Loss of PRMT1 in the central nervous system (CNS) induces reactive astrocytes and microglia during postnatal brain development

Misuzu Hashimoto1,2 | Ayako Kumabe1 | Jun-Dal Kim2 | Kazuya Murata3 | Sowmya Sekizar4 | Anna Williams4 | Weizhe Lu2 | Junji Ishida2 | Tsutomu Nakagawa1 | Mitsuharu Endo5 | Yasuhiro Minami5 | Akiyoshi Fukamizu2,6

Abstract

PRMT1, a major arginine methyltransferase, plays critical roles in transcription, DNA damage response, and cell proliferation. Although we have previously discovered the crucial roles of PRMT1 for oligodendrocyte lineage progression in the central nervous system of neural stem cell-specific PRMT1 conditional knockout (PRMT1-CKO) mice, the context of other glial cell states that may cause the hypomyelination phenotype in PRMT1-CKO mice has not been explored so far. Here, we performed RNA-seq of the neonatal cortices of PRMT1-CKO mice to reveal overall gene expression changes and show the up-regulation of inflammatory signaling which is generally mediated by astrocytes and microglia in advance of the myelination defects. In particular, qRT-PCR analyses revealed Interleukin-6 (Il-6), a major central nervous system cytokine, was dramatically increased in the PRMT1-CKO brains. The gene expression changes led to augmentation of glial fibrillary acidic protein and Vimentin protein levels in PRMT1-CKO mice, showing severe reactive astrogliosis after birth. We further show that IBA1-positive and CD68-positive activated microglia were increased in PRMT1-CKO mice, in spite of intact Prmt1 gene expression in purified microglia from the mutant mice. Our results indicate that PRMT1 loss in the neural stem cell lineage causes disruptive changes in all glial types perturbing postnatal brain development and myelination.

KEYWORDS

astrocyte, central nervous system (CNS), inflammation, microglia, PRMT1, transcriptome

1Laboratory of Biological Chemistry, Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan
2Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba, Japan
3Laboratory Animal Resource Center, Transborder Medical Research Center, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan
4MRC Centre for Regenerative Medicine, Institute for Regeneration and Repair, and MS Society Edinburgh Centre, Edinburgh bioQuarter, University of Edinburgh, Edinburgh, UK
5Division of Cell Physiology, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, Kobe, Japan
6The World Premier International Research Center Initiative (WPI), International Institute for Integrative Sleep Medicine, University of Tsukuba, Tsukuba, Japan

Correspondence
Akiyoshi Fukamizu, Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba 305-8577, Japan.
Email: akif@tara.tsukuba.ac.jp

Funding information
Japan Society for the Promotion of Science, Grant/Award Number: 17H01519, 17H06730, 18K05429 and 20K15913; Inamori Foundation; Mitsubishi Foundation

Abbreviations: CKO, conditional knockout; CNS, central nervous system; Cx3cr1, chemokine (C-X3-C motif) receptor 1; D, days post-injury; DEGs, differentially expressed genes; E, embryonic day; EAE, experimental autoimmune encephalomyelitis; FABP7, Fatty acid binding protein 7; GFAP, glial fibrillary acidic protein; GO, gene ontology; GSEA, gene set enrichment analysis; IBA-1, Ionized Calcium Binding Adapter Moloney Sarcoma Virus Oncogene Homolog 1; Il-6, Interleukin-6; KEGG, Kyoto Encyclopedia of Genes and Genomes; Nes-Cre, Nestin-Cre; NSCs, neural stem cells; OPCs, oligodendrocyte precursor cells; P, postnatal day; PRMT1, protein arginine methyltransferase 1; PRMT1-CKO, neural stem cell-specific PRMT1 conditional knockout; RPKM, reads per kilobase per million; RRID, Research Resource Identifier; STAT3, signal transducer and activator of transcription 3.
INTRODUCTION

Arginine methylation of proteins is widely conserved in plants, yeasts, and mammals. The function of this modification is widespread, affecting protein–protein interactions or localization that impact transcription and other cellular processes, including cell survival, proliferation, and differentiation (Bedford & Clarke, 2009; Blanc & Richard, 2017; Guccione & Richard, 2019). Arginine methylation is catalyzed by protein arginine methyltransferases (PRMTs), which consist of PRMT1-10. Type I PRMTs, which include PRMT1, 2, 3, 4, 6, 8, and METTL23, catalyze monomethylation and symmetric dimethylation of arginine (Bedford et al., 2009; Hatanaka et al., 2016). In contrast, type II PRMTs, PRMT5 and 9, catalyze monomethylation and symmetric dimethylation. PRMT7 is classified as a type III PRMT that can catalyze only monomethylation. PRMT1, the most common type I PRMT, is an evolutionarily conserved enzyme which regulates tissue development, lifespan, and stress responses (Blanc et al., 2017; Hirata et al., 2017; Takahashi et al., 2011). Conventional homozygous knockout of PRMT1 in mice results in failure of embryonic development, with survival only as heterozygotes (Pawlak, Scherer, Chen, Roshon, & Ruley, 2000). Studies with these PRMT1 heterozygotes suggest that methylation of KCNQ channel is important for suppressing basal activation of excitatory neurons in the brain (Kim et al., 2016).

In recent years, a growing number of studies have focused on generation of cell/tissue-specific knockout of PRMT1 (Blanc et al., 2017; Ishimaru et al., 2017; Murata et al., 2018) including in the neural stem cells (NSCs) of the central nervous system (CNS) (Hashimoto et al., 2016). In fact, we showed that oligodendrocyte lineage development is defective in the whole CNS of Prmt1flox/flox; Nestin-Cre mice designed with Nestin-Cre transgenic mice (B6.Cg-Tg(Nes-cre)1Kln/J, stock number: 003771. The Jackson Laboratory, RRID: IMSR_JAX:003,771). All animal experiments were done in compliance with and approved by the Institutional Animal Experiment Committee of the University of Tsukuba and Gifu University (protocol#18007). All these experiments were conducted in accordance with the Regulations for Animal Experiments at our institution and with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Pregnant mice were housed alone per cage in an environmentally controlled room temperature 25°C, 12/12h light/dark cycle (with lights on at 7 or 8 a.m.), and food and water available ad libitum. Experiments were done during light cycle. Postnatal day 0 (P0) or P8 mice were obtained without sex determination and PRMT1-CKO mice (Prmt1flox/flox; Nes-Cre genotype) were analyzed with Prmt1flox/flox as a control group.

This was a fundamental exploratory study and it was not pre-registered.

MATERIALS AND METHODS

Animals

Graphical timeline for all animal experiments in this study was shown in Figure 1a. Prmt1flox/flox; Nes-Cre mice were generated as previously described (Hashimoto et al., 2016). Briefly, Prmt1flox/flox mice designed by European Conditional Mouse Mutagenesis (EUCOMM) was crossed with Nestin-Cre transgenic mice (B6.Cg-Tg(Nes-cre)1Kln/J, stock number: 003771. The Jackson Laboratory, RRID: IMSR_JAX:003,771). All animal experiments were done in compliance with and approved by the Institutional Animal Experiment Committee of the University of Tsukuba and Gifu University (protocol#18007). All these experiments were conducted in accordance with the Regulations for Animal Experiments at our institution and with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

In our study, we carried out RNA-seq analysis on cortices from these CKO mice and have shown an increase in the inflammatory signals in the CNS. We have shown that these inflammatory pathways are activated in microglia and astrocytes in the CKO mice suggesting that PRMT1 is essential for proper brain development through suppressing such early brain inflammatory changes.

Quantitative RT-PCR (qRT-PCR)

P0 or P8 mice were rapidly decapitated without anesthetic and tissues were obtained and frozen in liquid nitrogen and then stored in a deep freezer until use. Frozen cortical tissues in ISOGEN II (Nippon Gene, Ltd., Tokyo, Japan #311-07361) were crushed with Micro Smash (Tomy Co., Ltd., Tokyo, Japan) and were processed for total RNA extraction following the manufacturer’s instructions. For RNA extraction from purified microglia or splenic leukocytes, cells obtained by methods as described...
below were collected by centrifugation and the total RNA was obtained by lysis with ISOGEN II. The total RNA was reverse transcribed into cDNA with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan #FSQ-301). Relative gene expression level was determined by SYBR Green-based quantitative RT-PCR (Bio-Rad Laboratories, Hercules, CA, USA #CFB3120EDU). Expression levels of target gene were corrected for Gapdh expression levels by using ddCt method. The amplification efficiency of primers for each target gene was confirmed to be equal by using serial dilutions of cDNA. For Figure S3, cDNA from stab wound brain injury model was prepared as
described previously (Endo et al., 2017). The primer sequences are listed on Table S2.

Prmt1-forward1 and -reverse1, which detect the longest isoform (NM_019830.3) of Prmt1, were used for the stab wound injury model (Figure S3) and Prmt1-forward2 and -reverse2 were used for the CD45 immunopanning experiments (Figure 5a).

2.3 | RNA sequencing and Functional enrichment analysis

For cDNA library preparation, total RNA was extracted from the cortices of P0 mice (n = 3) using ISOGEN II. RNA-seq analysis was performed as described previously (Ohkubo et al., 2018). Reads were mapped on mm10 mouse reference genome and estimated the expression pattern of transcripts between PRMT1-CKO and the control group, read counts were normalized by calculating number of reads per kilobase per million for each transcript in individual samples using the CLC Genomics Workbench software version 10.1.1 (QIAGEN). Filtering characteristics of fold change ≥2 or ≤−2 with a false discovery rate (p < .05) between the two groups were used to identify the differentially expressed genes (DEGs). Gene ontology (GO) analysis of DEGs, molecular function and biological process, and Kyoto encyclopedia of genes and genomes pathway was performed using online ToppGene Suite (http://toppgene.cchmc.org). RNA-seq datasets are deposited in the NCBI GEO under accession number GSE126127.

2.4 | Gene set enrichment analysis (GSEA)

GSEA analysis (Subramanian et al., 2005) was performed to investigate whether PRMT1 deficiency in the CNS is associated with astroglisis and microglisis using the computational software, GSEA_4.0.3 (http://www.broad.mit.edu/gsea). Annotated gene sets were downloaded from the Molecular Signatures Database (MsigDB, RRID: SCR_016863) under C5: Gene ontology (GO) categories (GO:0,048,708) and C7: accession number GSE126127 (Rock et al., 2005). The enrichment gene sets in GSEA were identified as statistically significant with a nominal p-value < .05 and a false discovery rate (FDR) <0.25.

2.5 | Western blot

P0 or P8 mice were rapidly decapitated without anesthetic and cortical tissues were obtained and frozen in liquid nitrogen. P8 cortices were processed as described previously (Hashimoto et al., 2016). P0 cortices were homogenized by sonication in ice-cold TNE buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, and 1 mM EDTA) with 1% Nonidet P-40 and a proteinase inhibitor mixture (Nacalai Tesque, Inc., Kyoto, Japan). Protein concentration was determined by Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories). Quantified total protein extracts were denatured with Laemml sample buffer supplemented with 2-mercaptoethanol. Proteins were separated by 10% SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA #IPVH00010). The membranes were blocked 30 min at 25°C in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH7.6) supplemented with 3% of non-fat–dried milk (Megmilk Snow Brand). Then, the membranes were incubated for 1 hr at 25°C or overnight at 4°C in primary antibodies listed on Table S3. 1% and 0.5% non-fat–dried milk were used as diluents for primary and secondary antibodies, respectively. After washing with TBST, incubations with HRP-conjugated secondary antibodies were carried out for 1 hr at 25°C. After washing with TBST, visualization was performed via chemiluminescent detection using SuperSignal West Femto Maximam Sensitivity Substrate (Thermo Fisher Scientific #30409) or ImmunoStar Zeta (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan #295-72404). Immunoreactive images were captured by LAS-3000 (Fujifilm, Tokyo, Japan). For glial culture, cells were washed with ice-cold phosphate-buffered saline (PBS), lysed and the proteins were denatured with Laemmli sample buffer supplemented with 2-mercaptoethanol. Total protein concentration was determined by Bradford assays with XL-Bradford (APRO Life Science Institute, Tokushima, Japan #KY-1030) and was analyzed by SDS-PAGE as described above.

2.6 | Immunohistochemistry

For immunostaining, P8 mice were rapidly decapitated without anesthetic and brains were fixed with 4% PFA overnight and embedded in O.C.T. compound (Tissue-Tek, Trance, CA, USA). Ten-µm cryosections were stained with primary antibodies after blocking with 10% goat serum donor herd (Sigma-Aldrich #G6767) in 0.1% TritonX-100/PBS. Primary antibodies used were listed on Table S3. Fluorescence images were obtained with fluorescence microscopes (BIOREVO BZ-9000, BZ-X710, and BZ-X810, Keyence, Osaka, Japan). Three to four mice from each group were analyzed for all stainings. For quantification of images, three to four mice per each genotype were used and same size areas were applied to manual cell counting with the ImageJ software.

2.7 | Purification of microglia

We purified microglia with anti-CD45 immunopanning method as previously described (Zhang et al., 2014). Briefly, P8 mice were deeply anesthetized with 10% pentobarbital and were transcardially perfused with PBS containing heparin to minimize leukocyte contamination. The brain cerebral cortices were processed with a neural tissue dissociation kit (P) (Miltenyi Biotech, Gladbach, Germany #130-092-628) and were passed through 40-µm cell strainer (BD Biosciences). The obtained single cell suspension was incubated for 20 min on a panning plate prepared with double coating with goat anti-rat IgG [heavy and light (H + L) chain] (2.4 µg in 1 ml of 50 mM
Tris-HCl solution pH8.5, Jackson ImmunoResearch #112-005-003, RRID: AB_2338090) and rat anti-mouse CD45 (0.104 μg in 1 ml of PBS/0.2% BSA; Biolegend #103101, RRID: AB_312966) as described previously (Zhang et al., 2014). The attached cells were designated as CD45+ fraction (purified microglia), whereas non-attached cells were called CD45− fraction (neuron, oligodendrocyte, and other types).

2.8 | Semi-quantitative RT-PCR (semi-qRT-PCR)

cDNA prepared as above from the splenic leukocytes and CD45+ cells was used for PCR with EmeraldAmpMAX PCR master mix (Takara Bio Inc., Shiga, Japan #RR320A) using thermal cycler (iCycler, Bio-Rad Laboratories). The PCR products were applied to 3% agarose gel containing ethidium bromide for electrophoresis and the images were obtained with a transilluminator.

The splenic leukocytes were obtained as described below and RNA was prepared as written above. The primer sequences are listed on Table S2.

As a positive control for leukocytes, splenic leukocytes were obtained from 3-month-old adult mouse (Prmt1flox/wt genotype). The spleen tissue was minced with forceps and single cells were prepared. Then, the red blood cells were lysed by water-based osmotic shock to enrich leukocytes. Then, the cells were passed through with 40-μm strainer.

2.9 | Primary glial culture

Brains from Prmt1flox/flox and Prmt1flox/wt, Nes-Cre mice at embryonic day (E) 19 were processed with a neural tissue dissociation kit (T) (Milteny Biotech #130-093-231). The cell suspension was passed through 40-μm cell strainer (BD Biosciences). The obtained single cell suspension was plated on poly-D-lysine (PDL) (Sigma-Aldrich #P-6407) coated 25 cm² flasks and was grown in Dulbecco’s modiﬁed Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/ml streptomycin in humidified, 37°C. The media were changed every 4 days. Cells became more than 90% confluency in 10–16 days. To increase purity of astrocyte lineage cells in the brain develop at E12.5 (Kessaris et al., 2014). As a positive control for leukocytes, splenic leukocytes were obtained as described below and RNA was prepared as written above. The primer sequences are listed on Table S2.

2.10 | siRNA-mediated Prmt1 knockdown

Passed glial culture from Prmt1flox/flox mouse was transfected with siRNA-targeting Prmt1 (Silencer Select, Life technologies, Carlsbad, CA, USA #4390771, siRNA ID #s67186 for siPrmt1#1 and #s67817 for siPrmt1#2) using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific #13778100) according to the manufacturer’s instructions. Non-transfected cells and non-targeting control siRNA (Silencer Select, Life technologies #4390843)-treated cells (siCtrl) were used as negative control samples. The siRNA sequences are as follows; siPrmt1#1 sense: 5’ GCAACUCCUGUUUCACAAatt 3’; siPrmt1#1 antisense: 5’ UUGUGAAACAUGGAGUUGCgg 3’; siPrmt1#2 sense: 5’ CUACUACUUGACUCCAAtt 3’; siPrmt1#2 antisense: 5’ AUAGGAGUCAAAGAUGGAtc 3’. Briefly, 5 μM siRNA and the transfection reagent were mixed in Opti-MEM (Thermo Fisher Scientific) and were incubated for 10 min at 25°C. Cells were then treated with the siRNA-reagent complex (final siRNA concentration in culture is 10 nM) and cultured for 2 days until media change. The cells were lysed at 98 hr after siRNA transfection for Western blot.

2.11 | Statistics

No randomization was performed to allocate animals in the study. No statistical methods were used to predetermine sample size before initiating this study. Instead, sample size conventions of the field were used (Huff et al., 2020). A minimum of three animals per group were used in all experiments. Normality was assessed using Shapiro–Wilk tests. Normality assessment was not applied to body weight and brain weight data (Figure 1b) and qRT-PCR data (Figure 2). ROUT method was used to identify outliers and no animals were specifically excluded from the study. Total number of mice used in this study is 79 and number of animals (n) for each data was described in Figure 1a and each figure legend. Since no animals were excluded from all experiments, n for all data are equal to initial numbers of animals. No blinding was performed in the study. Results are shown as mean ± SEM from n mice and were analyzed by a two-tailed Student’s t test or by Mann–Whitney U test. Data from body and brain weights were analyzed by Kruskal–Wallis test followed by Dunnett’s multiple comparisons test (Figure 1b). Data from stab wound brain injury were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test (Figure S3). Significance was considered at p < .05. Statistical analysis was performed using Prism 8 for macOS (Version 8.2.1 (279), RRID: SCR_002798).

3 | RESULTS

3.1 | Defective brain development and up-regulated inflammatory signaling in the neonatal PRMT1-CKO mice

We have previously demonstrated that PRMT1 deficiency in Nestin-positive cells in the CNS led to severe brain atrophy, hypomyelination, and an impaired behavioral phenotype characterized by tremor at postnatal stages (Hashimoto et al., 2016). As PRMT1 is depleted by Nestin-Cre–mediated recombination as early as E10, and oligodendrocyte lineage cells in the brain develop at E12.5 (Kessaris et al., 2014).
et al., 2006), loss of PRMT1 in other brain cell types derived from Nestin-positive NSCs could also be the trigger of the defective oligodendrocyte lineage development and subsequent hypomyelination. To clarify this, we compared the brain weight of these postnatal day (P) 0 neonates. Although the birth body weight of PRMT1-CKO mice was essentially the same as that of the littermate controls, the brain weight was significantly lower in PRMT1-CKO mice (Prmt1flox/flox; Nes-Cre genotype) (Figure 1b), which was not present in heterozygotes (Prmt1flox/wt; Nes-Cre), Prmt1flox/flox, and Prmt1flox/wt mice (Figure 1b). These results show that loss of PRMT1 by Nestin-Cre-mediated recombination affects brain development at least as early as birth and before myelination begins.

In order to identify which pathways or cell types are mostly affected by PRMT1 deficiency in the developing cortex, we performed RNA-seq analyses with PRMT1-CKO cortices at P0 with three biological replicates. The clustering heat map shows distinct groups of genes specifically expressed by each genotype, which is also evident in principal component analysis (Figure 1c–e). Based on a threshold of fold change ≥ 2 or ≤ −2 and FDR-adjusted p-value < .05, we identified 436 down-regulated and 455 up-regulated genes in PRMT1-CKO cortices compared to the control group (Figure 1d–f, Figure S2a and b). In the list of down-regulated genes in PRMT1-CKO mice, Pdgfra and Gpr17, markers for OPCs and pericytes were down-regulated in PRMT1-CKO mice (Figure 1d, Figure S2a and b, Table S1).
These data are consistent with our previous study, showing defects in oligodendrocyte lineage development by PRMT1 deficiency and imply that the transcriptome analysis is sensitive enough to capture the real phenotypic changes in PRMT1-CKO mice.

Next, we tried to classify genes based on signaling pathways using online-based TopGene Suite software, particularly focusing on the up-regulated genes in PRMT1-CKO group. In classification by biological process, the top five pathways were 'Defense response', 'Positive regulation of multicellular organismal process', 'Positive regulation of tumor necrosis factor superfamily cytokine production', 'Cellular calcium ion homeostasis', and 'Inflammatory response' (Figure 1f). In classification by molecular function, 'Transmembrane signaling receptor activity' was the top, in which many genes such as Cx3cr1 and Ccr5 were also detected in the above classifications (Figure 1f). Kyoto encyclopedia of genes and genomes pathway analysis also showed 'Cytokine-cytokine receptor interaction' was highly up-regulated in PRMT1-CKO cortices (Figure 1f). In this group, there were up-regulated 13 genes related to inflammation; Ccl12, Ccl3, Ccr5, Csfr1, Cx3cr1, Cxcl10, Edu2r, Fas, Il12rb2, Il-6, Il-7, Ngfr, and Thpo. Notably, we found several genes being common among the top groups in each classification. These included microglia-enriched genes (Ccl3, Csfr1, Cx3cr1, and Ccr5) (Zhao et al. 2018). In addition, some genes including Cxcl10 or Il-6 are highly expressed in microglia as well as in astrocytes and its relationships in neuroinflammation or astrogenesis are well studied (Clarner et al., 2015; Ertu, Quintana, & Hidalgo, 2012; Ko et al., 2014; Nakashima & Taga, 2002). We assume that those microglia and astrocytes-related genes highly contributed to the transcriptome changes in PRMT1-CKO. Collectively, RNA-seq analysis suggests that there is an overload of inflammatory signaling in the PRMT1-deficient cortices at P0 stage before developmental myelination begins.

### 3.2 Increased inflammation-related genes in the cortices by loss of PRMT1

Next, we validated these changes in gene expression by qRT-PCR. Cx3cr1 and C3ar1 showed a significant up-regulation at P8 in PRMT1-CKO group (Figure 2a and b). Most remarkably, Il-6 was up-regulated in cortices at P0 and P8 by 5–7 folds in PRMT1-CKO (Figure 2c). Furthermore, Il-10 and Tnf, the initial mediators of brain inflammatory responses, were significantly increased in PRMT1-CKO cortices at P8, although they were not yet elevated at P0 confirmed by RNA-seq and qRT-PCR (Figure 2d and e).

In addition, we found that an uracil-sensitive pyrimidinergic receptor called P2y4 which belongs to G protein-coupled receptor was significantly increased in PRMT1-CKO cortices by RNA-seq and this was confirmed by qRT-PCR (Figure 2f). Ligands for P2Y receptors are nucleotides such as ATP, which are known triggers for neuroinflammation (Di Virgilio, Ceruti, Bramanti, & Abbracchio, 2009). As these changes in inflammatory pathways in our RNA-seq analysis suggested reactive astrogliosis during brain development in PRMT1-CKO mice, we compared our dataset to those with previously validated astrogliosis (Zamanian et al., 2012). In these astrogliosis datasets, astrocytes were isolated from either an LPS-induced neuroinflammation model or an ischemia model induced by middle cerebral artery occlusion (MCAO) and microarray analyses performed. In our comparison analysis, we identified extracellular matrix (ECM) modification and adhesion molecules were highly up-regulated in these astrogliosis models and our PRMT1-CKO mice. For example, Col6a1, encoding an ECM protein, was up-regulated in these astrogliosis models, is also elevated in PRMT1-CKO mice at P8, and to a lesser extent at P0 (Figure 2g). Since Zamanian et al. identified that the up-regulated gene sets by LPS and MCAO are different, we speculated that the overlapping up-regulated genes from these two models may indicate general genes activated by gliosis. We identified 13 such genes among the top 50 genes up-regulated in each model and compared their reads per kilobase per million between control and PRMT1-CKO (Figure S1, Table S1). Six of the 13 of these genes also showed higher trends in PRMT1-CKO compared with control. These included Cd44, Aspg (asparaginase), Cxcl10, Gfap, Vim (Vimentin), and OsMr (oncostatin M receptor) (Figure S1). Gfap and Vim are well characterized as being increased when astrocytes are reactive. Interestingly, Cd44, a cell surface receptor involved in adhesion and migration, has been suggested to facilitate inflammatory responses (Dzwonek et al., 2015). In conclusion, our RNA-seq data validated by qRT-PCR suggested that PRMT1-CKO mice show changes in brain inflammation and astrogliosis. Also, we observed that those changes emerged prior to the dysmyelination phenotype in PRMT1-CKO, and further progressed until P8.

Since loss of PRMT1 induced these inflammatory gene up-regulations, we hypothesized that PRMT1 has a protective effect in neuroinflammation and its expression might be induced in response to inflammatory stimuli. To examine this, we measured Prmt1 mRNA expression in a stab wound brain injury model where acute neuroinflammation is induced (Endo et al., 2017). However, we found no increment of Prmt1 expression over 2 weeks post-injury by qRT-PCR (Figure S3), suggesting that PRMT1 may not serve as a protector from acute brain inflammation.

### 3.3 Reactive astrogliosis in the postnatal PRMT1-CKO mice

Next, we determined whether and how the up-regulation of inflammation-related gene expression links to the actual reactivity of astrocytes in PRMT1-CKO. Gene set enrichment analyses (GSEA) revealed up-regulated genes in PRMT1-CKO cortices to be positively associated with gene sets related to astrocyte development, differentiation, and activation (Figure S2c). By immunohistochemistry, control mice showed only a few Gfap-positive astrocytes under the pial surface of the cortices at P8 (Figure 3a, left panel). Also, in the gray matter of control cortices, Gfap-positive cells elongated radial fibers, which are probably astrocytes transformed from radial glia that support migration of newborn neurons for cortical plate formation (Tramontin, García-Verdugo, Lim, & Alvarez-Buylla, 2003; Woodhams, Basco,
Hajos, Csillag, & Balazs, 1981) (Figure 3a, upper panels, arrows). In contrast, in PRMT1-CKO mice, GFAP-positive cells covered almost entire area of the cortices (Figure 3a, right panel). Especially, GFAP-positive cells in the gray matter of PRMT1-CKO cortices appeared in a star-like filamentous shape which is a hallmark of reactive astrocytes (Figure 3a, lower panels). These cells were significantly increased in PRMT1-CKO (Figure 3a, graph), compared with control cortices (Figure 3a, left panel). In contrast to GFAP-expressing astrocytes, astrocytes labeled with glutamate transporter GLAST, a pan-astrocyte marker (Molofsky & Deneen, 2015), were distributed at comparable levels in control and PRMT1-CKO cortices (Figure 3b). These results indicate that astrocyte reactivity is induced by loss of PRMT1.

To further characterize astrogliosis by loss of PRMT1, we determined various protein levels by Western blot. Fatty acid binding
protein 7 (FABP7), a robust astrocyte marker, was also similarly expressed between control and PRMT1-CKO mice (Figure 3c and d). In contrast, we found that PRMT1-CKO cortices showed significant increases in an intermediate filament protein Vimentin, which is another marker protein for reactive astrocytes (Sofroniew, 2009), compared with the controls (Figure 3c and d). Similarly, signal transducer and activator of transcription 3 (STAT3) and its phosphorylated form pSTAT3 at threonine 705 (pSTAT3 (p705)), a transcription factor modulated in reactive astrocytes (Sofroniew et al., 2009), were increased in PRMT1-CKO. On the other hand, the STAT3 phosphorylation ratio, which is increased in scar-forming astrogliosis (Herrmann et al., 2008) or brains received postnatal hypoxic-ischemic insult (Hristova et al., 2016), was not affected by PRMT1 deficiency. These results indicate that reactive astrogliosis induced by loss of PRMT1 was relatively mild and gradually progressing during brain development, rather than acutely exacerbating as seen in brain injury. Indeed, GFAP protein expression was already increased in PRMT1-CKO cortices even at P0 (Figure 3e and f). Although we have reported that an AMPA receptor subunit GluA2 expressed by neurons is down-regulated in PRMT1-CKO cortices (Hashimoto et al., 2020), Tuj1, a major pan-neuronal marker, was detected equivalently between control and PRMT1-CKO cortices (Figure 3b and c). This implies that neuronal growth or cell death is not affected by the reactive astrogliosis by PRMT1 deficiency. Overall, these astrocytic phenotypes in postnatal PRMT1-CKO cortices are consistent with our transcriptome data, showing reactive astrogliosis in PRMT1-CKO cortices at an early time point prior to myelin formation and, therefore, did not occur as a consequence of hypomyelination.

3.4 | Microglial increase in PRMT1-CKO mice

Up-regulation of microglia/macrophage-specific genes such as Ccl3, Csf1r, and Cx3cr1 in PRMT1-CKO cortices suggests that microglia/
macrophages may also be affected in this region. It is known that cytokines or chemokines secreted by microglia or astrocytes activate each other leading to inflammatory signaling in the brain tissue (Sofroniew et al. 2009). GSEA analysis revealed that the genes up-regulated in PRMT1-deficient CNS is similar to the genes that are up-regulated by interferon (IFN)-γ treated human microglia (Figure S2d) (Rock et al., 2005). To explore this further, we examined the microglial population in PRMT1-CKO mice at P8. Immunostaining for IBA-1 revealed an increased number of IBA-1-positive microglia in PRMT1-CKO cortices compared to those in control mice (Figure 4a and c). CD68 is a lysosomal membrane-associated protein and is known to be a surrogate marker for reactive microglia (Zhao et al., 2018). CD68-positive reactive microglia were also increased in number (Figure 4b and c), indicating that microglial phagocytic activity is enhanced in PRMT1-CKO. CD45-positive microglia showed an increased trend in PRMT1-CKO as well (Figure S4a and b), indicating that microglial reactivity is increased by loss of PRMT1. In the hippocampal CA1 region of CKO brains, IBA1-positive microglia were amoeboid in shape, with large soma that are hallmarks of activated microglia, whereas cells in the control brains had sharp and thin processes (Figure 4d). These results in PRMT1-CKO mice are consistent with many other brain disease models where reactive astrogliosis is also accompanied by microglial activation.

3.5 Up-regulation of inflammatory signaling in microglia and other cells in PRMT1-CKO cortices

Although we have observed extensive microgliosis in Nestin-Cre-mediated PRMT1-CKO, it is generally considered that microglia do not express Nestin (Braun et al., 2012). To clarify why microglia was activated in PRMT1-CKO, we purified microglia from P8 cortices of PRMT1-CKO mice and performed qRT-PCR. Microglial purification was performed by immunopanning using an anti-CD45 antibody (see methods section for detail). We ensured that leukocyte contamination was minimal in the CD45-positive microglial fraction by semi-qRT-PCR (Figure S5). First, we confirmed that Prmt1 expression was intact in CD45-positive microglia, while it was significantly reduced in CD45-negative cells in PRMT1-CKO mice compared to control mice (Figure 5a). Next, to investigate which cell populations contributed to the increased level of the inflammation-related genes observed in PRMT1-deficient cortices (Figures 1 and 2), inflammatory gene expression levels were measured in each cell population. In both CD45-positive and CD45-negative fractions, Il-6 and Il-1β were significantly up-regulated in PRMT1-CKO cortices-derived cells compared with corresponding fractions derived from control mice (Figure 5b and c). These results show that the brain inflammatory environment in PRMT1 deficiency occurs by serial contributions of cells other than microglia followed by microglial activation. These data in combination with our transcriptomic analysis demonstrate that brain inflammation associated with astrogliosis and microgliosis is caused during brain development by loss of PRMT1 in the NSCs.
We still do not know how loss of PRMT1 in the CNS induces spontaneous inflammatory changes in the brain during development. It may be interesting to consider the reports about the roles of PRMT1 in other cells in terms of inflammation. TNF receptor-associated factor 6 (TRAF6) is an E3 ubiquitin ligase and its methylation by PRMT1 reduces toll-like receptor signaling in many culture cell lines (Tikhanovich et al., 2015). Similar to our observation in PRMT1-CKO brains, this report also demonstrated that arginine methylation by PRMT1 reduces inflammation under basal conditions. Conversely, PRMT1 expression was shown to be elevated by cytokines, exacerbating inflammation in fibroblasts in the asthmatic process (Sun et al., 2015), although there is no information whether this is true in the brain.

To give an insight on brain inflammation and PRMT1, we measured Prmt1 mRNA levels after an acute brain injury, stab wound. However, as we have shown in Figure S3, Prmt1 mRNA levels were not affected after injury, although the enzymatic activity of PRMT1 might be modulated. A previous study showed that FABP7 was up-regulated after cortical stab wound brain injury (Sharifi et al., 2011). However, the spontaneous gliosis in PRMT1-CKO cortices did not accompany any changes in FABP7 level (Figure 3c and d), suggesting that PRMT1 does not have a protective effect after acute inflammatory stimuli such as stab wound. It would be useful to investigate the role of PRMT1 under brain inflammation caused by various settings and developmental time points, since astrogliosis is a heterogeneous state (Molofsky et al., 2012). Since our results showed that CD45-positive microglia express a substantial level of Prmt1 in the developing cortices (Figure 5a), it would also provide developmental and clinical importance of PRMT1 in those cells. In summary, PRMT1 could act as either an inducer or suppressor of inflammation depending on tissue or context.

4.3 Potential link between purine/pyrimidine metabolism and gliosis

Our transcriptomic analysis in PRMT1-CKO cortices showed up-regulation of genes related to inflammatory signaling similar to previously reported gliosis models (Zamanian et al., 2012). However, not all of those genes overlapped with those in the gliosis models. In particular, P2ry4 was increased in PRMT1-CKO cortices but no changes in the purine/pyrimidine receptor pathways were seen in the previous gliosis models (Zamanian et al., 2012). It is noteworthy that nucleotides such as ATP are involved in cross-talk between neuron and glia, with various purinergic P2Y receptor family proteins involved in this process (Di Virgilio et al., 2009).

Clopidogrel is an anti-platelet drug inhibiting P2Y12 (Damman, Woudstra, Kuijt, de Winter, & James, 2012), therefore, it would be interesting to identify the role of purine/pyrimidine metabolism in brain inflammation and gliosis as well in terms of clinical importance.
4.4 | Interrelationship between brain inflammatory changes and myelination and oligodendrocyte development

From the present study, how can we explain the link of brain inflammation and hypomyelination in PRMT1-CKO mice? Recent studies extensively reveal glial crosstalk affecting developmental myelination, remyelination, and demyelination as reviewed elsewhere (Domingues, Portugal, Socodato, & Relvas, 2016; Molina-Gonzalez & Miron, 2019). During early postnatal stages, CD11c-positive cells, a subset of microglia, were shown to stimulate myelination and neurogenesis (Wlodarczyk et al., 2017), suggesting a supportive role of microglia in brain development. In addition, microglia cultured in astrogliosis-conditioned media suppressed secretion of TGFβ-1 which inhibits oligodendrocyte maturation (Nobuta et al., 2012). These studies support a positive influence of microglia and reactive astrocytes on myelination, respectively. Conversely, under pathological conditions such as CNS infections, hypoxia, or preterm birth, brain inflammation involving microglia and astrocytes causes defective myelination (Bradl & Lassmann, 2010; Gallo & Deneen, 2014). Thus, in our scenario, we have two working hypotheses, which may not be mutually exclusive. The loss of PRMT1 in OPCs may lead to poor myelination per se, with a concomitant inflammatory environment in the developing brain. Alternatively, the loss of PRMT1 may induce inflammatory signaling through astrocytes (and secondarily microglia) before OPC generation, which possibly directs damage of oligodendrocyte lineage cells and, hence, defective myelination (Figure 6).

To distinguish these hypotheses, we tried to culture primary OPCs from PRMT1-CKO mice by the immunopanning method (Emery & Dugas, 2013). However, interestingly, OPCs from PRMT1-CKO do not proliferate in culture and failed to survive compared with wild-type cells, suggesting that there is a direct effect of loss of PRMT1 on OPCs not secondary to other glial changes (data not shown).

We also tested primary glial cultures from PRMT1-CKO cortices to examine the effect of PRMT1 decrease in astrocytes. Surprisingly, there was no change in GFAP protein expression in astrocytes derived from either CKO cortices or wild-type cortices with siRNA-mediated Prmt1 knockdown compared to controls (Figure S6a and b). Therefore, from these results, it is speculated that inflammatory signals are attributed to PRMT1-deficient NSCs, and this might be sensed by astrocytes and microglia (Figure 6). Furthermore, these inflammatory shifts in the developing brain might have a further negative influence on oligodendrocyte differentiation/myelination.

The impact of arginine methylation on multiple sclerosis has been reviewed elsewhere (Webb & Guerau-de-Arellano, 2017). Supporting this notion is that methylthioadenosine (MTA), an inhibitor of pan-methyltransferase, has been shown to be effective for T-cell immunomodulatory activity in the experimental autoimmune encephalomyelitis rat model (Moreno et al., 2006). However, its direct effects on cells in the CNS have been unclear in terms of pathology of MS. Therefore, it is important to clearly define the roles of arginine methylation in various cell types including OPCs and immune cells. In this study, we have demonstrated that the requirement of PRMT1 in NSCs for suppressing abnormal inflammation during brain development. Our results indirectly suggest the importance of PRMT1 in neurodevelopmental diseases or MS.

In summary, we have identified loss of PRMT1 in the neural stem cell lineage in the CNS induces inflammatory signaling accompanied by activation of astrocytes and microglia prior to myelination. Our study suggests that excess of inflammatory signaling is a critical obstacle for successful brain development and that PRMT1 assists suppressing this and allowing normal function.

ACKNOWLEDGMENT

This work was supported by JSPS KAKENHI Grant-in-Aid for Research Activity Start-up (17H06730) to M.H., Grant-in-Aid for Young Scientists (20K15913) to M.H., Grant-in-Aid for Scientific Research (C) (18K05429) to J.-D.K., Grant-in-Aid for Scientific Research (A) (17H01519) to A.F., Inamori Foundation Research Grants to M.H., and by The Mitsubishi Foundation to A.F. We also appreciate technical advices from Dr Chiharu Suzuki-Nakagawa and Dr Yasuhiko Kizuka.

All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

We have no conflicts of interest disclosure.

ORCID

Akiyoshi Fukamizu https://orcid.org/0000-0002-8786-6020

REFERENCES

Bedford, M. T., & Clarke, S. G. (2009). Protein arginine methylation in mammals: Who, what, and why, Molecular Cell, 33, 1–13. https://doi.org/10.1016/j.molcel.2008.12.013

Blanc, R. S., & Richard, S. (2017). Arginine methylation: The coming of age. Molecular Cell, 65, 8–24. https://doi.org/10.1016/j.molcel.2016.11.003

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: Biology and pathology. Acta Neuropathologica, 119, 37–53. https://doi.org/10.1007/s00401-009-0601-5

Braun, T. P., Grossberg, A. J., Veleva-Rotse, B. O., Maxson, J. E., Szumowski, M., Barnes, A. P., & Marks, D. L. (2012). Expression of myeloid differentiation factor 88 in neurons is not requisite for the induction of sickness behavior by interleukin-1β. Journal of Neuroinflammation, 9, 229. https://doi.org/10.1186/1742-2094-9-229

Calabretta, S., Vogel, G., Yu, Z., Choquet, K., Darbelli, L., Nicholson, T. B., ... Richard, S. (2018). Loss of PRMT5 promotes PDGFRα degradation during oligodendrocyte differentiation and myelination. Developmental Cell, 46(426–440), e5. https://doi.org/10.1016/j.devcel.2018.06.025

Clarnier, T., Jansen, K., Nellessen, L., Stangel, M., Skipuletz, T., Krauspe, B., ... Kipp, M. (2015). CXCL10 triggers early microglial activation in the cuprizone model. The Journal of Immunology, 194, 3400–3413. https://doi.org/10.4049/jimmunol.1401459

Damman, P., Woudstra, P., Kuijt, W. J., de Winter, R. J., & James, S. K. (2012). P2Y12 platelet inhibition in clinical practice. Journal of Thrombosis and Thrombolysis, 33, 143–153. https://doi.org/10.1007/s11239-011-0667-5
stab-injured brain cortex and its role in astrocyte proliferation. *Histochemistry and Cell Biology*, 136, 501. https://doi.org/10.1007/s00418-011-0865-4

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in Neurosciences*, 32, 638–647. https://doi.org/10.1016/j.tins.2009.08.002

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., ... Mesirov, J. P. (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences*, 102, 15545–15550. https://doi.org/10.1073/pnas.0506580102

Sun, Q., Liu, L., Roth, M., Tian, J., He, Q., Zhong, B., ... Lu, S. (2015). PRMT1 upregulated by epithelial proinflammatory cytokines participates in COX2 expression in fibroblasts and chronic antigen-induced pulmonary inflammation. *The Journal of Immunology*, 195, 298–306. https://doi.org/10.4049/jimmunol.1402465

Takahashi, Y., Daitoku, H., Hirota, K., Tamiya, H., Yokoyama, A., Kako, K., ... Fukamizu, A. (2011). Asymmetric arginine dimethylation determines life span in *C. elegans* by regulating forkhead transcription factor DAF-16. *Cell Metabolism*, 13, 505–516.

Tikhanovich, I., Kuravi, S., Artigues, A., Villar, M. T., Dorko, K., Nawabi, A., ... Weinman, S. A. (2015). Dynamic arginine methylation of tumor necrosis factor (TNF) receptor-associated factor 6 regulates toll-like receptor signaling. *Journal of Biological Chemistry*, 290, 22236–22249. https://doi.org/10.1074/jbc.M115.653543

Tramontin, A. D., Garcia-Verdugo, J. M., Lim, D. A., & Alvarez-Buylla, A. (2003). Postnatal development of radial glia and the ventricular zone (VZ): A continuum of the neural stem cell compartment. *Cerebral Cortex*, 13, 580–587. https://doi.org/10.1093/cercor/13.6.580

Webb, L. M., & Guerra-de-Arellano, M. (2017). Emerging role for methylation in multiple sclerosis: Beyond DNA. *Trends in Molecular Medicine*, 23, 546–562. https://doi.org/10.1016/j.molmed.2017.04.004

Wlodarczyk, A., Holtman, I. R., Krueger, M., Yoge, N., Bruttger, J., Khoroski, R., ... Owens, T. (2017). A novel microglial subset plays a key role in myelination in developing brain. *EMBO Journal*, 36, 3292–3308. https://doi.org/10.15252/embj.201696056

Woodhams, P., Basco, E., Hajos, F., Csillag, A., & Balazs, R. (1981). Radial glia in the developing mouse cerebral cortex and hippocampus. *Anatomy and Embryology*, 163, 331–343. https://doi.org/10.1007/BF00315709

Zamanian, J. L., Xu, L., Foo, L. C., Nouri, N., Zhou, L., Giffard, R. G., & Barres, B. A. (2012). Genomic analysis of reactive astrogliosis. *Journal of Neuroscience*, 32, 6391–6410. https://doi.org/10.1523/JNEUROSCI.6221-11.2012

Zhao, X., Liao, Y., Morgan, S., Mathur, R., Feustel, P., Mazurkiewicz, J., ... Huang, Y. (2018). Noninflammatory changes of microglia are sufficient to cause epilepsy. *Cell Reports*, 22, 2080–2093. https://doi.org/10.1016/j.celrep.2018.02.004

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Hashimoto M, Kumabe A, Kim J-D, et al. Loss of PRMT1 in the central nervous system (CNS) induces reactive astrocytes and microglia during postnatal brain development. *J. Neurochem*. 2021;156:834–847. https://doi.org/10.1111/jnc.15149