—Original—

Altered Microglia in the Amygdala Are Involved in Anxiety-related Behaviors of a Copy Number Variation Mouse Model of Autism

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Background and Purpose: Autism spectrum disorder (ASD) is a neurodevelopmental disorder with a strong genetic basis. Although anxiety is a common major psychiatric condition in ASD, the underlying mechanisms of the anxiety are poorly understood. In individuals with ASD, evidence indicates a structural abnormality in the amygdala, a key component involved in anxiety and social behavior. Microglia, which are central nervous system-resident immune cells implicated in neurodevelopmental processes, are also reportedly altered in ASD. In the present study, we examined the involvement of microglia in the anxiety-related behaviors of ASD model mouse.

Methods: Mice that have a 6.3-Mb paternal duplication (*patDp/+*) corresponding to human chromosome 15q11-q13 were used as an ASD model. Iba1, a microglial activation marker, was examined in the amygdala using immunofluorescence. Effects of perinatal treatment with minocycline, a microglial modulator, on anxiety-related behaviors were examined in neonatal and adolescent *patDp/+* mice.

Results: In *patDp/+* mice, Iba1 was decreased in the basolateral amygdala at postnatal day 7, but not at postnatal days 37–40. Perinatal treatment with minocycline restored the Iba1 expression and reduced anxiety-related behaviors in *patDp/+* adolescent mice.

Conclusions: Perinatal microglia in the basolateral amygdala may play a pathogenic role in the anxiety observed in a mouse model of ASD with duplication of human chromosome 15q11-q13.

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Key words: amygdala, anxiety, autism spectrum disorder, microglia, minocycline

Introduction

Autism spectrum disorder (ASD) is a highly variable neurodevelopmental disorder with a strong genetic basis[1], although environmental factors are also suggested as an underlying etiology. ASD is characterized by impaired social interaction and communication skills, and unusual repetitive behavior[2]. The first signs of ASD appear in early childhood and the symptoms typically remain stable throughout adulthood[3,4]. However, more than 70% of individuals with ASD have prominent concurrent conditions. Among them, anxiety is a common major psychiatric condition across all age groups[2], and represents an important characteristic that induces several abnormal behaviors in ASD patients. A recent meta-analysis suggested that 40% of young people with ASD meet the criteria for anxiety disorders[5], while higher anxiety levels were found to be associated with more repetitive behaviors and lower quality of social relationships in children with ASD[6,7]. Although anxiety and impaired social function may be intimately linked and share a common neural mechanism in ASD[8], the underlying cellular and/or molecular mechanisms of anxiety in individuals with ASD are poorly understood.

The amygdala is a key component in the limbic system and connects to a number of brain regions involved in anxiety and social behavior[8]. There has been increasing

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interest in the role of the amygdala in socio-emotional impairments in ASD[9,10]. Abnormal amygdala structure and function have been widely reported in individuals with ASD[9–11]. Structural magnetic resonance imaging (MRI) studies indicated that the amygdala was enlarged in children, but not in adolescents, with ASD[12,13], while a significant decrease in the amygdala volume was observed in adolescents with ASD[14]. In meta-analyses of voxel-based morphometric studies, a decrease in the right amygdala volume was detected in individuals with ASD[15–17]. Moreover, altered functional connectivity between the amygdala and other brain regions was reported in individuals with ASD[10].

Microglia are central nervous system-resident immune cells derived from myeloid precursors with a mesodermal origin[18–21]. Microglial migration into the brain starts during early development in the fetus, peaks soon after birth, and then declines[22]. Microglia reside throughout the brain, but are preferentially recruited to specific regions that show dynamic development[20]. Microglia survey their local microenvironment and engage in tissue defense and repair through multiple mechanisms, including phagocytosis and cytokine secretion. However, recent studies have demonstrated that microglia are also implicated in neurodevelopmental processes including the regulation of cell death, synapse elimination, neurogenesis, and neuronal surveillance, and contribute to the maturation and plasticity of neural circuits that ultimately shape behavior[23]. Consistent with their role in the development of the nervous system, several studies have demonstrated microglial disturbances in different brain regions of individuals with ASD[24–26]. Thus, microglia are suggested to play an important role in the pathophysiology of ASD. In a subpopulation of patients with ASD, increased density and morphological changes of microglia were observed in their brains at postmortem[27–30]. In addition, an association of behavioral abnormality with altered microglia was reported in animal experiments[31,32]. In a mouse model of Rett syndrome, engraftment of wild-type (WT) microglia arrested numerous facets of the syndrome pathology[33].

In the present study, we examined the involvement of microglia in anxiety-related behaviors using ASD model mice with a 6.3-Mb paternal duplication (*patDp*/+) corresponding to human chromosome 15q11-q13[34]. Duplication of human chromosome 15q11-q13 is one of the most frequent cytogenetic causes of ASD, and may account for 1% of such cases. *patDp*/+ mice have been shown to exhibit several abnormal behaviors as seen in ASD patients, such as social abnormalities and anxiety-related behaviors[34,35].

Materials and Methods

Animals

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Review Committee of Nippon Medical School. We made efforts to minimize the number of animals used and their suffering. All mice were housed in a temperature and humidity-controlled vivarium with a 14-h/10-h light/dark cycle (lights on: 06:00 a.m. to 20:00 p.m.), and allowed food and water ad libitum. Mice with paternal inheritance of a 6.3-Mb duplication of mouse chromosome 7 equivalent to human chromosome 15q11-q13 duplication (*patDp*/+ mice) were used as the mouse model of ASD[34,35]. The mice were maintained on a genetic background of C57BL/6J at our animal facility. The presence of a vaginal plug was checked and the day of plug formation was considered to be day 0 of pregnancy. All experiments were performed in male mice, and WT littermates were used as controls.

Minocycline Administration

Minocycline (Sigma-Aldrich, Saint Louis, MO, USA) was added to the drinking water of pregnant mice from gestational day (GD) 17 and continued to be added to the mothers' drinking water until postnatal day (PD) 21. To administer a dose of 100 mg/kg/day, the minocycline concentration in drinking water was determined based on each mother's body weight and drinking volume. This minocycline administration method was previously shown to yield effective concentrations of minocycline in fetuses and pups[36–39]. Tap water only was used for control mice. After weaning at PD 21, the pups received tap water and food.

Ultrasonic Vocalizations (USVs)

USVs were recorded as described previously[40–42]. Pups were individually removed from the home cage and isolated from their mother and littermates. Each pup was placed in a plastic cup in a soundproof room and USVs were recorded for 5 min using a microphone (UltraSoundGate CM16; Avisoft Bioacoustics, Berlin, Germany). After the recording, the pups were returned to the home cage. The USVs were recorded at PDs 6, 7, 10, 14, 17, and 21, and analyzed using Avisoft-SAS Lab Pro (Avisoft Bioacoustics). The number of calls was counted for 3 min after 60 s of acclimation (i.e. between 60 s and 240 s after isolation). Calls with a frequency of 40−120 kHz and du-

ration of >10 ms were counted as USVs.

Open Field Test

Open field tests were performed at PDs 35–38. The open field chamber was 50 cm (length)×50 cm (width)×40 cm (height) (O'Hara & Co. Ltd., Tokyo, Japan). The field was illuminated at 40 lux. Each mouse was placed in a corner of the open field chamber and allowed to explore the novel open field for 15 min. The center of the field was defined as a central square of 30 cm×30 cm. The total center time, the total distance traveled and the number of rears were obtained using Image J OFC (O'Hara & Co. Ltd.), comprising modified software based on Image J. Image J OFC converted the image of open field to black-and-white duotone image and analyzed the cluster of black dots (i.e. mouse) to obtain the total center time and distance traveled. The number of rears was counted from the number of crossings of horizontal beams 65 mm over the ground, and was output to Image J OFC.

Immunofluorescence

Brain samples were collected at PDs 7 and 37–40. Briefly, mice were deeply anesthetized with intraperitoneal pentobarbital (58.9 mg/kg), and transcardially perfused with phosphate-buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde at 4°C overnight. After the post-fixation, the tissues were cryoprotected with 20% sucrose at 4°C for 3 days. Finally, the tissues were embedded in OCT compound, a formulation of water-soluble glycols and resins, immediately frozen, and stored at −80°C until analysis.

The brains were cut into 20-μm-thick coronal sections using a cryostat (CM1850; Leica Microsystems, Tokyo, Japan). The free-floating sections were blocked with 1% donkey serum for 2 h and incubated with a primary rabbit antibody against Iba1 (1:1,000 dilution; Wako, Osaka, Japan) at 4°C for 3 days in the presence of 0.3% Triton X-100. After three washes with PBS, the sections were incubated with an anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (1:2,000 dilution; Life Technologies, Tokyo, Japan) at room temperature for 2 h. After three further washes in PBS, the sections were mounted on slides. Fluorescence images were captured using a microscope equipped with a digital camera (DP-71; Olympus, Tokyo, Japan). For quantification of microglial density, the numbers of microglia in the basolateral amygdala (BLA) and central amygdala (CeA) were counted using Scion Image.

For quantification of Iba1 immunofluorescence, the fluorescence intensities in the microglial cell bodies were calculated using Image J. The average fluorescence intensity was obtained from all microglia in two sections that were randomly selected from individual mice.

Statistical Analysis

Values were expressed as means±SEM. All data except for those obtained from CeA were analyzed by two-way ANOVA followed by a Bonferroni test for post hoc multiple comparisons. Data obtained from CeA were analyzed by unpaired *t*-test. In all statistical tests, values of $P<0.05$ were considered to indicate statistical significance.

Results

Microglia Are Disturbed in the BLA of *PatDp*/+ Neonatal Mice

In rodents, microglial precursor cells enter the brain during early development until PD 10, while embryonic invasion of progenitor cells that give rise to the microglial population occurs between GDs 10 and 19[20,43]. Therefore, we treated *patDp*/+ and WT mice with minocycline from GD 17 to PD 21 to modulate the microglial functions, and performed histological analyses and behavioral tests as shown in **Figure 1A**. First, we examined the microglia of *patDp*/+ mice at PD 7 in the amygdaloid nuclei, BLA and CeA. In the BLA, the immunofluorescence intensity of Iba1, a microglial activation marker, was significantly decreased in *patDp*/+ mice treated with vehicle (**Fig. 1B and C**), while the number of microglia was unchanged (**Fig. 1B and D**). In the CeA, neither the immunofluorescence intensity of Iba1 nor the number of microglia was affected in *patDp*/+ mice (**Fig. 1B, E, and F**). Subsequently, we examined the effect of perinatal treatment with minocycline on the microglia in the BLA. The decrease in Iba1 immunofluorescence was blocked by perinatal minocycline treatment (**Fig. 1B and C**). In WT mice, perinatal minocycline treatment affected neither the Iba1 immunofluorescence nor the number of microglia in the BLA (**Fig. 1B–D**).

Perinatal Minocycline Has No Effect on USVs of *PatDp*/+ Neonatal Mice

As an anxiety-related behavior in neonatal mice, we recorded maternal separation-induced USVs[40,44] and examined the effects of minocycline on the USVs. In WT mice treated with vehicle, USVs emerged soon after birth and peaked at PD 7. Subsequently, the USVs decreased and had almost disappeared by PD 14 (**Fig. 2**). The USVs emitted by *patDp*/+ mice treated with vehicle were significantly greater than those emitted by WT mice, as previously described[34]. In *patDp*/+ mice, USVs emerged at PD 6 and peaked at PD 10. The peak of USVs was delayed compared with WT mice, and USVs were still

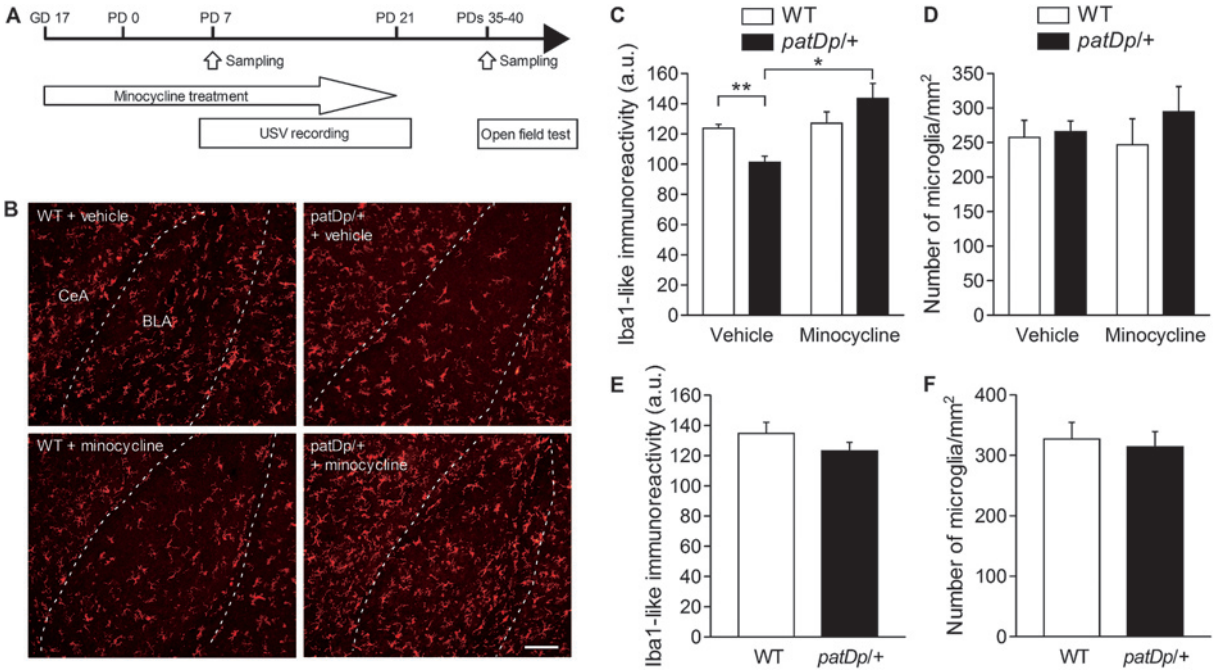

Fig. 1 Microglia in the amygdala of *patDp/+* mice at PD 7. (A) Schematic representation of the experimental protocol. (B) Representative micrographs of Iba1 immunofluorescence in the amygdala. Sections were obtained from WT and *patDp/+* mice treated with minocycline or vehicle. The dotted lines depict the areas of lateral nuclei of the amygdala. The scale bar indicates 100 μm. (C, E) Fluorescence intensities of Iba1 immunoreactivity in the BLA (C) and CeA (E). (D, F) Numbers of microglia in the BLA (D) and CeA (F). **P*<0.05, ***P*<0.01, by two-way ANOVA followed by a Bonferroni post hoc test in the BLA (C, D). No statistical significance was revealed by unpaired *t*-test in the CeA (E, F). *n*=4–6.

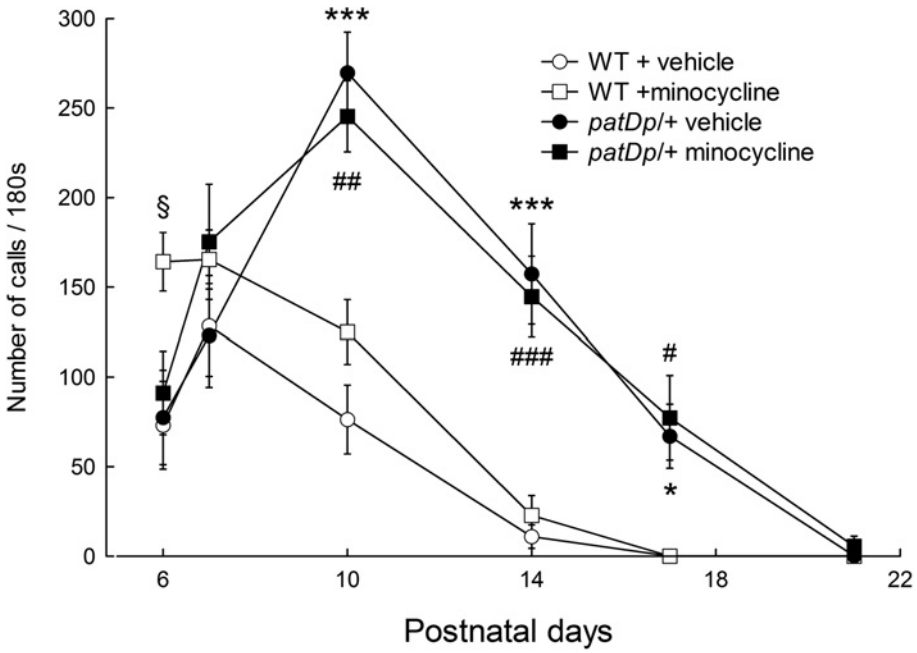

Fig. 2 Maternal separation-induced USVs in *patDp/+* mice. Pups were individually tested for USVs from PDs 6 to 21. **P*<0.05, ****P*<0.001, vs. WT mice treated with vehicle, #*P*<0.05, ##*P*<0.01, ###*P*<0.001, vs. WT mice treated with minocycline, and §*P*<0.05, vs. WT mice treated with vehicle, by two-way ANOVA followed by a Bonferroni post hoc test. *n*=11–12

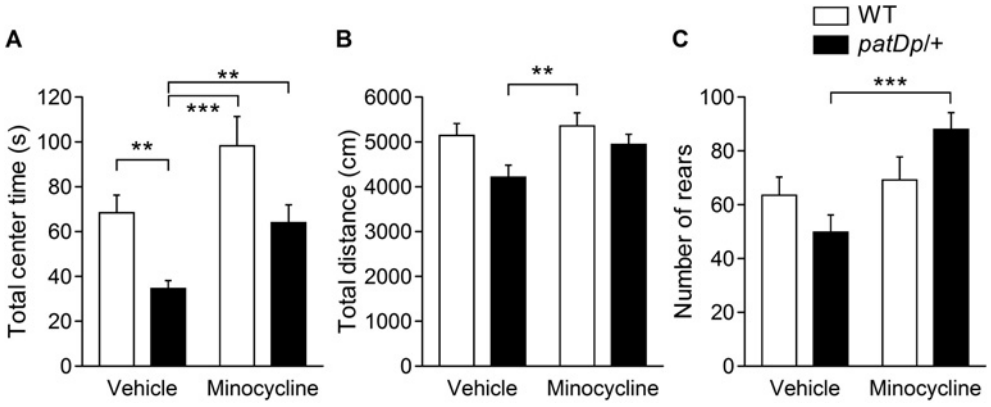

Fig. 3 Effects of perinatal minocycline treatment on the behaviors of *patDp*/+ mice at PDs 35–38 in open field tests. (A–C) The total center time (A), total distance (B), and number of rears (C) were evaluated in WT and *patDp*/+ mice treated with minocycline or vehicle at PDs 35–38. **P*<0.05, ***P*<0.01, ****P*<0.001, by two-way ANOVA followed by a Bonferroni post hoc test. *n*=16–20.

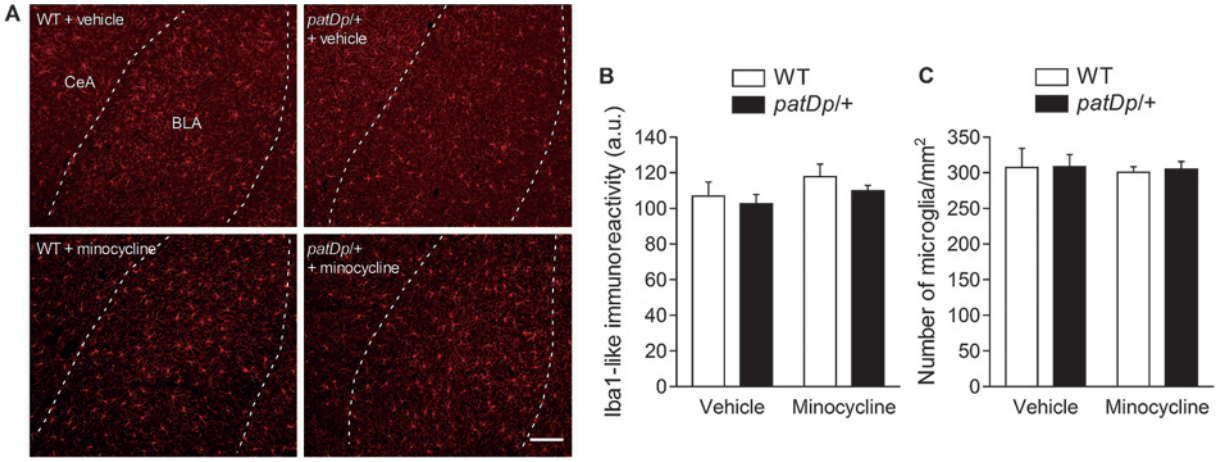

Fig. 4 Microglia in the BLA at PDs 37–40. (A) Representative micrographs of Iba1 immunofluorescence in the amygdala. Sections were obtained from WT and *patDp*/+ mice treated with minocycline or vehicle. The scale bar indicates 100 μm. (B) Fluorescence intensities of Iba1 immunoreactivity in the BLA. (C) Numbers of microglia in the BLA. *n*=4.

emitted at PDs 14 and 17. USVs were hardly observed at PD 21 (**Fig. 2**). Minocycline treatment had no effects on the USVs of both WT and *patDp*/+ mice (**Fig. 2**).

Perinatal Minocycline Increases the Total Center Time of *PatDp*/+ Adolescent Mice in Open Field Tests

To examine an anxiety-related behavior in adolescent mice, we performed open field tests. In the open field tests, *patDp*/+ mice treated with vehicle spent less time in the center area than WT mice treated with vehicle at PDs 35–38 (**Fig. 3A**), as previously shown[35]. The total distance and total number of rears were unaffected in *patDp*/+ mice (**Fig. 3B and C**). Perinatal minocycline treatment significantly increased the total center time and total number of rears, but not the total distance, in *patDp*/+ mice (**Fig. 3A–C**). In WT mice, minocycline treatment had no significant effects on the open field behaviors, although the total center time tended to increase (**Fig. 3A–C**).

Microglia Are Not Altered in the BLA of *PatDp*/+ Adolescent Mice

To examine the microglia in *patDp*/+ adolescent mice, Iba1 immunoreactivity was examined in the BLA at PDs 37–40. In the BLA of *patDp*/+ mice with perinatal vehicle treatment, the immunofluorescence intensity of Iba1 was unchanged at PDs 37–40 (**Fig. 4A and B**), in contrast to the findings for *patDp*/+ mice at PD 7 (**Fig. 1B and C**). The number of microglia was also unchanged in the BLA of *patDp*/+ mice at PDs 37–40 (**Fig. 4A and C**). Perinatal treatment with minocycline did not affect the Iba1-positive cell number or Iba1 immunoreactivity (**Fig. 4A–C**).

Discussion

Impaired Development of the Amygdala May Underlie the Anxiety in ASD

In this study, maternal separation-induced USVs, which are considered to reflect anxiety and fear in response to stress[40,44], were increased in *patDp*/+ mice at around PD 10, and *patDp*/+ mice at PDs 35–38 also showed an anxiety-related behavior in the open field test. The intensity of Iba1 immunoreactivity was decreased in the BLA of *patDp*/+ mice at PD 7, but not at PDs 37–40. In addition, although the underlying mechanisms of the minocycline effect on microglia are poorly understood, perinatal treatment with minocycline restored the Iba1 expression in the BLA at PD 7, and reduced the anxiety-related behavior of *patDp*/+ mice at PDs 35–38, but not at around PD 10. These results suggest that microglial alterations in the BLA during early development cause the anxiety-related behavior in adolescent mice. Because USVs upon maternal separation were not affected by perinatal minocycline treatment, the alterations in microglia in the BLA may not be crucial for anxiety at the early developmental stage. Perinatal exposure to stress-related events were found to cause an anxiety-like behavior associated with neurochemical changes in the amygdala[45–47]. Furthermore, human studies have indicated that the amygdala and its connections to other brain areas are affected in ASD[10]. Amygdala enlargement was reported in young children with ASD[11–13], while a decrease in the right amygdala volume was reported in child/adolescent subjects with autism[15–17]. A postmortem study reported a decrease in the number of amygdala neurons in adolescents and adults with ASD[48]. MRI studies indicated that the amygdala follows an aberrant trajectory of growth in individuals with autism[12]. The amygdala was found to have stronger functional connectivity with the ventromedial prefrontal cortex and weaker connectivity with the temporal lobe in patients with ASD[49]. Taken together with these previous observations, the present study further suggests that the BLA is an important subnucleus in the amygdala for the anxiety-related behaviors in *patDp*/+ mice, although the amygdala subnuclei are intricately connected with one another. BLA neurons also project to many other regions implicated in anxiety, including the medial prefrontal cortex, bed nucleus of the stria terminalis, and ventral hippocampus[8]. The BLA-ventral hippocampus circuit modulates anxiety-related behaviors as well as social interactions[8].

Microglial Alterations in the BLA during Early Development in *PatDp*/+ Mice

The period of minocycline treatment in the present study corresponds to the period when microglia actively enter and proliferate in the developing brain[20,43]. Microglia are closely associated with neurodevelopmental processes[50], such as the regulation of synapse elimination and neurogenesis, and the maturation and plasticity of neural circuits[23]. The present study showed a decrease in Iba1 immunoreactivity in the BLA, but no change was observed in the CeA, suggesting specific involvement of BLA. In addition, while this reduction implies reduced activity of the microglia in *patDp*/+ mice, many studies have indicated microglial activation in ASD. Therefore, microglial disturbances may vary depending on brain regions, ages, or etiologies. In humans, postmortem studies on patients with ASD revealed microglial activation in the cerebellum[25] and increased microglial density in the dorsolateral prefrontal cortex[28], fronto-insular cortex, and visual cortex[30]. Microglial dysfunction also varies depending on the ASD models examined[51]. In the ASD model of BTBR mice, the microglia showed no morphological changes[52]. In *Mecp2* knockout mice as a Rett syndrome model, glutamate was excessively released from microglia without microgliosis[53] and transplantation of WT bone marrow cells including WT microglia arrested the numerous pathologies of the syndrome[33]. In *Fmr1* knockout mice, a model for fragile X syndrome, there was no apparent microglial dysfunction, although minocycline improved the abnormal behaviors[39,54]. Therefore, the altered perinatal development mediated by malfunctioning microglia may underlie the anxiety in the *patDp*/+ adolescent mice.

In this study, microglia were altered in the BLA of *patDp*/+ mice during early development. Perinatal minocycline treatment restored the microglial malfunction in association with reduced anxiety-related behavior in adolescent mice. These findings suggest that perinatal microglia in the BLA play a pathogenic role in the anxiety observed in this mouse model of ASD with duplication of human chromosome 15q11-q13.

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