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Crystal Shula
Fatima Kahn
Sang Hoon Lee

See next page for additional authors

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Authors
Eri Iwasawa, Farrah N. Brown, Crystal Shula, Fatima Kahn, Sang Hoon Lee, Temugin Berta, David R. Ladle, Kenneth Campbell, Francesco T. Mangano, and June Goto
Neurobiology of Disease

The Anti-Inflammatory Agent Bindarit Attenuates the Impairment of Neural Development through Suppression of Microglial Activation in a Neonatal Hydrocephalus Mouse Model

Eri Iwasawa,1 Farrah N. Brown,1 Crystal Shula,1 Fatima Kahn,1 Sang Hoon Lee,2 Temugin Berta,2 David R. Ladle,3 © Kenneth Campbell,1,4 Francesco T. Mangano,1,5 and June Goto1,5

1Division of Pediatric Neurosurgery, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio, 45242, 2Pain Research Center, Department of Anesthesiology, University of Cincinnati Medical Center, Cincinnati, Ohio, 45242, 3Department of Neuroscience, Cell Biology, and Physiology, Wright State University, Dayton, Ohio, 45435, 4Division of Developmental Biology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, 45424, and 5Department of Neurosurgery, University of Cincinnati College of Medicine, Cincinnati, Ohio, 45424

Neonatal hydrocephalus presents with various degrees of neuroinflammation and long-term neurologic deficits in surgically treated patients, provoking a need for additional medical treatment. We previously reported elevated neuroinflammation and severe periventricular white matter damage in the progressive hydrocephalus (prh) mutant which contains a point mutation in the Ccdc39 gene, causing loss of cilia-mediated unidirectional CSF flow. In this study, we identified cortical neuropil maturation defects such as impaired excitatory synapse maturation and loss of homeostatic microglia, and swimming locomotor defects in early postnatal prh mutant mice. Strikingly, systemic application of the anti-inflammatory small molecule bindarit significantly supports healthy postnatal cerebral cortical development in the prh mutant. While bindarit only mildly reduced the ventricular volume, it significantly improved the edematous appearance and myelination of the corpus callosum. Moreover, the treatment attenuated thinning in cortical Layers II–IV, excitatory synapse formation, and interneuron morphogenesis, by supporting the ramified-shaped homeostatic microglia from excessive cell death. Also, the therapeutic effect led to the alleviation of a spastic locomotor phenotype of the mutant. We found that microglia, but not peripheral monocytes, contribute to amoeboid-shaped activated myeloid cells in prh mutants’ corpus callosum and the proinflammatory cytokines expression. Bindarit blocks nuclear factor (NF)-κB activation and its downstream proinflammatory cytokines, including monocyte chemoattractant protein-1, in the prh mutant. Collectively, we revealed that amelioration of neuroinflammation is crucial for white matter and neuronal maturation in neonatal hydrocephalus. Future studies of bindarit treatment combined with CSF diversion surgery may provide long-term benefits supporting neuronal development in neonatal hydrocephalus.

Key words: bindarit; microglia; neonatal hydrocephalus; white matter injury

Significance Statement

In neonatal hydrocephalus, little is known about the signaling cascades of neuroinflammation or the impact of such inflammatory insults on neural cell development within the perinatal cerebral cortex. Here, we report that proinflammatory activation of myeloid cells, the majority of which are derived from microglia, impairs periventricular myelination and cortical neuronal maturation using the mouse prh genetic model of neonatal hydrocephalus. Administration of bindarit, an anti-inflammatory small molecule that blocks nuclear factor (NF)-κB activation, restored the cortical thinning and synaptic maturation defects in the prh mutant brain through suppression of microglial activation. These data indicate the potential therapeutic use of anti-inflammatory reagents targeting neuroinflammation in the treatment of neonatal hydrocephalus.
Introduction
Neonatal hydrocephalus is a life-long incurable condition. Surgical diversion of excess CSF from the cerebral ventricles, via ventriculo-peritoneal shunt placement or endoscopic third ventriculostomy, often requires multiple revisions in young children. Sixty percent of surgically treated patients suffer from neurologic symptoms, including motor skills impairment, cognitive deficits, or epilepsy, throughout life (Pattisapu, 2001; Vinchon et al., 2012; Paulsen et al., 2015). This demonstrates an unmet need to develop early and/or supplemental medical interventions to prevent irreversible pathologic damage in the developing hydrocephalic brain (Robinson, 2012; Del Bigio and Di Curzio, 2016; Karimy et al., 2020a). The impact of hydrocephalus in perinatal human brains is significant as it affects organized neuronal connectivity, synaptogenesis, and myelination, which begins in the third trimester of gestation lasting until early childhood (Kostović and Jovanov-Milošević, 2006; Bercy and Macklin, 2015; Bouyssi-Kobar et al., 2016). In rodents, these cellular processes mostly occur during the early postnatal period (Ross et al., 2015).

Neuroinflammation is commonly described in animal models of hydrocephalus (Wys-Coray et al., 1995; Miller and McAllister, 2007; Deren et al., 2009; Lattke et al., 2012; Goulding et al., 2020; Klebe et al., 2020; Wu et al., 2020), along with pathophysiological and behavioral phenotypes representing neural developmental delays such as hypomyelination (Del Bigio, 2001; Ayannuga et al., 2016), less/shorter branches of the denticles with less denticritic spines (McAllister et al., 1985; Kriebel et al., 1993), and cognitive deficits (Suda et al., 1994; Jones et al., 1995).

Mononuclear myeloid cell activation has been described in periventricular tissue of fetal brains with hydrocephalus (Ulfig et al., 2004; Dominguez-Pinos et al., 2005) and rodent models (McAllister et al., 1998; Miller and McAllister, 2007; Goulding et al., 2020). Notably, several recent animal studies showed detrimental function of proinflammatory myeloid cells in hydrocephalus progression and tissue damage (McAllister and Miller, 2010; Lattke et al., 2012; Botfield, 2013; Abdi et al., 2018; Wu et al., 2020). However, the detailed definition of these active myeloid cells in pediatric hydrocephalus remains largely unknown (Del Bigio, 2010).

Motile cilia on ependymal cells of the ventricular walls are responsible for generating directional CSF flow within the ventricles. Genetic mutations affecting the development of multiciliated ependymal cells are responsible for a small proportion of congenital hydrocephalus cases (Badano et al., 2006; Feldner et al., 2017; Furey et al., 2018; Lalioti et al., 2019; Wallmeier et al., 2019; Robson et al., 2020). We previously described early and late neuroinflammation phenotypes ( Emmert et al., 2019; Goulding et al., 2020) in a robust rodent model of neonatal hydrocephalus, caused by loss of a motile cilia gene, coiled-coil domain-containing 39 (Ccdc39) and the subsequent intraventricular CSF flow retardation contributing to the progressive hydrocephalus (prh) phenotype (Abdelhamed et al., 2018). In the prh mutant rat, CD68-positive activated microglia cells start to accumulate in the periventricular tissue at early stages of ventriculomegaly, expressing monocyte chemoattractant protein-1 (MCP-1; Emmert et al., 2019). These myeloid cells also expressed other proinflammatory signals such as CD86 in the corpus callosum of the prh mutant mice (Goulding et al., 2020).

Bindarit (2-methyl-2-[1-(phenylmethyl)-1H-indazol-3-yl]methoxy]-propanoic acid) is a synthetic indazole derivative with an inhibitory effect on the nuclear factor (NF)-κB pathway on various inflammatory stimuli. It attenuates monocyte recruitment (Raghu et al., 2017) and myeloid cell activation (Shen et al., 2021) via reducing IkBα and p65 phosphorylation and the downstream target MCP-1 expression (Mora et al., 2012). Anti-inflammatory effects of bindarit have been demonstrated in various disease models, including arthritis (Rulli et al., 2009), pancreatitis (Bhatia et al., 2005), epilepsy (Cerri et al., 2016), ethanol-induced neuroinflammation (Zhang et al., 2018b), and experimental autoimmune encephalitis (EAE; Ge et al., 2012).

Here, we report the neuroprotective effects of bindarit in a genetic neonatal hydrocephalus model by suppressing proinflammatory microglia. Our findings demonstrate improvement in perinatal brain development and highlight the potential of early intervention to proinflammatory responses as a therapeutic strategy to provide an improved approach for treating neurologic symptoms in neonatal hydrocephalus.

Materials and Methods
Mice and body size measurements
The Ccdc39prh/prh allele (i.e., prh mice; Stottmann et al., 2011) was maintained on a mixed congenic strain background (50% CD1-background). Heterozygous Ccdc39prh/+ male and female were crossed, and age-matched for both sexes of homozygous Ccdc39 mutant (Ccdc39prh/prh, hereafter prh) and wild-type (Ccdc39+/+w, hereafter WT) mice were used. Mice were housed in specific pathogen-free conditions, and all experiments were performed according to the Institutional Animal Care and Use Committee guidelines of the Cincinnati Children’s Hospital Medical Center.

Body weight and head size were measured daily from postnatal day (P4) through P8. Head size was measured using a caliper as a sum of lengths in three directions of the head; top-bottom (parallel to chin direction), left-right (ear to ear), and rostral (just anterior to zygomatic bone)-caudal (posterior to occipital bone), in millimeter accurate to ±0.01 mm.

Genotyping
DNA was prepared from mouse toes by adding 50 μl NaOH, boiling at 95°C for 30 min, and finally adding Milli-Q water with pH 8 Tris buffer. Genotyping of the prh mouse line was done using TaqMan Sample-to- SNP kit (Applied Biosystems) for a single-nucleotide change at chr3: g.33731448A>T (assay ID: AH6R6X7).

Administration of drugs
Bindarit was obtained from Cayman Chemical Company, dissolved at 20 mg/ml in ethanol (for the experiment in Figs. 3, A–D) or 100 mg/ml at dimethylsulfoxide (DMSO; Sigma-Aldrich; for the experiment except above), and stored at −20°C for up to three months. DMSO was used over concerns of ethanol-related dermatitis, and the animals with severe dermatitis were removed from the experiment (<10% occurrence). qRT-PCR results showed no difference between the two vehicles in the expression level of Mcp-1 relative to a housekeeping gene Gapdh. On the day of administration, bindarit in ethanol was diluted in 5% Tween 80 (Sigma-Aldrich) and 5% polyethylene glycol 400 (Sigma-Aldrich), and bindarit in DMSO was diluted in 90% corn oil. P4 mouse pups of either sex were randomly assigned to the vehicle or bindarit treatment groups. Bindarit was given at 100 mg/kg body weight subcutaneously daily from P4 through P7. For the vehicle group, either the same amount of ethanol diluted in 5% Tween 80, 5% polyethylene glycol 400, or DMSO diluted in 90% corn oil was injected. The dose of bindarit was determined based on the previous reports (Cerri et al., 2016).

Pharmacokinetic analysis
For pharmacokinetic analysis, bindarit was given once at P7 or four times (P4, P5, P6, P7) subcutaneously to WT or prh mutant mice. Mice were killed at 0, 3, 12, 24, or 48 h after drug administration; blood was collected in lithium heparin tubes, and plasma was isolated by centrifugation at 5000 rpm for 5 min. Brains and livers were then quickly
removed and frozen in liquid nitrogen, and all the samples were sent to Charles River Laboratories, where further measurement was performed. Bindarit levels were determined after solid-phase extraction using liquid chromatography/tandem mass spectrometry (LC/MS/MS) on an API-6500 (Applied Biosystems) instrument. Organ samples were homogenized at 5 × in 80:20 in water:acetonitrile. Samples were then diluted at an additional 2 × into plasma for a final dilution factor of 10 × and run against a plasma curve.

RNA extraction and quantitative RT-PCR
Total RNA treated with DNase was extracted from a sagittal half hemisphere of the cerebrum of P8-P10 mice using RNeasy Plus Mini kit (QiAGEN). cDNA was synthesized using the SuperScript Vilo cDNA Synthesis kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed with QuantStudio 5 using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions, and mRNA expression relative to housekeeping gene Gapdh mRNA was calculated with two technical replicates for each biologically independent primer. Primer sequences are listed in Table 1.

nCounter mouse inflammation panel v2
Total RNA from nontreated WT and phr samples at P8 (n = 6 in each group) extracted above were sent to NanoString. Direct mRNA expression levels at 4°C. Samples were measured using the NanoString nCounter gene expression system. In each, all 100 ng of total RNA was hybridized with probes from the nCounter Mouse Inflammation v2 panel (NanoString) at 65°C for 16–19 h according to the nCounter Gene Expression Assay Manual. Excess probes were washed away using a two-step magnetic bead-based purification on the nCounter Prep Station (GEN1). The nCounter Digital Analyzer (GEN1) was used to count individual fluorescent barcodes and quantify target molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed. Gene expression data were analyzed using nSolver analysis software (NanoString) and the Molecular Signatures Database (MSigDB; Subramanian et al., 2005). Pathway scoring using nSolver was performed based on Reactome pathway database gene set (Fabregat et al., 2016), and the gene enrichment analysis (GSEA) using MSigDB was performed on C8: Cell type signature gene sets (http://www.gsea-msigdb.org/gsea/msigdb/).

Immunofluorescence staining, imaging, and quantification
Brains quickly removed from phr and WT mice aged embryonic day (E) 18 to P8 (n = 142) were fixed in 4% paraformaldehyde (PFA; in PBS) overnight at 4°C. Samples were then washed with PBS, and cryoprotected in 30% sucrose in PBS then immersed in NEG50 freezing medium (Thermo Fisher Scientific); 12-μm-thick cryosections were prepared. For the immunofluorescence staining, sections for nuclear staining (NeuN, SATB2, CTIP2, TBR1, DLX5, and Ki67) were permeabilized with 0.3% Triton X-100 (Thermo Fisher Scientific) in PBS for 30 min. Sections were then incubated with primary antibodies of either anti-mouse glial fibrillary acidic protein (GFAP; 1:500, Sigma, G3893), anti-rabbit IBA1 (1:500, Wako, 019-19741), anti-goat IBA1 (1:500, Abcam, ab5076), anti-rabbit OLG2 (1:500, Abcam, ab136253), anti-rabbit CNPase (1:100, Cell Signaling, 5664), anti-rat CD68 (1:100, Abcam, ab31630), anti-rabbit GLUT1 (1:100, Abcam, ab128033), anti-rabbit neurofilament H (NF-H; 1:500, Millipore AB1989), anti-rat C3AR (1:100, Bios, BS1101R), anti-rabbit Ki67 (1:1200, Abcam, ab15580), anti-mouse SATB2 (1:50, Abcam, ab51502), anti-rat CTIP2 (1:1000, Abcam, ab183032), anti-rabbit TBR1 (1:1000, Abcam, 183032), anti-rabbit neuronal nuclei (NeuN; 1:500, Cell Signaling, D4G40), anti-rabbit DLX5 (generously provided by Prof. John Rubenstein, University of California, San Francisco; Lindtner et al., 2019), anti-rabbit Calbindin D28K (CALB; 1:2000, Millipore, IHCRI013-6), anti-goat Calretinin (CALR; 1:2000, Millipore, AB1550), anti-guinea pig vesicular GABA transporter (VGAT; 1:500, Chemicon, AB5855), and anti-rabbit Gephyrin (GPHN; 1:500, Abcam, ab32206) overnight after the blocking in 0.25% normal donkey serum/0.25% Triton X-100/PBS for 1 h. After stringent washings and subsequent incubation with fluorophore-conjugated secondary antibodies for 2 h, the sections were counterstained with DAPI (Sigma-Aldrich) for 5 min and mounted with DAPI-Fluoromount-G mounting medium (Southern Biotech). For NF-kB p65 staining, antigen retrieval with citric buffer (pH 6) at 95°C for 45 min, along with tyramide signal amplification was performed. Briefly, sections were treated with 3% H2O2 solution for 1 h after the antigen retrieval, and blocking, and anti-rabbit NF-kB p65 (1:100, Cell Signaling, #8242) incubation overnight followed. On the second day, after stringent washings, anti-rabbit IgG biotinylated antibody was incubated for 2 h. ABC, biotin solution from VECTASTAIN ABC kit (Vector) for 1 h, and tyramide dissolved in 0.1 M borate were applied for 10 min with stringent washings between the incubations. Streptavidin Alexa Fluor 594 (Thermo Fisher Scientific) was then applied for 1 h, followed by DAPI and coverslip. For the cortical layer-specific markers (SATB2, CTIP2, and TBR1), since CTIP2 was clearly specific to Layer V, we defined Layer II–IV as SATB2-stained area bounded by CTIP2-positive Layer V, and Layer VI as TBR1-positive area bounded by CTIP1-positive area. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer’s protocol using ApopTag Fluorescein in situ apoptosis Detection kit (Millipore). To combine immunofluorescence signals with TUNEL, TUNEL assays were performed first, followed by blocking, primary and secondary antibody incubations as described above.

Fluorescently stained sections were observed under a confocal laser scanning microscope (Nikon A1RGAAsp inverted microscope) or Nikon Ti-E 90i upright widefield. Images were processed and analyzed using NIS Elements software (Nikon). For the quantification of OLIG2, GLUT1, NF-H, CD68, C3AR, SATB2, CTIP2, TBR1, DLX5, CALR, CALB, IBA1, TUNEL, NF-kB p65, Ki67-positive cells, the General Analysis (GA) tool in NIS Elements software was used, and the cell number was counted as an average of three regions of interest (ROIs) images captured with 20× optical lens for OLIG2, DLX5, CALR, TUNEL, and Ki67 positive cells. CD68 positive cells were counted based on each section per animal, GLUT1 and NF-H positive area were measured by NIS Elements software (Nikon), and the number of p65-expressing cells was counted as a sum in three ROIs. For the measurement of cortical, ventricular, and white matter area, three comparable sagittal DAPI-stained sections per animal were picked, and average area was calculated with ImageJ software (NIH). ImageJ software was also used to estimate the CNP positive area in the strictly comparable sections at which the rostral migratory system from the subventricular zone to olfactory bulb is clearly visible. For characterizing C3AR-positive cells, images were captured at three ROIs in white matter and gray matter with 20× optical lens, and the numbers of IBA1 positive round amoeboïd-shaped macrophages and ramified-shaped microglia were independently manually counted. For the above quantification, the evaluator was blinded to the drug treatment options and genotype.

In situ hybridization (RNAcope)
RNAcope in situ hybridization was performed according to the manufacturer’s protocol of RNAcope Multiplex Fluorescent v2 Assay (Advanced Cell Diagnostics). Briefly, slides with 12-μm-thick cryosections were washed in PBS for 5 min, and hydrogen peroxide solution was incubated for 10 min. Slides were then performed target retrieval in Target Retrieval Reagent at 95°C for 5 min, briefly washed in nuclease-free water for 10 s, moved into 100% ethanol for 3 min, and dried completely. After application of Protease III for 15 min at 40°C, a combination of the probes for purinergic receptor P2Y, G-protein coupled, 12 (P2y12; NM_025771.3, catalog #317601) mRNA, Gcr2 (C-C motif chemokine receptor 2; NM_009915.2, catalog #501681) mRNA, Mcp-1 (NM_011333.3, catalog #311791), and Tnem19 (NM_146162.2, catalog #472901) mRNA were hybridized for 2 h at 40°C in a HybEZ oven. After stringent wash in wash buffer and signal amplification steps, TSA plus Cyanine three or TSA Plus Cyanine 5 (PerkinElmer) were incubated for 30 min at 40°C, and horseradish peroxidase signals of each channel were developed. To combine RNAcope signals with immunofluorescence, slides were further washed after the development of RNAcope fluorophore signals, followed by blocking, primary and secondary antibodies
incubation as described above. Pictures of slides with RNAscope in situ hybridization were captured under Keyence BZ-X810.

Synapse quantification
Synapses were quantified according to the protocol previously described (Shi et al., 2015) with modification. Brain tissues were sectioned at 12-µm thickness. Three ROIs in comparable sections per animal were used for immunostaining with antibodies against the presynaptic protein, anti-guinea pig VGLUT2 (1:1000, Millipore, AB2251-I) and the postsynaptic protein anti-rabbit HOMER1 (1:1000, Millipore, ABN37). The stained sections were Images captured under Nikon A1R microscope with 100× oil objective lens with Nyquist focusing with pinhole 1.2 AU, gaining 0.11 µm/pixel resolution. Z-stack images of 21 serial optical frames (0.25-µm step, 5-µm total thickness from the surface of the tissue) were captured from the sensorimotor cortex in the area of Layer II/III and Layer IV. The denoised and deconvolved images were imported to Imaris 9.6.0 software, and 3D reconstructed images were analyzed with surface and spot creation algorithm. HOMER1 postsynaptic domain was defined with diameter of largest sphere at 0.6 µm, seed points diameter 0.5 µm with signal intensity threshold 28%. VGLUT2 presynaptic protein was defined with diameter of largest sphere at 0.6 µm, seed points diameter 0.6 µm, with signal intensity threshold 15%. Iso-surface was created for HOMER1 and was used to mask VGLUT2 signal, to define overlapping volume. Synapses were threshold of 15%. Iso-surface was created for HOMER1 and was used to

Table 1. Primers used for qRT-PCR experiments

<table>
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<tr>
<th>Gene</th>
<th>Forward primer (5′ – 3′)</th>
<th>Reverse primer (5′ – 3′)</th>
<th>GenBank #</th>
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<tr>
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<td>TGACTGCACTGATGGGACAC</td>
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<tr>
<td>Ikkα−1</td>
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<td>CAGGGAGCTGTCATTAGGGACATC</td>
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<td>TGAGACAGAGATTTGAGGAGTCA</td>
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Statistical analysis
All values are expressed as the mean ± SEM. Statistical computation of group differences among two drug treatment groups (vehicle and bindarit) and two genotype groups (WT and prh) was performed using a two-way ANOVA with Tukey’s post hoc test for multiple comparisons. Repeated measures of ANOVA with Sidak’s post hoc test was performed for behavioral tests with multiple time points. Survival data were analyzed using the log-rank procedure of Kaplan–Meier survival analysis. Body weights and head sizes normalized to body weights were analyzed by repeated measures using a two-way ANOVA, followed by Tukey’s post hoc test. The differences between two groups were compared with the Student’s t test. p < 0.05 was generally considered to be statistically significant, while p < 0.01 was used for NanoString pathway analysis. All statistical computations were performed using GraphPad Prism.

Results
Activation of myeloid cells in the prh hydrocephalic mouse brains
We found that several proinflammatory cytokine mRNAs that are elevated in other hydrocephalus models (Lattke et al., 2012; Kurt et al., 2010; Karimy et al., 2017; Zhang et al., 2018a) are also upregulated in the P8–P10 prh hydrocephalic brains; Mcp-1 (p = 0.0057), Trf-α (p = 0.019), Toll-like receptor 4 (Tlr−4; p = 0.0310), and Heme oxygenase-1 (Hmox-1; p = 0.0464, n = 5–6 in each group; Fig. 1A). Among these cytokines, Mcp-1 mRNA expression level was well correlated with the severity of ventriculomegaly calculated using the contralateral hemisphere (R² = 0.7385, n = 12; Fig. 1B). In order to comprehend the neuroinflammatory signature and identify active inflammatory cascades in the developing brain with hydrocephalus, we next performed nCounter Mouse Inflammatory panel assay using cerebral cortical RNAs (n = 6 in each group). Among 250 inflammatory genes tested, we found 28 genes upregulated...
Figure 1. Inflammation and myeloid cell involvement in prh hydrocephalic mouse brains. 

A, The Mcp-1 (**p = 0.0057), tumor necrosis factor (Tnf-α (*p = 0.019), Tlr-4 (p = 0.0310), Hmox-1 (p = 0.0464) mRNA expression relative to housekeeping gene Gapdh mRNA are significantly upregulated in prh relative to that in WT mouse brains (without treatment, P8–P10, Student’s t test, n = 5–6 in each group). 

B, Mcp-1 mRNA in nontreated prh is well correlated with the severity of ventricular size (R² = 0.7385, n = 12). 

C, Volcano plot of differentially expressed genes (DEGs) in P8 prh brains relative to WT (data based on n = 5–6 in each group). Adjusted p value < 0.5 is shown. 

D, DEGs with nominal p value < 0.01.
and 19 genes downregulated in *prh*, including upregulation of Ccl3, C3ar, Tyrobp, Trem2, C1qb, C1qa, Tlr4, and Cd86 [significantly differentially expressed genes with adjusted p value < 0.50 (Fig. 1C); genes with nominal p < 0.01 (Fig. 1D)]. nSolver pathway analysis identified 16 gene sets upregulated in *prh*, including activation of NF-kB transcription factor (p = 0.0027) and innate immune response (p = 0.0003; Fig. 1E, shown in blue). Also, gene enrichment analysis with cell type signature gene sets (C8) identified five gene sets significantly enriched in *prh* (False discovery rate q-value < 25%, nominal p value < 0.01; Fig. 1E, shown in blue), including developing heart macrophages (p < 0.001, Cui et al., 2019), major types of microglia (p < 0.001; Zhong et al., 2018; enrichment plot shown in Fig. 1F), and embryonic cortex microglia (p < 0.001; Fan et al., 2018). These microglia-signature and macrophage-signature gene sets annotated overlapping genes, most of which were also identified to be significantly upregulated in *prh* mutants as the volcano plot showed, along with *Mcp-1* (Fig. 1C). These results suggested that mononuclear myeloid cells are the major players in the inflammatory response in *prh*, which is in accordance with previous findings in other neonatal hydrocephalus models cited above.

The pharmacokinetics of subcutaneously administered bindarit in neonatal mice
In order to modulate the proinflammatory response, we evaluated the pharmacokinetics of a potent anti-inflammatory drug, bindarit, which inhibits myeloid cell activation by blocking inflammation-mediated nuclear translocation of NF-kB (Mora et al., 2012), in neonatal WT and *prh* mice. A single dose subcutaneous administration of bindarit (molecular weight 324.4 g/mol, 100 mg/kg) in P7 WT mice reached the brain within 3 h (85,400 ng/g = 263 µM) and was gradually metabolized, to 17,800 ng/g (54.9 µM) at 24 h (Fig. 2A). As bindarit significantly suppresses *Mcp-1* mRNA in cultured microglia at 50 µM (Ge et al., 2012), this result indicated successful delivery of bindarit to the neonatal mouse brain and maintained concentrations above the target level for 24 h. Once-a-day injections for four consecutive days did not change the tissue level of bindarit in the brain at 24 h from the last injection compared with that of a single injection (Fig. 2B), which indicated that bindarit does not accumulate in the brain after daily dosing and thus justifies the effectiveness of once-a-day injections of bindarit at 100 mg/kg. Furthermore, neither increasing the dose of bindarit to 200 mg/kg nor the *prh* genotype altered the bindarit level in the brain and the liver at 24 h (Fig. 2C). Therefore, the *prh* genotype did not affect the brain availability of bindarit at P7, as compared with WT.

Bindarit attenuates edematous swelling of white matter but only slightly improves ventricular enlargement in *prh* mutants
We performed daily injections of bindarit (100 mg/kg, s.c.) in WT and *prh* mice from P4 through P7 and evaluated tissue area of gray matter (i.e., total cerebral cortical area excluding white matter), white matter, and lateral ventricles measured from strictly comparable, DAPI-stained low power field pictures. The enlarged lateral ventricle size in vehicle-treated (veh-) *prh* (**p < 0.0001 to veh-WT) was significantly and mildly attenuated by bindarit treatment (**p = 0.0083), still exhibiting significantly larger ventricles in bindarit-treated (bin-) *prh* relative to WT (**p < 0.0001 to veh-WT; Fig. 2D, middle). The white matter was edematous and swollen in veh-*prh* relative to WT (**p < 0.0001 to veh-WT; Fig. 2D, right), E, reinforcing the finding that there is considerable neuroinflammation and tissue damage in the white matter of *prh* (Goulding et al., 2020). It is notable that this white matter swelling, because of edema, was dramatically improved in bin- *prh* (**p = 0.0008 compared with veh-*prh, n = 13 in each group; Fig. 2D, right). The total gray matter size was comparable among all groups (p = 0.800 for genotype, p = 0.468 for drug, two-way ANOVA, n = 13 in each group; Fig. 2D, left). Survival (Fig. 2F), body weight (Fig. 2G), and head circumference (Fig. 2H) in *prh* were not improved with bindarit treatment.

Myelination and vascularization in *prh* white matter is improved with bindarit treatment
To characterize the impact of white matter edema in *prh* and its alleviation by bindarit in myelination, we performed immunohistochemistry with two oligodendrocyte lineage cell-specific markers. The pan-oligodendrocyte lineage marker, Olig2, showed the significantly reduced density of total oligodendrocytes in veh-*prh* (**p < 0.0001 compared with veh-WT) and it was rescued by bindarit treatment (p = 0.0385 compared with veh-*prh, n = 12–13 in each group), most likely reflecting the edematous change in the white matter (Fig. 3A). Interestingly, mature oligodendrocyte and myelination marker CNP (2′,3′-cyclic nucleotide 3′ phosphodiesterase) staining showed a significant reduction of myelination in veh-*prh* (**p < 0.0001 compared with veh-WT), which was partially recovered in bin-*prh* (p = 0.0113 compared with veh-*prh, n = 12–13 in each group; Fig. 3B). These data indicate that bindarit treatment improves the maturation of oligodendrocytes and myelination in *prh* brains. To evaluate the axons adjacent to periventricular regions, NF-H staining was performed. It showed intact periventricular axon bundles even in veh-*prh* (Fig. 3C), implying that the observed myelin impairment precedes the axonal loss reported in neonatal hydrocephalus. Since the reduction of cerebro spinal fluid flow in white matter has been well documented in neonatal hydrocephalus patients (Shirane et al., 1992) and reduced vessel density in white matter has supported the finding (Jones et al., 1991), an endothelial cell marker GLUT1 staining was performed. It showed reduced vessel density in veh-*prh* compared with WT (p < 0.0004 compared with veh-WT), with significant recovery of vessel density in bin-*prh* (p = 0.0019 compared with veh-*prh, n = 6 in each group; Fig. 3D). The vessel density was also normalized to DAPI positive cells to adjust for the effect of the edematous swelling of the tissues, but it was still significantly decreased in the veh-*prh* (p = 0.0219 compared with veh-WT) and recovered with bindarit (p = 0.0253 compared with veh-*prh, graph not shown). These data demonstrate the protective effect of bindarit on white matter edema along with the improved myelination and preserved vascularization in the periventricular white matter in *prh* hydrocephalic brains.

Bindarit suppresses CD68+ activated myeloid cell infiltration and *Mcp-1* expression in the periventricular white matter
We next evaluated myeloid cell activation in *prh* brains. Consistent with our previous reports (Emmert et al., 2019;
Figure 2. Pharmacokinetics of bindarit and its effect on ventricular and white matter enlargement. A, Tissue concentration of bindarit in the brain, plasma, and liver at 0, 3, 12, 24, 36, and 72 h after the subcutaneous injection. Dotted line indicates 16,620 ng/g as effective tissue concentration. B, Tissue concentration of bindarit in brain and liver, 24 or 48 h after the single, once-a-day injection (1×) and once-a-day injection for four consecutive days (4×). C, Tissue concentration of bindarit in brain and liver 24 h after the single injection of 100 or 200 mg/kg in either WT or prh genotype. D, Tissue area size of gray matter (total cerebral cortical area excluding white matter), ventricular size relative to gray matter (***p, 0.0001 to vehicle-treated (veh-) WT, ‡‡‡‡p, 0.0001 to bindarit-treated (bin-) WT, **p = 0.0083 to veh-prh), and white matter size relative to gray matter (††††p, 0.0001 to veh-WT, ‡‡‡‡p, 0.0001 to bin-WT, ***p = 0.0008 to veh-prh) measured from DAPI-stained low power field pictures (two-way ANOVA followed by Tukey's test, n = 13 in each group). E, Low power field pictures of DAPI-stained animals. Double-headed arrow in veh-prh shows the swollen white matter (scale bars: 500 μm). GM, gray matter; WM, white matter; HC, hippocampus. F, Kaplan–Meier survival plot shows the survival rate of veh-WT and bin-WT are 93%, and veh-prh and bin-prh are 87.5% at P8 (nsignificant (ns) in 4 groups). G, Body weight analysis demonstrates that prh exhibit less weight growth compared with WT (§§§p < 0.001, prh vs WT) and no significant (ns) difference by treatment (repeated measures two-way ANOVA followed by Tukey's test, n = 8–10). H, Head circumference as a sum of the three directions (top-bottom, left-right, rostral-caudal, mm) normalized to body weight gradually declines in WT as they grow, but it stays relatively large in prh (§§§p < 0.001, prh vs WT), with no significant (ns) difference by drug treatment (repeated measures two-way ANOVA followed by Tukey's test, n = 8–12).
Goulding et al., 2020), there was a vast accumulation of CD68\(^{+}\)-activated myeloid cells (Boer et al., 2006; Van Vliet et al., 2012; Tanaka et al., 2013) in the periventricular white matter of the veh-prh, whereas there were very few CD68\(^{+}\) cells in veh-/bin-WT and remarkably, bin-prh cortex (Fig. 4A). We found that daily bindarit injections suppressed activated myeloid cell accumulation in the prh mutant brain (**\(p = 0.0040, n = 7\) in each group; Fig. 4A, graph) coincided with the reduced white matter

Figure 3. Myelination and vascularization in prh white matter with bindarit treatment. A, Number of pan-oligodendrocyte lineage marker OLIG2\(^{+}\) cells per WM shows reduced oligodendrocyte density in veh-prh and improvement in bin-prh (\(p = 0.0367, \dagger \dagger \dagger \dagger p < 0.0001\) vs veh-WT, \(\dagger \dagger \dagger \dagger p = 0.001\) vs bin-WT, \({ }^{*} p = 0.0385\) vs veh-prh; two-way ANOVA followed by Tukey’s test, \(n = 12-13\) in each group; scale bars: 100 \(\mu\)m). B, The mature myelination marker CNP positive staining area \% in WM shows significant reduction of myelination in veh-prh and partial recovery in bin-prh (\(\dagger \dagger \dagger \dagger p = 0.0001, \dagger \dagger \dagger \dagger p < 0.0001\) vs veh-WT, \(\dagger \dagger \dagger \dagger \dagger p = 0.002, \dagger \dagger \dagger \dagger p < 0.0001\) vs bin-WT, \({ }^{*} p = 0.0133\) vs veh-prh; two-way ANOVA followed by Tukey’s test, \(n = 10-13\) in each group; scale bars: 100 \(\mu\)m). C, Neurofilament-labeled axons (NF-H) positive area (\(\mu\)m\(^2\)) in WM shows no significant difference between veh-WT and veh-prh (\(p = 0.7244\); Student’s t test, \(n = 5\) in each group; scale bars: 200 \(\mu\)m). D, Endothelial cell marker, GLUT1 positive staining area per WM (\%) shows reduced vascular densities in veh-prh and significant recovery in bin-prh (\(\dagger \dagger \dagger \dagger p = 0.0004\) vs veh-WT, \(\dagger \dagger \dagger \dagger p = 0.0001\) vs bin-WT, **\(p = 0.0019\) vs veh-prh; two-way ANOVA followed by Tukey’s test, \(n = 6\) in each group; scale bars: 100 \(\mu\)m). WM, white matter; LV, lateral ventricles; HC, hippocampus.
Figure 4. Bindarit’s effect on myeloid cell activation in the periventricular white matter. A, CD68⁺-activated myeloid cells are rarely seen in veh-WT and bin-WT at P8, but significantly increased in white matter (WM) of veh-prh, which is significantly suppressed in bin-prh (†††p < 0.0001 vs veh-WT, ‡‡‡p < 0.0001 vs bin-WT, **p = 0.0040 vs veh-prh; two-way ANOVA followed by Tukey’s test, n = 6–7 in each group; scale bars: 100 μm). B, Mcp-1 mRNA relative to Gapdh mRNA is upregulated to 3.47 ± 0.82 times relative to veh-WT in veh-prh and is significantly suppressed in bin-prh (†††p = 0.0047 vs veh-WT, ‡‡‡p = 0.0016 vs bin-WT, **p = 0.0135 vs veh-prh; two-way ANOVA followed by Tukey’s test, n = 6 in each group; scale bars: 100 μm). C, Mcp-1 in situ hybridization shows exclusive Mcp-1 mRNA expression by ameboid-shaped IBA1⁺ myeloid cells in the periventricular WM and lower cortical layers of veh-prh.
injury described above (Figs. 2D, 3). Mcp-1 mRNA, the primary downstream target of bindarit, was 3.47 ± 0.82 times higher in the veh-prh brain than veh-WT (\(** p = 0.0055\)), but was significantly suppressed after bindarit treatment (1.31 ± 0.35 times to the veh-WT, \( p = 0.0979\) for bin-prh vs veh-WT, \(*** p = 0.0164\) for bin-prh vs veh-prh, \( n = 6\); Fig. 4B). To identify Mcp-1-expressing cells, we performed RNAseq in situ hybridization and found exclusive Mcp-1 expression by amoeboid-shaped IBA1 + myeloid cells in the prh mutants’ periventricular white matter and deep portions of the cortical plate (Fig. 4C), but not by astrocytes/pericytes/neurons, and at a negligible level in endothelial cells (data not shown). The number of Mcp-1 mRNA-expressing IBA1 + cells in prh brains was significantly reduced by bindarit treatment (Fig. 4C), in accordance with the reduction in the number of amoeboid-shaped myeloid cells. We further analyzed the cellular expression pattern of the highly upregulated gene, C3ar, detected in nontreated prh animals (Fig. 1). The complement system is a key regulator of innate immunity and C3AR, a G-protein-coupled receptor for C3A complement protein signaling, has been described as a central mediator of microglial activation in response to a variety of CNS diseases (Gasque et al., 1998; Zhang et al., 2020). We found that C3AR protein is almost solely expressed in the IBA1 + cells in both WT and prh brains (Fig. 4D). In WT, the C3AR signal was weakly expressed on the ramified-shaped homeostatic microglia in gray matter, whereas C3AR was strongly expressed in the IBA1 + amoeboid cells in veh-prh coinciding with C3ar mRNA upregulation in nontreated prh brains (Fig. 1C,D). Importantly, the number of C3AR-expressing IBA1 + amoeboid cells was significantly reduced by bindarit treatment (Fig. 4D, graph). Quantitative RT-PCR showed that, in addition to Mcp-1, the inflammatory genes related with microglia/macrophage activation including C3ar, Cd68, and Tlr4, as well as the proinflammatory microglia/macrophage activation (= classical activation; M1) marker Cd40 mRNA (Liu et al., 2013; Zhou et al., 2017) were significantly suppressed in bin-prh brains (Fig. 4E). In contrast, expression levels of the anti-inflammatory microbial/macroglial activation (= alternative activation; M2) markers Arginase 1 (Arg1) and Interleukin 4 (I4; Cherry et al., 2014; Abdelaziz et al., 2020), were not differentially expressed in nontreated prh relative to WT (data not shown), and were not changed by bindarit treatment (Fig. 4E). These results suggest that bindarit suppresses proinflammatory myeloid cells activation in the hydrocephalic brain.

Impaired development of cortical neurons in the prh mutant and the ameliorative effect of early postnatal bindarit treatment

In the early postnatal mouse cerebral cortex, postmitotic pyramidal neurons extend exuberant projections as callosal (Fame et al., 2011) and collateral axons (Larsen and Callaway, 2006) as well as dendrites which characterize their “pyramidal” shape (Larsen and Callaway, 2006; Kroon et al., 2019). In neonatal hydrocephalus (Del Bigio, 2010) and its models, axonal damage along with hypomyelination in the periventricular white matter (Del Bigio et al., 1994, 1997) and reduced dendrite arborization (Kriebel et al., 1993; Harris et al., 1996) have been reported as developmental deficits. To characterize the benefit of bindarit in cortical development with neonatal hydrocephalus, we evaluated the cortical layer thickness and neuronal cell densities of upper layers (II–IV) and lower layers (V and VI) using layer-specific markers; SATB2 (II–IV), Ctip2 (V), and Tbr1 (VI), respectively (Fig. 5A,B). The thickness of cortical Layer II through IV was significantly reduced in veh-prh (\(*** p < 0.0001\) compared with veh-WT; Fig. 5A, bottom left, B, top left), which was partially, but significantly, improved in bin-prh (\( * p = 0.011\) compared with veh-prh; Fig. 5A, bottom right, B, top left). Despite the improvement in Layer II–IV thickness, the cell density of SATB2 + neurons in these upper cortical layers were not significantly changed by bindarit treatment (\( p = 0.6875\), bin-prh vs veh-prh, \( n = 9–10\) in each group; Fig. 5B, bottom left). The Ctip2 + Layer V thickness was also reduced in prh brains (\(*** p = 0.0005\), prh vs WT), but was not recovered by bindarit (\( p = 0.9869\), bin-prh vs veh-prh, \( n = 9–10\) in each group; Fig. 5B, top middle). Conversely, Tbr1 + Layer VI thickness, as well as the cell density of Ctip2/Tbr1 positive Layer V/VI neurons, were not significantly reduced in prh brains and thus no effect of bindarit treatment was observed (Fig. 5B, bottom middle and right). These data indicate that (1) postnatal hydrocephalus does not reduce the neuronal cell densities at this stage (i.e., P8), and (2) cortical Layers II through V are vulnerable to the postnatal hydrocephalus insult leading to cortical thinning. However, Layers II–IV are more responsive to bindarit treatment, possibly because they are farther from the detrimental periventricular edema, and/or the later timing of active neuropil development than the lower layers (Kwan et al., 2012; Mukhtar and Taylor, 2018).

Embryonic neuronal cell migration and cortical thickness remains unchanged in prh, and no neuronal or astrocytic cell death is seen at P8

To rule out the possibility that the lack of ependymal ciliogenesis itself affects the embryonic neuronal cell migration and cortical development before hydrocephalus starts in the postnatal prh mouse, we performed cortical layers-specific analysis at E18 in WT and prh mutants. The thickness as well as neuronal cell density of each cortical layer was not significantly different between WT and prh brains (\( n = 3\) in each group; Fig. 6A). Moreover, TUNEL staining at P8 showed no difference in apoptotic neuronal cell death between WT and prh cortex (Fig. 6B). TUNEL signal was also not observed in GFAP + astrocytes in WT or prh (Fig. 6C). These data confirmed normal embryonic neuronal cell migration and intact presence of the postnatal neuronal soma in prh neonatal hydrocephalus mice.

Excitatory synapse maturation is impaired in prh brains, and improved by bindarit treatment

To determine whether synaptic maturation is affected in the prh mutant cortex, we evaluated excitatory synapse numbers defined by an overlap between the presynaptic protein VGLUT2 (Fujiyama et al., 2001; Nahmani and Erisir, 2005) and postsynaptic protein Homer1 (Gutierrez-Mecinas et al., 2016) at P8 in Layers II–IV of the somatosensory cortex. We focused on Layers II–III (Fig. 7A) and IV (Fig. 7B)
separately because the neuronal constituents of each of these layers are largely distinct. In the prh mutant, in line with the thinning of Layers II–IV (Fig. 5), we found reduced VGLUT2 (presynaptic) staining in the veh-prh cortex within Layers II–III (†††p = 0.0089 vs veh-WT; Fig. 7A, left) and Layer IV (†p = 0.0103 vs veh-WT; Fig. 7B, left). Similarly, HOMER1 (postsynaptic) staining was reduced in Layers II–III (†††p = 0.0003 vs veh-WT; Fig. 7A, middle) and Layer IV...
(†††p = 0.0007 vs veh-WT; Fig. 7A, right) and in Layer IV (****p < 0.0001 vs veh-WT; Fig. 7B, right; see also Movie 1 (veh-WT) vs Movie 2 (veh-prh) from Layer IV]. In Figure 5, we showed that bindarit treatment improved the thickness of Layers II–IV, and we hypothesized this could be because of enhanced neuronal matura-

tion including synaptogenesis. Indeed, bindarit treatment was observed to improve synapse numbers in Layers II–III (*p = 0.0317 bin-prh vs veh-prh; Fig. 7A, right) and in Layer IV [****p <
0.0001 bin-prh vs veh-prh; Fig. 7B, right; see also Movie 2 (veh-prh) vs Movie 4 (bin-prh) from Layer IV]. Despite the significant improvement in mature synapse numbers (i.e., VGLUT2/HOMER1 double staining) after bindarit treatment, the slight improvements in VGLUT2-only ($p = 0.2830$, bin-prh vs veh-prh in Layer II/III, $p = 0.1391$, bin-prh vs veh-prh in Layer IV) or HOMER1-only ($p = 0.1531$, bin-prh vs veh-prh in Layer II/III, $p = 0.0803$, bin-prh vs veh-prh in Layer IV) staining did not reach statistical significance (Fig. 7A,B, left and middle). These findings indicate that neonatal hydrocephalus leads to the impairment of synaptic maturation, and that the anti-inflammatory agent bindarit improves it via controlling myeloid cells functions in the neonatal hydrocephalic brain.

Interneuron morphology is less mature in prh cortex and is improved by bindarit treatment

One common complication of neonatal hydrocephalus patients is epilepsy (Vinchon et al., 2012). Epilepsy generally occurs when the balance of neuronal excitation and inhibitory counteraction is perturbed (Treiman, 2001), and thus various agonists of GABA, one of the main inhibitory neurotransmitters, have been prescribed as antiepileptic drugs (Macdonald and Kelly, 1995). As the postnatal cortical Layers II–V were affected and thinner in the prh mutant (Fig. 5), we next asked whether GABAergic interneurons, which compose around 20–30% of neocortical neurons in rodents (Markram et al., 2004), are altered in prh hydrocephalus mice. DLX5 is a transcription factor that regulates the migration and differentiation of parvalbumin-expressing cortical interneurons (Wang et al., 2010; Lim et al., 2018). Using
were reduced in veh-prh and bin-prh ($\dag\dag t p = 0.0025$, veh-prh vs veh-WT, $tp = 0.0380$, bin-prh vs veh-WT), and bindarit treatment did not achieve significant recovery ($p = 0.5564$, bin-prh vs veh-prh, $n = 4–5$ in each group; Fig. 8C). We attempted to evaluate GABAergic inhibitory synapses with presynaptic protein VGAT and postsynaptic protein GPHN in the upper cortical layers; however, both the immunoreactivities were very sparse even in WT at P8 as observed in a previous report (Virtanen et al., 2018), and therefore inhibitory synapses at this early postnatal age were unable to be compared accurately either between prh versus WT or with/without drug treatment. These results show that bindarit treatment can partially rescue interneuron maturation (i.e., dendritic development in CALR$^+$ neurons) in the upper cortical layers, potentially by rescuing the homeostatic microglial function.

**Bindarit suppresses microglial inflammatory activation and proliferation in the white matter and restores the ramified-shaped residential microglia in the gray matter**

We investigated whether these amoeboïd-shaped myeloid cells (Fig. 4A,C,D) in the white matter of prh mutants mainly originate from microglia, or peripheral monocyte-derived macrophages which infiltrate into the CNS after the injury. We performed *in situ* hybridization for the microglial signature genes *P2ry12* (purinergic receptor P2Y, G-protein-coupled 12) and *Tmem119* (transmembrane protein 119; Hickman et al., 2013; Butovsky et al., 2014; Bennett et al., 2016), as well as the monocyte signature gene *Ccr2* (chemokine (C-C motif) receptor 2), in combination with IBA1 immunohistochemistry. It has been shown that even after the inflammatory transformation of microglia, these signature genes’ expression remains expressed at low levels (Jordão et al., 2019). Strikingly, nearly all the IBA1$^+$ amoeboïd-shaped cells in the veh-prh white matter were positive for *P2ry12* (Fig. 9A, middle row) and *Tmem119* (Fig. 9B, middle row). Slightly decreased *P2ry12* and *Tmem119* expression in these cells compared with that of ramified-shaped mature microglia in veh-WT (Fig. 9A, top row, B, top row), suggested that they are activated and transformed microglia with proinflammatory phenotype (Jordão et al., 2019). In contrast, we did not observe tissue infiltrating Ccr2$^+$ monocytes in the prh mutant brain parenchyma at all, and no Ccr2 expression was detected in these activated IBA1$^+$ cells in the white matter (Fig. 9A, middle row). We confirmed that reliable Ccr2 signal was observed in monocytes in the blood and some of the IBA1$^+$ P2ry12-negative macrophages localized in the subarachnoid space and meninges (Fig. 9A, bottom row, arrowhead). Similarly, *Tmem119* was not expressed in meningeal IBA1$^+$ cells (Fig. 9B, bottom row, arrowhead), validating these mRNA probes for the specific labeling of microglia. Quantification from 100 amoeboïd-shaped IBA1$^+$ cells in the white matter ($n = 2–3$) showed that 98% and 85% were positive for *P2ry12* and *Tmem119*, respectively, but not Ccr2 (Fig. 9C). This result indicated that the majority of these amoeboïd-shaped myeloid cells originated from microglia.

Microglia undergo maturation during the postnatal period, during which time they acquire their homeostatic ramified morphology. The majority of the microglia migrate to the brain from the yolk sac (~E9.5), or from the fetal liver (~E13.5; Ginhoux et al., 2013), and keep their proliferative status at low levels in the postnatal brain under normal development (Arnoux et al., 2013; Smolders et al., 2019). In veh-prh, the amoeboïd-shaped IBA1$^+$ cells in the periventricular white matter often expressed cell proliferation marker Ki67, while only few IBA1$^+$ cells expressed Ki67 in WT and bin-prh (Fig. 9D). In addition, there were
significantly more TUNEL positive IBA1+ ramified-shaped microglia in the gray matter of veh-prh compared with WT (†††p = 0.0006 compared with veh-WT; Fig. 9F). Along with this observation, density of ramified-shaped homeostatic microglia in the gray matter was significantly decreased in veh-prh compared with WT (†††p = 0.0006 compared with veh-WT; Fig. 9E). Interestingly, the number of ramified-shaped microglia in gray matter was significantly restored in bin-prh as compared with veh-prh (*p = 0.0140 compared with veh-prh, n = 6 in each group; Fig. 9F), with significantly less TUNEL positive cells
Figure 9. Bindarit suppresses microglial activation in the white matter and restores the ramified microglia in the prh gray matter. A, The microglial signature gene P2ry12 (purinergic receptor P2Y, G-protein-coupled 12) is expressed on ramified-shaped IBA1<sup>+</sup> cells in WTs at P8 (top row). The amoeboid-shaped IBA1<sup>+</sup> cells in the white matter (WM) of veh-prh at P8 also express P2ry12 to a lesser extent, but do not express the monocyte signature gene Ccr2 [chemokine (C-C motif) receptor 2; middle row]. The Ccr2 probe is validated, and its signal is observed on IBA1<sup>+</sup> cells outside the brain in veh-WT at P8, which do not have P2ry12 signal (bottom row, arrowhead), whereas P2ry12 is expressed on cortical microglia on the same slide (bottom row, arrow; n = 3–4; scale bars: 50 μm for low-magnification pictures, 10 μm for high-magnification pictures). B, Another microglial signature gene Tmem119 is expressed on ramified-shaped IBA1<sup>+</sup> cells in veh-WT at P8 (top row). The amoeboid-shaped IBA1<sup>+</sup> cells in the WM of veh-prh at P8 also express Tmem119 to a lesser extent (middle row). Tmem119 is not expressed on IBA1<sup>+</sup> cells outside the brain in veh-WT at P8 (bottom row, arrowhead), whereas cortical microglia express Tmem119 on the same slide (bottom row, arrow; n = 2; scale bars: 50 μm for low-magnification pictures, 10 μm for high-magnification pictures). C, Graph shows the quantification of P2ry12 (96%, 98%), Tmem119 (89%, 85%), and Ccr2 (0%, 0%) expression in 100 IBA1<sup>+</sup> cells (n = 2–3) in the WM of veh-WT and veh-prh, respectively. All 100 IBA1<sup>+</sup> cells quantified in veh-prh are amoeboid-shaped cells. D, Double-staining of IBA1 and the proliferation marker Ki67 shows very few double labeled cells in the WM of veh-WT (top) but significantly more proliferating amoeboid-shaped IBA1<sup>+</sup> cells (arrows) in the WM of veh-prh (bottom; †††p = 0.0009 vs veh-WT, ††p = 0.0003 vs bin-WT). This effect is suppressed in bin-prh brains (***p = 0.0009, two-way ANOVA followed by Tukey’s test, n = 6 in each group; scale bars: 100 μm). E, TUNEL assay with IBA1 staining shows significantly more TUNEL positive (i.e., dying) ramified microglia (arrows) within the gray matter of veh-prh cortex (††p = 0.0082 vs veh-WT, †††p = 0.0004 vs bin-WT). Bindarit treatment significantly reduces this cell death in bin-prh cortex (*p = 0.0411 vs veh-prh; two-way ANOVA followed by Tukey’s test, n = 4–6 in each group; scale
Effect of bindarit on suppressing NF-kB p65 activation in myeloid cells

As bindarit has been reported to specifically downregulate the stimulus-induced inflammatory cytokine expression including Mcp-1, via inhibiting NF-kB p65 phosphorylation, subsequent nuclear translocation, and DNA binding (Mora et al., 2012), we performed NF-kB p65 staining to determine its cellular localization. The active nuclear p65 signals were prominent in the IBA1+ amoeboid-shaped cells in veh-prh (Fig. 10C), while bin-prh showed weak p65 signals mainly in the cytoplasm of the IBA1+ cells to a lesser extent than veh-prh, as well as ependymal cells and a fraction of neural progenitor cells in the subventricular zone, SVZ (Fig. 10D), similar to WT (Fig. 10A). The number of p65-expressing IBA1+ cells was significantly reduced in bin-prh (Fig. 10E, ***p < 0.0001 relative to veh-prh). Moreover, the ratio of IBA1+ cells showing nuclear p65 signal was significantly lower in bindarit-treated prh cortex (27.6 ± 3.2% in veh-prh vs 6.1 ± 10% in bin-prh, **p = 0.0028; Fig. 10F). In the WT brain, at a homeostatic state, p65 localized in the cytoplasm of ependymal cells (Fig. 10A, short thick arrows), blood vessels (Fig. 10A, arrowhead), and GFAP+ astrocytes with fusiform morphology in the ventricular-subventricular zone (Fig. 10A, left, long arrows, right, clear arrows; Plate and Bordey, 2016). The low level and steady expression of p65 protein in these cell types was in accordance with Rela mRNA expression found in the comprehensive mouse brain single-cell RNA sequencing data (http://www.mousebrain.org), validating the specificity of the p65 antibody. Together, these findings indicate that NF-kB signal activation in myeloid cells drives the accumulation of proinflammatory amoeboid-shaped cells and edema formation in the corpus callosum of the neonatal prh brain. Importantly, the targeted inhibition of NF-kB activation by bindarit supports a multitude of early postnatal neural cell development processes that are severely affected in the neonatal hydrocephalus and thus may inform an important therapeutic approach for this brain condition.

Bindarit partially improves the swimming locomotor phenotype in prh mutants

Motor skills impairment including spasticity and a variable level of paralysis has been identified in pediatric hydrocephalus patients (Rekate, 1999; Pattisapu, 2001). To evaluate the therapeutic significance of the bindarit in early neonatal brain functions, we performed an array of developmental reflex and locomotion tests available in neonatal mice. We performed the surface righting reflex (P4, P6, P8), the grasp reflex (P4, P6, P8), negative geotaxis (P4, P6, P8), and a swim test (P8) in untreated WT (n = 12) and prh mutants (n = 18 for P4 and P6, n = 15 for P8). The prh mutant showed comparable levels of reflexes tested in the surface righting reflex (Fig. 11A), negative geotaxis test (Fig. 11B), and the forelimb grasp reflex (data not shown) relative to WT. Both genotypes showed improvement over time in surface righting (p < 0.0001) and negative geotaxis (p = 0.0023 between P6 and P8; repeated-measures of ANOVA followed by Sidak’s test). In contrast, the prh mutants showed distinct locomotor phenotypes in the swim test. We found a temporary spastic paraplegia-like phenotype in the water. The spastic phenotype was characterized by either a significant delay when starting hindlimb movements (12 out of 15) or temporary pause during (three out of 15) the sequence of left-right hindlimb alternating movements in the water (7.23 ± 0.79 s for the first trial, and 3.64 ± 0.97 s for the second trial) compared with WT (1.43 ± 0.41 s for first trials and 0.18 ± 0.14 s for second trials) (Fig. 11C, ****p < 0.0001 for first trial, *p = 0.0235 for second trial, Student’s t test; Movies 5 and 6). During this time, the mutants extended hindlimbs with toes spread, which is similar to adult rats exhibiting spasticity phenotypes as a consequence of injury to upper motor neurons (Ryu et al., 2017, 2021) or neonatal V2b-ablated mice with pronounced extension of the hindlimbs and impairment of hindlimb flexion movement in the tail suspension test (Britz et al., 2015). Following this temporary spastic phenotype, prh mutants eventually started to move their hindlimbs but showed reduced frequency of alternating hindlimbs relative to WT (Fig. 11D, ****p < 0.0001 for first trials, *p = 0.0127 for second trials, Student’s t test). Both of these phenotypes became less obvious in the second trial, which could be explained by habituation or learning. In fact, in WT, the frequency of alternating hindlimbs becomes slower in second trials than first trials (p = 0.0030, Student’s t test), probably reflecting habituation or learning in the swim test. Bindarit treatment improved the spastic phenotype in the mutants during the first trial compared with veh-prh (Fig. 11E, **p = 0.0086, one-way ANOVA followed by post hoc Sidak’s test) (Movie 7). Frequency of alternating hindlimbs was not reduced in both veh-prh and bin-prh compared with veh-WT (Fig. 11F), mainly because the frequency was overall reduced in veh-WT relative to untreated WT (Fig. 11D), as daily injection may have affected the baseline behavior in veh-WT. From these results, we showed that prh mutant had a locomotor defect in the swim test which resembles mild spasticity and paraplegia phenotypes in nonweight-bearing environments (i.e., swimming) and bindarit partially, but significantly, improved this phenotype.

Discussion

Notwithstanding the common finding of neuroinflammation in pediatric hydrocephalus (Savman et al., 2002; Ullig et al., 2004; Schmitz et al., 2007; Habiyaremye et al., 2017), the role of inflammation and activated myeloid cells in hydrocephalic brains remains largely unknown. The common histologic changes in neurons in neonatal hydrocephalus, including reduced dendritic arborization and axonal growth, have been considered to be secondary to ventriculomegaly and tissue compression (Del Bigio, 2010; Femi-Akinlosotu et al., 2019). Therefore, the clinical treatment for hydrocephalus has been almost solely focused on CSF diversion (Kahle et al., 2016). Here, we examined myeloid cell activation in a robust neonatal hydrocephalus model and showed that inhibiting neuroinflammation via suppressing NF-kB activation significantly improves neural cell development and motor behavior deficits, despite ongoing ventriculomegaly in these brains.

To examine inflammatory gene expression in the neonatal hydrocephalic brain, we identified a number of upregulated and
downregulated genes, which represented microglia/macrophage activation, NF-kB activation, and specific cytokine upregulation (e.g., Mcp-1, Tnfα, Ccl3) in prh mutants. We demonstrated that bindarit, a potent anti-inflammatory drug that suppresses NF-κB pathways (Mora et al., 2012; Oddi et al., 2019), significantly reduced proinflammatory cytokine production, including Mcp-1 in amoeboid-shaped IBA1⁺ cells in the prh brain. Furthermore, this treatment

Figure 10. Bindarit works on activated microglia through suppressing NF-κB p65 activation in the white matter. A, In P8 WT brains, NF-κB p65 signals are seen in the cytoplasm of ependymal cells (short arrows), vessels (arrowhead), and GFAP⁺ astrocytes with fusiform morphology in the ventricular-subventricular zone (long arrows; high power, far right, open arrows; scale bars: 50 μm). B, In veh-prh sections stained with no primary antibody as a control, only red blood cell auto-fluorescence is observed which is easily identified by the morphology and size of the cells (scale bars: 50 μm). C, p65 signals in veh-prh are seen in IBA1⁺ amoeboid-shaped cells in the white matter, both within the nucleus and cytoplasm (arrows), along with cytoplasmic signals in the ependymal, vessel, and some radial glial cells (scale bars: 50 μm). D, p65 signals in bin-prh sections are mainly within cytoplasm of the ependymal and fusiform-shaped astrocytic cells as in WT brain, or to a lesser extent in IBA1⁺ cells than that in veh-prh (scale bars: 50 μm). E, The total number of p65-expressing IBA1⁺ cells per three ROIs per animal is significantly reduced in bin-prh cortex (**p < 0.0001 relative to veh-prh, Student’s t test, n = 5). F, The percentage of p65 nuclear expression (shown as veh-prh) rather than cytoplasmic expression (bin-prh) in all p65-expressing IBA1⁺ cells is 27.6 ± 3.2% in veh-prh, and this is significantly reduced (6.1 ± 10%) in bin-prh cortex (**p = 0.0028 relative to veh-prh, Student’s t test, n = 5 in each group, total >200 p65⁺ cells; scale bars: 5 μm).
Figure 11. Bindarit partially improves the swimming locomotor phenotype in prh mutants. A, Both untreated WT and prh show improvement in surface righting reflex over time (**p < 0.0001) and there is no difference between WT and prh (p = 0.8627; n = 12–18 in each group, repeated measures ANOVA, followed by post hoc Sidak’s test). B, There is no difference in the time for negative geotaxis between untreated WT and prh (p = 0.1914) and both genotypes have significant improvement over time between P6 and P8 (p = 0.0023; n = 12–18 in each group, repeated measures ANOVA, followed by post hoc Sidak’s test). The frequency of falling from the surface during the negative geotaxis test is similar in WT (8%) and prh mutants (10%). C, During swim testing, untreated prh show significantly longer “time without hindlimb alternation” or spastic time, relative to WT, both in first trials and second trials on putting prh into the warm water (****p < 0.0001 for first trial, *p = 0.0235 for second trial, n = 12–15 in each group, Student’s t test). D, Frequency of alternating hindlimbs is significantly less in prh relative to WT (****p < 0.0001 for first trial, *p = 0.0127 for second trial, Student’s t test). E, Vehicle-treated (veh-)prh show significantly longer time without hindlimb alternation, relative to veh-WT (††††p < 0.0001), and bindarit treatment significantly improved and shortened this spastic time compared with veh-prh, in the first trials (****p = 0.0086, one-way ANOVA followed by post hoc Sidak’s test, n = 8–13 in each group), but there is no significant difference among the groups in the second trials (p = 0.2647, one-way ANOVA). F, There is no difference among veh-WT, veh-prh, and bin-prh in the frequency of alternating hindlimbs, excluding the time without hindlimb alternation, in the first (p = 0.3339) or second trials (p = 0.0861; n = 8–13 in each group, one-way ANOVA).
reduced edema formation in the periventricular white matter of the hydrocephalic prh brain. The activated myeloid cells observed in the prh periventricular white matter at P8 were actively proliferating and we propose that they are most likely microglia in origin, based on our findings in microglia-specific gene expression and the absence of parenchymal infiltrating monocytes in this model (Fig. 9A–C). Our results suggest that microglia in neonatal hydrocephalic brains use the NF-kB/MCP-1 pathway to transform from homeostatic/ramified states to proinflammatory/amoeboid states, which has been observed in other neuronal insult models (Hanisch and Kettenmann, 2007; Graeber and Streit, 2010). These cells acquire expression of immature microglial gene signatures (Moyon et al., 2015) and become more proliferative and migratory, with the production of proinflammatory cytokines (Kaur et al., 2007). Although the direct upstream cellular events of NF-kB/MCP-1 activation in hydrocephalus remain unclear, we showed therapeutic potential of NF-kB p65 inhibition in attenuating the subsequent proinflammatory responses caused by a broad range of damage-associated molecular patterns (DAMPs) signals and their receptors including TLR4.

Bindarit is a small-molecule drug available both orally and by subcutaneous/intravenous/intraperitoneal injection and has shown its benefits in the CNS disease models (Cerri et al., 2016). We showed that subcutaneously-injected bindarit reaches the brain at physiologically effective (>50 μM) levels (Fig. 2A–C). Recent work showed that bindarit binds to Fatty acid-binding protein (FABP)4 to exert its NF-kB modulating effect (Oddi et al., 2019), and thus FABP4 (Duffy et al., 2017) in microglia could be the target of bindarit. Possible effects and mechanism of bindarit on other cell types remain for future investigation.
Our findings of targeting microglia align with some previous findings in doxycycline-analog, minocycline treatment for kaolin-induced hydrocephalus models (McAllister and Miller, 2010) and other neonatal brain injuries (Dommergues et al., 2003; Fox et al., 2005). Minocycline is not recommended for treating children especially with hydrocephalus because of its possible side effects of raising intracranial pressure. On the other hand, specific modulation of the TLR4/NF-kB signaling in hydrocephalus may be more beneficial as it also may decrease CSF overproduction in the choroid plexus and blood-brain barrier breakdown reported in other neonatal brain injury models (Karimy et al., 2017, 2020a,b). Blocking TLR4/NF-kB signaling prevents posthemorrhagic vasospasm and platelet-derived IL-6 release and neutrophil invasion (Karimy et al., 2020b). While taking advantage of a broad function of bindarit in NF-kB modulation (Mora et al., 2012; Oddi et al., 2019), our study demonstrated that blocking a proinflammatory response is beneficial for neural cell development in pediatric hydrocephalus. Higher levels of MCP-1 protein have been noted in the CSF of hydrocephalus patients (Killer et al., 2010), and Mcp-1 mRNA in prh brains directly correlated with the severity of ventriculomegaly in this model. Mcp-1 is a well-known downstream target of the TLR4/NF-kB pathway and a mediator of the microglia/monocyte responses (Platten et al., 2003; Deng et al., 2009). Therefore, MCP-1 may become a good biomarker to monitor neuroinflammation levels in hydrocephalus patients.

One of the most drastic neuroprotective effects of bindarit in neonatal hydrocephalus was the preservation of cortical Layer II–IV thickness, independent of ventricular size restoration. Similar to previous findings in other hydrocephalus models (Del Bigio and Zhang, 1998), we found no apoptotic neurons nor astrocytes. Importantly, however, our data showed that gray matter microglia undergo apoptotic cell death, further validating the loss of homeostatic microglia in perinatal hydrocephalus. The mouse cortex completes neuronal cell migration around P4 and proceeds to elaborate dendrites and synapse formation during the first three postnatal weeks (Innocenti and Price, 2005; Kast and Levitt, 2019). Microglia in the postnatal brain play a vital role in regulating brain development through supporting axon guidance, myelination, synaptic patterning, and neurogenesis (Schafer and Stevens, 2015; Mosser et al., 2017; Lenz and Nelson, 2018; Rosin et al., 2018; Cserep et al., 2021). Therefore, the observed microglial dysfunction in prh hydrocephalic brains likely affects cortical neuronal maturation and synaptogenesis. Indeed, we found that the number of mature excitatory synapses, double stained with presynaptic VGLUT2 and postsynaptic HOMER1 markers, were severely reduced in veh-prh. Nevertheless, it was significantly recovered by bindarit treatment along with the restoration of homeostatic microglia. This result suggests that bindarit treatment improved synaptogenesis by preserving the function of ramified-shaped microglia, as proposed in adult Layer II/III neurons (Akiyoshi et al., 2018). The effects of bindarit on enhancing GABAergic interneuron maturation in CALR+ interneurons also suggest the necessity of microglial physiological function on the development of inhibitory neurons and cortical networks (Favuzzi et al., 2021), as seen in the microglia-depleted embryonic brain (Squarzoni et al., 2014). As we observe no detrimental effect of bindarit in the developing WT brains, we propose that the proinflammatory microglia-targeting therapy (e.g., bindarit) may be more beneficial for brain development than removal of all microglia (e.g., using colony-stimulating factor-1 receptor inhibitor) to alleviate neuroinflammation and promote healthy brain development.

Similarly, we noticed that white matter is significantly injured with reduced myelination and less vascular densities in prh brains. Accordingly, periventricular white matter injury and myelination delay has been described in neonatal hydrocephalus patients (Gadson et al., 1979; Hanlo et al., 1997) and rodent models (Del Bigio et al., 1997; Khan et al., 2006). Two lines of evidence that microglia play essential roles in oligodendrocyte regulation and myelination are: (1) microglial ablation leads to a reduced number of oligodendrocyte precursor cells (OPCs; Hagemeyer et al., 2017), and (2) inflammatory microglia aggressively phagocytose OPCs in the white matter of Cnp knock-outs (Garcia-Aguado et al., 2019). It is noteworthy that bindarit-treated hydrocephalus restored the number of resident microglia, which may have contributed to the improved myelination observed.

Lastly, our novel finding of locomotor defects in the swim test using prh mice suggests a hydrocephalus-related spasticity. Interestingly, previous reports using a rat model of spinal cord injury that quantitatively evaluated “spasticity” found similar postural findings in a swim test (Ryu et al., 2017, 2021). Spasticity has been reported in pediatric hydrocephalus populations (Pattisapu, 2001). Theoretically, any injury to, or delay in development of the descending innervation from cortex to spinal motor neuron, including corticospinal tract (CST) and corticoreticular input to the medulla, or potentially the ascending innervations, can cause spasticity (Wieters et al., 2021). The CST develops by around P8 in mice (Gianino et al., 1999; Namikawa et al., 2015). Spinthalamic and thalamocortical pathways, which carry ascending sensory information and can also affect motor function, develop embryonically (Fitzgerald, 2005; Davidson et al., 2010). We also noted that the frequency of alternating hindlimbs in prh was mildly reduced, likely indicating their reduced motor function in the no-weight-environment in addition to spasticity. Bindarit significantly shortened the spastic time in water, which suggests that reducing inflammatory damage improved the development of motor circuits, though further investigation is needed. The negative findings of general developmental reflexes in prh could be because of their compensatory mechanism in intracranial pressure with abnormally enlarged cranial volume (Fig. 2H; Abdelhamed et al., 2018), as suggested in some forms of human neonatal hydrocephalus (Amiel-Tison et al., 2002; Kiesler and Ricer, 2003).

In the current study, we demonstrate that activated myeloid cells contribute to altered cortical development in prh mutant brains and that suppression of this neuroinflammation with bindarit ameliorates microglial, neuronal, and behavioral alterations in this mouse genetic model of neonatal hydrocephalus. In our continued efforts to achieve better neurologic outcomes in neonatal hydrocephalus patients, this study provides molecular insights and potential translatable therapeutic targets, which may redefine the standard care for this condition. The limitation of this study is that prh mutants die at a young age (Abdelhamed et al., 2018), and thus, we could not observe the long-term effect of bindarit treatment. Nevertheless, our findings suggest that modulation of neuroinflammation, in combination with CSF diversion surgery, may provide a significant improvement for treatment of pediatric hydrocephalus.

References


