CX3C-chemokine receptor 1 modulates cognitive dysfunction induced by sleep deprivation

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Abstract

Background: Microglia plays an indispensable role in the pathological process of sleep deprivation (SD). Here, the potential role of microglial CX3C-chemokine receptor 1 (CX3CR1) in modulating the cognition decline during SD was evaluated in terms of microglial neuroinflammation and synaptic pruning. In this study, we aimed to investigat whether the interference in the microglial function by the CX3CR1 knockout affects the CNS's response to SD.

Methods: Middle-aged wild-type (WT) C57BL/6 and CX3CR1$^{-/-}$ mice were either subjected to SD or allowed normal sleep (S) for 8 h to mimic the pathophysiological changes of middle-aged people after staying up all night. After which, behavioral and histological tests were used to explore their different changes.

Results: CX3CR1 deficiency prevented SD-induced cognitive impairments, unlike WT groups. Compared with the CX3CR1$^{-/-}$ S group, the CX3CR1$^{-/-}$ SD mice reported a markedly decreased microglia and cellular oncogene fos density in the dentate gyrus (DG), decreased expression of pro-inflammatory cytokines, and decreased microglial phagocytosis-related factors, whereas increased levels of anti-inflammatory cytokines in the hippocampus and a significant increase in the density of spines of the DG were also noted.

Conclusions: These findings suggest that CX3CR1 deficiency leads to different cerebral behaviors and responses to SD. The inflammation-attenuating activity and the related modification of synaptic pruning are possible mechanism candidates, which indicate CX3CR1 as a candidate therapeutic target for the prevention of the sleep loss-induced cognitive impairments.

Keywords: Sleep deprivation; Cognitive dysfunction; Microglia; CX3CR1 deficiency

Introduction

Sleep is an important and indispensable physiological process to maintain human health. As modern fast-paced life often disrupts the sleep cycle of the general public, sleep deprivation (SD) is increasingly prevalent worldwide, especially in the middle-aged population, who are under tremendous pressure.[1] Recent studies have shown that SD leads not only to declined immunity[2,3] increasing incidence of cerebrovascular diseases[4,5] but also to deteriorated cognitive functions.[6,7] The incidence of Alzheimer disease (AD) in people with sleep disorders is 1.4 times higher than that in people without sleep disorder,[8] and sleep disorders are strongly associated with multiple system atrophy.[9] At the same time, accumulating evidence implies a close causal relationship between SD and cognitive disorders.[10,11]

Up to date, accumulative evidence has documented that SD results in an abnormal microglial activity in the brain. In addition, an increasing number of studies have reported that SD can lead to both cognitive impairments and abnormal immune and inflammatory responses.[12] The chemokine (C-X3-C motif) ligand 1 (CX3CL1), synthesized as a transmembrane protein, is the only member of CX3C chemokine family, which is highly expressed in hippocampus, the cognitive- and emotional-related brain area. Previous views suggest that CX3CL1 is mainly an

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inflammatory factor and participate in inflammatory response through selectively binding with the CX3C-chemokine receptor 1 (CX3CR1), the only receptor mainly expressed in microglia.[13] *In vitro* studies have shown that CX3CL1 can protect neurons in lipopolysaccharide-activated microglia by reducing the level of pro-inflammatory factors.[14] The blockage of the CX3CL1/CX3CR1 signal axis by CX3CR1 knockout has been found to increase the neuroinflammation.[15] New research found that CX3CL1 can not only influence the number and function of microglia but also affect synaptic pruning and cognitive function.[16-18] The interaction and mechanism between CX3CL1 secreted by neurons and CX3CR1 on microglia are a key axis on the role of microglia.

Synaptic plasticity is the basis of learning and memory. The long-term potentiation recorded from Schaffer collateral CA1 synapses has been found to be impaired in rats subjected to forced locomotion-induced SD for 12 h,[19] which supports that SD attenuates synaptic plasticity in the hippocampus. Recent studies have shown that synaptic pruning is involved in the development of sleep.[20] Within the brain, synaptic pruning is mainly executed by microglia. The process of synaptic pruning involves microglial distinguishing, phagocytosis, digestion, clearance, and regeneration of synaptic components (dendritic spines, presynaptic terminals, synaptic vesicle transporters, etc) to promote synaptic remodeling.[16] However, it remains blurred whether microglial synaptic pruning has a crucial involvement in the pathological process of sleep disorders.

Based on the aforementioned evidence, the mechanism behind cognitive impairment caused by SD may involve microglia inflammation and synaptic pruning which are mainly executed by microglia within the brain, and CX3CR1 is likely to participate in the process, affecting the integrity of central nervous system's (CNS) structure and functional circuits. However, the underlying mechanism remains elusive. In this study, the middle-aged CX3CR1$^{-/-}$ mice and age-matched wild-type (WT) C57BL/6 mice were subjected to acute SD, which mimics the pathophysiological changes of middle-aged people after staying up all night in daily life. We investigated here whether the interference in the microglial function by the CX3CR1 knockout affects the CNS's response to SD, which will help to uncover the role of microglial CX3CR1 signal-mediated physiopathogenesis in the cognition loss caused by sleep disorders.

Methods

Ethics approval

All animal procedures and all experiments observed The National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Care and Use of Fujian Medical University (FJMU IACUC 0314).

Animals and groups

WT male C57BL/6 mice (aged 13-month-old) and age-matched CX3CR1$^{-/-}$ mice were used in this study. The CX3CR1$^{-/-}$ mice were introduced from Jackson Laboratory (Bar Harbor, ME, USA; Stock: 005582) and generated on the C57BL/6 genetic background, in which the *CX3CR1* gene was replaced by a green fluorescent protein (GFP) reporter gene such that homozygote CX3CR1$^{GFP/GFP}$ (CX3CR1$^{-/-}$, knockout) mice are lack functional CX3CR1. Mice were housed in a 12 h light/dark cycle and allowed free access to food and water. Both groups were exposed to two experimental environments, respectively[21]: (1) normal sleeping (S) group; (2) SD group.

Eight-hour sleep-deprived paradigm

SD group mice were exposed to gentle handling and running platforms during the light phase to achieve a total deprivation from 8:00 AM to 4:00 PM. S group mice were exposed to the same environment but with a normal sleep phase. After the deprivation, both groups were sacrificed at the same time (4:00 PM), except those who will undergo behavioral tests. Independent mice of S and SD groups were used for molecular and histological studies.

Behavioral tests

The mice participated in the behavioral tests 2 h after 8 h SD. They were placed in the experimental environments and allowed adjustment for more than half an hour before tests. The apparatus was thoroughly cleaned with 95% alcohol and dried after each test to keep it clean and odorless.

Open field test

The open field test was performed as previously described.[22] Briefly, mice were placed in a square arena ($50 \times 50 \times 50\,cm^3$) of opaque black plexiglass with a central area of $18 \times 18\,cm^2$. Each mouse underwent a single 10-min trial. All performances were recorded with a camera positioned above the apparatus and analyzed automatically with the software and by experimenters blinded to the treatment conditions. The traveled distance and the speed spent in the central and peripheral zones of the arena were recorded.

The fear conditioning test

The fear conditioning test was conducted as previously indicated.[21] Briefly, the fear conditioning apparatus (Sansbio, China) was placed in a quiet room. On the day 1, mice were placed in the conditioned fear box for 2 min (phase A), and the freezing time of animals in the first 2 min was recorded as a baseline. Then a click noise of 80 dB was added for 30 s (phase B). Subsequently, the mice were shocked by 0.35 mA electric current that lasted for 2 s (phase C). After one round of training was completed, another two rounds of the three-phase training (phases A, B, and C) were performed. During the whole training period, the animal's freezing time was recorded. The context test phase took place on the day 2 after training. Mice were put into the same box used on the day 1, and the computer automatically recorded the animal's stagnation behavior. The animal's contextually conditioned fear was measured by recording the freezing time in the operating box for 5 min.

Immunohistochemistry

Three stages, including brain collection, sectioning, and immunolabeling, were performed during immunohistochemical analysis. Although CX3CR1$^{+/-}$ mice are often used to represent mice with normal CX3CR1 function, in strict consideration, we chose WT mice as the normal control to ensure the complete retention of CX3CR1 function. To compensate for the difference of GFP fluorescence between WT and CX3CR1$^{-/-}$ mice, we used ethanol dehydration and paraffin embedding to quench GFP fluorescence as previously described.[22] After anesthesia, mice were first transcardially perfused with 20 mL 0.1mol/L pre-cooled phosphate-buffered saline (PBS) until colorless liquid drained out of the right auricle. Then, pre-cooled 4% polyformaldehyde (PFA) was perfused to fix the tissues. The whole brain was removed and post-fixed in 4% PFA at 4°C overnight. Then, the samples were dehydrated with sucrose and embedded in paraffin. Subsequently, serial coronal sections (5 μm in thickness) were cut from bregma −1.46 to −2.46 mm.

For all the antibodies, the immunolabeling procedure was the same. The paraffin slices were baked at 65°C for 2 h. After three washes with PBS, antigen retrievals were performed in an ethylenediaminetetraacetic acid buffer by microwaving for 10 min. Slices were appropriately cooled at room temperature and then incubated in a 3% hydrogen peroxide solution at room temperature for 10 min in the absence of light, followed by blocking with 5% bovine serum albumin for 10 min. Immunolabeling was then conducted by incubating the slices with different antibodies at 4°C overnight: rabbit anti-ionized calcium-binding adaptor molecule-1 (Iba-1) (1:100, Cambridge, MA, USA), rabbit anti-cellular oncogene fos (c-fos) (1:300, Abcam). After washing, sections were then incubated with specific secondary antibodies, respectively, at 37°C for 50 min. The negative control was treated without primary antibody, which was performed at the same time for accuracy. The slices were examined under an inverted microscope (Olympus Corp., Tokyo, Japan, IX51) with a Micropublisher Q-imaging system. To evaluate the density of microglia and c-fos-positive neurons, the dentate gyrus (DG) of the hippocampus was measured using the Image J software (Version 1.52s, National Institutes of Health, Bethesda, MD, USA). The operators of all analyses were blinded to genotypes and groups.

Golgi staining

Golgi staining was performed using a Hito Golgi-Cox OptimStain^{TM} Kit (Hitobiotec Corp., Kingsport, TN, USA) according to the user's instructions and previous studies.[23] After deep anesthesia, the brain tissue was carefully and quickly removed and transferred to a prepared soaking solution (equal amount of kit solutions 1 and 2) of at least five times volume and stored at room temperature overnight. Next day, the soaking solution was refreshed and the brain tissue was placed at room temperature for 2 weeks without light. Afterward, the tissue was transferred to solution 3 of at least five times volume and stored at 4°C in a lightproof storage. Solution 3 was refreshed 12 h later and 48 h later, successive coronal sections of 150 μm were cut with a freezing microtome (CM1950, Leica, Germany). The sections were examined under a Zeiss LSM 780 confocal microscope with a 100× oil objective. The images of dendritic branches in the hippocampus were thus obtained. The spine density was determined by operators blinded to genotype and groups using Image J.

Western blot

After the removal of the brain tissue, the hippocampus was quickly separated on ice. They were put into the pre-marked cryopreservation tube and immediately transferred to liquid nitrogen for quick freezing. Then, they were quickly transferred to the freezer at −80°C for storage and subsequent use.

The Western blotting was performed with the following primary antibodies: anti-brain-derived neurotrophic factor (BDNF) (1:1000, Abcam), anti-cAMP-responsive element binding protein (CREB) (1:500, Danvers, MA, USA), anti-phosphorylated cAMP-responsive element binding protein (p-CREB) (1:500, CST), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000, Abcam). Forty micrograms of proteins were loaded in each lane. Protein concentrations were measured by the bicinchoninic acid method. The level of each protein was standardized based on GAPDH expression.

Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using the following reaction system: SYBR Green II (Rox) 10 μL, forward primer (300 nmol/L) 0.6 μL, reverse primer (300 nmol/L) 0.6 μL, RNase-free water 6.8 μL, template complementary DNA 2 μL at 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. A total of 40 cycles were executed. Primers were summarized in Table 1. GAPDH was used as an internal control and the relative quantitative method was used to calculate the $2^{-\Delta\Delta CT}$ value of each sample, the ratio of other groups to WT group, as the control group, was used for group comparison.

Statistical analysis

All results were presented as the mean ± standard error of the mean. All analyses were performed with GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA). The data were analyzed by one-way analysis of variance (one-way analysis of variance [ANOVA]). When assessing statistical effects was required by the experiment, one-way ANOVA with repeated measures was performed. Statistical significance was set at P value < 0.050.

Results

CX3CR1 deficiency prevents SD-induced cognitive decline

To investigate the effect of CX3CR1 on hippocampus-dependent learning and memory, the fear conditioning test was performed. Mice were trained and tested with a

Table 1: Primers used in qRT-PCR.

Genes	Primers	Sequences
IL-1β	Forward	ATGCCACCTTTTGACAGTGATG
	Reverse	TGATGTGCTGCTGCGAGATT
Arg-1	Forward	CAATGAAGAGCTGGCTGGTG
	Reverse	GGCCAGAGATGCTTCCAACT
IL-4	Forward	GAACGAGGTCACAGGAGAAGG
	Reverse	AATATGCGAAGCACCTTGGAA
Cx3cr1	Forward	GAGTATGACGATTCTGCTGAGG
	Reverse	CAGACCGAACGTGAAGACGAG
Trem2	Forward	CTGGAACCGTCACCATCACTC
	Reverse	CGAAACTCGATGACTCCTCGG
C3	Forward	CTGGCCCTGATGAACAAACT
	Reverse	GGATGTGGCCTCTACGTTGT
CR3	Forward	GGCTTTGGACAGAGTGTGGT
	Reverse	ACACTGGTAGAGGGCACCTG

Arg-1: Arginase-1; C3: Complement C3; CR3: Complement receptor3; IL-4: Interleukin-4; qRT-PCR: Real-time fluorescence quantitative polymerase chain reaction; Trem2: Triggering receptor expressed on myeloid cells 2.

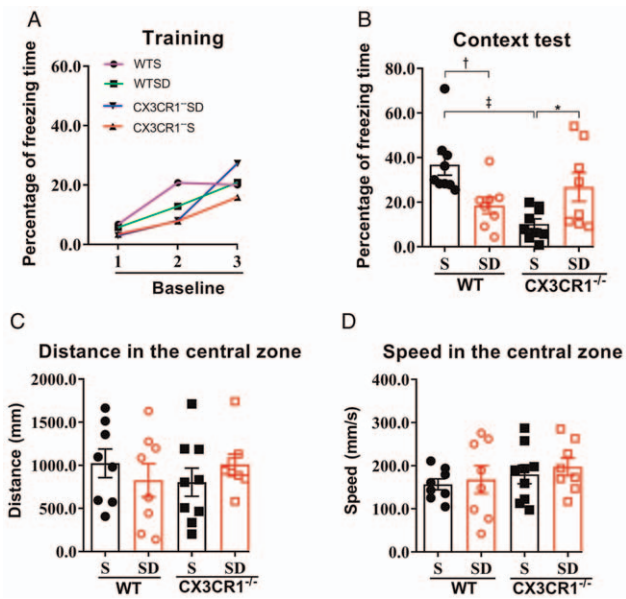

Figure 1: Effects of CX3CR1 deficiency on the SD-induced cognitive decline. (A) The percentage of freezing time of mice in each group during the training period; (B) The freezing response of mice tested 24 h after the training in the conditional fear test; (C) Distances traveled in the center zone in the open field test; (D) Speed traveled in the center zone in the open field test. All data are expressed as mean ± standard error of the mean. $n = 8$–9/group. $CX3CR1^{-/-}$ mice were compared with age-matched WT mice with or without SD. $^{*}P < 0.050$; $^{\dagger}P < 0.010$; $^{\ddagger}P < 0.001$. CX3CR1: CX3C-chemokine receptor 1; SD: Sleep deprivation; WT: Wild-type C57BL/6 mice.

standard protocol as described before.[24] During the training period, the percentage of freezing time in each group was comparable ($P > 0.050$) [Figure 1A], suggesting a similar cognitive function at the basal line. Twenty-four hours after the training phase, significant differences were found among groups in the conditional fear test. In WT groups, the freezing time of SD-treated mice was significantly shorter than that of the normal sleep group ($P < 0.010$) [Figure 1B]. Surprisingly, although the cognitive function of CX3CR1-deficient mice was significantly impaired when compared with that of WT-S mice ($P < 0.001$) [Figure 1B], the freezing time increased significantly in CX3CR1-deficient mice after SD ($P < 0.050$) [Figure 1B], indicating that CX3CR1 deficiency induces a different response to SD and thereby preserving the cognitive function.

To examine the anxiety condition, all groups were tested in the open field task. No differences were observed in either traveled distance or speed in the center zone among groups ($P > 0.050$) [Figure 1C and 1D]. Neither the loss of function of $CX3CR1^{-/-}$ gene nor SD causes excessive anxiety-like behavior in this study. Together, all these results imply that the absence of CX3CR1 prevents the SD-induced cognitive impairment.

Elevated BDNF and CREB phosphorylation in the hippocampus of CX3CR1-deficient mice after SD

The expression of selected neurotrophins BDNF, p-CREB, and CREB in the hippocampus was evaluated by Western blotting [Figure 2A–E]. Compared with the WT mice, the level of BDNF was significantly reduced in $CX3CR1^{-/-}$ mice ($P < 0.010$) [Figure 2B]. Of note, this reduction was dramatically reversed in $CX3CR1^{-/-}$ mice after SD ($CX3CR1^{-/-}$ SD *vs.* $CX3CR1^{-/-}$ S group, $P < 0.050$) [Figure 2B]. In contrast, although there was no significance, a downward trend in BDNF was found in the WT-SD group when compared with WT-S group ($P > 0.050$) [Figure 2B]. No significant differences in CREB expression were found among groups ($P > 0.050$) [Figure 2C]. Regarding the CREB activation, the phosphorylation of CREB was assessed. The $CX3CR1^{-/-}$ SD mice displayed a higher expression level of p-CREB than the $CX3CR1^{-/-}$-S group ($P < 0.001$) [Figure 2D]. When WT-SD was compared, we also observed a significant upward trend in the $CX3CR1^{-/-}$ SD group [$P < 0.001$, Figure 2D]. In addition, the ratio of p-CREB/CREB was also significantly increased in the hippocampus in $CX3CR1^{-/-}$ SD mice when compared with $CX3CR1^{-/-}$ S mice and WT-SD mice ($P < 0.050$, for both) [Figure 2E].

The microglial density in the DG of CX3CR1-deficient mice decreases after SD

Representative activity and distribution of microglia in the DG were illuminated by Iba-1, a marker of microglia [Figure 3A]. Representative images showed an increased Iba-1-positive immunoreactivity in the DG of WT mice after SD. The number of microglia increased 1.8 folds in response to the SD in the WT mice ($P < 0.001$) [Figure 3B], whereas the effect was offset when the function of CX3CR1 was deleted. A notable decrease of Iba-1-positive cells in the DG was observed in CX3CR1-deficient mice after SD ($P < 0.001$, for both) [Figure 3B]. Meanwhile, the absence of CX3CR1 induced a marked increase in Iba-1-positive cells in the DG area when compared with that of the WT mice ($P < 0.010$) [Figure 3B]. These findings indicate that CX3CR1 mediates the SD-induced microglial overactivation, which can be attenuated by CX3CR1 deficiency.

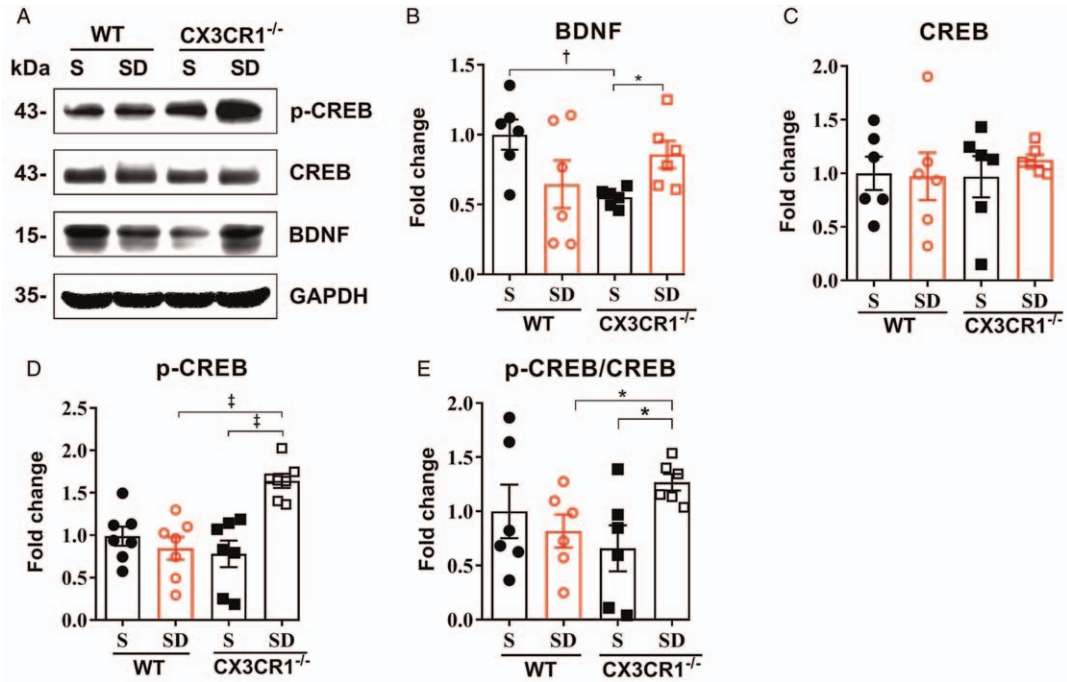

Figure 2: Elevated levels of BDNF and p-CREB proteins in the hippocampus of CX3CR1$^{-/-}$ mice after SD. (A) Representative bands of the BDNF, CREB, p-CREB proteins; (B–E) Quantifications of the relative optical densities of hippocampal BDNF, CREB, p-CREB, and the ratio of p-CREB/CREB. The GAPDH was used as a referral loading protein. All data are expressed as mean ± standard error of the mean. $n = 6$. $^{*}P < 0.050$; $^{\dagger}P < 0.010$; $^{\ddagger}P < 0.001$. BDNF: Brain-derived neurotrophic factor; CREB: cAMP-responsive element binding protein; CX3CR1: CX3C-chemokine receptor 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; p-CREB: Phosphorylated cAMP-responsive element binding protein; SD: Sleep deprivation.

Diverse levels of c-fos expression in the DG are observed in WT and CX3CR1-deficient mice after SD

Immediate early genes (IEGs) refer to a group of genes that are first expressed in neurons after stimulation and are closely related to inflammation. The most characteristic and important one is *c-fos* gene.[1] The expression of c-fos in the DG region of the hippocampus was detected by labeling of c-fos [Figure 3C]. After SD, the average positive staining area percentage of c-fos in the DG was significantly higher in the WT-SD group than that in WT-S mice ($P < 0.010$) [Figure 3D]. Interestingly, in CX3CR1$^{-/-}$ groups, the c-fos expression was lower in SD group than that in S group ($P < 0.050$) [Figure 3D]. After SD, the c-fos level in CX3CR1-deficient mice was significantly lower than that of WT mice ($P < 0.001$) [Figure 3D]. These results indicate a differential alteration in microglia-neuron interaction and neuronal activity between the WT and CX3CR1-deficient mice after SD and that the CX3CR1-deficient mice fail to react to the SD-induced neuronal stress response, thus preserving the neuronal functions.

Altered levels of pro-inflammatory and anti-inflammatory cytokines in the hippocampus of CX3CR1-deficient mice after SD

To further evaluate the SD-triggered inflammatory response in the hippocampus, we examined the expression levels of pro-inflammatory and anti-inflammatory cytokines in the hippocampus using qRT-PCR. As expected, an upward trend of pro-inflammatory levels was found in the WT group after SD ($P < 0.010$) [Figure 4A]. The interleukin-1 beta (IL-1β) expression in the DG was markedly decreased in CX3CR1-deficient mice after SD when compared with the CX3CR1$^{-/-}$ S group and WT-SD group ($P < 0.010$, for both) [Figure 4A]. Arginase-1 (Arg-1) and interleukin-4 (IL-4) were chosen as typical representatives of anti-inflammatory factors. Compared with the CX3CR1$^{-/-}$-S group, CX3CR1$^{-/-}$-SD mice showed a significant increase in Arg-1 ($P < 0.010$) [Figure 4B]. The mRNA level of Arg-1 significantly increased in the CX3CR1-deficient mice after SD (CX3CR1$^{-/-}$-SD *vs.* WT-SD, $P < 0.001$) [Figure 4B]. A similar trend of IL-4 was found in the CX3CR1-deficient mice. The level of IL-4 in CX3CR1$^{-/-}$-SD group was higher than that in the CX3CR1$^{-/-}$-S group, although with no statistical difference. Moreover, CX3CR1 deficiency significantly reduced the level of IL-4 ($P < 0.050$) [Figure 4C]. Taken together, these data support the notion that SD-induced pernicious neuroinflammation is decreased in CX3CR1-deficient mice.

The spine density in the DG is better retained and accompanied with the decrease of phagocytosis-related factors in CX3CR1-deficient mice after SD

We next determined whether memory restoration in CX3CR1-deficient mice after SD was reflected at the spine construction. Mid-dendritic segments of the hippocampal DG area, which are the third dendrite branches of granular layer cells, were analyzed using the Golgi staining [Figure 5A–D]. The analysis showed a remarkable decrease in the spine density in middle-aged WT-SD mice when compared with that of WT-S mice ($P < 0.010$) [Figure 5E]. In the CX3CR1-deficient groups, opposite noteworthy

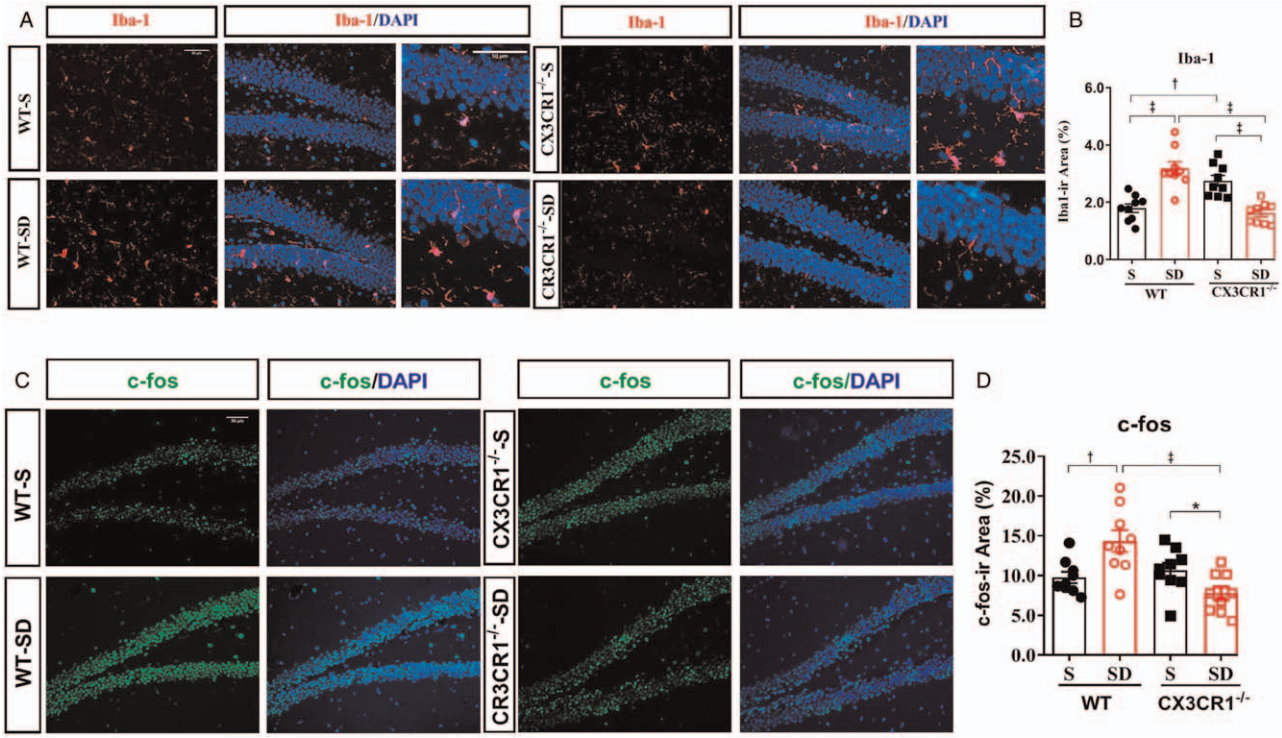

Figure 3: Decreased microglial density and c-fos expressions in the DG area of CX3CR1$^{-/-}$ mice after SD. (A) Representative images of Iba-1 immunofluorescence in the DG region; (B) Percentage of Iba-1-positive immunoreaction in the DG; (C) Representative images of c-fos immunofluorescence in the DG area; (D) Percentage of c-fos positive immunoreaction in the DG. All data are expressed as the mean ± standard error of the mean. Scale bar: 50 μm. $n = 9$. $^{*}P < 0.050$; $^{\dagger}P < 0.010$; $^{\ddagger}P < 0.001$. c-fos: Cellular oncogene fos; CX3CR1: CX3C-chemokine receptor 1; DG: Dentate gyrus; Iba-1: Ionized calcium-binding adaptor molecule-1; SD: Sleep deprivation.

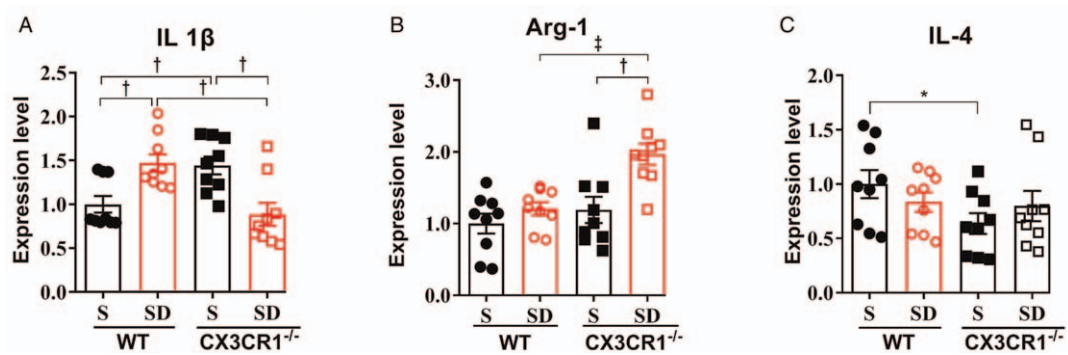

Figure 4: Alterations in inflammatory cytokines in the hippocampus of WT or CX3CR1$^{-/-}$ mice after SD. Quantifications of the relative expressions of mRNA level of IL-1β (A), Arg-1 (B), and IL-4 (C) in the hippocampus. All data are expressed as mean ± standard error of the mean. $n = 9$. $^{*}P < 0.050$; $^{\dagger}P < 0.010$; $^{\ddagger}P < 0.001$. Arg-1: Arginase-1; CX3CR1: CX3C-chemokine receptor 1; IL-1β: Interleukin-1 beta; IL-4: Interleukin-4; SD: Sleep deprivation; WT: Wild-type C57BL/6 mice.

differences were found between SD mice and S mice; a significantly higher spine density was observed in CX3CR1$^{-/-}$-SD mice when compared with that of the CX3CR1$^{-/-}$-S mice ($P < 0.050$) [Figure 5E]. Meanwhile, the CX3CR1 deficiency significantly reduced the spine density (CX3CR1$^{-/-}$-S *vs.* WT-S, $P < 0.001$) [Figure 5E], while after SD, the spine density was even higher in CX3CR1$^{-/-}$-SD mice when compared with WT-SD mice, although without significance. Altogether, these data indicate that CX3CR1 deficiency partially restores the reduction of synapses in the DG area of the SD mice.

Besides the CX3CL1/CX3CR1 system, several other microglia-specific pathways are required in the process of synaptic elimination, including the classical complement signaling and triggering receptor expressed on myeloid cells 2 (TREM2) in microglia. We first tested the expression level of CX3CR1 in all groups by qRT-PCR. As expected, almost no expression of CX3CR1 was seen in CX3CR1$^{-/-}$ groups and the CX3CR1 expression significantly increased in the WT mice after SD ($P < 0.050$) [Figure 6A]. The TREM2 is required for the phagocytosis of microglia. In WT mice, the upregulated expression of TREM2 was evident in the SD group ($P < 0.010$) [Figure 6B], whereas in CX3CR1-deficient mice, the decrease was significant in SD mice when compared with S mice ($P < 0.001$) [Figure 6B]. CR3 is the classical receptor of complement C3 and the specific pattern

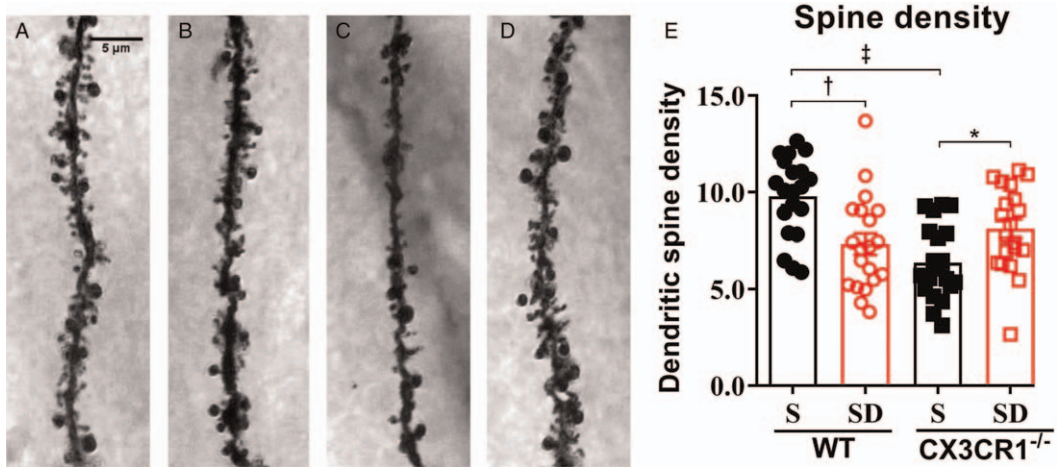

Figure 5: Increased spine density in the DG area of CX3CR1$^{-/-}$ mice after SD. (A–D) Representative images of Golgi-Cox stained dendrites in the DG area of CX3CR1$^{-/-}$ and WT mice in each group. (A) WT-S mice; (B) WT-SD mice; (C) CX3CR1$^{-/-}$-S mice; (D) CX3CR1$^{-/-}$-SD mice; and (E) Quantification of the spine density in the DG area using Image J. All data are expressed as the mean ± standard error of the mean. Scale bar: 5 μm. $n = 20$ dendritic segments/group. $^{*}P < 0.050$; $^{\dagger}P < 0.010$; $^{\ddagger}P < 0.001$. CX3CR1: CX3C-chemokine receptor 1; DG: Dentate gyrus; S: Normal sleep; SD: Sleep deprivation; WT: Wild-type C57BL/6 mice.

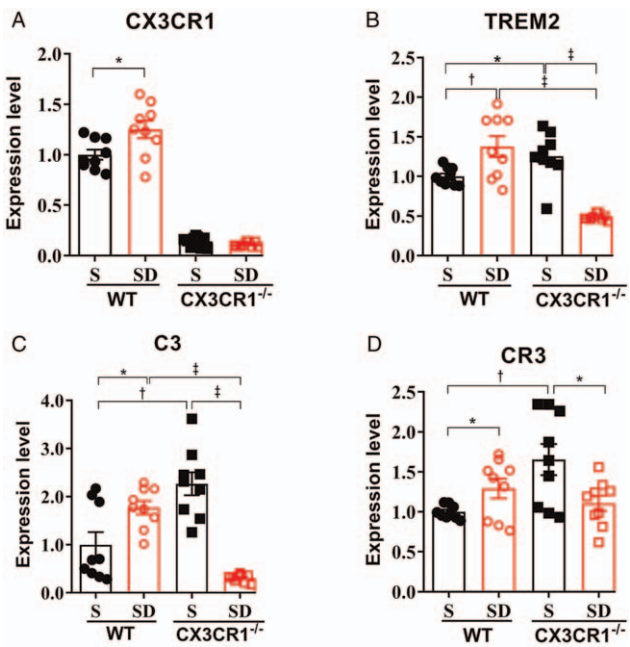

Figure 6: Alterations in phagocytosis-related factors in the hippocampus of WT or CX3CR1$^{-/-}$ mice after SD. Quantifications of the relative expression mRNA levels of CX3CR1 (A), TREM2 (B), C3 (C), and CR3 (D) in the hippocampus. All data are expressed as mean ± standard error of the mean. $n = 9$. $^{*}P < 0.050$; $^{\dagger}P < 0.010$; $^{\ddagger}P < 0.001$. C3: Complement C3; CR3: Complement receptor 3; CX3CR1: CX3C-chemokine receptor 1; SD: Sleep deprivation; TREM2: Triggering receptor expressed on myeloid cells 2; WT: Wild-type C57BL/6 mice.

recognition receptor of microglia. The C3/CR3 signaling pathway also plays an important role in microglial phagocytosis. A similar trend with the former two factors was found: in the groups of CX3CR1$^{-/-}$, C3 and CR3 levels decreased significantly after SD (respectively, $P < 0.001$ and $P < 0.050$) [Figure 6C and 6D]; conversely, a significant increase in C3 and CR3 expression in the hippocampus was found after SD in WT mice ($P < 0.050$, for both) [Figure 6C and 6D]. Thus, we confirm that the deficiency of CX3CR1 in the middle-aged WT mice can cause completely different changes of microglial phagocytic capacity.

Discussion

It has become a consensus that sleep disorders impair cognitive function, although the mechanism is still unclear.[21] Tens of thousands of people suffer from insomnia and many middle-aged people are forced to stay up all night because of various pressures. Obviously, understanding the mechanisms by which SD leads to cognitive impairments can help those who suffer from SD to combat the deleterious effects.

The present study rarely reveals a pivotal role of CX3CR1 through microglial activity in the suppression of hippocampal-related memory decline after the SD in middle-aged mice. As we all know, a lack of sleep can lead to serious cognitive impairment, whereas in this study, unlike the WT mice, the cognitive function of CX3CR1-deficient mice was improved rather than impaired after SD. At the same time, the inhibition of microglial activity, the decreased pernicious neuroinflammation, and the upregulated hippocampal BDNF, p-CREB level, were found in the CX3CR1-deficient mice after SD. Consistent with the improved cognitive function, CX3CR1 deficiency restored the abnormal synaptic pruning process and the density of the spines in the DG after SD. The modulation of the microglial phenotype by targeting the CX3CR1 pathway might serve as a way to restore the microglial homeostasis and to treat sleep disorder-associated cognition loss.

Previous studies have shown that hippocampus-dependent memory is particularly sensitive to SD.[25] In the present study, middle-aged mice were selected for 8-h acute SD and received the fear conditioning test, which enables researchers to examine hippocampus-dependent cognitive function by testing their ability to connect electric shock to a purposeful environment. WT-SD mice showed a

significant cognitive decline in the conditioned fear test as expected. However, although CX3CR1 knockout showed cognitive impairment in the normal sleep group, unexpectedly, unlike the WT mice, the cognitive function in CX3CR1-deficient mice after SD was distinctively improved, which, is a rare study of its kind in CX3CR1 knockout mice. Maggi *et al*[26] found that enriched environment may attenuate cognitive loss and improve synaptic plasticity but may not affect CX3CR1-deficient mice. It seems that CX3CL1/CX3CR1 signal axis plays an important role in the process of environmental intervention on cognitive function.

For SD experiment, stress is an inevitable factor. The spontaneous activity and anxiety state of mice were evaluated by the open field test, which found no significant differences among groups. As mice had similar freezing time in the training phase in the fear conditioning test, it is unlikely that stress leads to the changed cognitive function after SD.

We observed an improvement in BDNF levels, a neurotrophic factor widely distributed in the CNS, in the hippocampus along with the ameliorative hippocampus-dependent cognitive function in CX3CR1-deficient mice after SD, which is the opposite of the WT group. BDNF is one of the most important regulators of synaptic plasticity, closely related to cognitive function.[27] It has been confirmed to modulate ligand-gated channels such as *N*-methyl-D-aspartic acid and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionicacid receptors. Studies show that serum BDNF levels in patients with early stage of AD can be increased by donepezil treatment [28] and more recent studies evidence its role in sleep regulation.[29] Wong *et al*[30] have found that the deletion of p75, a receptor possibly interacting with BDNF precursor, prevents SD-mediated decrease in the hippocampal BDNF and improves the cognitive behavior. CREB is a transcription factor directly related to BDNF expression. In the present study, we found no differences in the level of hippocampal CREB among groups, but, after SD, the CX3CR1-deficient mice had a notable elevated level of p-CREB. Abundant experiments have proved that the CREB–BDNF pathway plays a key role in learning and memory processes and that, using plasmid transfection, the increased level of CREB can significantly reduce the apoptosis of neurons and promote the survival of neurons, which is closely related to the level of p-CREB.[31] Taken together, in the present study, the absence of CX3CR1 leads to the resistance to the harmful effects of SD on cognitive function at both behavioral and protein levels.

IEGs are a group of informative neural activity markers, of which c-fos is the most characteristic and important.[32] c-fos is called "the third messenger" for it transforms external signals into gene expression and has the characteristics of signal transmission. We validated hippocampal neuron response by c-fos immunofluorescence and found that the c-fos-positive neurons were activated by SD in WT mice. However, most intriguingly, the c-fos-positive neurons reached a much lower level after SD in CX3CR1-deficient mice. The SD influences on c-fos level are consistent with previous studies in WT mice, and increased c-fos level is observed to be independent of age, various SD durations, and SD patterns.[33] It should be noted that the high expression of c-fos occurs not only during SD but also during spontaneous wake, indicating that its elevation is not due to the stress of SD.[34] As a famous third messenger, the protein expressed by *c-fos* gene regulates the response of the body to external stimulation by activating or inhibiting its target genes,[35] including inflammatory response. Zhang *et al*[36] have reported that both c-fos and pro-inflammatory genes were promoted by high salt, whereas the upregulation of pro-inflammatory factors can be notably eliminated by the selective inhibitor of c-fos. In the present study, the activation of c-fos after SD was significantly reduced by CX3CR1 knockout, according with the ameliorated memory ability. These data indicate that CX3CR1 deficiency may mediate the excessive inflammatory response caused by SD through the activation of c-fos, thereby leading to cognitive impairment.

Sleep is closely related to the immune system. Microglia are the most common macrophages in the CNS and a loyal guardian against the stimulation of external environment.[18] In this study, a prominent increase in number of the Iba-1-positive microglia in DG regions was found in the WT mice after SD, which tends to decrease in the absence of *CX3CR1* gene. CX3CR1 belongs to the fractalkine receptor family and is supposed to be exclusively expressed on the microglia in the brain, which is important for mediating neuron–microglia interaction under both pathological and physiological conditions.[37] CX3CR1 is the unique receptor of neuronally expressed CX3CL1.[38] Neuroinflammation occurs widely in a variety of CNS diseases related to cognitive impairment. As previously shown, some pro-inflammatory phenotypes are seen in the DG area in the absence of CX3CR1.[39] In AD, deficiency of CX3CL1/CX3CR1 axis leads to an increasing expression of pro-inflammatory molecules especially IL-1β which triggers a massive cell death.[40,41] IL-1β is the first cytokine found to be able to promote sleep, which triggers a lot of inflammatory cascades in addition to participating in sleep regulation.[12] In the detection of pro-inflammatory factors represented by IL-1β, our study has obtained similar results. SD causes the overactivation of microglia and oversecretion of pro-inflammatory factors in the WT group. Surprisingly, we found a rather low expression of IL-1β and high level of anti-inflammatory factors Arg-1 and IL-4 in the hippocampus in CX3CR1-deficient mice after SD. The reason for these results may be that CX3CR1 deficiency prevented the normal increase of sleep promoting factors such as IL-1β after SD, thus inhibiting the subsequent abnormal inflammatory cascade. Meanwhile, inflammatory microglia have been found to inhibit the recovery of CNS, increase excitotoxicity, increase oxygen free radicals, and aggravate necrosis and apoptosis of nerve cells.[42] Based on the aforementioned information, the microglial activation may be involved in the SD-induced cognitive impairment through the modulation of inflammatory factors, as CX3CR1 and the previously mentioned inflammatory cytokines are candidates underlying the SD resistance in the $CX3CR1^{-/-}$ mice. CX3CR1 knockout can prevent this abnormal inflammatory response and improve cognitive function after SD.

In general terms, the overactivation of CX3CR1 in microglia can induce inflammatory damage, but a limited activation of CX3CR1 may play a neuroprotective role, in which synaptic pruning plays an important role, a process that can clear the dead cells and synapses in physiological and pathological condition, thus promoting the regeneration of synapses and myelin sheaths in the process of brain injury repairmen.[43] Electron microscopy and two-photon imaging *in vivo* research have found many synapses and spines become smaller and disappear after contacting microglia, which directly confirms that microglia have significant synaptic pruning effect. After dark stimulation, microglia change their morphology and synaptic contact mode, indicating that microglia participate in the brain synaptic pruning and remodeling after an environmental stimulation, in which, sleep is thought to affect the synaptic pruning process of microglia.[44] Other researchers reconstructed and analyzed 6920 synapses in the brain of mice using serial scanning 3D microscopy, and measured their sizes. Of note, both in middle-aged WT-SD and age-matched CX3CR1$^{-/-}$ S mice, the activated microglia were related to fewer synapses in the DG area. A previous study showed that the inhibition of microglial overactivation significantly reduced the extent of early synapse loss in adult AD mice.[45] Yang *et al*[46] also found that after learning different skills, new dendritic spines appeared in mice. A reasonable explanation for our results is that the overactivated microglia performed an incorrect synaptic pruning procedure, and the synapses are abnormally engulfed and eliminated, thus affecting the growth of new synapses. Unexpectedly, this abnormal synaptic pruning process was offset in CX3CR1$^{-/-}$ SD mice in the present study. Our data showed that CX3CR1$^{-/-}$ SD mice had more synapses and better cognitive function. Giri *et al*[47] proved that SD reduced neuronal dendritic length, branching, arborization, and spine density in hippocampus, which is in line with our results. It was also found that compared with the waking state, the sleeping state is more conducive to the correct implementation of synaptic pruning.[48] It is believed that the binding of soluble CX3CL1 to its receptor CX3CR1 maintains the "synapse pruning" function of microglia.[49] Thus, the knockout of CX3CR1 in the present study may prevent overactivated microglia from recognizing normal synapses, thus inhibiting the subsequent abnormal after SD due to the deficiency of CX3CL1/CX3CR1 pathway. Strong evidence shows that enough synapses are extremely important for the formation and maintenance of learning and memory. Our findings support the notion that, in CX3CR1-deficient mice, the microglial activation and the related abnormal excessive synaptic pruning induced by SD are reduced, eventually improving the related cognitive function. Interestingly, Milior's study showed that CX3CR1-knockout mice did not respond to depression behaviors in harsh environments such as chronic stress.[37] The aforementioned findings suggest that the deficiency in CX3CR1/CX3CL1 signaling pathway may prevent the harmful reconstruction of brain structure and function and the change of cognitive behavior caused by environmental stimulation.

A previous study found that the number of microglia and dendritic spine density increased in CX3CR1-knockout mice at the age of 2 to 3 weeks. However, at 40 days after birth, the number of microglia and dendritic spine density returned to the same level as that of WT mice.[17] As far as we know, little data are available regarding the microglial status of middle-aged and old CX3CR1$^{-/-}$ mice. The present study showed that compared with the WT mice, the absence of CX3CR1 showed a marked increase in the number of microglia in the DG area and decrease in dendritic spine density in the middle-aged mice. The synaptic pruning declines in the microglia during aging. The CX3CR1/CX3CL1 pathway is a pivotal signal path to initiate the "find me" phase, after which the microglia can bind with target synapses to perform phagocytosis and clearance.[50] In the present study, CX3CR1$^{-/-}$ SD mice showed better cognitive function, associated with higher synaptic density. The rationale for the previously mentioned facts is that, in middle-aged mice, the absence of CX3CR1 partially reverses the overactivation of microglia and the corresponding abnormal synaptic pruning after SD. For those who are activated, the lack of "find me" signal limits their inappropriate phagocytosis of synapses, thus combining with more synapses and protecting the integrity of synaptic links and neural networks. Therefore, this change in microenvironment prevents the disadvantages caused by SD.

To further observe the microenvironmental changes related to "synaptic pruning" in the brain of the middle-aged CX3CR1$^{-/-}$ mice after SD, we focused on other factors closely related to the "synaptic pruning" function of microglia, which also links microglial responses to specific stimuli. The TREM2 is uniquely expressed on microglia and is required for microglial synaptic pruning other than CX3CR1.[51] We found a significant decrease in the level of TREM2 in the hippocampus after SD in CX3CR1$^{-/-}$ mice, which had a higher dendritic spine density. This result is consistent with a previous study that documents that the decreased expression of TREM2 is related to lower phagocytic properties using cell culture system.[52] Similar to TREM2, C3, another factor involved in synaptic pruning-complement proteins, was also reduced in CX3CR1$^{-/-}$ mice after the stimuli of SD. It has been proved that C3 deposit can provoke microglial phagocytosis of synapses. In this regard, during SD, the lack of CX3CR1 may give rise to a low expressing level of phagocytosis-related factors such as TREM2 and C3, eventually reducing the synaptic pruning exerted by microglia. Therefore, CX3CR1 deficiency might lock microglia in a homeostatic state and block essential functions of microglia during the progression of sleep disorders.

In conclusion, our results indicate that the CX3CR1 has an extremely important role in regulating the CNS response to SD and the interplay between external stimulation and brain function. The deficiency of CX3CR1 contributes to the recovery from the SD-induced cognitive dysfunction. Although the underlying mechanism is not fully understood, the inflammation-attenuating activity and the related modification of synaptic pruning indicated in the present study make CX3CR1/CX3CL1 a candidate therapeutic pathway for the prevention of cognitive impairment associated with sleep loss.

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Conflicts of interest

None.

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