Sex-Dependent Gliovascular Interface Abnormality in the Hippocampus following Postnatal Immune Activation in Mice

Maryam Ardalan\textsuperscript{a, b}, Tetyana Chumak\textsuperscript{a}, Alexandra Quist\textsuperscript{a}
Seyyedeh Marziyeh Jabbari Shiadeh\textsuperscript{a, b}, Anna-Jean Mallard\textsuperscript{a}
Ali Hoseinpoor Rafati\textsuperscript{b}, Carina Mallard\textsuperscript{a}

\textsuperscript{a}Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; 
\textsuperscript{b}Translational Neuropsychiatry Unit, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

Abstract
The neuro-gliovascular unit is a crucial structure for providing a balanced well-functioning environment for neurons and their synapses. Activation of the immune system during the developmental period is believed to affect the gliovascular unit, which may trigger neurodevelopmental and neurological/neuropsychiatric diseases. In this study, we hypothesized that vulnerability of the male brain to a neonatal insult was conditioned by sex-dependent differences in the impairment of the hippocampal gliovascular unit. Male and female C57BL/6J pups received lipopolysaccharide (LPS) (1 mg/kg) or saline on postnatal day (P) 5. Brains were collected at P12 and morphological quantifications of hippocampal fibrillary glial acid protein (GFAP\textsuperscript{+}) astrocytes and ionized calcium-binding adaptor molecule 1 protein (Iba1\textsuperscript{+}) microglia were performed by using 3-D image analysis together with measuring the length of CD31\textsuperscript{+} and aquaporin-4 (AQP4\textsuperscript{+}) vessels. We found a significant increase in the length of CD31\textsuperscript{+} capillaries in the male LPS group compared to the saline group; however, coverage of capillaries by astrocytic end-feet (AQP4\textsuperscript{+}) was significantly reduced. In contrast, there was a significant increase in AQP4\textsuperscript{+} capillary length in female pups 1 week after LPS injection. GFAP\textsuperscript{+} astrocytes via morphological changes in the hippocampus showed significant enhancement in the activity 1 week following LPS injection in male mice. We propose that neonatal inflammation could induce susceptibility to neurodevelopmental disorders through modification of hippocampal gliovascular interface in a sex-dependent manner.

Introduction
Brain development is a long sexually dimorphic process which in human starts from the third gestational week and continues after birth until late adolescence \[1\]. Brain development is paralleled and supported by development and maturation of brain microvasculature which...
starts from embryogenesis and continues until the first few weeks after birth in mice [2]. The proper function of neurons and their synapses depends on a well-balanced environment, requiring normal structure and function of the blood-brain barrier (BBB) [3]. Function of the BBB is highly dependent on signaling and interaction between astrocytes, microglia, and endothelial cells, termed the gliovascular unit [4, 5]. Astrocytes as part of a gliovascular unit modulate local blood flow and calcium (Ca2+) signaling via their processes, so-called “end-feet” [6].

Every year, over 15 million infants are born preterm (<37 weeks of gestation) and over 1 million die [7]. A significant number of surviving infants born preterm suffer from long-term neurological disabilities, and two of the main etiological factors are exposure to intrauterine or neonatal infections [8].

Indeed, activation of the immune system during early development is believed to affect the gliovascular unit, leading to abnormal cognition and therefore a potential important trigger of neurodevelopmental disorders such as autism [9, 10]. The timing of the immune activation during pregnancy and neonatal period together with other factors such as genetics, sex, and type of infection have been suggested to influence the vulnerability to neurodevelopmental disorders [11]. Specifically, sexual dimorphism impacts astrocyte proliferation and maturation during brain development which varies in different brain regions [11]. We previously showed enhanced microgliaogenesis and reduced neurogenesis in the developing hippocampus following lipopolysaccharide (LPS) administration in neonatal (P5) mice [12].

While basic and translational research have shed some light on underlying mechanisms and effects of neonatal immune activation on the developing brain, there is still a lack of knowledge on effects on the hippocampal gliovascular interface. Further, little is known about sex-specific mechanisms underlying such changes in the developing brain following early life inflammation. The current study focused on the hippocampus since increasing evidence indicates the impact of inflammation particularly on the hippocampus and as one of the main brain regions involved in cognitive functions, memory, and learning abilities [13]. Thus, this project was designed to identify sex-dependent alterations in the hippocampal gliovascular interface following early life inflammation. Specifically, we systematically investigated the various cellular compartments and their interactions by comparing alterations in the hippocampal vasculature, astrocyte, and microglial activation between male and female mice after neonatal LPS administration.

### Materials and Methods

#### Animals

All animals were kept on a 12 h dark-light cycle and standard housing conditions with ad libitum food access. On postnatal day 5 (P5), C57Bl/6J mice were randomly assigned to 4 experimental groups: LPS (1 mg/kg, #423, List Biological Laboratories, Campbell, CA, USA)-injected mice ($n = 12$, $\sigma = 6$, $\Phi = 6$) and saline (1 mg/kg, Sigma Aldrich, St. Louis, MO, USA)-injected mice ($n = 12$, $\sigma = 6$, $\Phi = 6$). Injections were performed intraperitoneally. This age in mice represents a critical brain developmental stage equivalent to preterm human infants [14]. Morphological analysis was performed 7 days after injection (P12). The P12 time point was studied as it corresponds to a stage in mouse brain development with high rate of brain growth, including gliogenesis, increasing axonal and dendritic density, and with significant brain plasticity activity [14]. Animals were anesthetized deeply with an intraperitoneal injection of pentobarbital (20 µL per animal, 60 mg/mL), followed by transcardial perfusion with histolox (Histolab products AB, Vatra Frölunda, Sweden) and collection of brains. One brain hemisphere (left or right) was randomly selected for each animal to have equal number of each hemisphere per group, immersed in the same fixative solution, and stored at 4°C for further brain structural quantification.

#### Tissue Preparation

The perfused-fixed brain hemispheres were placed in a 30% sucrose solution (Sigma Aldrich) for 48 h, followed by snap-freezing using isopentane (Sigma Aldrich). Brain hemispheres were cut into 40-µm sections with coronal orientation on a cryostat (Leica, CM 3050 S). The first section was selected randomly with a section sampling fraction 1/8 based on a systematic sampling method [15] which included 7–8 sections per set. Five sets of sections were used for Nissl staining with 0.25% thionin solution (Sigma T3387, France), glial fibrillary acidic protein (GFAP) (astrocytes), ionized calcium-binding adaptor molecule 1 (Iba1) (microglia), CD31 (capillary), and aquaporin-4 (AQP4) (end-feet of astrocytes) immunohistochemistry staining.

#### Immunohistochemistry

Free-floating brain sections were washed in phosphate-buffered saline (PBS; Gibco Invitrogen, Waltham, MA, USA) for 20 min. Sections were then incubated in target retrieval solution (Dako, Glostrup, Denmark) at 85°C for 40 min. Afterward, sections were washed for 20 min in PBS, followed by blocking of endogenous peroxidases (3% H2O2 in PBS, Sigma Aldrich) for 10 min and then washed with PBS containing 0.02% Triton-X-100 (PBS-T; Sigma Aldrich). Next, sections were incubated with polyclonal primary rabbit anti-GFAP (1:500; Dako), rabbit anti-lba1 (1:1,000; Sigma), rabbit anti-CD31 (1:500, Abcam), or polyclonal rabbit anti-AQP4 (1:500; Boster Biological Technology, Ref#PB9475) overnight at 4°C. The next day, sections were washed in PBS-T and subsequently incubated in polyclonal secondary biotinylated goat-anti-rabbit antibody (1:250; Vector Laboratories, Olean, NY, USA) in PBS-T for 2 h at room temperature. Sections were washed in PBS-T followed by incubation in ABC elite solution (1.5% solution A + 1.5% solution B in PBS, Vector Laboratories) for 1 h at room temperature. Afterward, sections were washed in PBS-T for 20 min, and immunolabeling was performed by using 3,3-diaminobenzidine solution (Acros
Organics, Geel, Belgium). Sections were washed in distilled H2O and PBS; mounted on gelatin-coated slides; dehydrated in 95%, 99% alcohol, xylene; and coverslipped.

**Delineation of Hippocampal Molecular Layer of Dentate Gyrus**

The area of interest in this project was the molecular layer of dentate gyrus (DG) (MDG) due to the anatomical proximity of this area with GCL (the area with the high rate of neurogenesis in adulthood) and also the effect of neonatal LPS administration on the neurogenesis in the granule cell layer of DG [12]. This area is occupied mainly by the dendrites of the dentate granule cells, axons, astrocytes, microglia, and vessels [16].

**Quantification of Hippocampal MDG Volume**

The volume of the hippocampal MDG subregion was estimated on the Nissl-stained sections by point counting using the Cavalieri estimator method applying the Cavalieri estimator method using point counting. The captured images were analyzed using Filament Tracers algorithm of the Imaris software (version 8.4, Bitplane AG, Zurich, Switzerland). Analyzed morphological parameters were (1) number of cellular branches, (2) total length of the branches, and (3) sholl analysis based on the radial distance from the center of the cell soma in 5-µm intervals for microglia and 10-µm intervals for astrocytes. To measure the sphericity of microglia and astrocyte soma and also the area of astrocyte soma, as indicators of activation, the captured images were analyzed using Surface module of the Imaris software, as described previously [20]

**Estimation of the Length Density of CD31+ and AQP4+ Capillaries**

The length density (Lc) of the CD31+ and AQP4+ capillaries was measured by applying the global spatial sampling method allowing quantification of the length of the capillaries within a three-dimensional sampling box [21] with a x63 oil immersion objective lens as described before (Fig. 3a, b) [22].

The following formula was used for measuring the length density of the capillaries:

\[ L_c (\text{capillaries}) = \frac{2 \times p (\text{box}) \times \Sigma Q (\text{capillaries})}{\text{avg } a (\text{plane}) \times \Sigma P}, \]

where \( L_c \) (capillaries) is the length density of the capillaries; \( \Sigma Q \) (capillaries) is the sum of intersections between the test lines and the capillaries; \( p (\text{box}) \) is the number of box corners; \( \text{avg } a (\text{plane}) \) is the average of the plane area; \( \Sigma P \) is the sum of the box corners hitting the area of interest.
Effect of Postnatal Inflammation on the Morphology of Astrocytes in the MDG

We found a significant main effect of LPS treatment on the soma size of astrocytes, while sex effect was not significant ($F_{(1, 20)} = 9.186, p = 0.007$; $F_{(1, 20)} = 0.446, p = 0.512$). Tukey post hoc analysis showed significantly larger size of the astrocytes in male LPS mice compared to the male saline group ($p = 0.034$), while LPS did not affect the size of astrocytes in the female group ($p = 0.569$) (Fig. 2d). Regarding the sphericity of astrocyte soma, there was a significant main effect of LPS treatment on the sphericity of astrocytes, while sex effect was not significant ($F_{(1, 20)} = 7.804, p = 0.012$; $F_{(1, 20)} = 0.401, p = 0.534$). Importantly, we observed significantly less sphericity of astrocytes in female LPS mice versus female saline group ($p = 0.025$).
while it was not significant in male mice ($p = 0.874$) (Fig. 2e, f).

Our results demonstrated a significant main effect of LPS treatment and sex on the total length and number of astrocyte processes ($F_{(1, 20)} = 10.07$, $p = 0.005$; $F_{(1, 20)} = 8.71$, $p = 0.008$; $F_{(1, 20)} = 25.64$, $p = 0.000$; $F_{(1, 20)} = 5.39$, $p = 0.031$), respectively. Tukey post hoc analysis showed significantly longer astrocyte processes in the male LPS group compared to the saline-treated males ($p = 0.024$) without significant effect in female mice ($p = 0.548$) (Fig. 2g). Both male and female mice showed significantly greater number of astrocytic processes 7 days after LPS compared to saline injection groups, with a stronger effect in male mice ($p = 0.002$ in males vs. $p = 0.048$ in females) (Fig. 2h). Sholl analysis showed a higher complexity of astrocytes processes in male LPS compared to the male saline-treated mice ($p = 0.006$) (Fig. 2i) at 20-μm distance from the soma. Sholl analysis did not reveal any significant difference in complexity of astrocyte arbors between saline- and LPS-treated females ($p = 0.832$) (Fig. 2i).

**Effect of Perinatal Inflammation on the Length Density of CD31+ Capillaries in the MDG**

The length density of the capillaries in the MDG was significantly influenced by sex and LPS treatment ($F_{(1, 20)} = 4.78$, $p = 0.041$; $F_{(1, 20)} = 15.58$, $p = 0.001$). Tukey post hoc analysis indicated that length density of capillaries in the MDG was significantly higher in the male LPS mice versus male saline group ($p = 0.017$) (Fig. 3c); however,
Effect of Perinatal Inflammation on the Length Density of AQP4+ Capillaries in the MDG

Assessment of hippocampal AQP4+ capillaries showed a significant sex × LPS treatment interaction effect on the AQP4+ capillaries’ length density ($F_{(1, 20)} = 21.83, p = 0.000$). Interestingly, the length density of AQP4+ capillaries was significantly shorter in male LPS mice compared to the male saline group ($p = 0.038$), while female LPS mice showed significantly longer AQP4+ capillaries versus female saline mice ($p = 0.007)$. At the baseline level, the length density of AQP4+ capillaries was shorter in female mice compared to the males ($p = 0.054$) (Fig. 3d).

Effect of Perinatal Inflammation on Microglia Morphology in the MDG

Morphological analysis of microglia indicated that sphericity of microglia soma was significantly influenced by sex × LPS treatment interaction ($F_{(1, 20)} = 8.44, p = 0.008$) with significantly less sphericity in male LPS mice versus male saline group ($p = 0.029$) (Fig. 4a, e). Moreover, a significant main effect of LPS treatment on the length and number of microglia branches was observed ($F_{(1, 20)} = 182.97, p = 0.000; F_{(1, 20)} = 189.69, p = 0.000$). Tukey post hoc analysis showed shorter and lower number of processes, indicative of increased activity of microglia, in both male and female mice 1 week after LPS injection ($p = 0.000$) (Fig. 4b, c, f, g). The sholl analysis showed a significant reduction in microglia arborization 7 days following LPS injection in both males and females with significantly lower number of branching intersections 10–30 μm from the cell soma ($p < 0.05$) (Fig. 4d).

Correlation between Altered Biological Parameters of Hippocampus following Perinatal Inflammation

There was a significant positive correlation between the total length of astrocyte processes, number of astrocyte processes, and the volume of MDG ($r = 0.62, p = 0.001; r = 0.70, p = 0.000$) (Fig. 5a, b). A significant positive correlation between the length density of CD31+ capillaries from the cell soma was significantly lower in LPS group versus saline group at P12 independent of sex (Fig. 5c).
illaries, the volume of MDG ($r = 0.68$, $p = 0.000$), and the morphological changes of astrocytes including the length and number of astrocytic branches ($r = 0.59$, $p = 0.002$; $r = 0.66$, $p = 0.000$) was observed (Fig. 5c–e). There was also a significant correlation between the length density of AQP4⁺ capillaries and the sphericity of microglia soma ($r = 0.46$, $p = 0.045$) (Fig. 5f).

**Discussion**

The main result of the study was that LPS-induced inflammation postnatally in mice resulted in sex-dependent effects on the gliovascular unit, particularly on vascular and astrocytic components. We found (i) increased density of capillaries, (ii) increased activation of astrocytes, and (iii) reduced coverage of astrocytic end-feet on capillaries in males after LPS injection, while the overall size of MDG and microglia activation were increased in both males and females following LPS injection. Interestingly, the density of AQP4⁺ capillaries (astrocyte end-foot coverage) was significantly increased in females after LPS, which was opposite to that found in males.

Studies suggest that perinatal inflammation can lead to pathological development of the brain that contributes to neurodevelopmental disorders such as autism [23]. An activated neuroimmune system has been found in post-mortem brain tissue from autistic patients [24, 25]. Further, it has been suggested that the combination of genetic predisposition with environmental factors such as preterm birth and infections may increase vulnerability of infant brain to lifelong neuropathological impairments [26]. We recently demonstrated that neonatal LPS exposure in mice resulted in autistic-like behavior in adolescent mice [27]. The interplay between the peripheral immune system and neuroglia has been suggested as an important indicator for proper brain development; once the balance is disturbed, both can substantially contribute to pathogenesis of the neurodevelopmental and neurodegenerative diseases that occur later in life [28, 29]. Therefore, it is important to know how postnatal systemic immune activation influences the structure of the gliovascular unit, which is a key component in the interaction between the periphery and CNS [12].

In our study, we performed volumetric analysis of MDG, the hippocampal subregion, which contains the
dendrites and axons of the dentate granule cells as well as astrocytes and microglia cells and found that the size, under physiological conditions, was significantly larger in male than in female mice and with a significant increase in both male and female mice after perinatal inflammation. This enlargement of the MDG may be explained by an increase in morphological complexity of astrocytes and microglia found in this study. Further, maternal immune activation by LPS was previously shown to induce a significant increase in the number of spines in the granule cells of the DG accompanied by ASD-like behavior [30]. This study and our results are in line with clinical evidence showing that the hippocampal volume is enlarged in ASD patients compared to controls [13, 31].

Our findings indicated that microglia were significantly activated in LPS-injected mice compared to the control groups. Microglia maturation is associated with morphological alteration; however, the time of transition from amoeboid to ramified microglia morphology in the developing brain is not clear yet and that could be the possible explanation for age-dependent differences in the microglial response to insults such as inflammation and hypoxic-ischemic injury in the developing brain [32, 33]. We have recently found long-term (P45) activation of microglia following neonatal LPS injection [27]. Similarly, following antenatal LPS exposure in rabbits, proinflammatory microglial phenotypes were shown to persist postnatally through P14–17 [34], suggesting sustained activation of microglia. While there are studies showing that perinatal inflammation results in microglia activation specifically in males [11], others indicate that maternal inflammation induces activation of the TLR4 signaling pathway with the consequence of abnormal activation of microglia without considering sex [35]. In support, we found that alteration of most of the morphological indicators of activation (e.g., microglia branching pattern and process morphology) was not sex dependent.

The reactive phenotype of astrocytes is indicated by cellular hypertrophy via upregulation of intracellular GFAP [36]. While number of astrocytic processes increased after LPS in both males and females, hypertrophy (soma size) and the length of processes only increased in males in our study. In particular, outgrowth of long processes has been associated with “reactive” phenotype of astrocytes [37], suggesting a more profound activation in male mice in our study. It was previously shown that at the age of P5, astrocytes proliferate in both males and females [11]; however, female astrocytes were more branched and matured earlier than male astrocytes [38]. Potentially, this difference in development may have influenced the sex-dependent effects of perinatal inflammation on astrocyte morphology that we observed. This could hypothetically also result in long-term effects as astrocytes in the developing brain are permanently differentiated by neonatal estradiol, leading to permanent sex-differences in morphology and function of astrocytes [39]. For instance, male LPS-treated astrocytes exhibited higher mRNA levels of TNF-α, and IL-1β [40].

Astrocytes play an important role in BBB maintenance. Among other, factors produced by astrocytes regulate an efflux transporter P-glycoprotein (P-gp) in brain endothelial cells [41]. P-gp regulates transport of several endogenous and exogenous compounds across the BBB, mainly removing potentially toxic substances from the brain, and deterioration of this mechanism with age can contribute to development of Alzheimer’s disease and Parkinson’s disease. Abnormal maturation of astrocytes decreased P-gp at the BBB with the consequence of increased transfer of endogenous and exogenous P-gp substrates into the brain, which was suggested to lead to failure of this transport mechanism, in turn contributing to long-term neurodevelopmental/neurological deficits [42]. Indeed, an earlier study on the gliovascular unit showed significantly altered astrocytic markers in the brains of patients with autism [43] and also in a genetic mouse model of Fragile X syndrome [44]. This could point to the fact that the reactive state of astrocytes, which we found in our study, can affect astrocyte function and maturation which may contribute to the development of an ASD phenotype following perinatal inflammation. In addition, activation of astrocytes has often been referred to as the neuroinflammatory neurotoxic response to neuropathological abnormalities in neurodegenerative disorders [45]. It was shown that there is a bidirectional relationship between microglia and astrocyte activation in neuropathological abnormalities [46]. Further, BBB breakdown may occur as a result of glia activation [47].

Our results showed increased vascularization of MDG in LPS-injected male mice compared to the control group, while there was no significant difference between the female groups. It has been shown that endothelial dysfunction causes deficits in brain vascular development which is associated with autistic-like behaviors, particularly in males [48]. Moreover, it was shown that at the baseline level, women have different vascularity level from men during cognitive activity [49]. This difference could be underlying the different vascular responses to the LPS exposure between male and female mice. In addition, due to the regulation of brain vasculature via steroid hormones during the developmental period, the sex-depen-
dent differences in vascularization of the hippocampus in our study may relate to the modulation of gliovascular maturation by sex hormones [50]. Importantly, there is a high expression of estrogen receptors in the developing brain with the consequence of sex-dependent effect on astrocytes development and alteration in gliovascular unit [51].

One of the important findings in our study is the opposite effect of perinatal inflammation on the expression of AQP4, a water-selective membrane transport protein in the end-feet of astrocytes. While we observed a higher astrocytic end-feet coverage of capillaries in female mice 1 week after LPS injection, male mice had significantly lower AQP4 coverage of capillaries. It has been reported that at the age of P14, more than 95% of astrocytes were positive for GFAP and AQP4, without considering sex [42]. AQP4 is expressed in brain perivascular astrocyte end-feet that makes contact with the brain microvasculature [52]. It is suggested that different types of reactive astrocytes could be classified based on their AQP4 expression levels [53]. AQP4 also modulates astrocyte-tomicroglia communication during neuroinflammation. Indeed, AQP4 does not act just as a water channel protein but also as an adhesion molecule involved in functional regulation of synaptic plasticity and learning/memory [54]. The interaction between AQP4 and glutamate receptors on astrocytes has a role in pathophysiology of Alzheimer’s disease [55], and AQP4 deficiency-induced gliosis has been reported in experimental Parkinson’s disease [56]. There is also evidence that AQP4 expression was significantly decreased in the cerebellum of ASD cases [43]. Loss of AQP4 polarization in perivascular astrocytic end-feet leads to BBB breakdown, which may contribute to pathology [57]. Regarding the role of AQP4 during early development, sporadic obstructive hydrocephalus was reported in AQP4-deficient mice, but without considering sex [58]. One clinical study analyzed the sex distribution of AQP4 in autoimmunity and showed significantly higher number of AQP4-IgG-seropositive females than males, which suggested one possible mechanism underlying the higher prevalence of autoimmune CNS disorders in women [59]. The lower vascular coverage by AQP4 in male mice after LPS injection in this study may suggest increased vulnerability in males for neurodevelopmental/neurodegenerative disorders or autoimmune disorders in females later in life, although this remains to be explored.

A significant positive correlation between the volume of MDG and astrocyte arborization complexity indicated the possible involvement of astrocyte morphological changes in the volume alteration of MDG. Gliovascular changes in this hippocampal subregion are important, as the microvasculature of the DG is a key contributor to hippocampal neurogenesis. It was shown that angiogenesis is modulated by the signaling of vascular endothelial growth factor (VEGF), which is expressed by astrocytes in the hippocampus [60]. The significant correlation between the length density of capillaries (vascularization) and astrocytic branching complexity indicates that perinatal inflammation may impact hippocampal microvasculature by changing the morphology of astrocytes.

**Conclusion**

The current study showed that LPS-induced inflammation postnatally modified the hippocampal gliovascular interface in a sex-dependent manner by changing the activation of astrocytes and hippocampal vascularization more pronouncedly in males. We speculated that the altered vascular network during development may contribute to long-term neurodevelopmental/neurodegenerative disorders.

**Statement of Ethics**

All animal experiments were approved by the Gothenburg Animal Ethical Committee (No. 663/2017).

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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**Author Contributions**

Maryam Ardalan and Carina Mallard contributed to the main idea and design of the study. Data collection has been done by Maryam Ardalan, Tetyana Chumak, Alexandra Quist, Seyedeh Ardalan/Chumak/Quist/Jabbari Shiadeh/Mallard/Rafati/Mallard...
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Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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