Maternal Oligodendrogenesis by Maternal Undernutrition and Inflammation

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Maternal undernutrition and infection during pregnancy may impair development of oligodendrocytes, thereby increasing risks of neuropsychiatric disorders of their children. We analyzed the effects of those risk factors on oligodendrogenesis in fetal and neonatal brains. Female mice were given low-protein or regular food for 2 weeks before their pregnancy. On the 14th day of pregnancy, they received a transcervical injection of lipopolysaccharide to induce inflammation or control solution, consisting of four groups, depending on nutritional conditions with or without vaginal inflammation. We collected fetal brains on embryonic day (E) 17 for evaluating oligodendrocyte precursor cells (OPCs) and neonatal brains on postnatal day (P) 7 for evaluating mature oligodendrocytes. OPCs and mature oligodendrocytes were identified as positive immunostaining for oligodendrocyte-lineage transcription factor 2 and myelin basic protein, respectively. There was no difference in the number of OPCs in E17 brains among the four groups, suggesting that nutritional restriction with or without inflammation exerts no noticeable influence on the differentiation of OPCs. However, the number of mature oligodendrocytes was decreased in P7 brains obtained from nutrient-restricted mice with inflammation, suggesting that their combination impairs oligodendrogenesis in the neonatal brain. We also analyzed reactive astrocytes that express both glial fibrillary acidic protein and nestin for evaluating brain inflammation. The population of reactive astrocytes was increased in P7 brains derived from mice with LPS injection, irrespective of nutritional restriction, indicating that maternal vaginal inflammation induces neonatal brain inflammation. The maternal management of both nutrition and infection is crucial to prevent neuropsychiatric disorders of the children.

Keywords: undernutrition; intrauterine infection; oligodendrocyte; neuropsychiatric disorders; neonatal period


Maternal undernutrition during pregnancy increases risks for neuropsychiatric disorders, such as schizophrenia, depression, and bipolar disorder of their children (Hulshoff Pol et al. 2000). In addition, prenatal infection with bacterial or viral agents during pregnancy has been shown to increase risks of neuropsychiatric disorders in adulthood of their children (Brown et al. 2009). Some animal studies investigated the relation between each risk factor and the incidence of mental disorders (Almeida et al. 1996; Fortier et al. 2004; Baharnoori et al. 2009; Markham and Koenig 2011). However, each of maternal undernutrition and infection during pregnancy cannot explain the increased risk of neuropsychiatric disorders. Therefore, we wanted to investigate the combined effects of maternal undernutrition and inflammation on the development of oligodendrocytes using a mouse model.

Oligodendrogenesis in mouse forebrain begins on embryonic day (E) 12.5 and is completed by postnatal day (P) 10 (Kessaris et al. 2006). We therefore collected fetal brains on E17 to evaluate the differentiation of oligodendrocyte precursor cells (OPCs) and neonatal brains on P7 to measure the number of mature oligodendrocytes. Especially, we focused on oligodendrocytes as an indicator of neuropsychiatric disorders, because oligodendrocyte dysfunction in the white matter is strongly linked with the onset of neuropsychiatric disorders (Hakak et al. 2001; Tkachev et al. 2003).

In this study, to evaluate maternal multifactorial influences on the oligodendrogenesis in fetal brain, we analyzed the effects of maternal undernutrition and inflammation on
development of oligodendrocytes in the fetal brain by administration of lipopolysaccharide (LPS) to nutrient-restricted pregnant mice.

Materials and Methods

Animal and Drugs

This experiment was conducted on the basis of Center for laboratory animal research, Tohoku University approval. Virgin female C57BL/6N mice (bred at Clea Japan, Inc., Japan; 12 hour light cycle, 24°C Sweekes old) were transferred to single cages, fed ad libitum, and their food consumption and body weight were recorded everyday.

On the 14th day of gestation, pregnant mice in each group were further divided into two groups; in one group the mice were transvaginally given 30 μl of 0.1 mg/ml LPS (E. coli. Serotype O55:B5; Sigma Chemical Co., St. Louis, MO) diluted in sterilized PBS (−) (LPS group) to induce minor infection, while in the other group sterilized PBS (−) was given (PBS group) as a negative control. Hence, mice were divided into four groups: L-LPS, L-PBS, N-LPS, and N-PBS.

Three pregnant mice from each group were used for the assessment of LPS effect after 24 hours of the injection. Expression of oligodendrocyte-lineage transcription factor 2 (Olig2) was analyzed as a marker for oligodendrocyte precursor cells (OPCs) (Furusho et al. 2006). Rabbit anti-human Olig2 IgG affinity purified (IBL, Takasaki, Japan) was diluted at 1:500 in PBS (−) with 0.3% Triton X-100 (Wako, Osaka, Japan) and 1% bovine serum albumin (BSA: Wako). We used FITC-labeled TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling: MEBSTAIN Apoptosis TUNEL Kit II, MBL, Nagoya, Japan) stain for detection of apoptosis (Duran et al. 2004). Donkey anti-rabbit IgG 546 (1:500; Molecular Probes, Eugene, OR) was used as the secondary antibody. It was diluted in PBS (−) with Bis-benz-amine (DAPI; 0.5 μg/ml; Sigma Chemical Co.) to identify the nuclei. Myelin basic protein (MBP) was used for detection of mature oligodendrocytes (Buser et al. 2009). Rat anti-MBP antibody (Millipore, Billerica, MA) was diluted at 1:200 in PBS (−) with 0.1% Triton X-100 and 1% bovine serum albumin. Cy-3® Donkey anti-rat IgG (1:500; Jackson Immuno Research, West Grove, PA) was used as the secondary antibody. It was diluted in PBS (−) with DAPI (0.5 μg/ml; Sigma Chemical Co.) to identify the nuclei. Expression of glial fibrillary acidic protein (GFAP) and nestin was analyzed to detect reactive astrocytes (Suzuki et al. 2003; Brunet et al. 2004). GFAP is a marker of astrocytes. Monoclonal mouse anti-GFAP (Sigma Chemical Co.) and rabbit anti-nestin antibodies (Millipore) were used to stain reactive astrocytes. Reactive astrocytes appear in response to inflammation (Luna et al. 2010). Alexa Fluor® 488 goat anti-mouse IgG (1:500; Molecular Probes) for the mouse anti-GFAP antibody and Alexa Fluor® 546 goat anti-rabbit IgG 546 (1:500; Molecular Probes) for the rabbit anti-nestin antibody were used as the secondary antibodies. They were diluted in PBS (−) with DAPI (0.5 μg/ml; Sigma Chemical Co.) to identify the nuclei.

Reactive astrocytes were analyzed to evaluate the inflammation of the neonatal brain (Luna et al. 2010). According to Nakagawa et al. (2005), we used the ratio of reactive astrocyte (GFAP- and nestin-positive) fibers to the total number of astrocyte (GFAP-positive) fibers as an index of astrocyte infiltration.

Table 1. Food composition.

<table>
<thead>
<tr>
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<th>AIN-93G (N)</th>
<th>Modified AIN-93G (L)</th>
<th>L / N</th>
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<tbody>
<tr>
<td>Water</td>
<td>9%</td>
<td>10.41%</td>
<td>1.16</td>
</tr>
<tr>
<td>Crude protein</td>
<td>17.9%</td>
<td>8.67%</td>
<td>0.48</td>
</tr>
<tr>
<td>Crude fat</td>
<td>7%</td>
<td>7.15%</td>
<td>1.02</td>
</tr>
<tr>
<td>Crude ash</td>
<td>2.5%</td>
<td>3.05%</td>
<td>1.22</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>3%</td>
<td>5%</td>
<td>1.67</td>
</tr>
<tr>
<td>Nitrogen free extract</td>
<td>60.6%</td>
<td>65.2%</td>
<td>1.08</td>
</tr>
<tr>
<td>Calorie</td>
<td>377 kcal/100 g</td>
<td>352.6 kcal/100 g</td>
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fibers. The ratio was used for evaluating the degree of inflammation caused by LPS injection.

All images were captured on a fluorescence microscope (Leica Microsystems, DM5000B).

Statistical analysis

Quantitative data were expressed as mean values ± SEM for each group. In this study, we used the two independent events (maternal undernutrition and LPS injection). Results were analyzed using the two-factor factorial ANOVA. The two-factor factorial ANOVA could detect effect of maternal undernutrition, effect of LPS injection and interaction effect of maternal undernutrition with LPS injection. The significance level was set at \( p \leq 0.05 \). All statically analyses were performed by Prism 4.0c for Mac. OS (GraphPad Software Inc., San Diego, CA).

Results

Gestational condition

After 24 hours of LPS injection, infiltration of neutrophils and macrophages were detected in the vagina, but not in the chorion and amnion, in the LPS-injected groups (N-LPS and L-LPS) (Fig. 1a-d). Such infiltration was not detectable in the PBS-treated groups (N-PBS and L-PBS). We thus established a mouse model that mimics inflammatory vaginitis.

The maternal body weights during gestational period showed similar increase in each group (Fig. 2a). There were no significant differences between N and L groups in the food consumption; therefore, the protein consumption of the L groups were approximately the half of the protein consumption of the N groups (Fig. 2b). There was no intrauterine fetal death on E17.

The mean fetal body weight of the L groups was significantly lower than that of N groups on E17 (Fig. 2c). In addition, the mean neonatal body weight of the L groups was significantly lower than that of the N groups on P7 (Fig. 2d). It should be noted that LPS injection exerted no noticeable effect on the mean body weights of fetuses and neonates, irrespective of nutritional conditions.

Immunohistochemistry

E17

There was no significant difference in the number of OPCs (olig2-positive cells) (Fig. 3a-d). The apoptotic cells (TUNEL-positive cells) were not found in all groups (Fig. 3a-d).

P7

The number of mature oligodendrocytes (MBP-positive cells) was significantly decreased only in the L-LPS group (Fig. 4a-e). There was no significant difference in the number of astrocyte fibers (GFAP-positive) in each group (Fig. 4a-e). However, there was significant increase in the ratio of reactive astrocyte fibers (GFAP- and nestin-positive) to the GFAP-positive fibers (total astrocytes) by maternal LPS injection (Fig. 4f).

Discussion

In this study, we investigated the combined effects of
Fig. 2. Evaluation of pregnancy, fetal and neonatal growth.
(a) Maternal daily body weight gain.
(b) Maternal daily protein intake.
The protein intake of the L groups (L-PBS and L-LPS) was about the half of the N groups (N-PBS and N-LPS).
(c) Fetal body weight on E17.
(d) Neonatal body weight on P7.
The fetal (c) and neonatal (d) body weights were decreased by maternal undernutrition.
N: N group and L: L group.
The numbers within each column indicate the number of samples. Values are expressed in mean ± s.e. †p < 0.05 compared to N group by two-factor factorial ANOVA.

Fig. 3. Detection of apoptotic cells and OPCs.
(a) N-PBS, (b) N-LPS, (c) L-PBS and (d) L-LPS.
The white matter on E17 was stained with TUNEL (green) and olig2 (red). Nuclei (blue) were stained with DAPI.
TUNEL-positive (apoptotic) cells were not detectable in all groups. There was no difference in the number of olig2-positive cells (OPCs) among all groups. Scale bar, 50 µm.
Elavitation of oligodendrocyte differentiation.

(a) N-PBS, (b) N-LPS, (c) L-PBS and (d) L-LPS.

(e) Number of MBP-positive cells (red).
The white matter on P7 was stained for MBP (red). Nuclei (blue) were stained with DAPI. The number of MBP-positive cells (mature oligodendrocyte) was decreased only in L-LPS mice. Scale bar, 100 µm.

N: N group and L: L group.
The number, shown below each bar graph, indicates the number of samples.
Values are expressed in mean ± s.e. *p < 0.05 compared to N-PBS, N-LPS and L-PBS groups by two-factor factorial ANOVA.
Fig. 5. Evaluation of inflammation of neonatal brain.
The neonate white matter on P7 was stained with GFAP (green) and nestin (red). Nuclei (blue) were stained with DAPI.
(a) N-PBS, (b) N-LPS, (c) L-PBS and (d) L-LPS.
(e) Number of GFAP-positive fibers.
(f) Ratio of GFAP- and nestin-positive fibers to GFAP-positive fibers.
Regions of the brain are shown in (a). Scale bars indicate 100 µm.
There was no change in the number of astrocyte (GFAP-positive fibers). However, there was a significant increase in the ratio of the reactive astrocyte (GFAP- and nestin-positive fibers: yellow; enclosed by a white broken line oval) to the total astrocyte (GFAP-positive fibers) by LPS injection.
N: N group and L: L group. SVZ: subventricular zone.
The numbers, shown below each bar graph, indicates the number of samples. Values are expressed in mean ± s.e. †p < 0.05 compared to PBS group by two-factor factorial ANOVA.
maternal undernutrition and vaginal inflammation on the development of oligodendrocytes in mice. We thus found that the number of mature oligodendrocytes was decreased by about 50% in the P7 neonatal brain, derived from mice with undernutrition and transvaginal LPS injection, whereas no significant effects were detected on the OPCs in the E17 brain. These results suggest that the combination of maternal undernutrition and vaginal inflammation impairs the maturation of oligodendrocytes.

Wright et al. (2010) reported that the decrease in MBP-positive cells detected on P7-14 did not fully recover in adulthood, suggesting that the decreased number of oligodendrocytes in the neonatal period may remain unchanged. It is therefore conceivable that the decrease in the number of oligodendrocytes detected on P7 (see Fig. 4) may be irreversible. Such impaired maturation of oligodendrocytes may increase the risk of neuropsychiatric disorders.

It is also noteworthy that the number of the mature oligodendrocytes was decreased only in L-LPS group, suggesting that maternal undernutrition increases vulnerability of fetal OPCs to inflammation. Insulin-like growth factor (IGF) has been identified as a key factor that is responsible for maturation of the OPCs (El-Khattabi et al. 2003), and the expression of IGF proteins in the fetus was suppressed under maternal undernutrition (Ye and D’Ercole 1999). These results suggest that maternal undernutrition and inflammation may impair the maturation of OPCs by decreasing the expression of IGF.

There was no difference in the differentiation of OPCs in the white matter on E17, irrespective of maternal undernutrition with or without LPS injection. These results suggest that the oligodendrogenesis in the fetus is not affected by the maternal undernutrition and vaginal inflammation. In contrast, the combination of maternal undernutrition with vaginal inflammation resulted in the decrease in the number of mature oligodendrocytes in the P7 white matter. These results suggest that maternal undernutrition may increase the vulnerability to inflammation of OPCs, which leads to the inhibition of oligodendrocytes maturation.

After 24 hours of LPS injection, infiltration patterns of neutrophils and macrophages were similar in L-LPS group and N-LPS group (see Fig. 1), suggesting that maternal immune response was maintained in L-LPS animals. Regardless of the maternal nutrition, the LPS injection resulted in the increase in the population of reactive astrocytes in the neonatal white matter of P7 (see Fig. 5). Thus, the LPS-mediated vaginitis is able to cause inflammation in the neonatal brain. However, the relative number of reactive astrocytes showed no difference between the L-LPS group and N-LPS group, indicating that the maternal nutrition does not affect the severity of the neonatal brain inflammation associated with the LPS-mediated vaginitis.

Under the maternal undernutrition, the fetal brain damage is enhanced even by maternal vaginal inflammation. Therefore, the maternal management of both nutrition and infection is crucial to prevent neuropsychiatric disorders of the children, even with the mild infection.

Acknowledgments
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Conflict of Interest
We have no conflicting interest.

References


