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Inhibition of IL-6 trans-signaling in the brain increases sociability in the BTBR mouse model of autism

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ABSTRACT

Autism is a severe neurodevelopmental disorder with a large population prevalence, characterized by abnormal reciprocal social interactions, communication deficits, and repetitive behaviors with restricted interests. The BTBR T^{+} $Itpr3^{tf}$ (BTBR) mice have emerged as strong candidates to serve as models of a range of autism-relevant behaviors. Increasing evidences suggest that interleukin (IL)-6, one of the most important neuroimmune factors, was involved in the pathophysiology of autism. It is of great importance to further investigate whether therapeutic interventions in autism can be achieved through the manipulation of IL-6. Our previous studies showed that IL-6 elevation in the brain could mediate autistic-like behaviors, possibly through the imbalances of neural circuitry and impairments of synaptic plasticity. In this study, we evaluate whether inhibiting IL-6 signaling in the brain is sufficient to modulate the autism-like behaviors on the BTBR mice. The results showed that chronic infusion of an analog of the endogenous IL-6 trans-signaling blocker sgp130Fc protein increased the sociability in BTBR mice. Furthermore, no change was observed in the number of excitatory synapse, level of synaptic proteins, density of dentitic spine and postsynaptic density in BTBR cortices after inhibiting IL-6 trans-signaling. However, inhibition of IL-6 trans-signaling increased the evoked glutamate release in synaptoneurosomes from the cerebral cortex of BTBR mice. Our findings suggest that inhibition of excessive production of IL-6 may have selective therapeutic efficacy in treating abnormal social behaviors in autism.

1. Introduction

Autism is a severe neurodevelopmental disorder with a large population prevalence, characterized by impairments in social interaction, deficits in verbal and non-verbal communication, and repetitive behavior and restricted interests. The Centers for Disease Control and Prevention estimates 1 in 68 U.S. children has autism (www.cdc.gov/autism). Although a few pharmacological treatments appear to reduce some of the associated symptoms, there are no therapeutic options that target the core symptoms of autism. It has been widely accepted that autism involves a complex interplay of both genetic and environmental risk factors. However, the exact pathological mechanism of this disorder is unclear. Emerging evidence suggests that aberrant neuroimmune response may contribute to phenotypic deficits and could be appropriate targets for pharmacologic intervention of autism [1]. The neuroimmune network includes astrocytes and microglia, immune mediators as well as other classical immune pathways. The function of neuroimmune factors play an important role in brain development and is critical for all processes of neuronal migration, axonal growth, neuronal positioning, cortical lamination, as well as dendritic and synaptic formation, reviewed in [2].

Interleukin (IL)-6, one of the most important neuroimmune factors, was shown to be involved in physiological brain development and in several neurological disorders such as schizophrenia, major depression and Alzheimer's disease [3–5]. Increasing studies have suggested an association of IL-6 with autism. Vargas et al. demonstrated that IL-6 was increased in the anterior cingulated gyrus of autistic brains and also in the cerebrospinal fluid of autistic children [6]. Our previous study found that IL-6 was significantly increased in the frontal cortices and cerebellum of autistic subjects as compared with the age-matched control subjects [7,8]. However, the exact mechanism by which IL-6 alteration contributes to the pathophysiology of autism remains undefined. We developed a mouse model of over-expressing IL-6 in the brain with an adenoviral gene delivery approach and showed that IL-6 elevation in the brain could mediate autistic-like behaviors, possibly through the imbalances of neural circuitry and impairments of synaptic plasticity [9]. Using high-resolution magnetic resonance

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imaging, we further found that mice with elevated IL-6 in the brain displayed an increase in total brain volume and could mediate neuroanatomical abnormalities [10].

The BTBR T^{+} $Itpr3^{tf}$ (BTBR) mice are an inbred strain and have emerged as strong candidates to serve as models of a range of autism-relevant behaviors, showing deficiencies in social behaviors and reduced or unusual ultrasonic vocalizations as well as increased repetitive self-grooming. There are increasing investigations targeted to ameliorating the behavioral phenotypes of autism in BTBR mice [11–13]. And one study that examined the expression of various cytokines in the whole brain and brain regions of BTBR mice showed a trend toward an increased production of IL-6 compared to C57BL/6J (B6) control mice [14]. Therefore the purpose of this study was to extend these studies and evaluate whether inhibiting IL-6 signaling in the brain is sufficient to modulate the autism-like behaviors on the BTBR mice. The results showed chronic infusion of IL-6 trans-signaling blocker sgp130Fc protein increased the sociability in BTBR mice, possibly through the increase of the evoked glutamate release in the cerebral cortex. These findings suggest that inhibition of excessive production of IL-6 through its signaling pathways may be helpful in treating the behavioral deficits in autism.

2. Materials and methods

2.1. Animals and surgical procedure

BTBR mice breeding pairs were purchased from Model Animal Research Center of Nanjing University (Nanjing, China) and bred at Shanxi Medical University. Subject mice were weaned at 21 ± 1 days of age, then group housed by sex and strain in standard mouse cages containing 2–4 mice. Standard rodent chow and tap water were available ad libitum. The colony room was maintained on a 12:12 light/dark cycle, with lights on at 7:00 AM. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. The mice at the age of 6 weeks were anesthetized and placed in a stereotaxic apparatus. A hole was drilled through the skull according to stereotaxic coordinates (from bregma: −0.5 mm posterior, −1.1 mm lateral and −2.5 mm ventral), and a cannula (Brain infusion kit 3, ALZET, Durect Corp., Cupertino, CA) was lowered into the right lateral ventricle of the brain. The animals received a continuous infusion of sgp130Fc protein (R&D Systems) or sterile saline containing 0.1% BSA as control for 14 d, by an ALZET osmotic pump (Model 2002, release rate 0.5 μl/h, filled with 200 μl containing 1.5 μg of sgp130Fc protein, Durect Corp), following which the behavioral test was performed. The osmotic pump was implanted subcutaneously on the back of the mice, slightly posterior to the scapulae.

2.2. Behavioral assays

Behavioral experiments were conducted in dedicated behavioral testing rooms during the standard light phase, as previously described [13]. Mice were brought to a holding room in the hallway of the testing area at least 1 h prior to the start of the behavioral test. Testing began at ages 8 weeks. Each group consisted of 6–10 mice, with approximately equal numbers of males and females throughout.

2.2.1. Sociability

This experiment has two habituation phases (center and all 3 chambers) followed by the sociability testing phases. The test compares the preference for a social stimulus versus an inanimate object. Social approach behaviors were tested in an apparatus with 3 chambers in a single 30-min session, divided into 3 phases. The subject mouse was acclimated to the apparatus for 10 min in the center chamber (phase 1), and then for an additional 10 min with access to all 3 empty chambers (phase 2). The subject was then confined to the middle chamber, while the novel object (an inverted wire cup) was placed into one of the side chambers, and the stranger mouse, inside an identical inverted wire cup, was placed in the opposite side chamber. The B6 mice of the same sex with the subject mouse, aged 8 weeks old were used as the stranger mice. The location (left or right) of the novel object and stranger mouse alternated across subjects. The chamber doors were opened simultaneously, and the subject had access to all 3 chambers for 10 min (phase 3). Video tracking with SuperMaze automatically scored the time spent in each of the 3 chambers, time spent sniffing, and number of entries into each chamber during each 10-min phase of the test. The stranger mice were habituated to the testing chamber for 30-min sessions on 3 consecutive days and were enclosed in the wire cup to ensure that all social approach was initiated by the subject mouse. Both end chambers maintained a lighting level of 26–27 lx with 2 desk lamps angled away from the maze.

2.2.2. Self-grooming

Mice were scored for spontaneous repetitive self-grooming behavior. Briefly, each mouse was placed individually into a clean, empty mouse cage without bedding. Each mouse was given a 10-min habituation period in the empty cage and then rated for 10 min for cumulative time spent grooming all body regions. The investigator sat approximately 2 m from the test cage and recorded cumulative time spent in grooming with a stopwatch. Previous studies demonstrated that no sex differences in sociability or self-grooming in BTBR mice [12,15]. Therefore, male and female mice were used in the experiments in approximately equal proportions.

2.3. ELISA

The mouse cerebral cortices were lysed in ice cold lysis buffer and protein concentrations were determined using the BCA protein assay according to manufacturer's protocol. Samples from IL-6 blocked BTBR mice and control BTBR mice were analyzed for brain IL-6 using commercially available enzyme-linked immunosorbent assay kit according to manufacturer's guidelines (ExCell Biology, Beijing, China).

2.4. Western blotting

Western blotting procedures were performed as described previously [16]. The mouse cerebral cortices were homogenized in RIPA buffer. A Bradford assay was performed to calculate protein yield and the lysate concentration was then normalized and denatured in loading buffer at 95 °C and stored at −20 °C until use. 100 μg of the lysate was resolved via electrophoresis on 10% SDS-PAGE gels. The gels were transferred to PVDF membranes, and incubated in a solution of 5% non-fat milk for 2 h at room temperature. Blots were incubated with primary antibody overnight at 4 °C, washed four times for 15 min in PBS with 0.1% Tween 20, followed by 1 h incubation with a horseradish peroxidase conjugated goat anti-rabbit (1:20,000, EarthOx), goat anti-guinea pig (1:5000, Beijing Biosynthesis Biotechnology CO., LTD.) or goat anti-mouse (1:20,000, EarthOx). Blots then were washed four times for 15 min and visualized using chemiluminescent substrate (Millipore). Antibodies used were as follows: rabbit anti-JAK2 (1:1000, Cell Signaling Technology), rabbit anti-STAT3 (1:1000, Cell Signaling Technology), rabbit anti-Shank3 (1:1000, Synaptic Systems), guinea pig anti-VGLUT1 (1:4000, Synaptic Systems) and mouse anti-beta actin (1:1000, ABGENT). For VGLUT1 protein, samples were not boiled, as VGLUT1 protein aggregates after boiling. Quantification of band intensity was performed using ImageJ (NIH, Bethesda, Maryland) and presented relative to the loading control (beta actin). The Western blot analysis was performed twice and representative data were shown.

2.5. Immunofluorescence assay

Animals were sacrificed by decapitation and rapidly the brains were fixed in 4% PFA by direct immersion overnight at 4 °C. Then the brains were coronally sectioned (200 μm thick) using a vibratome at room temperature and stored in antifreeze solution (30% ethylene glycol, 20% glycerol, and 50% 0.05 M phosphate buffer) at −20 °C until use.

Synapse quantification was performed as previously described [17]. Briefly, sections were washed three times for 10 min in PBS and then placed in 0.01% Triton X-100 in PBS solution for 15 min at room temperature and incubated in blocking solution containing 10% goat serum for 1 h at room temperature. Rabbit anti-Shank3 (1:250, Synaptic Systems) to label glutamatergic postsynaptic compartments and guinea pig anti-VGLUT1 (1:500, Synaptic Systems) to label glutamatergic presynaptic terminals were added to the PBS, and sections were incubated overnight at 4 °C. For some sections, the primary antibody was omitted as a control. Tissue was washed three times for 15 min in PBS, and was incubated in secondary antibody solution consisting of goat anti guinea-pig Alexa Fluor 488 (1:1000, Invitrogen) and goat anti rabbit Alexa Fluor 647 (1:1000, Invitrogen) for 1 h at room temperature in the dark. The sections were washed five times in PBS and incubated in DAPI solution. Sections were washed in PBS and mounted with ProLong Antifade (Invitrogen). Images were collected using a 60× oil-immersion objective lens on a confocal laser scanning microscopy (FV1000, Olympus). As previously described, at least 3 animals were included for each group [17]. Synapses were quantified using Z-stacks confocal planes in somatosensory cortex. Colocalized puncta were identified using the Puncta Analyzer plug-in in ImageJ as described [18]. For each section, in both the 488 and 635 channels, we image serial optical sections at 0.3 μm intervals over a total depth of 2.7 μm for a total of 9 optical sections. Maximum intensity projections (MIPs) are generated and quantified from groups of 3 consecutive sections yielding 3 MIPs representing 0.9 μm of depth each.

For the immunofluorescence assay of STAT3 and p-STAT3, sections were incubated with rabbit anti-STAT3 (1:100, BOSTER) and rabbit anti-phospho-STAT3 (1:100, Cell Signaling Technology), respectively. The secondary antibody was goat anti rabbit Alexa Fluor 647 (1:1000, Invitrogen). Images were collected using a 40× objective lens with a 2× digital zoom factor on a confocal laser scanning microscopy (FV1000, Olympus). The original Olympus OIB images were split into Alexa Fluor 647 channel and DAPI channel. Mean gray value was measured on Alexa Fluor 647 MIP images using ImageJ software. The pseudo-color images were shown. The measurements were averaged for each animal and the mean value was used for statistical analysis.

2.6. Dendritic spine assay

According to the method described in our previous work [9], the brains were fixed and coronally sectioned (200 μm thick) using a vibratome at room temperature. Sections were incubated with Vybrant-DiI cell-labeling solution (1:150, Invitrogen) for 36 h at 4 °C to allow DiI to diffuse fully along the neuronal membranes. Then the sections were bathed in the PBS for 48 h to allow more time for diffusion and mounted on glassslides with ProLong Gold antifade reagent (Invitrogen). All images were taken within 7 days after coverslipping using a 60× oil-immersion objective lens with a 2× digital zoom factor on a confocal laser scanning microscopy (FV1000, Olympus). The DiI labeled pyramidal neurons in the somatosensory cortex were analyzed. Spine quantification and measurements were done as described previously [19,20]. Dendrite length was measured using a Freehand-line tool on a maximum intensity projection of the Z-stacks. Spines were counted by scrolling through the Z-stack and marking each spine. Spine density was computed as number of spines per dendrite length in 10 μm. The spines were counted in 5–6 neurons/mouse and 4 mice/group. For each neuron, 1–4 dendrites were analyzed and the measurements were averaged for each animal and the mean value was used for statistical analysis.

2.7. Electron microscopy

Mice were deeply anesthetized and transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde (PFA) in phosphate buffer. The brains were removed and the somatosensory cortex was dissected and post-fixed overnight in PFA 4%, then transferred into a 2% glutaraldehyde solution for 2 h on ice. Samples were washed twice for 10 min with 0.1 M PBS, fixed for 1 h in 1% OsO_4. Samples were gradually dehydrated with a 10 min wash of 50–100% ethanol. Samples were then treated for 20 min twice with Propaline oxide and impregnated with 50:50 Propaline oxide:Epon resin overnight at 4 °C. Epon resin 812 was used to embed the samples and cured at 55 °C for 2–3 d. Thin sections (100 nm) were cut using Leica UC6 ultramicrotome and then stained in uranyl acetate and lead citrate. TEM images were taken using a FEI Tecnai Spirit transmission electron microscope. The postsynaptic density (PSD) measurements were performed using ImageJ by an observer that was blinded to the genotype of the samples [21].

2.8. Glutamate release from synaptoneurosomes (SNs)

SNs were prepared from mouse cerebral cortex as described previously [18,22]. Briefly, the cerebral cortex was homogenized in SN buffer at 4 °C using a Teflon-glass mechanical tissue grinder (0.25 mm clearance). From this step forward the homogenate was kept ice-cold at all times to minimize proteolysis throughout the isolation procedure. The sample was loaded through three layers of a pre-wetted 147 μm pore nylon filter. The resulting filtrate was placed in a 50 ml polycarbonate tube and centrifuged at 1000 ×*g* for 10 min. The pellet obtained corresponded to the SN fraction. The isolated SNs were characterized by electron microscopy as previously described [23]. The glutamate release assay was performed using enzyme-linked fluorescent detection of released glutamate [24,25]. In brief, SNs were stored on ice and diluted to 2 mg/ml protein using Krebs-like solution as described. For each well of a 96 well plate 25 μl SN solution was added to 175 μl calcium-free Krebs-like solution (118 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM Glucose, pH 7.4). After 60 s, 1 mM NADP+ (Sigma) was added ±1.2 mM calcium chloride as required. After ~6 min, five units of glutamate dehydrogenase (Sigma) were added and allowed to equilibrate inside the instrument for at least 10 min. Fluorescence at 460 nm emission was continuously measured using a BioTek Synergy 4 Microplate reader set at the following parameters: 80–120 cycles (number of times a complete plate is read), 21 s cycle time (time between each cycle), FlashMode (high sensitivity), excitation filter 360-40, emission filter 460-40, injection cycle 60, shaking after each cycle for 1 s, 37 °C. Depolarisation stimulus (30 mM KCl) was added by pausing the instrument.

2.9. Statistical analysis

All data were analyzed using commercially available statistical software packages (StatView 5.0 and GraphPad Prism 5). For the social approach task, the times spent in each of the 3 chambers were not independent; for the analysis, only times spent in the side chambers (containing the stranger mouse and novel object) were compared. Time spent in the center chamber is shown in the graphs to illustrate where the subject mouse spent time during the entire 10-min phase. Chamber time, time spent sniffing the novel object versus the stranger mouse, the entries to the side chambers in the social approach test and the glutamate release data were analyzed by two-way ANOVA with repeated measures with Bonferroni's post hoc analysis. The unpaired *t*-test (two-tailed) was conducted to determine the significant differences for the synaptic assay, dendritic spine assay, ELISA and Western blotting data. The Kolmogorov-Smirnov test was used to

compare the patterns of cumulative frequency of PSD thickness. The data was shown as mean ± SEM. For all findings, the statistically significant *P* values are shown as $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Inhibition of IL-6 trans-signaling increased social sniffing in BTBR mice

In the present study, the level of IL-6 expression in the brain cortex was decreased after the continuous infusion of sgp130Fc protein into the lateral ventricle of the BTBR brain for 14 days (Fig. 1A, $t = 4.40$, $P < 0.01$). And the expression of downstream factor JAK2 was also lower than that in the control mice determined using the Western blotting (Fig. 1B, $t = 5.42$, $P < 0.01$). However, there is no significant change in the level of STAT3 (Fig. 1C, $t = 1.07$, $P = 0.31$). We next detected the STAT3 and phospho-STAT3 expression using immunofluorescence method. As shown in the Fig. 1D and E, while no significant change was observed in the expression of STAT3 ($t = 1.40$, $P = 0.23$), the expression of phospho-STAT3 and the ratio of phospho-STAT3/STAT3 in IL-6 blocked BTBR mice were significantly reduced than that in controls ($t = 3.92$, $P < 0.05$ and $t = 3.47$, $P < 0.05$, respectively). The IL-6 trans-signaling in the brain was successfully inhibited.

Fig. 2 illustrates the sociability scores from the automated 3-chambered social approach task following a continuous infusion of sgp130Fc protein or vehicle in BTBR mice. Sociability, defined as spending more time in directed sniffing to the stranger mouse than in sniffing the novel object in the side chambers, was significant increased in IL-6 blocked BTBR mice as compared to controls. There was a significant difference of treatment ($F_{1, 32} = 4.99$, $P < 0.05$) but not of chamber time ($F_{1, 32} = 2.23$, $P = 0.15$) or interaction of treatment and chamber time ($F_{1, 32} = 0.69$, $P = 0.41$) (Supplementary Fig. 1A). There were significant effects of sniff time ($F_{1, 32} = 12.60$, $P < 0.01$) and treatment ($F_{1, 32} = 6.24$, $P < 0.05$), but not of interaction of treatment and sniff time ($F_{1, 32} = 3.57$, $P = 0.07$). Bonferroni's post hoc analysis indicated that the IL-6 blocked BTBR mice spent significant more time sniffing the stranger mouse as compared to controls (Fig. 2A, $P < 0.01$). There was a significant difference of treatment ($F_{1, 32} = 13.07$, $P < 0.01$) but not of entries ($F_{1, 32} = 0.68$, $P = 0.42$) or interaction of treatment and entries ($F_{1, 32} = 0.43$, $P = 0.52$) (Supplementary Fig. 1B). Furthermore, there was a trend that inhibition of IL-6 trans-signaling decreased the

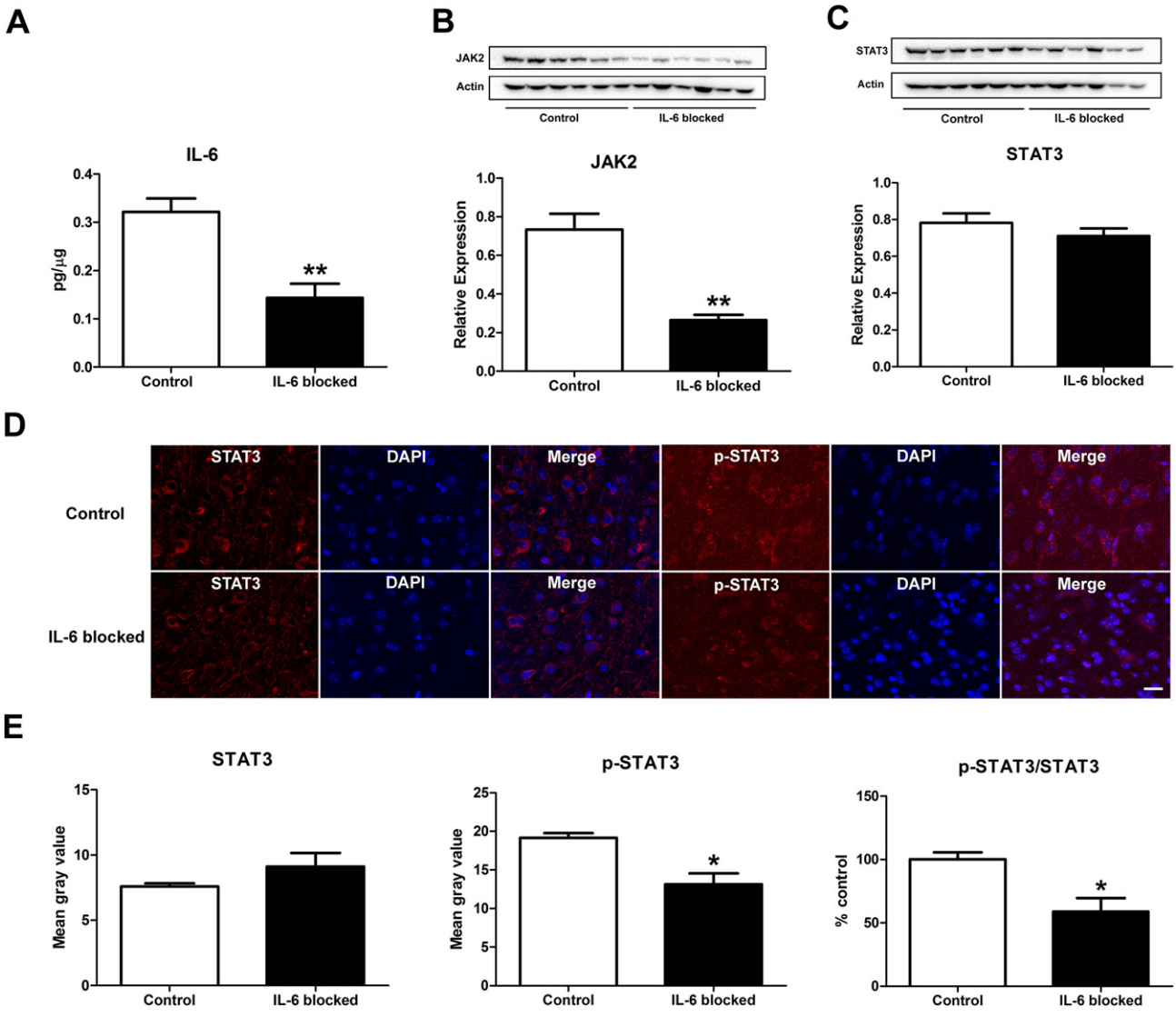

Fig. 1. IL-6 trans-signaling was inhibited by chronic infusion of sgp130Fc protein into mouse brains. (A) The level of IL-6 protein in the brain cortex was decreased after the continuous infusion of sgp130Fc protein into the brain of the BTBR for 14 days. $n = 6$ mice per group. (B) The expression of JAK2 in IL-6 blocked BTBR mice was lower than that in controls. $n = 6$ mice per group. (C) No significant change was observed in the expression of STAT3 using Western blotting. $n = 6$ mice per group. (D) Representative immunofluorescence images of sections of the somatosensory cortex from IL-6 blocked BTBR mice and controls labeled with antibodies to STAT3 and phospho-STAT3, respectively. Scale bar, 20 μm. The legend in one of the panels applies to all panels. (E) Quantitative analysis of immunofluorescence images. $n = 3$ mice per group. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

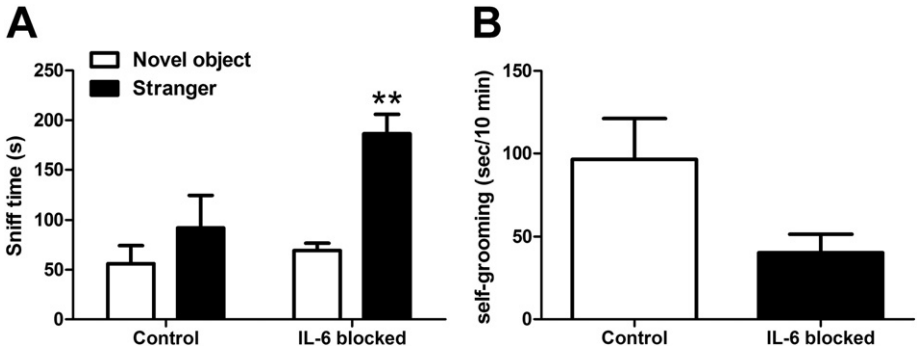

Fig. 2. Inhibition of IL-6 trans-signaling increased social sniffing in BTBR mice (A) IL-6 blocked BTBR mice spent significant more time sniffing the stranger mouse as compared to controls. (B) There was a trend that IL-6 blocked BTBR mice displayed less self-grooming than that in controls ($P = 0.06$). The behavioral assays were conducted after the continuous infusion of sgp130Fc protein for 14 days. Data are shown as mean ± SEM. $n = 9$ mice per group. $^{**}P < 0.01$.

repetitive self-grooming behavior. IL-6 blocked BTBR mice placed in a clean empty standard mouse cage for 10 min displayed less self-grooming than controls (Fig. 2B, $t = 2.07$, $P = 0.06$).

3.2. No change in the number of excitatory synapse and level of synaptic proteins after the inhibition of IL-6 trans-signaling

Numerous studies support synapse dysfunction as a core pathological feature of autism [26]. Inhibitory and excitatory synapses play fundamental roles in information processing in the brain. Inappropriate loss of synaptic stability could lead to the disruption of neuronal circuits and to brain dysfunction. Because of the close relationship between excitatory synapse and dendritic spine, the present study focused on the change of the excitatory synapse. To investigate whether the inhibition of IL-6 trans-signaling could have an effect on the number of the excitatory synapse in the brain of BTBR mice, we double labeled sections of IL-6 blocked BTBR mice and control mice using antibodies to the pre-synaptic protein VGLUT1 and the post-synaptic protein Shank3 (Supplementary Fig. 2A). By image analysis, no change in the number of colocalized pre- and post-synaptic puncta was observed in IL-6 blocked BTBR brains as compared to controls (Supplementary Fig. 2B, $t = 1.67$, $P = 0.15$). In order to confirm the result of the morphological image analysis for the number of excitatory synapse, Western blotting was employed to test the expressions of VGLUT1 and Shank3 synaptic proteins (Supplementary Fig. 3A). Quantitative analysis indicates that both VGLUT1 and Shank3 proteins were not changed between IL-6 blocked BTBR mice and controls (Supplementary Fig. 3B, VGLUT1: $t = 1.74$, $P = 0.11$; Shank3: $t = 0.69$, $P = 0.51$).

3.3. No change in the density and PSDs of dendritic spine and after the inhibition of IL-6 trans-signaling

Most excitatory synapses occur on small protrusions along dendrites called dendritic spines that contain the postsynaptic machinery. Dendritic spines are the major site for excitatory transmission in the brain. The plasticity of the dendritic spine has been proved to be involved in the pathology of autism. Our previous study showed IL-6 elevation stimulated the formation of mushroom-shaped dendritic spines in IL-6 overexpressed mice [9]. In this study we employed DiI labeling to outline dendritic spines in pyramidal neurons of the somatosensory cortex (Supplementary Fig. 4A). Our results showed that the density of dendritic spines was not altered in IL-6 blocked BTBR mice as compared to controls (Supplementary Fig. 4B, $t = 1.60$, $P = 0.16$). Excitatory synapses are characterized by a morphological and functional specialization of the postsynaptic membrane called PSD, which is usually located at the tip of the dendritic spine [27]. Next, we analyzed PSD morphology by electron microscopy (Supplementary Fig. 4C). We found no difference in mean thickness of PSDs in IL-6 blocked BTBR mice relative to controls (Supplementary Fig. 4D, $P = 0.32$).

3.4. Inhibition of IL-6 trans-signaling increased the evoked glutamate release in the cerebral cortex of BTBR mice

The PSD contains the glutamate receptors that are activated by the glutamate neurotransmitter released from the presynaptic terminal. Glutamate binds to glutamate receptors, giving rise to synaptic transmission. In order to uncover a potential effect of inhibition of IL-6 on evoked neurotransmitter release, SNs were purified from mouse cerebral cortex. The SNs retain pre- and postsynaptic characteristics, which make them useful in the study of neurotransmitter assays [28]. In the present study, electron micrographs of isolated SNs showed a dense cellular mass in which mitochondria and vesicular structures were seen together with densely stained membranes representing PSDs (Fig. 3A and B). The SNs were stimulated to release glutamate by the addition of depolarizing agent. There was a significant effect of time ($F_{15, 128} = 31.51$, $P < 0.01$), treatment ($F_{1, 128} = 232.88$, $P < 0.01$) and interaction of treatment and time ($F_{15, 128} = 5.77$, $P < 0.01$). Bonferroni's post hoc analysis indicated that the evoked glutamate release was significantly increased in the IL-6 blocked BTBR mice as compared to controls begin at 16 min after detection (Fig. 3C, $P < 0.01$).

4. Discussion

This study evaluated the therapeutic effects of sgp130Fc protein on the autism-like behaviors in the BTBR mouse model of autism. These experiments first demonstrate that the low level of sociability inherent in the BTBR strain can be increased with a continuous infusion of sgp130Fc protein through the inhibition of IL-6 trans-signaling in the brain. At present there is still no gold standard, and in fact no reliable compound, that improves sociability in autism. Evidence from animal studies suggests that several compounds including mGluR5 antagonist MPEP [12], AMPA receptor modulator AMPAKINE [29] and fluoxetine [30] can reverse the deficits in sociability or self-grooming in mouse model of autism. IL-6 is a pleiotropic cytokine that plays a key role in interaction between immune and nervous system. A growing body of research is focusing on the pharmacological treatments that modulate IL-6 signaling in the mental disorders.

Cellular communication by IL-6 is mediated by two distinct modes: IL-6 classic signaling and IL-6 trans-signaling. While classic IL-6 signaling is mainly regenerative and protective, IL-6 trans-signaling is rather pro-inflammatory [5]. It has been shown that trans-signaling is a dominant mechanism for the pathogenic actions of IL-6 in the brain [31]. IL-6-neutralizing antibodies to IL-6 or IL-6R block both classical and trans-signaling of IL-6, while sgp130Fc protein blocks IL-6 trans-signaling with no effect on IL-6 classical signaling [32,33]. The JAK2/STAT3 signaling pathway is the major pathway of IL-6 [5]. Here STAT3 phosphorylation in the brain cortex was reduced treated with continuous infusion of sgp130Fc protein. This result further confirmed

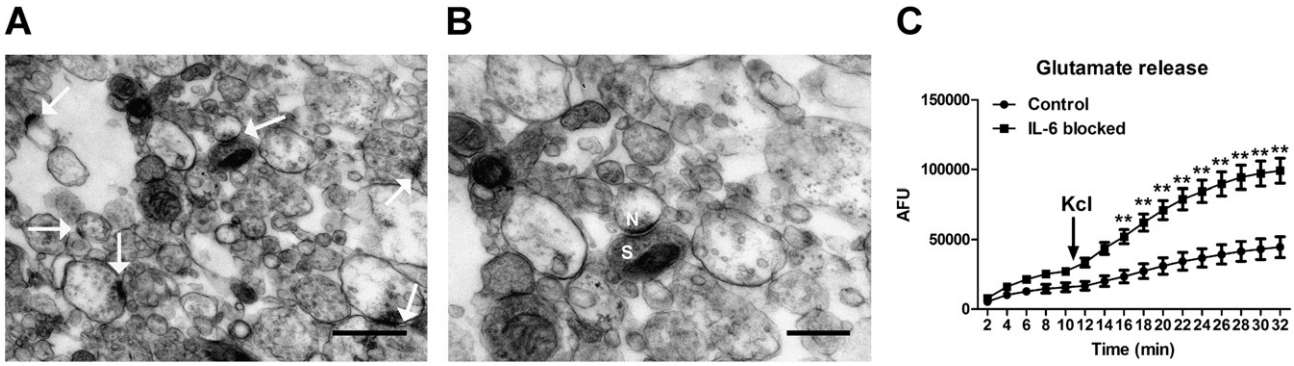

Fig. 3. Inhibition of IL-6 trans-signaling increased the evoked glutamate release in the cerebral cortex of BTBR mice. (A) Electron micrographs of representative sample of SNs isolated from BTBR mouse cortex. Arrow indicates synaptic junctions. Scale bar, 1 μm. The tissue samples were obtained after the behavioral assays. (B) Higher magnification of the synaptic site where the pre-synaptic terminal displays vesicles and a post-synaptic membrane contains PSDs. Synaptosome (S) and neurosome (N) are shown. Scale bar, 500 nm. (C) Comparison of glutamate release in SNs between control and IL-6 blocked BTBR mice. AFU: arbitrary fluorescence units. Data are shown as mean ± SEM. $n = 5$ mice per group. $^{**}P < 0.01$.

the previous findings that sgp130Fc protein could attenuate STAT3 phosphorylation of IL-6 signaling [31,34,35]. The decreased level of STAT3 phosphorylation may be caused by the lower levels of JAK2 protein. Activation of STAT proteins is mostly mediated by JAK family members. Regulative experiment using specific inhibitors indicated JAK2 protein was responsible for the STAT3 activation in cancer cells [36]. In addition, our result showed that the levels of IL-6 and JAK2 treated with continuous infusion of sgp130Fc protein were reduced. It has been reported that sgp130Fc protein decreased the lipopolysaccharide-induced IL-6 mRNA and protein levels in the hippocampus [35]. And evidence from tumor studies suggested the presence of a growth regulatory loop in IL-6–JAK2–STAT3 axis [36,37]. The decreased levels of IL-6 and JAK2 may be due to STAT3 regulation of IL-6 and JAK2 transcriptions. However, whether the acute injection has an inhibitory effect on the IL-6 signaling in BTBR mice is a topic of great value for future study. Further, a genetically modified sgp130 mouse based on the BTBR strains should be powerful to verify the role of IL-6 trans-signaling as compared to the chronic infusion.

Several reports have indicated the treatment effectiveness of targeting IL-6 signaling. Maternal immune activation (MIA) yields offspring displaying mouse versions of the three core symptoms of autism [38]. Smith et al. found that coadministration of an anti-IL-6 antibody in the poly (I:C) model of MIA which causes a global IL-6 blockade prevents the social deficits caused by poly (I:C) and normalizes the associated changes in gene expression in the brains of adult offspring [39]. And diosmin, a member of the flavonoid family was able to oppose MIA-induced abnormal behavior and neuropathological abnormalities in MIA/adult offspring via targeting JAK2/STAT3 signaling [40]. Futhermore, IL-6 has been associated with other mental disorders, such as depression and schizophrenia [3]. IL-6 trans-signaling may be intimately linked to the processes that form the biological underpinnings of depression. Targeting IL-6 trans-signaling is a promising direction in depression [41]. Burton et al. reported that inhibition of IL-6 trans-signaling with repeated intracerebroventricular injection of sgp130Fc protein in the brain facilitates recovery from lipopolysaccharide induced sickness behavior in mice [34,35]. All these evidences from animal models suggest that elevation in the levels of IL-6 may play a key role in a number of neuropsychiatric conditions including autism, schizophrenia, anxiety disorders and depression. Our previous studies have revealed that IL-6 was significantly increased in the frontal cortices and cerebellum of autistic subjects and elevated IL-6 in the brain of wild type mouse displayed many autistic features. These results further support our hypothesis that the detrimental effects of IL-6 in the etiology of conditions triggered by concomitant genetic predisposition and unfavorable environmental factors could be an important pathogenic factor involved in the mediation of autism-like behaviors [2].

The previous study indicated that IL-6 elevation in the brain mediated autistic-like behaviors in mice, possibly through the imbalances of neural circuitry and impairments of synaptic plasticity [9]. Several studies support the view that pathologic synaptic rearrangement in autism might be caused by an increase in the strength of local excitatory glutamatergic synapses, and/or by a decrease in the number or effectiveness of local GABAergic synapses [42]. Garcia-Oscos et al. reported that stress-induced IL-6 shifts the balance between synaptic inhibition and excitation and the synaptic inhibition/excitation ratio was prevented by prior intraventricular injection of IL-6 trans-signaling blocker sgp130Fc protein [43]. However, in this study, we didn't observe a change of the number of excitatory synapse and level of synaptic proteins in BTBR cortex after the inhibition of IL-6 trans-signaling. And the density and PSDs of dendritic spine in cortex were also not altered between IL-6 blocked BTBR mice and controls. These findings suggest that the therapeutic efficacy of the inhibition of IL-6 trans-signaling in treating social deficits in autism may not attribute to the change of the number and structure of excitatory synapses. One possible reason for this result is that the neurobiological factors that account for the BTBR sociability levels may be not caused by the structural abnormalities of neuronal synapses. Recently, we compared the morphology of excitatory synapses between the BTBR mice with low level sociability and B6 mice with high level sociability in old age stage. The results revealed that these two strains have the same number of excitatory synapses and dendritic spine shape [44]. It is understandable that for autism, as well as other neuropsychiatric diseases, their profound consequences are not necessarily the direct effects of immediate cellular or biochemical damage, but typically are secondary to sometimes years-long chains of psychological, biochemical and synaptic modifications that may be very difficult to revert [42].

An interesting finding in the present study is that inhibition of IL-6 trans-signaling increased the evoked glutamate release in SNs from the cerebral cortex of BTBR mice. Glutamate is the major excitatory neurotransmitter in the brain and may be a key neurotransmitter involved in autism. There have been several studies of glutamate concentration in autism using proton magnetic resonance spectroscopy (1H-MRS). However, there is no consistent conclusion. As summarized in a recent review, while 8 of the published MRS studies of glutamate and glutamine signal have reported increases in autism relative to controls, 4 have reported the reverse effect and a single study has reported no difference [45]. There is a cumulating body of evidences suggest that the glutamate system exerts an important role in regulating the balance between neuronal excitation and inhibition. D'Arcangelo et al. found that acute IL-6 treatment inhibited glutamate release from cortical synaptosomes, suggesting that IL-6 may act as a neuronal modulator of excitatory neurons [46]. On the other hand, Garcia-Oscos et al. found that IL-6 superfusion decreased inhibitory post-synaptic current (IPSC) amplitude from pyramidal cells in the medial prefrontal cortex, and slice incubation and superfusion with sgp130Fc protein prevented the inhibitory effect of IL-6 on IPSCs [47]. A limitation of this study is

that it did not test the effect of inhibition of IL-6 trans-signaling on the structure and function of inhibitory synapses. However, it is conceivable that IL-6 may have diverse effects on the synaptic transmission in various animal conditions. Furthermore, it is helpful to investigate the effect of inhibition of IL-6 trans-signaling on the animals without autistic behaviors such as B6 mice. Whether the inhibition of IL-6 trans-signaling affect any other types of behaviors that are not affected in BTBR mice such as anxiety-related or depressive-like behaviors also needs to be explored.

Altogether, the results in our study showed that chronic infusion of IL-6 trans-signaling blocker sgp130Fc protein increased the sociability in autism model, possibly through the increase of the evoked glutamate release in the cerebral cortex. Inhibition of excessive production of IL-6 may have selective therapeutic efficacy in treating abnormal social behaviors in autism. The therapeutic implications of IL-6 blockade in schizophrenia, depression and sickness behavior, combined our finding in autism, suggest that targeting IL-6 is a promising field for translation medicine in mental diseases. The current study extends the body of literature and shows the effectiveness of sgp130Fc protein in inhibiting IL-6 trans-signaling in brain of animal. Since trans-signaling is a dominant mechanism for the pathogenic actions of IL-6 in the central nervous system, specific blockade of this signaling pathway by sgp130Fc protein might be superior to global IL-6 blockade by neutralizing antibodies directed at IL-6 or IL-6R. Physiological barriers like the blood-brain barrier and blood-cerebrospinal fluid barrier as well as various efflux transporter proteins make a huge challenge for effective delivery of drugs to the central nervous system [48]. Further studies are needed to investigate how to overcome the barriers and deliver the sgp130Fc protein into the brain.

Disclosure

The authors declare no conflict of interest.

Transparency document

The transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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