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The Ameliorative Effect of Fluoxetine on Neuroinflammation

Induced by Sleep Deprivation

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Sleep deprivation; astrocytes; NLRP3 inflammasomes; STAT3; fluoxetine

Abbreviations used:

SD, sleep deprivation; NLRP3, nucleotide-binding domain and leucine-rich repeat protein-3; SSRIs, specific serotonin reuptake inhibitors; MDD, major depressive disorder; STAT3, signal transducer and activator of transcription 3; NLR, nucleotide-binding oligomerization domain-like receptor; ASC, apoptosis-associated speck-like protein containing a CARD; CUMS, chronic unpredictable mild stress; GSK-3β, glycogen synthase kinase-3β; PI3K, phosphatidylinositol-3-kinase; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; P2X7R-KO, P2X7 receptor knockout; RTK, receptor tyrosine kinase; DAPI, 4',6-diamidine-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; dBcAMP, dibutyryl cyclic AMP.

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Abstract

It is well known that sleep disorders are harmful to people’s health and performance, and growing evidence suggests that sleep deprivation (SD) can trigger neuroinflammation in the brain. The nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasome is reported to be relevant to the neuroinflammation induced by SD, but the regulatory signaling that governs the NLRP3 inflammasome in SD is still unknown. Meanwhile, whether the regulatory action of antidepressants in astrocytes could affect the neuroinflammation induced by SD also remains obscure. In this study, we were the first to discover that the antidepressant fluoxetine, a type of specific serotonin reuptake inhibitor (SSRI) widely used in clinical practice, could suppress the neuroinflammation and neuronal apoptosis induced by SD. The main findings from this study are as follows: 1) SD stimulated the expression of activated NLRP3 inflamasomes and the maturation of IL-1β/18 via suppressing the phosphorylation of STAT3 in astrocytes; 2) SD decreased the activation of AKT and stimulated the phosphorylation of GSK-3β, which inhibited the phosphorylation of STAT3; 3) the NLRP3 inflammasome expression stimulated by SD was partly mediated by the P2X7 receptor; 4) an agonist of STAT3 could significantly abolish the expression of NLRP3 inflamasomes induced by an agonist of the P2X7 receptor in primary cultured astrocytes; 5) the administration of fluoxetine could reverse the stimulation of NLRP3 inflammasome expression and function by SD through elevating the activation of STAT3. In conclusion, our present research suggests the promising possibility that fluoxetine could ameliorate the neuronal impairment induced by SD.
**Introduction**

Sleep is indispensable for human life, reduced or disrupted sleep is harmful to cognition, emotions and overall health. Sleep deprivation (SD) impairs several physiological processes, including immunity and cognition (Van Cauter et al., 2008; Irwin et al., 2008). Recently, growing evidence has suggested that SD can trigger neuroinflammation (Zielinski et al., 2017). The nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasome contributes to neuronal damage in several pathological states, in particular in mood disorders and cognitive diseases as well as in SD, although peculiarities of the regulation of the NLRP3 inflammasome in the latter pathology remain unclear (Sahin et al., 2016; VanItallie, 2017; Zielinski et al., 2017). Treatment with fluoxetine (a widely used antidepressant) inhibits activation of NLRP3 inflammasome in major depressive disorder (Alcocer-Gómez et al., 2017). Based on this evidence we addressed the question of whether the activation of NLRP3 inflammasome induced by SD could be reversed by fluoxetine.

Astroglia, the homeostatic cells of the brain (Verkhratsky and Nedergaard, 2016), contribute to the majority of neurological disorders (Pekny et al., 2016). In particular, astrocytes play a primary role various neuropsychiatric diseases; in major depressive disorder (MDD) astrocytes undergo atrophy and pathological remodelling (Wang et al., 2017, Verkhratsky et al., 2014). Our previous studies demonstrated that antidepressant fluoxetine, a type of specific serotonin reuptake inhibitor (SSRI), exerts therapeutic action by directly stimulating astroglial 5-HT$_{2B}$ receptors (Peng et al., 2014; Hertz et al., 2015). Antidepressants have been also considered clinically effective for treating the SD (Giedke et al., 1990). The rapid eye movement (REM) latency has been suggested an indicator of depression (Kupfer and Foster, 1972), and long-term sleep disturbance causes health damage; for example, it elevates the risk of metabolic and mental disorders (Dworak et al., 2011; Armitage, 2007). Moreover,
in a long-term SD model, the underlying regulatory effects of fluoxetine on astrocytes remain poorly understood. The nucleotide-binding oligomerization domain-like receptor (NLR) protein family includes the NLRP1-14 and ICE-protease-activating factor (IPAF) subfamilies, which form inflammasome complexes in response to various forms of physiological or pathological stimulation (Wong et al., 2016; Jian et al., 2016). In the NLRP3 inflammasome, the NLRP3 scaffold interacts directly with the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD), which promotes the activation of cysteine protease caspase-1 from the inactive precursor pro-caspase-1. Activated caspase-1, in turn, triggers the maturation of pro-IL-1β to IL-1β or of pro-IL-18 to IL-18 and the secretion of the product (Zielinski et al., 2017). Extracellular ATP, acting through the ionotropic P2X₇ receptor (P2X₇R), leads to K⁺ efflux, which is required for NLRP3 inflammasome activation (Pelegrin et al., 2007; Karmakar et al., 2016). Activation of the P2X₇ receptor with subsequent stimulation of the NLRP3 inflammasome in the mouse hippocampus induced depression-like behaviors (Yue et al., 2017). The level of ATP in the cortex increases during sleep deprivation (Dworak et al., 2010).

However, whether P2X₇R is involved in the activation of the NLRP3 inflammasome in SD remains unknown. In addition, chronic unpredictable mild stress (CUMS) increases the activation of glycogen synthase kinase-3β (GSK-3β), and the latter inhibits the phosphorylation of STAT3 (signal transducer and activator of transcription 3) and up-regulates the expression of NLRP3 (Liu et al., 2015). Moreover, fluoxetine can activate the signaling pathways of phosphatidylinositol-3-kinase (PI3K)/AKT and MAPK/ERK₁/₂ by transactivating the epidermal growth factor receptor (EGFR) in astrocytes (Li et al., 2008; Ren et al., 2015). In astrocytic or C2C12 cells, there is cross-talk between PI3K/AKT and MAPK/ERK₁/₂ (Ren et al., 2015; Pijet et al., 2013), and these two signaling pathways both regulate the activation of STAT3 (Pijet et al., 2013; Liu et al., 2015). However, the exact regulatory effect of fluoxetine on the phosphorylation of STAT3 is still unknown in astrocytes, particularly under the pathological condition of sleep deprivation.

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In the present study, we found that SD induced P2X7R-mediated formation of NLRP3 inflammasomes in astrocytes, which promoted astroglial release of IL-1β and IL-18 that exerted neurotoxicity. The antidepressant fluoxetine increased the activation of STAT3 and suppressed formation of the NLRP3 inflammasome, thus preventing SD-induced neuronal impairment.

**Experimental Procedures**

**Animals**

As in our previous research (Xia et al., 2017), C57BL/6 (B6; #000664, RRID:IMSR_JAX:000664), FVB/N-Tg(GFAPGFP)14Mes/J (GFAP-GFP; #003257, RRID:IMSR_JAX:003257) and B6.129P2-P2rx7tm1Gab/J (P2X7R-KO; #005576, RRID:IMSR_JAX:005576) mice (all purchased from the Jackson Laboratory, Bar Harbor, ME, USA) were used. Newborn B6 mice were bred in the experimental animal center of China Medical University. Experiments were performed on 3 month old male mice (~25 g), which were kept in standard housing conditions (22 ± 1°C; light/dark cycle of 12 h/12 h) with food and water available *ad libitum*. All B6, GFAP-GFP and P2X7R-KO mice were randomly assigned to different experimental groups with a random number table. All operations were carried out in accordance with the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023) and its 1978 revision. All experimental protocols were checked and approved by the Institutional Animal Care and Use Committee of China Medical University. The study was not pre-registered. To minimize the numbers of animals used, we improved the experimental design and statistical analysis used in this study. The number of animals used in every experimental group was six, it is the lowest number for the experimental design *in vivo*, considering the effective statistical analysis. The built model of sleep deprivation in this study was a
non-invasive method which could not trigger the pain sense of animals. Unless the anesthesia was operated, the mice were intraperitoneally injected with ketamine (80 mg/kg) and xylazine (10 mg/kg).

**Materials**

Most chemicals, including fluoxetine (F132), BzATP (B6396), DNaseI (AMPD1), U0126 (U120), LY294002 (L9908), DAPI (4′,6-diamidine-2-phenylindole) dihydrochloride (D8417), and a primary antibody raised against β-actin (A5441; RRID:AB_476744) were purchased from Sigma (USA). Other primary antibodies were acquired from Millipore (USA). Alexa Fluor-conjugated or horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA). Colivelin (#3945) was supplied by Tocris Bioscience (UK). Chemicals for the preparation of cell sorting medium were obtained from Gibco Life Technology Invitrogen (USA).

**Sleep Deprivation (SD) and Drug Treatment**

SD was induced by “gentle handling” according to standard protocols (Franken et al., 1991), including the introduction of new objects into the cage or gentle touching with a brush to keep the mice awake. SD was induced for 6 hours, which began at 7 a.m. and ended at 1 p.m. During SD, the mice continually had access to food and water. Animals in the sham group were kept undisturbed in a separate room with the same light/dark cycle as the SD group. The treatment with SD was continued for 1-5 weeks. This study adopted the commonly used time point of 3 weeks; the experimental timeline is shown in Supplementary Figure 1. The mice were treated with sham or SD stimulation for 3 weeks. During the third week, U0126 (10 μmol in 2 μL for intracerebroventricular (ICV) infusion), LY294002 (10 μmol in 2 μL for ICV
infusion), or vehicle (artificial cerebrospinal fluid, ACSF) was injected each day; 30 min later, fluoxetine (10 mg/kg) or normal saline was injected intraperitoneally.

**Primary Culture of Astrocytes**

Astrocytes were cultured as described previously (Li et al., 2008 and 2011); in brief, primary cultures of mouse astrocytes were prepared from the cerebral hemispheres of newborn C57BL/6 mice. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 7.5 mM glucose. For the entire third week, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. All dishes of primary cultured astrocytes were labeled with numbers and randomly separated into different experimental groups with a random number table.

**Immunohistochemistry**

The brain tissue was fixed by immersion in 4% PFA and cut in 100 µm slices. Immunohistochemistry was performed as previously described (Xia et al., 2017). The following primary antibodies were used: mouse anti-phosphorylated STAT3 (1:200), rabbit anti-NeuN (1:250) (MABN140; RRID:AB_2571567), chicken anti-GFAP (1:200) (MAB3402C3; RRID:AB_10916759). The slices were incubated with Alexa Fluor-conjugated secondary antibodies for 2 hours at room temperature (1:450). DAPI (1:2000) was used to identify cell nuclei. Immunofluorescence was imaged using a confocal scanning microscope (IX81, Olympus, Tokyo, Japan). To evaluate the level of phosphorylated STAT3 (p-STAT3), we measured the mean intensity of p-STAT3 immunofluorescence within cortical regions. The background intensity of each image was measured in cell-free parenchyma in the same field of view and subtracted from the total immunofluorescence intensity. The intensity of p-STAT3 immunofluorescence from each group was normalized to the intensity of the control group.

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TUNEL Staining and Analysis

To evaluate neuronal apoptosis in the frontal cortex, we performed TdT-mediated dUTP-biotin nick end labeling (TUNEL) in conjunction with immunofluorescent staining for NeuN as described by Chen et al. (2017). Briefly, the formalin-fixed brain slices were incubated overnight at 4°C with anti-NeuN antibody (1:250) (MABN140; RRID:AB_2571567) followed by incubation with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:400) (sc-2040; RRID:AB_631743) for 2 hours at room temperature. TUNEL staining was performed using an in situ cell death detection kit (#11684795910) purchased from Roche-Sigma (USA), following the manufacturer’s protocol. Finally, the sections were incubated with DAPI (1:2000). The number of cells double-labeled with TUNEL and NeuN signal in each field was calculated at ×40 magnification imaged via a confocal scanning microscope (IX81, Olympus, Tokyo, Japan) by an investigator blinded to the experimental design. The ratio of TUNEL and NeuN double positive cells/NeuN positive cells were totaled for five brain slices from every mouse. Similar cortical sections from six mice in every experimental group were assessed, and the average percentage of TUNEL+ and NeuN+ /Total NeuN+ was statistically analyzed.

Dissociation and Fluorescence-activated Cell Sorting (FACS)

FVB/N-Tg(GFAPGFP)14Mes/J mice were used for isolating astrocytes. A single-cell suspension from the cortex and hippocampus was prepared as previously described (Xia et al., 2017). In brief, tissue from 3 mice was pooled for one sample. Wavelengths of 488 nm and 530/30 nm were used for GFP excitation and emission, respectively. GFP+ cells were sorted and collected. The purity of astrocytes sorted by this method has been confirmed in our previous works (Fu et al. 2012) by measuring the mRNA expression of cell markers of astrocytes, neurons and oligodendrocytes.

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Western blotting

Western blotting was performed as previously described (Li et al., 2011; Li et al., 2016). After being blocked with powdered skim milk, sections were cultured for 2 h with the primary antibodies at room temperature. After the sections were washed, specific binding were detected with goat anti-rabbit (sc-2007; RRID:AB_631740) or goat anti-mouse (sc-2005; RRID:AB_631736) horseradish peroxidase-conjugated secondary antibodies. Staining was visualized with ECL detection reagents, and images were acquired with an electrophoresis gel imaging analysis system. Band density was measured in Windows AlphaEase FC 32-bit software.

Enzyme-Linked Immunosorbent Assay (ELISA)

As we have described previously (Li et al., 2016; Xia and Zhu, 2015), IL-1β, IL-18 and TNF-α in the culture supernatant or in the tissues were measured with mouse IL-1β (MLB00C), IL-18 (#7625) and TNF-α (MTA00B) immunoassay kits (R&D Systems, MN, USA). In brief, the measurement was conducted according to the manufacturer’s protocol. Samples were collected in pyrogen/endotoxin-free tubes. The lower limits of detection for IL-1β, IL-18 and TNF-α were <5 pg/ml, <25 pg/ml and <8 pg/ml, respectively.

Statistics

Western blotting, ELISA and the quantification of immunofluorescence and TUNEL staining were performed by an investigator blinded to the experimental conditions. Differences between multiple groups with one or two variables were evaluated by one-way or two-way analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD) or a Tukey-Kramer post hoc multiple comparison test for
unequal replications using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Sample size was not predetermined by formal power calculation, and no samples or data were excluded from the analysis. All statistical data in the text are expressed as the mean ± SEM; the level of significance was set at p < 0.05.

Results

Sleep deprivation induces expression of NLRP3 inflammasome-related proteins

To characterize effect of SD on astrocytes, (Fig. 1A), we used FACS to purify astrocytes from FVB/N-Tg(GFAPGFP)14Mes/J transgenic mice, which express green fluorescent protein (GFP) in a subpopulation of astroglia. Fluorescence images of the cortex of GFAP-GFP mice are shown in Fig. 1B. Chronic treatment with SD for 1-5 weeks increased the expression of astroglial caspase-1 in the time dependent manner (Fig. 1C). After one or two weeks SD, expression of caspase-1 protein increased by 75% ± 5.2% or 117% ± 4.9% (n=6), respectively, of the level in the sham group. After 3 to 5 weeks of SD, the level of caspase-1 was elevated by approximately 200%, with no significant difference among 3, 4 and 5 weeks groups (Fig. 1D). According to this result, we chose three weeks of SD treatment for subsequent tests. Three weeks of SD significantly increased expression of proteins of the NLRP3 inflammasome and pro-caspase-1 (Fig. 1E). Expression of NLRP3 protein increased by 146% ± 7.3% (n=6) while no significant changes in the levels of ASC or pro-caspase-1 were detected (Fig. 1F).

Fluoxetine reduces neuroinflammatory markers induced by sleep deprivation
Sleep deprivation of 3 weeks elevated the levels of IL-1β and IL-18 in FACS sorted astrocytes to 1634% ± 75.2% (n=6) and 2503% ± 128.5% (n=6) of control group, respectively (Fig. 2A). Intraperitoneal daily injections of 10 mg/kg fluoxetine during 3rd week of SD treatment (Li et al., 2013), partly alleviated the effect of SD on the up-regulation of IL-1β and IL-18. The level of IL-1β was approximately 9 times that of the control group, and the level of IL-18 was 1706% ± 93.1% (n=6) of control group (Fig. 2A-B). The inhibiting effect of fluoxetine on IL-1β and IL-18 levels was dose dependent (Supplementary Figure 2A,B). Treatment with fluoxetine at 1, 10 or 100 mg/kg significantly suppressed the SD-induced elevation of IL-1β and IL-18 in purified astrocytes.

We also quantified the neuronal impairment triggered by SD by using a TUNEL assay. As shown in Fig. 2C and D, the apoptosis of neurons labeled by the marker NeuN was significantly increased in the frontal cortex of the SD group (red arrows); the average percentage of TUNEL+ and NeuN+/Total NeuN+ was 7.9% ± 0.91% (n=6), whereas in the control group it was only 1.4% ± 0.47% (n=6). Treatment with fluoxetine at 10 mg/kg significantly decreased the neuronal apoptosis induced by SD (Fig. 2F); the percentage of TUNEL+ and NeuN+/Total NeuN+ decreased to 5.5% ± 0.52% (n=6) (Fig. 2G). However, treatment with fluoxetine alone had no effect on the level of apoptosis (Fig. 2E).

**Effects of SD and fluoxetine treatment on ERK1/2, AKT, GSK-3β and STAT3**

Sleep deprivation does not affect the phosphorylation of ERK1/2 (Fig. 3A-C), while significantly decreasing phosphorylation of AKT (by 50% ± 2.3%, n=6 Fig. 3D), and increasing phosphorylation of GSK-3β at Tyr216 (excitatory phosphorylation site) (by 115% ± 9.2% (n=6), Fig. 3E). At the same time phosphorylation of STAT3 at Tyr705 (excitatory phosphorylation site) was suppressed by 52% ± 7.3%, n=6 (Fig. 3F).

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However, animals treated with fluoxetine alone had almost inverted effects compared with the SD-treated group; treatment with fluoxetine increased the phosphorylation of AKT by $112\% \pm 5.1\%$ (n=6) of the level in the sham group, while treatment with both fluoxetine and SD enhanced AKT phosphorylation by $160\% \pm 11.7\%$ (n=6) of the level observed in the group that received SD only (Fig. 3D).

Conversely, fluoxetine reduced the phosphorylation of GSK-3β by $40\% \pm 2.4\%$ (n=6) of the sham group value and by $22\% \pm 5.1\%$ of the SD group value (Fig. 3E). Similarly, fluoxetine elevated the phosphorylation of STAT3 by $133\% \pm 10.9\%$ (n=6) of the sham group level and by $214\% \pm 17.3\%$ (n=6) of the SD group (Fig. 3F). Meanwhile, the stimulatory effect of fluoxetine on the phosphorylation of STAT3—which counteracted the effect of SD was also dose dependent (Supplementary Figure 2C,D). Moreover, fluoxetine induced the phosphorylation of ERK$_{1/2}$ by $39\% \pm 7.5\%$ (n=6) and $46\% \pm 9.0\%$ (n=6) of the sham group value, and fluoxetine with or without SD treatment also had a similar effect on the phosphorylation of ERK$_{1/2}$ (Fig. 3A-C).

We also used immunofluorescence to examine the phosphorylation level of STAT3, as shown in Fig. 3G-K. In the frontal cortex, the astrocytic fluorescence intensity of p-STAT3 was identified by the specific marker GFAP. The phosphorylation of STAT3 increased in astrocytes, marked by red arrows, and in non-astrocytes, indicated by white arrows. The background intensity of each image was measured in cell-free parenchyma in the same field of view and subtracted from the total immunofluorescence intensity. The intensity of p-STAT3 immunofluorescence in astrocytes from the different groups was normalized to the intensity of the sham group. As shown in Fig. 3, in the SD group (Fig. 3H), the intensity of p-STAT3 was significantly decreased by $29\% \pm 7.5\%$ (n=6) of the sham group value (Fig. 3G). However, the intensity was increased by $157\% \pm 12.2\%$ (n=6) in the fluoxetine-treated group (Fig. 3I), and the intensity was increased by $101\% \pm 10.8\%$ (n=6) in the SD animals treated with fluoxetine (Fig. 3J).
Effects of fluoxetine on primary cultured astrocytes

In cultured cerebral astrocytes, fluoxetine increased the phosphorylation of ERK$_{1/2}$ and AKT at 10 μM (Fig. 4A, D); furthermore, 10 μM U0126 (an inhibitor of MAPK) and 10 μM LY294002 (an inhibitor of PI3K) both abolished the effect of fluoxetine on p-ERK$_{1/2}$ and p-AKT (Fig. 4A-E). However, the regulatory effect of fluoxetine on the activation of p-GSK-3β was the opposite of its effect on p-AKT (Fig. 4E). Fluoxetine suppressed the phosphorylation of GSK-3β by 66% ± 2.2% (n=6) of the control group value (Fig. 4F), and LY294002 (LY) completely abolished the effect of fluoxetine. Moreover, fluoxetine elevated the phosphorylation of STAT3 by 102% ± 9.1% (n=6) of the control group value (Fig. 4H), while U0126 and LY partly inhibited the activation of STAT3 induced by fluoxetine (Fig. 4G, H).

The regulation of P2X7R in the expression of NLRP3 inflammasome

To verify contribution of P2X7Rs to the activation of the NLRP3 inflammasome in SD, we used P2X7 receptor knockout (P2X7R-KO) mice. Total protein from the cortex and hippocampus was collected for the western blot (Fig. 5A). The effect of SD significantly increased the expression of NLRP3 and caspase-1 by 153% ± 6.9% (n=6) and 115% ± 7.1% (n=6) of the sham group level, respectively (Fig. 5B). However, in P2X7R-KO mice, SD only elevated the levels of NLRP3 and caspase-1 by 65% ± 9.2% (n=6) and 67% ± 5.7% (n=6) of the sham group level, respectively (Fig. 5B), and SD still had no effect on the protein levels of ASC and pro-caspase-1.

We further examined the effect of STAT3 activation on the NLRP3 inflammasome by using 100 μM BzATP, an agonist of P2X7R (Yue et al., 2017), to stimulate expression of the NLRP3 inflammasome (Fig. 5C). BzATP increased the protein expression of NLRP3 and caspase-1 by 127% ± 7.3% (n=6) and 202% ± 7.1% (n=6) of the control group level (Fig. 5D). However, a selective STAT3 activator colivelin administred at 10 μM
nM (Chiu et al., 2016)) significantly suppressed the effect of BzATP; the expression of NLRP3 was decreased by 38% ± 4.7% (n=6) of the level found in the BzATP-treated group, and the level of caspase-1 was reduced by 41% ± 5.3%, n=6 (Fig. 5D).

**Effect of fluoxetine on the expression and function of the NLRP3 inflammasome in an SD model**

To further corroborate the findings from cultured astroglia, we investigated FACS sorted astrocytes obtained from mice subjected to SD. As shown in Fig. 6A, fluoxetine significantly decreased the expression of NLRP3 and caspase-1, respectively, by 41% ± 4.8% and 46% ± 5.4% (n=6) of the levels in the SD-treated group (Fig. 6B and D). However, U0126 and LY partly suppressed effects of fluoxetine. After pretreatment with U0126 or LY, respectively, the expression of NLRP3 was increased by 34% ± 5.3% and 67% ± 7.3% (n=6) of the level in the SD plus fluoxetine group (Fig. 6B), and the level of caspase-1 was elevated by 23% ± 6.9% and 19% ± 4.1% (n=6) of the level in the SD plus fluoxetine group (Fig. 6D). However, SD treated animals with or without fluoxetine had no significant effect on the expression of ASC or procaspase-1 (Fig. 6C and E).

Fluoxetine suppressed the level of IL-1β and IL-18, respectively, by 44% ± 4.7% and 36% ± 5.5% (n=6) of the values in the SD-treated group (Fig. 7A, B), and U0126 and LY both partly decreased the effect of fluoxetine. However, the level of TNF-α in the sorted astrocytes was affected neither in the SD group nor in animals treated with fluoxetine (Fig. 7C).
Discussion

In this study, we discovered that SD increased the expression of NLRP3 inflammasomes and the levels of IL-1β and IL-18 in astrocytes; furthermore, the SD increased neuronal apoptosis. Sleep deprivation stimulated formation of NLRP3 inflammasomes, with subsequent induction of the maturation of IL-1β and IL-18 (Fig. 8), whereas treatment with the antidepressant fluoxetine inhibited the adverse effects of SD. We further demonstrated that P2X7R was involved in the SD-induced formation of NLRP3 inflammasomes, which may be attributed to the SD-promoted increase in extracellular ATP that activates P2X7R (Dworak et al., 2010; Karmakar et al., 2016). The SD also decreased the activation of AKT and STAT3 but increased the activation of GSK-3β. Moreover, phosphorylated AKT inhibited the activation of GSK-3β, which suppresses the phosphorylation of STAT3 (Liu et al., 2015). Therefore, the activation of STAT3 was decreased in the SD model; furthermore, stimulating the phosphorylation of STAT3 inhibited the expression of NLRP3 inflammasomes. However, treatment with fluoxetine stimulated the phosphorylation of STAT3 by activating the phosphorylation of ERK1/2 and AKT; the phosphorylation of AKT induced by fluoxetine suppressed the activation of GSK-3β, thus increasing the activation of STAT3. The up-regulation of STAT3 activation by fluoxetine, in turn, suppressed the expression of NLRP3 inflammasomes and maturation of IL-1β and IL-18 in astrocytes. Moreover, fluoxetine decreased the neuronal apoptosis induced by SD. Consequently, treatment with fluoxetine suppressed astroglial expression of neuroinflammatory markers induced by SD.

We further demonstrate that chronic SD stimulated the expression of the NLRP3 inflammasome and increased the levels of IL-1β and IL-18 in astrocytes purified from the cortex (Fig. 1). Total IL-1β was previously found to be increased in the cortex after SD (Zielinski et al., 2017). Similar results were obtained regarding the acute effects of SD on the elevation of caspase-1 and IL-1β in the cortex (Zielinski et al., 2017).
It is well known that sleep loss elevates the levels of sleep regulatory substances (SRS) including IL-1β and TNF-α (Clinton et al., 2011). At the same time, injection of IL-1β or TNF-α can induce symptoms associated with sleep deprivation (Clinton et al., 2011). In the present study, the administration of fluoxetine effectively decreased the levels of IL-1β and IL-18 in astrocytes by inhibiting the expression of activated NLRP3 inflammasomes. However, the level of TNF-α in astrocytes was not changed by SD. In previous studies, the relationship between SD and fluoxetine was mainly examined in relation to depressive disorders. Moreover, there is little evidence that the antidepressant fluoxetine can suppress the neuroinflammation induced by SD. In clinical experiments, patients treated with fluoxetine in addition to SD have shown greater improvement of depressive symptoms (Benedetti et al., 1997). However, SD exerts its antidepressant activity mainly by stimulating the accumulation of serotonergic or noradrenergic neurotransmission, and depriving patients of sleep is clearly not suitable for long-term therapeutic treatment of depressive disorders (Hipólide et al., 2005; Conti et al., 2006). Long-term sleep deprivation is harmful to health and causes brain injury (Dworak et al., 2011; Armitage, 2007). The timing of the short-term or long-term SD is yet to be identified, it has been suggested that the short-term is less than 24 hours, while long-term SD exceeds 48 hours (Junek et al., 2010).

P2X₇R is shown to be expressed in neurons and astrocytes not only in vitro but also in vivo (Arbeloa et al., 2012; Hirayama et al., 2015; Ohishi et al., 2016, Illes et al., 2012). Under mechanical trauma, stimulation of P2X₇R triggers the activation of the NLRP3 inflammasome and the elevation of IL-1β in astrocytes (Albalawi et al., 2017). Extracellular release of ATP is required for the sleep drive and occurs in response to a sleep deficit, with astrocytes being important for the wakefulness-dependent release of ATP (Schmitt et al., 2012). In this work, we discovered that SD increased the astrocytic levels of IL-1β and IL-18 by stimulating the expression of the NLRP3 inflammasome, which could also be induced by BzATP activation of P2X₇R in astrocytes.
astrocytes. Extracellular ATP, associated with neuronal and glial signalling via P2X7R, has a role in the release of IL-1β and TNF-α (Clinton et al., 2011), however we did not find SD to have any effect on the level of TNF-α in astrocytes. Some nerve injuries such as neuropathic pain and SD cause the activation of microglia, which then release pro-inflammatory cytokines, e.g., IL-6, IL-1β and TNF-α (Zhao et al., 2017). The regulatory effect of SD on astrocytic expression of the NLRP3 inflammasome is the key point of this study; the effect of SD with or without fluoxetine on the microglia remains the matter for further research.

In this study, we found that SD decreased the activation of STAT3 and the administration of fluoxetine increased the activation of STAT3 in the cerebral astrocytes. Meanwhile, we also identified the activation of ERK1/2 and AKT as being involved in the phosphorylation of STAT3 induced by fluoxetine in vivo and in vitro. However, as Fig. 3 shows, the increased phosphorylation of STAT3 in response to fluoxetine occurred not only in astrocytes (red arrows) but also in non-astrocytes (white arrows), which we speculate to be mainly neurons based on their morphology. There have not been any reports that fluoxetine can increase the activation of p-STAT3 in neurons, although this topic merits further investigation because some anti-inflammatory cytokines (such as IL-10) decrease neuronal apoptosis by activating the JAK-STAT3 pathway (Zhu et al., 2017). Whether the stimulation of STAT3 phosphorylation by fluoxetine could have neuroprotective effects and whether the neuroprotective effect of fluoxetine is related to its therapeutic function for depressive disorder or sleep deprivation remain obscure. In a chronic depressive stress model, levles of 5-HT2B receptors were selectively decreased, and fluoxetine restored their expression in astrocytes (Dong et al., 2015). According to previous reports, astrocytes are the key location where the antidepressant fluoxetine exerts its therapeutic effects (Peng et al., 2014; Hertz et al., 2015). Fluoxetine acutely increased the phosphorylation of ERK1/2 in primary cultured astrocytes, it directly stimulated 5-HT2B receptors and transactivated the...
phosphorylation of epidermal growth factor (EGF) receptor (Li et al., 2008; Peng et al., 2014). In addition, stimulation of EGF receptor causes phosphorylation of receptor tyrosine kinases (RTK); the latter activate two major intracellular signaling pathways, namely, MAPK-ERK and PI3K-AKT. We found that the activation of ERK1/2 and AKT in response to fluoxetine both increased the phosphorylation of STAT3, but the activated AKT inhibited the phosphorylation of GSK-3β, which, in turn, suppressed the activation of STAT3. Similar results, in which GSK-3β inhibited the stimulation of STAT3, have been reported in a rat model of depression (Liu et al., 2015).

Sleep loss and the associated fatigue severely affect quality of life (Lee et al., 2009), and the rhythm of sleep is gradually being recognized as an indicator contributing to many diseases (Grandner, Sands-Lincoln, Pak, & Garland, 2013), to emotional health and mood (Goldstein & Walker, 2014). In this study, we discovered that the regulatory effect of fluoxetine on cerebral astrocytes played a key role in inhibiting the neuroinflammation induced by SD. Furthermore, the activation of STAT3 was an important target that regulated the expression of NLRP3 inflammasomes in an SD model treated with or without fluoxetine. Our present research suggests the possibility that fluoxetine could suppress the neuronal impairment induced by SD. However, whether the antidepressant fluoxetine can improve the quality of sleep and reduce the adverse effect of sleep deprivation still requires in-depth clinical research.
Involves human subjects:
If yes: Informed consent & ethics approval achieved:
=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

ARRIVE guidelines have been followed:
Yes
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Conflicts of interest: none
=> if 'none', insert "The authors have no conflict of interest to declare."
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**Figure Legends**

**Figure 1. The expression and function of NLRP3 inflammasomes in an SD model treated with or without fluoxetine.** Astrocytes were dissociated and separated from FVB/N-Tg(GFAPGFP)14Mes/J mice, which selectively express green fluorescent protein (GFP), in order to conduct the measurements (A). Representative immunofluorescence image of brain sections from GFAP-GFP mice, showing GFP (green) and DAPI (blue) in the cerebral cortex (B). Scale bar, 20 μm. After GFAP-GFP mice were treated with SD for 1-5 weeks, protein expression of caspase-1 was present, as shown in this representative blot; bands of 10 and 42 kDa represent caspase-1 and β-actin, respectively (C). The average protein level was quantified as the ratio of caspase-1 to β-actin in the astrocytes purified from GFAP-GFP mice (D). *p<0.05, statistically significant difference compared with the sham, 1-week, or 2-week group; #p<0.05, statistically significant difference compared with any other group. Data represent the mean ± SEM. The number of animals in every group was 6, n=6. GFAP-GFP mice treated with SD for 3 weeks were used for the following experiments (E). Representative immunoblots for NLRP3, ASC and pro-caspase-1 in astrocytes sorted from GFAP-GFP mice treated with SD for 3 weeks are shown in E; bands of 120, 25, 45 and 42 kDa represent NLRP3, ASC, pro-caspase-1 and β-actin, respectively. Average protein levels were normalized to the level of β-actin (F). *p<0.05, statistically significant difference compared with the sham group. Data represent the mean ± SEM. The number of animals in every group was 6, n=6.
Figure 2. Neuroinflammation and neuronal apoptosis in SD mice treated with or without fluoxetine. GFAP-GFP mice or B6 wild-type mice treated with SD for 3 weeks with or without fluoxetine injection in the final week were used for ELISA measurement (A-B) and neuronal apoptosis assays (C-G). The levels of IL-1β (A) and IL-18 (B) were measured in the sorted astrocytes. *p<0.05, statistically significant difference compared with any other group. Data represent the mean ± SEM. The number of animals in every group was 6, n=6. The neuronal apoptosis levels in the frontal cortex were measured via a TUNEL assay (green indicates TUNEL signal); in addition, NeuN (red) was stained as a specific marker of neurons, and DAPI was used as a marker of nuclei (blue). Scale bar, 200 μm. Red arrows indicate the apoptotic neurons. The percentage of TUNEL and NeuN double positive cells/Total NeuN+ cells (G). Data represent the mean ± SEM. The number of animals in every group was 6, n=6. *p<0.05, statistically significant difference compared with any other group.

Figure 3. The phosphorylation of ERK1/2, AKT, GSK-3β and STAT3 in SD mice treated with or without fluoxetine. GFAP-GFP mice were treated with sham or SD stimulation for 3 weeks and were injected with normal saline or fluoxetine in the third week. Then, the astrocytes from GFAP-GFP mice were purified via FACS and were used to measure protein expression via western blotting (A-F). Representative blots for p-ERK1/2 and ERK1/2, p-AKT and AKT, p-GSK-3β and GSK-3β, and p-STAT3 and STAT3 are shown in A, D, E and F, respectively. Average phosphorylation levels were quantified as the ratios of p-ERK1 to ERK1 (B), p-ERK2 to ERK2 (C), p-AKT to AKT (D), p-GSK-3β to GSK-3β (E), and p-STAT3 to STAT3 (F). *p<0.05, statistically significant difference compared with the sham and SD groups (B and C); #p<0.05, statistically significant difference compared with any other group (D, E and F). Data represent the mean ± SEM. The number of animals in every group was 6, n=6. In addition, immunofluorescent staining for p-STAT3 in the frontal cortex was present, as shown in G-J. Immunohistochemical staining of phosphorylated STAT3 (green) is shown in cortical astrocytes (GFAP; red), with DAPI (blue) as a nuclear marker (G-J). Scale bar,
50 μm. p-STAT3 immunolabeling intensity normalized to the intensity of the control group was analyzed in K. *p<0.05, statistically significant difference compared with any other group. Data represent the mean ± SEM. The number of animals in every group was 6, n=6.

**Figure 4. The regulatory effect of fluoxetine in primary cultured astrocytes.** After pretreatment in serum-free medium with or without U0126 or LY294002 for 30 min, the astrocytes were cultured in the absence of any drug (Control) or in the presence of 1 μM fluoxetine for 3 days. Representative blots for p-ERK\(_{1/2}\) and ERK\(_{1/2}\), p-AKT and AKT, p-GSK-3β and GSK-3β, and p-STAT3 and STAT3 are shown in A, D and G, respectively. Average phosphorylation levels were quantified as the ratios of p-ERK\(_{1}\) to ERK\(_{1}\) (B), p-ERK\(_{2}\) to ERK\(_{2}\) (C), p-AKT to AKT (E), p-GSK-3β to GSK-3β (F), and p-STAT3 to STAT3 (H). *p<0.05, statistically significant difference compared with any other group (B, C, E, F and H); #p<0.05, statistically significant difference compared with the Control, Fluoxetine, U0126 or LY group (H). Data represent the mean ± SEM. The number of cell-culture dishes in every group was 6, n=6.

**Figure 5. The activation of STAT3 was required for the stimulation of NLRP3 inflammasome expression by a P2X7 receptor agonist.** B6 wild-type mice and P2X7R-KO mice were treated with SD for 3 weeks (A). After pretreatment in serum-free medium with or without colivelin for 30 min, the astrocytes were cultured in the absence of any drug (Control) or in the presence of BzATP for 3 days (B). The protein expression of NLRP3 inflammasomes in the cortex (A) or in cultured astrocytes (B) was measured by western blotting. Data represent the mean ± SEM. The number of animals in every group was 6, n=6. Representative blots for the expression of NLRP3, ASC, caspase-1 and pro-caspase-1 are shown in A and C. *p<0.05, statistically significant difference compared with the sham, P2X7R-KO sham or P2X7R-KO plus SD group (B); †p<0.05, statistically significant difference compared with...
with the sham, SD or P2X7R-KO sham group (B); *p<0.05, statistically significant difference compared with the control, colivelin or colivelin plus BzATP group (D); 

*p<0.05, statistically significant difference compared with the control, BzATP or colivelin group (D). Data represent the mean ± SEM. The number of cell-culture dishes in every group was 6, n=6.

Figure 6. The regulatory effect of fluoxetine on the expression of NLRP3 inflammasomes via stimulation of ERK_{1/2} and AKT in an SD model. GFAP-GFP mice were treated with sham or SD stimulation for 3 weeks, and during the third week, U0126, LY294002 or vehicle (ACSF) was ICV injected each day; 30 min later, fluoxetine or normal saline was injected intraperitoneally. Then, astrocytes were purified from GFAP-GFP mice via FACS and were used to measure protein expression (A). Representative blots for the expression of NLRP3, ASC, caspase-1 and pro-caspase-1 are shown in A. *p<0.05, statistically significant difference compared with the sham, fluoxetine (Flux), SD plus Flux, SD plus U0126 and Flux or SD plus LY and Flux group (B-E); 

*p<0.05, statistically significant difference compared with any other group (B-E); 

*p<0.05, statistically significant difference compared with the sham, SD, Flux, SD plus Flux, SD plus U0126 or SD plus LY group (B-E). Data represent the mean ± SEM. The number of cell-culture dishes in every group was 6, n=6.

Figure 7. The regulatory effect of fluoxetine on the function of NLRP3 inflammasomes via stimulation of ERK_{1/2} and AKT in an SD model. GFAP-GFP mice were treated with sham or SD stimulation for 3 weeks, and in the third week, U0126, LY294002 or vehicle (ACSF) were ICV injected each day; 30 min later, fluoxetine or normal saline was injected intraperitoneally. Then, astrocytes were purified from the GFAP-GFP mice via FACS and were used for ELISA (A-C). The levels of IL-1β (A), IL-18 (B) and TNF-α (C) were measured by ELISA in astrocytes. *p<0.05, statistically significant difference compared with the sham, fluoxetine (Flux), SD plus Flux, SD

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plus U0126 and Flux or SD plus LY and Flux group (A-B); $^p<0.05$, statistically significant difference compared with any other group (A-B); $^5p<0.05$, statistically significant difference compared with the sham, SD, Flux, SD plus Flux, SD plus U0126 or SD plus LY group (A-B). Data represent the mean ± SEM. The number of animals in every group was 6, n=6.

**Figure 8. The schematic diagram.** SD activated the expression of NLRP3 inflammasomes in astrocytes, and the production of IL-1β and IL-18 correspondingly increased. SD inhibited the phosphorylation of AKT, which decreased the activation of GSK-3β; the latter, in turn, suppressed the phosphorylation of STAT3. In addition, the suppression of STAT3 phosphorylation was involved in the neuroinflammation stimulated by SD because the activation of STAT3 could inhibit the expression of NLRP3 inflammasomes. P2X7R was involved in the effect of SD on the expression of NLRP3 inflammasomes. Administration of the antidepressant fluoxetine reversed the neuroinflammation induced by SD. Fluoxetine stimulated the activation of ERK1/2 and AKT; both of them increased the phosphorylation of STAT3, and the increased activation of AKT elevated the phosphorylation of STAT3 by inhibiting the activation of GSK-3β. Fluoxetine decreased the expression of NLRP3 inflammasomes and the production of IL-1β and IL-18 by stimulating the activation of STAT3.

**Supplementary Figure 1. The experimental timeline.** B6, GFAP-GFP or P2X7R-KO mice were treated with sham or SD stimulation for 3 weeks. During the third week, vehicle (artificial cerebrospinal fluid, ACSF), U0126 or LY294002 was infused ICV in a volume of 2 μL. After 30 min, fluoxetine (10 mg/kg) or normal saline (NS) was injected intraperitoneally. The above injections were repeated every day during the third week.

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Supplementary Figure 2. The regulatory effect of fluoxetine on the function of SD was concentration dependent. GFAP-GFP mice were treated with sham or SD stimulation for 3 weeks and were injected with normal saline (fluoxetine at 0 mg/kg/day) or fluoxetine at 1, 10 or 100 mg/kg/day throughout the third week. Then, astrocytes were purified from the GFAP-GFP mice via FACS and were used for ELISA (A-B) and for the measurement of protein expression (C). The levels of IL-1β (A) and IL-18 (B) were measured by ELISA in astrocytes. *p<0.05, statistically significant difference compared with any other group (A-B); #p<0.05, statistically significant difference compared with the sham, SD plus Flux at 0 or 1 mg/kg group (A-B).

Representative levels of p-STAT3 and STAT3 are shown in C. The average phosphorylation level was quantified as the ratio of p-STAT3 to STAT3 (D). *p<0.05, statistically significant difference compared with any other group (C); "p<0.05, statistically significant difference compared with the SD plus Flux at 0, 10 or 100 mg/kg group (C). Data represent the mean ± SEM. The number of animals in every group was 6, n=6.

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