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Effects of Sleep Deprivation (SD) on Rats via ERK1/2 Signaling Pathway

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Sleep deprivation (SD) is common in humans, and sleep loss has a significant influence on health and produces related diseases. Orexin-A has been demonstrated to play a role in physiological processes, including feeding, sleep/wake cycle, and energy metabolism. The aim of this study was to investigate the effect of SD on rats and to define the underlying mechanism.

Material/Methods: We constructed an SD rat model. The Morris water maze test was used to assess rat learning and memory. Imaging of hippocampus and hippocampal tissue in rats were captured by magnetic resonance imaging or electron microscopy. We used the CCK-8 kit to assess cell viability. The level of protein was measured using Western blot analysis, and qRT-PCR was used to evaluate mRNA level.

Results: SD rats had poorer learning and memory and had damage to the hippocampus. SD resulted in shrinkage of hippocampal volume and encephalocele size. SD increased the expression of Orexin-A, OX1R, OX2R, and PARP-1, and decreased the expression of ERK1/2 and p-ERK1/2. Orexin-A (0–10 μM) improved neuron viability, whereas orexin-A (10–100 μM) attenuated neuron viability. SB334867 treatment reduced the viability of neurons treated with orexin-A. NU1025 treatment increased cell viability, especially in neurons treated with orexin-A. SB334867 treatment decreased the p-ERK1/2 levels in neurons treated with orexin-A. NU1025 increased the expression of p-ERK1/2 in neurons treated with orexin-A.

Conclusions: SD decreases learning and memory through damage to the hippocampus. Higher concentrations of orexin-A had a major negative effect on hippocampal neurons via OX1R and PARP-1 through inhibition of the ERK1/2 signaling pathway.

MeSH Keywords: Hippocampus • MAP Kinase Signaling System • Orexins • Sleep Deprivation

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Background

Living organisms have an accurate internal biological clock that times daily cycles of events ranging from sleep to wakefulness [1]. In the central suprachiasmatic nucleus (SCN) of the hypothalamus, neuronal circadian oscillators are regarded as a major pacemaker for driving rhythms in activity of humans [2]. Sleep deprivation (SD) is common in humans, and it is reported that 20% of adults suffer from SD [3]. Ward et al. found that sleep-deprived juvenile rats were slower to learn the location of a hidden platform than controls; however, adult performance was not impaired [4].

Studies suggest that sleep loss has significant effects on the cardiovascular, endocrine, immune, and nervous systems [5,6]. SD also increases the risk of developing false memory and enhances avoidance learning, which suggests SD impedes memory and learning, but its key mechanism is unclear [7,8]. A study has shown that SD predominantly affects functions of memory and learning mediated via the prefrontal cortex [9]. The hippocampus has an important role in learning and the formation of memory, as well as emotions such as fear, anxiety, and happiness [10]. The hippocampus may have a link with SD, and a recent study has indicated that SD changes the hippocampus. In the present study we investigated the effects of SD on the hippocampus, focusing on submicroscopic structure and molecular biology.

Orexins, which are peptide transmitters, including orexin-A and orexin-B, are produced by hypothalamic neurons [11] and are restricted to the lateral hypothalamus, and they have been demonstrated to have important effects on many physiological processes such as feeding, sleep/wake cycle, and energy metabolism [12–14]. Orexins work on 2 specific G protein-coupled receptors (GPCRs) – orexin-1 (OX1) and orexin-1 (OX2) – in target cells [11,15]. A recent study showed that orexin-A attenuated the impairment of learning and memory in the hippocampus [16]. Moreover, orexin-A affects the gastric distention-sensitive neurons via the hippocampus [17]. Based on results of recent studies, we hypothesized that orexin-A has a connection with the hippocampus, and we explored the effect of orexin-A on hippocampal neurons.

Material and Methods

The rat model of sleep deprivation

This animal study was approved by the Animal Care and Use Committee of Qianfoshan Hospital Affiliated to Shandong University (No. DW20171208006). Sprague-Dawley rats, food, and water were purchased from Guangdong Medical Laboratory Animal Center. Rats were housed individually in standard plastic cages at 24±1°C and 40–70% of humidity. The rats adapted to the animal room environment on a 12-h light-dark cycle for 3 days. A Columbux device (YuYan, Shanghai, China), setting 10 cm/s and stopping 12 s every 3 s, was used for depriving sleep from 8: 00 to 20: 00 for 12 h. Rats could drink and eat freely. The sleep deprivation experiment lasted 5 weeks.

Morris water maze (MWM) test

The MWM test was used to assess learning and memory [18]. The MWM test was performed in a round, black pool, 160 cm in diameter and 60 cm deep. The pool was filled to a depth of 55 cm with water mixed with milk, which made water milky white. The temperature of water in the pool was balanced at 25±0.5°C by addition of warm water. The escape platform, 12 cm in diameter and 25 cm deep, was placed in the center of one quadrant of the pool, 20 cm away the pool edge and submerged 1 cm below the water surface, and this quadrant designated the goal/target quadrant. The location of devices, containing a camera and the escape plate, were in the same position during learning trials and probe test.

The rats were allowed to stay on the platform for 20 s to learn direction. Rats were lowered tail-first into pool, facing the wall of the pool at the first quadrant, second quadrant, third quadrant, and fourth quadrant, in turn. A smart digital tracking system (Version 2.5, Panlab, Barcelona, Spain) recorded the trials. Maximum swim time was set at 120 s. If the rat found the platform within 120 s, the rat was removed from the pool. If the rat found the platform after 120 s, the rat was guided to the platform and the rat stayed on the platform for 20 s to re-orient, and the escape latency was recorded as 120 s. After removal from the pool, rats were dried with a towel and placed in a warming cage before being returned to the home cage. Rats were tested twice every day, and all testing was performed at the same time each day. The test lasted 4 days.

The probe test was used to examine spatial reference memory. The platform was removed from the pool and the rat was put in one quadrant. We recorded the number of times a rat crossed the platform, the length of time the rat stayed in the goal quadrant, and the time it took the rat to find the platform.

Imaging of hippocampus and hippocampal neurons culture and identification

The hippocampus was imaged by magnetic resonance imaging (NiuMai, Suzhou, China). An electron microscope (Thermo, Waltham, MA, USA) was used to image neurovascular units of the hippocampus. The hippocampal neurons of normal rats were extracted by separation of hippocampal tissue with scissors and Hank's buffer solution (Gibco, Carlsbad, CA, USA), and digestion with trypsin (Gibco, Carlsbad, CA, USA),

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and implantation or culture with DMEM/F-12 medium (Gibco, Carlsbad, California, USA) attached 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% 10 000 units/ml penicillin-10000 µg/ml streptomycin (Gibco, Carlsbad, California, USA). Cells were seeded in a 75-mm dish (Corning, Corning, USA) for 24 h and then washed twice with phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA). Then, cells were incubated at room temperature for 2 h with NSE antibody (ab53025, Abcam, Cambridge, UK) mixed with TBST, and the dilution of ratio was performed according to the manufacturer's instruction. Then, the goat anti-rabbit antibody (ab150077, Cambridge, UK) incubated cells for 2 h after the cells were washed for 3 times with TBST. ECL kit (Invitrogen, Carlsbad, California, USA) added to cell, then the cells were washed with PBS. DAPI mixed with PBS stained the cells for 30 min. The fluorescence of the cells was observed by fluorescence microscope (Olympus, Tokyo, Japan).

Cell viability

Hippocampal neurons were planted in 96-well plates (Corning, Corning, USA) for 24 h. Different reagents were added to cells for 24 h or 48 h. SB334867, an OX1 receptor (OX1R) antagonist [19], was obtained from Selleck Chemicals (Houston, TX, USA). TCSOX229, the OX2 receptor (OX2R) antagonist [20,21], and orexin-A were purchased from MCE (NJ, USA). NU1025, an inhibitor of PARP [22], was obtained from Selleck Chemicals (Houston, TX, USA). The CCK-8 kit (Invitrogen, Carlsbad, CA, USA) mixed with free FBS DMEM/F-12 medium (Gibco, Carlsbad, CA, USA) was added to the wells after discarding medium. The plates were read using a microplate reader (Thermo, Waltham, MA, USA) at the wavelength of 490 nm.

Western blot

Protein of hippocampal tissue was extracted with tissue extraction reagent (Invitrogen, Carlsbad, CA, USA). RIPA Lysis (Thermo Scientific, Waltham, MA, USA) was applied in collecting protein of hippocampal neurons. The BCA kit (Thermo Scientific, Waltham, MA, USA) was used to measure the concentration of protein following the manufacturer's instructions. Separation of protein was performed by SDS-PAGE. The protein was transferred to PVDF membranes (Sigma-Aldrich, St. Louis, MO, USA) by electrophoresis. Orexin-A antibody (AB3098, Sigma-Aldrich, St. Louis, MO, USA), OX1R antibody (AB3092, Sigma-Aldrich, St. Louis, MO, USA), OX2R antibody (ab183072, Abcam, Cambridge, UK), Poly (ADP-ribose) polymerase-1 (PARP-1) antibody (SAB2104087, Sigma-Aldrich, St. Louis, MO, USA), extracellular-regulated kinase (ERK) 1/2 (ERK1/2) antibody (#4695, CST, Danvers, MA, USA), p-ERK1/2 antibody (#4370, CST, Danvers, MA, USA), and GAPDH (#5174, CST, Danvers, MA, USA) were incubated with protein membranes for 12 h at 4°C before washing membranes with TBST solution contained Tween-20 (Invitrogen, Carlsbad, CA, USA). Anti-rabbit antibody with HRP (#7074, CST, Danvers, MA, USA) was incubated protein membranes for 2 h. All antibodies were diluted with TBST, and the ratio was as specified in the manufacturer's instructions. ECL (Invitrogen, Carlsbad, CA, USA) was applied for coloration.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Primer and probes were designed specifically for the target genes of Orexin-A, OX1R, OX2R and PARP-1 using PrimerBlast (NCBI, Bethesda, MD, USA), purchased from Sangon Biotech (Shanghai, China). We amplified 600 ng of cDNA using TaQMan Universal PCR Master Mix (Life Sciences, Grand Island, NY, USA). The concentration of forward and reverse primers (200 nM) and probe (60 nM) were used in reverse transcription-polymerase chain reaction. Initial denaturation of cDNA was performed at 95°C for 6 min, followed by 40 cycles of denaturation (95°C for 30 s), annealing (50–55°C for 1.5 min), and extension (72°C for 90 s) were performed in qRT-PCR. We used the $2^{(-\Delta\Delta Ct)}$ method to analyze the relative level of gene expression.

The following primers were used: Orexin-A primer (forward: 5'-AGAAGACGTGTTCCTGCCGT-3'; reverse: 5'-CGTGGTTACCGTTGGCCTGAA-3'), OX1R primer (forward: 5'-CAGATGAACTCTACCCTAA-3'; reverse: 5'-TTCTCCG AACGTGTCACGT-3'), OX2R primer (forward: 5'-TTGGGGTTCA TCGTCGTCAA-3'; reverse: 5'-TCGGTCAATGTCCAATGTTC-3'), PARP-1 primer (forward: 5'-ACGCACAATGCCTATGAC-3'; reverse: 5'-CCAG CGGAACCTCTACAC-3') and β-actin primer (forward: 5'-CCAACTG GGACGATATGGAG-3'; reverse: 5'-CAGAGGCATACAGGGACAAC-3').

Statistics and analysis

All values are presented as mean ± standard deviation. Protein stains were analyzed by Image J software, and these tests confirmed that repeated-measures data were normally distributed. Data analyzed by ANOVA with *t* tests in SPSS 22.0 (IBM, Armonk, NY, USA) were assessed for comparison between experimental groups or control group. Time%=(the time that the rat stayed in the goal quadrant)/(the time that the rat found the location of the platform). Differences were deemed as statistically significant at $p<0.05$.

Results

Effects of sleep deprivation on rat learning and memory

Escape latency decreased with increasing number of training days (Figure 1A) in both the control group and sleep deprivation (SD) group. However, escape latency in the SD group was longer than in the control group, showing that the level

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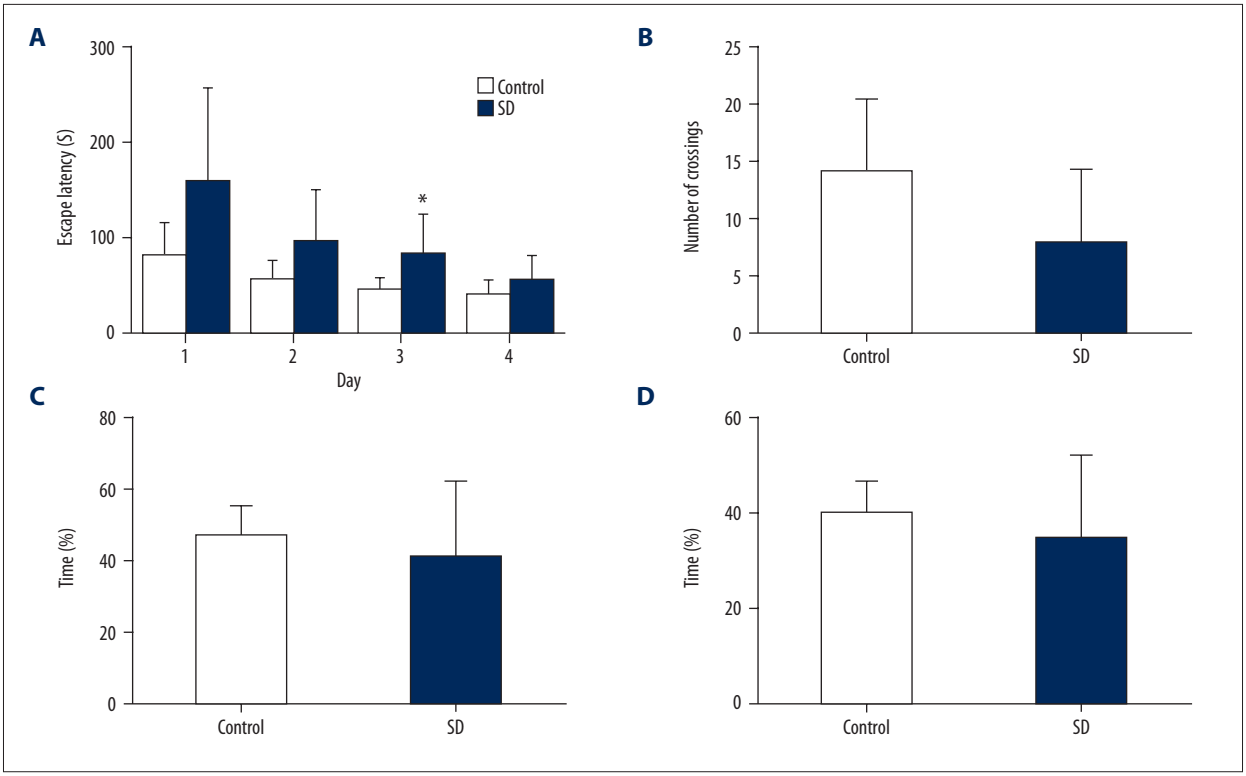

Figure 1. (**A–D**) Sleep deprivation (SD) decreases rat learning and memory. Rats were housed individually in standard plastic cages at 24±1°C and 40–70% humidity. The SD rat model (N=20) was established by setting the parameter of the Columbux device from 8: 00 to 20: 00 daily for 5 weeks. The Morris water maze (MWM) test was used to assess learning and memory. Learning trials lasted for 4 days, and escape latency was recorded. A probe test was used to test rat memory, and we recorded the number of platform crossings, time spent in the goal quadrant, and time needed to find the platform. ANOVA with *t* tests was used to analyze the data (* *vs.* control group; * $p<0.05$).

of learning in SD rats was lower than in normal rats. In the rat memory experiment, SD rats made fewer crossings and spent less time in the target quadrant compared to control rats (Figure 1B–1D). In the memory trials, the indicators suggested that the SD rats had worse memory.

Effects of sleep deprivation on rat hippocampus and hippocampal neurons

Hippocampal volume of rats in the SD group was smaller than in normal rats (31.34±1.85 mm^3 *vs.* 38.95±1.97 mm^3) (Figure 2A), and encephalocele size of rats was reduced in the SD group (Figure 2B). Hippocampal tissues were examined using a 30 000× and 10 000× electron microscope, showing that cells of hippocampal tissue in SD rats were disorderly and more debris appeared in hippocampal neurons (Figure 3A). There was more hippocampal tissue fluid in SD rats than in normal rats, and the staining was darker than in the control group (Figure 3B).

Effects of sleep deprivation on expression of Orexin-A, OX1R, OX2R, PARP-1, ERK1/2, and p-ERK1/2 in rat hippocampal tissue

Sleep deprivation affected hippocampal tissue and hippocampal neurons. We investigated the related protein expression and mRNA expression in hippocampal tissue. SD increased the protein levels of Orexin-A, OX1R, OX2R, and PARP-1 in hippocampal tissues of rats, which was accompanied by increased mRNA of Orexin-A, OX1R, OX2R, and PARP-1 (Figure 4). However, SD decreased ERK1/2 expression and activation of ERK1/2.

Effects of orexin-A combined with SB334867, TCSOX229, or NU1025 on hippocampal neuron viability

Orexin-A increased hippocampal neuron viability at a relative lower concentration (0–10 μM), but orexin-A inhibited hippocampal neuron viability at a relative higher concentration (Figure 5B). SB334867 did not obviously affect hippocampal neuron viability in the control group, whereas SB334867 reduced the viability of hippocampal neurons treated with orexin-A regardless of concentration (Figure 5C).

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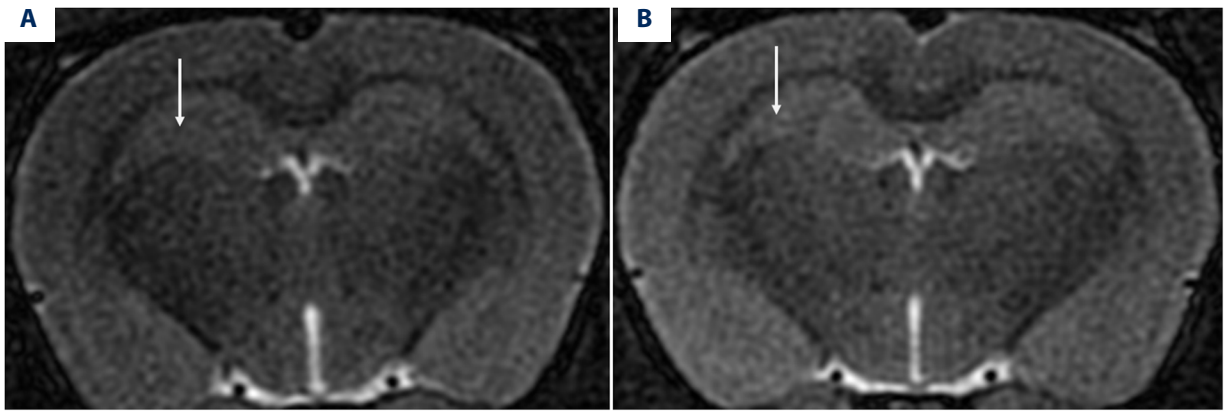

Figure 2. Sleep deprivation (SD) reduces the rat hippocampal volume and encephalocele size. Hippocampus and encephalocele of SD rats were observed via magnetic resonance imaging. Change in hippocampus and encephalocele were assessed by comparing them to the sizes in normal rats. The images of hippocampus (**A**) and encephalocele (**B**) were taken by magnetic resonance imaging.

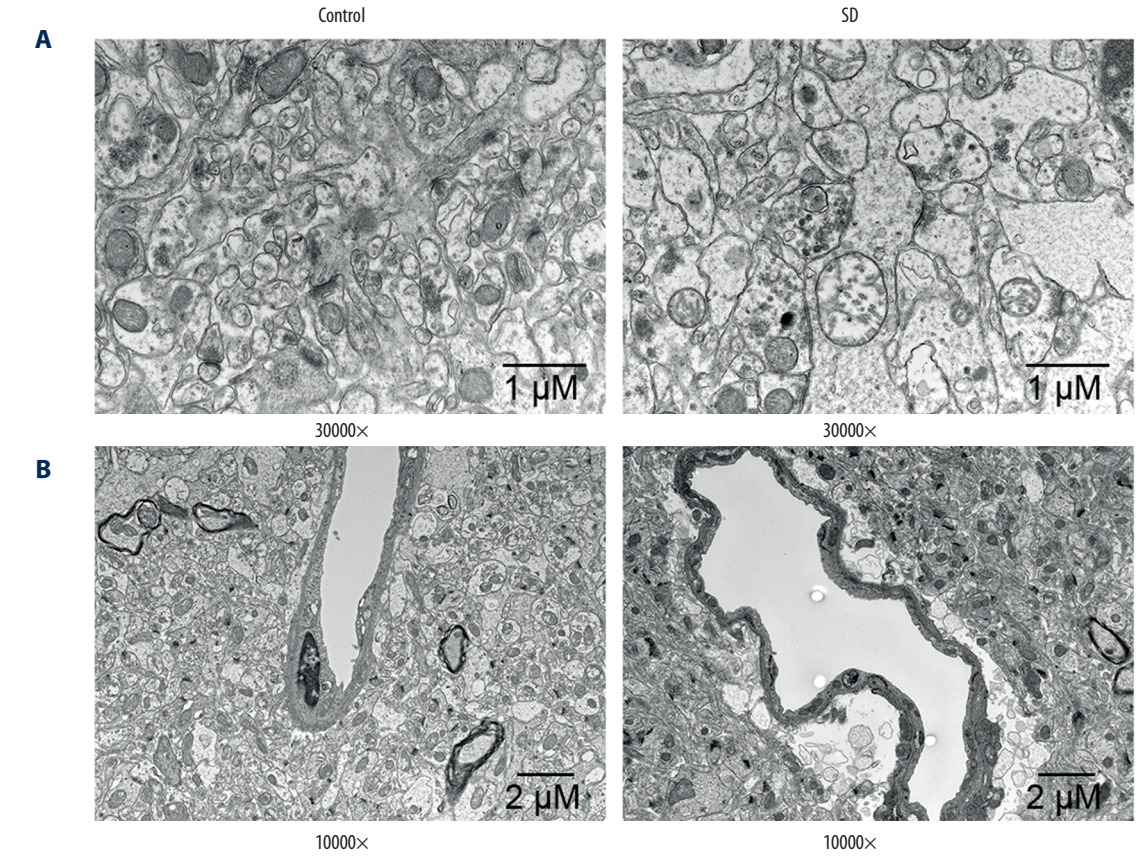

Figure 3. Sleep deprivation (SD) damage to hippocampal neurons. Hippocampal tissue of SD rats and normal rats were collected, and cells in hippocampal tissue were observed using an electron microscope. The images of hippocampal tissue were taken at 30 000× (**A**) and 10 000× (**B**) using an electron microscope.

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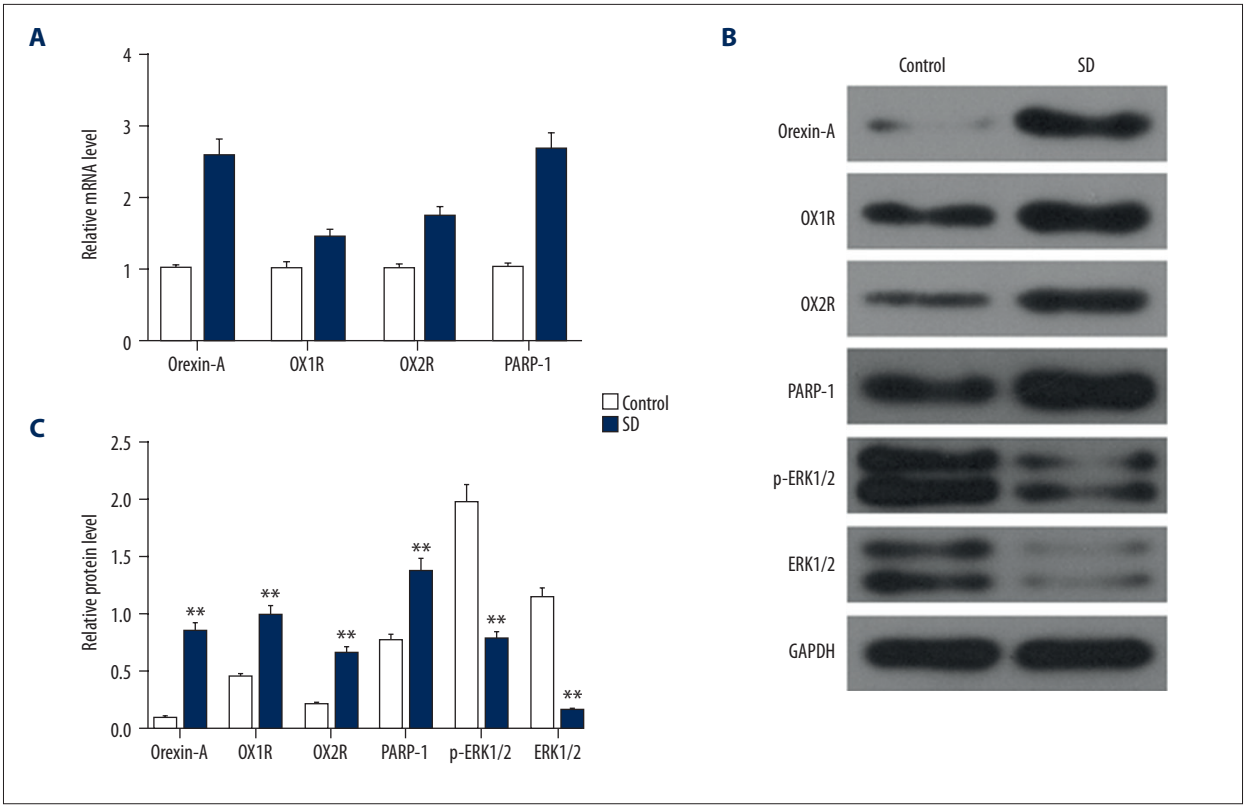

Figure 4. Effects of sleep deprivation (SD) on the expression of Orexin-A, OX1R, OX2R, PARP-1, ERK1/2, and p-ERK1/2 in rat hippocampal tissue. Total protein and total RNA were collected from hippocampal tissue using tissue extraction reagent. Western blot analysis was used to assess total protein, separation of protein, protein transference, incubation of primary antibody and secondary antibody, and coloration. We used qRT-PCR for cDNA synthesis and amplification. (**A**) Relative mRNA level was measured using qRT-PCR. (**B, C**) Relative protein level was evaluated using Western blot analysis. ANOVA with *t* tests was used to analyze data (* *vs.* control group; * p<0.05, ** p<0.01).

TCSOX229 had not apparent effect on hippocampal neuron viability in the control group and in rats treated with orexin-A (Figure 5D). NU1025 increased cell viability, especially in hippocampal neurons treated with the orexin-A for 24 h or 48 h (Figure 5E). We selected 2 combination groups – orexin-A combined with SB334867 and orexin-A combined with NU1025 – for subsequent experiments because TCSOX229 did not clearly affect viability of cells treated with orexin-A.

Effects of orexin-A combined SB334867 on the levels of OX1R, ERK1/2, and p-ERK1/2 in rat hippocampal neurons

SB334867 inhibited the expression of OX1R at both the higher concentration in the orexin-A group and in the lower concentration in the orexin-A group (Figure 6). Orexin-A caused increased expression of OX1R. The expression of ERK1/2 did not change greatly in any groups, whereas the higher concentration of orexin-A resulted in reduction of p-ERK1/2, and lower dosage of orexin-A increased the level of p-ERK1/2. SB334867 decreased the activation of ERK1/2 in hippocampal neurons treated with the lower dosage of orexin-A and the higher dosage of orexin-A, and it had not obvious influence on p-ERK1/2 compared to the control group.

Effects of orexin-A combined with NU1025 on levels of PARP-1, ERK1/2, and p-ERK1/2 in rat hippocampal neurons

The higher dosage of orexin-A caused higher expression of PARP-1 and lower expression of ERK1/2 and p-ERK1/2 in hippocampal neurons (Figure 7). NU1025 decreased the level of PARP-1, whereas it increased the expression of ERK1/2 and p-ERK1/2 regardless of orexin-A treatment, suggesting that the mechanism of NU1025 may be the same as with SB334867.

Discussion

SD reduced the learning and memory of rats, as shown by increased escape latency, decreased number of crossings, and reduction of goal quadrant time (Figure 1). SD reduced hippocampal volume and the encephalocele size, suggesting that SD affected brain and hippocampus function (Figure 2).

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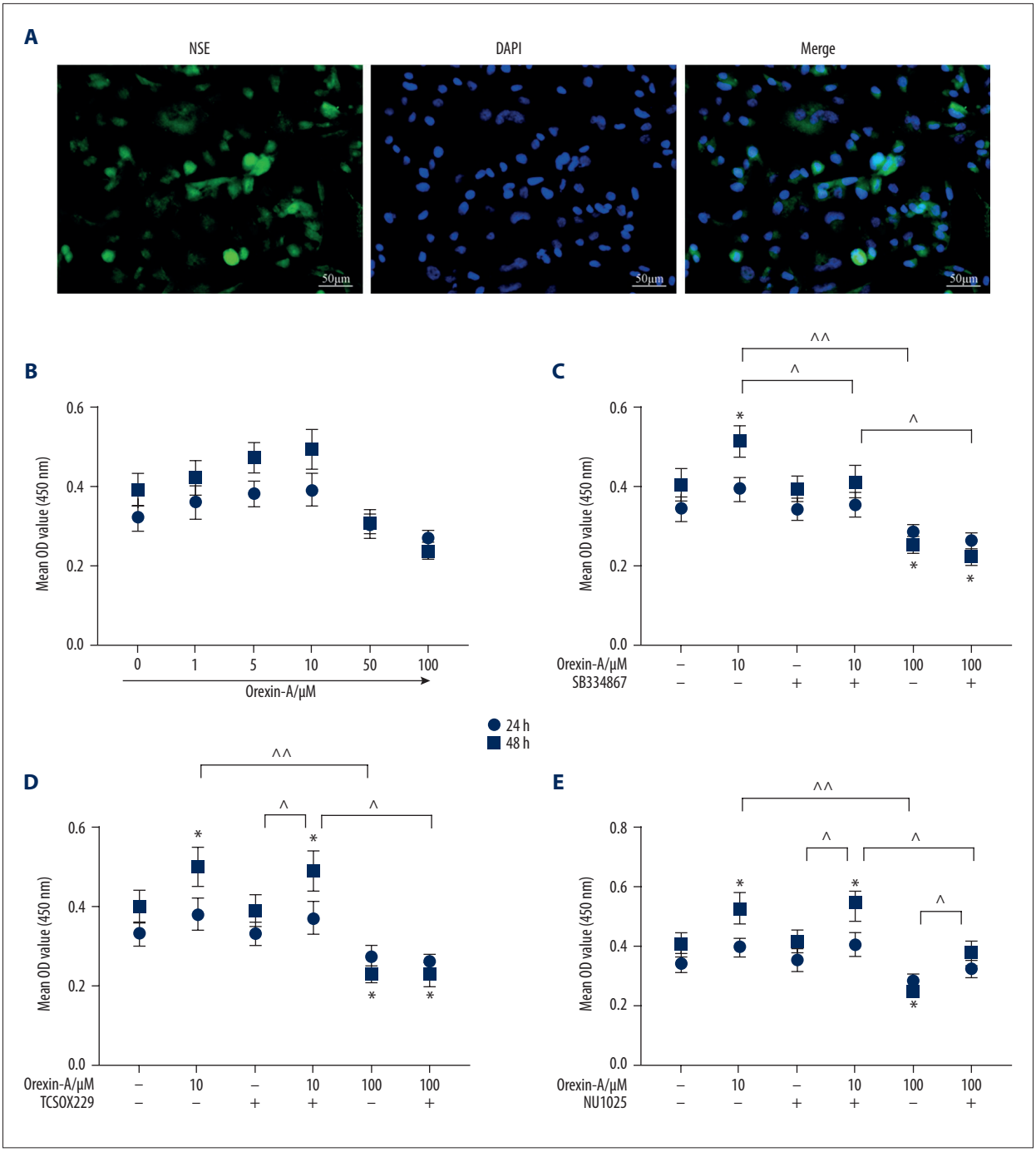

Figure 5. Effects of orexin-A combined with SB334867, TCSOX229, or NU1025 on hippocampal neuron viability. (**A**) Hippocampal neurons were extracted from normal rats. Antibody antigen immune method was used to mark NSE, and hippocampal neurons were dyed with DAPI. The fluorescence of cells was observed by fluorescence microscopy. (**B**) Different concentrations of orexin-A in treated hippocampal neurons at 24 h or 48 h, then the CCK-8 kit was used to detect cell viability with a microplate reader at the wavelength of 490 nm. (**C–E**) 10 μM or 100 μM orexin-A and 10 μM SB334867 (or 10 μM TCSOX229, or 0.2 mM NU1025) alone or in combination were used to treat hippocampal neurons for 24 h or 48 h, and the CCK-8 kit was used to assess cell viability. ANOVA with *t* tests was used to analyze the data (* *vs.* control group; ^, * p<0.05, ^^, ** p<0.01).

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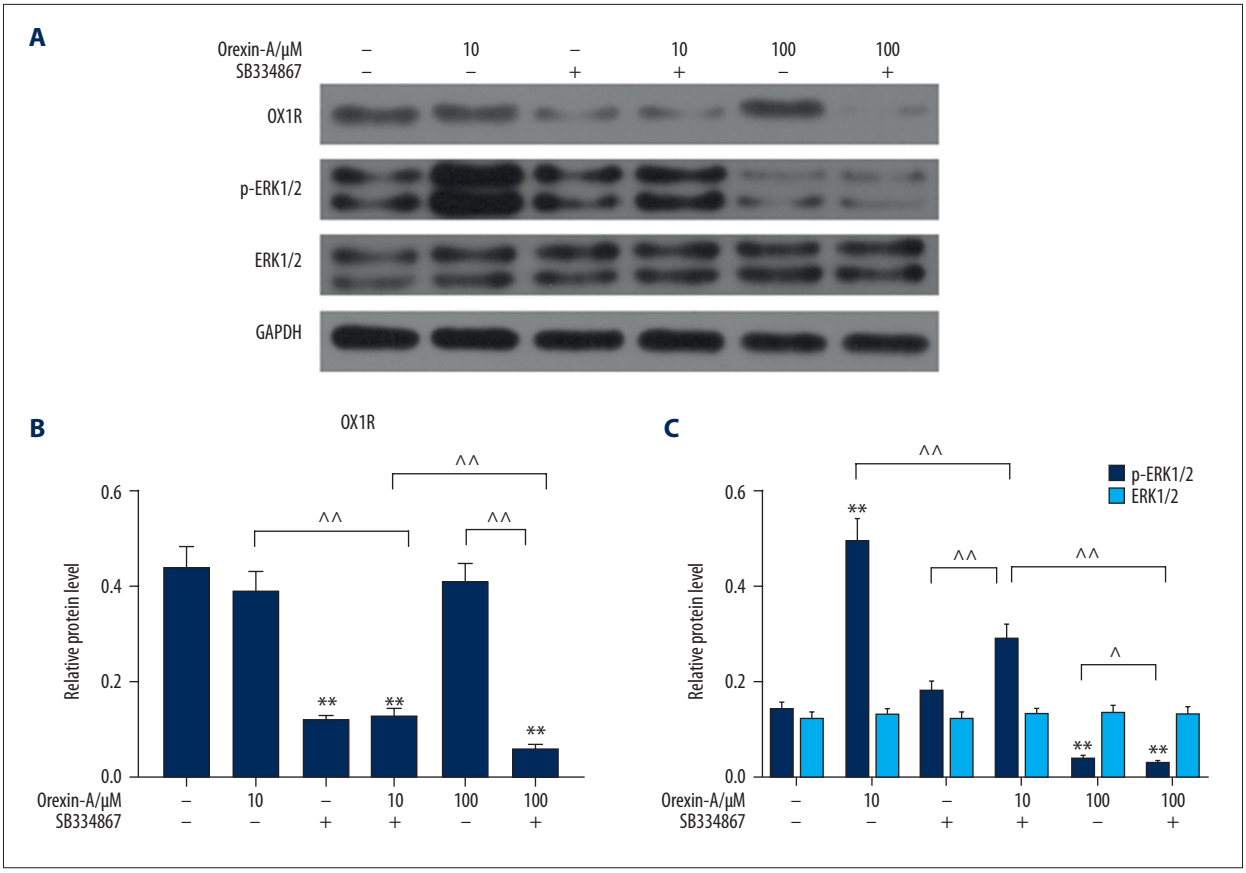

Figure 6. (**A–C**) Effects of orexin-A combined with SB334867 on the levels of OX1R, ERK1/2, and p-ERK1/2 in rat hippocampal neurons. Total protein was collected from hippocampal neurons with cell lysis solution after we used the reagents (10 μM and 100 μM orexin-A and 10 μM SB334867) to treat cells for 48 h. Western bolt analysis was used to assess the levels of OX1R, ERK1/2, and p-ERK1/2, followed by detection of total protein, separation of protein, protein transference, incubation of primary antibody and secondary antibody, and coloration. ANOVA with *t* tests was used to analyze data (* *vs.* control group; ^, * p<0.05, ^^, ** p<0.01).

We observed the submicroscopic structure of hippocampal neurons in tissue, and found obvious damage to hippocampal tissue, with disordered arrangement of cells, increased cell fragments, and dark color (Figure 3), suggesting that SD caused hippocampal injury in rats.

Orexin-A and orexin-B, called hypocretins, originate from the same precursor synthesized by hypothalamic neurons [11,23]. Orexin-A, a neuropeptide that promotes wakefulness, effectively enhances pyramidal neuron activity in the prefrontal cortex [24]. The activities of orexins are mediated by 2 membrane-bound G-protein-coupled receptors – OXR1 and OXR2 – which are found in nerves and peripheral organs, including the hypothalamus, adrenal glands, gastrointestinal tract, and the pancreas [11,13,25,26]. OXR1 or OXR2 result in apoptosis and cell growth in cancer cell lines, including human neuroblastoma cells and human colon cancer cells [27,28]. Studies have also shown that orexin-A inhibits gastric cancer cell apoptosis via OXR1 through the Akt signaling pathway [29].

In our study, we found increased orexin-A levels in hippocampal tissue of SD rats, and determined that adequate levels of orexin-A promote hippocampal neuron health (Figures 5, 6). Extracellular-regulated kinase 1/2 (ERK1/2) is a member of the mitogen-activated protein kinase family, and higher p-ERK1/2 expression is an independent factor for poor overall survival in non-small cell lung cancer patients [30]. Many studies have reported that blockage of ERK1/2 inhibits cancer cell viability, invasion, and migration [31,32]. However, one study has demonstrated that orexin-A upregulates OX1R and improves the proliferation of gastric cancer cells through the ERK signaling pathway [33]. However, it is clear that cell viability is affected by orexin-A, OX1R, OX2R, and ERK1/2. We found that SD decreased the levels of p-ERK1/2 and ERK1/2 and growth of orexin-A, OX1R, and OX2R (Figure 4). Inhibition of OX1R and OX2R had no obvious effect on hippocampal neuron viability, but OX1R inhibition decreased hippocampal neuron viability in rats treated with higher concentrations of orexin-A via decreasing expression of p-ERK1/2 (Figures 5, 6), which shows

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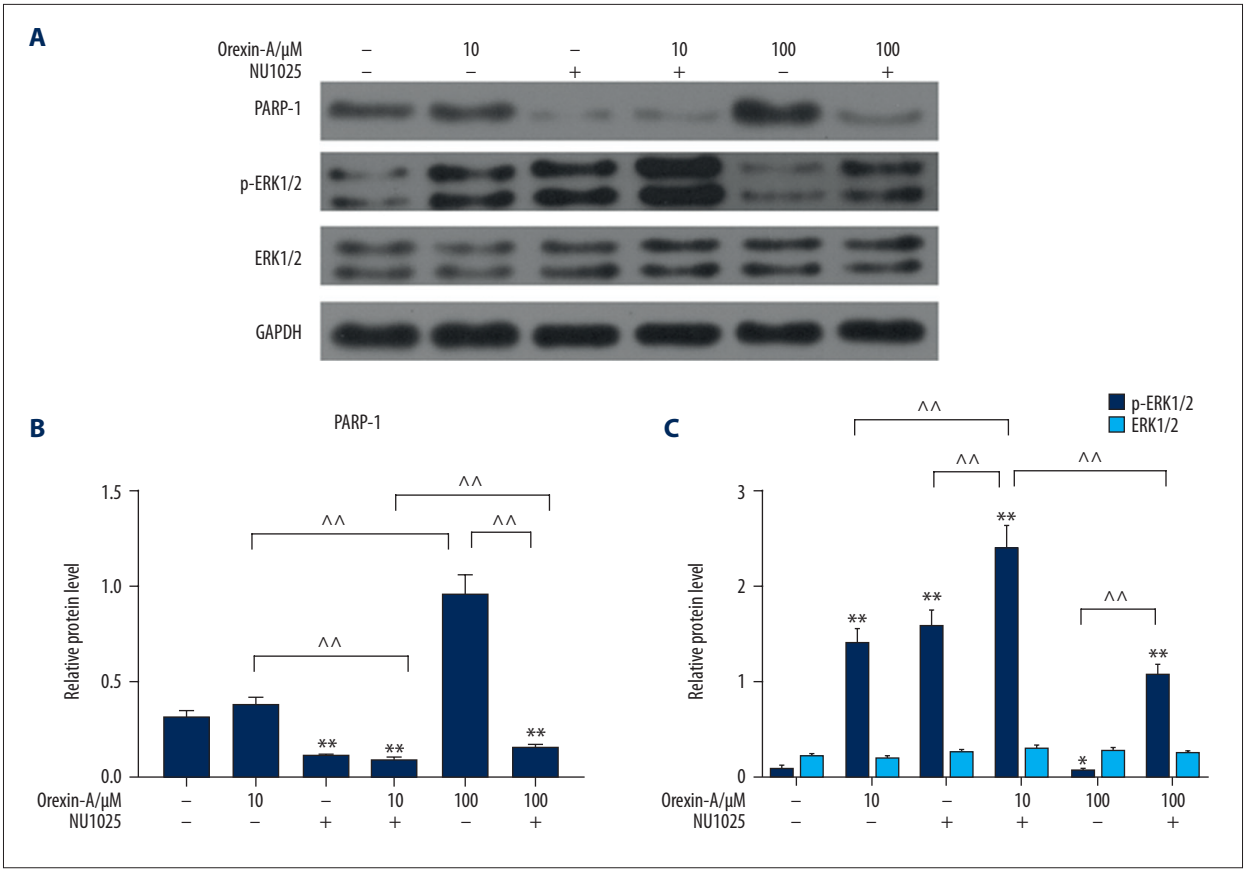

Figure 7. (**A–C**) Effects of orexin-A combined with NU1025 on the levels of PARP-1, ERK1/2, and p-ERK1/2 in rat hippocampal neurons. Total protein was extracted from hippocampal neurons with cell lysis solution after we treated cells with reagents (10 μM and 100 μM orexin-A and 0.2 mM NU1025) for 48 h. Western blot analysis was used to assess levels of PARP-1, ERK1/2, and p-ERK1/2, followed by detection of total protein, separation of protein, protein transference, incubation of primary antibody and secondary antibody, and coloration. ANOVA with *t* tests was used to analyze the data (* *vs.* control group; ^, *p < 0.05, ^^, ** p<0.01).

that OX1R plays a major role in hippocampal neuron with a higher concentration of orexin-A induced.

Poly(ADP-ribose) polymerase-1 (PARP-1), an enzyme, catalyzes the covalent attachment of polymers of ADP-ribose (PAR) moieties and its target proteins [34]. The 3 zinc fingers on PARP-1 are responsible for DNA binding and DNA binding-dependent activation of catalytic activity [35,36], and it has been reported that zinc deficiency can increase DNA damage, decrease DNA repair ability, and increase cancer risk [37]. PARP-1 activation promotes DNA repair, and high levels of PARP-1 causes cell death; therefore, inhibition of PARP-1 should benefit high-risk cancer patients [38–40]. SD increased levels of PARP-1 in hippocampal tissue (Figure 4). Decreased PARP-1 improved cell viability and higher concentrations of orexin-A increased expression of PARP-1 and decreased expression of p-ERK1/2 (Figures 5, 7), which further shows that PARP-1 inhibition improved viability of hippocampal neurons treated with higher concentrations of orexin-A via upregulation of p-ERK1/2.

Conclusions

We found that SD decreases learning and memory through damage to the hippocampus. Higher concentrations of orexin-A had a major negative effect on hippocampal neurons via OX1R and PARP-1 through inhibition the ERK1/2 signaling pathway. These findings may provide ideas and evidence for improvement of SD treatment.

Conflict of interest

None.

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