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Mechanisms of Memory Impairment Induced by Orexin-A via Orexin 1 and Orexin 2 Receptors in Post-traumatic Stress Disorder Rats

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Abstract—Post-traumatic stress disorder (PTSD) patients exhibit abnormal learning and memory. Axons from orexin neurons in the lateral hypothalamus innervate the hippocampus, modulating learning and memory via the orexin 1 and 2 receptors (OX1R and OX2R). However, the role of the orexin system in the learning and memory dysfunction observed in PTSD is unknown. This was investigated in the present study using PTSD animal model-single prolonged stress (SPS) rats. Spatial learning and memory in the rats were evaluated with the Morris water maze (MWM) test; changes in body weight and food intake were recorded to assess changes in appetite; and the expression of orexin-A and its receptors in the hypothalamus and hippocampus was examined and quantified by immunohistochemistry, western blotting and real-time PCR. The results showed that spatial memory was impaired and food intake was decreased in SPS rats; this was accompanied by downregulation of orexin-A in the hypothalamus and upregulation of OX1R and OX2R in the hippocampus and of OX1R in the hypothalamus. Intracerebroventricular administration of orexin-A improved spatial memory and enhanced appetite in SPS rats and partly reversed the increases in OX1R and OX2R levels in the hippocampus and hypothalamus. These results suggest that the orexin system plays a critical role in the memory and appetite dysfunction observed in PTSD.

Key words: post-traumatic stress disorder (PTSD), orexin-A, orexin 1 receptor (OX1R), orexin 2 receptor (OX2R), hippocampus.

INTRODUCTION

One of the main symptoms of post-traumatic stress disorder (PTSD) is recurrent, involuntary, intrusive, and distressing memories of the traumatic event(s). The hippocampus is an important structure in the limbic system that regulates learning and memory. Numerous studies have demonstrated that hippocampal volume is reduced in patients with PTSD (Karl et al., 2006; Woon and Hedges, 2011), and magnetic resonance spectroscopy imaging has revealed altered neurometabolite ratios and concentrations in the hippocampal neurons of PTSD patients (Karl and Werner, 2010). Single prolonged stress (SPS) enhances negative feedback in the hypothalamic–pituitary–adrenal (HPA) axis and alters the transcript levels of glucocorticoid receptor, which reproduces the neuroendocrine features of PTSD (Liberzon et al., 1997, 1999). Thus, SPS is a paradigm that has been used to model PTSD in an experimental setting (Yamamoto et al., 2009). SPS-exposed animals show spatial learning and memory deficits in the Morris water maze (MWM) test and apoptosis of hippocampal neurons (Wen et al., 2017; Zheng et al., 2017), suggesting a dysfunction in memory-related neural circuits.

Orexins are paired peptides (i.e., orexin-A/-B) produced by hypothalamic neurons that are ligands of orphan G protein-coupled receptors (GPCRs) expressed in the rat brain (Sakurai et al., 1998). Orexin neurons have been identified in the lateral hypothalamus (LH), posterior hypothalamus (PH), perifornix nucleus (PeF), and dorsomedial hypothalamic nucleus (DMH) (Peyron et al., 1998; Sakurai et al., 1998; Nambu et al., 1999). Orexin 1 and 2 receptors (OX1R and OX2R, respectively) (Sakurai and Mieda, 2011) are GPCRs; the former has higher affinity for orexin-A than for orexin-B, whereas OX2R has similar affinities for both peptides. Orexin neurons project along most of the neuroaxis excluding the cerebellum (Peyron

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Abbreviations: ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; CSF, cerebrospinal fluid; DG, dentate gyrus; DMH, dorsomedial hypothalamus; GPCRs, G-protein-coupled receptors; HPA, hypothalamic–pituitary–adrenal; i.c.v., intracerebroventricular; LH, lateral hypothalamus; mRNA, messenger RNA; MWM, Morris water maze; OD, optical density; OX1R, orexin 1 receptor; OX2R, orexin 2 receptor; PCR, polymerase chain reaction; PeF, perifornix nucleus; PFA, paraformaldehyde; PH, posterior hypothalamus; PNT, place navigation test; PTSD, post-traumatic stress disorder; SABC, streptavidin-biotin peroxidase complex; SPS, single prolonged stress; SPT, spatial probe test.

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et al., 1998; Nambu et al., 1999), and OX1R and OX2R are distributed throughout the central nervous system and regulate a variety of physiological functions including feeding and energy homeostasis (Edwards et al., 1999; Volkoff et al., 1999; Yamada et al., 2000; Leinninger et al., 2011; Sakurai and Mieda, 2011; Yokobori et al., 2011; Goforth et al., 2014), the sleep-wake cycle (Carter et al., 2009; Tsujino and Sakurai, 2013), reward processing and addictive behavior (Rao et al., 2013; Baimel et al., 2015), stress response, and learning and memory (Stanley and Fadel, 2012; Yang et al., 2013; Palotai et al., 2014).

The axons of orexin neurons in the hypothalamus project to the hippocampus where orexin receptors are highly expressed (Trivedi et al., 1998). Orexin/ataxin-3-transgenic mice in which orexin neurons degenerate by 3 months of age exhibit deficits in long-term social memory (Yang et al., 2013), which is restored by enhancing hippocampal synaptic plasticity and cyclic adenosine monophosphate (cAMP) response element-binding protein phosphorylation through nasal administration of orexin-A (Yang et al., 2013). Rats treated with the selective OX1R antagonist SB-334867-A exhibit impaired memory acquisition, consolidation, and retrieval in the MWM test compared to control rats, suggesting that orexin-A plays an important role in spatial learning and memory (Akbari et al., 2006). Many studies have shown that the orexin system regulates learning and memory processes; however, how this is impaired in PTSD is unknown.

To answer this question, in the present study we investigated changes in spatial memory in a rat model of SPS-induced PTSD. We quantified the expression of orexin-A and its receptors in the hypothalamus and hippocampus by immunohistochemistry, western blotting, and real-time polymerase chain reaction (PCR), and examined the effect of orexin-A administration on these parameters. Finally, we monitored weight and food intake to determine whether these were altered by SPS exposure and orexin-A treatment. This study aimed to further elucidate the role of the orexin system in memory impairment and the dysregulation of appetite in PTSD.

EXPERIMENTAL PROCEDURES

Animals and grouping

A total of 152 male Wistar rats were obtained from the Experimental Animal Center of China Medical University and were housed three to a cage, with free access to water and food under controlled conditions (12:12-h light/dark cycle, 23 °C ± 1 °C, and 55% ± 5% humidity).

In Experiment 1 (examining the effects of SPS), 120 rats with a body weight range of 170–200 g were randomly divided into the following five groups according to the time between SPS exposure and sacrifice: control (n = 30), 1 day post-SPS (SPS-1 d; n = 20), 4 days post-SPS (SPS-4 d; n = 20), 7 days post-SPS (SPS-7 d; n = 20), and 14 days post-SPS (SPS-14 d; n = 30). Ten rats each from the control and SPS-14 d groups were used for body weight, food intake measurements and in the MWM test; and 20 rats per group were used for immunohistochemistry (n = 6/group), western blotting (n = 6/group), and real-time PCR (n = 6/group).

In Experiment 2 (orexin-A treatment), 32 rats with a body weight range of 200–230 g were randomly divided into four groups of eight rats each: SPS + vehicle, SPS + orexin-A, non-SPS + vehicle, and non-SPS + orexin-A. All the rats in four groups were used in the body weight measure, food intake measure, MWM and western blotting. The detailed experimental arrangement in experiment 2 was showed in Fig. 1.

SPS procedure

The SPS procedure was always performed in the morning. Rats were immobilized for 2 h in a plastic bottle that was the same size as their body with an open mouth that allowed normal breathing. The animals were exposed to forced swim stress (water depth, 40 cm; 24 °C ± 1 °C) for 20 min with a 15-min rest period; they were then anesthetized with ether until they were unconscious, and returned to their home cages. All experimental procedures involving the animals were approved by the Ethics Committee of China Medical University.

In Experiment 1, rats were exposed to SPS (day 0). Control rats were left in their home cages without handling and were sacrificed on day 14. In Experiment 2, the SPS + vehicle and SPS + orexin-A groups underwent the SPS procedure whereas the non-SPS + vehicle and non-SPS + orexin-A groups did not. All rats were sacrificed on day 13.

Measurement of body weight

In Experiment 1, the body weight of rats in the control and SPS-14d groups (n = 10 rats/group) were recorded on days 0, 7, and 14 post-SPS; food intake over two experimental stages (days 0–7 and 7–14 after SPS) in the control and SPS-14d groups (n = 10 rats/group) was recorded. In Experiment 2, body weight and food intake in the four groups were recorded on day 13.

Surgery and orexin-A administration

In Experiment 2 (Fig. 1), SPS + vehicle, SPS + orexin-A, non-SPS + vehicle, and non-SPS + orexin-A groups (n = 8 each) underwent surgery for intracerebroventricular (icv) administration of orexin-A or vehicle (0.9% saline). One day after SPS exposure, the rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g) and placed in the prone position. The head of each rat was fixed on a stereotaxic instrument. After preparing and disinfecting the skin, a 0.5- to 1.0-cm incision was made at the center of the head to expose the skull. According to the stereotaxic coordinates of 0.8 mm posterior and 1.4 mm lateral to bregma and 4.5 mm below the dural surface, puncture cannulas were implanted into the right lateral ventricle and fixed to the skull with dental cement. Rats were individually housed after the surgery and behavioral experiments were performed 7 days later. The place

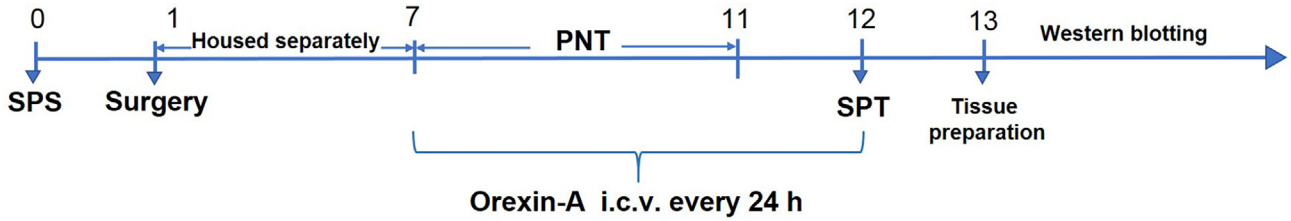

Fig. 1. Protocol for the MWM test and orexin-A administration in Experiment 2. All four groups (SPS + vehicle, SPS + orexin-A, non-SPS + vehicle, and non-SPS + orexin-A) were tested in the MWM and were treated with orexin-A or vehicle through icv administration (n = 8 rats/group).

navigation test (PNT) of the MWM was completed on days 7–11 after SPS, and the spatial probe test (SPT) was performed on day 12. Orexin-A (Absin, Shanghai, China) was dissolved in sterile 0.9% saline. Over the 6 days of the MWM test, the SPS + orexin-A and non-SPS + orexin-A groups were treated with 5 μl (5 nmol) of orexin-A every 24 h by icv delivery via the cannula, while SPS + vehicle and non-SPS + vehicle groups received 5 μl of 0.9% saline. The rats were decapitated after the behavioral tests to obtain brain tissue samples for western blot analysis. The orexin-A treatment was administered between 09:00 and 11:00 h.

MWM

The MWM was used to assess spatial memory and learning in rats. The MWM consisted of a circular pool with a camera and behavioral analysis system. The experiment had two parts: the PNT was conducted in the first 5 days, and the SPT was performed on day 6. In the PNT, In the PNT, the rat was placed in the water at the midpoint of four different quadrant edges of the pool with its head facing to the wall of the pool. If the rat found the hidden platform (2 cm below the water surface) within 2 min, it was allowed to remain on the platform for 20 s. If it failed to find the platform within this time frame, it was guided to the platform and allowed to remain there for 20 s. Each rat was tested in a 2-min learning trial (with a 20-s interval) four times a day. We recorded the swimming path of the rats and swimming distance before finding the platform to determine spatial learning ability. In the SPT, the platform was removed, and the rats were placed in the water on the opposite side of the target quadrant (where the platform was previously located).

In Experiment 1, the control and SPS-14d group (n = 10 rats/group) were tested in the MWM (PNT each day from day 2 to 6 after SPS and SPT on day 7), and were sacrificed on day 14. In Experiment 2 (Fig. 1), all four groups (n = 8 rats/group) were tested in the MWM (PNT each day from day 7 to 11 and SPT on day 12).

Immunohistochemistry

Rats in the control and SPS groups (n = 6 per group) were anesthetized on days 1, 4, 7, and 14 with Nembutal (Abbott Laboratories, Irving, TX, USA; 40 mg/kg by intraperitoneal [ip] injection) between 09:00 and 11:00 h and transcardially perfused through the left ventricle with 300–400 ml prewarmed saline and 300–400 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The whole brain was removed and immersed in 4% PFA at 4 °C for 6–10 h and then transferred to 40% sucrose solution for 2 days. Sections of brain tissue frozen in liquid nitrogen were cut at a thickness of 20 μm and stored at −20 °C for immunohistochemistry.

The streptavidin–biotin peroxidase complex (SABC) method was used for immunohistochemistry. Frozen sections were washed three times for 5 min with PBS and treated with 5% bovine serum albumin at 37 °C for 30 min. The sections were then incubated overnight at 4 °C with goat anti-orexin-A primary antibody (1:200; Santa Cruz Biotechnology, CA, USA; cat. no. H-104). After washing three times for 5 min with PBS, the sections were incubated at room temperature for 2 h with horseradish peroxidase (HRP)-conjugated anti-goat IgG (1:1000; Boster Biotechnology, Wuhan, China), then treated with SABC at 37 °C for 30 min and incubated with diaminobenzidine (DAB). The staining of neurons was observed under a microscope. When the brown color appeared, all sections were immediately washed three times for 5 min with PBS to terminate the reaction simultaneously. Finally, the sections were dehydrated and mounted with neutral balsam. In the immunohistochemical experiment, the concentration and incubation time of all the reagents were strictly controlled to assure that all sections were treated the same way. Orexin neurons are located in the LH, PH, PeF, and DMH; the LH was photographed for analysis of orexin-A expression. Image-pro plus 6.0 was used to measure the mean optical density (OD), the number and size of orexin-A-positive cells. After calibrating the intensity and spatial scales, the count/size command was selected to perform counting and measurement operations. Optical density of 50 orexin-A-positive cells per rat were measured and the average value of 50 cells were calculated as OD of per rats. Then 6 rats per group were calculated as the average OD of each group. For orexin-A-positive cells, cross-sectional area is used to evaluate cell size with square micron (μm^2) as unit. The investigators performing experiments and analyzing the data were blinded to group assignment.

Preparation of brain tissue for western blotting and real-time PCR

Rats were anesthetized with Nembutal (40 mg/kg ip) and decapitated between 09:00 and 11:00 h. The whole brain was immediately removed and placed in an ice-cold dish.

The hypothalamus and hippocampus were rapidly dissected, frozen in liquid nitrogen, and stored at −80 °C for western blotting and real-time PCR analyses. In Experiment 1, rats in the control and SPS groups were sacrificed on days 1, 4, 7, and 14 and in Experiment 2, rats in all four groups were sacrificed on day 13.

Western blotting

Protein was extracted from the hippocampus and hypothalamus using precooled radio immunoprecipitation assay lysis buffer containing protease inhibitor. The protein concentration of the lysate was evaluated with the Coomassie Brilliant Blue method. Samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane that was blocked with 5% skim milk for 2 h and washed with Tris-buffered saline with 0.1% Tween-20 (TBST), then incubated overnight at 4 °C with goat anti-OX1R (cat. no. sc-8072, 1:500) and anti-OX2R (cat. no. sc-8074, 1:200) antibodies (both from Santa Cruz Biotechnology). After washing with TBST, the membrane was incubated with HRP-conjugated anti-goat IgG as the secondary antibody (1:1000; Boster Biotechnology, Wuhan, China)

Table 1. The sequence of primers

Gene	Forward primer	Reverse primer	Product length (bp)
Orexin-A	GCTCCAGACACCATGAACCT	TTCGTAGAGACGGCAGGAAC	162
OX1R	TCAGAGCAACTGGAAGCTCA	AAACCAGCAGAACCACCATC	136
OX2R	GGTTCATCGTCGTCAAGGAG	GCTGCTGGGAGTGTGCTTAT	150

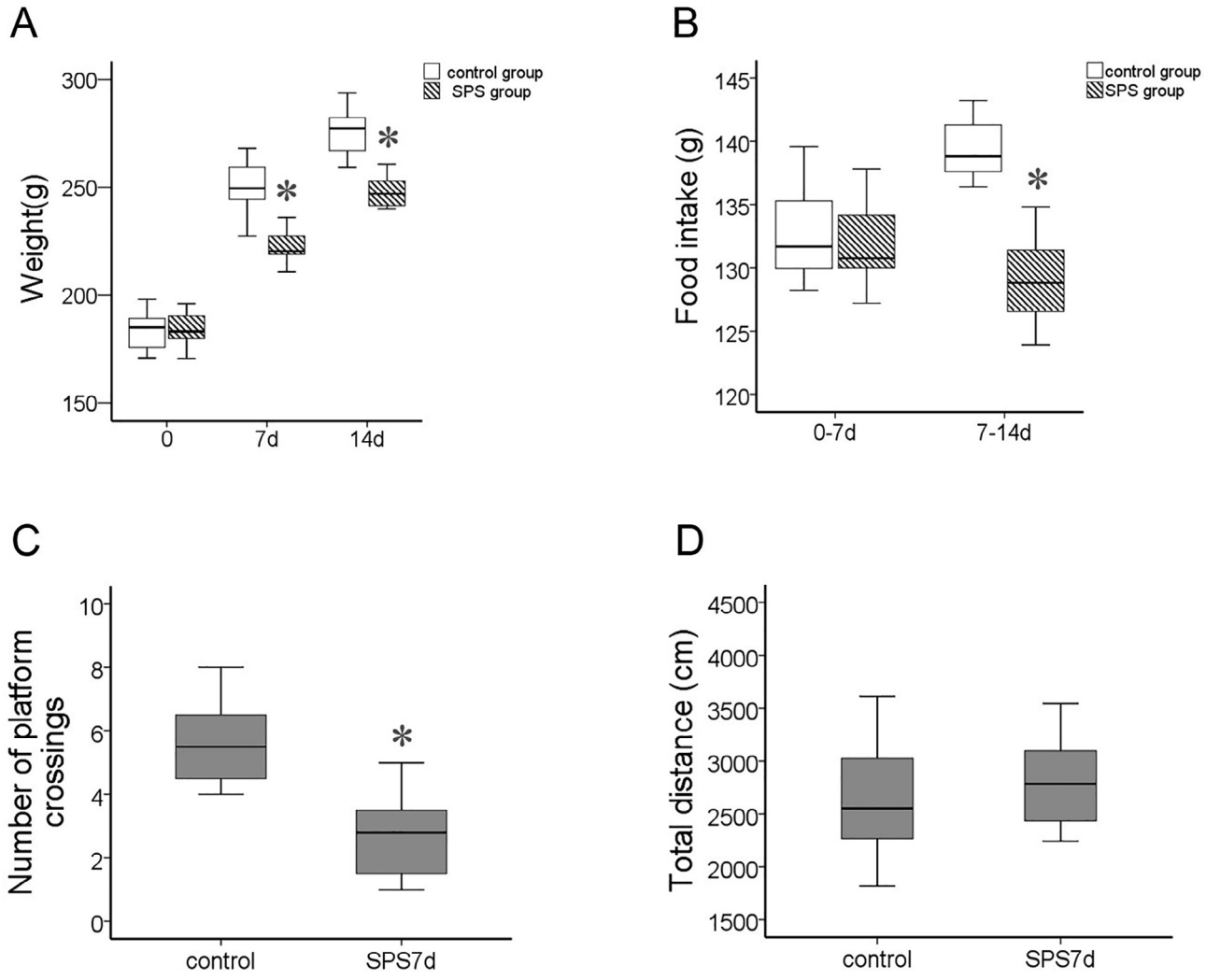

Fig. 2. Changes in body weight, food intake, and behavior in the MWM test in rats subjected to SPS. **(A)** Body weight (g) (n = 10/group). **(B)** Food intake (g) (n = 10/group). **(C)** Number of platform crossings (n = 10/group). **(D)** Total swimming distance (cm) (n = 10/group). *P < 0.01 vs control (Student's t test).

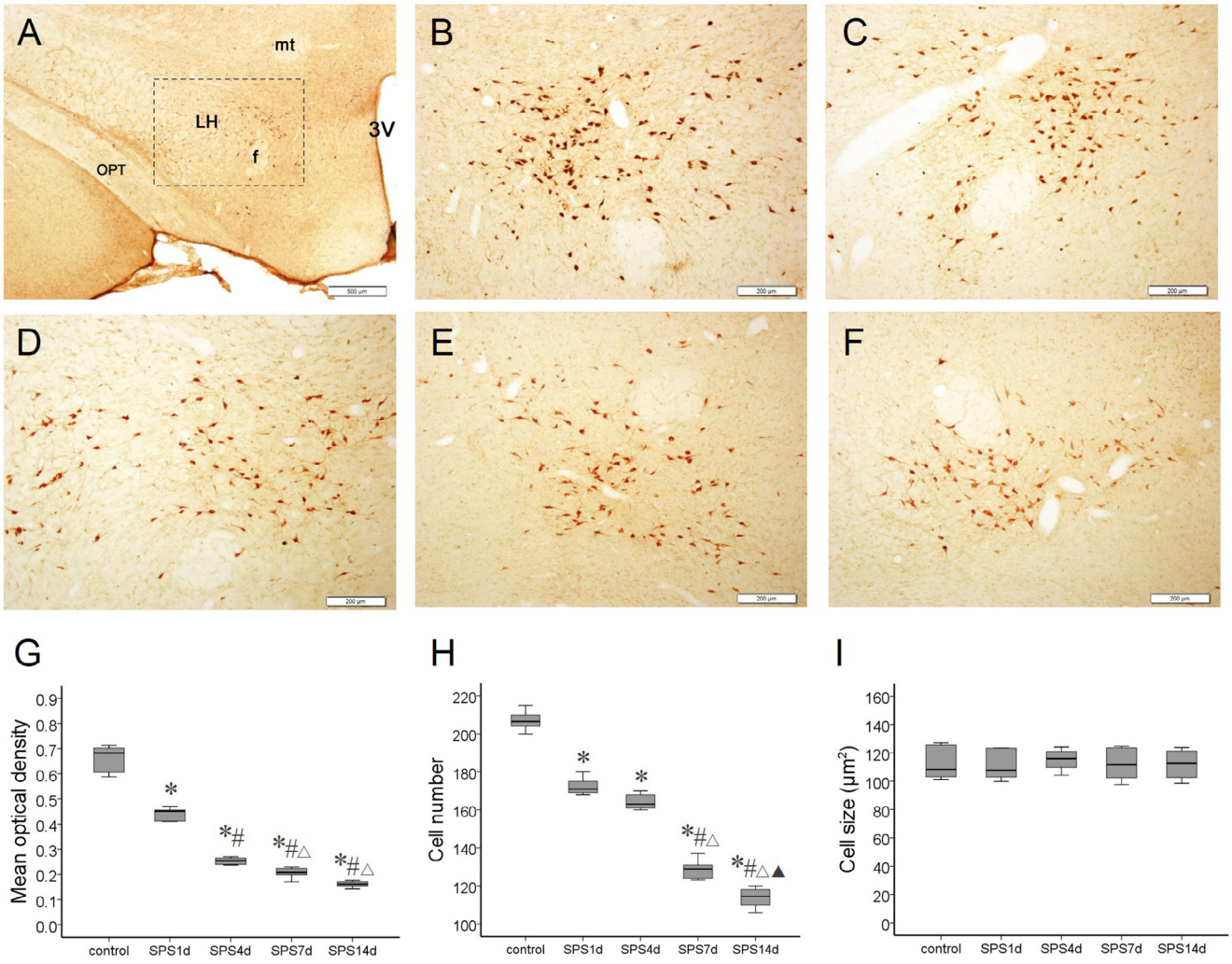

Fig. 3. Immunohistochemical analysis of orexin-A expression in LH neurons. **(A)** Control group. Orexin-A-positive neurons were observed in the LH. **(B)–(F)** High magnification view of orexin-A-positive neurons in the LH. **(B)** Control group. **(C)** SPS-1 d group. **(D)** SPS-4 d group. **(E)** SPS-7 d group. **(F)** SPS-14 d group. **(G)** The mean integral optical density of orexin-A in hypothalamus. **(H)** Cell count of orexin-A-positive neurons. **(I)** Cell size of orexin-A-positive neurons (μm^2). Scale bars = 500 μm **(A)** and 200 μm **(B–F)**. 3 V, 3rd ventricle; f, fornix; mt, mammillothalamic tract; LH, lateral hypothalamus; OPT, optic tract. Data represent mean ± SD (n = 6 per group). *P < 0.05 vs control; $^{\#}P$ < 0.05 vs SPS-1d; $^{\Delta}P$ < 0.05 vs SPS-4d; $^{\blacktriangle}P$ < 0.05 vs SPS-7d (Tukey's test).

for 2 h at room temperature. Protein expression was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was stripped and re-probed with an antibody against the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Experiment 1) or β-actin (Experiment 2). The orexin receptor bands were identified based on molecular weight (OX1R, 56 kDa; OX2R, 38 kDa) using the marker. The optical density (OD) of target proteins, GAPDH and β-actin were analyzed on the Gel Image Analysis System (Model 2500R; Tanon Science and Technology, Shanghai, China). We used the ratio of OD (target protein/GAPDH or β-actin) for statistical analysis. The procedures were repeated three times per rat and then calculation of six rats per group to obtain the average value of each group.

Quantitative real-time PCR

Total RNA was extracted from the hippocampus and hypothalamus using TRIzol reagent (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. RNA concentration and purity were determined with an ultramicro spectrophotometer based on an OD ratio of A260/A280. Total RNA was reverse transcribed into cDNA using an RT-PCR kit (Takara Bio). PCR primers were designed and synthesized by Sheng Gong (Shanghai, China); the sequences are shown in Table 1. PCR was performed using SYBR Premix Ex Taq II (Takara Bio). The *orexin-A*, *OX1R*, and *OX2R* genes were amplified on a LightCycler 480 (Roche, Indianapolis, IN, USA) and relative transcript levels were calculated with the comparative cycle threshold method and normalized to that of the internal reference *GAPDH*.

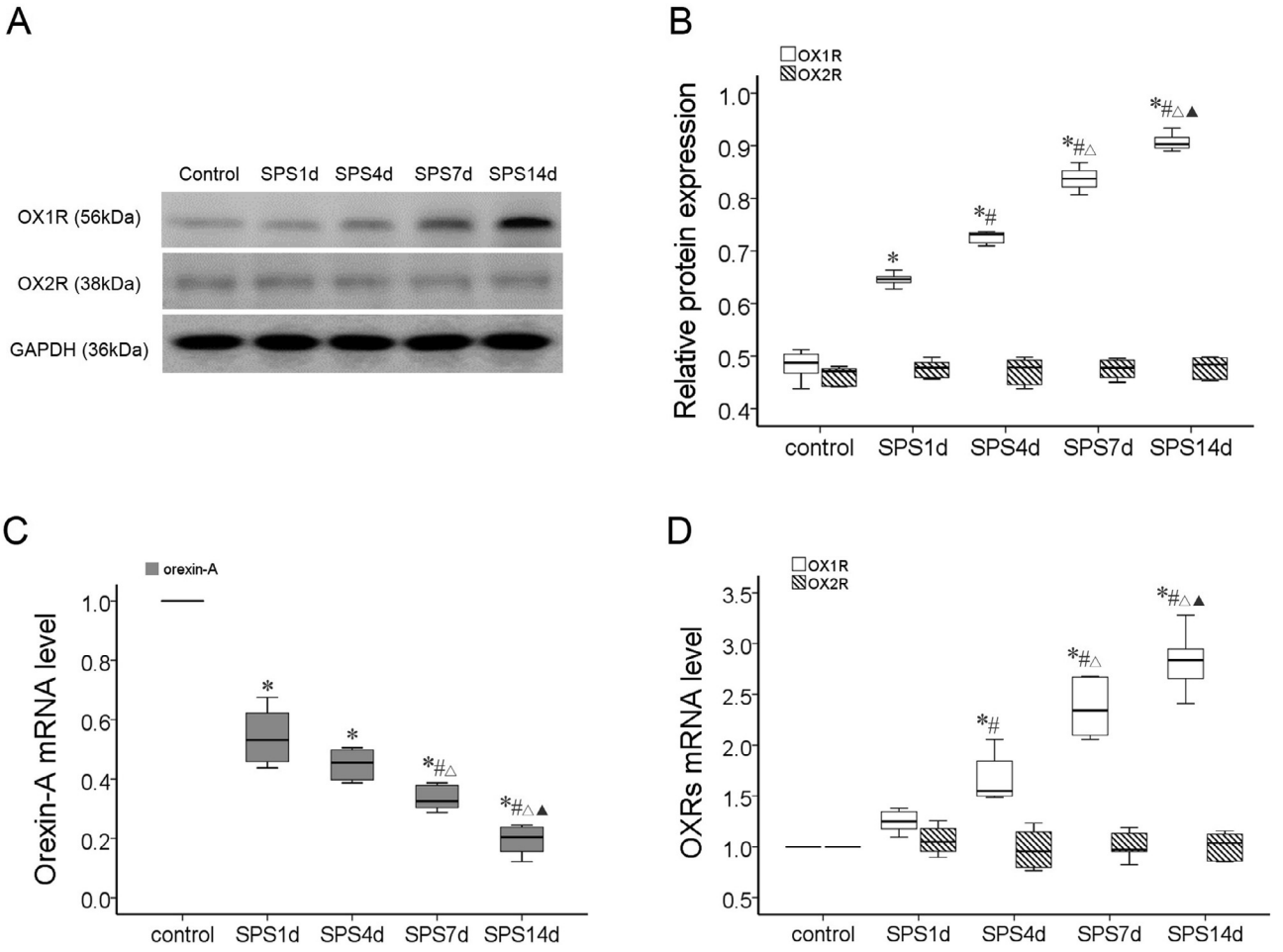

Fig. 4. Western blot and qRT-PCR analyses of orexin-A and orexin-A receptor expression in the hypothalamus. **(A)**, **(B)** OX1R and OX2R protein levels detected by Western blotting. **(C)**, **(D)** *Orexin-A* **(C)** and *OX1R* and *OX2R* **(D)** mRNA levels detected by qRT-PCR. Data represent mean ± SD (n = 6 per group). *P < 0.05 vs control; $^{\#}P$ < 0.05 vs SPS-1d; $^{\triangle}P$ < 0.05 vs SPS-4d; $^{\blacktriangle}P$ < 0.05 vs SPS-7d (Tukey's test).

Statistical analysis

In the present study, the blinded studies used in performing experiment and data analyzing from behavior test, immunohistochemistry, protein and real-time PCR results. Data are expressed as mean ± SD and were analyzed using SPSS v18.0 software (IBM, Armonk, NY, USA). The means of two groups were compared with the unpaired Student's t test. Differences between groups in Experiment 1 were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to correct for multiple comparisons. The effects of orexin A administration in Experiment 2 were assessed by two-way ANOVA followed by Bonferroni's post-hoc test to determine the significance of post-hoc differences between individual groups. P < 0.05 was considered statistically significant.

RESULTS

Changes in weight, food intake and memory behavior in the MWM following SPS

We recorded changes in body weight of rats in the control and SPS-14d groups on days 1, 7, and 14 and total food intake between days 1–7 and days 7–14 post-SPS. Rats exposed to SPS gained weight at a slower rate (P < 0.01; Fig. 2A) and showed decreased food intake on days 7–14 (P < 0.01; Fig. 2B) compared to controls.

In the MWM test, the SPS group showed fewer platform crossings than control rats (P < 0.01; Fig. 2C). There was no significant difference in the total swimming distance between the two groups (Fig. 2D).

SPS alters the expression of orexin-A and its receptors in the hypothalamus and hippocampus

Immunohistochemical staining results. The immunohistochemical analysis revealed that orexin-A-positive neurons were distributed in the LH of control rats (Fig. 3A). At higher magnification (Fig. 3B–F), positive orexin-A immunoreactivity was observed in the cytoplasm and fibers of neurons. SPS decreased the mean integral optical density and the number of orexin-A-positive cells (P < 0.05; Fig. 3G, H), but the cells size doesn't change compared to the neurons in control rats (Fig. 3I).

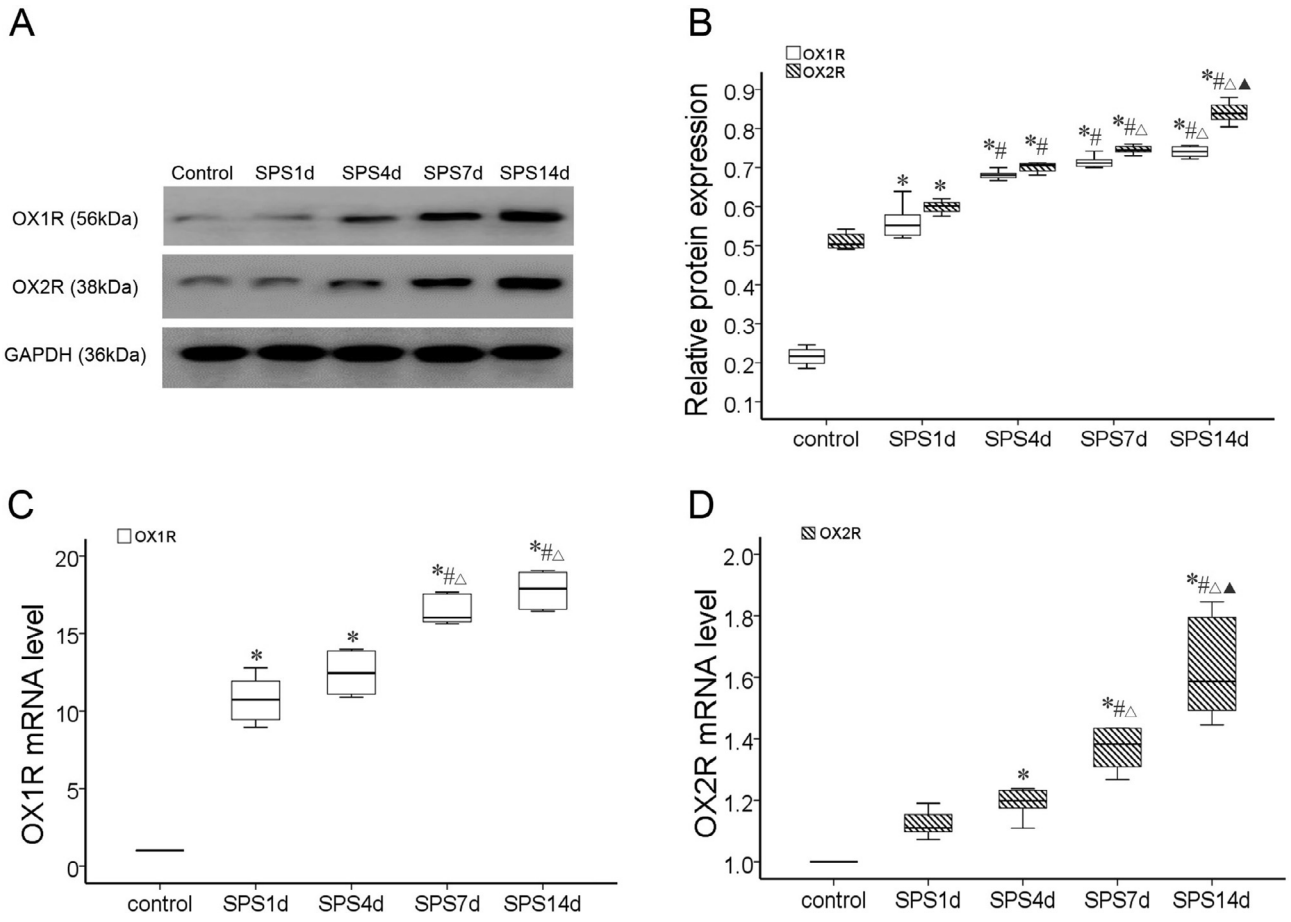

Fig. 5. Western blot and qRT-PCR analyses of OX1R and OX2R expression in the hippocampus. **(A)**, **(B)** OX1R and OX2R protein levels detected by Western blotting. **(C)**, **(D)** *OX1R* **(C)** and *OX2R* **(D)** mRNA levels detected by qRT-PCR. Data represent mean ± SD (n = 6/group). *P < 0.05 vs control; $^{\#}P$ < 0.05 vs SPS-1 d; $^{\Delta}P$ < 0.05 vs SPS-4 d; $^{\blacktriangle}P$ < 0.05 vs SPS-7 d (Tukey's test).

Western blotting and qRT-PCR results. OX1R protein expression in the hypothalamus increased after SPS (P < 0.05; Fig. 4A, B), whereas no change was observed for OX2R (Fig. 4A, B). *Orexin-A* mRNA level was reduced (P < 0.05; Fig. 4C) whereas *OX1R* mRNA was upregulated (P < 0.05; Fig. 4D) in the hypothalamus. There were no obvious changes in *OX2R* mRNA expression at any time point (Fig. 4D).

Western blot analysis revealed that OX1R and OX2R protein levels in the hippocampus gradually increased over time following SPS (P < 0.05; Fig. 5A, B). Similar trends for *OX1R* and *OX2R* mRNA were detected by qRT-PCR (P < 0.05; Fig. 5C, D).

Orexin-A administration restores body weight and memory performance

Body weight (day 13 post-SPS) and food intake (days 7–13 post-SPS) were recorded during orexin-A administration (days 7–13 post-SPS). Two-way ANOVA showed significant main effects of SPS and orexin-A on body weight (F[1,28] = 96.80, P < 0.01; F[1,28] = 13.90, P < 0.01) and food intake (F[1,28] = 130.32, P < 0.01; F[1,28] = 39.18, P < 0.01) (Fig. 6A, B). On the other hand, the interaction between SPS and orexin-A was not significant for body weight (F[1,28] = 2.60, P = 0.118) and food intake (F[1,28] = 3.04, P = 0.09). A Bonferroni post-hoc test showed that body weight (Fig. 6A) and food intake (Fig. 6B) were increased in the SPS + orexin-A group compared to the SPS + vehicle group (P < 0.01) and decreased in the SPS + vehicle group relative to the non-SPS + vehicle group (P < 0.05). Meanwhile, food intake (P < 0.05; Fig. 6B) was increased in the non-SPS + orexin-A group compared to the non-SPS + vehicle group.

In the MWM test, two-way ANOVA revealed significant main effects of SPS and orexin-A on the number of platform crossings (F[1,28] = 67.95, P < 0.01; F[1,28] = 8.22, P < 0.01) (Fig. 6C, D). The interaction between SPS and orexin-A was significant (F[1,28] = 4.624, P = 0.04). A post-hoc analysis showed that the number of platform crossings was higher in the SPS + orexin-A group than in the SPS + vehicle group (P < 0.01) and lower in the SPS + vehicle group compared to the non-SPS + vehicle group (P < 0.01) (Fig. 6C). However, there was no statistically significant difference between the non-SPS + vehicle and non-SPS + orexin-A groups (Fig. 6C), and total swimming distance was similar across groups (Fig. 6D).

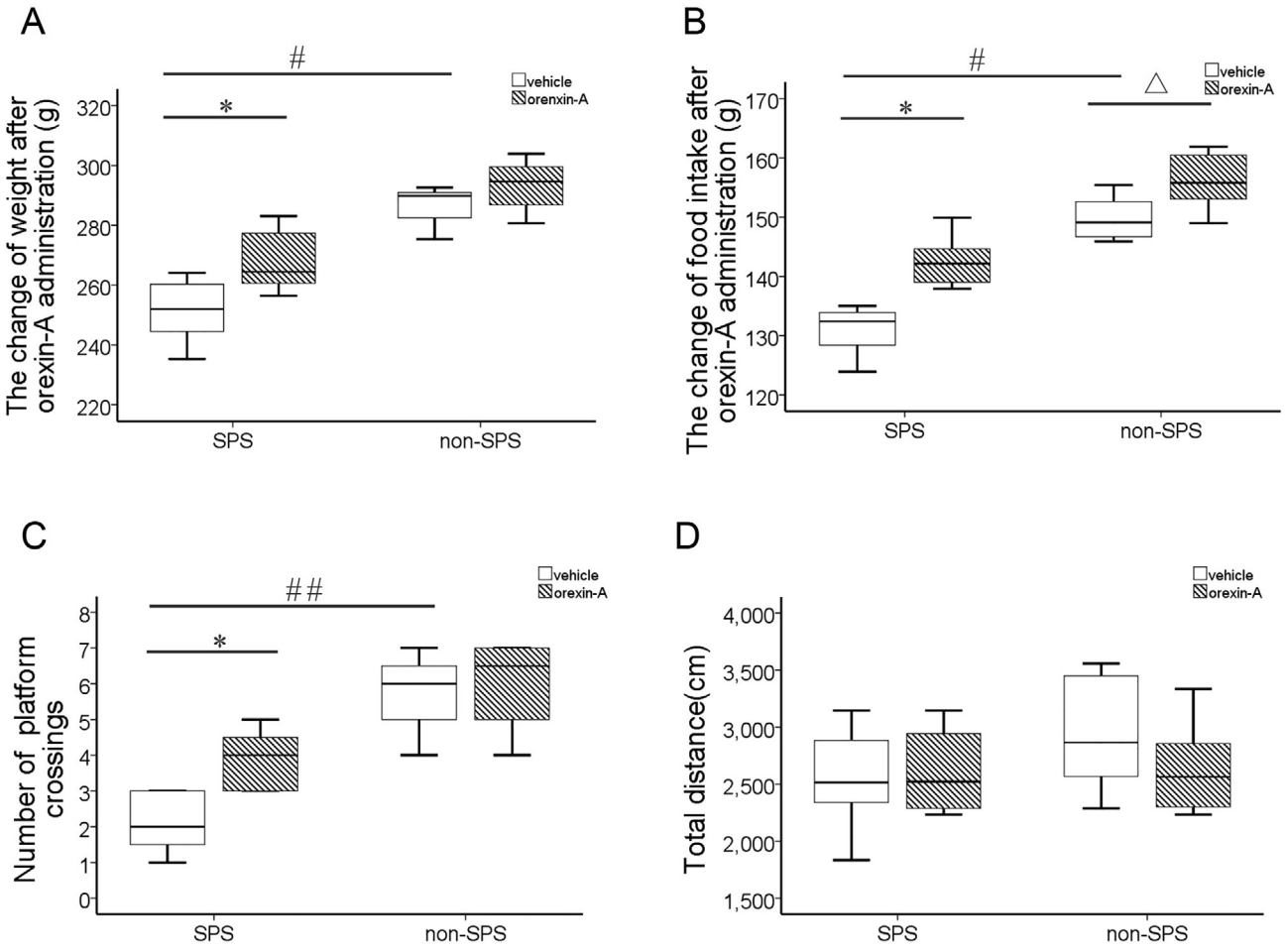

Fig. 6. (A), (B) Body weight and food intake following orexin-A administration in rats subjected to SPS. **(A)** Body weight (g). **(B)** Food intake (g). **(C), (D)** Results of the MWM test following orexin-A administration. **(C)** Number of platform crossings. **(D)** Total swimming distance (cm). Data represent mean ± SD (n = 8/group). *P < 0.01 vs SPS + vehicle; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 vs non-SPS + vehicle; $^{\Delta}P$ < 0.05 vs non-SPS + vehicle (two-way ANOVA with Bonferroni post-hoc test).

Orexin-A administration alters orexin receptor expression

In the hypothalamus (Fig. 7A–C), the main effect of SPS and orexin-A administration on OX1R protein expression (F[1,28] = 185.19, P < 0.01; F[1,28] = 23.33, P < 0.01) was statistically significant. The interaction between SPS and orexin-A was also significant (F[1,28] = 23.38, P < 0.01). The post-hoc test revealed that OX1R was upregulated in the SPS + vehicle group compared to the non-SPS + vehicle group (P < 0.01) but downregulated in the SPS + orexin-A group relative to the SPS + vehicle group (P < 0.01). In contrast, OX2R level did not differ across groups.

In the hippocampus (Fig. 7D–F), OX1R and OX2R protein levels showed main effects for SPS (F[1,28] = 90.85, P < 0.01; F[1,28] = 7.027, P = 0.013) and orexin-A (F[1,28] = 74.39, P < 0.01; F[1,28] = 7.78, P < 0.01). The interaction between SPS and orexin-A was significant for OX1R and OX2R expression levels (F[1,28] = 7.66, P = 0.01; F[1,28] = 8.356, P < 0.01). The post hoc test showed that OX1R and OX2R protein levels were higher in the SPS + vehicle group than in the non-SPS + vehicle group (P < 0.01), whereas OX1R and OX2R levels were lower in the SPS + orexin-A group than in the SPS + vehicle group (P < 0.01).

DISCUSSION

Orexins are involved in the regulation of various physiological functions including learning and memory. Orexin A facilitates learning, consolidation of learning and also retrieval processes (Telegdy and Adamik, 2002) and controls bidirectional long-term synaptic plasticity (Selbach et al., 2010) in the hippocampus. The results of the present study show that SPS decreased orexin-A and increased OX1R and OX2R expression in rats, which was associated with spatial learning and memory impairment. Injection of orexin-A into the brain improved memory performance and restored OX1R and OX2R levels in the hippocampus. Orexins may mediate the relationship between memory and behavior by altering hippocampus function and connectivity (Selbach et al., 2010; Stanley and Fadel, 2012). Abnormal orexin signaling is closely associated with the development of stress-

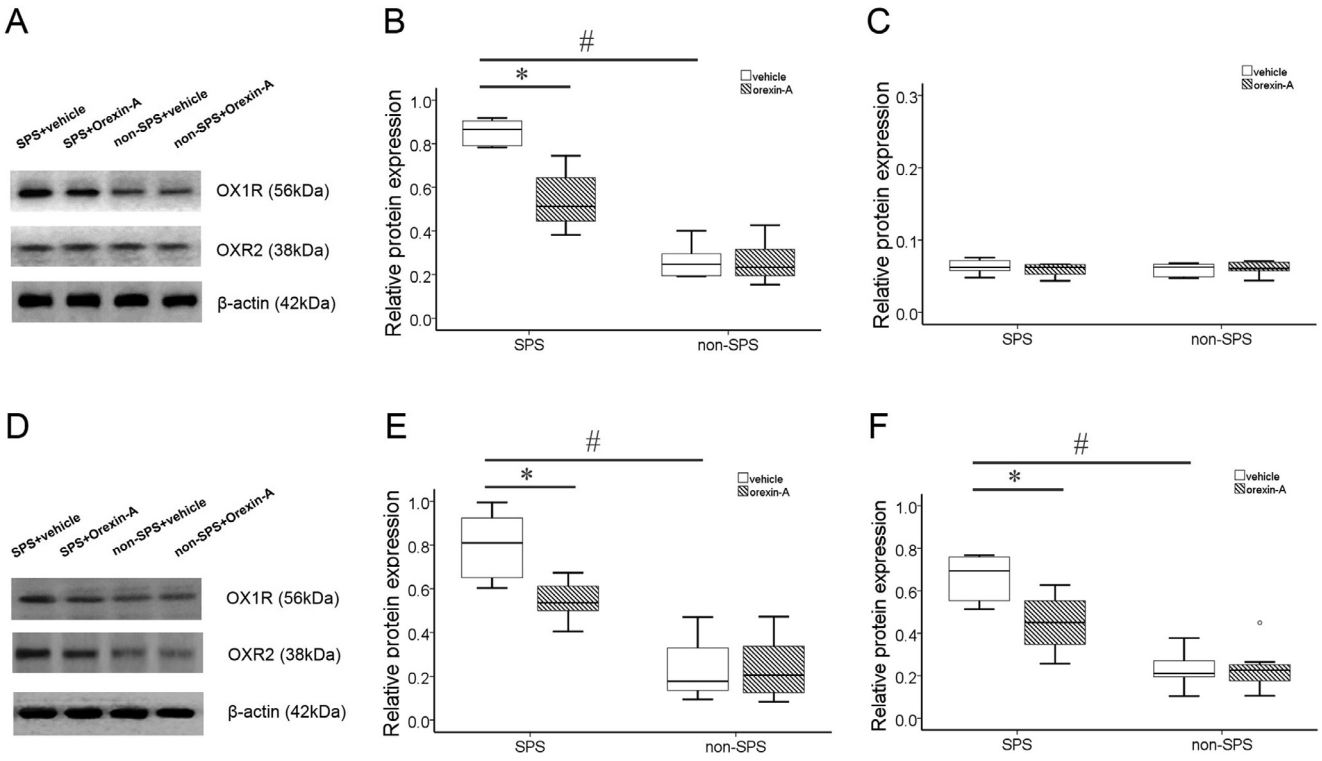

Fig. 7. Orexin receptor expression following orexin-A administration. **(A)** OX1R and OX2R protein levels in the hypothalamus detected by western blotting. **(B), (C)** OX1R **(B)** and OX2R **(C)** protein expression analysis in the hypothalamus. **(D)** OX1R and OX2R protein levels in the hippocampus detected by western blotting. **(E)**, **(F)** OX1R **(E)** and OX2R **(F)** protein expression analysis in the hippocampus. Data represent mean ± SD (n = 8/group). $^{*}P < 0.01$ vs SPS + vehicle; $^{\#}P < 0.01$ vs non-SPS + vehicle (two-way ANOVA with Bonferroni post-hoc test).

related disorders such as PTSD, depression, and panic disorder, as evidenced by the reduced orexin levels in anxiety patients with severe depression compared to normal subjects (Johnson et al., 2010) and low orexin-A activity in PTSD patients with combat experience (Strawn et al., 2010). It was also suggested that downregulation of orexin in the hippocampus contributes to age-related learning and memory impairment (Stanley and Fadel, 2012).

The role of OX1R and OX2R in the hippocampus of SPS rats

The action of orexins is mediated via OX1R and OX2R. In our study, icv injection of orexin-A reversed the effects of SPS on behavior and orexin receptor expression. This is consistent with previous reports demonstrating that orexin-A alleviated spatial learning and memory dysfunction via OX1R (Zhao et al., 2014) and that performance in the MWM test was impaired by blocking OX1R (García-Brito et al., 2018). In addition, hippocampal OX1R is thought to be involved in the disturbance of learning and memory induced by pain (Kooshki et al., 2017) and morphine (Alijanpour et al., 2016; Farahmandfar et al., 2016). Intrusive persistent memories are a key feature of PTSD, and the inability to suppress recall of intrusive memories is different from spatial memory and learning dysfunction. Injection of the OX1R antagonist SB-334867-A into the CA1 area of the hippocampus was shown to prevent memory acquisition, consolidation, and retrieval (Akbari et al., 2006), whereas injection into the dentate gyrus affected only the first two processes (Akbari et al., 2007, 2011), implying that OX1R plays distinct roles in different stages of learning and memory.

Few studies to date have investigated how OX2R affects learning and memory. One study found that endogenous orexins inhibited hippocampal synaptic N-methyl-D-aspartate receptor function and memory formation via OX2R (Perin et al., 2014); in addition, injection of OX1R and OX2R antagonists into the CA1 area of the hippocampus prevented morphine-induced conditioned place preference, but the effect size was three times larger upon inhibition of OX1R compared to OX2R (Alizamini et al., 2018). Orexin neuron activation induced a passive coping phenotype during social defeat leading to memory deficits, but this did not involve OX2R (Eacret et al., 2019). Thus, OX1R plays a more important role than OX2R in the regulation of learning and memory; but our results suggest that both receptors are involved, which may be due to different memory type.

The role of OX1R and OX2R in the hypothalamus of SPS rats

In the present study, SPS reduced the rate of weight gain and food intake in rats; these changes were associated with decreased orexin-A and increased OX1R expression in the hypothalamus, which was partly reversed by orexin-A administration. Other studies have shown that rats exposed to SPS showed decreased

body weight (Xiao et al., 2018; Ding et al., 2019) and loss of appetite (Xiao et al., 2018). Orexins stimulate food consumption (Sakurai et al., 1998; Edwards et al., 1999; Benoit et al., 2005; Yokobori et al., 2011; Li et al., 2015) whereas fasting results in an upregulation of pre-pro-orexin mRNA level (Sakurai et al., 1998). Conversely, orexin-deficient mice exhibit narcolepsy, inactivity, and obesity (Hara et al., 2001); and orexin deficiency in individuals with narcolepsy is linked to higher body mass (Schuld et al., 2000; Wang et al., 2016). Thus, orexins promote negative energy balance. The effects of orexin on food intake are influenced by circadian rhythm: orexin treatment increased daytime feeding and reduced nocturnal feeding, resulting in no net change in food intake over 24 h (Haynes et al., 1999). Orexin-A is thought to be more effective than orexin-B in mediating food intake (Sakurai et al., 1998; Edwards et al., 1999; Volkoff et al., 1999). OX1R activation is necessary for food-reinforced responses and motivation in mice (Sharf et al., 2010). Administration of a selective orexin-1 receptor antagonist (Haynes et al., 2000) or anti-orexin antibody reduced food intake (Yamada et al., 2000). Additionally, OX1R signaling was shown to regulate cue-induced feeding in rats (Cole et al., 2015). However, OX2R signaling may also play a role in energy homeostasis: enhanced orexin signaling via OX2R conferred resistance to diet-induced dysregulation of energy metabolism by enhancing leptin sensitivity (Funato et al., 2009). In the present study, only OX1R and not OX2R was upregulated in SPS rats. It is possible that OX1R level returned to the baseline at some time point after SPS that was beyond the temporal resolution of our experiment. In future studies, this possibility will be investigated by examining additional time points (e.g., 21 and 28 days post-SPS exposure).

The limitation of OX1R and OX2R

There has been a long-standing problem in the specificity of OX1R and OX2R antibodies. To our knowledge, all attempts to find OXR antibodies that are knockout-verified have failed. Commercial OXR antibodies typically stain multiple bands. The anti-OX1R and -OX2R antibodies used in this study are polyclonal antibodies (from Santa Cruz), resulting in the appearance of non-specific bands possibly. It is a limitation of western in the present study. In future studies, more specific antibodies or other experimental methods will be used to obtain more convincing data.

In summary, our results indicate that perturbation of the orexin system may play a critical role in memory impairment and appetite disturbance in PTSD. Also, the changes in behavior and orexin receptor expression in SPS rats were partly reversed by orexin-A administration; these findings suggest that orexin-A has therapeutic potential for the treatment of PTSD.

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AUTHOR CONTRIBUTIONS

YS designed the study with FH and DH. DH and SZ performed surgeries and collected tissues. DH and LW performed immunohistochemistry, western blotting and real-time PCR, and carried out behavioral testing in the MWM. DH wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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