Social Defeat Stress in Adolescent Mice Induces Depressive-like Behaviors with Reduced Oligodendrogenesis

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Abstract—Strong stress related to adverse experiences during adolescence can cause mental disorders, as well as affecting brain structure and function. However, the underlying neurobiological mechanisms remain largely unknown. To investigate whether stress induced by adverse experience during adolescence affects oligodendrocyte (OL) remodeling, social defeat stress was applied to 6-week-old adolescent mice for 10 days, followed by behavioral tests and assessments of oligodendrogenesis. Socially defeated mice showed depressive-like behaviors in behavioral experiments. Stress led to a decrease in the number of newly born OLs in the anterior cortical region and the number of proteolipid protein-positive mature OLs in the corpus callosum and posterior cerebral cortex. Fewer bromodeoxyuridine-incorporated CC1-positive mature OLs were observed in these regions in socially defeated mice. To assess whether decreased oligodendrogenesis caused by social defeat stress is related to depressive-like symptoms under stress, clemastine, a drug that induces OL generation, was administered to socially defeated adolescent mice, resulting in the rescue of the behavioral abnormalities accompanied by increased oligodendrogenesis. These findings suggest that oligodendrogenesis in adverse environments during adolescence plays a role in psychiatric disorders, and clemastine may provide a potential therapeutic drug for adolescent mental disorders, targeting OLs. © 2020 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: Social defeat stress, Oligodendrocyte, Depressive-like behavior, Oligodendrogenesis.

INTRODUCTION

Adolescence is a sensitive time, and the pubertal environment can have a substantial impact on brain structure and function. With adverse events experienced during adolescence, the influence on brain function can be long-lasting, accompanied by a higher risk of psychiatric diseases in adulthood (Chapman et al., 2004; Anda et al., 2007). Recently, substantial attention has been paid to the influence of experiences in adolescence on depression. However, the mechanisms by which stress during adolescence affects mental disorders remain to be fully elucidated.

During adolescence, psychosocial stresses are increased by various environmental changes, such as an increase in independence and social interactions. Age-specific effects of stress have been proposed to explain why most psychiatric disorders appear during the adolescent period (Arnett, 1999). In addition, among psychosocial factors, bullying in the adolescent period was found to be related to depressive symptoms (Kaltiala-Heino and Fröjd, 2011). Animal studies can provide useful information for clarifying the causal link between stress-induced behavioral changes and the underlying molecular mechanisms, enabling examination of the specific effects of adolescent adversity on brain function. A mouse model of social defeat stress (SDS) has been suggested to approximate the effects of bullying in human adolescents, with the aggressor mouse being equivalent to a bully and the defeated submissive mouse being equivalent to a bullied individual.

Oligodendrocytes (OLs) are glial cells that myelinate neuronal axons in the central nervous system (CNS). Recent studies have reported that neuronal activity can regulate OL proliferation, differentiation and myelination in vivo (Gibson et al., 2014; Mitew et al., 2018). In addition, recent reports have demonstrated that OL remodeling in postnatal mammals depends on environmental conditions. Human diffusion tensor imaging (DTI) studies have revealed changes in white matter structure after visuomotor skill learning (Bengtsson et al., 2005; Scholz et al., 2009). Richardson and colleagues found that inhibition of OL differentiation to mature OLs in the adult mouse brain resulted in impaired motor learning in a wheel running task with irregularly-spaced rungs.
In addition, social isolation has been found to lead to behavioral and cognitive dysfunction, as well as impaired myelination in prefrontal cortex (Liu et al., 2012; Makinodan et al., 2012). Conversely, administration of drug treatment that enhances myelination in socially isolated mice was found to rescue behavioral abnormalities and improve myelination of the prefrontal cortex (Liu et al., 2016). Thus, previous studies indicate that OLs respond to external environments and play important roles in higher brain function.

To clarify the neurobiological mechanisms underlying the onset of depressive-like behaviors during adolescence, we applied SDS to 6-week-old mice as a model for examining stress-related disorders in adolescent rodents, focusing on oligodendrogenesis. We revealed that SDS induced depressive-like behaviors in adolescent mice and led to a decrease in the number of Enpp6-positive newly born OLs in the anterior cortex and the number of proteolipid protein (PLP)-positive mature OLs in the corpus callosum and posterior cerebral cortex. The results also revealed that the number of bromodeoxyuridine (BrdU)-incorporated CC1-positive mature OLs was decreased in these regions in socially defeated mice, whereas it was recovered by clemastine administration. Interestingly, clemastine treatment also rescued behavioral abnormalities, suggesting that promotion of oligodendrogenesis can ameliorate depressive-like symptoms caused by SDS during adolescence.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male mice of a C57BL/6 and an ICR (Institute of Cancer Research) genetic background were purchased from Japan SLC (Shizuoka, Japan). Mice were housed under controlled temperature conditions (22–23 °C).
and a 12 h light–dark cycle, with free access to food and water. Experimental procedures were approved by the Committee of Animal Experimentation of Nagoya City University Medical School and were conducted in accordance with the animal care guidelines of Nagoya City University.

Behavioral experiments

Social defeat stress. Social defeat stress was applied as described previously (Krishnan et al., 2007) with minor modifications. Briefly, male ICR mice were used as aggressor mice in the social defeat stress paradigm. One week before the start of the social defeat stress, each 5-week-old male C57BL/6 mouse to be defeated was individually housed. The 6-week-old male C57BL/6 mouse to be defeated was introduced into the home cage of a resident aggressor male ICR mouse for 10 min/day for 10 consecutive days, and then singly brought back to the home cage. The pair of C57BL/6 and aggressor ICR mice was changed each day to average the aggressiveness of aggressor mice. Control mice stayed in the home cage of another C57BL/6 mouse for 10 min/day for 10 consecutive days, and then singly brought back to the home cage.

Mice were treated daily with clemastine (10 mg/kg, Tocris Bioscience, 14976-57-9) by oral gavage (Liu et al., 2016; Cree et al., 2018) for the last 5 consecutive days, accompanied with social defeat stress. BrdU (50 mg/kg) was intraperitoneally injected to SDS mice or control mice for five consecutive days prior to perfusion fixation. Both clemastine and BrdU were applied immediately prior to social defeat stress on each day of the five consecutive days.

The social interaction test, the open field test and the forced swim test were performed as described below on the next day of the last application of social defeat stress. The social interaction test was performed as described previously (Krishnan et al., 2007) with minor modifications. To familiarize mice with the testing environment, each socially defeated or control mouse was kept for 150 sec in the open field (38 × 22 cm) with an empty wire cylinder (7 × 13.5 cm) put on one side of the field. The same mouse was then put into an open field with a wire cylinder occupied by an unfamiliar ICR mouse for 150 sec. A bordering area 10 cm away from the wall opposite to the wire cylinder was defined as the social avoidance zone. The time spent in the avoidance zone was estimated as an index of susceptibility to social defeat stress.

Open field test. The open field test was performed as described previously (Walsh et al., 2017) with minor modifications. Mice were individually introduced to an open field (40 × 40 cm²) chamber under standard light condition for 5 min. Their movements were recorded with a video camera and analyzed using the SMART video tracking system (Panlab Harvard Apparatus). Locomotor activity was evaluated as the total distance traveled. The center entry frequency or the duration spent in the central zone (13 × 13 cm²) was analyzed.

Forced swim test. The forced swim test was performed as described previously (Mombereau et al., 2004) with minor modifications. Mice were placed in a transparent cylinder (20 cm in diameter, 50 cm in height) containing water at a temperature of 22–25 °C (14 cm deep). Each mouse was kept for 6 min in the cylinder and the duration of immobility was examined during the last 4 min. Mouse behavior was evaluated by the time spent immobile while floating in the water.

Immunostaining

Mice were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). Mouse brains were obtained and post-fixed with 4% PFA overnight at 4 °C followed by cryoprotection with 20% sucrose. The brains were embedded in optimal cutting temperature compound (Sakura Finetek), and cut into 20 μm slices using a cryostat (CM3050, Leica) for immunohistochemistry and in situ hybridization. Cryosections were blocked in 5% normal goat serum in phosphate-buffered saline and 0.1% Triton X-100 (PBST), then incubated with primary antibodies (mouse monoclonal APC (CC1) antibody (Merck Millipore, #OP80) or rat anti-myelin basic protein (MBP) antibody (Millipore, MAB386)) overnight at 4 °C. After rinsing in PBST, the sections were incubated with secondary antibodies. The secondary antibodies were: Alexa 488- or 594-conjugated goat anti-mouse, anti-rabbit or anti-rat IgG (Molecular Probes). The sections were coverslipped with ProLong Gold Antifade Mountant (Thermo-Fisher Scientific). Slides were observed using an AX70 microscope (Olympus).

For double-immunostaining with mouse monoclonal APC (CC1) antibody (Merck Millipore, #OP80) or rabbit anti-Olig2 antibody (Millipore, #AB9610) and rat anti-BrdU antibody (abcam, ab6326), sections were soaked in 10 mM citrate buffer (pH 6.0) for 5 min, and heated to 90 °C in a microwave. DNA was then denatured to expose the antigen by incubating the sections in 2 N HCl for 60 min at 37 °C. To neutralize the acid, sections were immersed in 125 mM borate buffer (pH 8.4) for 20 min and rinsed three times with PBST. Sections were blocked with 10% normal goat serum in PBST, and then incubated with rat anti-BrdU antibody (abcam, ab6326), sections were soaked in 10 mM citrate buffer (pH 6.0) for 5 min, and heated to 90 °C in a microwave. DNA was then denatured to expose the antigen by incubating the sections in 2 N HCl for 60 min at 37 °C. To neutralize the acid, sections were immersed in 125 mM borate buffer (pH 8.4) for 20 min and rinsed three times with PBST. Sections were blocked with 10% normal goat serum in PBST, and then incubated with rat anti-BrdU antibody (abcam, ab6326), sections were soaked in 10 mM citrate buffer (pH 6.0) for 5 min, and heated to 90 °C in a microwave. DNA was then denatured to expose the antigen by incubating the sections in 2 N HCl for 60 min at 37 °C. To neutralize the acid, sections were immersed in 125 mM borate buffer (pH 8.4) for 20 min and rinsed three times with PBST. Sections were blocked with 10% normal goat serum in PBST, and then incubated with rat anti-BrdU antibody (abcam, ab6326), sections were soaked in 10 mM citrate buffer (pH 6.0) for 5 min, and heated to 90 °C in a microwave. DNA was then denatured to expose the antigen by incubating the sections in 2 N HCl for 60 min at 37 °C. To neutralize the acid, sections were immersed in 125 mM borate buffer (pH 8.4) for 20 min and rinsed three times with PBST. Sections were blocked with 10% normal goat serum in PBST, and then incubated with rat anti-BrdU antibody (abcam, ab6326), sections were soaked in 10 mM citrate buffer (pH 6.0) for 5 min, and heated to 90 °C in a microwave. DNA was then denatured to expose the antigen by incubating the sections in 2 N HCl for 60 min at 37 °C. To neutralize the acid, sections were immersed in 125 mM borate buffer (pH 8.4) for 20 min and rinsed three times with PBST. Sections were blocked with 10% normal goat serum in PBST, and then incubated with rat anti-BrdU antibody (abcam, ab6326).
the pixel number above the threshold was considered to be positive staining.

**In situ hybridization and cell count**

Cryosections were treated with 40 μg/ml proteinase K for 30 minutes, then post-fixed in 4% PFA for 15 min at room temperature. After the samples were washed with PBS, they were treated with 0.1 M triethanolamine-HCl (pH 8.0) followed by addition of acetic anhydride. Hybridization and post-hybridization washing were performed as described previously (Shimizu et al., 2005). The samples were pre-incubated in blocking solution consisting of 10% heat-inactivated sheep serum in PBST for 1 hour and incubated with 1/2000 diluted alkaline phosphatase (AP)-conjugated anti-digoxigenin antibodies (1/2000, Roche Diagnostics Corp.) in blocking solution at 4°C overnight. After washing three times in MABT buffer (0.1 M maleic acid, 0.15 M NaCl and 0.1% Tween-20; pH 7.5), the samples were exposed to twice of 10-minute treatments with NTMT buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.05 M MgCl2 and 0.1% Tween-20; pH 9.5). Nitro blue tetrazolium (NBT, Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche) were used as the substrate for AP. The probes for PLP, Enpp6 and PDGFRA were synthesized by PCR reaction with the previously described primer sets (Kagawa et al., 1994 for PLP, Ma et al., 2006 for PDGFRA) or as follows: Enpp6: Forward primer: ATGATCGGCAACTACATGTG, Reverse primer: TACTTCAGGACAGTGTCCAC.

The number of cells positive for *in situ* hybridization signal was counted in several fields from each mouse brain. The number of fields counted and animals used in each experiment can be found in Figure legends. Cell count in the posterior cerebral cortex (bregma; −1.75 to −2.3 mm) was done for the field including the whole six layers of the cortex. Cell count was also done in the corpus callosum (bregma; −1.75 to −2.3 mm). Cell count was also done in anterior brain regions (bregma; 2.05 to 1.75 mm) corresponding to the mPFC, anterior cingulate cortex and secondary motor cortex.

**RNA preparation and Real-time quantitative RT-PCR**

Total RNA was isolated using Sepasol reagent (Nacalai, Kyoto, Japan). Samples were prepared from the anterior cortical region of control and SDS mice for analysis of FcγR2B, arginase1, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and from their corpus callosum for analysis of Jagged1, Nkx2.2. The first-strand cDNA was synthesized with 3 μg of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan). Real-time quantitative RT-PCR was performed with 10 μl SYBR master mix reagent (Takara, Otsu, Japan). Mixed material (0.05 μM specific primer sets for Jagged1, Nkx2.2 and β-actin, primer sequences are shown below), and 1 μl of the prepared cDNA. Real-time quantitative RT-PCR analysis was performed using a StepOne analyzer (Life Technologies). The temperature profile consisted of 40 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 1 min. To distinguish specific amplification from nonspecific amplification, the melting curve was analyzed after each PCR reaction. Two replicates were averaged for each sample measurement on a 96 well plate. To determine the cDNA amount applied for this assay, purified PCR products were serially diluted and used as standards. The primer sets used in this study were as follows:

**FcγR2B:** Forward primer: AGCTGGTGTCTCTTG-GAAPG, Reverse primer: TGATGGTGACAGGCTTGGAC.

arginase1: Forward primer: TTGGCCTTGGAGAGCTAGAC, Reverse primer: TCGGCCCTTCTCTCCTCCC.

TNF-α: Forward primer: GCCTCTTCTCATTCCGGTG, Reverse primer: CTTGTGTTTTGTGCTACGAGC.

IL-6: Forward primer: AAGTCGGAGAGGAGGACTCTC, Reverse primer: CCAAGATTTCCCAGAGAAC.

Jagged1: Forward primer: ATTCGCTTCTGGCTGGATG, Reverse primer: GGTGCTAGCACACTCCAC.

**Fig. 2.** Social defeat stress reduces oligodendrogenesis in adolescent mice. (A–D) Schematic drawing showing the brain regions for (A) and (B). Corpus callosum and posterior cerebral cortex sections from control (A) and SDS (B) mice were analyzed using *in situ* hybridization for PLP (positive signals are blue-colored dots): a mature OL marker. (C) The number of positive cells/field was quantified in the corpus callosum (*P* < 0.001 compared with control values, Student’s *t*-test; *n* = 10 fields counted from *n* = 6 mice). (D) The number of positive cells/field was quantified in the posterior cerebral cortex (*P* < 0.01 compared with control values, Student’s *t*-test; *n* = 10 fields counted from *n* = 6 mice). (E) Immunohistochemistry with an anti-CC1 antibody was performed in the corpus callosum of SDS and control mice. The number of positive cells/field was quantified in the corpus callosum (*P* < 0.001 compared with control values, Student’s *t*-test; *n* = 11 fields counted from *n* = 6 mice). (F, G) Schematic drawing shows the mPFC region we analyzed. The mPFC containing prelimbic cortex (PrL) and infralimbic cortex (IL) regions from control and SDS mice was analyzed using *in situ* hybridization for PLP: a mature OL marker (F), and Enpp6: a newly born OL marker (G). The number of positive cells/field was quantified (N.S.: no significant difference by Student’s *t*-test; *n* = 15 fields counted from *n* = 7 mice for PLP, *n* = 15 fields counted from *n* = 5 mice for Enpp6). (H–K) Schematic drawing shows the anterior cortical region we analyzed. The anterior cortex sections from control (H) and SDS (I) mice were analyzed using *in situ* hybridization for Enpp6 (positive signals are shown as blue dots): a newly born OL marker. (J) The number of positive cells/field was quantified (N.S.: no significant difference by Student’s *t*-test; *n* = 8 fields counted from *n* = 4 mice). (K) The number of Enpp6-positive cells/field was quantified (*P* < 0.001 compared with control values, Student’s *t*-test; *n* = 11 fields counted from *n* = 4 mice). Arrows indicate positive cells (L, M) OL precursor cells positive for PDGFRA were analyzed using *in situ* hybridization in the corpus callosum and posterior cerebral cortex (L) and the anterior cortex (M) of control and SDS mice. The number of positive cells/field was quantified (N.S.: no significant difference by Student’s *t*-test; *n* = 13 fields counted from *n* = 7 mice for the corpus callosum and posterior cerebral cortex, *n* = 16 fields counted from *n* = 5 mice for the anterior cortex). Scale bar = 500 μm.
Nkx2.2: Forward primer: GAGGACTCGATCCTTACC
AC, Reverse primer: TTCAAGGGAGAGCGAT
G. 

β-actin: Forward primer: CTCCCTGAGAGAGCT-
TATG, Reverse primer: GCACGTGTTGGCATAGAG
G.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). For comparison of two groups, Student’s t-test was used. P values < 0.05 were considered significant. Data for multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test using GraphPad InStat 3 (GraphPad Software, San Diego, CA, USA). A multiple comparison post-test was performed only if P < 0.05. The level of significance was P < 0.05.

RESULTS

Social defeat stress induces depressive-like behaviors in adolescent mice

We first investigated whether SDS in adolescent mice at 6 weeks of age causes behavioral changes. In the open field test (Crawley, 1999), we found that SDS mice spent a longer time in the periphery and exhibited a lower frequency of entering into the center area (Student’s t-test, P = 0.00112, Fig. 1A). The forced swim test (Can et al., 2012) was performed to examine depressive-like behaviors in socially defeated mice. In the forced swim test, immobility time of SDS mice was significantly longer than that of control mice (Student’s t-test, P = 0.00798, Fig. 1B). Thus, the results indicated that SDS in adolescent mice induced depressive-like symptoms. SDS did not change the mouse body weight (Student’s t-test, P = 0.977, Fig. 1C).

To assess whether SDS causes neuroinflammation, we examined microglial activation and inflammatory cytokines in the anterior cortical region using quantitative RT-PCR analysis with primers for activated microglial markers (FcγR2B, arginase1, Tang and Le, 2016) and inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], interferleukin-6 [IL-6]). The expression levels of those genes in the anterior cortical region of SDS mice were comparable to those of controls (Student’s t-test; FcγR2B P = 0.854; arginase1 P = 0.944; TNF-α P = 0.614; IL-6 P = 0.721, Fig. 1D), indicating that SDS did not induce neuroinflammation.

Social defeat stress reduces oligodendrogenesis

As various external environments influence OL development and myelination in mice postnatally (Scholz et al., 2009; Liu et al., 2012; Makinodan et al., 2012), we investigated whether oligodendrogenesis was affected by SDS in adolescent mice. Mature OLs were visualized using in situ hybridization for PLP. The results revealed that the number of PLP-positive OLs was decreased in the corpus callosum and the posterior cerebral cortex in SDS mice (Student’s t-test; Corpus callosum P = 1.54E–05; cerebral cortex P = 0.00713, Fig. 2A–D). Immunohistochemistry with an anti-CC1 antibody further showed that the number of CC1-positive mature OLs was decreased in the corpus callosum in SDS mice (Student’s t-test, P = 0.000376, Fig. 2E). These results indicate that the number of mature OLs decreased in white matter and gray matter of SDS mice.

To examine whether the decrease in PLP+ mature OL number coincides with impairment of myelination, immunostaining for MBP was performed. The decrease in MBP+ signal was observed in the somatosensory cortex of SDS group compared with control group (Fig. S1A, B). Semi-quantification using ImageJ software revealed the intensity of MBP+ signal significantly decreased in the upper layer of the somatosensory cortex of SDS group (Fig. S1C). Thus, SDS also led to altered myelination in the somatosensory cortex.

Several previous studies reported that myelination along neuronal fibers in the medial prefrontal cortex (mPFC) is affected by SDS, but the number of mature OLs in this region was not changed by stress (Zhang et al., 2016; Lehmann et al., 2017). In addition, impacts on oligodendrogenesis by SDS during adolescence in the mPFC have not been evidently demonstrated. First, we checked the number of PLP-positive mature OLs in the mPFC containing prelimbic cortex (PrL) and infralimbic cortex (IL) regions. Since no change of the number of PLP-positive mature OLs was observed in the mPFC (Student’s t-test, P = 0.925, Fig. 2F) consistent with the previous reports (Zhang et al., 2016; Lehmann et al.,...
Also focused on the other anterior brain region corresponding to the anterior cingulate cortex and secondary motor cortex (hereafter, we focused on this anterior cortical region, which we refer to as the anterior cortex), which have been reported to be involved in psychological disorders such as schizophrenia (Höistad et al., 2013) and bipolar disorder (Keshavarz, 2017). Although the number of PLP-positive OLs in the anterior cortex of SDS mice was comparable to that in control mice (Student’s t-test, P = 0.224, Fig. 2J), the signal pattern of PLP hybridization was different; two distinctive populations of cells with larger or smaller morphology were found in controls but fewer cells with the larger morphology in SDS mice (Student’s t-test, P = 0.00116, Fig. S1D–F). This finding suggests the possibility that cells with the larger PLP-positive signal were newly forming OLs, as reported in MBP-positive cells detected by in situ hybridization in previous studies (McKenzie et al., 2014; Xiao et al., 2016). We thus investigated another OL lineage marker, Enpp6, which is a transiently expressed marker for early differentiating OLs (Xiao et al., 2016). Enpp6 in situ hybridization revealed that the number of Enpp6-positive cells was decreased in the anterior cortex of SDS mice compared with control mice, but not in the mPFC (Student’s t-test; anterior cortex P = 0.000545; mPFC P = 0.384, Fig. 2G–K), suggesting that SDS prevents OPC differentiation at an early stage in the anterior cortex. The staining for another OL lineage marker, PDGFRα, revealed that the number of PDGFRα-positive OPCs in SDS mice was comparable to that in control mice (Student’s t-test; corpus callosum and posterior cerebral cortex P = 0.576; anterior cortex P = 0.965, Fig. 2L, M), suggesting that SDS to adolescent mice did not actively modulate the proliferative state of OPCs.

### Promotion of oligodendrogenesis by clemastine ameliorates the depressive-like behaviors caused by SDS

If impairment of oligodendrogenesis contributes to depressive-like symptoms by SDS, promotion of OL generation in the SDS mouse brain might be useful for suppressing them. In addition, although small-molecule compound clemastine has been reported to rescue myelination defects in the prefrontal cortex of socially isolated mice (Liu et al., 2016), the effects of clemastine on SDS have not been reported. To investigate this issue, we administered clemastine, which is known to promote OPC differentiation in vitro and remyelination in demyelinating lesions in both mice and humans (Mei et al., 2014; Green et al., 2017), after mice were subjected to SDS for the first five consecutive days (Fig. 3A). Mice were treated with clemastine for the last five consecutive days, accompanied by SDS.

BrdU was given to SDS mice or control mice for five consecutive days during clemastine treatment to assess the differentiation of proliferative OPCs into mature OLs in the corpus callosum and the anterior cortex (Fig. 3A). The ratio of CC1 + BrdU+ cells/BrdU+ cells was decreased by SDS in the corpus callosum and the anterior cortex (Fig. 3D, E), consistent with the finding that the number of Enpp6+ newly born OLs was decreased by SDS (Fig. 2), suggesting that SDS led to blocking of OPC differentiation at an early stage. Clemastine increased the ratio of CC1 + BrdU+ cells/BrdU+ cells in both regions in SDS mice, compared with SDS mice without clemastine (one-way ANOVA; Corpus callosum cont vs SDS, P < 0.0001; cont + clemastine vs SDS, P < 0.0001; SDS vs SDS + clemastine, P < 0.0001; anterior cortex cont vs SDS, P = 0.0002; cont + clemastine vs SDS, P = 0.0032; SDS vs SDS + clemastine, P = 0.0056, Fig. 3B–E). The number of BrdU-positive cells itself was not significantly changed in either group (Fig. 3F, G).

To assess the proliferative state of OL lineage cells in the corpus callosum and the anterior cortex, we performed double-immunostaining for BrdU and Olig2, an OL lineage marker (Fig. 3H, I). The ratio of Olig2+ cells/BrdU+ cells was not significantly changed in the corpus callosum and the anterior cortex of SDS + clemastinized group, compared to SDS group (Fig. 3J, K), indicating that clemastine administration to adolescent mice did not actively modulate the proliferative state of OPCs under SDS condition, but rather promotes OPC differentiation.

We next counted the number of newly forming OLs and mature OLs in the corpus callosum, posterior cerebral cortex and anterior cortex after clemastine treatment accompanied by SDS. Brain sections from SDS and control mice were analyzed by in situ hybridization for Enpp6, a newly born OL marker, and...
PLP, a mature OL marker. Clemastine rescued the decrease of PLP+ mature OLs caused by SDS in the corpus callosum and posterior cerebral cortex (one-way ANOVA; corpus callosum cont vs SDS, $P < 0.0001$; cont + clemastine vs SDS, $P < 0.0001$; SDS vs SDS + clemastine, $P < 0.0001$; cerebral cortex cont vs SDS, $P = 0.0014$; cont + clemastine vs SDS, $P = 0.001$; SDS vs SDS + clemastine, $P = 0.0039$).

Fig. 5. Clemastine treatment rescued the depressive-like behaviors caused by SDS. (A) Mouse behavior in the open field. (B) Center entry frequencies in the open field were analyzed for 5 min (***$P < 0.01$, *$P < 0.05$, by one-way ANOVA with a Tukey's post hoc test; $n = 10$ mice). (C) The ratio of center zone duration to total distance traveled in the open field (***$P < 0.01$, *$P < 0.05$, by one-way ANOVA with a Tukey's post hoc test; $n = 10$ mice). (D) Total distance (mm) traveled in the open field (N.S.: no significant difference by one-way ANOVA with a Tukey's post hoc test; $n = 10$ mice). (E) Clemastine administration did not change the mouse body weight (N.S.: no significant difference, by one-way ANOVA with a Tukey's post hoc test; $n = 10$ mice). (F) Immobility duration (sec) in forced swim test were analyzed for the last 4 min. Clemastine treatment rescued the increased immobile time of forced swim test caused by SDS (***$P < 0.001$, *$P < 0.05$, by one-way ANOVA with a Tukey's post hoc test; $n = 12$ mice).
Fig. 4A–F) and restored the decrease of Enpp6+ newly born OLs in the anterior cortex (one-way ANOVA; anterior cortex cont vs SDS, $P = 0.0492$; cont + clemastine vs SDS, $P = 0.0121$; SDS vs SDS + clemastine, $P = 0.0355$, Fig. 4G–K).

We further examined the effects of clemastine on the depressive-like behaviors caused by SDS. Effects of clemastine on SDS have not been previously reported. Clemastine-treated SDS mice spent more time in the center area of the open field, compared with SDS mice without clemastine (one-way ANOVA; number of center zone entry cont vs SDS, $P = 0.0037$; cont + clemastine vs SDS, $P = 0.0222$; SDS vs SDS + clemastine, $P = 0.0438$; % of center zone duration cont vs SDS, $P = 0.0033$; cont + clemastine vs SDS, $P = 0.0164$; SDS vs SDS + clemastine, $P = 0.0129$, Fig. 5A–C), indicating that clemastine treatment rescued the behavioral abnormalities. However, no significant differences in total distance traveled were detected between the four groups (Fig. 5D), indicating that locomotion itself was not significantly influenced by clemastine. Clemastine did not cause a reduction of body weight in SDS mice (Fig. 5E). Although the immobility time of SDS mice was longer than that of control mice in the forced swim test, clemastine administration to SDS mice significantly reduced immobility time (one-way ANOVA; cont vs SDS, $P = 0.0453$; cont + clemastine vs SDS, $P = 0.01$; SDS vs SDS + clemastine, $P = 0.0002$, Fig. 5F). These findings indicated that clemastine rescued depressive-like behaviors by SDS, suggesting that enhanced oligodendrogenesis can ameliorate the behavioral abnormalities caused by SDS during adolescence.

**Expression of OL regulatory genes was changed in SDS mice, and was not rescued by clemastine administration**

To assess the molecular mechanisms underlying the impairment of oligodendrogenesis by SDS, we investigated the expression of OL regulatory genes that are reported to regulate OPC proliferation, OL differentiation and OL maturation. Real-time quantitative RT-PCR was performed with total RNA isolated from socially defeated mice.

Among various OL regulatory genes we tested, we found changes in the expression levels of just two genes, Jagged1 and Nkx2.2. The expression level of Jagged1 (Wang et al., 1998) was significantly increased in the corpus callosum, whereas that of Nkx2.2 (Qi et al., 2001; Zhu et al., 2014) was decreased in response to SDS (Fig. 6). These results suggest that up-regulation of Jagged1 maintains OL progenitor state, while down-regulation of Nkx2.2 reduces OL differentiation under SDS conditions. Since clemastine rescued behavioral abnormalities, it might reverse changes in the expression of these two genes. However, we were not able to observe significant differences in the expression level of those two OL differentiation regulatory genes between the clemastine-treated samples and the clemastine-untreated samples (one-way ANOVA; Jagged1 cont vs SDS, $P = 0.0075$; cont vs SDS + clemastine, $P = 0.0435$; cont + clemastine vs SDS, $P = 0.0081$; cont + clemastine vs SDS + clemastine, $P = 0.0471$; Nkx2.2 cont vs SDS, $P = 0.0002$; cont vs SDS + clemastine, $P = 0.0015$; cont + clemastine vs SDS, $P < 0.0001$; cont + clemastine vs SDS + clemastine, $P = 0.0009$, Fig. 6). Thus, the effects of clemastine on behavioral recovery did not appear to be due to a change in the expression of those OL differentiation regulatory genes. Rather, these effects may have been caused by changes in the signaling pathway of the muscarinic receptor, on which clemastine acts (Cree et al., 2018).

**DISCUSSION**

We examined OL generation and OL remodeling in adolescent mice under SDS. A decrease of Enpp6-positive newly born OLs was observed in the anterior cortical region corresponding to the anterior cingulate cortex and secondary motor cortex. In addition, a decrease in the number of PLP-positive mature OLs was found in the corpus callosum and posterior cerebral cortex, indicating that oligodendrogenesis during adolescence was affected by SDS. SDS mice exhibited more depressive-like behaviors in behavioral experiments. The effects of clemastine on SDS have not been previously reported. Clemastine rescued depressive-like behaviors of socially defeated mice accompanied by enhanced oligodendrogenesis, suggesting that abnormal oligodendrogenesis contributes to the onset of depressive-like behaviors.

An important role of the mPFC in major depression has been reported (Drevets, 2000), and neuronal morphological changes in the mPFC accompanied by behavioral abnormalities have been observed in experiments using repeated restraint stress (Shansky and Morrison, 2009). In addition, although it was reported that myelination along neuronal fibers in the mPFC is affected by SDS, the number of mature OLs was not found to be changed by the stress (Zhang et al., 2016; Lehmann et al., 2017). In addition, influences on oligodendrogenesis by SDS during adolescence in the mPFC have not been evidently demonstrated. In our study, we observed no change in the number of PLP-positive mature OLs or the number of Enpp6-positive newly forming OLs in the mPFC. Therefore, we focused on the different anterior brain region corresponding to the anterior cingulate cortex and secondary motor cortex in this study. Our results newly revealed a decrease in the number of Enpp6+ newly born OLs (Fig. 2) and a difference in the signal pattern of PLP in situ hybridization (Fig. S1) in the anterior cortex, indicating that not only the mPFC containing pre-limbic cortex (PrL) and infralimbic cortex (IL) region but also the anterior cingulate cortex and secondary motor cortex in the anterior cortical region are affected by SDS.

Direct reciprocal connections between the secondary motor cortex and posterior parietal cortex (Zingg et al., 2014) and the connection of the retrosplenial cortex to both the secondary motor cortex and posterior parietal cortex in mice (Yamawaki et al., 2016) have been previously reported, suggesting a functional interaction between the secondary motor cortex, hippocampal
working memory, and fear learning. In addition, reduction of the c-Fos response to external simulation has been reported in the secondary motor cortex of mouse depression models, such as repeated forced swimming (Stone et al., 2007). Thus, impaired function in the secondary motor cortex of the anterior cortex appears to be related to depressive-like behaviors in rodents. Communication between the anterior cingulate cortex and hippocampus is frequently implicated in schizophrenia (Reid et al., 2016). These reports suggest that the anterior cortical region corresponding to the anterior cingulate cortex and secondary motor cortex might be involved in symptoms of psychological disorders.

OL morphogenesis and developmental myelination after birth have been reported in mice; the critical period for OL morphogenesis induced by the external environment ranges from 3 to 5 weeks of age (Makinodan et al., 2012), and developmental myelination in the mouse forebrain approaches a plateau by 5 weeks of age, followed by gradual completion by 8 weeks of age. Although SDS was induced in 6-week-old mice (after the plateau of myelination) in our experiment, a small amount of subsequent developmental myelination could be modulated by stress (psychosocial stresses during adolescence). These reports suggest that the anterior cortical region corresponding to the anterior cingulate cortex and secondary motor cortex might be involved in symptoms of psychological disorders.

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Our study assessed whether OLs could be a target for depression therapy. Depression is associated with decreased neuronal synapses in brain regions that regulate mood and cognition, and antidepressants can reverse these neuronal deficits (Duman and Aghajanian, 2012). A large number of previous studies focused on impairments of neuronal function. Several recent studies have suggested that microglia play an important role in psychiatric disorders (Kato and Kanba, 2013; Monji et al., 2013). Minocycline, an antibiotic and inhibitor of microglial activation, has been reported to have therapeutic efficacy in schizophrenia. In the current study, we examined microglial activation and inflammatory cytokines using quantitative RT-PCR analysis with primers for activated microglial markers and inflammatory cytokines, FcγR2B, arginase1, TNF-α and IL-6. The expression levels of these four genes in SDS mice were comparable with those of controls.

**Fig. 6.** Expression of OL regulatory genes were affected in SDS mice, which was not rescued by clemastine administration. (A) Transcription level of an OL regulatory gene Jagged1 was changed, examined using real-time qRT-PCR analysis of samples prepared from the corpus callosum of control mice and SDS mice, normalized against β-actin expression. The quantified value was scaled to that of the control mouse sample which was set at 1. Clemastine administration did not recover the alteration of the gene expression (**P < 0.01, *P < 0.05, by one-way ANOVA with a Tukey’s post hoc test; n = 5 mice). (B) The transcription level of an OL regulatory gene Nkx2.2 was changed, examined with real-time qRT-PCR analysis using samples prepared from the corpus callosum of control mice and SDS mice, normalized against β-actin expression, and the quantified value was scaled to that of the control mouse sample which was set at 1. Clemastine administration did not recover the alteration of the gene expression (**P < 0.001, *P < 0.01, by one-way ANOVA with a Tukey’s post hoc test; n = 6 mice).
These results indicate that SDS did not cause microglial activation or neuroinflammation.

The density of OLs has been reported to be significantly decreased in both white and gray matter of the prefrontal cortex in patients with schizophrenia (Uranova et al., 2004; Uranova et al., 2007). Gene expression profiling of RNA extracted from homogenized white and gray matter of the cerebral cortex has revealed that various OL- and myelin-associated genes are differentially expressed in schizophrenia patients (Sequeira et al., 2012). Thus, OLs are known to play a role in the cause of psychiatric disorders to some extent. However, the effects of OL remodeling and/or oligodendrogenesis are yet to be fully elucidated. Our results revealed that clemastine treatment rescued the behavioral abnormalities caused by SDS during adolescence, suggesting that promotion of oligodendrogenesis can ameliorate depressive-like behaviors. Adolescence in humans is thought to be a period in which psychosocial stresses are increased by various environmental changes, including bullying, increased independence and social interactions. Our studies indicate that adverse events during adolescence affect oligodendrogenesis, involving the cause of mental disorders. SDS applied to adolescent mice may be a useful tool to assess a novel therapeutic treatment for stress-related disorders in adolescence.

Although clemastine has been reported to rescue myelination defects in the prefrontal cortex of socially isolated mice (Liu et al., 2016), the effects of clemastine on SDS have not been previously reported. Our results revealed that clemastine ameliorated depressive-like symptoms caused by SDS. Our findings suggest a potential new approach for developing novel treatments for depression caused by SDS, targeting oligodendrocytes rather than neurons. Clemastine is a potential candidate for this therapeutic approach. Clemastine is known to act as a muscarinic receptor antagonist that directly promotes OL differentiation (Cree et al., 2018). We showed that clemastine administration to adolescent mice did not actively modulate the proliferative state of OL lineage cells under SDS condition (Fig. 3H–K), indicating that the behavioral improvement by clemastine was caused by promoting OPC differentiation. Previous studies also reported that clemastine administration did not significantly change the number of OPCs in various rodent models (Li et al., 2015; Cree et al., 2018). However, we cannot exclude the possibility that clemastine also affects other proliferating cells such as astrocyte, microglia and pericyte in the adolescent forebrain under SDS condition. Among various OL regulatory genes we tested, we found changes in the expression levels of just two genes, Jagged1 and Nkx2.2, induced by SDS. The expression levels of those two genes were not influenced by clemastine administration. However, clemastine was able to rescue the behavioral abnormalities caused by SDS. Therefore, recovery from SDS-induced depressive-like symptoms by clemastine might be caused by the signaling pathway initiated by the muscarinic receptor.

It is well known that depressive-like behavior in humans is more common in females. Therefore, we have to note the fact that the model used in our experiments was limited to male mouse only, although this is an inevitable result of social defeat test that is designed for male-type stress. It is very important to say that sex hormone differences should be taken into the consideration to understand the mechanism of the onset of depression-like behavior. Further study will be performed to examine whether the effect of oligodendrogenesis on depression-like behavior is different between both sexes.

Our results revealed that socially defeated mice exhibited depressive-like behaviors in the behavioral experiments. This stress led to a decrease in the number of Enpp6-positive newly born OLs in the anterior cortical region and the number of PLP + mature OLs in the corpus callosum and posterior cerebral cortex. Promotion of oligodendrogenesis by clemastine administration largely ameliorated the depressive-like symptoms of SDS. Taken together, these findings suggested that oligodendrogenesis in psychosocial environments during adolescence plays a role in psychiatric disorders.

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AUTHOR CONTRIBUTIONS

T.S. and H.H. designed the experiments. T.S. performed the experiments. T.S. analyzed the data. T.S. and H.H. wrote the manuscript. A.I., M.H., Y.U., A.H. and N.T. advised the experimental processes.

DECLARATIONS OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2020.07.002.