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Inhibitory Effect of LPS on the Proliferation of Oligodendrocyte Precursor Cells through the Notch Signaling Pathway in Intrauterine Infection-induced Rats*

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Summary: Periventricular white matter injury (PWMI) is very common in survivors of premature birth, and the final outcomes are a reduction in myelinated neurons leading to white matter hypomyelination. How and (or) why the oligodendrocyte lineage develops abnormally and myelination is reduced is a hot topic in the field. This study focuses on the effect of intrauterine inflammation on the proliferation of oligodendrocyte lineage cells and the underlying mechanisms. Lipopolysaccharide (LPS) (300 μg/kg) was intraperitoneally injected into pregnant Sprague-Dawley rats at embryonic days 19 and 20 to establish a rat model of intrauterine infection-induced white matter injury. Corpus callosum tissues were collected at postnatal day 14 (P14) to quantify the number of oligodendrocytes, the number and proliferation of oligodendrocyte precursor cells (OPCs), and the expression of myelin proteins (MBP and PLP). Furthermore, the expression of Wnt and Notch signaling-related proteins was analyzed. The results showed that the number of oligodendrocytes in the corpus callosum tissues of LPS-treated rats was reduced, and the expression levels of myelinating proteins were down-regulated. Further analysis showed that the Notch signaling pathway was down-regulated in the LPS-treated group. These results indicate that intrauterine LPS may inhibit the proliferation of OPCs by down-regulating the Notch rather than the Wnt signaling pathway, leading to hypomyelination of white matter.
Key words: oligodendrocyte precursor cells; intrauterine infection; hypomyelination; lipopolysaccharide; signaling pathway

A large number of clinical and animal model studies have proven that infection, especially intrauterine infection, is one of the main important causes leading to brain injury in preterm infants[1–4]. In survivors of premature birth, periventricular white matter injury (PWMI) is the dominant form of brain white matter injury causing long-term neurological disabilities, which can manifest in the offspring as hypomyelination, cystic lesions, palsy and microcephaly[5, 6].

The underlying mechanisms of PWMI are still unknown because of its complicated etiology and unknown contributing factors. The candidate mechanisms may be related to ischemia-reperfusion injury, massive activation of microglia, production of oxygen free radicals, and damage to oligodendrocytes (OLs) and their precursor cells, which influences myelin development and maturation[7, 8]. Microglial activation, OL and OL precursor cell (OPC) dysfunction and the interaction between glial cells and

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the microenvironment are all involved. In particular, disturbances to normal OL cell lineage development are central to the development of any preterm white matter injury[9].

Our previous studies illustrated that lipopolysaccharide (LPS) can induce maternal infection and central hypomyelination and that activated protein C can protect against white matter injury in offspring[10, 11]. However, OPCs can proliferate and differentiate into mature OLs, the latter of which produce myelin basic protein (MBP) and proteolipid protein (PLP), which can wrap axons. Injury to OPCs and OLs can irreversibly impair myelination and white matter development. Taken together, these observations have led us to hypothesize that intrauterine inflammation may influence the maturation and proliferation of OPCs, leading to hypomyelination. Accordingly, we aimed to study how OPCs and OLs change in LPS-induced neonatal white matter injury.

1 MATERIALS AND METHODS

1.1 Animals and Brain Tissue Preparation

We previously established an animal model of LPS-induced intrauterine infection[10]. In brief, 3 adult female rats were caged overnight with 1 fertile male rat, and the next morning, upon the presence of a vaginal plug observed by the naked eye, was designated embryonic day 1 (E1). Pregnant rats were randomly divided into the control and LPS groups (n=10 per group). The animals in the LPS group were intraperitoneally injected with 300 μg/kg LPS (*E. coli*, serotype 055:B5, Sigma, USA) at E19 and E20 to establish the LPS-induced intrauterine infection rat model. The animals in the control group were given the same doses of sterile saline. Neonatal pups were raised normally and were anesthetized and killed at postnatal day 14 (P14) for further experiments. Rat brains were harvested after perfusion, fixed with 4% PFA/DEPC overnight at 4°C, cryoprotected with 30% sucrose and embedded in optimal cutting temperature compound (OCT). Subsequently, 12-μm-thick sections were prepared for histological staining.

The experimental protocol was approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (China).

1.2 BrdU Injection and Staining for Proliferation Experiments

P13 pups were intraperitoneally injected with BrdU (50 mg/kg dissolved in PBS). Twenty-four h after BrdU injection (P14), the brain tissues were harvested and fixed as described above. Brain sections were processed for immunohistochemistry (IHC) with anti-BrdU antibodies and the OPC marker PDGFRα.

For BrdU staining, after antigen retrieval in Retrievagen A solution (BD Pharmingen, USA), the sections were incubated in 2 mol/L HCl for 10 min at 37°C and washed with 0.1 mol/L borate buffer for 10 min at room temperature. Then, the IHC staining protocol was performed.

1.3 IHC Staining

The fixed P14 brain tissues were cut coronally at 12 μm (n=6 for each group). After antigen retrieval in Retrievagen A solution, sections were washed with PBS and blocked with 10% goat serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 1 h at room temperature before incubation with the primary antibody overnight at 4°C. The sections were washed with PBS 3× for 5 min each wash, incubated with the appropriate fluorophore-conjugated secondary antibody for 1 h at room temperature and again washed with PBS 3× for 5 min each wash. After counterstaining with DAPI (1:1000, Invitrogen, USA) for 5 min, the sections were examined under a fluorescence microscope.

1.4 Antibodies

The following primary antibodies were used for IHC or Western blotting: rat anti-BrdU (1:100), rabbit anti-PDGFRa (1:300), rabbit anti-GST-π (1:500), rat anti-MBP (1:1000), rabbit anti-PLP (1:2000), goat anti-Notch-1 (1:200), rat anti-Hes-1 (1:200), rat anti-Wnt3a (1:200), mouse anti-β-catenin (1:500) and mouse anti-β-actin (1:5000). Secondary antibodies included goat anti-mouse or anti-rat conjugated with either Alexa 488 (1:1000) or Alexa 546 (1:1000), goat anti-rabbit conjugated with Alexa 546 or 555 (1:1000), and goat anti-mouse or -rabbit IgG HRP (1:3000).

1.5 Immunofluorescence *In Situ* Hybridization

Immunofluorescence *in situ* hybridization (FISH) was performed according to the protocol previously described[12–14]. In brief, probes targeting PLP and Pdgfrα (Addgene, USA) were generated by digesting the plasmids with *Eco*RⅠ and *Hind*Ⅲ, respectively. DIG-labeled RNA probes were generated using Sp6 and T7 polymerase *in vitro* transcription (Roche Applied Science; DIG RNA Labeling Kit, USA) according to the manufacturer's instructions. Hybridization occurred at 68°C overnight. The tissue was then washed at 65°C with 5×SCC for 5 min and then with 500 mL 0.2×SCC until most of the solution had evaporated (almost 2–3 h). To detect the DIG-labeled probes, the TSA-Plus Cyanine 3 labeling system (Perkin Elmer, USA) was used according to the manufacturer's instructions.

1.6 Western Blotting

The corpus callosum was dissected under a Leica stereo microscope (Germany), followed by washes in PBS and lysis in ice-cold RIPA buffer (1% Nonidet P-40, 50 mmol/L Tris pH 7.6, 120 mmol/L NaCl, 1 mmol/L EDTA) containing protease inhibitor cocktail set 1 (Calbiochem, USA). Protein concentration was determined by the Bio-Rad protein assay (Bio-

Rad), and equal amounts of proteins were used for Western blotting analysis. MBP and PLP were used as myelination markers, and Wnt3a, β-catenin, Notch-1 and Hes-1 were detected to test the signaling pathway. β-actin was used as an internal standard, and band intensity was quantified with the ImageJ image analysis system. The ratio of protein to total protein was calculated. For each experiment, 3 to 5 independent repeats were performed.

1.7 Statistical Analysis

Images were blindly quantified. All data are represented as $\bar{x}\pm s_{\bar{x}}$. Statistical analysis was conducted by GRAPHPAD Prism Software (GraphPad Software version 5, USA) using unpaired *t*-tests. A *P* value <0.05 was defined as statistically significant.

2 RESULTS

2.1 LPS-induced Decreases in Oligodendrocytes

To detect whether the pups had hypomyelinated white matter, we examined mature OLs in the corpus collosum using the OL markers PLP and GST-π by FISH and IHC, respectively. The number of PLP+ cells and GST-π+ cells was significantly less in the LPS group than that in the control group ($P<0.05$ for PLP+ cells and $P<0.01$ for GST-π+ cells, fig. 1 and fig. 2). The data demonstrated that LPS exposure decreased the number of OLs, potentially resulting in less myelin protein and hypomyelination.

2.2 Marked Downregulation of MBP and PLP Protein Expression in the Corpus Collosum after LPS Injection

To confirm whether the reduction in the number of OLs resulted in less myelin production, MBP and PLP expression was detected in the corpus callosum by Western blotting. The protein expression of MBP and PLP at P14 in pups exposed to LPS injection was significantly lower than that in control pups ($P<0.05$ for both, fig. 3). Therefore, the data revealed that after LPS exposure, the number of OLs decreased significantly,

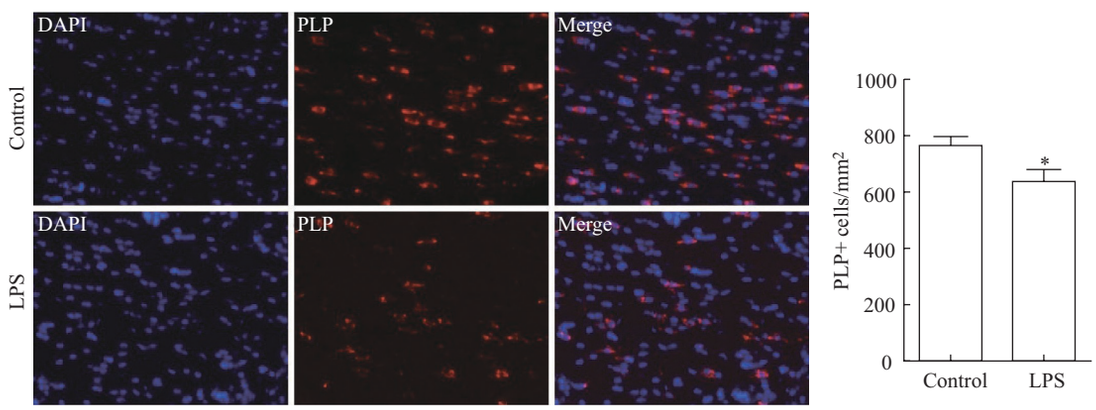

Fig. 1 The decreased number of PLP+ cells in the corpus callosum at P14 after LPS injection
The number of PLP+ cells in the corpus callosum at P14 was significantly less in the LPS group (634.2±41.68/mm^2) than in the control group (766.1±28.45/mm^2) (t=2.61, $^*P$=0.026, n=6 per group). DAPI: blue; PLP: red

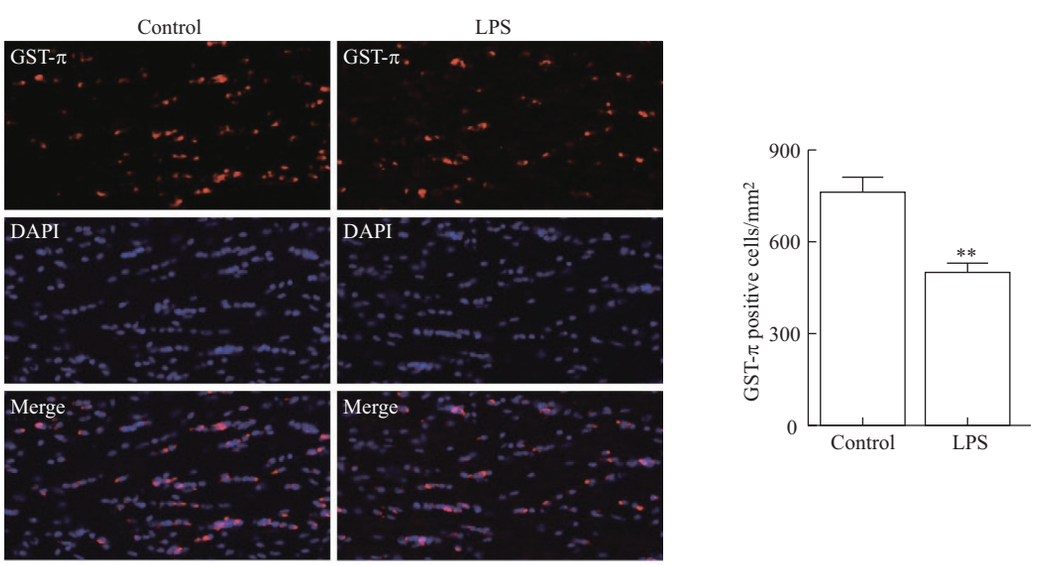

Fig. 2 The decreased number of GST-π+ cells in the corpus callosum at P14 after LPS injection
Representative images show significantly fewer GST-π+ cells in the LPS group than in the control group (493.7±33.80/mm^2 *vs.* 762.0±50.83/mm^2, t=4.39, $^{**}P$=0.001, n=6 per group). DAPI: blue; GST-π: red

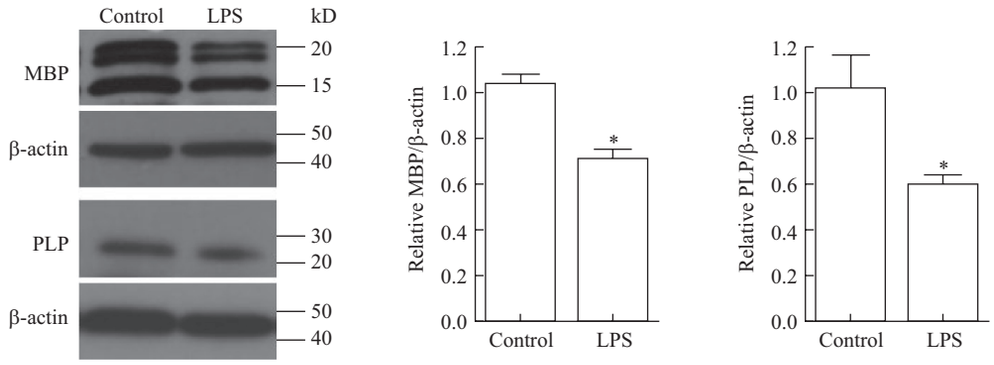

Fig. 3 Downregulation of MBP and PLP expression in the corpus callosum after LPS injection
The MBP and PLP protein expression levels in the corpus callosum at P14 were significantly lower in the LPS group than in the control group (n=3 for each group; for MBP protein: t=3.97, $^*P$=0.02; for PLP protein: t=2.84, $^*P$=0.04). β-actin was used as a loading control.

resulting in less myelin protein production and hypomyelination in the corpus callosum.

2.3 Relationship between Reduction in OPCs and Decrease in OPC Proliferation

Hypomyelination resulted from a decrease in the number of mature OLs, which are formed from OPC differentiation and proliferation. Accordingly, we hypothesized that OPCs might be altered by intrauterine LPS injection. First, we quantified the number of OPCs in the corpus callosum by FISH using a probe targeting Pdgfrα. There were significantly fewer Pdgfrα+ cells in the LPS group than in the control group (P<0.05, fig. 4), indicating that the decreased number of OLs might result from a reduction in OPC proliferation. Furthermore, the proliferative ability of OPCs was detected by BrdU injection. BrdU and Pdgfrα double staining by IHC was carried out to detect the proliferation of OPCs. As hypothesized, the LPS group exhibited fewer BrdU and Pdgfrα double-positive cells than the control group (P<0.05, fig. 5).

2.4 Inhibitory Effects of LPS on OPC Proliferation via the Notch Rather Than the Wnt Signaling Pathway

Many signaling pathways have been reported to be involved in OPC proliferation. To investigate the signaling mechanism, we examined the expression of proteins in two common signaling pathways, Notch and Wnt. Wnt3a and β-catenin expression in the corpus collosum was not different between the two groups (n=5, P>0.05 for both proteins, fig. 6). However, Notch-1 and Hes-1 expression was significantly lower in the LPS group than in the control group (n=5, P<0.05 for both proteins, fig. 7).

3 DISCUSSION

Intrauterine infection and inflammation are considered two major causes closely associated with high risks of PWMI. In a follow-up of very-low-birth-weight neonates, 9.4% had PWMI[15]. The offspring also developed hypomyelination and some other symptoms, such as neurological and intellectual impairments[16, 17]. There are many mechanisms that could result in these outcomes, such as epigenetic alterations and inflammation. The candidate mechanisms include inflammatory cytokines that are secreted by microglia and destroyed neurons and OLs[18–20]. Our previous data showed that LPS-

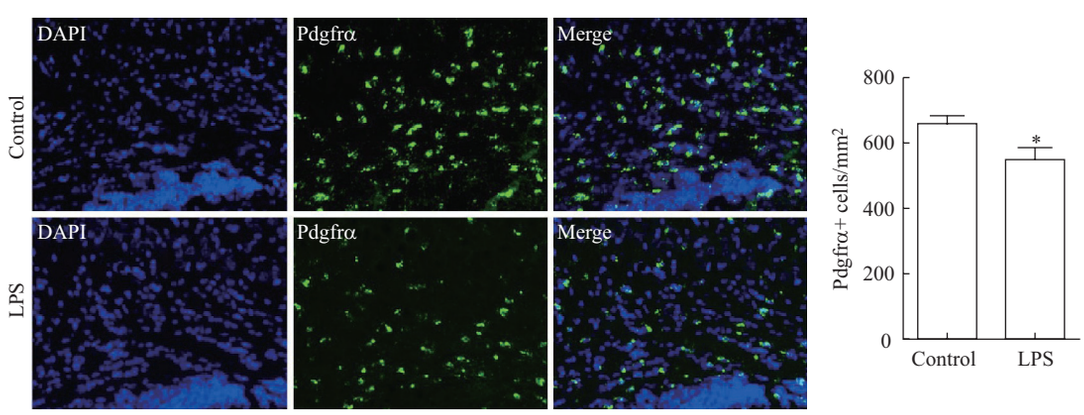

Fig. 4 Pdgfrα+ OPCs in the corpus callosum at P14 by FISH
The number of Pdgfrα+ OPCs in the corpus callosum was significantly less in the LPS group (518.7±37.88/mm^2) than in the control group (657.0±23.59/mm^2) (n=6 per group, t=3.10, $^*P$=0.01). DAPI: blue; Pdgfrα: green

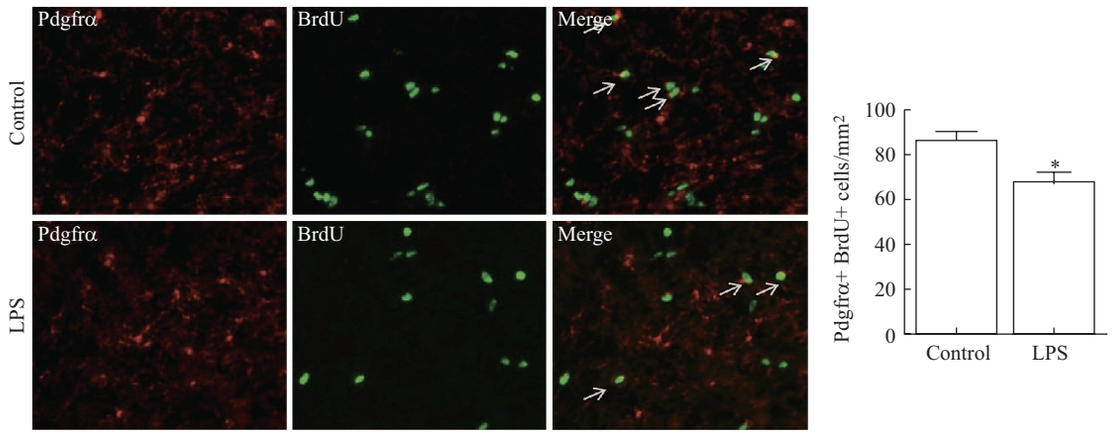

Fig. 5 The inhibitory effects of LPS injection on the proliferation of OPCs
The proliferation of OPCs in the corpus callosum at P14 was detected by BrdU staining. There were fewer Pdgfrα and BrdU double-positive cells in the LPS group (67.5±4.16/mm^2) than in the control group (86.1±3.43/mm^2) (n=6 per group, t=3.45, $^*P$=0.013). Pdgfrα: red; BrdU: green

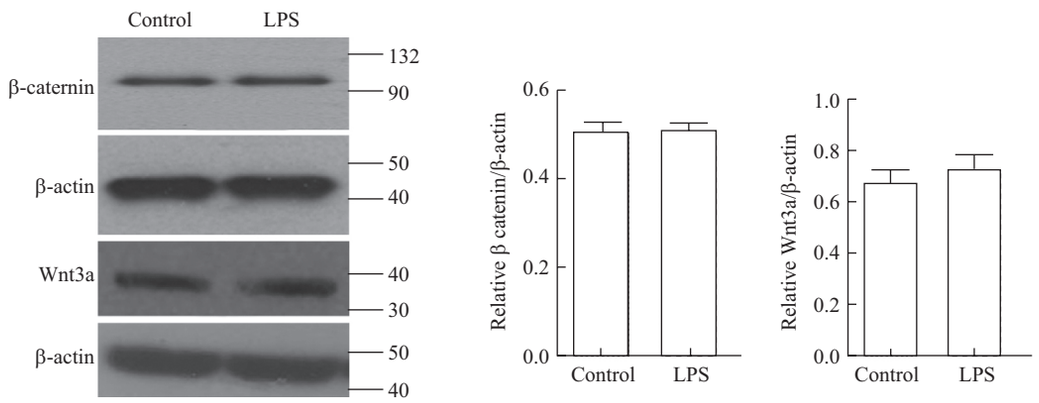

Fig. 6 No change in the Wnt signaling pathway
There was no significant difference in Wnt3a or β-catenin protein expression in the corpus callosum at P14 between the two groups (n=5 for each group; for Wnt3a, t=0.74, P=0.48; for β-catenin protein, t=0.14, $^*P$=0.89). β-actin was used as a loading control.

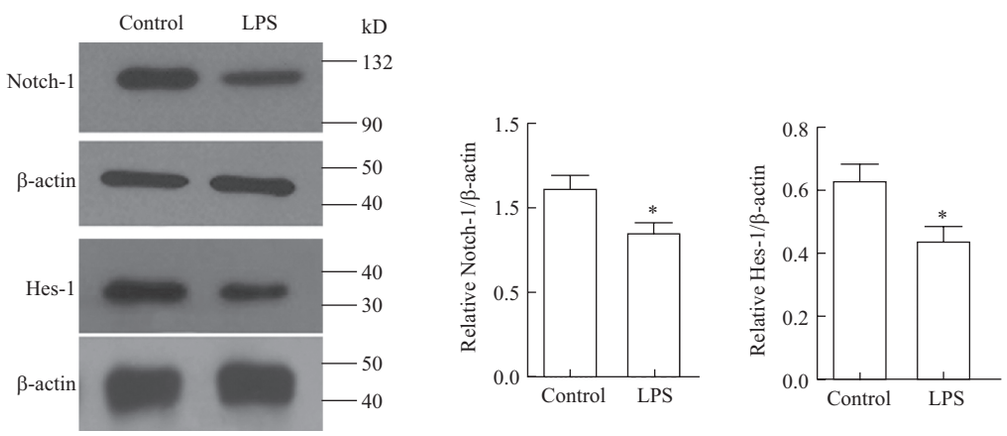

Fig. 7 Involvement of the Notch signaling pathway in the reduced proliferation of OPCs
The relative expression levels of Notch-1 and Hes-1 proteins in the LPS group in the corpus callosum at P14 were significantly lower than those in the control group (n=5 for each group; for Notch-1, t=2.96, $^*P$=0.02; for Hes-1, t=2.98, $^*P$=0.02). β-actin was used as a loading control.

induced intrauterine infection could transport across the blood-brain barrier, resulting in hypomyelination in the offspring, with activated microglia expressing protease-activated receptor 1 (PAR1) and a significant reduction in MBP expression in the periventricular region[10, 11]. However, how and why hypomyelination

occurrs is still unknown.

The myelination of axons by OLs forms an electrical insulator that allows rapid signal transmission. To myelinate axons, OPCs exit the cell cycle and differentiate into premyelinating OLs, when they begin expressing more major myelin proteins [e.g., MBP, PLP and myelin oligodendrocyte glycoprotein (MOG)]. Once differentiation begins, OLs undergo complex morphological changes to extend numerous processes. Any change in the steps involved in myelination will influence myelination. OPCs are generated at E16, and in our model, LPS was injected at E19 and E20; therefore, we examined whether LPS injection affected OPC development. In this study, we focused on the changes in the OL lineage. After LPS injection, there were fewer mature OLs and lower myelin protein expression, resulting in the myelination of fewer axons. Many studies have reported on the mechanisms underlying maternal inflammation exposure in fetal rats, such as programmed cell death apoptosis in white matter and delayed development and differentiation arrest of OPCs[21, 22]. A reduction in OLs results from a chance in OPC proliferation, and our data showed lower OPC proliferation in the LPS group, with fewer BrdU/Pdgfrα double-positive cells than in the control group. The reduction in the proliferation of OPCs might result from OPC injury alone or from other interactions between glia and the microenvironment. The crosstalk between different glial cell types, such as microglia-OPCs, extracellular matrix (ECM), OLs and astrocyte-OPCs, is now receiving an outstanding amount of attention[23, 24].

The Wnt signaling pathway plays a critical role in embryonic development, such as through the proper regulation of cell proliferation and migration. This process increases nuclear and cytoplasmic β-catenin, which can initiate transcriptional activation of proteins such as cyclin D1 and c-myc. These two proteins control the G1-to-S phase transition in the cell cycle. Recently, studies have shown that the Wnt/β-catenin signaling pathway participates in neonatal brain injury and human OPC proliferation[25, 26]. First, we aimed to measure how Wnt/β-catenin expression changed in our model. The data showed that the Wnt/β-catenin signaling pathway did not participate in OPC proliferation. Then, we focused on the Notch signaling pathway because it is a highly conserved cell signaling system present in most multicellular organisms. The Notch signaling pathway promotes proliferative signaling during neurogenesis and plays a major role in the regulation of embryonic development[27]. The Notch pathway is primarily critical for neural progenitor cell (NPC) maintenance and self-renewal. In recent years, other functions of this pathway, including glial cell specification, neurite development and learning and memory, have been identified. Our data showed that the expression levels of the two major proteins in this pathway, Notch-1 and Hes-1, were lower in the LPS group than in the control group, illustrating that the Notch signaling pathway is involved in the observed OPC proliferation dysfunction.

In conclusion, the present study illustrates that after LPS exposure, decreased OPC proliferation is the main cause of the reduction in the number of OLs and the resulting hypomyelination. Furthermore, the Notch signaling pathway might be involved in the underlying mechanism of this reduction in proliferation.

Conflict of Interest Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1 Araujo Júnior E, Carvalho FH, Tonni G, *et al*. Prenatal imaging findings in fetal Zika virus infection. Curr Opin Obstet Gynecol, 2017,29(2):95-105
2 Saliba E, Marret S. Cerebral white matter damage in the preterm infant: pathophysiology and risk factors. Semin Neonatol, 2001,6(2):121-133
3 Simonsen KA, Anderson-Berry AL, Delair SF, *et al*. Early-onset neonatal sepsis. Clin Microbiol Rev, 2014,27(1):21-47
4 Graf AE, Haines KM, Pierson CR, *et al*. Perinatal inflammation results in decreased oligodendrocyte numbers in adulthood. Life Sci, 2014,94(2):164-171
5 Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res, 2001,50(5):553-562
6 Leviton A, Paneth N. White matter damage in preterm newborns—An epidemiologic perspective. Early Hum Dev, 1990,24(1):1-22
7 Wakabayashi A, Sawada K, Nakayama M, *et al*. Targeting interleukin-6 receptor inhibits preterm delivery induced by inflammation. Mol Hum Reprod, 2013,19(11):718-726
8 Thomas W, Speer CP. Chorioamnionitis: important risk factor or innocent bystander for neonatal outcome? Neonatology, 2011,99(3):177-187
9 Back SA, Riddle A, Dean J, *et al*. The instrumented fetal sheep as a model of cerebral white matter injury in the premature infant. Neurotherapeutics, 2012,9(2):359-370
10 Jin SJ, Liu Y, Deng SH, *et al*. Protective effects of activated protein C on neurovascular unit in a rat model of intrauterine infection-induced neonatal white matter injury. J Huazhong Univ Sci Technolog Med Sci, 2015,35(6):904-909
11 Jin SJ, Liu Y, Deng SH, *et al*. Neuroprotective effects of activated protein C on intrauterine inflammation-induced neonatal white matter injury are associated with the downregulation of fibrinogen-like protein 2/ fibroleukin prothrombinase and the inhibition of pro-inflammatory cytokine expression. Int J Mol Med,

2015,35(5):1199-1212
12 Dugas JC, Cuellar TL, Scholze A, *et al*. Dicer1 and miR-219 are required for normal oligodendrocyte differentiation and myelination. Neuron, 2015,65(5): 597-611
13 Bialas AR, Stevens B. TGF-beta signaling regulates neuronal C1q expression and developmental synaptic refinement. Nat Neurosci, 2013,16(12),1773-1782
14 Giera S, Deng Y, Luo R, *et al*. The adhesion G protein-coupled receptor GPR56 is a cell-autonomous regulator of oligodendrocyte development. Nat Commun, 2015,6:6121
15 Tsimis ME, Johnson CT, Raghunathan RS, *et al*. Risk factors for periventricular white matter injury in very low birthweight neonates. Am J Obstet Gynecol, 2016,214(3):380.e1-6
16 Van Well GTJ, Daalderop LA, Wolfs T, *et al*. Human perinatal immunity in physiological conditions and during infection. Mol Cell Pediatr, 2017,4(1):4
17 Peng CC, Chang JH, Lin HY, *et al*. Intrauterine inflammation, infection, or both (Triple I): A new concept for chorioamnionitis. Pediatr Neonatol, 2018,59(3):231-237
18 Deng Y, Xie D, Fang M, *et al*. Astrocyte-derived proinflammatory cytokines induce hypomyelination in the periventricular white matter in the hypoxic neonatal brain. PLoS One, 2014,9(1):e87420
19 Zhang Z, Jyoti A, Balakrishnan B, *et al*. Trajectory of inflammatory and microglial activation markers in the postnatal rabbit brain following intrauterine endotoxin exposure. Neurobiol Dis, 2017,111:153-162
20 Lin W, Lin Y. Interferon-γ inhibits central nervous system myelination through both STAT1-dependent and STAT1-independent pathways. J Neurosci Res, 2010,88(12):2569-2577
21 Hossain S, Bhowmick S, Jahan S, *et al*. Maternal lead exposure decreases the levels of brain development and cognition-related proteins with concomitant upsurges of oxidative stress, inflammatory response and apoptosis in the offspring rats. Neurotoxicology, 2016,56:150-158
22 French HM, Reid M, Mamontov P, *et al*. Oxidative stress disrupts oligodendrocyte maturation. J Neurosci Res, 2009,87(14):3076-3087
23 Kempuraj D, Selvakumar GP, Zaheer S, *et al*. Cross-talk between glia, neurons and mast cells in neuroinflammation associated with parkinson's disease. J Neuroimmune Pharmacol, 2018,13(1):100-112
24 Diaz A, Merino P, Manrique LG, *et al*. A cross talk between neuronal urokinase-type plasminogen qctivator (uPA) and astrocytic uPA receptor (uPAR) promotes astrocytic activation and synaptic recovery in the ischemic brain. J Neurosci, 2017,37(43):10 310-10 322
25 Fancy SP, Harrington EP, Baranzini SE, *et al*. Parallel states of pathological Wnt signaling in neonatal brain injury and colon cancer. Nat Neurosci, 2014,17(4):506-512
26 Chen HL, Chew LJ, Packer RJ, *et al*. Modulation of the Wnt/beta-catenin pathway in human oligodendroglioma cells by Sox17 regulates proliferation and differentiation. Cancer Lett, 2013,335(2):361-371
27 Aguirre A, Rubio ME, Gallo V. Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. Nature, 2010,467(7313):323-327

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