4800, two-way Anova with Tukey post hoc test), the number of c-Fos$^+$/OXR1$^+$ cells (Fig. 3B2; main effect of drug formation F (1, 16) = 1.029, main effect of drug challenge F (1, 16) = 0.02625, interaction of drug formation * drug challenge F (1, 16) = 0.07944, two-way Anova with Tukey post hoc test) as well as the percentage of c-Fos$^+$/OXR1$^+$ cells among c-Fos$^+$ cells (Statistical graph is not shown;main effect of drug formation F (1, 16) = 0.7722, main effect of drug challenge F (1, 16) = 0.1593, interaction of drug formation * drug challenge F (1, 16) = 0.04861, two-way Anova with Tukey post hoc test) between every group were observed. Results also showed that there was no significant change in the number of OXR1$^+$ cells between the NAc core and the shell (Fig. 3C1; main effect of group F (3, 33) = 1.087, main effect of brain region F (1, 33) = 0.4889, interaction of group * brain region F (3, 33) = 0.1786, two-way Anova with Tukey post hoc test), the c-Fos$^+$/OXR1$^+$ cells number in the NAc core was significantly more than that in the shell of the coc + coc group (Fig. 3**C2**; a significant main effect of group F (3, 33) = 4.968, a significant main effect of brain region F (1, 33) = 12.67, a significant interaction of group * brain region F (3, 33) = 6.054, two-way Anova with Tukey post hoc test), and no significant change of the percentage of c-Fos$^+$/OXR1$^+$ cells among c-Fos$^+$ cells between the NAc core and the shell was observed (Fig. 3**C3**; main effect of group F (3, 33) = 0.4775, main effect of brain region F (1, 33) = 0.03246, interaction of group * brain region F (3, 33) = 0.1717, two-way Anova with Tukey post hoc test). The above results indicate that OXR1$^+$ neurons in the NAc core but not the shell is involved in chronic cocaine-induced

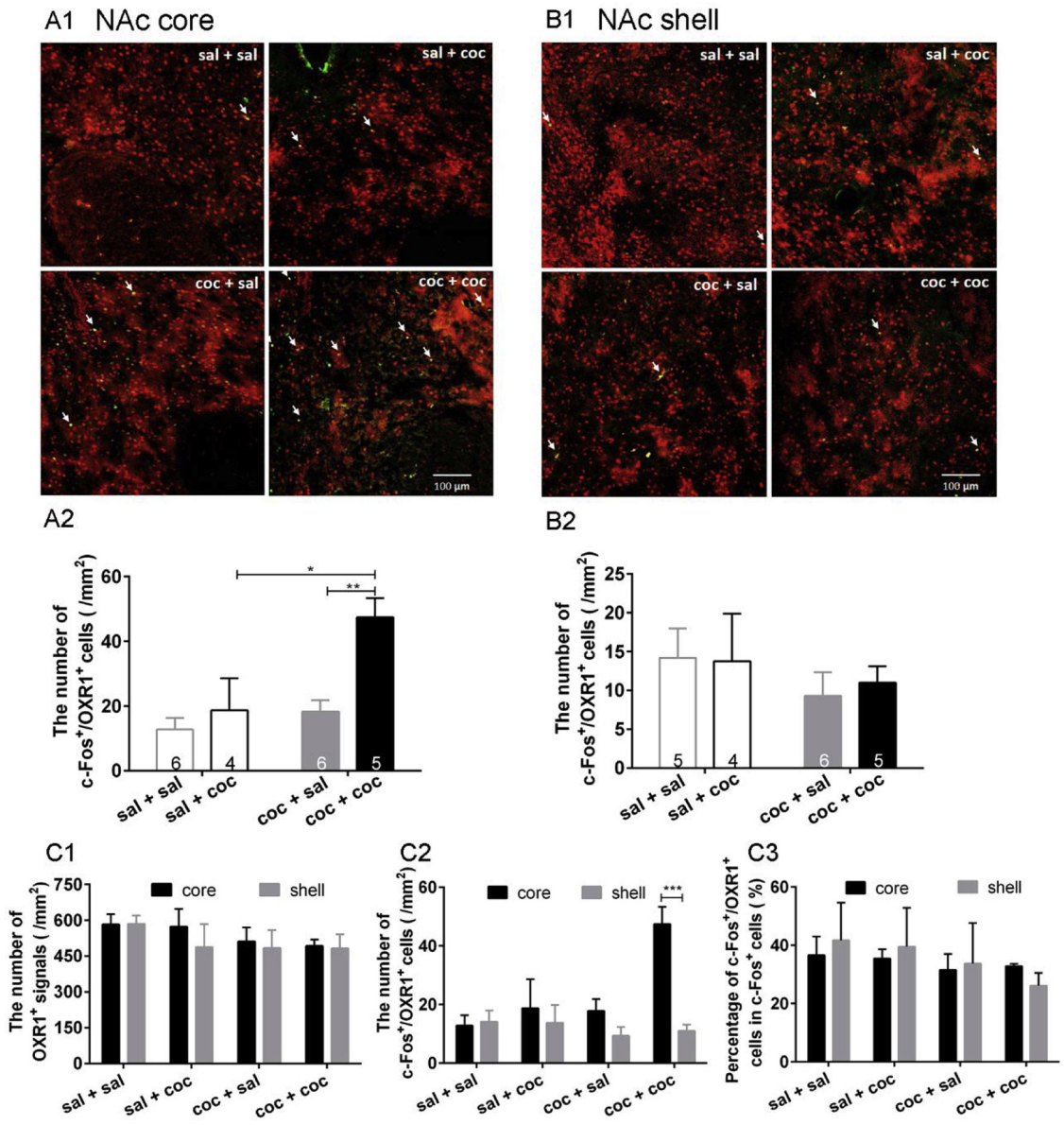

Fig. 3. The number of c-Fos$^+$/OXR1$^+$ cells in the NAc core was specifically increased following expression of locomotor sensitization. A1. c-Fos and OXR1 staining in the NAc core (green: c-Fos; red: OXR1). A2. The number of c-Fos$^+$/OXR1$^+$ cells in the NAc core of the four groups (* $p < 0.05$, ** $p < 0.01$, two-way ANOVA with Tukey post hoc test). B1. c-Fos and OXR1 staining in the NAc shell (green: c-Fos; red: OXR1). B2. The number of c-Fos$^+$/OXR1$^+$ cells in the NAc shell of the four groups. C1. The number of OXR1$^+$ cells in the NAc core and the shell of the four groups. C2. The number of c-Fos$^+$/OXR1$^+$ cells in the NAc core and the shell of the four groups (*** $p < 0.001$, two-way ANOVA with Tukey post hoc test). C3. The percentage of c-Fos$^+$/OXR1$^+$ cells among c-Fos$^+$ cells in the NAc core and the shell of the four groups. sal: saline; coc: cocaine.

locomotor sensitization expression in rats. However, only about 33 % of the activated cells are OXR1$^+$ cells, which indicates that OXR1 may only participate in but not play a direct role in locomotor sensitization. These results are consistent to other scientists' findings that OXR1 may play an indirect role by interaction with DAR and AMPA/NMDA receptors (AMPAR/NMDAR) [35].

3.4. The level of OXR1 membrane protein in the NAc core was selectively increased following expression of locomotor sensitization

For further study on OXR1 in the NAc core and the shell mediating chronic cocaine-induced locomotor sensitization, we tested the level of OXR1 total and membrane protein in the NAc core and the shell of the rats after challenge. As shown in Fig. 4**A1**, there was no significant change of the OXR1 total protein level in the NAc core (Fig. 4A2; a significant main effect of drug formation F (1, 16) = 7.257, a significant main effect of drug challenge F (1, 16) = 11.79, interaction of drug formation * drug challenge F (1, 16) = 0.6415, two-way Anova with Tukey post hoc test) and the shell (Fig. 4A3; main effect of drug formation F (1, 16) = 0.05156, main effect of drug challenge F (1, 16) = 1.372, interaction of drug formation * drug challenge F (1, 16) = 0.07946, two-way Anova with Tukey post hoc test) between every group, and no significant change of OXR1 total protein level of every group between the NAc core and the shell was observed (Fig. 4A4; main effect of group F (3, 32) = 0.09946, a significant main effect of brain region F (1, 32) = 6.331, interaction of group * brain region F (3, 32) = 1.512, two-way Anova with Tukey post hoc test). As shown in Fig. 4B1, the OXR1 membrane protein level in the NAc core of the coc + coc group was significant higher than that in the sal + coc group and the coc + sal group (Fig. 4B2; a significant main effect of drug formation F (1, 16) = 8.409, a significant main effect of drug challenge F (1, 16) = 6.305, interaction of drug formation * drug challenge F (1,

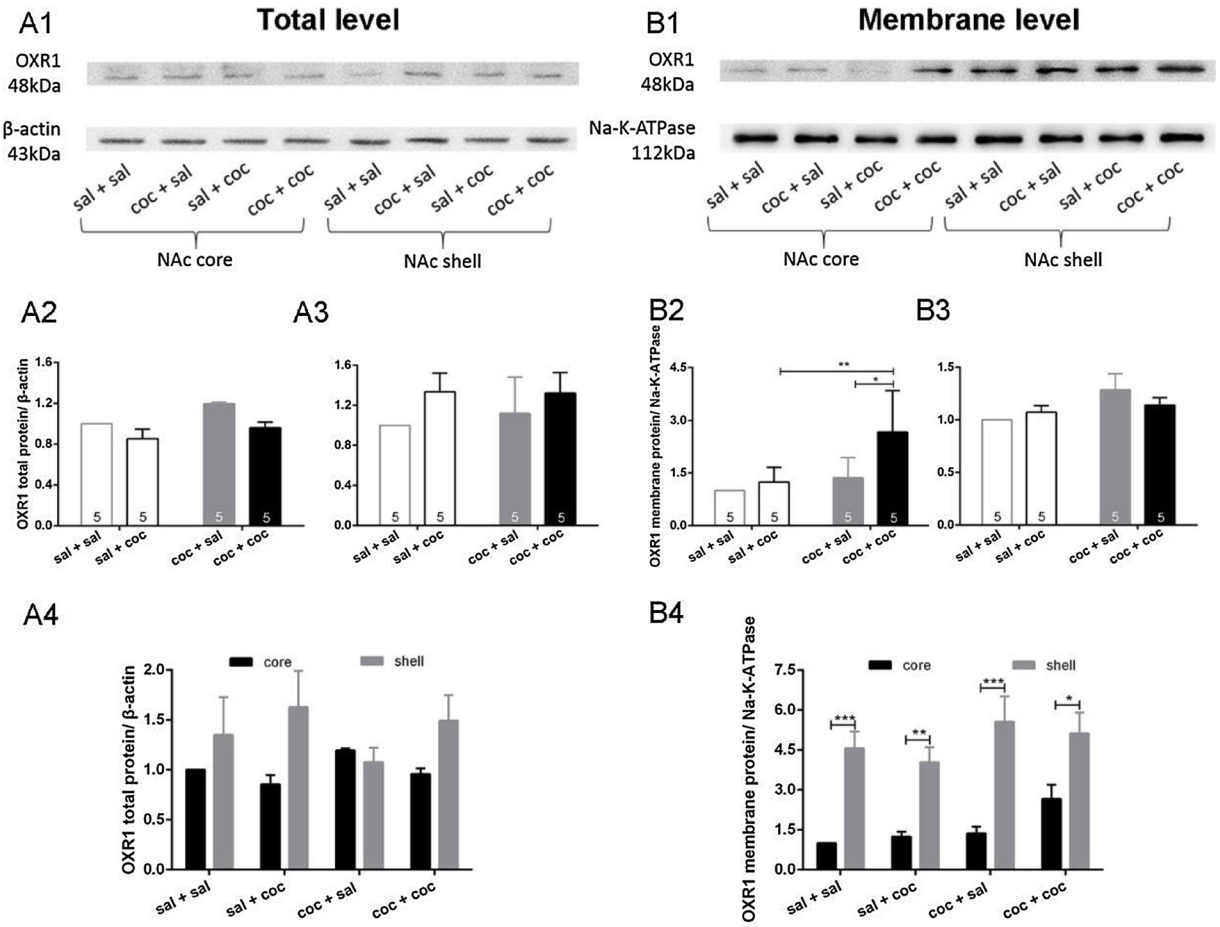

Fig. 4. The level of OXR1 membrane protein in the NAc core was selectively increased following expression of locomotor sensitization. A1. Expression of OXR1 total protein level in the NAc core and the shell of the four groups detected by Western Blot. A2. OXR1 total protein level in the NAc core of the four groups. A3. OXR1 total protein level in the NAc shell of the four groups. A4. OXR1 total protein level in the NAc core and the shell of the four groups. B1. Expression of OXR1 membrane protein level in the NAc core and the shell of the four groups detected by Western Blot. B2. OXR1 membrane protein level in the NAc core of the four groups (* $p < 0.05$, ** $p < 0.01$, two-way ANOVA with Tukey post hoc test). B3. OXR1 membrane protein level in the NAc shell of the four groups. B4. OXR1 membrane protein level in the NAc core and the shell of the four groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA with Tukey post hoc test). sal: saline; coc: cocaine.

16) = 2.953, two-way Anova with Tukey post hoc test), but there was no significant change of OXR1 membrane protein level in the NAc shell between every group (Fig. 4B3; main effect of drug formation F (1, 16) = 3.797, main effect of drug challenge F (1, 16) = 0.1493, interaction of drug formation * drug challenge F (1, 16) = 1.458, two-way Anova with Tukey post hoc test), and the OXR1 membrane protein level in the NAc shell was significantly higher than that in the core (Fig. 4B4; main effect of group F (3, 32) = 2.110, a significant main effect of brain region F (1, 32) = 65.14, interaction of group * brain region F (3, 32) = 0.9331, two-way Anova with Tukey post hoc test). The above results illustrate that OXR1 up-regulation on the membrane in the NAc core could mediate chronic cocaine-induced locomotor sensitization expression. However, the OXR1 membrane protein level in the NAc shell was significantly more than that in the core, without a significant change of OXR1 total or membrane protein level in the NAc shell between each group, indicating that OXR1 in the shell may mediate other physiologic functions.

3.5. OXR1 selective antagonist micro-infusion into the NAc core specifically attenuated the expression of locomotor sensitization in rats

To further certificate whether OXR1 in the NAc participates in the expression of chronic cocaine-induced locomotor sensitization in rats, we tested the effect of specific OXR1 antagonist, SB-334867, micro-infusion into the NAc on this behavior. The dosage of SB-334867 was 20 nmol/μl, 0.5 μl per side, which was based on previous reports and pre-experiment [30–33]. The experimental procedures are illustrated in Fig. 5A1 and Fig. 6A1. The baseline horizontal travel distance during the 15 min recording period (Fig. 5B1) was not affected by the SB-334867 micro-infusion into the NAc core, however, a significant decrease of horizontal travel distance compared to the DMSO treated rats (control group) was observed, for all observed time intervals after cocaine injected (Fig. 5B1; a significant main effect of group F (2, 25) = 5.392, a significant main effect of time F (8, 200) = 22.96, a significant interaction of group * time F (16, 200) = 2.892, repeated measures two-way Anova with Tukey post hoc test). Pre-treatment with SB-334867 into the NAc core significantly impaired the enhanced expression of chronic cocaine-induced locomotor sensitization compared to the Sham group and the DMSO treated group (Fig. 5B2; F (2, 25) = 5.469, $p = 0.0107$, n = 9–10; * $p < 0.05$, one-way ANOVA with Tukey post hoc test). However, SB-334867 micro-infusion into the NAc shell had no effect on the expression of locomotor sensitization (Fig. 6B1; main effect of group F (2, 25) = 1.062, a significant main effect of time F (8, 200) = 40.50, interaction of group * time F (16, 200) = 0.6248, repeated measures two-way Anova with Tukey post hoc test; Fig. 6B2; F (2, 25) = 0.5964, $p = 0.5584$, n = 9–10; one-way ANOVA with Tukey post hoc test). Spontaneous locomotor activity of the rats were not affected by the SB-334867 micro-infusion into the NAc core (Fig. 5**C1**; main effect of group F (8, 208) = 29.96, a significant main effect of time F (2, 26) = 0.4031, a significant interaction of group * time F (16,

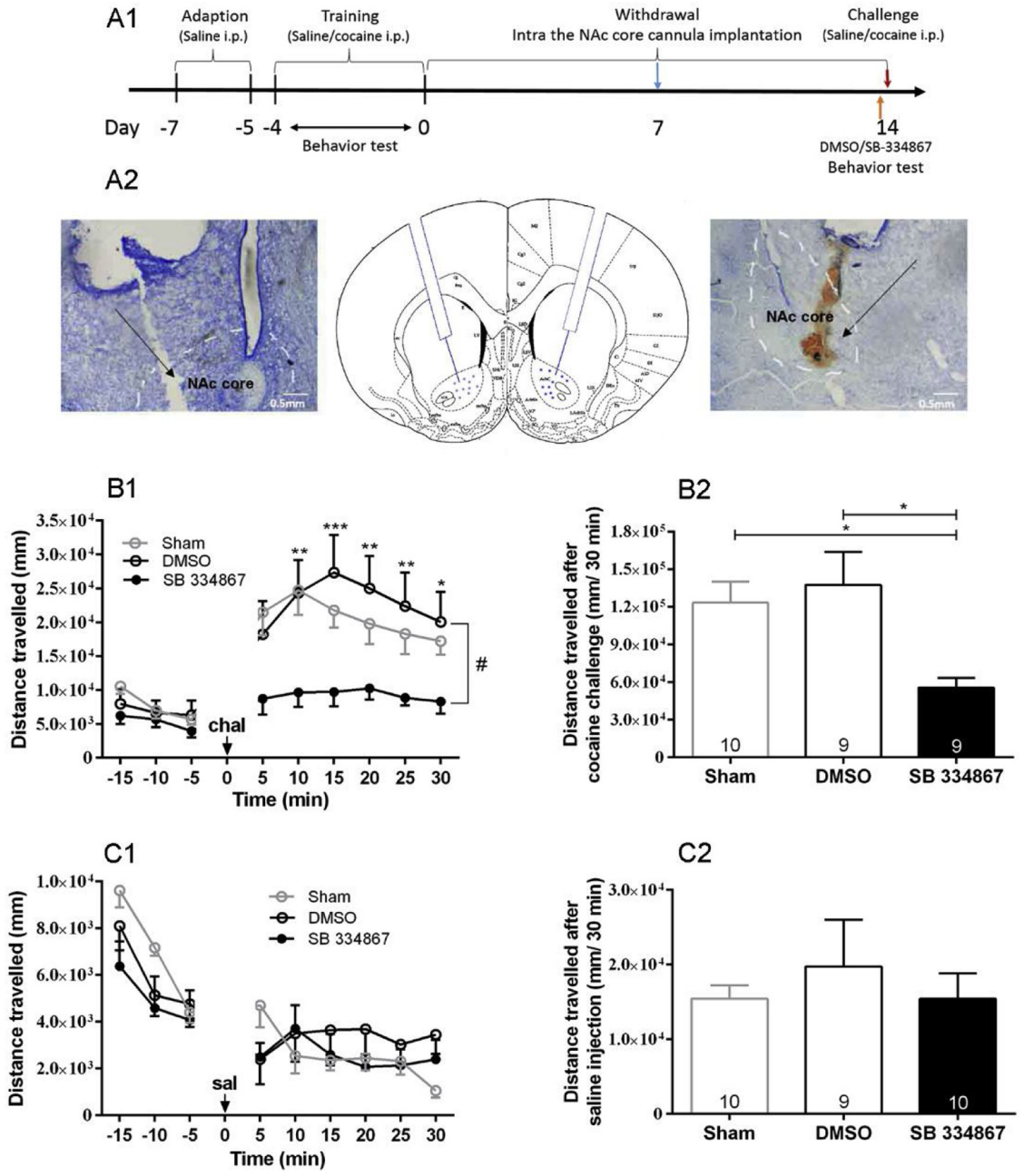

Fig. 5. OXR1 selective antagonist micro-infusion into the NAc core significantly attenuated the expression of locomotor sensitization in rats. A1. The experimental procedure. A2. The injection site of intra-NAc core microinjection verified by Nissl staining. B1. Horizontal travel distance in every 5 min before and after cocaine challenge of the three groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$, repeated measures two-way ANOVA with Tukey post hoc test). B2. Horizontal travel distance 30 min after cocaine challenge of the three groups (* $p < 0.05$, one-way ANOVA with Tukey post hoc test). C1. Horizontal travel distance in every 5 min before and after saline injection of the three groups. C2. Horizontal travel distance 30 min after saline injection of the three groups. sal: saline; chal: challenge.

208) = 2.817, repeated measures two-way Anova with Tukey post hoc test; Fig. 5C2; F (2, 26) = 0.3590, p = 0.7018, n = 9–10; one-way ANOVA with Tukey post hoc test) or the shell (Fig. 6C1; main effect of group F (2, 25) = 0.8296, a significant main effect of time F (8, 200) = 27.19, interaction of group * time F (16, 200) = 1.579, repeated measures two-way Anova with Tukey post hoc test; Fig. 6C2; F (2, 25) = 0.2406, p = 0.7880, n = 9–10; one-way ANOVA with Tukey post hoc test). These results suggest that OXR1 in the NAc core plays an important role in the expression of chronic cocaine-induced locomotor sensitization in rats. The injection sites of intra-NAc core and intra-NAc shell micro-infusions were verified by Nissl staining as illustrated in Figs. 5A2 and 6 A2, respectively.

4. Discussion

The present study demonstrates that the NAc core OXR1 is selectively involved in the expression of chronic cocaine-induced locomotor sensitization in rats. Specifically, following the expression of cocaine-induced locomotor sensitization, the OXR1 positive neurons were activated, and the level of membrane OXR1 protein was also increased in the NAc core but not the shell. Meanwhile, micro-infusion of OXR1 selective antagonist SB-334867 into the NAc core, but not the shell before the cocaine challenge significantly attenuated locomotor sensitization expression in rats. These results suggest that NAc core OXR1 is selectively involved in the expression of chronic cocaine-induced locomotor sensitization.

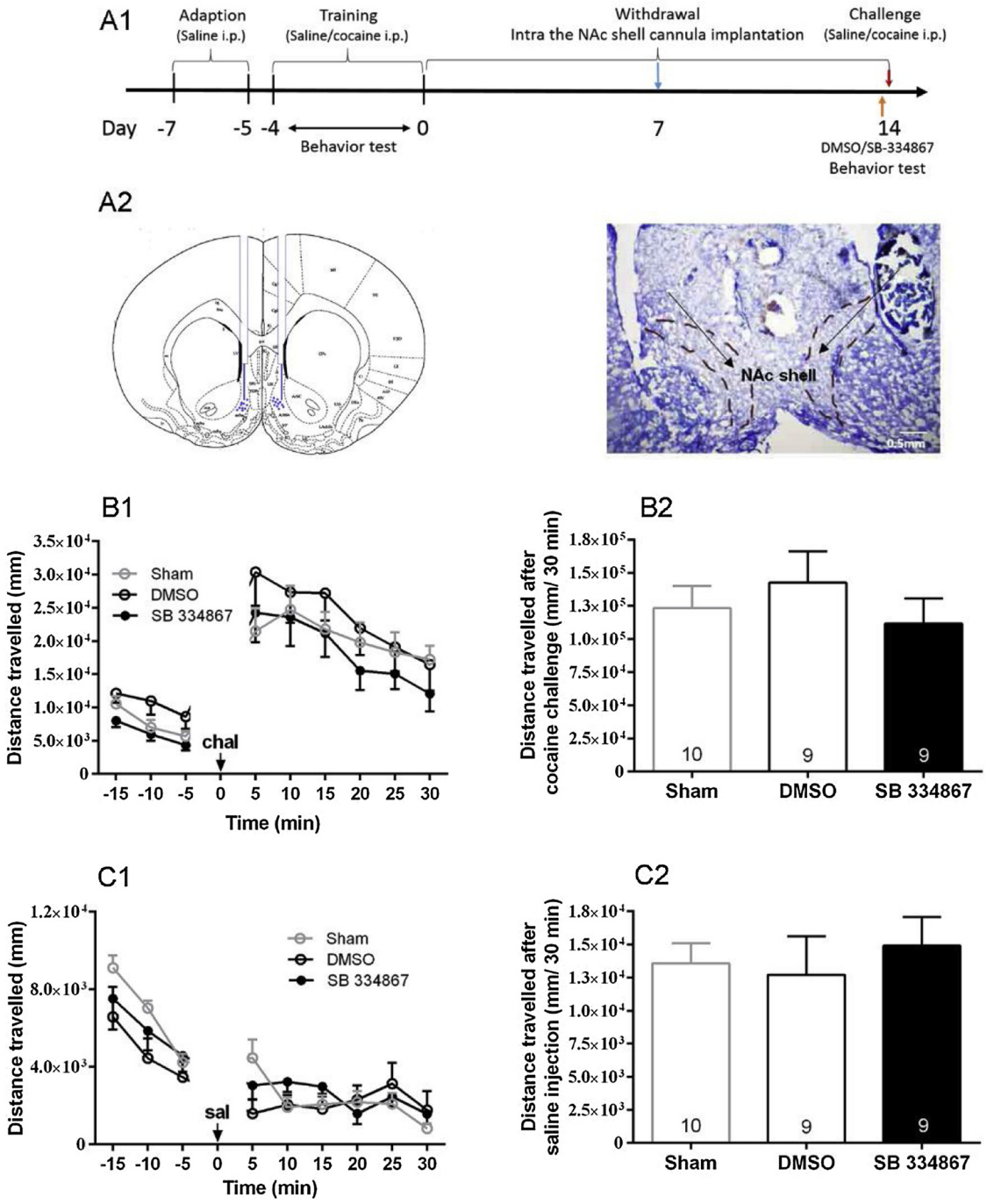

Fig. 6. OXR1 selective antagonist micro-infusion into the NAc shell did not affect the expression of locomotor sensitization in rats. A1. The experimental procedure. A2. The injection site of intra-NAc shell microinjection verified by Nissl staining. B1. Horizontal travel distance in every 5 min before and after cocaine challenge of the three groups. B2. Horizontal travel distance 30 min after cocaine challenge of the three groups. C1. Horizontal travel distance in every 5 min before and after saline injection of the three groups. C2. Horizontal travel distance 30 min after saline injection of the three groups. sal: saline; chal: challenge.

Psychoactive substance-induced locomotor sensitization is a simple animal model and is widely used in investigating the neuroplasticity invoked by relapse after withdrawal [36,37]. Consistent with previous reports [28,34,38], our study showed that chronic cocaine-induced locomotor sensitization in SD rats has an individual difference. 72.2 %, not all, of the rats were sensitized to cocaine treatments. It has been reported that drug addiction susceptibility is related to the enhanced DA release from the VTA to the NAc [39], and the uprate of AMPAR subunits GluR1 and GluR2/3 membrane level in the NAc after drug re-exposure [27]. However, to achieve our target, the experiments were only conducted on cocaine-sensitized rats.

It has been shown that OXR1 selective antagonist SB-334867 systemic administration reduces amphetamine-evoked DA outflow in the NAc, and decreases the expression of amphetamine sensitization [9], implying that the effect of SB-334867 reducing amphetamine sensitization is related to its inhibition of DA outflow in the NAc. In the present study, we found that the OXR1 positive neurons were activated, and the level of membrane OXR1 protein was also increased in the NAc core but not the shell after cocaine re-exposure in the cocaine-sensitized rats, but not after a single cocaine exposure in the saline-treated rats. These findings indicate that OXR1 in the NAc core participates in locomotor sensitization of individuals with chronic drug abuse experience

re-exposed to drug after withdrawal, but not in mediating the excitatory effect of a single exposure to cocaine.

It is worth noting that OXR1 membrane protein level in the NAc shell was not changed before and after cocaine re-exposure in the cocaine-treated rats, but the baseline level of OXR1 membrane protein here was significantly higher than that in the core. This is consistent with study of Quarta et al. that the NAc shell receives more orexinergic neuron projection from the LHA, so more OXR are expressed there, mediating ingestion, awake and sleep processes [9].

To certificate the NAc core OXR1′s role in drug re-exposure-induced locomotor sensitization expression, a selective OXR1 antagonist, SB-334867, was administrated respectively into the NAc core and the shell of cocaine-treated rats before cocaine challenge. We found that blockage of OXR1 in the NAc core, but not in the shell significantly attenuate the locomotor sensitization expression, with no effect on the spontaneous locomotor activity of the rats. At present, clinical use of OXR antagonist includes Suvorexant [40,41] and Almorexant [15,42], both are unselective antagonist of OXR1 and OXR2, treated for insomnia. Recently, it has been shown that systemic Almorexant injection reduces alcohol intake of alcohol-addicted rats [41], which provides probability for OXR antagonist used as treatment of drug addiction. However, more research is needed on the clinical use of small molecules such as OXR1 selective antagonist to prevent and treat relapse after drug withdrawal.

Additionally, we found in the present work that only about 33 % of the activated neurons were OXR1 positive neurons in the NAc core, which were not the majority of the activated neurons in chronic cocaine-induced rat locomotor sensitization expression. However, OXR1 membrane protein level in the NAc core was significantly elevated following the expression of locomotor sensitization, and inhibiting OXR1 in the NAc core could significantly impair the expression of chronic cocaine-induced locomotor sensitization. These findings indicate that OXR1 plays a non-dominant but important role in chronic cocaine-induced rat locomotor sensitization expression. The follows are some research explain it: DAR [40] and ionotropic glutamate AMPAR/NMDAR [43] have been reported to play dominant role in psychoactive substance-induced locomotor sensitization expression. Moreover, Patyal et al. and Zhang et al. also reported that OXA bonded to OXR1 in the NAc has to interact with DAR to accomplish its function. Specifically, OXA stimulation increases DA release in the NAc slices with a slow time course, reaching a maximum at 25 min after OXA application, and the progress can be impaired by AMPAR/NMDAR antagonist, indicating that OXR1′ function is via regulating the activation of AMPAR/NMDAR [44,45]. Obviously, the interaction between OXR1 and DAR or AMPAR/NMDAR in drug-induced locomotor sensitization expression needs to be study further.

In conclusion, the present study demonstrates that re-exposure to cocaine after withdrawal could evoke the expression of locomotor sensitization in the cocaine-treated rats, followed with the activation of the OXR1 positive neurons and the elevation of membrane OXR1 protein in the NAc core but not the shell. Further, the microinjection of SB-334867, an OXR1 selective antagonist into the NAc core rather than the shell before the re-exposure could significantly attenuate the expression of cocaine-induced rat locomotor sensitization. These results suggest that OXR1 in the NAc core plays an important role in cocaine-induced locomotor sensitization, and provide a potential molecular clue for the intervention to relapse after drug re-exposure.

Authors contribution

Cailian Cui, Liuzhen Wu and Mingda Yang conceived and designed the research; Mingda Yang performed the experiments; Hui Ma and Meng Jia provided technical support; Mingda Yang drafted the manuscript; Cailian Cui, Liuzhen Wu, Yijing Li and Degen Miao edited and revised the manuscript.

Declaration of Competing Interest

The authors declare no competing financial interests.

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