Brain, Behavior, and Immunity 61 (2017) 375–385

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

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

Full-length Article

Microglia-derived neuregulin expression in psychiatric disorders

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ARTICLE INFO

Article history:
Received 22 August 2016
Received in revised form 5 January 2017
Accepted 8 January 2017
Available online 10 January 2017

Keywords:
Neuregulin
Microglia
Autism spectrum disorder
PBMCs
Cytokine

ABSTRACT

Several studies have revealed that neuregulins (NRGs) are involved in brain function and psychiatric disorders. While NRGs have been regarded as neuron- or astrocyte-derived molecules, our research has revealed that microglia also express NRGs, levels of which are markedly increased in activated microglia. Previous studies have indicated that microglia are activated in the brains of individuals with autism spectrum disorder (ASD). Therefore, we investigated microglial NRG mRNA expression in multiple lines of mice considered models of ASD. Intriguingly, microglial NRG expression significantly increased in BTBR and socially-isolated mice, while maternal immune activation (MIA) mice exhibited identical NRG expression to controls. Furthermore, we observed a positive correlation between NRG expression in microglia and peripheral blood mononuclear cells (PBMCs) in mice, suggesting that NRG expression in human PBMCs may mirror microglia-derived NRG expression in the human brain. To translate these findings for application in clinical psychiatry, we measured levels of *NRG1* splice-variant expression in clinically available PBMCs of patients with ASD. Levels of *NRG1* type III expression in PBMCs were positively correlated with impairments in social interaction in children with ASD (as assessed using the Autistic Diagnostic Interview-Revised test: ADI-R). These findings suggest that immune cell-derived NRGs may be implicated in the pathobiology of psychiatric disorders such as ASD.

1. Introduction

Studies have increasingly revealed that the immune system exhibits broad patterns of dysfunction in individuals with psychiatric disorders such as autism spectrum disorder (ASD), schizophrenia (SCZ), and major depressive disorder (MDD). Such studies have reported abnormal cytokine profiles, reduced lymphocyte numbers, weakened T-cell mitogen responses, imbalanced serum immunoglobulin levels, and aberrant autoimmune-related gene expression in these patients (Ashwood et al., 2006; Estes and McAllister, 2015; Hughes et al., 2016; Muller et al., 2015; Onore et al., 2013).

Consistent with the human data, recent studies in animal models have also indicated abnormal immune system function in

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http://dx.doi.org/10.1016/j.bbi.2017.01.003
0889-1591/

psychiatric disorders (Heo et al., 2011; Leonard and Song, 2002). Most of these studies, however, have focused on systemic immune system components such as plasma and peripheral blood mononuclear cells (PBMCs). In order to dissect the immune-related pathobiology of psychiatric disorders, research must focus on the role of microglia, the resident immune cells of the central nervous system (CNS) that originate from the yolk sac (Ginhoux et al., 2010; Schulz et al., 2012) and account for 5–20% of non-neuronal glial cells in the CNS (Lawson et al., 1990). Intriguingly, several studies using positron emission tomography (PET) have suggested that microglial activation is increased in the brains of patients with ASD, SCZ, and MDD relative to controls, although the translocator protein (TSPO) radiotracers used in these studies are not specific to microglia (Hafizi et al., 2016; Kato et al., 2013; Kenk et al., 2015; Suzuki et al., 2013; van Berckel et al., 2008). Postmortem brain examinations of psychiatric disorders also demonstrated microglial activation (Bayer et al., 1999; Busse et al., 2012; Fillman et al., 2013; Radewicz et al., 2000; Vargas et al., 2005; Wierzba-Bobrowicz et al., 2005).

In pathological conditions such as injury, microglia alter their morphology from a ramified to an amoeboid form (Giulian, 1987). These "activated" microglia are responsible for numerous functions, including phagocytosis of cellular debris, release of inflammatory molecules, and synaptic stripping via contact with injured neurons (Blinzinger and Kreutzberg, 1968; Hanisch and Kettenmann, 2007; Kreutzberg, 1996; Ransohoff and Perry, 2009). Along with activated microglia, ramified/resting microglia also play important roles in synaptic plasticity (Ji et al., 2013; Parkhurst et al., 2013; Tremblay et al., 2010). Thus, loss of the regular function of resting microglia may disturb synaptic function and neurogenesis (Schafer et al., 2012), particularly in developing brains.

In addition to increasing secretion of pro-inflammatory cytokines, microglial expression of neurotrophic factors such as brain-derived neurotrophic factor, glial-derived neurotropic factor, and basic fibroblast growth factor may further affect brain function (Chamak et al., 1995; Elkabes et al., 1996; Merson et al., 2010; Narantuya et al., 2010).

Neuregulins (NRGs) are members of the epidermal growth factor family, and consist of NRG1-4. Among these subtypes, NRG1, NRG2, and NRG3 are substantially expressed in the CNS, where they bind to ErbB family receptors (Carraway et al., 1997; Meyer et al., 1997; Zhang et al., 1997). NRG1 plays a major role in axon guidance, glial cell development, myelination, synapse formation, synaptic plasticity, and neuronal survival (Corfas et al., 2004; Mei and Xiong, 2008). NRG1 also has numerous alternative splicing variants that are generated by different promotors, predominantly types I-VI (Falls, 2003; Steinthorsdottir et al., 2004). The most abundant NRG1 splicing variant in the adult cerebral cortex is type III, followed by type II and type I in the adult cerebral cortex (Liu et al., 2011). NRG1 type I is a chemoattractant for the tangential migration of medial ganglionic eminence-derived interneurons to the cortex in the postnatal period (Flames et al., 2004). NRG1 type II, also known as glial growth factor, is considered necessary for radial glia formation and migration in the developing cerebral cortex (Anton et al., 1997). NRG1 type III is required for normal sensory gating and memory-related behaviors (Chen et al., 2008), promotes oligodendrocyte myelination (Taveggia et al., 2008), and controls the ventral hippocampus-induced electrophysiological activity in the nucleus accumbens (Nason et al., 2011), suggesting its possible role in psychiatric disorders. Genetic, postmortem, animal, and cellular studies have also reported a link between NRGs and SCZ (Hashimoto et al., 2004; Nicodemus et al., 2009; Roy et al., 2007; Stefansson et al., 2002). However, in almost all such studies, the source of NRGs had been presumed to be neurons or astrocytes (Liu et al., 2011; Pankonin et al., 2009; Yin et al., 2013), rather than microglia.

Microglia are highly motile in the CNS, facilitating easy access to their targets. Moreover, these cells can physically attach to where NRGs are needed; thus, NRGs from microglia most likely affect neuronal function at least as much as neuronal NRGs (Meyer et al., 1997). Both decreases and increases in ubiquitous NRG expression lead to abnormal behaviors, such as hyperactivity, impaired prepulse inhibition, poor working memory, and decreased social behavior (Chen et al., 2008; Kato et al., 2010). Therefore, it is possible that aberrant NRG expression in microglia is involved in the behavioral alterations observed in many psychiatric disorders.

In the present study, we examined NRG expression in the microglia of multiple animal models of ASD and in clinically available peripheral blood mononuclear cells (PBMCs) of patients with ASD in order to evaluate the role of immune cells-derived NRG expression in the development of ASD symptoms.

2. Materials and methods

2.1. Mice

Male C57BL/6J (C57) mice and male BTBR *T*$^{+}$*tf*/J (BTBR) mice (Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed in a temperature- and humidity-controlled animal facility under a reversed light–dark cycle (lights on 8:00–20:00). All animals were provided with food and water *ad libitum* throughout the experiments. Experimental protocols followed the guidelines of the Animal Care Committee of Nara Medical University, in accordance with the policies established in the NIH Guide for the Care and Use of Laboratory Animals.

The social isolation protocol utilized in the present study has been previously reported (Makinodan et al., 2012). Briefly, male C57 mice were randomly divided into two different housing conditions at the time of weaning (postnatal day 21; P21): regular environment (RE) or isolation (IS-RE). In the isolation condition, mice were exposed to 2 weeks of isolation (P21 to P35) and were then housed with other isolated mice. In the regular environment condition, four mice were housed in a standard cage after weaning. In comparison to RE mice, IS-RE mice exhibit impaired social interaction and working memory (Makinodan et al., 2012). In order to produce an animal model in which maternal immune-activation (MIA) leads to ASD-like and schizophrenia-like symptoms in offspring (MIA mice), as based on epidemiological findings (Knuesel et al., 2014; Makinodan et al., 2008), pregnant mice were intraperitoneally injected with polyinosinic-polycytidylic acid (poly-IC, 20 mg/kg) (Sigma-Aldrich, St Louis, MO, USA), or phosphate-buffered saline (PBS) as a vehicle, on embryonic day 9.5 (E9.5). Male offspring were used for this experiment at P8 (Suppl. Fig. 1b).

2.2. Microglial purification

We used a magnetic-activated cell sorting (MACS) system for microglial purification. Mice were deeply anaesthetized and transcardially perfused with PBS to remove blood cells (particularly CD11b^{+} macrophages). Microglia were isolated from microdissected whole brains of BTBR and MIA mice, at P8, when both mouse lines exhibit abnormal behaviors (Scattoni et al., 2008; Schwartzer et al., 2013). Alternatively, microglia were isolated from the cerebral cortex of socially isolated mice at P58 P65, when abnormal behaviors are observed in this mouse line (Makinodan et al., 2012). Dissected tissues were dissociated into single-cell suspensions using a Neural Tissue Dissociation kit (Miltenyi Biotec,

Bergisch Gladbach, Germany), and myelin debris was removed from the single-cell suspensions with Myelin Removal Beads II (Miltenyi Biotec). CD11b$^+$ microglia were isolated with anti-CD11b microbeads (Miltenyi Biotec). Separations were performed using a QuadroMACS Separator with an LS column (Miltenyi Biotec), and all procedures were conducted in accordance with the manufacturer's instructions.

2.3. Microglial activation

In vitro activation (Suppl. Fig. 1d upper): Following MACS purification, microglia were suspended in culture media (DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution) and plated in a 6-well culture plate at a density of 1×10^6 cells/well. Cells were then cultured with lipopolysaccaride (LPS, 1.0 mg/mL) (Sigma-Aldrich, Tokyo, Japan) or PBS. After 24 h of incubation, microglia were scraped off using a cell scraper, centrifuged at 300 *g* for 15 min, and used for quantitative RT-PCR analysis.

In vivo activation (Suppl. Fig. 1d lower): At P8, C57 mice were intraperitoneally injected with LPS (Sigma-Aldrich, Tokyo, Japan) at a dose of 2 mg/kg body weight. Mice were deeply anesthetized 24 h after LPS injection, following which the brains were removed for subsequent immunohistochemical analysis.

2.4. Immunocytochemistry

Following MACS separation, microglia were suspended in culture media (DMEM + 10% fetal bovine serum, and 1% antibiotic-antimycotic) and spread on poly-L-lysine-coated glass coverslips (Matsunami Glass, Osaka, Japan) inside a 24-well plate at a density of 1×10^5 cells/well. These cells were cultured for 96 h, fixed with 4% paraformaldehyde for 15 min, and then rinsed three times with PBS. Cells were then blocked and permeabilized for 1 h in blocking buffer (PBS containing 5% bovine serum albumin and 0.2% TritonX-100). Microglia were immunostained using rabbit anti-NRG1 antibody (1:200 dilution; Abcam, Tokyo, Japan) and goat anti-Iba1 antibody (1:500 dilution; Abcam, Tokyo, Japan) diluted in blocking buffer at room temperature for 1 h. After rinsing three times with PBS, Alexa 488- or Alexa 555-conjugated secondary antibodies (Life Technologies, Tokyo, Japan) were added at room temperature for 1 h. After again rinsing three times with PBS, slides were prepared using VECTASHIELD Hard-Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Control samples were incubated without primary antibodies using the aforementioned methods. No immunoreactivities were observed in controls (data not shown). Images were randomly acquired using a Leica DMI 4000B microscope (Leica, Tokyo, Japan) with a 40× objective lens using LAS AF Version 2.6.0.7266 acquisition software.

2.5. Immunohistochemistry

After 24 h LPS treatment, hippocampal slices (200 μm) were sectioned using a Leica VT 1200S (Leica, Tokyo, Japan), and the slices were then fixed with 4% paraformaldehyde for 1 h, followed by rinsing with PBS three times. Slices were then blocked and permeabilized for 1 h in blocking buffer (PBS containing 5% BSA and 0.3% TritonX-100), and immunostained with rabbit anti-NRG1 antibody (1:200 dilution; Abcam, Tokyo, Japan) and goat anti-Iba1 antibody (1:200 dilution; Abcam, Tokyo, Japan) diluted in blocking buffer at 4 °C for 48 h. After rinsing three times with PBS, Alexa 488-or Alexa 555-conjugated secondary antibodies (Life Technologies, Tokyo, Japan) were added at room temperature for 1 h. After sections were counterstained with DAPI (1 μg/mL) (Invitrogen, Carlsbad, CA, USA), slides were prepared using ProLong® Gold (Thermo Fisher Scientific, Kanagawa, Japan). To verify the expression of NRG1 in microglia, representative z-stack images were obtained using a Nikon C2 confocal laser microscope (Nikon, Tokyo, Japan) with a 100× objective lens and NIS-Elements AR (Nikon, Tokyo, Japan).

2.6. Murine PBMC samples

At P58 65, whole-blood samples were collected via transcardial perfusion with PBS from RE or IS-RE mice prior to microglial purification. PBMCs were isolated using Lympholyte®-Mammal (Cedarlane Laboratories Ltd., Burlington, Canada) after sampling, and all procedures were conducted in accordance with the manufacturer's instructions.

2.7. Induction of human microglia-like (iMG) cells from human peripheral blood cells

Human iMG cells were produced according to a previously described method (Ohgidani et al., 2014). Briefly, peripheral blood was collected from healthy adult volunteers using a heparinized tube. PBMCs were isolated using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA) density-gradient centrifugation. PBMCs were resuspended with RPMI-1640 (Nacalai Tesque, Kyoto, Japan), 10% heat-inactivated fetal bovine serum (FBS; Japan Bio Serum, Hiroshima, Japan), and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). PBMCs were plated onto culture chambers at a density of 4×10^5 cells/mL and cultured overnight under standard culture conditions (37 °C, 5% CO_2). After overnight incubation, culture supernatant and non-adherent cells were removed. The adherent cells were cultured with RPMI-1640 Glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 1% antibiotic-antimycotic solution and a mixture of the following candidate cytokines in order to develop iMG cells: recombinant human GM-CSF (10 ng/mL; R&D Systems, Minneapolis, MN, USA) and recombinant human IL-34 (100 ng/mL; R&D Systems). Levels of *NRG1* mRNA expression were measured following exposure to standard culture conditions for 14 days.

2.8. Human PBMC samples

The present study was approved by the ethics committee of the Hamamatsu University School of Medicine. All participants and their guardians were given a complete description of the study and provided written informed consent prior to enrollment.

In order to isolate PBMCs from human blood samples, we recruited 30 male patients with ASD (mean age: 11.6 ± 2.67 years). Since boxplots (prepared using SPSS software) identified one patient as an outlier, we included only 29 male patients for the remaining analyses. All participants were of Japanese descent and had been born and lived in central Japan, including Aichi, Gifu, and Shizuoka Prefectures.

Based on interviews and available information including hospital records, diagnoses of ASD were made by an experienced child psychiatrist based on criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition-Text Revision (DSM-IV-TR). The Autism Diagnostic Interview-Revised (ADI-R) was also conducted by qualified child psychiatrists. The ADI-R is a semi-structured interview conducted with a parent for the confirmation of ASD diagnoses. Domain A of the ADI-R quantifies impairments in social interaction at the age of 4 years, while domain B quantifies impairments in communication. Domain C quantifies restricted, repetitive, and stereotyped patterns of behaviors and interests. Comorbid psychiatric illnesses were excluded by means of the Structured Clinical Interview for the DSM-IV (SCID).

Participants were excluded from the study if they had any symptoms of inflammation, a diagnosis of fragile X syndrome,

epileptic seizures, obsessive-compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. None of the participants had ever received psychoactive or anti-inflammatory medications prior to this study. All 30 male control participants (mean age: 11.1 ± 2.34 years) underwent a comprehensive assessment of their medical history to eliminate individuals with any neurological or other medical disorders. The SCID was also used to identify any personal or family history of past or present mental illness. None of the comparison participants initially recruited fulfilled any of the exclusion criteria. Whole-blood samples were collected by venipuncture from all participants. PBMCs were isolated from blood samples by means of the Ficoll-Paque gradient method within 2 h after sampling.

2.9. Quantitative RT-PCR (qRT-PCR)

Levels of mRNA were quantified in microglia and PBMCs isolated from the whole brains of P8 mice, or the cerebral cortex of

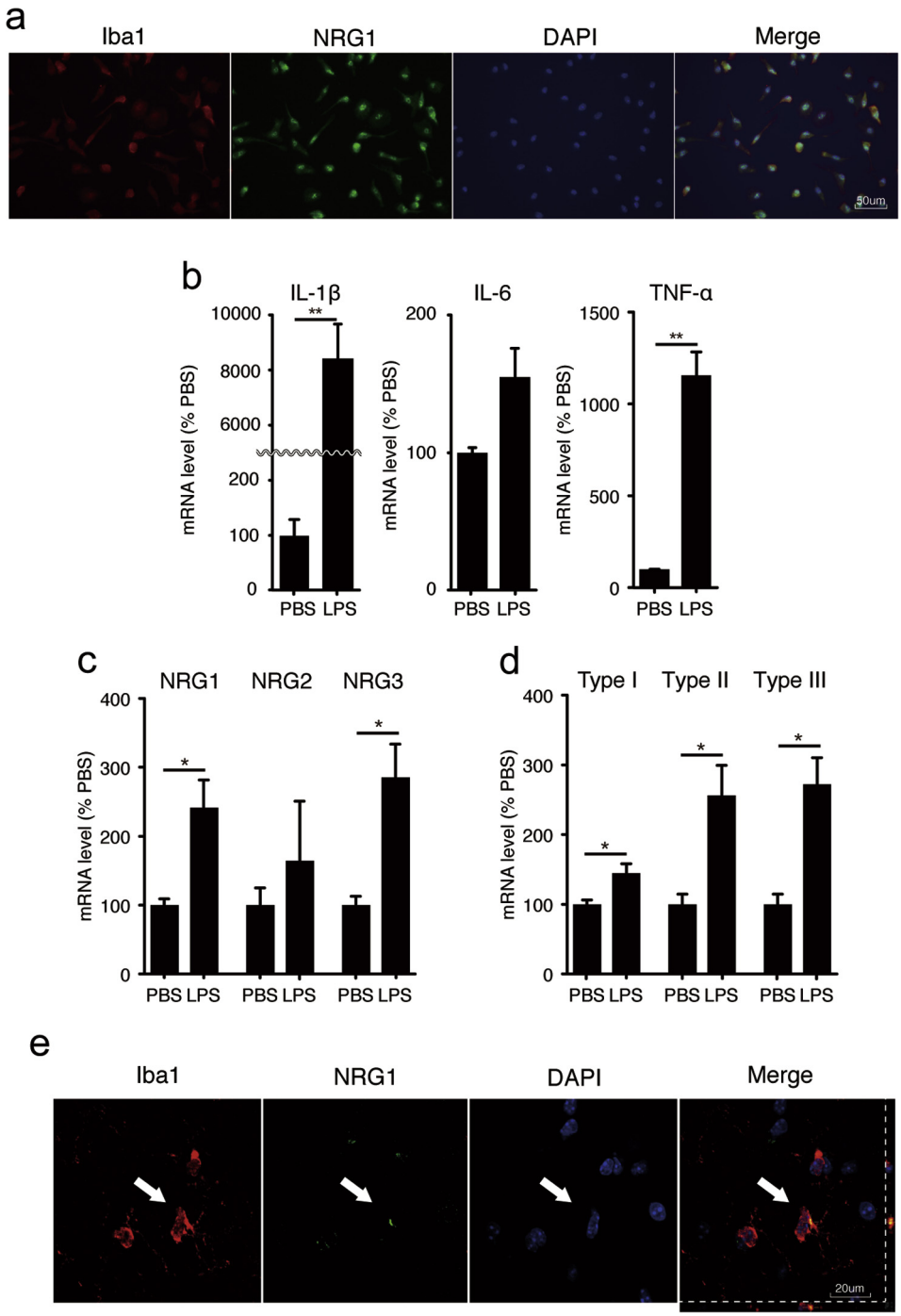

Fig. 1. NRG1 expression and elevation in murine microglia following microglial activation (n = 3 in each group). (a) Using immunocytochemistry, we observed that NRG1 (green) was expressed in purified Iba1 $^{+}$ /DAPI $^{+}$ microglia (red/blue). Scale bar: 50 μm. (b, c, d) Microglia activated with LPS treatment for 24 h expressed markedly high levels of *IL-1β* and *TNF-α* (*IL-1β*, $p < 0.01$; *TNF-α*, $p < 0.01$), as well as *NRG1*, *NRG3*, and *NRG1* type I, type II, and type III (*NRG1*, $p < 0.05$; *NRG3*, $p < 0.05$; type Ⅰ, $p < 0.05$; type Ⅱ, $p < 0.05$; type Ⅲ, $p < 0.05$). However, no increases in *IL-6* or *NRG2* were observed (*IL-6*, $p > 0.05$; *NRG2*, $p > 0.05$). (e) Iba1 $^{+}$ /DAPI $^{+}$ murine microglia (red/blue) expressed NRG1 (green) in the hippocampus following treatment with LPS for 24 h *in vivo*. The arrow indicates the microglia. An ortho-view of z-stack merge images confirmed intracellular distribution of NRG1 in microglia. Scale bar: 20 μm. NRG: neuregulin; IL-1β: interleukin-1 beta; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha.

P58 65 mice, by means of qRT-PCR. Tissue RNA was isolated using Direct-zol™ RNA MiniPrep (ZYMO RESEARCH, Irvine, CA, USA) following the manufacturer's protocol. RNA quantity was determined by absorbance at 260 nm. First-strand cDNA was synthesized from 100 ng of RNA using an iScript kit (Bio-Rad Laboratories, Hercules, CA, USA), and qRT-PCR was performed using SYBR Green® Premix Ex Taq™ II (TAKARA BIO INC., Otsu, Shiga, Japan). The *18S* ribosomal RNA was chosen as an internal control for use in the delta-delta C_T method. To measure mRNA levels of NRGs in human PBMCs, total RNA was isolated from cells using TRIzol® reagent (Invitrogen), and the RNA samples were further purified using the RNeasy Micro Kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized from the RNA samples using the SuperScript III First-Strand Synthesis System (Invitrogen), and qRT-PCR analysis was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Relative quantification of NRG expression levels was performed using the delta-delta C_T method using the constitutively expressed genes; Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Actin Beta (ACTB), Glucose-6-Phosphate Dehydrogenase (G6PD), as an internal control. Primer sequences are available upon request.

2.10. Statistical analysis

Quantitative RT-PCR data are presented as mean percent changes (relative to control) ± SEM. Comparisons were performed using Student's *t* test, and differences were considered significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$. Evaluation of the relationships between gene expression levels and clinical variables was performed using Pearson's correlation coefficient. Spearman's rank correlation coefficient was used for the correlation of gene expression levels between PBMCs and microglia in mice. *P* values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. NRG expression and elevation via LPS-induced activation of microglia

After sorting and incubation of $CD11b^+$ microglia from C57 mice, we confirmed successful microglial isolation using the specific marker Iba1, which was expressed in 97.4% of $DAPI^+$ cells, consistent with the findings of a previous study (Marek et al., 2008). Surprisingly, NRG1 was expressed in all microglia ($Iba1^+$ $DAPI^+$ cells) (Fig. 1a). Since previous studies have reported activation of microglia in the brains of patients with psychiatric disorders (Kato et al., 2013; Suzuki et al., 2013; van Berckel et al., 2008), we examined whether microglial activation led to changes in NRG mRNA expression. Treatment of microglia with LPS for 24 h markedly elevated levels of the pro-inflammatory cytokine *IL-1β* and *tumor necrosis factor* (*TNF*)-α (Fig. 1b) as well as mRNA of the following NRGs: *NRG1* (types I III) and *NRG3* (Fig. 1c, d). A trend for increased gene expression of *IL-6* was observed in LPS-treated microglia ($p = 0.057$, Fig. 1b). No elevation in *NRG2* levels was observed (Fig. 1c). Furthermore, we confirmed NRG1 expression

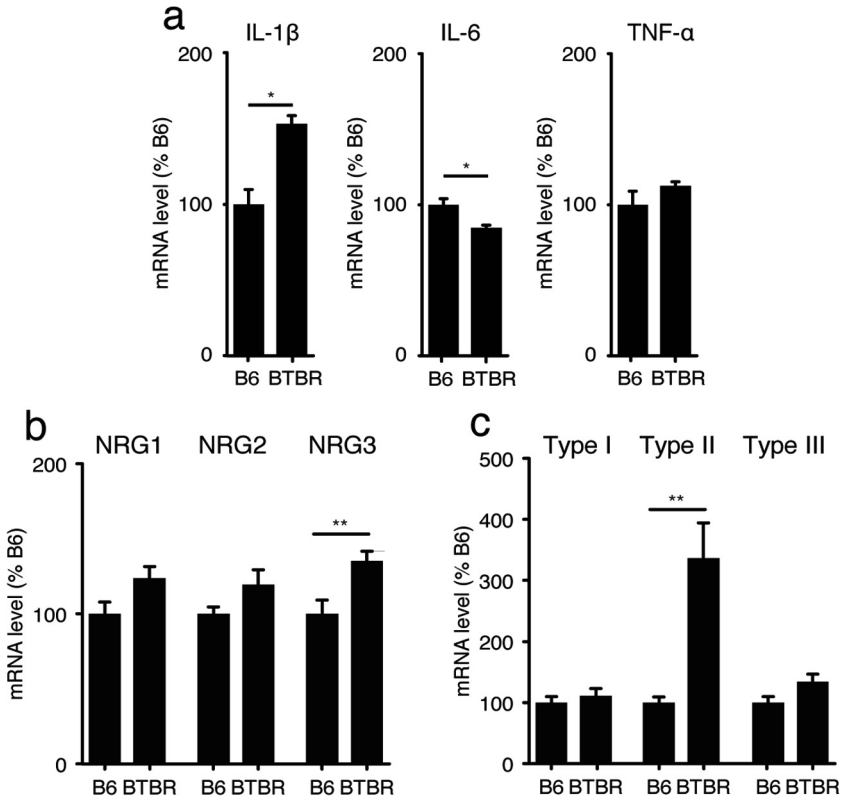

Fig. 2. Cytokine and NRG expression in the microglia of C57 and BTBR mice (n = 8 in each group). (a) *IL-1β* mRNA expression was significantly higher in BTBR mice than in C57 mice ($p < 0.05$), whereas *IL-6* mRNA was lower ($p < 0.05$). *TNF-α* mRNA expression was identical between C57 and BTBR mice ($p > 0.05$). (b) *NRG1* mRNA expression showed a tendency of increase in the microglia of BTBR mice compared to C57 mice ($p = 0.051$). *NRG3*, but not *NRG2*, mRNA expression was significantly higher in the microglia of BTBR mice than in those of C57 mice (*NRG2*, $p > 0.05$; *NRG3*, $p < 0.01$). (c) Among the *NRG1* splicing variants, only Type II was markedly increased in the microglia of BTBR mice compared to C57BL/6J mice (Type I, $p > 0.05$; Type II, $p < 0.01$; Type III, $p > 0.05$). NRG: neuregulin; IL-1β: interleukin-1 beta; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha.

in activated (ameboid) microglia (Iba1 $^{+}$ NRG1 $^{+}$ DAPI $^{+}$ cell) in the hippocampus of mice *in vivo* (Fig. 1e).

3.2. NRG expression in the microglia of animal models of psychiatric disorders

We observed NRG expression in the microglia of mice, which was elevated by microglial activation. Therefore, we hypothesized that NRG expression would be elevated in the microglia of animal models of psychiatric disorders.

BTBR inbred mice are commercially available and represent the most frequently utilized model of ASD in animal studies. These mice exhibit deficits in social interaction, unusual vocalizations in the early postnatal period, and repetitive stereotyped behaviors (McFarlane et al., 2008; Moy et al., 2008; Scattoni et al., 2008), which correspond to three defining symptoms of ASD, aberrant reciprocal social interactions, qualitative impairments in communication and restricted/repetitive and stereotyped patterns of behavior (Shishido et al., 2014), respectively. While research has indicated that pro-inflammatory cytokines are elevated in the brains of BTBR mice (Heo et al., 2011), no studies have reported levels of cytokine expression derived from the microglia of these mice. Therefore, we measured and compared cytokine expression in microglia purified from C57 and BTBR mice. While microglial *IL-1β* expression was significantly increased in BTBR mice relative to levels observed in C57 mice ($p < 0.05$), microglial *IL-6* expression was unexpectedly reduced in BTBR mice ($p < 0.05$; Fig. 2a). No significant differences in microglial expression of *TNF-α* were observed ($p > 0.05$; Fig. 2a). Thus, the purification of microglia revealed that pro-inflammatory cytokine expression was not necessarily increased in the microglia of BTBR mice as compared to C57 mice.

Since microglial activation increases NRG expression, we hypothesized that NRG expression would also be higher in the microglia of BTBR mice compared to those in C57 mice. *NRG1* levels showed a trend toward an increase ($p = 0.051$), while *NRG3* levels were significantly higher ($p < 0.01$) in the microglia of BTBR mice relative to C57 mice, but this was not true for *NRG2* ($p > 0.05$; Fig. 2b). In order to further evaluate *NRG1* expression, we measured levels of *NRG1* types I–III and observed that only *NRG1* type II expression was markedly higher in BTBR mice than in C57 mice (*NRG1* type II, $p < 0.01$; *NRG1* type I, $p > 0.05$; *NRG1* type III, $p > 0.05$; Fig. 2c). In summary, the expression of *NRG1* type II and *NRG3* was significantly higher in the microglia of BTBR mice than in those of C57 mice. These data indicate that NRG expression was influenced by the activation pattern of microglia.

MIA is associated with a variety of psychiatric disorders and, over the last decade, several animal models have been developed that mimic the mechanisms that underlie such disorders (Chen et al., 2016; Estes and McAllister, 2015). For example, maternal viral infection and poly-IC-induced immune activation lead to ASD-like or SCZ-like behaviors in offspring (Schwartzer et al., 2013; Shi et al., 2003). In the present study, we observed increases in *IL-1β* expression, although no such increases were observed for *IL-6* or *TNF-α* (*IL-1β*, $p < 0.001$; *IL-6*, $p > 0.05$; *TNF-α*, $p > 0.05$; Suppl. Fig. 2a). Furthermore, we observed no differences in the expression of microglial NRGs between control mice and MIA mice (*NRG1*, $p > 0.05$; *NRG2*, $p > 0.05$; *NRG3*, $p > 0.05$; *NRG1* type I, $p > 0.05$;

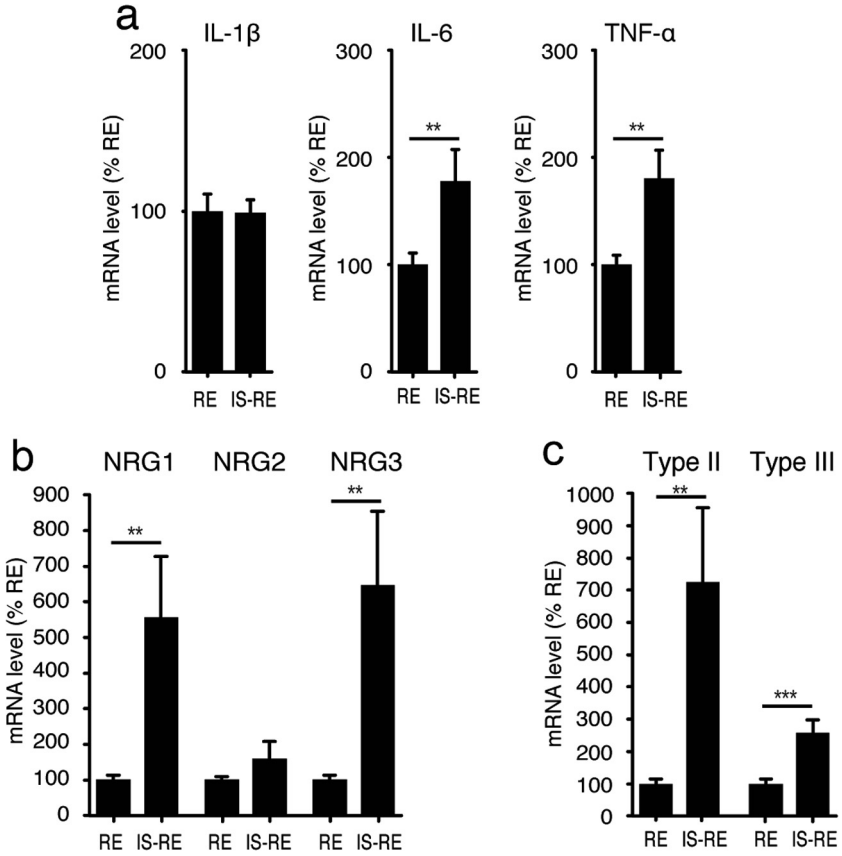

Fig. 3. Cytokine and NRG expression in the microglia of mice after juvenile social isolation (n = 12 in RE mice group, n = 11 in IS-RE mice group). (a) *IL-1β* mRNA expression was identical between RE and IS-RE mice. *IL-6* and *TNF-α* mRNA expression was significantly increased in the microglia of IS-RE mice compared to RE mice (*IL-6*, $p < 0.01$; *TNF-α*, $p < 0.01$). (b) Levels of *NRG1* and *NRG3*, but not *NRG2*, mRNA were significantly higher in the microglia of IS-RE mice than in RE mice (*NRG1*, $p < 0.01$; *NRG3*, $p < 0.01$; *NRG2*, $p > 0.05$). (c) Among *NRG1* splicing variants, the mRNA of types II and III was increased in the microglia of IS-RE mice as compared to RE mice (type II, $p < 0.01$; Type III, $p < 0.001$). Type I expression was not determined. RE: Regular-Environment (four mice per a cage); IS-RE: Isolation (four mice per a cage after social isolation in juveniles); NRG: neuregulin; IL-1β: interleukin-1 beta; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha.

NRG1 type II, $p > 0.05$; *NRG1* type III, $p > 0.05$, compared to control mice; Suppl. Fig. 2b, c).

Juvenile social isolation is considered to produce, or at least modify, ASD-like, SCZ-like, and depressive behaviors in mice (Haj-Mirzaian et al., 2016; Heitzer et al., 2013; Jiang et al., 2013; Koike et al., 2009). Moreover, ASD, SCZ, and MDD traits are correlated with adverse events during the juvenile period, as compared to healthy controls (Bernet and Stein, 1999; Roberts et al., 2015; Velikonja et al., 2015). Therefore, we utilized juvenile socially isolated mice (Makinodan et al., 2012) as a model of psychiatric disorder related to the experience of traumatic events at a young age, such as neglect. As little as 2 weeks of social isolation resulted in significant elevation of pro-inflammatory cytokine and NRG expression in microglia (*IL-1β*, $p > 0.05$; *IL-6*, $p < 0.01$; *TNF-α*, $p < 0.01$; *NRG1*, $p < 0.01$; *NRG3*, $p < 0.01$; *NRG1* type II, $p < 0.01$; *NRG1* type III, $p < 0.001$, compared to RE mice; Fig. 3a–c). Levels of *NRG1* type I were not determined due to insufficient expression. These results indicate that transient social isolation disturbs pro-inflammatory cytokine and NRG expression into adulthood.

3.3. NRG1 expression in PBMCs of patients with ASD

Although human iMG cells (Ohgidani et al., 2014) also express a substantial amount of *NRG1* (Suppl. Fig. 3), it is difficult to investigate a statistically sufficient number of iMG cells from human patients. Therefore, we instead measured mRNA levels of NRGs in human PBMCs, which play crucial roles in the immune system and are broadly utilized in clinical studies. We observed that only *NRG1* type III among the splicing variants of *NRG1* (types I–VI), but not *NRG2* or *NRG3*, was expressed in human PBMCs (data not shown). We then compared the expression of *NRG1* type III in the PBMCs of patients with ASD to with ADI-R scores (domains A, B, and C). Most importantly, we observed a significant positive correlation between levels of *NRG1* type III and impairments in social

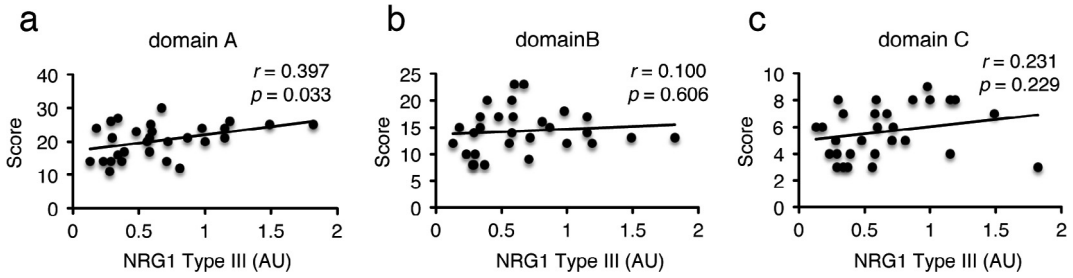

Fig. 4. Correlations between *NRG1* type III expression in PBMCs and ADI-R scores of patients with ASD. (a) Levels of *NRG1* type III mRNA expression were positively correlated with scores on domain A of the ADI-R (domain A, n = 29, $p = 0.033$, $r = 0.397$). (b, c) There were no correlations between levels of *NRG1* type III mRNA expression and scores on domain B or C of the ADI-R (domain B, n = 29, $p > 0.05$, $r = 0.100$; domain C, n = 29, $r = 0.231$). NRG: neuregulin; PBMCs: peripheral blood mononuclear cells; ADI-R: Autism Diagnostic Interview-Revised; ASD: autism spectrum disorder; AU: arbitrary unit.

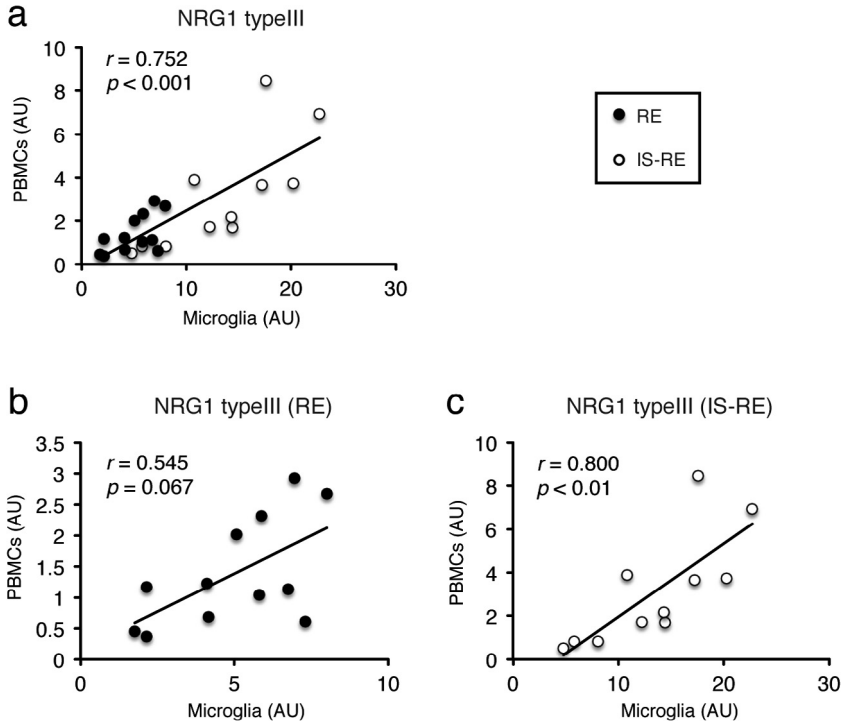

Fig. 5. Correlation between expression of *NRG1* type III mRNA in PBMCs and microglia in mice (n = 12 in RE mice group, n = 11 in IS-RE mice group). (a) There was a strong positive correlation between levels of *NRG1* type III mRNA expression in PBMCs and microglia ($p < 0.001$, $r = 0.752$). (b, c) The correlation between the two was preserved only in IS-RE mice when considered separately. RE: Regular-Environment (four mice per a cage); IS-RE: Isolation (four mice per a cage after social isolation in juveniles); NRG: neuregulin; PMBCs: peripheral blood mononuclear cells; AU: arbitrary unit.

interaction at the age of four (domain A score of ADI-R; *GAPDH*, $p < 0.033$, $r = 0.397$; Fig. 4a). However, no significant correlations were observed between *NRG1* type III levels and either impairment of communication (domain B score of ADI-R; *GAPDH*, $p > 0.05$, $r = 0.100$; Fig. 4b) or restricted, repetitive, and stereotyped patterns of behaviors and interests at the age of four (domain C score of ADI-R; *GAPDH*, $p > 0.05$, $r = 0.231$; Fig. 4c). Even when the other two internal controls were used, identical results were obtained (domain A; *ACTB*, $p = 0.012$, $r = 0.462$; *G6PD*, $p = 0.029$, $r = 0.405$: domain B; *ACTB*, $p = 0.478$, $r = 0.137$; *G6PD*, $p = 0.840$, $r = 0.039$: domain C; *ACTB*, $p = 0.312$, $r = 0.195$; *G6PD*, $p = 0.277$, $r = 0.209$; Suppl. Fig. 4a–f).

3.4. NRG1 expression in PBMCs of socially isolated mice

Accordingly, we then investigated PBMCs in RE and IS-RE mice. PBMCs and microglia were separated from identical mice. Consistent with the results of microglial analysis, only 2 weeks of social isolation resulted in significant elevation of pro-inflammatory cytokine and NRG expression in PBMCs (*IL-1β*, $p > 0.05$; *TNF-α*, $p < 0.05$; *NRG1*, $p < 0.01$; *NRG3*, $p < 0.01$; *NRG1* type II, $p < 0.01$; *NRG1* type III, $p < 0.05$, compared to RE mice; Suppl. Fig. 5a–c). *IL-6*, *NRG2* and *NRG1* type I was not determined due to low or absent expression.

3.5. The positive correlations of NRGs expression between PBMCs and microglia

We demonstrated a correlation between impairments in social interaction and levels of *NRG1* type III in the PBMCs of patients with ASD. Although there is a missing link between central and peripheral immune cells, it is impossible to obtain microglia from patients with ASD. Hence, we investigated the correlation between levels of *NRG1* type III in the PBMC and microglia of RE mice and IS-RE mice. Fig. 5a depicts the strong positive correlation observed between the level of *NRG1* type III mRNA expression in PBMCs and that in microglia ($p < 0.001$, $r = 0.752$). Strong correlations were also observed with regard to *NRG1*, *NRG3*, and *NRG1* type II mRNA expression in PBMCs and microglia (Suppl. Fig. 6a, d, g). If divided to RE and IS-RE mice, these correlations were preserved only in IS-RE mice (*NRG1* type III, $p < 0.01$, $r = 0.800$; Fig. 5b, c; *NRG1*, $p < 0.001$, $r = 0.873$; *NRG3*, $p < 0.01$, $r = 0.845$; Suppl. Fig. 6b,c,e,f), except for *NRG1* type II (Suppl. Fig. 6h,i), indicating that only stress-induced NRG expressions is correlated between PBMCs and microglia.

4. Discussion

In the present study, we observed for the first time that murine microglia in the CNS substantially express NRGs. In contrast to neurons or astrocytes, microglia can rapidly move in the CNS parenchyma (Ohsawa and Kohsaka, 2011) and likely enhance the efficiency and influence of NRGs on development by physically approaching, and even attaching to, local sites on neuronal soma, synapses, and oligodendrocytes (Corfas et al., 2004; Mei and Xiong, 2008; Ohsawa and Kohsaka, 2011). Whereas a recent PET study using a novel second-generation ligand showed no difference in microglial states between healthy controls and patients with schizophrenia (Hafizi et al., 2016), a number of studies have also implicated microglial function in neuropsychiatric disorders such as ASD, SCZ, and MDD (Steiner et al., 2008; Suzuki et al., 2013). Although other glial cells such as astrocytes have gained recent attention in the field of psychiatry (Moraga-Amaro et al., 2014; Yamamuro et al., 2015), we focused on microglial states in the brains of three animal models of psychiatric disorders and observed that the expression of cytokines and NRGs in microglia of these animals was significantly different from that of controls, except in MIA mice. BTBR mice and IS-RE mice were chosen as genetic and environmental models of ASD, respectively. MIA mice were examined as a model of ASD based on our expectation that microglia would be highly affected because the disturbance of immune system function is a primary feature of this model.

Liu et al. reported that NRG1 expression depends on neuronal activity (Liu et al., 2011), and further research indicates that postnatal social experience decreases NRG1 expression in the murine medial prefrontal cortex (mPFC) (Makinodan et al., 2012). Together, these findings suggest that NRG1 expression is activity-dependent and can be affected by postnatal social experiences. Thus, psychosocial factors as well as genomic sequences should be taken into consideration when investigating NRG1 expression. Interestingly, in the current study, we purified microglia and observed that social experience increased NRG expression in microglia, but that there was no difference in NRG expression in the brain as a whole between RE mice and IS-RE mice (data not shown). These results may be due to the effects of re-socialization. Microglia-derived NRG1 may compensate for reductions in neuronal or astrocytic NRG1 expression in the mPFC following social isolation. These results suggest that cell-type specific evaluation may yield insights into the role of NRGs in psychiatric disorders compared to conventional methods using whole-brain tissue.

Previous research has reported that transient exposure of neonatal mice to NRG1 induces hyperdopaminergic states associated with the pathophysiology of SCZ (Kato et al., 2010), congruent with the microglial NRG1 overexpression observed in neonatal BTBR mice in the present study. Other reports have indicated that overexpression of NRG1 in mice leads to SCZ-like symptoms and synaptic dysfunction (Luo et al., 2014; Yin et al., 2013). Along with that of NRG1, NRG3 overexpression in the early postnatal period, as shown in BTBR mice, leads to an anxiogenic-like phenotype and deficits in social behaviors, similar to those observed in patients with ASD (Paterson and Law, 2014). NRG3 is also abundantly expressed in the CNS (Carteron et al., 2006), and multiple lines of evidence have implicated a role for both NRG1 and NRG3 in psychiatric disorders (Mei and Nave, 2014). For example, a specific *NRG3* genotype is related to SCZ (Faraone et al., 2006; Kao et al., 2010; Xu et al., 2009) as well as prefrontal cortex dysfunction and delusions (Loos et al., 2014; Morar et al., 2011; Tost et al., 2014). Given that microglia can rapidly migrate and approach target brain regions with high concentrations of NRG3, an elevation of NRG3 expression in the microglia of BTBR mice could have strong effects on behavioral phenotypes. These findings indicate that a genetic mouse model of ASD (BTBR mice) exhibits high levels of NRG expression in microglia, which may be associated with the pathology of ASD.

Research has indicated that MIA mice exhibit abnormal behaviors, such as those observed in patients with ASD and SCZ (Schwartzer et al., 2013; Shi et al., 2003). Therefore, we hypothesized that microglia is activated and that NRG expression is increased in MIA mice, given that the primary pathogenic mechanism in these mice is immune-related. However, levels of gene expression in microglia remained unaltered in poly-IC mice, suggesting that NRG expression in microglia is not implicated in the behavioral changes observed in poly-IC mice. While both BTBR and poly-IC mice exhibit abnormal ultrasonic vocalization around P10 (Scattoni et al., 2008; Schwartzer et al., 2013), NRG expression in MIA microglia is identical to that in controls at P8, and BTBR mice express higher NRG levels in microglia than do controls. This result indicates that altered NRG expression in microglia is not necessary for abnormal ultrasonic vocalization around P8. Consistent with our poly-IC mice data, Giovanoli and colleagues

demonstrated that MIA did not influence microglial status under non-stressful conditions in peripuberty (Giovanoli et al., 2016a, b). These studies suggest that peripubertal stress in addition to MIA induces microglial activation and behavioral alterations. Furthermore, it is possible that MIA-induced microglial activation becomes apparent earlier than P8 (Matcovitch-Natan et al., 2016). Therefore, in order to elucidate the effects of microglial NRG expression on the behavior of MIA mice, further experiments with additional peripubertal stressors involving mice of various developmental stages are required.

Children who experience abuse appear to be vulnerable to developing a variety of psychiatric disorders (Putnam et al., 2013), and research has indicated that inflammation is a long-lasting biomarker of adverse events during the juvenile period, and that IL-6 and C-reactive protein (CRP) may be stable biomarkers for adults independent of the current severity of psychiatric symptoms (Danese et al., 2008, 2007). Based on these findings, we hypothesized that juvenile socially isolated mice would have long-lasting microglial activation and elevated microglial NRGs expression. Intriguingly, only 2 weeks of social isolation produced microglial activation and an increase in NRGs expression in these cells, which persisted even into adulthood. These findings are consistent with those of epidemiological studies concerning child maltreatment (Nemeroff, 2016) and indicate that microglial NRG expression is also elevated in an environmental mouse model of ASD. Further investigation may reveal that NRGs expression in microglia or immune cells can serve as a biomarker and a target molecule for childhood maltreatment.

While research has revealed that genetic factors, embryonic environments, and juvenile social experiences are related to the development of psychiatric disorders, the results of the present study indicate that microglial status and NRGs expression may be more subject to genetic background (BTBR mice) and social experience influences during the juvenile period (socially-isolated mice) than inflammation in embryonic brains (poly-IC mice). Since ASD displays significant heterogeneity, an increase in microglia-derived NRG expression may be associated with a certain subtype of ASD, and differing profile of NRG expression may account for the heterogenous pathophysiology of ASD. Further studies using other animal models are needed to elucidate the implication of microglia-derived NRG in the pathobiology of ASD. Mice with a paternal duplication of 15q11-13 (Nakatani et al., 2009) and neuroligin-3 deficient mice (Tabuchi et al., 2007) may be useful models because both mouse lines exhibit similar phenotypes of synaptic formation to BTBR mice (Isshiki et al., 2014).

Several postmortem studies have reported microglial activation and aberrant expression of cytokines—such as IL-1β, IL-6, and TNF-α—in the brains of patients with ASD, SCZ, and MDD (Morgan et al., 2010; Radewicz et al., 2000; Vargas et al., 2005). Further, by utilizing a radiotracer for activated microglia, PET analyses have also confirmed microglial activation in the brains of patients with ASD and SCZ (Suzuki et al., 2013; van Berckel et al., 2008). Considering that activated microglia express higher levels of NRGs than non-activated microglia, it is plausible that microglial NRGs expression in the brains of patients with ASD and SCZ may be elevated compared to typically developing individuals.

Finally, in order to translate our findings in animal models to humans, we measured cytokine and NRG mRNA levels in the PBMCs of patients with ASD. PBMCs are accessible and feasible resource in humans and have been employed for identifying biomarkers for ASD (Napoli et al., 2013; Onore et al., 2009). A few studies have investigated *NRG1* expression in lymphocytes (Chagnon et al., 2008; Yamamori et al., 2011) and in PBMCs (Belzeaux et al., 2010); however, these studies have not evaluated *NRG1* splicing variants. In the current study, we examined the major *NRG1* splicing variants (type I VI) and observed that only *NRG1* type III was substantially expressed in human PBMCs. Intriguingly, there was also a positive correlation between *NRG1* type III levels and scores on domain A of the ADI-R, indicating that patients with ASD who had higher levels of *NRG1* type III exhibited greater impairments in social interaction during childhood. While these findings suggest that the expression of NRGs in microglia may be involved in the pathophysiology of psychiatric disorders such as ASD, thereby potentially reflecting the influence of social interaction during the juvenile period, it is possible that NRG expression in microglia is unrelated to ASD-like behaviors. Thus, levels of NRG expression may have been only coincidentally elevated in the animal models of ASD utilized in the present study. However, as we did not employ behavioral measures to evaluate the potential causal relationship between NRG expression in microglia and ASD-like symptoms, further studies utilizing such behavioral assessments are warranted.

Acknowledgments

We would like to thank all the participants and families for their time and efforts as well as Mr. Michael Angland for critical reading of the manuscript. This work was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (15K19743, 15K15432), the Uehara Memorial Foundation, the Osaka Medical Research Foundation for Intractable Disease, the Naito Foundation, and the Takeda Science Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2017.01.003.

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