Dysfunction of Microglial STAT3 Alleviates Depressive Behavior via Neuron–Microglia Interactions

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Neuron–microglia interactions have a crucial role in maintaining the neuroimmune system. The balance of neuroimmune system has emerged as an important process in the pathophysiology of depression. However, how neuron–microglia interactions contribute to major depressive disorders has been poorly understood. Herein, we demonstrated that microglia-derived synaptic changes induced through STAT3 knockout (KO) (STAT3fl/fl;LysM-Cre+−) mice. We found that microglia-specific STAT3 KO mice showed antidepressive-like behavior in the forced swim, tail suspension, sucrose preference, and open-field tests. Surprisingly, the secretion of macrophage colony-stimulating factor (M-CSF) was increased from neuronal cells in the brains of STAT3fl/fl;LysM-Cre+− mice. Moreover, the phosphorylation of antidepressant-targeting mediators and brain-derived neurotrophic factor expression were increased in the brains of STAT3fl/fl;LysM-Cre+− mice as well as in neuronal cells in response to M-CSF stimulation. Importantly, the miniature excitatory postsynaptic current frequency in the medial prefrontal cortex was increased in STAT3fl/fl;LysM-Cre+− mice and in the M-CSF treatment group. Collectively, microglial STAT3 regulates depression-related behaviors via neuronal M-CSF-mediated synaptic activity, suggesting that inhibition of microglial STAT3 might be a new therapeutic strategy for depression.

Neuropsychopharmacology (2017) 42, 2072–2086; doi:10.1038/npp.2017.93; published online 7 June 2017

INTRODUCTION

Neuron–microglia interactions have a crucial role in maintaining the neuroimmune system (Rogers et al, 2011; Wake et al, 2013; Zhan et al, 2014). Recent evidence has focused on the imbalance of the neuroimmune system in association with psychiatric disorders, such as major depression (Couch et al, 2013). Presumably, microglial dysfunction causes disturbances in synaptic regulation, resulting in major depression. However, the cellular and molecular mechanisms of major depression underpinning the bidirectional interplay between neurons and microglia remain unclear.

For decades, many studies focused on novel therapeutic approaches for major depression (Domino et al, 2008; Goodyer et al, 2007). The intracellular signaling pathways of extracellular signal-regulated kinase (ERK)1/2, Akt and glycogen synthase kinase-3β (GSK3β) have been identified as the potent targets for antidepressants (Duman et al, 2016). According to postmortem studies, ERK1/2 signaling, which is downregulated in the brains of patients with major depression (Dwivedi et al, 2005; Dwivedi et al, 2001), has been implicated in antidepressant treatment (Duman et al, 2012b; Einat et al, 2003; Tardito et al, 2006). Although the blockade of the ERK signaling pathway leads to depression-related behaviors, antidepressant treatments increase ERK phosphorylation (Gourley et al, 2007; Hisaoka et al, 2007). In addition, GSK3β is regarded as a key factor involved in psychiatric diseases (Beurel et al, 2015; Chuang et al, 2011; Jope and Roh, 2006). Recent studies showed increased GSK3β activity in the cortical regions of postmortem brains of suicide victims who had suffered from depression (Karege et al, 2007). However, the inactivation of GSK3β using lithium or valproate may alleviate unipolar depression (Chen et al, 2000; Cipriani et al, 2006; Davis et al, 1996). GSK3β is.
also negatively regulated by phosphatidylinositol 3-kinase-mediated Akt activation (Fang et al., 2000).

Despite having a sufficient knowledge of ERK1/2 and Akt/GSK3β signaling pathways, the etiological factors leading to major depression remain unknown. Recent studies showed that neuroinflammation caused by stress-induced activation of microglia leads to depressive-like behaviors (Brites and Fernandes, 2015; Steiner et al., 2011; Streit et al., 2004). In addition to microglial activation, microglial senescence and decline can negatively affect neurogenesis, causing major depression (Caldeira et al., 2014; Kreisel et al., 2014). These findings suggest the importance of microglial activation status in depression.

Microglia provide pro- and anti-inflammatory cytokines as mediators for the neuroimmune system. Signal transducer and activator of transcription 3 (STAT3) is one of the transcription factors for cytokine production (El Kasmi et al., 2006), such as soluble intracellular cell adhesion molecule-1 (sICAM-1) (Park et al., 2013), interleukin (IL)-6 (Mori et al., 2011), IL-10 (Riley et al., 1999), tumor necrosis factor-α (TNF-α) (Chabot et al., 1997; Riazi et al., 2008) and IL-1β (Clausen et al., 2008). These cytokines have been identified as mediators for the neuroimmune system of depression (Hodes et al., 2015). For example, the IL-6/STAT3 signaling pathway was shown to be involved in depressive-like behavior (Kong et al., 2015). In addition, pathological levels of these cytokines contribute to behavioral deficits such as depressive-like behavior (Audet and Anisman, 2013; Felger and Lotrich, 2013; Khairova et al., 2009; Schiepers et al., 2005), requiring a more sophisticated manipulation of cytokine levels.

In this study, we hypothesized that STAT3 signaling in microglia affects neuron–microglia interactions via secreted cytokines, resulting in synaptic and behavioral changes. To investigate the key factors regulated by microglial STAT3 in neuron–microglia interactions, we used microglia-targeted STAT3-deficient mice and analyzed the molecular mechanisms and their behavioral phenotypes. As a result, we revealed that STAT3 dysfunction in microglia led to antidepressive-like behavior via crosstalk between neurons and microglia, suggesting a novel therapeutic avenue for major depression.

**MATERIALS AND METHODS**

**Experimental Animals and Genotyping**

Mice homozygous for the loxP-flanked (floxed) Stat3 gene (Stat3fl/fl) were kindly gifted from Dr S Akira. Mice carrying a Cre transgene under the control of the distal LysM promoter (LysM-Cre/+), were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice with a STAT3 deletion in myeloid cells were generated by crossing mice with the floxed STAT3 allele with mice expressing Cre under the control of the LysM promoter. Genomic DNA was isolated from tail tips using a NucleoSpin genomic DNA purification kit (Macherey-Nagel GmbH, Duren, North Rhine-Westphalia, Germany). The PCR reaction was performed using AccuPower PCR premix (Bioneer, Daejeon, Korea) with the primers, which are specific for exons 22 and 23 of STAT3 and Cre transgene, according to the manufacturer’s instructions. All experiments were performed with male mice aged 8–10 weeks. Experimental animals were maintained under specific pathogen-free conditions and 22 ± 1°C with a reversed 12 h light–dark cycle (lights on at 0700 h). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the College of Medicine, Seoul National University.

**Primary Microglia Cell Culture**

Primary microglia cells were isolated from primary mixed glial cells of 2-day-old mice. To obtain mixed glial cells, cerebral cortices were dissected, carefully stripped of their meninges, and dissociated into a single-cell suspension by trituration. Cells were cultured on poly-l-lysine (Sigma-Aldrich, St Louis, MO)-coated 100 mm² culture dish in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Gibco, Grand Island, NY), and incubated at 37°C in 5% CO₂. On the third day of culture, cells were vigorously washed with pipetting and the media was replaced to remove debris. After 6 days in vitro, cells were transferred to a T-75 flask and incubated for 1–3 weeks. Then, the conditioned media were replaced with fresh media to achieve complete confluence. To isolate microglia, the T-75 flask was rotated (200 rpm, 37°C, 5 h) using a temperature-controlled, non-humidified shaker, and then supernatant media containing microglia was centrifuged (215g, 5 min). The microglial pellet was suspended and seeded onto poly-l-lysine-coated 60 mm² cover glass-bottom dish (SPL Life Sciences, Pocheon, Korea), and incubated at 37°C in 5% CO₂.

**Cell Culture and Co-Culture Experiments**

Mouse microglia cell line BV2 and mouse hippocampal neuronal cell line HT22 (ATCC, Manassas, VA) were cultured in DMEM containing 10% FBS and 1% penicillin–streptomycin solution, and incubated at 37°C in 5% CO₂. HT22 cells were starved before stimulation for 8 h and then stimulated with macrophage colony-stimulating factor (M-CSF) (40 ng/ml, ProSpec, East Brunswick, NJ) in a time-dependent manner or 24 h. Before the co-culture experiment, BV2 cells were seeded in six-well dishes at 4 × 10⁵ cells/well and transfected with STAT3 siRNAs for 24 h. The cells were washed twice with PBS and then incubated in fresh DMEM containing 10% FBS after transfection. Then, the HT22 cells were cultured on 0.4 μm pore-size Falcon cell culture inserts (Corning, Durham, NC) at 2 × 10⁷ cells/well in DMEM containing 10% FBS and co-cultured with BV2 cells for 24 h. To confirm the effect of macrophages on neurons, HT22 cells were treated with culture medium of RAW264.7 cells with or without STAT3 inhibition for 24 h.

**siRNA Transfection**

BV2 cells and RAW264.7 cells were cultured in the growth condition to a density of 2 × 10⁵ cells in six-well culture plate. The cells were transfected with the siRNAs using HiperFect transfection reagent (Qiagen, Hilden, Germany) according to the fast-forward protocol of manufacturer’s instructions. STAT3 siRNAs targeting two different regions of STAT3 (SI01435287 and SI01435294) and a negative control siRNA (1027280) were purchased from Qiagen.
Immunohistochemistry

Brains of mice were perfused with buffer containing 4% paraformaldehyde, fixed for 2 days in 4% paraformaldehyde at 4 °C, and embedded in paraffin. The paraffin blocks were cut using a microtome (4 μm, Finesse E+, Thermo Shandon, Runcom, UK). Paraffin slices were mounted on the silane-coated micro slides (Muto Pure Chemicals, Tokyo, Japan) and then allowed to air dry at room temperature in the dark for 24 h. Before immunostaining, the slides were deparaffinized in xylene, dehydrated through graded alcohols, and heated in citrate buffer for 10 min. Nonspecific binding was blocked with 5% normal goat serum (Bovogen, East Keilor, VIC, AUS) in PBS. The fluorescent immunostaining was performed with the primary antibodies for p-STAT3 (1:200, Cell Signaling Technology, MA), STAT3 (1:200, Abcam, Cambridge, UK), Iba-1 (1:200, Wako, Japan), Iba-1 (1:200, Novus Biologicals, CO), and NeuN (1:200, Merck Millipore, MA), and visualized using Cy3 goat anti-mouse IgG, 488 goat anti-mouse IgG, Cy3 donkey anti-rabbit IgG (1:200, Biolegend, CA), 488 donkey anti-rabbit IgG (1:200, Abcam), and 488 bovine anti-goat IgG (1:200, Santa Cruz Biotechnology, CA, USA). The slides were mounted with 4',6-diamidino-2-phenylindole, and images were collected using the LSM510 program on a confocal microscope (Carl Zeiss MicroImaging, München, Germany).

Restraint Stress Procedure

Mice were transferred to a behavior analysis test room before initiating chronic stress procedures. For the chronic stress, the experimental group was immobilized in a rodent restrainer (Ø 30 × 95 mm) and separated from the control group for the duration (2 h a day for 14 consecutive days). After the chronic restraint stress, the mice were returned to their home cages for a day and then behavioral experiments were conducted.

Behavioral Experiments

Behavioral experiments were performed independently according to the behavioral test paradigm and separate groups were used for each behavioral test paradigm.

Experimental mice were subjected to tail suspension and forced swimming tests at intervals of 1 day after 14 days of chronic restraint stress to assess despair-based behavior. A separate cohort of mice was used to conduct sucrose preference test to assess reward-based behavior under normal and chronic stress conditions. Another group was used to assess behavior based on anxiety and locomotion; the following series of behavioral experiments were conducted at intervals of 2 days: handling, elevated plus maze test, open-field test, and rotarod test. All mice were killed immediately after the behavioral experiment and all brain tissues of the mice used in the behavioral experiments under chronic restraint stress were extracted.

Forced Swim Test

The forced swim test was performed as described previously (Page et al., 1999). Briefly, mice were placed in a glass beaker (2 l beaker), which was filled to a depth of 18 cm with water (25 °C). The water was regularly changed between subjects. The duration of immobility was recorded and measured with a video camera for 6 min.

Tail Suspension Test

Mice were suspended by their tails from a steel bar using adhesive tape in a chamber with opaque walls. The distance between the floor of the chamber and the steel bar was ~ 40 cm. Mice that climbed onto their tails or fell off during the test were excluded from analyses. Mice movements were videotaped for 6 min and the total duration of immobility recorded.

Sucrose Preference Test

The sucrose preference test was conducted as described previously (Strekalova et al., 2004). After food and water deprivation for 24 h, mice were given free access to both water and 1% sucrose solution in individual cages for 24 h. The position of each bottle was switched after 12 h to rule out side preference. The consumption of water and sucrose solution was measured by weighing the bottles. The preference for sucrose solution was calculated as a percentage of the volume of consumed sucrose solution over the total volume of liquid drunk.

Open-Field Test

Mice were placed in the center of an open field box (40 × 40 × 40 cm), illuminated by the light of 20 lx intensity,
and the mice movements were recorded with a video camera for 30 min. The total distance traveled and time spent in the center of the entire open field (20 × 20 cm) were calculated using video tracking software (EthoVision XT 8.5, Noldus).

### Elevated Plus Maze Test

The elevated plus maze consisted of two open arms (30 × 5 cm) and two closed arms of the same size, with 15 cm high side walls. The four arms and central square were...
50 cm above the ground. Mice were placed in the central square of the maze (5 × 5 cm), facing the open arms. The mice movements were recorded during a 5 min test period. The number of entries and the time spent in the open and closed arms were calculated using video tracking software (EthoVision XT 8.5, Noldus).

**Rotarod Test**

The rotarod test was performed by a coordination test system (Rotamex 5, Columbus). The mice were placed on a rotating rod (3 cm in diameter) accelerated from 3 to 50 rpm for 6 min.

**Cytokine Array and Enzyme-Linked Immunosorbent Assay (ELISA)**

The expression of cytokines and chemokines in the culture supernatant was assessed with a mouse cytokine antibody array panel A (R&D Systems, MN) according to the manufacturer’s instructions. Total M-CSF in the conditioned medium of the cultured cells was measured using the quantikine ELISA kit (R&D Systems) according to the manufacturer’s instructions. The data were presented as absorbance units at 450 nm and correction absorbance units at 540 nm from three independent experiments.

**RNA Isolation and Quantitative Real-Time PCR**

Total RNA was isolated from BV2 and HT22 cells using an RNAliso Plus reagent (Takara, Shiga, Japan) and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed using the EvaGreen qPCR Mastermix (Applied Biological Materials), and the results were normalized to the signals of GAPDH expression. Primers for CCL2 (QT00167832), TNF-α (QT00148750), ICAM-1 (QT00155078), STAT3 (QT00148750), GSK3β (QT00167832), α-tubulin (QT00167832), and GAPDH (QT00167832), and GAPDH (QT00167892) were purchased from Qiagen.

**Western Blotting**

Cells were washed with the cold PBS and then lysed in the Triton lysis buffer containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.4), 0.35 M NaCl, 0.5% Nonidet P-40, 10% glycerol, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na3VO4, 1 mM PMSF, and 0.5 mM NaF. Brain tissue was homogenized and lysed in the triton lysis buffer. After incubation for 30 min on ice, insoluble debris was removed by centrifugation at 16 000 g for 10 min at 4°C. The lysates were resolved in SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Pittsburgh, PA). The membranes were blocked in 5% skim-milk (LPS Solutions, Daejeon, Korea) and probed with the primary antibodies for phospho-STAT3, STAT3, phospho-ERK1/2, ERK1/2, phospho-Akt, Akt (1:1000, Cell Signaling Technology), brain-derived neurotrophic factor (BDNF), phospho-GSK3β, GSK3β (1:1000, Santa Cruz Biotechnology), α-tubulin (1:1000, Thermo Scientific, Grand Island, NY), VGLUT1, PSD95 (1:2000, 1:1000, Synaptic Systems, Göttingen, Germany), Synaptophysin (1:1000, Epitomiks, Burlingame, CA), GFAP (DAKO, CA), and Iba-1 (Novus Biologicals) for the target molecules, followed by HRP-conjugated secondary antibodies for goat anti-mouse IgG, goat anti-rabbit IgG (1:10 000, Enzo Life Science, NY), and donkey anti-goat IgG (1:10 000, Santa Cruz Biotechnology). The membranes were visualized using an ECL detection kit (SurModic, Eden Prairie, MN).

**Slice Preparation and Electrophysiology**

Mice were anesthetized with isoflurane and decapitated, and the brains were immediately removed and placed in ice-cold slicing solution (0–4°C) containing the following artificial cerebrospinal fluid: 124 mM NaCl, 2.5 mM KCl, 1 mM NaH2PO4, 1.3 mM MgCl2, 2.5 mM CaCl2, 26.2 mM NaHCO3, and 20 mM d-glucose, bubbled with a gas mixture of 5% CO2/95% O2 to maintain a pH of 7.4. Coronal slices containing medial prefrontal cortex (mPFC) were obtained from hekera (slice thickness 250 μm; Leica VT1200S; Leica, Nussloch, Germany). After cutting, the slices were kept for 30 min at 35°C and stimulated with M-CSF (10 nM) for synaptosomal preparations and whole-cell recordings. Whole-cell recordings in the mPFC pyramidal cells were performed in the voltage-clamp mode using an amplifier (HEKA Instruments, Lambrecht/Pfalz, Germany). The signal was acquired at 10 kHz and low-pass filtered at 5 kHz. For recording the spontaneous miniature excitatory post synaptic currents (mEPSCs), the recording electrodes (resistance 2–4 MΩ) were filled with a solution containing (in mM) 135 mM Cs-methane sulfonate, 10 mM CsCl, 10 mM HEPES, 4 mM Mg2ATP, 0.4 mM Na3GTP, and 0.2 mM EGTA (pH 7.25). Synaptic responses were analyzed by Mini Analysis Program, Synaptosoft.
Golgi Staining

Dendritic spine density was analyzed using an FD Rapid GolgiStain Kit (FD Neurotechnologies, Baltimore, MD) was used according to the manufacturer's instructions. In brief, mice were anesthetized with isoflurane and decapitated. The brains were immediately removed, rinsed, and immersed in the impregnation solution, and stored at room temperature for 2 weeks in the dark. The brains were transferred into a solution containing sucrose and stored at room temperature in the dark for at least 3 days. Frozen slices of the brain were cut using a freezing microtome (100 μm, Cryotome FE,
Therm Shandon). The slices were mounted on gelatin-coated microscope slides and then allowed to air dry at room temperature in the dark for 3 days. Slides were then rinsed with distilled water before being dehydrated in absolute alcohol, cleared with xylene, and covered with non-acidic synthetic balsam and cover slips.

Brain Synaptosomal Preparation

Brain synaptosomal preparation was performed as described previously (Kamat et al., 2014). In brief, the prefrontal cortex was homogenized in 10% (w/v) of 0.32 M sucrose-HEPES buffer on ice and the homogenate was centrifuged at 600 g for 10 min at 4–8 °C. The HEPES buffer was composed of 145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM glucose, and 5 mM HEPES (pH 7.4). The supernatant was diluted 1:1 with 1.3 M HEPES sucrose, to yield a suspension with a final concentration of 0.8 M HEPES sucrose. This suspension was further centrifuged at three times with HEPES buffer at 12 000 g for 15 min at 4 °C. The pellet consisting of synaptosomes was suspended in RIPA buffer (mixed with a protease inhibitor and PMSF) along with 0.2% TritonX-100 and centrifuged at 20 000 g for 30 min. The resulting synaptosomes were immediately used for the western blotting.

Statistical Analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of cytokine array. Multiple comparisons were investigated via Tukey-Kramer’s post hoc test. Two-way ANOVA (genotype × stress) was applied to assess behavioral experiments with chronic restraint stress. Bonferroni’s post hoc test was performed if applicable. Repeated-measures ANOVA (genotype × trial session) was used to analyze locomotive activity, and learning and memory. Group comparisons of immunofluorescence intensity, RNA and protein expressions, and electrophysiology data were performed by Student’s t-tests. The data were assessed to ensure normality with the Shapiro-Wilk test, and where violations of the normality assumptions were found, non-parametric statistics were conducted. All data are presented in means ± SEM. All statistical analyses were conducted using SigmaPlot software (ver. 13, Systat Software, San Jose, CA).

RESULTS

Targeting STAT3 in Microglia by Using STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> Mice

Microglia are unique glial cells derived from common myeloid progenitors during developmental stages of the CNS, possibly explaining the experimental paradigm for neuron-microglia interactions. In this study, we targeted STAT3 in microglia, a key transcription factor for immune responses, due to its relevance in the regulation of cytokine expression levels. We employed the myeloid cell-specific STAT3-deficient mouse model by breeding STAT3 floxed mice and LysM-Cre mice. We denoted STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> as wild-type (WT) and STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> as KO (Figure 1a; see Materials and Methods).

To verify a STAT3 depletion in microglia, STAT3 gene KO was first confirmed by genotyping (Figure 1b). The absence of microglial STAT3 expression (red, anti-STAT3; green, anti-Iba-1) in the prefrontal cortex of STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> mice was verified using tissue immunostaining. The profile of immunofluorescence intensity showed that STAT3 was not expressed in microglia (Figure 1c). In primary cultured microglial cells (red, anti-Iba-1) obtained from the STAT3-deficient mouse model, the expression of STAT3 (green, anti-STAT3) was completely depleted in STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> mice, as well as shown in quantitative data (Figures 1d and e; p < 0.001). From the immunoblotting analysis, we also confirmed that the STAT3 expression was depleted specifically in microglia, but not in neurons and astrocytes that were isolated from each primary cultured cell (Figure 1f; p < 0.001). In addition, depletion of STAT3 in microglia had no effect on the expression of STAT3 in neurons and on the number of both neurons and microglia in STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> mice (Supplementary Figure S1).

Loss of Microglial STAT3 Leads to Antidepressive-Like Behavior

To investigate the behavioral correlates of neuron–microglia interactions, we examined mood-related behaviors in the model mice. Interestingly, we observed antidepressive-like behaviors in the tail suspension test, forced swim test, sucrose preference test, and open-field test. As animal models of stress-induced depression to validate the stress-resistant behavior, we induced chronic restraint stress for 2 h a day for 2 weeks. The level of STAT3 phosphorylation were increased in the WT, but not in STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> mice under chronic stress conditions (Supplementary Figure S2). In the tail suspension and forced swim tests, the immobility time was significantly reduced in STAT3<sup>fl/fl</sup>;LysM-Cre<sup>−/−</sup> mice both in control and in chronic stress conditions (Figure 2a; genotype × stress interaction: F<sub>(1,38)=</sub> 1.443, p = 0.237; genotype effect: F<sub>(1,38)=</sub> 24.906, p < 0.001; stress effect: F<sub>(1,38)=</sub> 8.469, p = 0.006, and Figure 2b; genotype × stress interaction: F<sub>(1,36)=</sub> 0.415, p = 0.523; genotype effect: F<sub>(1,36)=</sub> 23.718, p < 0.001; stress effect: F<sub>(1,36)=</sub> 38.4,
Under chronic stress conditions, the immobility time of STAT3fl/fl;LysM-Cre+/− mice was increased in the forced swim tests, but not in the tail suspension tests (Figure 2b). These results indicate that STAT3fl/fl;LysM-Cre+/− mice had intrinsic resistance owing to genetic modification and showed partial resilience to chronic stress conditions. To ensure consistency among WT groups, immobility time was further compared in the tail suspension and forced swim test. The WT groups did not differ from each other (Supplementary Figure S3). Corroborating the generality of these findings, the WT mice subject to chronic restraint stress exhibited a significant decrease in sucrose preference. However, the sucrose preference of STAT3fl/fl;LysM-Cre+/− mice was higher than WT mice under chronic stress conditions (Figure 2c; genotype × stress interaction: \( F_{(1,35)} = 12.295, p < 0.001 \); genotype effect: \( F_{(1,35)} = 15.764, p < 0.001 \); stress effect: \( F_{(1,35)} = 15.601, p < 0.001 \)). Both WT and STAT3fl/fl;LysM-Cre+/− mice showed similar amounts of liquid intake under both control and stressed conditions (Figure 2d; genotype × stress interaction: \( F_{(1,35)} = 0.573, p = 0.454 \); genotype effect: \( F_{(1,35)} = 0.177, p = 0.677 \); stress effect: \( F_{(1,35)} = 4.071, p = 0.051 \)). Lastly, we verified reduced locomotor activity of STAT3fl/fl;LysM-Cre+/− mice in the open-field test (Figure 2e; \( p < 0.05 \)), but not the exploration time in the center area (Figure 2f). A previous study demonstrated that the stressed mice showed enhanced locomotor activity under the light of modest brightness (Strekalovala et al, 2004). Our results suggest that antidepressive-like behavior was observed in STAT3fl/fl; LysM-Cre+/− mice. Collectively, dysregulation of STAT3 in microglia successfully alleviated depressive and stress-induced behaviors.

In addition, we found no differences in body weight, anxiety-related, and motor behaviors of STAT3fl/fl;LysM-Cre+/− mice (Supplementary Figure S4a and Figures 2g and h; trial session effect: \( F_{(2,30)} = 83.245, p < 0.001 \); genotype effect: \( F_{(1,15)} = 0.008, p = 0.931 \), demonstrating that deletion of STAT3 in microglia had solely contributed to antidepressive effects. We also tested whether the antidepressive effects could affect other cognitive functions associated with learning and memory, but found neither cognitive impairment nor enhancement in STAT3fl/fl;LysM-Cre+/− mice (Supplementary Figure S4b–f).

**Downregulation of Microglial STAT3 Increases Neuronal M-CSF Levels in Neuron-Microglia Interactions**

To identify key factors affecting antidepressive-like behavior caused by depletion of microglial STAT3, we first examined the cytokine levels in the brain tissue of both WT and STAT3fl/fl;LysM-Cre+/− mice using a cytokine array. The data showed that M-CSF was increased solely in STAT3fl/fl; LysM-Cre+/− mice (Figures 3a and b; one-way ANOVA: \( F_{(1,4)} = 9.304, p < 0.05 \)). The effect of M-CSF in association with antidepressive-like behavior was highly localized to several brain regions, such as prefrontal cortex, cerebellum, and hippocampus, where M-CSF receptors were primarily expressed (Figure 3c; \( p < 0.001 \), Figure 3d; \( p < 0.01 \), and Figure 3e; \( p < 0.05 \)). However, we could not detect any change in the levels of canonical cytokines, such as ICAM-1, IL-1β, TNF-α, IL-6, and IL-10 (Figure 3b and Supplementary Figure S5). These results suggest that M-CSF may be a key factor for regulating antidepressive-like behavior. To determine whether peripheral macrophages had an effect on increasing M-CSF, we applied the same cytokine array to peritoneal macrophages isolated from both WT and STAT3fl/fl;LysM-Cre+/− mice (Figure 3f and Supplementary Figure S6a). sICAM-1 and CCL2 were increased, but CXCL13 was decreased in macrophages of STAT3fl/fl;LysM-Cre+/− mice (Figure 3g; CXCL13: \( F_{(1,2)} = 71.092, p < 0.05 \); sICAM-1: \( F_{(1,2)} = 22.346, p < 0.05 \); CCL2: \( F_{(1,2)} = 71.906, p < 0.05 \)). These factors were expressed at the similar level in the brain tissue, indicating that M-CSF-mediated antidepressive-like behavior was not associated with peripheral macrophages. In short, the results indicate that M-CSF production regulates antidepressive-like behaviors through neuron–microglia interactions.

To mimic the neuron–microglia interactions of STAT3fl/fl; LysM-Cre+/− mice model, we performed in vitro coculture using transwell inserts with both the neuronal cell line HT22 and the microglia cell line BV2 (Figure 3h). Endogenous STAT3 level was silenced by transfection with specific small interfering RNA for STAT3 (siSTAT3) in BV2 cells with the maintenance of cell viability (Supplementary Figure S6b and c). Although the mRNA level of STAT3 and ICAM-1 in BV2 cells decreased in the co-culture system, the M-CSF, IL-1β, and TNF-α levels remained unchanged in the cells (Figure 3i; STAT3: \( p < 0.001 \), ICAM-1: \( p < 0.01 \)). Importantly, the mRNA level of M-CSF increased in HT22 cells co-cultured with STAT3 silenced BV2 cells, but no change was observed in the STAT3, ICAM-1, IL-1β, and TNF-α mRNA levels (Figure 3j; \( p < 0.01 \)).

Based on the results, we hypothesized that the secretion of M-CSF may occur mainly in neuronal cells. To examine this, we measured the concentration of M-CSF using ELISA in the co-culture system. The amount of secreted M-CSF was highly increased in the co-culture medium of HT22 cells with STAT3-silenced BV2 cells (Figure 3k; \( p < 0.001 \)), however,
that of M-CSF secretion had no difference between the control and STAT3-silenced BV2 cells (Figure 3l). Thus, the data suggest that ablation of STAT3 in microglia is crucial for the increase in M-CSF production in neuronal cells and that these mechanisms may have a crucial role in neuronal functions, leading to the antidepressive-like behavior in STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mice.

**M-CSF Upregulates Antidepressant Signaling Pathways and BDNF Expression**

To identify how the secreted M-CSF triggers the activation of antidepressant signaling pathways, we investigated BDNF levels and signaling cascades of ERK1/2 and Akt/GSK3β in the STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mouse model. We observed that the BDNF expression as well as ERK1/2 and Akt/GSK3β phosphorylation were increased in several brain regions of STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mice, including the prefrontal cortex, cerebellum, and hippocampus (Figure 4a; all <i>p</i> < 0.05). In synaptosomes from the prefrontal cortex of STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mice, the phosphorylation of ERK1/2 and Akt/GSK3β was enhanced, along with increased BDNF expression; however, we found no changes in the levels of pre-/post-synaptic proteins, such as synaptophysin, VGLUT1, and PSD95 (Figure 4b; all <i>p</i> < 0.05). Lastly, we verified the direct effects of M-CSF on ERK1/2 and Akt/GSK3β signaling pathways in a time-dependent manner in synaptosomes from cortical slices of the WT mice. M-CSF stimulation gradually increased phosphorylation of ERK1/2 and Akt/GSK3β in synaptic level (Figure 4c; ERK1/2 at 30, 60 min, Akt and GSK3β at 30 min: <i>p</i> < 0.05, Akt at 15 min and GSK3β at 60 min: <i>p</i> < 0.01). The results imply that M-CSF activates antidepressant pathways, along with BDNF production.

We further confirmed the results in an in vitro co-culture system. The expression of BDNF as well as phosphorylation of ERK1/2 and Akt/GSK3β was increased only in HT22 cells co-cultured with STAT3-silenced BV2 cells (Figure 4d; all <i>p</i> < 0.05). As expected, BDNF expression was persistently increased and both ERK1/2 and Akt/GSK3β remained phosphorylated after M-CSF stimulation for 24 h in HT22 cells (Figure 4e; all <i>p</i> < 0.05). Finally, we observed the direct effects of M-CSF within an hour on ERK1/2 and Akt/GSK3β signaling pathways in HT22 cells (Figure 4f; ERK1/2 at 60 min, Akt at 30 min, and GSK3β at 15, 30 min: <i>p</i> < 0.05, ERK1/2 at 15, 30 min, Akt at 15 min, and GSK3β at 60 min: <i>p</i> < 0.01). However, we found no direct effects of peritoneal macrophages on neuronal cells. ERK1/2 and Akt/GSK3β signaling pathways did not induce any change in HT22 cells with macrophage cell line RAW264.7 medium regardless of whether STAT3 was downregulated or not (Figure 4g and Supplementary Figure S6d and e). We reasoned that the effects of M-CSF stimulation on microglia should be tested since M-CSF was one of the well-known inducers for intracellular signaling in microglia (Imaizumi and Kohsaka, 2002). Data showed that the phosphorylation of ERK1/2 and Akt/GSK3β did not increase in STAT3-silenced BV2 cells (Supplementary Figure S7). Collectively, our data strongly indicate that M-CSF may be critically involved in antidepressive-like behavior by upregulating BDNF expression through a direct effect on the ERK1/2 and Akt/GSK3β signaling cascades.

**Depletion of Microglial STAT3 Enhances M-CSF-Mediated Glutamatergic Neurotransmission**

As neuronal mechanisms of depression, the increased synaptic transmission could be a potent mechanism from a therapeutic point of view (Duman and Aghajanian, 2012a). To explore the microglia-derived effects on neurotransmission, we investigated the excitatory synapses of the pyramidal cells (layer 5) in the mPFC by recording α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated mEPSCs. As the representative traces demonstrated (Figure 5a), the cumulative probability distributions of the mEPSC frequency increased in STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> model mice (Figure 5b; <i>p</i> < 0.05); however, those of the mEPSC amplitudes were not statistically different (Figure 5c). These results imply that microglia-derived effects could facilitate the release of neurotransmitters on presynaptic synapses.

The above results led us to further investigate whether M-CSF could affect the release of neurotransmitters on presynaptic synapses. We incubated brain slices with M-CSF for 10 min and measured the mEPSCs. As the representative traces illustrated (Figure 5d), the cumulative probability distributions of the mEPSC frequency were increased in the presence of M-CSF (Figure 5e; <i>p</i> < 0.05); however, those of the mEPSC amplitudes were not statistically different (Figure 5f).

Furthermore, we investigated whether the increase in the mEPSC frequency caused by M-CSF was involved in the presynaptic effect. By stimulating the layer 2/3 of the mPFC, we measured the paired-pulse ratio in the excitatory synapses of the pyramidal cells (layer 5). As the representative traces illustrated (Figure 5g), the probability of neurotransmitter release was increased in STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mice (Figures 5h and i; all <i>p</i> < 0.01). In addition, we analyzed the shape of individual traces of AMPA receptor-mediated EPSCs, but found no differences in decay time and 10%–90% rise time in both STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mice (Figures 5h and i). In morphological perspectives, Golgi staining showed no differences in the number of dendritic spines between the WT and STAT3<sup>−/−</sup>;LysM-Cre<sup>−/−</sup> mice (Figures 5j and k). We concluded that elevated M-CSF secretion in neuronal cells caused by interaction with STAT3-deficient microglia presynaptically enhanced glutamatergic neurotransmission.

**DISCUSSION**

In the present study, we explored the mechanisms of neuron–microglia interactions. Primarily, we used a microglia-targeted STAT3-deficient mouse model. From an immunological perspective, STAT3 signaling has a major role in immune responses and microglia are CNS-resident immune cells that sense stress in the microenvironment. However, microglia were shown to modulate synaptic activity through functional changes (Wake et al, 2013). In the current study, we demonstrated that dysfunction of STAT3 in microglia enhanced M-CSF action on neural

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Figure 5  Depletion of microglial signal transducer and activator of transcription 3 (STAT3) enhances macrophage colony-stimulating factor (M-CSF)-mediated glutamatergic neurotransmission. (a) Representative traces of the miniature excitatory postsynaptic currents (mEPSCs) in the wild-type (WT) and knockout (KO) groups (n = 10 and 9 neurons/group). Each asterisk indicates synaptic events. (b) Graphs for the cumulative probability of the mEPSC frequency; bar graphs for an average of frequency (1.2 ± 0.27 Hz vs 2.14 ± 0.5 Hz in the WT and KO mice, respectively). (c) Graphs for the cumulative probability of the mEPSC amplitudes; bar graphs for an average of amplitudes (17.185 ± 1.306 pA vs 18.846 ± 2.186 pA in the WT and KO mice, respectively). (d) Representative traces of the mEPSCs in the control and the M-CSF treatment groups (n = 6 neurons/group). (e) Graphs for the cumulative probability of the mEPSC frequency; bar graphs for an average of frequency (1.18 ± 0.19 Hz vs 2.22 ± 0.41 Hz in the control and M-CSF treatment groups, respectively). (f) graphs for the cumulative probability of the mEPSC amplitudes; bar graphs for an average of amplitudes (13.538 ± 2.765 pA vs 11.651 ± 2.620 pA in the control and M-CSF treatment groups, respectively). (g) Representative traces for paired pulse ratio measurement in WT and KO mice (n = 6 and 5 neurons/group). (h) The graph for paired pulse ratio at the 100 ms intervals (paired-pulse ratio (PPR): the ratio of EPSC2/EPSC1) (1.365 ± 0.076 vs 0.935 ± 0.058 in the WT and KO mice, respectively). (i) Dependence of PPR on interstimulus intervals at the 50, 100 and 150 ms (1.252 ± 0.035 vs 1.013 ± 0.038 at 50 ms, 1.365 ± 0.076 vs 0.935 ± 0.058 at 100 ms, 1.018 ± 0.017 vs 0.96 ± 0.03 at 150 ms in the WT and KO mice, respectively). (j) Representative images of Golgi staining in the WT and KO groups (n = 5 mice/group). (k) Quantitative data for the number of dendritic spines (0.543 ± 0.022 μm vs 0.556 ± 0.015 μm in the WT and KO mice, n = 17 and 23 neurons/group, respectively). Scale bar = 40 μm. Scale bar of the enlarged image = 20 μm. Data are means ± SEM and *p < 0.05, **p < 0.01, and ***p < 0.001.
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functions along with upregulation of BDNF expression through antidepressant pathways and consequently alleviated depression-related behaviors. These findings could aid in the development of novel pharmaceutical antidepressant drugs.

Our findings on the behavioral association of neuronomicroglia interactions show that the STAT3 pathway in microglia may be strongly associated with antidepressive-like behavior. These behavioral experimental results could be categorized as the positive valence system according to Research Domain Criteria for emotional behavior. Despair-based behavior represented by tail suspension and forced swim tests was responsible for willingness to overcome stressful responses, by measuring motility. Similarly, behavioral processes related to hedonic responses were examined in the sucrose preference test for probing the neural systems for reward seeking behavior. Each experimental design may demonstrate partial aspects of depressive symptoms mimicking human patients' behavior; however, it is quite plausible for newly built criteria on human mental researches.

For many decades, accumulating evidence has reported a positive relationship between depression and cytokines and/or chemokines, such as TNF-α, IL-1, IL-6, and the CC chemokine ligand (CCL) family (Khairova et al, 2009). A recent study suggested that manipulation of the microglia activation status with microglial stimulators, such as lipopolysaccharide and M-CSF, could be a potent etiological therapies for depression symptomatology (Kreisel et al, 2014). For example, M-CSF could reduce the depressive-like behavior by activating microglia status under chronic stress conditions. In our microglial STAT3-deficient model, the microglial STAT3 as a sensor of the cytokine profiles was effectively depleted. Notably, the expression of M-CSF was increased in the brains of STAT3fl/fl;LysM-Cre−/− mice. The elevated mRNA levels and the secretion of M-CSF were detected in neuronal cells in in vitro co-culture system. These results implied the novel actions of M-CSF in the absence of microglial STAT3. Under altered circumstances by depletion of microglial STAT3, M-CSF can be synthesized in neuronal cells and at the same time affect the neuronal cells themselves. For example, M-CSF is produced with an autocrine/paracrine mechanism of action in distinct brain regions, including the dorsal forebrain, and cerebellum, and has an essential role in neural progenitor cell maintenance and maturation (Chitu et al, 2016). In this study, we suggest that M-CSF is involved in autocrine loops in neuronal cells.

Based on these results, we hypothesized that M-CSF actions in neuronal cells could activate certain signaling pathways for antidepressant drugs. We discovered that Akt/GSK3β was phosphorylated by stimulation with M-CSF in neuronal cells. Shared with the mechanisms of conventional antidepressant drugs, our findings could be helpful to take less risk for developing novel therapeutics. Thus, these results indicate that the regulation of Akt/GSK3β pathways by M-CSF can be a strong candidate for the development of antidepressants.

In relation to BDNF signaling, we first verified that BDNF expression, as well as the phosphorylation of ERK1/2 and Akt/GSK3β was increased in neuronal cells both in STAT3fl/fl;LysM-Cre−/− mice and in in vitro co-culture system in response to secreted M-CSF. M-CSF-induced ERK1/2 and Akt/GSK3β signaling pathways potentially work as the molecular mechanisms to regulate antidepressive-like behaviors. Many studies showed that the ERK1/2 and Akt/GSK3β signaling subsequently induce BDNF expression (Jope and Roh, 2006; Obata et al, 2003). BDNF production may also affect presynaptic neurotransmitter release rather than morphological changes of synapses. For this reason, it is important to note that M-CSF regulates antidepressant-targeting mediators and BDNF expression.

In order to examine which factors from microglia could increase the release of M-CSF from neurons, we analyzed the cytokine/chemokine profiles of microglia in the STAT3-deficient mouse model. The results showed that the expression of CCL2 was reduced in primary microglia isolated from STAT3fl/fl;LysM-Cre−/− mice, as well as in STAT3-silenced BV2 cells from in vitro co-culture system (Supplementary Figure S9a-c). We assumed that CCL2 in microglia contributed to the increase of M-CSF in neurons. However, the following results indicated that CCL2 had no effects on the changes of M-CSF levels in neurons (Supplementary Figure S9d). Besides, the expression level of CCL2 had no differences between the brains of WT and STAT3fl/fl;LysM-Cre−/− mice (Supplementary Figure S9e). These results indicate that CCL2 was not involved in the M-CSF production. Considering the modulation of M-CSF expression, alternatively, nuclear factor-κB (NF-κb) can be an intermediate factor in neuron–microglia interactions. It was reported that NF-κb was implicated in the transcriptional regulation of the M-CSF (Rajavashishth et al, 1995). Therefore, further studies are needed to investigate whether the STAT3-targeted factors in microglia regulate neuronal M-CSF production via NF-κb signaling.

When it comes to neuron–microglia cross-talk, cell-to-cell interactions have a pivotal role in intercellular communication. For example, CX3CR1/CX3CL1 signaling between microglia and neurons is involved in synaptic engulfment, resulted in synaptic elimination (Ueno et al, 2013; Zhan et al, 2014), and in the modulation of cytokine production and glutamatergic neurotransmission (Rogers et al, 2011; Scianni et al, 2013). CD200 and its receptor, CD200 receptor, also have critical roles in the interplay between neurons and microglia by controlling anti-inflammatory signaling and by maintaining them in a resting state (Hoek et al, 2000). Presumably, however, our data indicated that de novo mechanisms of neuron-microglia cross-talk would exist. Ablation of STAT3 in microglia did not alter the number of synaptic dendritic spines. The cytokine array and ELISA experiments also provided that M-CSF production occurs in neuronal cells but not IL-1β, TNF-α, IL-6, and IL-10.

As a novel antidepressant mechanism, rapid responses mainly occur through NMDA and AMPA receptor-mediated glutamatergic neurotransmission (Duman et al, 2012b). Our findings indicated that AMPA receptor-mediated synaptic activity was enhanced presynaptically without morphological changes in response to M-CSF. As previously mentioned, we determined the increase of ERK1/2 phosphorylation by stimulation of M-CSF. Regarding the ERK1/2 signaling pathways, the mEPSC frequency was decreased in the inhibition of phosphorylated ERK1/2 (Kushner et al, 2005). Thus, this implicated that M-CSF-induced ERK1/2 signaling could enhance the release probability of neurotransmitters. The findings that M-CSF enhanced glutamatergic
neurotransmission can help elucidate novel antidepressant mechanisms for a rapid-acting antidepressant response.

In conclusion, microglial STAT3 was essential for promoting M-CSF actions on synaptic transmission, which led to antidepressive-like behavior. We propose that ERK1/2 and Akt/GSK3β signaling pathways are involved in BDNF-dependent antidepressant behaviors via M-CSF secretion in neuronal cells. These findings may provide a novel therapeutic approach for alleviating major depressive disorders.

FUNDING AND DISCLOSURE
This study was supported by grants from the NRF funded by the Korea government (MISP; 2012R1A5A2A44671346 and MESF; 2012R1A2A2A01012897 and 2014R1A2A1A11053203 to SKY and 2012R1A5A2A44671346 to SK), the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (0720540 to SKY and A120476 to SK), and Seoul National University Hospital (SNUH) Research Fund (3420130270 and 0320140100 to SKY), S-HK received a scholarship from the BK21-plus education program provided by the National Research Foundation of Korea (NRF). The authors declare no conflict of interest.

ACKNOWLEDGMENTS
We thank Jaerong Ahn, Haeri Lee, Joohan Woo, Seung-Eon Roh, Kyung-Jin Kim, and Chung-Hyun Cho for technical support and for reading the manuscript.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)