Biochemical and Biophysical Research Communications 472 (2016) 319–323

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Prenatal minocycline treatment alters synaptic protein expression, and rescues reduced mother call rate in oxytocin receptor-knockout mice

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ARTICLE INFO

Article history:
Received 19 February 2016
Accepted 25 February 2016
Available online 27 February 2016

Keywords:
Oxytocin receptor
Microglia
Autism spectrum disorder
Medial amygdaloid nucleus
Lateral septal nucleus

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired communication, difficulty in companionship, repetitive behaviors and restricted interests. Recent studies have shown amelioration of ASD symptoms by intranasal administration of oxytocin and demonstrated the association of polymorphisms in the oxytocin receptor (*Oxtr*) gene with ASD patients. Deficient pruning of synapses by microglial cells in the brain has been proposed as potential mechanism of ASD. Other researchers have shown specific activation of microglial cells in brain regions related to sociality in patients with ASD. Although the roles of *Oxtr* and microglia in ASD are in the spotlight, the relationship between them remains to be elucidated. In this study, we found abnormal activation of microglial cells and a reduction of postsynaptic density protein PSD95 expression in the *Oxtr*-deficient brain. Moreover, pharmacological inhibition of microglia during development can alter the expression of PSD95 and ameliorate abnormal mother–infant communication in *Oxtr*-deficient mice. Our results suggest that microglial abnormality is a potential mechanism of the development of Oxt/Oxtr mediated ASD-like phenotypes.

1. Introduction

Microglia are the resident immunocompetent cells of the central nervous system (CNS), involved in phagocytic events in the CNS under inflammatory conditions, such as strokes, ischemia and infectious disease [1]. Although the role of microglia in the immune system in the CNS has been well characterized, researchers currently believe that in addition to the immunological function, microglia also contribute to brain physiology and the pathology of many mental disorders. Recent studies have shown the associations between the disruptions of microglia-expressed genes and mental disorders, including autism spectrum disorders (ASD) [2–4].

ASD is one of the most common mental disorders. ASD comprises a group of developmental disabilities that can cause significant defect in social communication and behavioral challenges as well as morbid repetitive behaviors and restricted interests [5,6]. ASD is common in all racial, ethnic, and socioeconomic groups; it is, however, almost five times more common among boys than girls. It is thought that about 1% of children are affected with ASD [7], but no effective cures has been identified to date. Because of this situation, ASD is the subject of public concern, and ASD research has grown a great deal in recent years. Recently, several reports demonstrated a notable amelioration in ASD episodes by intranasal administration of oxytocin (Oxt) [8–10]. Moreover, clinical application of oxytocin to ASD patients has begun.

Oxt is a nonapeptide neurohormone synthetized in the supraoptic nucleus and paraventricular nucleus and secreted into the blood by the pituitary gland. The most noted function of Oxt in its history is in peripheral. Peripherally secreted Oxt has long been known as a reproductive hormone during pregnancy and parturition [11], and recent studies demonstrated a function of central Oxt in prosocial behaviors, such as trust, social memory, maternal behavior, and pair bonding [12–15]. Interestingly, these are some of the behaviors that are often impaired in patients with ASD. Mice

Abbreviations: Oxt, oxytocin; Oxtr, oxytocin receptor; ASD, autism spectrum disorder; MeA, medial amygdaloid nucleus; LS, lateral septal nucleus; mPFC, medial prefrontal cortex; CNS, central nervous system; PFA, paraformaldehyde; PBS, phosphate-buffered saline; TBS, tris-buffered saline; TBST, TBS with 0.1% Tween-20.

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http://dx.doi.org/10.1016/j.bbrc.2016.02.109
0006-291X/

deficient in the *Oxt* gene, generated by our group, showed social amnesia [16]. In Oxt receptor (*Oxtr*) knockout mice, we also reported notable abnormal phenotypes of aggressive behavior, impaired maternal behavior, and suppressed ultrasonic vocalization by infant mice [17]. *Oxt* and *Oxtr*- deficient mice can therefore serve as useful animal models of human ASD.

Interestingly, a recent study suggested that deficient pruning of excess synapses by microglial cells in the brain can be one potential mechanism of ASD development [18]. Here, we analyzed the pathological phenotype of microglial cells in sociality-related brain regions of *Oxtr* deficient mice. Inhibition of abnormal microglial activation by minocycline can ameliorates PSD95 expression and mother–infant communication in the *Oxtr* deficient mice. Our results suggest that microglial abnormality is a potential mechanism of the development of Oxt/Oxtr mediated ASD-like phenotypes.

2. Materials and methods

2.1. Mice

Oxtr-deficient mice used in this work have been described previously [16]. Only male mice were used for behavioral analyses. Mice were fed a standard chow diet and water ad libitum, and kept at 25 °C in a room with a 12-h light/dark cycle. All animal experiments were performed according to the Tohoku University guidelines for animal experimentation.

2.2. Drug administration

Minocycline (Wako; 100 mg/kg/day) was dissolved into drinking water at the required concentration, which was determined based on each dam's body weight and drinking volume, and given to pregnant mice from gestational day 15.5 (E15.5) until postnatal day 21 (P21).

2.3. Tissue preparation

Mice were deeply anesthetized by intraperitoneal injection of tribromoethanol (0.4 mg/g body weight) and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at the same time. The brains were dissected, post-fixed with 4% PFA, and cryoprotected in 30% sucrose. The brains were then embedded in OCT compound (Sakura), and 20-μm-thick coronal sections were prepared with a cryostat (Leica CM1520) and stored at −80 °C until used.

2.4. Immunohistochemistry

Tissue sections were permeabilized with 0.5% Triton X100 in PBS and blocked with 5% normal horse serum in PBS containing 0.3% Triton X100. The tissue sections were stained with an Iba1 rabbit polyclonal antibody (Wako; 1:200) in blocking solution and incubated overnight at 4 °C. The sections were rinsed and incubated with a secondary rabbit IgG antibody conjugated to Alexa Fluor 488 (Molecular Probes; 1:1000) for 2 h at 22–24 °C. The sections were then rinsed with PBS and mounted. Images were taken using a confocal laser microscope (Zeiss LSM780).

2.5. Image analysis

To measure the lengths of activated microglial processes, we created maximum-intensity projection images using the Zen 2012 software (Zeiss). Three-dimensional z-stacks were collected from more than 10 cells for each genotype. To quantify immunostaining intensities, we prepared three sections per individual animal and used at least three animals for each genotype. The fluorescence intensity was measured using Image J (NIH).

2.6. Ultrasonic vocalization analysis

Pups were isolated individually from the mothers on P5 to induce ultrasonic vocalization. Each pup was placed in a sound-proof chamber (Metris smart chamber, Metris), and ultrasonic vocalizations were evaluated for 5 min. Vocalization was recorded and analyzed using Sonotrack (Metris) with a sampling rate of 200 kHz. Frequencies from 25 kHz to 100 kHz were counted as pup vocalizations. Data from mice that emitted no calls were excluded from evaluation.

2.7. Immunoblotting

For protein extraction, whole brains were placed in ice-cold homogenizing buffer (0.32 M sucrose, 4 mM HEPES and 1 mM EDTA) containing complete protease inhibitor cocktail (Roche) and homogenized using a Dounce tissue homogenizer. The homogenates were centrifuged at 800 g for 15 min at 4 °C, and the supernatants were transferred to new microfuge tubes and centrifuged at 9000 g for 15 min at 4 °C. The pellets were dissolved in resuspension buffer (20 mM Tris–HCl, pH 7.5, 10% (w/v) sucrose). Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce). Ten micrograms of protein combined with 2 × SDS sample buffer was loaded onto a 10% polyacrylamide gel. The samples were separated at 100 V for 1.5 h. The proteins were transferred from the gel onto PVDF membrane at 30 V overnight at 4 °C. The membrane was washed several times with Tris-buffered saline (TBS, pH 7.4) containing 0.1% (v/v) Tween-20 (TBST) and blocked with 5% skim milk in TBST for 30 min at 22–24 °C. The membrane was then incubated with a rabbit polyclonal PSD95 antibody (1:1000; Abcam, 18258), a β-actin antibody (1:1000; Santa Cruz, C4), or both. All antibody incubations were performed overnight at 4 °C. The antibodies were diluted in blocking solution. The membrane was washed with TBST and incubated with HRP-conjugated secondary antibodies against mouse IgG (1:5000; Jackson Immunoresearch, 715-035-151) and rabbit IgG (1:1000; Vector, PI-1000) for 30 min at 22–24 °C. Peroxidase activity was detected using Luminata Forte Western chemiluminescent HRP substrate (Millipore) and analyzed on the ChemiDoc XRS system (Bio-Rad).

2.8. Statistical analysis

Statistical analyses were conducted using Graphpad Prism version 6.0 for Windows (GraphPad Software). Data were compared using two-tailed unpaired Student's t-tests or one-way ANOVA followed by Tukey's post hoc test.

3. Results

Synaptic scaffolding protein expression is decreased in brains of adult *Oxtr*-deficient mice.

Recent studies reported that the number of synapses is altered in ASD patients and several ASD model animals. Therefore, we first assessed the expression of PSD95, a major synaptic scaffolding protein, in *Oxtr*-deficient mouse brains. Surprisingly, the expression level of PSD95 was markedly reduced in the *Oxtr* null brain (Fig. 1 A, B).

Excessive microglial activation was observed in several social-behavior-related brain regions in Oxtr deficient mice.

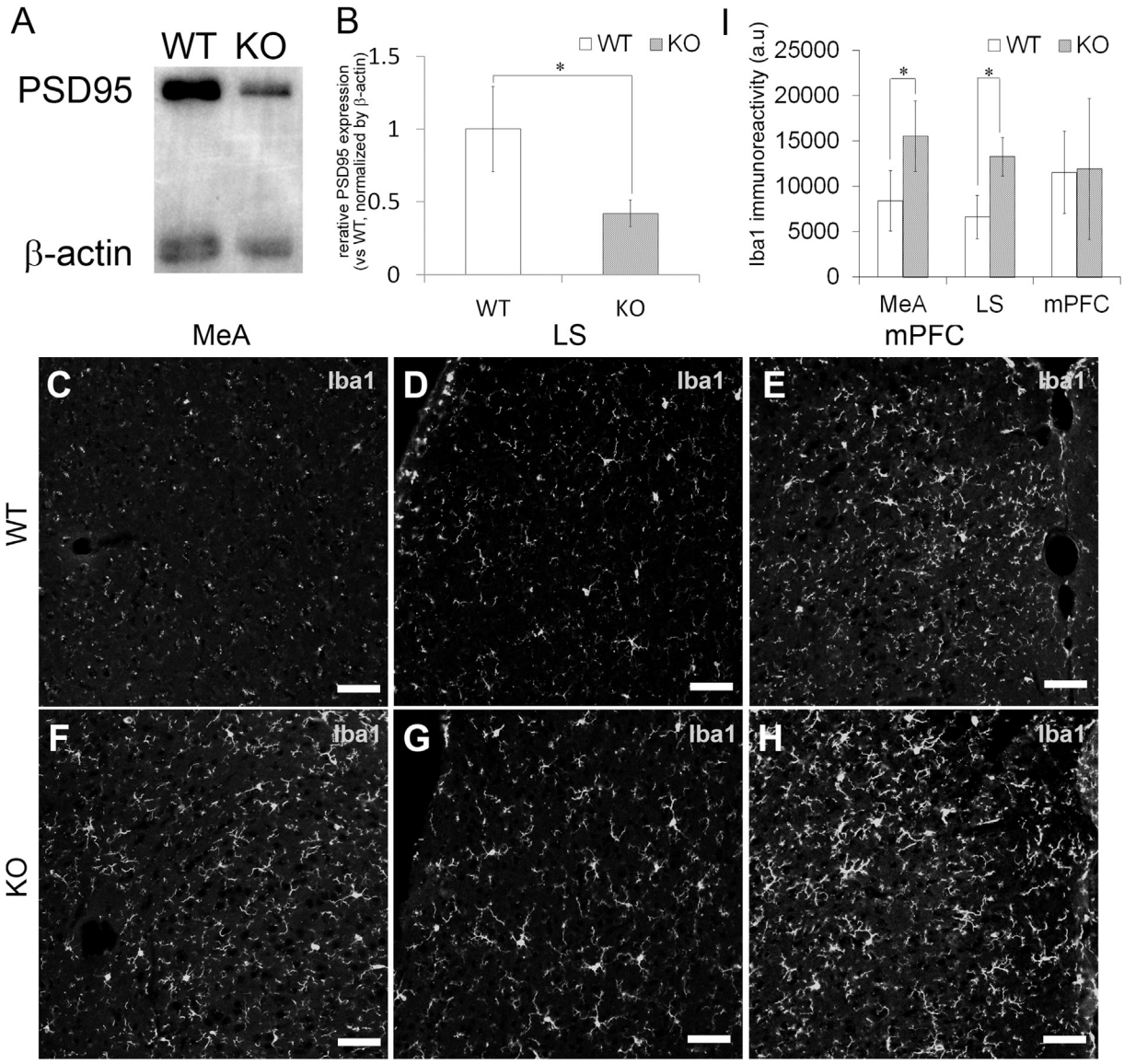

Fig. 1. Phenotypic analyses of adult Oxtr-deficient mice. An immunoblot of PSD95 in wild-type (WT) and Oxtr knockout (KO) brains is shown in (A) and summarized in (B). Immunostaining images of microglial cells in WT and Oxtr-KO mice are shown in (C–H). The staining intensities were quantified and are summarized in (I). MeA, medial amygdaloid nucleus; LS, lateral septal nucleus; mPFC, medial prefrontal cortex. Scale bars: 50 μm (C–H). In (B) and (I), statistically significant differences are indicated with * ($p < 0.01$). Data are presented as mean ± standard deviation of the mean.

Next, we examined microglia to address the possible effects of the PSD95 reduction in Oxtr-deficient mice. Using these mice that displayed autism related behaviors, we focused on brain areas important for sociality to study how changes in microglial cells might be correlated with these behaviors. We found that, in the medial amygdaloid nuclei (MeA) of the mutant brains, the intensity of Iba1 staining—a microglial marker—was approximately twice as high in the wild type brains (Fig. 1 C, F, I). Further analysis of the morphologies of these microglial cells from the Oxtr-deficient MeA revealed obvious increases in cell body volume as well as in the number of branching processes. These results clearly show abnormal hyperactivation of microglial cells in a sociality related region of the Oxtr-deficient brain. We then carried out similar analyses of the lateral septal nuclei (LS) and medial prefrontal cortex (mPFC), two other brain regions that contribute to sociality [19,20]. In the *Oxtr*-deficient LS, we detected enhanced Iba1 staining similar to that in the MeA (Fig. 1 D, G, I). The morphologies of the microglial cells also showed enlarged cell bodies and increased numbers of branches. In addition, the average branch length was significantly increased compared to the wild type microglia. Surprisingly, despite the activation of microglial cells in the LS, we did not observe microglial activation in the mPFC of the mutant brains (Fig. 1 E, H, I). These results suggest that microglial activation may be restricted to the MeA and LS regions in *Oxtr*-deficient mice.

Pharmacological inhibition of microglia improved PSD95 expression and mother–infant communications in Oxtr deficient mice.

To assess whether the PSD95 downregulation was caused by microglial activation in *Oxtr*-deficient mice, we treated *Oxtr*-deficient mice with minocycline, a tetracycline derivative known to reduce microglial activity [21], and examined PSD95 expression on P28 and mother–infant communication activities on P5. Invasion of

microglia progenitors to the CNS occurs starting from E10, and Oxtr expression commence at E15.5 [22]. Therefore, we administered minocycline from gestational day 15.5 to P21 (Fig. 2 A). Mother–infant communication was assessed by pup's ultrasonic vocalization (USV) analysis at P5 (Fig. 2 B). The number of mother call was significantly decreased in *Oxtr*-deficient pup; however, it was rescued by the minocycline treatment. Immunohistological staining showed suppression of microglia in the minocycline treated *Oxtr*-deficient brains (Fig. 2 C–H), and the PSD95 expression was significantly elevated in immunoblotting analyses (Fig.2 I).

4. Discussion

Oxt administration has recently been reported to ameliorate ASD episodes [9]; however, the molecular, biochemical, and physiological mechanisms that underlie this improvement are still unknown. Oxt is thought to function through the activation of neurons via the release of Ca^{2+} ions from intracellular stores, which, in turn, activates protein kinase C. Interestingly, functional magnetic resonance imaging analyses of patients with ASD indicate that nasal administration of Oxt activates nuclei that are usually inactive in ASD brains [23]. These reports imply that Oxt/Oxtr system not only behaves as a neurotransmitter or neuromodulator, but also as a growth factor, cytokine, or even indirect regulator.

In this study, we found a downregulation of PSD95 protein expression in adult *Oxtr*-deficient mouse brains. PSD95 is a major synaptic scaffold protein, and the reduction of PSD95 expression strongly implies a reduction of synapses. Thus, this result suggests that synaptic loss occurs in the *Oxtr*-deficient brain. This is in accord with previous studies on postmortem brains from human ASD patients [24]. Given these findings, we focused on the relationship between Oxt and microglia. In the brain, apart from immunological functions, microglial cells contribute to neuronal function through regulation of synapse pruning, which influences synaptic plasticity [2]. Recent studies suggest that microglia are activated in ASD brains and play an important role in ASD pathology [4]. By immunohistochemical analysis, we revealed that microglia were activated in several regions of *Oxtr*-deficient brains. This finding implies that abnormal microglial activation could be a potential mechanism of synaptic loss in *Oxtr*-deficient brains. To investigate it, we assessed whether PSD95 downregulation was caused by microglial activation. We found that pharmacological suppression

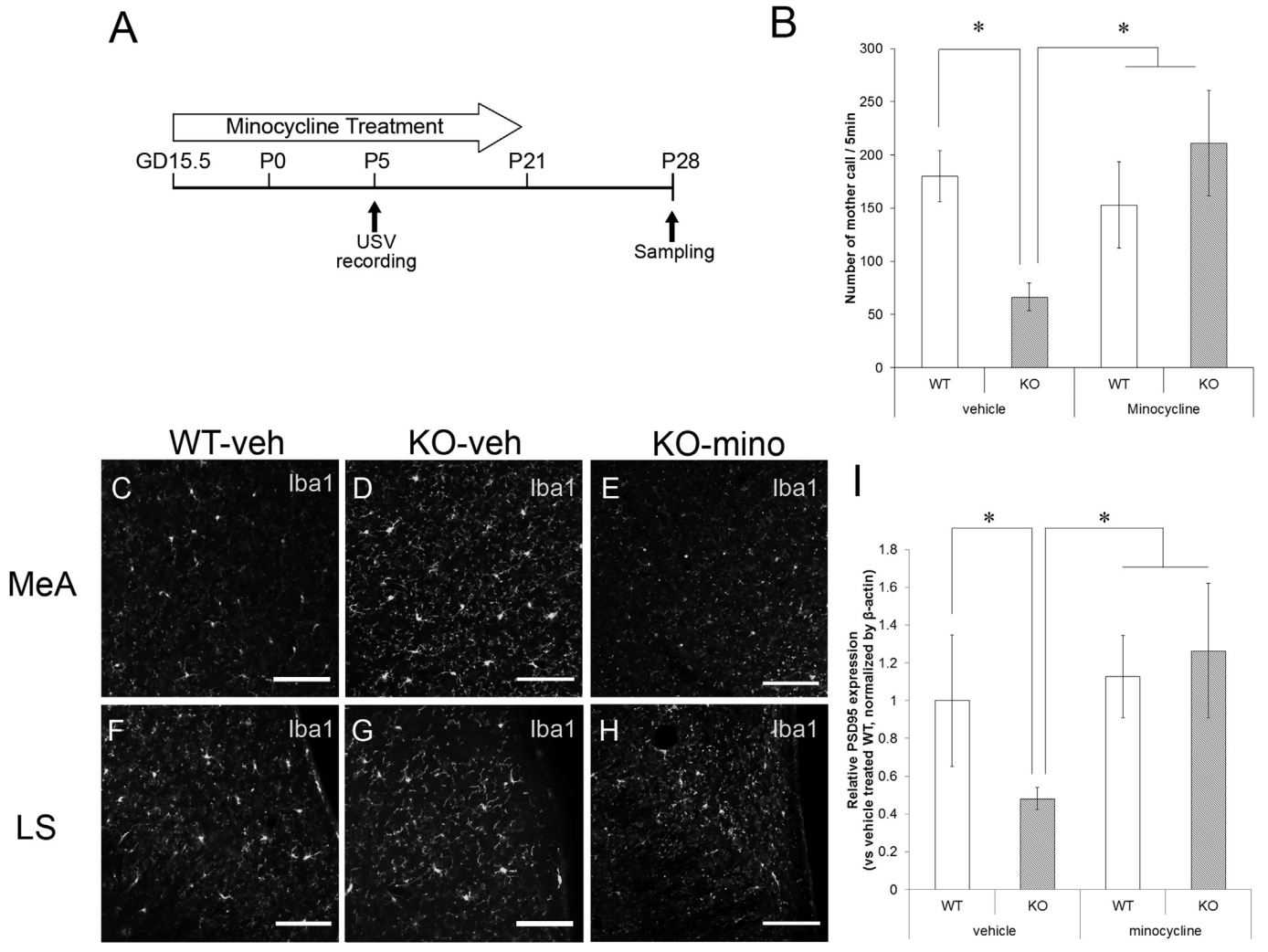

Fig. 2. Minocycline treatment ameliorates mother–infant communication in Oxtr-deficient pups and elevates PSD95 expression. A schematic diagram of the minocycline treatment experiment is shown in (A). The results of USV analysis at P5 are summarized in (B). Representative images of immunohistochemical staining of the medial amygdaloid nucleus (MeA) and lateral septal nucleus (LS) in minocycline-treated Oxtr-deficient (KO) and wild-type (WT) mice at P28 (C–H). Quantitated data from immunoblotting analyses of PSD95 expression in minocycline or vehicle-treated wild-type (WT) and Oxtr knockout (KO) brains are shown in (I). Scale bars: 100 μm (C–H). In (B) and (I), statistically significant differences are indicated with * ($p < 0.01$). Data are presented as mean ± standard error of the mean (B) or standard deviation of the mean (I).

of microglia by minocycline treatment rescued adult PSD95 expression and mother–infant communication in *Oxtr*-deficient pups. These results indicate that Oxtr regulates synaptic maturation during neuronal development via microglial activation and is necessary for mother–infant communication, which is compromised in ASD patients. Taken together, our results suggest that patients with ASD associated with Oxtr malfunction may have abnormally activated microglial cells and a perturbed balance between the excitatory and inhibitory synaptic functions, which are under the control of the Oxt/Oxtr system in the normal brain.

A recent study showed that synaptic development is impaired in several transgenic ASD mouse model [25], which exhibit autism-like behaviors. Therefore, many researchers now believe that synaptic abnormality is one of the underlying mechanisms of ASD pathology. Nevertheless, the mechanisms of these synaptic alterations are still controversial. Our data yield new insight into potential pathogenic mechanisms underlying ASD symptoms in patients associated with *Oxtr* mutations. As described above, microglial activation occurs not only in the developing brain but also in adult patients. Microglia can facilitate long-term potentiation by releasing brain-derived neurotrophic factor, which may also increase the excitatory input in fully developed brain. Hence, we can infer that microglial activation induced by the lack of normal Oxtr activity may be a key mechanism of ASD pathology in adult patients.

However, molecular mechanisms underlying the link between Oxtr function and microglial activation were not identified in this study. We also did not assess the effects of minocycline treatment on social behaviors in adult animals. Therefore, further investigations are needed to explore the regulatory mechanisms of microglial activation in ASD patients, and we anticipate that they will be key in understanding the pathogenic and pathological mechanisms of ASD.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

SM, YH and KN performed this research. YH designed this research. SM and YH performed the experiments. SH contributed essential reagents of this research. SM and YH analyzed the data. YH and KN wrote the paper. All authors have read and approved the manuscript for publication.

Acknowledgments

This work was supported by a Grant-in Aid from the Japan Society for the Promotion of science of Japan (23380055, 15H02442), and part of this study is the result of “Integrated Research on Neuropsychiatric Disorders” carried out under the Strategic Research Program for Brain Sciences by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.02.109.

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