Microglial ASD-related genes are involved in oligodendrocyte differentiation

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Autism spectrum disorders (ASD) are associated with mutations of chromodomain-helicase DNA-binding protein 8 (Chd8) and tuberous sclerosis complex 2 (Tsc2). Although these ASD-related genes are detected in glial cells such as microglia, the effect of Chd8 or Tsc2 deficiency on microglial functions and microglia-mediated brain development remains unclear. In this study, we investigated the role of microglial Chd8 and Tsc2 in cytokine expression, phagocytosis activity, and neuro/gliogenesis from neural stem cells (NSCs) in vitro. Chd8 or Tsc2 knockdown in microglia reduced insulin-like growth factor-1 (Igf1) expression under lipopolysaccharide (LPS) stimulation. In addition, phagocytosis activity was inhibited by Tsc2 deficiency, microglia-mediated oligodendrocyte development was inhibited, in particular, the differentiation of oligodendrocyte precursor cells to oligodendrocytes was prevented by Chd8 or Tsc2 deficiency. These results suggest that ASD-related gene expression in microglia is involved in oligodendrocyte differentiation, which may contribute to the white matter pathology relating to ASD.

Autism spectrum disorders (ASDs) are highly inherent neurodevelopmental disorders characterized by repetitive behaviors, restricted interest, and deficits in social interaction1. It has been reported that a large number of neurons, excessive synapses, and myelin abnormalities are observed in the brain of patients with ASD, indicating that developmental anatomical abnormalities are considered to be associated with ASD pathology2–4.

ASD is known to be closely associated with genetic variation. Recent studies have identified several genes that are frequently mutated in human ASD and have the potential to regulate the expression of other ASD-risk genes. For example, chromodomain-helicase DNA-binding protein 8 (Chd8), which is an ATP-dependent chromatin remodeling protein that controls epigenetic and transcription regulation5, represents one of the most high-risk susceptibility genes in ASD5,6. Missense, nonsense, and frameshift mutations have all been found in Chd8 alleles, and several missense mutations in Chd8 confer loss-of-function7. This is supported by the fact that Chd8-haploinsufficient mice showed behavioral abnormalities associated with ASD. In addition, Chd8 deficiency in oligodendrocytes leads to impaired oligodendrocyte development and hypomyelination8,9. Other genes related to ASD, such as Tsc2 (also known as Tuberin), which is a negative regulator of the mammalian target of rapamycin (mTOR) signaling by dephosphorylating Rheb, a G-protein which activates mTOR complex 1 (mTORC1)10. Tuberous sclerosis complex (TSC) is a genetic disease characterized by benign tumors, epilepsy, ASD, and loss of function of Tsc2 due to missense mutations in TSC patients11. Tsc2 haplodeficient mice also showed neurological deficits, including ASD-like behaviors, and Tsc2 conditional knockout mice showed hypomyelination in oligodendrocyte lineage cells12,13. Thus, loss of function of both Chd8 and Tsc2 are considered to be associated with ASD pathology and myelin abnormalities. Although the recent findings about the function of ASD-related genes have been mainly interpreted by neuronal changes, the role of ASD-related genes in glial cells that also express Chd8 and Tsc2 remains unclear.

Microglia are central nervous system (CNS) resident immune cells that play crucial roles in CNS homeostasis14. Microglia also contribute to brain development e.g., development of neurons and oligodendrocytes from the subventricular zone15; myelin formation in the neonatal brain16, and regulation of the number of neural precursor cells (NPCs) by promoting cell proliferation and/or engulfing cells17,18. In addition, microglia...
Results

Chd8 or Tsc2 was effectively down-regulated by siRNA treatment in primary microglia. To examine the role of ASD-related genes in microglia, we established gene knockdown experiments in primary microglia by siRNA treatment. Initially, we transduced fluorescent-labeled siRNA to primary microglia to determine the efficiency of siRNA transduction and measured the ratio of fluorescent-labeled microglia 94.7% of microglia represented red fluorescence. (a) Representative images show the gating strategy of flow cytometry analysis. Primary microglia were stained with DAPI and observed under fluorescent microscopy (n = 3, biologically independent experiments, F (3,8) = 1.005, df = 8, p = 0.4390 for main effect of group assessed by one-way ANOVA). (c,d) Chd8 (e) and Tsc2 (d) mRNA expression levels were analyzed by quantitative RT-PCR in primary microglia treated with control siRNA, Chd8 siRNA and Tsc2 siRNA. Expression levels of Chd8 and Tsc2 were normalized to that of Gapdh (n = 6, p < 0.01 assessed by the one-way ANOVA, followed by Tukey–Kramer tests). (e) Gapdh expression levels were analyzed by quantitative RT-PCR by normalizing to Actb (n = 4, biologically independent experiments, F (2,9) = 0.2393, df = 9, p = 0.7920 for main effect of group, p = 0.7789 for control siRNA vs. Chd8 siRNA, p = 0.9012 for control siRNA vs. Tsc2 siRNA assessed by the one-way ANOVA, followed by Tukey–Kramer tests). (f-i) mRNA expression levels of Tnf (f, n = 5, biologically independent experiments, F (3,16) = 27.19, df = 16, p < 0.001 for main effect of group, p < 0.001 for w/o LPS vs. control siRNA, p > 0.9999 for control siRNA vs. Chd8 siRNA, p = 0.5328 for control siRNA vs. Tsc2 siRNA), Il1b (g, n = 5, biologically independent experiments, F (3,16) = 108, df = 16, p < 0.001 for main effect of group, p < 0.001 for w/o LPS vs. control siRNA, p = 0.3573 for control siRNA vs. Chd8 siRNA, p = 0.8292 for control siRNA vs. Tsc2 siRNA), Spp1 (h, n = 4, biologically independent experiments, F (3,12) = 22.36, df = 12, p < 0.001 for main effect of group, p < 0.001 for w/o LPS vs. control siRNA, p = 0.9558 for control siRNA vs. Chd8 siRNA, p = 0.9880 for control siRNA vs. Tsc2 siRNA), and Iglf (i; n = 5, biologically independent experiments, F (3,16) = 47.97, df = 16, p < 0.001 for main effect of group, p < 0.001 for w/o LPS vs. control siRNA, p = 0.0232 for control siRNA vs. Chd8 siRNA, p = 0.0491 for control siRNA vs. Tsc2 siRNA) in microglia treated with control siRNA, Chd8 siRNA and Tsc2 siRNA were analyzed with quantitative RT-PCR. Statistical analysis was performed by the one-way ANOVA, followed by Tukey–Kramer tests. NS: not significant. *p < 0.05, **p < 0.001. Error bars represent mean ± SEM.

Knockdown of Tsc2 affected phagocytic activity. Microglia are involved in brain development by engulfing excessive neurons or synapses during brain development16,20. To examine whether the deficiency of Chd8 and Tsc2 in microglia affects phagocytosis activity, we performed phagocytosis assays using fluorescent-labeled latex beads. Quantification of the number of fluorescently-labeled beads in Isoclin B4-labeled micro-
glia indicated a low number of fluorescent-labeled beads in Tsc2 siRNA-treated microglia compared with control (Fig. 2a,b), whereas microglia treated with Chd8 siRNA did not change. These results indicate that Tsc2, but not Chd8, plays a role in phagocytosis activity in primary microglia.

Deficiency of Chd8 or Tsc2 in microglia led to the impairment of oligodendrocyte development. Microglia promote the differentiation of neurons and oligodendrocytes from NSCs during brain development. Next, we examined whether deficiency of Chd8 or Tsc2 affects the microglia-mediated differentiation of neurons and oligodendrocytes. NSCs were prepared by dissociating neurospheres, and co-cultured with siRNA-treated microglia across the transwell membranes (Fig. 3a). After 7 days, we counted the number of βIII-Tubulin (Tuj1)+ neurons, glial fibrillary acidic protein (GFAP)+ astrocytes, platelet-derived growth factor receptor α (PDGFRα)+ oligodendrocyte precursor cells (OPC), and myelin basic protein (MBP)+ oligodendrocytes by immunocytochemistry. The number of neurons, OPC, and oligodendrocytes, but not astrocytes, significantly increased in the presence of microglia (Fig. 3b–e) consistent with the previous reports. In addition, the number of MBP+ oligodendrocytes significantly decreased in the presence of Chd8 or Tsc2 siRNA-treated microglia compared with control siRNA-treated microglia (Fig. 3e). No change was detected in the number of neurons and OPC in the presence of Chd8 and Tsc2 siRNA-treated microglia (Fig. 3b,d). These results suggest that microglia require Chd8 or Tsc2 to promote the oligodendrocyte differentiation.

As OPCs proliferate and differentiate into oligodendrocytes, we asked whether Chd8 or Tsc2 in microglia is involved in OPC proliferation. To this end, we co-cultured OPCs with microglia treated with siRNA and performed immunocytochemistry for Ki67 (proliferation marker), PDGFRα, and oligodendrocyte transcription factor 2 (Olig2) after 3 days (Fig. 3f). We counted the number of Ki67+ proliferating cells in PDGFRα and Olig2 double-positive OPCs, and we found that Chd8 or Tsc2 deficiency in microglia did not affect the percentage of Ki67+/PDGFRα+ Olig2+ proliferating OPCs (Fig. 3g,h). There was no significant difference about total number of Olig2- cells between the groups (Fig. 3i). To determine whether Chd8 or Tsc2 deficiency in microglia affects oligodendrocyte differentiation, we co-cultured siRNA-treated microglia and NSCs for 7 days and counted the number of MBP+/Olig2+ oligodendrocyte-lineage cells. The percentage of MBP+/Olig2+ cells was lower in the presence of Chd8 or Tsc2 siRNA-treated microglia than in control siRNA-treated microglia (Fig. 3j,k). These results suggest that microglia promote the differentiation from OPC to oligodendrocyte in a Chd8- or Tsc2-dependent manner, with no effect on OPC proliferation.

Chd8 or Tsc2 knockdown by shRNA in microglia reduced oligodendrocyte differentiation. To assess the sustainable effects of ASD-related genes on microglia, we used lentivirus encoding short hairpin RNA (shRNA) against Chd8 or Tsc2 (Fig. 4a). We confirmed the reduction of Chd8 and Tsc2 expression by quantitative RT-PCR after 7 days of infection (Fig. 4b,c). We then prepared NSCs obtained from neurospheres prepared...
from the P1 subventricular zone (SVZ), where oligodendrocytes are generated\(^\text{2,3}\) and developed during the neonatal period. We cultured lentivirus-infected microglia co-cultured with NSCs from the SVZ and counted the number of MBP\(^\text{+}\) oligodendrocytes in DAPI\(^\text{+}\) cells. The number of MBP\(^\text{+}\) oligodendrocytes was reduced in the presence of microglia infected with lentivirus encoding either Chd8 or Tsc2 shRNA compared with microglia infected with scramble shRNA (Fig. 4d,e). Moreover, we stained with myelin-associated glycoprotein (MAG), which starts to be expressed before MBP in differentiating cultures. The number of MAG\(^\text{+}\) cells was also reduced in the presence of microglia infected with lentivirus encoding either Chd8 or Tsc2 shRNA (Fig. 4f,g). These results support our finding that Chd8 and Tsc2 play a role in microglia-mediated oligodendrocyte differentiation.

Oligodendrocyte differentiation was also regulated by other neural cells.\(^\text{44}\) Therefore, it is possible to suppose that Chd8 or Tsc2 regulates microglia-mediated oligodendrocyte differentiation through other cells. To determine the possibility, we observed the number of neurons, astrocytes, NPCs, and OPC after co-culturing cells in PDGFR\(\alpha\)+ OPC was increased in the presence of microglia, Chd8 or Tsc2 knockdown in microglia did not affect the number of OPC, and Ki67\(^\text{+}\) proliferating OPC compared with microglia infected with scramble shRNA- coding lentivirus (Fig. 4d,e). Moreover, we stained with myelin-associated glycoprotein (MAG), which starts to be expressed before MBP in differentiating cultures. The number of MAG\(^\text{+}\) cells was also reduced in the presence of microglia infected with lentivirus encoding either Chd8 or Tsc2 shRNA (Fig. 4f,g). These results support our finding that Chd8 and Tsc2 play a role in microglia-mediated oligodendrocyte differentiation.

**Chd8 or Tsc2 regulates numerous gene expression in microglia.** To investigate the molecular profiles of microglia with Chd8 or Tsc2 deficiency, we performed RNA sequencing (RNA-seq) in primary microglia treated with control, Chd8, or Tsc2 siRNA after LPS stimulation for three days. Principal component analysis (PCA) showed the difference of transcriptome among control siRNA, Chd8 siRNA, and Tsc2 siRNA treatment microglia (Fig. 5a). We analyzed differentially expressed genes (DEGs) using volcano plots and found that the expression of 83 genes was significantly reduced and 89 genes were increased in Chd8 siRNA-treated microglia (Fig. 5b,d). Alternatively, the expression of 82 genes was significantly reduced, and 69 genes were increased in Tsc2 siRNA-treated microglia (Fig. 5c,e). To investigate the properties of DEGs, we classified 172 genes (for vs. Chd8 siRNA) and 151 genes (vs. Tsc2 siRNA) by Gene Ontology (GO) analysis of the biological process. Numerous GO terms such as "Cellular localization", "Cellular response to stress", "Protein localization", "Chromatin organization", "Organelle localization", and "Protein modification process" were rich in DEGs of Chd8 siRNA (Fig. 5f). Alternatively, GO terms "Cellular component biogenesis", "Regulation of signal transduction", "Cellular response to stress", "Protein biogenesis", "Demethylation", and "Cell motility" were rich in DEGs of Tsc2 siRNA (Fig. 5g). From these results, both Chd8 and Tsc2 regulate numerous biological processes, especially protein synthesis and localization. We also confirmed the reduction of Tsc2 or Chd8 expression in Tsc2 siRNA- or Chd8 siRNA treated microglia, respectively (Fig. 5h). Moreover, Igf1 expression was also reduced in Chd8 or Tsc2 siRNA treatment (Fig. 5i). These results suggest that either Chd8 or Tsc2 contributes to the expression of genes related to numerous biological processes including Igf1.

Tsc2 deficiency impairs phagocytosis activity in microglia. To determine whether Tsc2 regulates the expression of phagocytosis-related genes, we analyzed the expression of Itgam, Becn1, Itgb2, and Mfge8, which are annotated with phagocytosis engulfment. However, the expression of these genes was not significantly different under Tsc2 siRNA treatment, suggesting that impairment of phagocytosis activity during Tsc2 deficiency was not due to changes in the expression of phagocytosis-related genes (Fig. 5i). Microglia play a role in the engulfment of excessive myelin and synapses.\(^\text{35}\) We also analyzed the expression of synapse- or myelin recognition-related genes, such as Msr1, Marco, Cd36, and Trem2. However, there were no significant differences between the groups (Fig. 5j). These results indicate that Tsc2-regulated phagocytosis activity is independent of the expression change of genes that are well-established for phagocytosis recognition.

**Chd8 or Tsc2 deficiency in microglia impaired oligodendrogensis in vivo.** To examine the effect of Chd8 or Tsc2 deficiency in microglia on oligodendrocyte differentiation in vivo, we used AAV6, which has triply mutated capsid variants (mAAV6)\(^\text{36}\) to reduce Chd8 or Tsc2 expression in microglia. We integrated enhanced green fluorescent protein (EGFP) and miR-30-based shRNA into the AAV construct under the CD68 promoter to enhance specificity for microglia (Fig. 6a). At E14, we injected mAAV6 into the embryo lateral ventricle, and performed histological analysis at P12. We confirmed EGFP fluorescence in ionized calcium binding adapter protein 1 (Iba1)\(^\text{+}\) microglia in the corpus callosum (Fig. 6b). Additionally, we purified EGFP\(^\text{+}\) microglia from P12 brain and found that the expression levels of Chd8, Tsc2, and Igf1 in microglia were reduced in Chd8 or Tsc2 siRNA treatment, which was consistent with in vitro studies (Fig. 6c).

To label oligodendrocytes sparsely by immunohistochemistry, we used CC1 monoclonal antibody that recognizes adenosomatous polyposis coli (APC). As CC1 monoclonal antibody also recognizes Quaking 7 protein, which is expressed in oligodendrocytes and some astrocytes, we defined oligodendrocytes as CC1 and Sox10 (specific marker of oligodendrocyte-lineage cells) double-positive cells. We counted the number of CC1\(^\text{+}\) Sox10\(^\text{+}\) oligodendrocytes and found that a low number of CC1\(^\text{+}\) Sox10\(^\text{+}\) oligodendrocytes were detected in the corpus callosum (Fig. 6d,e), anterior commissure (Fig. 6f,g), and striatum (Fig. 6h,i) of mice treated with Chd8 or Tsc2 shRNA compared with control (scramble shRNA treated) mice. To examine the possibility that the reduction of oligodendrocyte number by Chd8 or Tsc2 shRNA was due to the oligodendrocyte death, we observed Cleaved Caspase-3\(^\text{+}\) Olig2\(^\text{+}\) double positive cells (apoptotic oligodendrocyte-lineage cells) in the Chd8 or Tsc2 shRNA-treated brain. However, there were few Cleaved Caspase-3\(^\text{+}\) Olig2\(^\text{+}\) double positive cells even in Chd8 or Tsc2 KO or Tsc2 KO.
Microglia are key players in causing structural abnormalities in the brain during development, and they play a key role in the pathogenesis of neuronal disorders. Regarding the involvement of ASD pathology, microglia engulf synapses of neurons undergoing synapse formation during brain development37,38, and this developmental microglial dysfunction causes abnormal behavioral phenotype such as social interaction deficits39,40. Considering the presence of ASD-related gene expression in microglia, in this study, we asked whether microglial ASD-associated gene expression is autonomously involved in microglial functions of the cell.

Discussion

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We found that Tsc2 deficiency impaired phagocytosis activity in primary microglia. Tsc2 forms a TSC complex with Tsc1, which inhibits mTORC1 activity41. Knocking out microglial Tsc1 increases phagocytosis activity42. Consistent with this finding, mTOR deletion in microglia led to the impairment of phagocytosis both in vitro and in vivo43. In contrast, Tsc2−/− mice showed impaired autophagic activity44, and our study showed Tsc2-deficient microglia had reduced phagocytosis activity. This discrepancy may be explained by the difference in signal transduction between Tsc1 and Tsc2. One of the well-established differences between Tsc1 and Tsc2 is the function of Tsc1 in Smad2/3 phosphorylation in the presence of TGF-β, which controls epithelial-to-mesenchymal transition45. However, in microglia, TGF-β and Smad3 activation increases phagocytosis activity46; therefore, TSC-mediated intracellular signaling may have cell-type specific regulation. Further studies on the intracellular mechanism will contribute to the understanding of Tsc2-mediated intracellular signaling that regulates phagocytosis.

In this study, we found a decrease in the number of oligodendrocytes generated from NSCs or OPCs co-cultured with Chd8- or Tsc2-deficient microglia. However, we did detect that Tsc2-deficient microglia showed altered phagocytosis activity. As we used transwells for co-culture experiments, the decrease in the number of
oligodendrocytes that we observed does not depend on the phagocytic function of microglia in vitro. However, we should note that the recent study showed that microglia regulate myelination by engulfment of OPC in developing brain. It is generally accepted that microglia eliminate synapses and myelin during brain development in a cell–cell contact-dependent manner. Molecules involved in phagocytosis of synapses and myelin by microglia include complement receptor-3 (Itgam), scavenger receptor (Msr1, Marco, Cd36), and Trem2. Although our RNAseq analysis showed that Chd8 or Tsc2 deficiency did not affect the expression of the well-established genes which is associated with the phagocytosis of synapses and myelin, there is still the possibility that ASD-related microglia contribute to phagocytosis of synapses and myelin because the expression of some of the above molecules is dramatically regulated by several conditions (such as developmental regulation of Trem2).

Previous studies have demonstrated that myelin dysfunction is observed in ASD patients, and genetic analysis showed that myelin development-related genes were associated with ASD. Therefore, dysfunctional myelin development may be key to understanding ASD pathology. Regarding Chd8 and Tsc2, oligodendrocyte-specific loss of function experiments showed the impairment of oligodendrocyte differentiation and causes hypomyelination through the epigenetic modulation of gene expression, which is involved in oligodendrocyte proliferation and myelination. In this study, we focused on the expression of ASD-related genes in microglia and revealed a novel function of ASD-related genes in myelin pathology. However, we should note that the expression of ASD-related genes is not limited to the brain, but is also detected systemically. Recently, we found that changes in systemic environments control myelin development directly via circulating factors, providing more opportunities to understand the contribution of ASD-related genes to neuropathology.

Materials and methods

Animals. Pregnant mice at embryonic day (E) 14 and E18 of C57BL/6J mice were purchased from SLC Japan. All experiments procedures were approved by the Animal Care Committee of National Center of Neurology and Psychiatry (2018034R9). The mice were housed in an air-conditioned room at 23 ± 1 °C with a 12-h light–dark cycle under specific pathogen-free conditions and had free access to water and food. The mice were randomly assigned to groups. All experiments were conducted according to the relevant guidelines and regulations including Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines.

Microglia and OPC primary culture. Whole brains were dissected from E18 mice, and incubated with 0.25% trypsin (Gibco) in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) for 15 min at 37 °C, followed by treatment with DNase I (Sigma Aldrich) for 1 min at 37 °C. Cell suspension was washed by DMEM containing 10% fetal bovine serum (FBS) and centrifuged at 450×g for 10 min. The isolated cells were resuspended with...
DMEM containing 10% FBS, and filtered with 70 μm nylon cell strainer. Cells were plated on poly-l-lysine pre-coated 75 cm² tissue culture flask (IWAKI) in DMEM supplemented with 10% FBS and penicillin–streptomycin (Thermo Fisher Scientific). After 12–15 days, culture flasks were gently shaking for 30 min, and supernatant was centrifuged at 1500 rpm for 10 min. The cell pellet was resuspended with microglia culture medium supplemented with 50% of the supernatant, 10% FBS in DMEM, and plated to 96 well plate. Each biological replicate represents a culture prepared from a distinct mouse brain. All experiments were repeated at least three times.

For primary OPCs, mixed glial cells with removed microglia were detached from the culture flask by treatment with 0.05% trypsin for 5 min and replated to fresh culture flasks. After 30 min of incubation, the culture medium was centrifuged at 1500 rpm for 10 min. The cell pellet was resuspended with microglia culture medium supplemented with 50% of the supernatant, 10% FBS in DMEM, and plated to 96 well plate. Each biological replicate represents a culture prepared from a distinct mouse brain. All experiments were repeated at least three times.

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siRNA treatment for microglia. Primary microglia were transduced with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Briefly, 0.5 pmol of siRNA for BLOCK-it Alexa Fluor Red Fluorescent control (14750100: Thermo Fisher Scientific), negative control (4390843: Thermo Fisher Scientific), Chd8 (4390771: Thermo Fisher Scientific), and Tsc2 (AM16708: Thermo Fisher Scientific) were diluted with OPTI-MEM (Invitrogen), and mixed with 0.15 μL of RNAiMAX Reagent, which were diluted with OPTI-MEM. Complex was added to primary microglia plated on 96 well plate. After 4 h, culture medium was changed to new microglia culture medium.

Lentivirus production. The scramble shRNA plasmid was a gift from David Sabatini (Addgene plasmid #1864, http://www.addgene.org/1864/; RRID:Addgene_1864). To generate the shRNA construct, the scramble sequence was digested with EcoRI and AgeI, and shRNA sequences against Chd8 and Tsc2 were inserted with the DNA Ligation Kit Ver 2.1 (Takara). The shRNA target sequences of Chd8 and Tsc2 were TCGGGAGCC TTGCCATATTAT and CCTCTCTCTTAAAACTTATA, respectively. pLenti, pCAG-HIVgp, and p-CMV-VSV-G plasmids were transfected into Lenti-X 293 T cells (Takara) with polyethyleneimine (PEI) Max (Polysciences). After 3 days of transfection, the supernatant was centrifuged at 1000×g for 10 min, and filtered with 0.45 μm filter. Then, the supernatant was centrifuged at 50,000×g for 2 h (SW28, Beckman). The pellet was resuspended in PBS and concentrated with an Amicon Ultra-15 (Millipore), pCAG-HIVgp and p-CMV-VSV-G plasmids were generated by Dr. Hiroyuki Miyoshi and purchased from RIKEN Bioresource Research Center.

AAV injection of microglia. pAAV plasmids encoding EGFP and miR-30-based siRNA under the CD68 promoter were prepared by VectorBuilder. The shRNA sequences were as follows: scramble shRNA: ccttaagg-taatacgactcactatAGTTGTAGCTCAATTCG; Chd8 shRNA: atgaacgtattgattggcagagttagtaagctggagca-gtattgtgcaagtgtcgg; Tsc2 shRNA: ctgacgacagaactgtcagtcagtcagcttcgcgct; pHelper, and modified AAV6 capsids were transfected to AAV293 cells (Takara) by PEI Max. After 3 days, AAV was purified with an AAVpro Purification kit (Takara) and concentrated with an Amicon Ultra-15 (Millipore). To inject AAV into the mouse brain, E14 pregnant mice were anesthetized with isoflurane (3%), and 1 μL of AAV containing 0.01% fast green was injected into the lateral ventricle of the embryonic mouse brain, and P12 neonatal mice were analyzed.

Flow cytometry. Primary microglia, which were transduced fluorescent-labeled siRNA, were treated with APC conjugated anti-CD11b (1:200; Biolegend) on ice for 30 min. After three times wash, primary microglia were carried out flow cytometry analysis by FACSCantoII fluids system (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star).

Phagocytosis assay. Three days after siRNA transduction, microglia phagocytic activities were measured with Phagocytosis Assay Kit (Cayman). Microglia were incubated for 24 h with latex beads coated with fluorescently-labeled rabbit IgG, followed by 1 min incubation with trypan blue. Microglia were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. Microglia were incubated with Isolectin B4 Conjugates (10 μg/mL; Invitrogen) in PBS containing 0.1% Triton X-100 for 2 h followed by three times PBS washing. Counterstaining were carried out with DAPI (1:2000, DOJIDO). Images were acquired using a confocal laser scanning microscope (FV3000, Olympus).

Co-culture of microglia and NSCs. For generating neurospheres, telencephalons were dissected from E14 or P0 of C57BL/6j mice and the tissue was dispersed into single cells by triturating several times. Cells were plated on 100 mm dish (CELLSTAR) at 1.0 × 10⁶ cells/dish in DMEM/Nutrient Mixture F-12 Ham medium (DMEM/F12; Sigma Aldrich) supplemented with B27 supplement (1:200; Thermo Fisher Scientific), Penicillin–Streptomycin (1:100; Thermo Fisher Scientific), Epidermal Growth Factor (EGF, 20 ng/mL; Peprotech) and basic Fibroblast Growth Factor (bFGF, 20 ng/mL; PEPROTECH) and cultured for 7 days.

For co-culturing microglia and NSCs, neurospheres were incubated with 0.25% trypsin (Gibco) in DMEM/F12 for 3 min at 37 °C, and dissociated NSCs were plated on poly-ornithine (30 μg/mL)-precoated 96 well plate (Corning) at a density of 2 × 10⁴ cells/well in DMEM/F12 supplemented with Penicillin–Streptomycin (1:100). Control siRNA, Chd8 siRNA, and Tsc2 siRNA treated microglia were activated with LPS (10 μg/mL) for 6 h and washed carefully to remove residual LPS. Microglia were not stimulated by LPS in the experiments shown in Figs. 1b,c–e, 4b,c. For lentivirus infection, microglia were cultured in the presence of lentivirus and polybrene (8 μg/mL; Nacalai Tesque) for 24 h. Culture media containing lentivirus were replaced with fresh media, and microglia were incubated for an additional 2 days. Microglia were plated on 96 well Transwell plates (pore size 0.4 μm; Corning), which were plated on NSCs at a density of 2.0 × 10⁴ cells/well. NSCs and microglia were co-cultured for 7 days, and immunocytochemistry was performed.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde (PFA; Merck) at room temperature for 30 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. Then, cells were blocked with 3% normal donkey serum (Sigma Aldrich) for 1 h at room temperature and treated with primary antibody overnight at 4 °C. Primary antibodies were used as follows; rabbit anti-βIII-Tubulin (1:2000, PRB-435P; Biolegend), rat anti-MBP (1:500, ab7349; Abcam), rabbit anti-MAG (1:500; 9043; Cell Signaling Technology), mouse anti-GFAP (1:1000, GA-5, Sigma Aldrich), rat anti-GFAP (1:500, 2.2B10; Thermo Fisher Scientific), goat anti-PDGFPrA (1:1000, AF1062; R&D Systems), rabbit anti-Ki67 (1:500, ab16667; Abcam), mouse anti-OLig2
Figure 6. Chd8 or Tsc2 deficiency in microglia inhibits oligodendrocyte development in vivo. (a) Triply mutated AAV6 particles encoding EGFP and miR-30-based shRNA under control of the CD68 promoter were intraventricularly injected to embryonic mice brain at E14. The following analyses was performed at P12. (b) Representative immunohistochemistry images for EGFP (green) and Iba1 (red) at P12. White arrowheads represent EGFP+ microglia. Scale bar: 100 μm. (c) Graph showing the mRNA expression level of Chd8, Tsc2, and Iglf1 in EGFP+ CD45+CD11b+ microglia purified from the brain of P12 neonatal mice by flow cytometry (Scramble shRNA: n = 3; Chd8 shRNA: n = 3; Tsc2 shRNA: n = 3, biologically independent experiments, Chd8: F(2, 6) = 14.66, df = 6, p = 0.0049 for the main effect of the group, p = 0.0053 for scramble shRNA vs Chd8 shRNA, Tsc2: F(2, 6) = 21.43, df = 6, p = 0.0019 for main effect of group, p = 0.0046 for scramble shRNA vs Tsc2 shRNA, Iglf1: F(2, 6) = 9.084, df = 6, p = 0.0153 for main effect of group, p = 0.0374 for scramble shRNA vs Chd8 shRNA, p = 0.0171 for scramble shRNA vs Tsc2 shRNA, assessed by one-way ANOVA, followed by Tukey–Kramer tests). (d) Representative immunohistochemical images for Sox10 (cyan), CC1 (red) and DAPI (blue) in the corpus callosum (cc). Scale bar: 100 μm. (e) Graph of the number of CC1+Sox10+ oligodendrocytes in the corpus callosum (Scramble shRNA: n = 6; Chd8 shRNA: n = 6; Tsc2 shRNA: n = 4, biologically independent experiments, F(2, 11) = 7.296, df = 12, p = 0.0122 for main effect of group, p = 0.0195 for scramble shRNA vs. Chd8 shRNA, p = 0.0329 for scramble shRNA vs Tsc2 shRNA, assessed by one-way ANOVA, followed by Tukey–Kramer tests). (f) Immunohistochemical images for Sox10 (cyan), CC1 (red) and DAPI (blue) in the anterior commissure. Scale bar: 100 μm. (g) Graph of the number of CC1+Sox10+ oligodendrocytes in the anterior commissure (Scramble shRNA: n = 5; Chd8 shRNA: n = 5; Tsc2 shRNA: n = 4, biologically independent experiments, F(2, 11) = 6.818, df = 11, p = 0.0119 for main effect of group, p = 0.0138 for scramble shRNA vs. Chd8 shRNA, p = 0.0431 for scramble shRNA vs. Tsc2 shRNA, assessed by one-way ANOVA, followed by Tukey–Kramer tests). (h) Immunohistochemical images for Sox10 (cyan), CC1 (red) and DAPI (blue) in the striatum. Scale bar: 100 μm. (i) Graph of the number of CC1+Sox10+ oligodendrocytes in the striatum (Scramble shRNA: n = 6; Chd8 shRNA: n = 5; Tsc2 shRNA: n = 4, biologically independent experiments, F(2, 11) = 7.296, df = 12, p = 0.0084 for main effect of group, p = 0.0105 for scramble shRNA vs. Chd8 shRNA, p = 0.0390 for scramble shRNA vs. Tsc2 shRNA, assessed by one-way ANOVA, followed by Tukey–Kramer tests). (j) Immunohistochemistry for GFAP (cyan) and CC1 (red) in the corpus callosum, anterior commissure, and striatum. Scale bar: 200 μm. (k) Immunohistochemistry for GFAP (cyan) in the corpus callosum (cc), anterior commissure (ac) and striatum in the brain of scramble, Chd8 and Tsc2 shRNA-treated mice. Scale bar: 100 μm. (l) Graph of the area of GFAP+ astrocytes in the corpus callosum, anterior commissure, and striatum (Scramble shRNA: n = 5; Chd8 shRNA: n = 5; Tsc2 shRNA: n = 5, biologically independent experiments, F(2, 12) = 0.9855, df = 2, p = 0.4015 for main effect of group, assessed by two-way ANOVA). (m) Immunohistochemistry for PDGFRα (red) and Sox10 (cyan) in the corpus callosum (cc), anterior commissure, and striatum. Scale bar: 100 μm. (n) Graph showing the number of PDGFRα+ Sox10+ cells in the corpus callosum (cc), anterior commissure and striatum in the brain of scramble, Chd8 and Tsc2 shRNA-treated mice (Scramble shRNA: n = 5; Chd8 shRNA: n = 5; Tsc2 shRNA: n = 5, biologically independent experiments, F(2, 12) = 0.5092, df = 2, p = 0.6134 for main effect of group, assessed by two-way ANOVA). NS not significant, *p < 0.05. **p < 0.01. Error bars represent mean ± SEM.

Immunohistochemistry. Mice were anesthetized with a mixture of medetomidine hydrochloride (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). Mice were transcardially perfused with ice-cold PBS then 4% PFA (Merck). Dissected brains were immersed in 4% PFA overnight at 4 °C, followed by 30% sucrose (pH 7.2) overnight at 4 °C. Brains were embedded in optimal cutting temperature compound (Tissue-Tek) and cut to a thickness of 30 μm on a cryostat (Leica). For immunohistochemistry, sections were treated with PBS containing 0.1% Triton X-100 for 10 min twice, and blocked with 3% normal donkey serum at room temperature for 1 h. Sections were treated with primary antibodies: rabbit anti-Iba1 (1:3000, 019-19741; Wako), rat anti-GFP (1:500, 04404-84; Nacalai Tesque), mouse CC1 monoclonal antibody (1:500, OP80; Calbiochem), rabbit anti-Sox10 (1:1000, ab155279; Abcam), rabbit anti-GFAP (1:1000, ab207165; Abcam), goat anti-PDGFRα (1:1000, AF1062; R&D Systems), goat anti-Olig2 (1:1000, AF2418; R&D Systems), rabbit anti-Cleaved Caspase-3 (1:500, 9661; Cell Signaling Technology) overnight at 4 °C. After three washes, sections were treated with secondary antibodies: Alexa Fluor 488-conjugated donkey anti-rat IgG, Alexa Fluor 568-conjugated donkey anti-rabbit IgG, Alexa Fluor 647-conjugated donkey anti-mouse IgG (all: 1:500; Thermo Fisher Scientific), and DAPI (1:2000; DOJINDO), and sections were mounted with ProLong Glass Antifade Mountant (Thermo Fisher Scientific). Images were acquired using a confocal microscope (FV3000, Olympus). GFAP area was calculated by dividing the GFAP-positive area in the region of the white matter (corpus callosum and anterior commissure) or total area (striatum).

RNA-seq. After 3 days of treatment with siRNA against primary microglia, RNA was isolated using TRIzol Reagent (Invitrogen). RNA quality was checked with an Agilent Bioanalyzer and RNA with an RNA integrity number > 8 were analyzed. Libraries were generated using Illumina standard Total RNA Prep and sequenced using Novaseq 6000 (Illumina) at a depth of 20 million 150 bp paired-end reads. Read counts and TPM were...
calculated with salmon (v0.14.1) on the mm10 mouse genome reference and Tximport package (v1.14.2) on R (v3.6.3). Normalization was performed with the DESeq2 (v 1.26.0), and DEGs were defined by a false discovery rate (FDR) < 0.05 and fold-change > 1.5. PCA was conducted by scikit-learn module (v0.22.2, post1) on Python (v3.7.1). GO analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8)55,56.

Quantitative RT-PCR. Six hours after LPS treatment, microglia RNA was extracted with TRIzol Reagent (Invitrogen) and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied biosystems). Quantitative RT-PCR was conducted with KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) or KAPA PROBE Fast qPCR kit (Kapa Biosystems). PCR reactions were analyzed with CFX Connect Real-time PCR Detection System (Bio-rad). Gene expressions were calculated by ΔΔCt method. Sequences of PCR primers are as follows: Chd8: 5′-cagagggaggttgtaagac-3′ (forward) and 5′-gagtgctacacagtgtcgcg-3′ (reverse); Tsc2: 5′-aagattcggctgagag-3′ (forward) and 5′-gacctcactcaagtctctc-3′ (reverse); Gapdh: 5′-agtggctgtggagaggtc-3′ (forward) and 5′-tgtagtacctcatgccg-3′ (reverse); Actb: 5′-ggctgtattcccctccatcg-3′ (forward) and 5′-ccagttgtaacaatgccatgt-3′ (reverse). Probes used were as follows: Gapdh (Assay ID: Mm99999915_g1, ThermoFisher Scientific), Tnf (Assay ID: Mm00443258_m1, ThermoFisher Scientific), Spp1 (Assay ID: Mm00440110_m1, ThermoFisher Scientific), Il1b (Assay ID: Mm00434228_m1, ThermoFisher Scientific), and Igf1 (Assay ID: Mm00439560_m1, ThermoFisher Scientific).

Cell sorting of microglia. P12 mice were anesthetized by intraperitoneal administration of a cocktail of domitor (0.3 mg/kg), dromicium (4 mg/kg), and butorphanol (5 mg/kg). Mice were transcardially perfused with ice-cold PBS, and dissected brains were digested with collagenase D (1 mg/mL; Roche) containing 2.5 mM calcium chloride at 37 °C for 30 min. After trituration, digested tissues were resuspended with 30% percoll (GE Healthcare), and 70% percoll was layered underneath, followed by centrifugation at 2000 rpm for 30 min at room temperature. Cells were treated with APC conjugated anti-CD45 (1:200; Biolegend) on ice for 30 min. GFP+ CD11b+ CD45mid microglia were sorted with cell sorter SH800 (Sony). Total RNA was purified from sorted microglia by Trizol Reagent (Thermo Fisher Scientific).

Statistics. The data are presented as mean ± standard error of the mean (SEM). Student’s t-tests, one-way analysis of variance (ANOVA) and Tukey–Kramer post-hoc tests, two-way ANOVA tests were performed with GraphPad Prism 7 (GraphPad). p < 0.05 was considered as significant.

Data availability. RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) under the accession number GSE172014.

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References
1. Abrahams, B. S. & Geschwind, D. H. Advances in autism genetics: On the threshold of a new neurobiology. Nat. Rev. Genet. 9, 341–355 (2008).
2. Courchesne, E. et al. Neuron number and size in prefrontal cortex of children with autism. JAMA J. Am. Med. Assoc. 306, 2001–2010 (2011).
3. Hutsler, J. J. & Zhang, H. Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. Brain Res. 1309, 83–94 (2010).
4. Deoni, S. C. L. et al. White-matter relaxation time and myelin water fraction differences in young adults with autism. Psychol. Med. 45, 795–805 (2015).
5. Barnard, R. A., Pomaville, M. B. & O’Roak, B. J. Mutations and modeling of the chromatin remodeler CHD8 define an emerging autism etiology. Front. Neurosci. 9, 1–13 (2015).
6. Katayama, Y. et al. CHD8 haploinsufficiency results in autistic-like phenotypes in mice. Nature 537, 675–679 (2016).
7. Hoffmann, A. & Spengler, D. Chromatin remodeler CHD8 in autism and brain development. J. Clin. Med. 10, 366 (2021).
8. Maree, C. et al. Oligodendrocyte precursor survival and differentiation requires chromatin remodeling by Chd7 and Chd8. Proc. Natl. Acad. Sci. U. S. A. 115, E8246–E8255 (2018).
9. Kawamura, A. et al. Oligodendrocyte dysfunction due to Chd8 mutation gives rise to behavioral deficits in mice. Hum. Mol. Genet. 29, 1274–1291 (2020).
10. Inoki, K., Li, Y., Xu, T. & Guan, K. L. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 17, 1829–1834 (2003).
11. Hoochmandi, M., Wong, C. & Khoutorsky, A. Dysregulation of translational control signaling in autism spectrum disorders. Cell. Signal. 75, 109746 (2020).
12. Sato, A. et al. Rapamycin reverses impaired social interaction in mouse models of tuberous sclerosis complex. Nat. Commun. 3, 1–9 (2012).
13. Carson, R. P. et al. Hypomyelination following deletion of Tsc2 in oligodendrocyte precursors. Ann. Clin. Transl. Neurol. 2, 1041–1054 (2015).
14. Bohlen, C. J., Friedman, B. A., Dejanovic, B. & Sheng, M. Microglia in brain development, homeostasis, and neurodegeneration. Annu. Rev. Genet. 53, 263–288 (2019).
15. Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J. E., Sekino, Y. & Sato, K. Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone. J. Neurosci. 34, 2231–2243 (2014).
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**Author contributions**
Y.T. and S.T. performed most of the experiments and analyzed the data. S.T. designed the experiments and coordinated the project. D.K. conducted preliminary experiment of phagocytosis assay. M.K., T.S., and H.B. supported the experiments. R.M. wrote the manuscript and directed the project. All authors approved of the manuscript.

**Competing interests**
The authors declare no competing interests.

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