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Abnormal Behavior Controlled via GPR56 Expression in Microglia

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During pregnancy, maternal immune activation (MIA) from infection increases the risk of neurodevelopmental diseases, including schizophrenia and autism spectrum disorders. MIA induced by polyinosinic-polycytidylic acid (poly (I:C)) and lipopolysaccharide (LPS) in animal experiments has led to offspring with abnormal behaviors and brain development. In addition, it has recently been reported that microglia, which reside in the brain and function as immune cells, play an important role in behavioral abnormalities and brain development in MIA-induced offspring. However, the underlying mechanism remains unclear. In this study, we investigated whether microglia-specific inhibition of GPR56, a member of the G protein-coupled receptor (GPCR) family, causes behavioral abnormalities in brain development. First, MIA induction did not affect the microglia population, but when examining the expression of microglial GRP56 in MIA-induced fetuses, GPR56 expression was inhibited between embryonic days 14.5 (E14.5) and E18.5 regardless of sex. Furthermore, microglial GPR56-suppressed mice showed abnormal behaviors in the MIA-induced offspring, including sociability deficits, repetitive behavioral patterns, and increased anxiety levels. Although abnormal cortical development such as that in the MIA-induced offspring were not observed in the microglial GPR56-suppressed mice, their brain activity was observed through c-fos staining. These results suggest that microglia-specific GPR56 deficiency may cause abnormal behaviors and could be used as a biomarker for the diagnosis and/or as a therapeutic target of behavioral deficits in MIA offspring.

Key words : Abnormal behavior, GPR56, maternal immune activation, microglia, neurodevelopment

Introduction

Epidemiological studies implicate a correlation between virus infection during pregnancy and an increased risk of postnatal developmental disease including schizophrenia and autism spectrum disorder (ASD) [15, 24]. Several rodent models have shown that maternal immune activation (MIA) alone is sufficient to induce fetal brain developmental abnormalities and behavioral deficits in offspring [34]. However, the underlying pathophysiological mechanisms by which MIA-induced inflammation induces neurodevelopmental and behavioral abnormalities in the offspring remain poorly understood. Recently, clinical and preclinical investigations have suggested that microglial dysfunction is a critical cellular mechanism linked to impaired brain development and aberrant postnatal behaviors [21].

Microglia, the resident immune cells of the brain, play important role in the proper development of the central nervous system (CNS) and are involved in a variety of pathological functions. Several studies have shown that the introduction of poly (I:C) into gestational dams results in persistent changes such as an increase in inflammatory cytokines and morphological changes in the microglia of offspring [6, 14].

GPR56/ADGRG1 belongs to the member of G protein-coupled receptor (GPCR) family and is expressed in the brain, heart, thyroid, kidney, testes, pancreas, and skeletal muscles [19, 33]. In addition, GPR56 is expressed in Tuj1+ migrating neurons, Cajal-Retzius cells, radial glial cells, and multiple cell types from the prelate and marginal zones, including the ventricular and sub-ventricular zones of the developing brains [16, 17]. GPR56 is involved in various physiological processes. The most studied are in the central and peripheral nervous systems (CNS/PNS) [1, 2, 28], the immune system [8, 30], the differentiation of hematopoietic stem and progenitor cells (HSPCs) [27, 32], the reproductive system [12], and muscular hypertrophy [38]. In addition, it has been reported that GPR56 participates to the cortical lamination, and repair

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as well as myelination. GPR56 regulates brain cortical patterning and proper cerebral cortex development, rostral cerebellar development, nerve axon myelination and myelin repair in both CNS and PNS [5, 18, 22, 25, 29].

Previous reports identified areas of abnormal cortical morphology within the cerebral cortex of ASD patients [9], and MIA offspring patches were observed in the S1DZ region in the cerebral cortex [13]. GPR56 plays an important role in cortical formation [40]. Therefore, in this study, we investigated MIA-like behavioral deficits and cerebral cortical malformations by specifically inhibiting GPR56 expression in the microglia of MIA offspring.

Materials and Methods

Animals

All mice were obtained from Taconic Biosciences (Rensselaer, NY, USA), and floxed GPR56 and, CX3CR1 Cre mice were obtained from Dr. Piao (UCSF University). Mice were maintained in standard housing conditions (12 hr light/dark cycle, temperature 23-25℃, humidity 50~60%). Food and water were provided *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Maternal immune activation model

Seven to twelve-week old C57Bl/6N females and male mice were mated at a ratio of 2:1. On the next day, a vaginal plug was defined as embryonic day 0.5 (E0.5). At E12.5, pregnant mice were intraperitoneal injected with 20 mg/kg (dissolved in sterile phosphate buffer saline (PBS) of the viral mimetic poly (I:C) (potassium salt, Sigma-Aldrich, Cat No. 9582) or vehicle (PBS). Pregnant females were randomly assigned to either the vehicle or the poly (I:C) group. Offspring were caged with littermates of the same sex on postnatal day 21 (PD 21). All tests were performed on the male offspring.

Cell sorting of microglia

E13.5, E14.5, and E18.5 embryonic brains from pregnant mice were collected and homogenized in cold Dulbecco's phosphate-buffered saline (DPBS) on ice. Brains were pooled from identical pups and homogenized. Brain homogenates were centrifuged at 300× g for 10 min at 4℃ to obtain cell pellets. Cell pellets were washed and resuspended as single-cell suspensions in Fluorescence-Activated Cell Sorting (FACS) buffer (Hanks Balanced Salt Solution + 0.5% bovine serum albumin (BSA)). The single-cell suspension was blocked with rat anti-mouse CD16/32 (1:100) for 20 min and fluorescently conjugated with rat anti-mouse CD45 (1:50) and, rat anti-mouse CD11b (1:50) and dye with LIVE/DEAD markers (DraQ7 far red, 1:100). A BD FACS Aria II cell sorter (BD Biosciences, New Jersey, USA) with a 70-μm nozzle was used for microglia sorting. DraQ7-CD45mediCD11b + cells were collected as microglia, and DraQ7-CD45-CD 11b- cells were collected as non-microglia. Cells were directly sorted in RLT lysis buffer from the RNeasy Mini Kit (QIAGEN, Germany, Cat no.74104) or resuspended in TRIzol (Invitrogen life Technology, Massachusetts, USA) and stored at -80℃ for RNA extraction.

Real-time PCR

Total RNA was isolated from microglia at E13.5, E14.5 and E18.5, as well as from MIA and vehicle controls. RNeasy Mini Kit or TRIzole was used according to the manufacturer's instructions. 2 μg of RNA was reversed to cDNA using oligo-dT using a synthesis kit (TAKARA Bio Inc., Japan). A polymerase chain reaction (PCR) detection system (Bio-Rad CFX96, California, USA) was used according to the manufacturer's instructions. The relative gene was normalized to beta-actin. The primers used were GPR56: 5'-TTGCTGCCTACCTACCTCTGCTCC-3' and 5'-AGCAGGAAGACAGCGGACAG-3' and beta-actin: 5'-CAGCAAGCAGGAGTACGATGAGTC-3' and 5'-CAGTAACAGTCCGCCTAGAAGCAC-3'.

Immunofluorescence

Mice were anesthetized with ketamine, perfused with 4% paraformaldehyde (PFA), and their brains were isolated. After dehydration in 30% sucrose, frozen sections were cut to 30 μm using a cryomicrotome (Leica Microsystems, Wetzlar, Germany) and the samples were stored at 4℃ until processing. Primary antibodies were mouse anti-SATB2 (1:50, Abcam, Cambridge, UK) and rabbit anti-c-fos (1:200, Cell Signaling Technology, Massachusetts, USA). Antibody reactions were incubated overnight at 4℃, secondary antibodies (Jackson ImmunoResearch, Philadelphia, USA) DyeLight 488-conjugated anti-rabbit and 568-conjugated anti-mouse were reacted at room temperature (RT) for 2 hr. 4',6'-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Images were captured using Image J software a Leica confocal microscope (Leica Biosystems, Wetzlar Germany).

Open field test

All experimental mice in the behavioral tests were 2-3-

month-old males and housed at two to five mice per cage at all times, unless otherwise specified. The arenas were thoroughly cleaned with 70% ethanol between testing sessions. All the mice were habituated to the experimental room for 1 hr prior to the experiment. During the experiment, mice were placed in the center of white Plexiglas box, in an open testing arena (arena size, 40×40 cm), and allowed to explore the entire arena freely for 15 min. The center of the arena was defined as a 20×20 cm. Infrared photon_beam breaks were recorded, and movement metrics were tracked and analyzed using Noduls software (EthoVision). The percentages of time spent in the center and the total distance were analyzed by the tracking software.

Three-chamber social tests

One week after the open-field test, a two-day three-chamber sociability test was conducted on the experimental mice. On day 1, experimental mice were single-housed for 1 hr in separate clean holding cages in the experimental room for habituation and then placed into a three-chamber arena (23.5 ×17.5 inches) with two empty object/mouse holders located in the left and right chambers. Experimental mice were allowed to explore the arena and holders for 10 min and were then placed back in their home cages. On day 2, one holder contained an unfamiliar C57BL/6N mouse (social animal) of a similar age to that of experimental mice, and the other holder contained a plastic mouse toy of a similar size (an inanimate object). The experimental mice were placed in the center chamber without access to the left and right chambers and were only allowed to explore the center. Five minutes later, the gates to the lateral chambers were lifted, and the mice were allowed to explore the entire arena freely for 10 min. The interaction was recognized when the experimental mice approached either object holder within 2 cm, showing attention to the object holder. The sessions were video-recorded and analyzed using the SMART Video Tracking system. Each social mouse was used no more than four times. The preference for the stranger mouse over the empty cage was assessed by calculating the percentage of time spent making contact with the stranger mouse over the sum of the time spent with both S and the E. After each mouse, the cages and chambers were cleaned with 5% ethanol to avoid olfactory cues.

Marble-burying test

All mice were placed for 10 min in a standard cage filled with woodchip bedding to a depth of 5 cm with 20 evenly spaced marbles. After 15 min, the number of marbles was measured and the marble was considered buried if 2/3 of the marble was covered with bedding. Twenty glass marbles were placed in 4×5 array for 15 min. After the exploration period, mice were gently removed and returned to their home cages. The testing cages were recorded and manually counted as follows: 1 point for marbles covered more than 50%, 0.5 points for marbles approximately 50% covered, and 0 for anything less.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 8 software (GraphPad Software Inc., San Diego, CA, USA). The results were analyzed using one-way analysis of variance (ANOVA) or unpaired Student's t-tests, as described in the figure legends. The sample size n is defined in the figure legends as well as the individual dots in the bar graphs. Each embryonic experiment was conducted in at least two litters for each condition. All data are shown as means ± standard error (SE) in bar graphs [not significant (n.s.)]. or $p<0.05$, $^{**}p<0.01$, and *** or $p<0.001$ unless specified.

Results

GPR56 expression in microglia in MIA-induced offspring

To investigate whether there was a change in the microglia population in the MIA-induced animal model, fetal brain tissues were isolated and sorted using $CX3CR1^{+}/CD11b^{+}$ microglia at E18.5. The microglia population did not differ between the poly (I:C) injected dams and PBS control groups. The microglial population in the fetal brains of GPR56 floxed/floxed; CX3CR1 Cre/+ mice, in which the expression of microglia-specific GPR56 was inhibited, was not statistically significant (Fig. 1). These results suggest that microglia did not affect brain cortical development, behavior, or microglial activation in MIA-induced offspring or offspring with GPR56 deficits.

Neuronal proliferation and migration play important roles in complex structures within the brain. Previous results suggested that poly (I:C) may affect the timing at which layer-specific neurons are generated [13]. Therefore, it was investigated the time-dependent expression of GPR56 mRNA in sorted microglia from MIA-induced fetal brains. MIA was induced at E12.5, and microglia were sorted from embryonic brains at E13.5, E14.5, and E18.5, to examine the GPR56

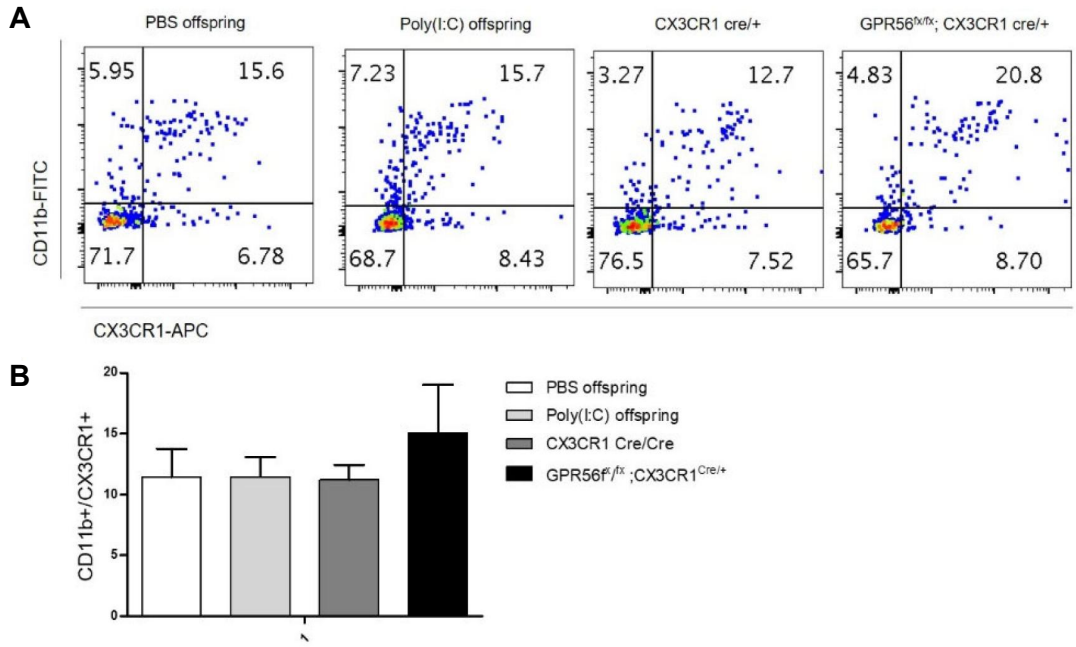

Fig. 1. Microglia population of fetal brain at E18.5 in offspring PBS or poly (I:C) injected dam and CX3CR1 cre/+, and GPR56 fx/fx; CX3CR1 cre/+ mice. Fetal brain microglia sorted by Dump^{-}CD45^{int}CD11b^{high} CX3CR1^{+} (n=4 each). Shows the FACs plot (A) and graph (B) of CX3CR1^{+}CD11b^{+} microglia.

mRNA expression. E13.5 showed no statistically significant difference in GPR56 mRNA expression compared to the PBS control group. However, at E14.5 and E18.5, the expression of GPR56 mRNA was significantly suppressed in microglia by MIA induction (Fig. 2A). Additionally, suppression of GPR45 mRNA expression was not sex-dependent (Fig. 2B). It is not clear at E13.5; however, previous studies have shown that cortical formation occurs continuously between E14.5 and E16.5 [36]. Microglial in the brains of MIA-induced offspring provide information about neural circuits through synaptic pruning [34]. These results suggest that microglial GPP56 expression plays an important role in cortical formation in MIA-induced offspring.

Abnormal behavior caused by microglia specific GPR56 deficiency

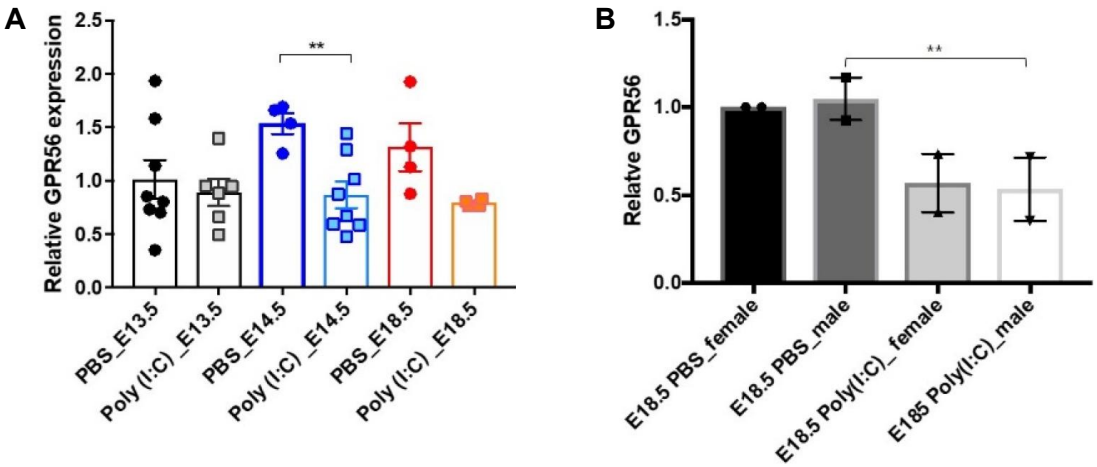

Fig. 2. Embryonic day dependent relative GPR56 mRNA expression in fetal brain microglia from PBS- or poly (I:C)-injected mother (A). And sex-dependent relative GPR56 mRNA expression at E18.5 fetal brain microglia (B). Fetal brain microglia sorted by Dump^{-}CD45^{in}tCD11b^{high}CX3CR1$^{+.}$ (n=2 PBS, n=3 poly (I:C) treated mothers). *p<0.05, **p<0.01 as calculated by t-test.

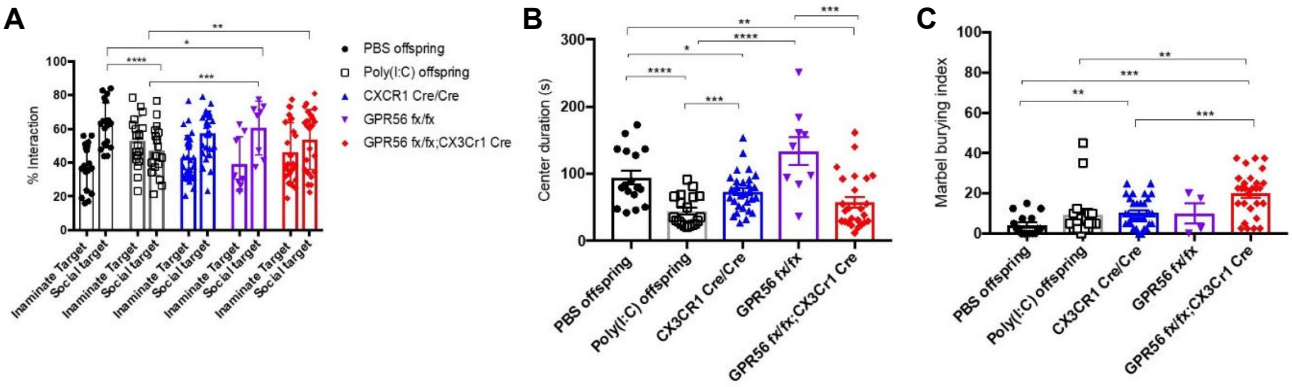

Fig. 3. GPR56 deletion in brain microglia (CX3CR1^{+}) promote abnormal behavior associated with MIA offspring. (n=17 CX3CR1 wt, n=14 CX3CR1 mt). A, the sociability index (% interaction or percentage of time spent investigating the social or inanimate stimulus out of the total exploration time of both objects during the 10 min sociability test) B, the time spent in the center of an open field (during the 15 min open field test) and, C, the marble burying index (the percentage of marbles buried during the 15 min marble burying test). *p<0.05, **p<0.01, ***p<0.001 as calculated by t-test (a, b, c and e), or one-way ANOVA with Tukey test.

Next, it was investigated whether microglia specific GPR56 suppressed animals showed behavioral phenotypes similar to the abnormal behavior in MIA offspring. Social behavior (Fig. 3A), open field (Fig. 3B), and repetitive behavior (Fig. 3C) were examined using the MIA offspring as the positive control group. MIA-induced offspring were induced abnormal behavior phenotypes like three-chamber social behavior test, open field, and marble burying tests. Similarly, autism-like behavioral patterns were observed in mice with a specific GPR56 deficiency in microglia (CX3CR1 Cre/+;GPR56 floxed/floxed mice). Thus, these results suggest that GPR56 may influence neural circuits formation during development and beyond and may affect behavior.

Previous studies have reported that abnormal morphology of the cortical S1DZ1 region was observed in MIA-induce offspring [13], and GPR56 deficiency caused cortical malformations [22, 25]. Next, cortical formation was investigated in microglial specific deficit GPR56 animals using an AT-rich sequence-binding protein (SATB2)-specific antibody that recognize specific cortical layers. As previously reported, SATB2 expression is specifically inhibited in the S1DZ, primary somatosensory cortex, region of MIA-induced offspring, resulting in abnormal cortical formation. However, abnormal cortical formation was not observed in animals in which GPR56 expression was specifically suppressed in the microglia (Fig. 4A). However, c-fos activity was observed in the cortical region S1DZ of GPR56-deficient animals (Fig. 4B). c-fos expression indicates neuronal activity. Although microglia-specific deficiency of the GPR56 gene is not involved in abnormal cortical formation, this suggests that it affects neuronal activity.

These results suggest that microglia-specific GPR56 gene deficiency affects autism-like behavioral phenotypes by affecting the formation of neural circuits, rather than the formation of the cortex.

Discussion

Microglia are derived from primitive myeloid precursors in the embryonic yolk sac. They undergo self-renewal and

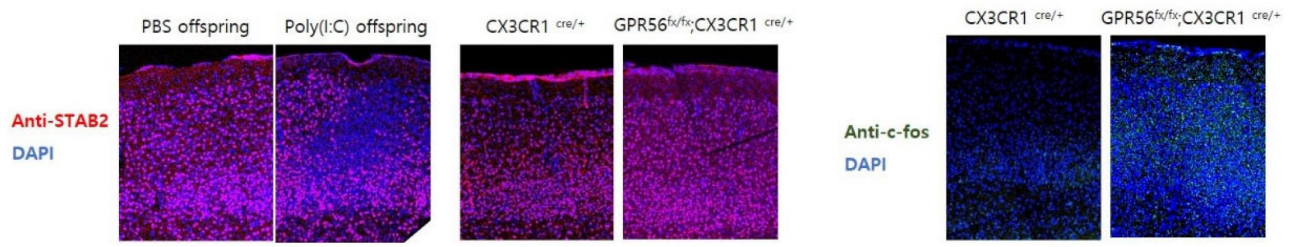

Fig. 4. GPR56 deletion in brain microglia (CX3CR1^{+}) was stained the S1DZ cortical region using STAB2 antibody and cortical activation was verified using the c-fos antibody.

play an important role in early brain development [4]. Under physiological conditions, microglia continuously survey the CNS microenvironment and responds sensitively to homeostasis [31, 35, 39]. Microglial priming is considered a major outcome in offspring with MIA and is associated with neurological dysfunction and abnormal behavior [10, 11]. Therefore, it is important to study the activation and function of microglia in offspring with MIA.

In this study, GPR56 expression was observed specifically in microglia, and GPR56 suppression in microglia was observed during cortical formation and abnormal behavior. Several recently studies have reported the dual roles of microglia that depend on the M1 and M2 phenotypes. In addition, microglia exert neurotoxic or neuroprotective effects by regulating the hippocampal neurogenic niche, depending on their activation profile [26, 37]. Moreover, the administration of minocycline, a semisynthetic second-generation tetracycline, for 2 weeks in an animal model of schizophrenia alleviate hyperactivity, sensory gating, and cognitive behavioral deficits. And inhibits microglia activation in the MIA induced offspring [3, 41]. However, the molecular signals that regulate microglial activation in offspring with MIA remain unknown.

The current study confirmed that changes in GPR56 expression in microglia are involved in abnormal autism-like behavior in offspring with MIA, and in MIA-induced pathological processes during brain development. However, further mechanistic studies are required to identify the exact role of GPR56 in brain development and abnormal behavior.

GPR56 is also involved in many biological functions in the brain related to the pathophysiology of neurological disorders such as depression. Recently, GPR56 was found to be a major treatment response biomarker in patients with depression. In particular, treatment with antidepressants induces upregulated GPR56 expression. Mice exposed to chronic stress show a significant decrease in GPR56 expression in the blood, prefrontal cortex and dorsal hippocampal areas, but recover from depression caused by downregulated GPR56 expression after antidepressant treatment [6]. These findings imply that the GRP56 functions as a regulator of behavioral development through various pathways. Additionally, GPR56 has been found to be involved in microglia-mediated synaptic remodeling by acting as a receptor phosphatidylserine (PS) exposed on the synaptic membrane during apoptosis and promoting uptake and elimination by microglia [20, 23]. GPR56 is also involved in the formation and repair of myelin in neuron via the $G\alpha_{12/13}$-RhoA signaling pathway [1].

Therefore, further investigation of the role of GPR56 in various aspects, such as synaptic remodeling and the $G\alpha_{12/13}$-RhoA various signaling pathways, as well as behavioral changes according to the GPR56 expression in MIA offspring. In the current study, explored novel pathological changes regulated by the gene expression of microglia-specific GPR56 associated with induced autism-like behavior in offspring with MIA. In addition, GPR56 is involved in MIA-induced pathological processes during brain development.

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Conflict of interest statement

The author declares no conflicts of interest.

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초록 : 미세아교세포에서 GPR56 발현에 의한 이상 행동

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임신 중 감염에 의한 산모의 면역 활성화는 조현병과 자폐 스펙트럼 장애를 포함한 신경 발달 질환의 위험을 증가시킨다. 여러 연구에서 poly (I:C) 또는 LPS를 사용하여 모체 면역 활성화 유도한 자손에서 비정상적인 행동과 뇌 발달을 관찰하였다. 또한 최근 뇌에 상주하며 면역 세포로 기능하는 미세아교세포가 MIA 유발 자손의 행동 이상과 뇌 발달에 중요한 역할을 한다는 것이 보고되고 있으나 아직 메커니즘은 명확하지 않다. 본 연구에서는 GPCR의 구성원인 GPR56의 미세아교세포 특이적 억제가 행동 이상과 뇌 발달을 유발하는지 여부를 조사하였다. 먼저, MIA 유도는 발달 중인 뇌의 미세아교세포 집단에 영향을 미치지 않으나, 미세아교세포를 분리하여 GRP56의 발현을 조사한 결과, MIA 유도 태아에서 성별에 관계없이 E14.5와 E18.5 사이에서 GPR56 발현이 억제됨을 관찰하였다. 그리고 미세아교세포 특이적 GPR56 억제는 MIA 유도 자손에게서 나타나는 사교성 결손, 반복적인 행동 패턴 및 증가된 불안 수준과 같은 비정상적인 행동을 관찰하였다. 미세아교세포 GPR56 억제 마우스에서는 MIA 유도 자손과 같은 비정상적인 피질 발달이 관찰되지 않았지만, c-fos 염색을 통해 뇌 활동이 관찰되었다. 따라서 본 연구는 미세아교세포 특이적 GPR56 결핍이 이상 행동을 유발함을 시사하며, 추후 연구를 통해 MIA 자손의 행동 결손 진단 및/ 치료 표적을 위한 바이오마커로 활용될 수 있음을 시사한다.