ผลของความเครียดขณะตั้งครรภ์ต่อการถ่ายทอดสัญญาณของ Reelin และการย้ายถิ่นของเซลล์ประสาทประสานงานแบบยับยั้งในเปลือกสมองส่วนหน้าของลูกหนู

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ความเครียดขณะตั้งครรภ์ส่งผลต่อการพัฒนาสมองของตัวอ่อนและเพิ่มความเสี่ยงในการเกิดโรคทางจิตเวชภายหลัง สมองส่วนหน้ามีหน้าที่สำคัญในการคิดระดับสูงซึ่งบกพร่องไปในผู้ป่วยจิตเวช เซลล์ประสาทประสานงานชนิดยับยั้ง (GABAergic interneuron) เป็นเซลล์ที่เคลื่อนย้ายเข้าไปในเปลือกสมองช้าที่สุด มีหน้าที่ทำให้เกิดสมดุลระหว่างการกระตุ้นและยับยั้งวงจรประสาท โปรตีน Reelin มีหน้าที่นำทางเซลล์ประสาทเกิดใหม่ให้ย้ายเข้าไปในเปลือกสมอง ที่สำคัญคือความบกพร่องของ Reelin และความไม่สมดุลระหว่างการกระตุ้นและการยับยั้งวงจรประสาทในเปลือกสมองใหญ่มีความเกี่ยวข้องกับการเกิดโรคทางจิตเวช อย่างไรก็ตาม ผลของความเครียดในแม่หนูตั้งครรภ์ต่อการถ่ายทอดสัญญาณของ Reelin และการย้ายถิ่นของเซลล์ประสาทประสานงานชนิด GABA ในสมองส่วนหน้ายังไม่ทราบแน่ชัดนัก งานวิจัยนี้ศึกษาผลของความเครียดในแม่หนูตั้งครรภ์ต่อการส่งสัญญาณของ Reelin และการอพยพของเซลล์ประสาทประสานงานชนิด GABA เข้าไปในเปลือกสมองส่วนหน้าของลูกหนู โดยแบ่งแม่หนูเป็นสองกลุ่มคือ 1) กลุ่มควบคุม และ 2) กลุ่มที่เหนี่ยวนำให้เครียด จากนั้นศึกษาเปรียบเทียบการกระจายตัวของเซลล์ประสาทประสานงานชนิด GABA และวัดระดับโปรตีนที่เกี่ยวข้องกับการส่งสัญญาณของ Reelin ในเปลือกสมองของลูกหนูเปรียบเทียบระหว่างกลุ่ม ผลการวิจัยพบว่าลูกหนูกลุ่มที่แม่มีภาวะเครียดขณะตั้งครรภ์มีเซลล์ประสาทประสานงานชนิด GABA ลดลงในเปลือกสมองส่วนหน้าและมีปริมาณโปรตีน Reelin เพิ่มขึ้นชั่วคราวในช่วงแรกเกิด แต่กลับลดลงอย่างมีนัยสำคัญทางสถิติเมื่ออายุ 7 วัน นอกจากนั้นยังพบว่าระดับโปรตีนตัวรับของ Reelin เช่น VLDLR และ Dab1 ลดลงอย่างมีนัยสำคัญทางสถิติเมื่อลูกหนูอายุ 7-14 วัน ผลการทดลองนี้แสดงว่าความเครียดขณะตั้งครรภ์ส่งผลระยะยาวต่อการถ่ายทอดสัญญาณของ reelin และการย้ายถิ่นของเซลล์ประสาทประสานงานชนิด GABA เข้าไปในเปลือกสมองส่วนหน้าของลูกหนู ดังนั้น การลดลงของ Reelin และโปรตีนตัวรับร่วมกับการลดจำนวนเซลล์ประสาทประสานงานชนิด GABA ในเปลือกสมองส่วนหน้าอาจเป็นกลไกที่เชื่อมโยงระหว่างความเครียดในช่วงต้นของชีวิตกับการเกิดโรคทางจิตเวชในภายหลัง

คำสำคัญ: ความเครียดขณะตั้งครรภ์ การส่งสัญญาณของโปรตีน Reelin เซลล์ประสาทประสานงานชนิด GABA เปลือกสมองส่วนหน้า

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Effects of maternal stress on Reelin signaling and migration of GABAergic interneurons in the prefrontal cortex of postnatal rats

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Abstract

Stress during pregnancy affects fetal brain development and increases the risk of subsequent neuropsychiatric diseases. The prefrontal cortex (PFC) plays important role in higher cognition and its impairment is associated with neuropsychiatric disorders. GABAergic interneurons are the last group of neurons that migrate into the cortex and plays a crucial role in modulating cortical output for proper sensory gating function and cognition. Reelin is an extracellular matrix protein that plays role in neural migration and its dysfunction causes the abnormal positioning of neurons in the cortex. Importantly, both the dysfunction of reelin and the abnormal function of GABAergic interneurons are associated with the pathology of neuropsychiatric disorders. However, it is still not clear how maternal stress alters GABAergic interneurons migration in the brains of the rat pups. The present study investigates the effect of MS on reelin signaling and the migration of GABAergic interneurons in the PFC among the rat pups. The pregnant rats were divided into two groups; maternal stress (MS) and the control group. The distribution of GABAergic interneurons and the expression level signaling proteins for the reelin pathway were measured and compared between groups at a statistically significant level of 0.05. The results revealed that MS significantly decreased GABA-immunopositive cells in the PFC of rat pups as compared to the control. Besides, MS temporarily increases reelin expression in the PFC of newborn pups, but later decreased at postnatal day (P)7. In addition, MS-induced a significant decrease in VLDLR and Dab1 expression in the PFC of rat pups at P7-P14. Altogether, the results indicated that MS has long-term effects on GABAergic interneurons migration in the prefrontal cortex of rat pups and the mechanism might be related to the dysfunction of reelin signaling. In conclusion, reelin dysfunction and abnormal positioning of GABAergic interneurons might be the mechanism that links early-life stress and the emergence of neuropsychiatric disorders later in life.

Keywords: maternal stress, Reelin signaling, GABAergic interneurons, prefrontal cortex

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Introduction

Maternal stress (MS) has detrimental effects on neurodevelopmental outcomes in young children and increased the vulnerability of neuropsychological problems that could be observed until adulthood[1]. For example, MS is associated with cognitive, behavioral, and emotional issues in neuropsychological disorders such as attention deficit hyperactivity disorder (ADHD), depression, and schizophrenia[2-5]. The adverse effects of MS on cognitive, behavioral, and psychosocial functions are mediated by maternal glucocorticoid that has a programming effect on fetal brain development. For example, MS reduces dendritic arborization and induces a synaptic loss in the prefrontal cortex (PFC) of the rodent offspring[6]. Moreover, stress-induced morphological changes in the hippocampus, amygdala, and PFC are associated with impairment of learning and memory and alter emotional responses in the rat pups[7]. The PFC plays important role in higher cognitive functions[8] which is highly vulnerable to early life stress[9]. In the cortex, GABAergic interneurons play a crucial role in modulating cortical output to prevent sensory overload and cognitive deficits. The imbalance between the excitatory-inhibitory function caused by abnormal development of GABAergic interneurons is associated with neuropsychiatric disorders such as schizophrenia and autism spectrum disorders[10].

The extracellular matrix glycoprotein Reelin is produced by the Cajal-Retzius cells, which are the earliest neurons in the marginal zone of the developing cortex. During early brain development, reelin plays a vital role in corticogenesis by guiding the neural migration and positioning of the projection neurons into the cortical plate. In the adult brain, reelin is produced from a subset of cortical GABAergic interneurons that plays an important role in the maintenance of synaptic function and plasticity[11,12]. Reelin binds to the receptors called ApoER2 and VLDLR to trigger intracellular signaling cascade by phosphorylation the adapter protein-1 (Dab-1), which activates the cytosolic kinases and phosphatases leading to the growth of the axon and dendritic spine, synaptic formation, and synaptic plasticity[13]. Moreover, abnormal expression of reelin is a common feature found in various neuropsychiatric diseases e.g., schizophrenia, bipolar disorder, major depressive disorder, and autism[14-17], and neurodegenerative disorder such as Alzheimer's disease[17]. Alteration in reelin expression was hypothesized as one factor that causes an impairment of neuronal connectivity underlying the cognitive deficits in those psychological disorders[18]. Although the mechanism underlying abnormal expression of reelin is still unclear, some evidence shows that early developmental insults might lead to abnormal reelin processing[19]. During the early postnatal period, interneurons tangentially migrate into the cortical plate and begin to switch from the tangential to a radial mode and integrate into the specific cortical layer[20, 21]. Therefore, reelin and its signaling proteins might play a critical role in the radial migration of interneurons into a specific layer of the developing cortex.

MS has a detrimental effect on brain development and increases the risk of neuropsychiatric disorder in later life, but the mechanism is still unclear. Disturbance of GABAergic interneurons associated with the pathology of neurodevelopmental disorders; however, it is still not known how MS alters GABAergic interneurons positioning and reelin signaling in the PFC of rat pups. We hypothesize that MS might disturb the expression of reelin and its signaling proteins, and lead to adverse consequences on GABAergic interneurons positioning in the developing prefrontal cortex. In this study, we investigate the effects of MS on the distribution of GABAergic interneurons and the expression of proteins involved with the reelin signaling pathway in the PFC of rat pups.

Materials and Methods

Animals

Pregnant Sprague Dawley rats (n=4/group) and their offspring (n=6/group/experiment) were used in this study. Eight pregnant rats were obtained from the National Experimental Animals Center of Mahidol University, Salaya, Thailand. There were housed in a single housing condition in a temperature- and humidity-controlled environment and maintained on a 12-hour light/dark cycle with free access to food and water. Pregnant rats were weighed daily on gestation day (GD) 7-21 before any manipulations. On the morning of GD 21, the pregnant rats were checked twice daily for the appearance of litter. The day a litter discovered was designated as postnatal day (P)0 and the length of gestation was noted. The equal number of male and female rat pups at P0, P7, and P14 were used for the western blot study (n=6/group) and immunofluorescence study (n=6/group). The humane endpoint was done by injection with double doses of anesthesia and follow by decapitation or transcardial perfusion. All experiments were conducted according to the Guidelines for Care and Use of the Laboratory Animals. The experimental protocol was approved by the Experimental Animal Ethics Committee of the Institute of Molecular Biosciences, Mahidol University, Thailand (COA. MB-ACUC 2015/003). Every effort was taken to minimize the number of animals used and their suffering.

Maternal stress

Pregnant rats were randomly divided into two groups: the control group, and the maternal stress (MS) group. The pregnant rat in the MS group was immobilized individually in a Plexiglas restrainer in which the length and diameter can be adjusted to accommodate the size of the animal. The immobilization stress is a model widely used to induce psychological stress in the rodent and has been proved to increase circulating corticosteroids[22,23]. The immobilization stress was performed 4 hours/day during GD 14-21 as previously described[24]. During the restrained

period, each pregnant rat was observed every half an hour and if the rat showed a sign of restlessness or suffering, it will be free and exclude from the experiment. Control dams were left undisturbed throughout the gestation period. The GD14–21 was selected because this is the most sensitive period to the teratogenic effects of prenatal stress, moreover, this is the period of pyramidal and non-pyramidal neuron migration within the cortical wall[25].

Tissues preparation

Rat pups were deeply anesthetized with sodium pentobarbital (30 mg/kg) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed rapidly and postfixed immediately in the same fixative overnight at 4 °C. The brain tissues were frozen in PBS and embedded in paraffin, then cut into 7 µm thickness in a coronal plane. Thereafter, the slices were immediately transferred to a submerged-type slice chamber and permanently preserved.

Immunofluorescence staining

Immunofluorescence staining for GABA was performed using a rabbit polyclonal anti-GABA antibody (ab9446, Abcam, Cambridge, UK) and the nucleus was stained with the anti-ToPRO3 antibody (T3605, Thermo Fisher Scientific, MA, USA). First, the dried tissue sections were deparaffinized and rehydrated by immersing slides through xylene and graded alcohol. Then, the sections were rinsed two times, 5 min each with 0.1 M PBS, and incubated for 40 min with 0.1 M PBS containing 1% glycine and 0.4% Triton X-100, washed three times in 0.1 M PBS for 10 min each and then blocked with 10% normal goat serum diluted in PBST (PBS containing 0.4% Triton X-100) for 2 h at room temperature. Sections were incubated with primary antibodies (1:500) at 4 °C overnight, washed with PBST and PBS, and then incubated with the secondary antibodies, anti-rabbit IgG-Alexa 488 (ab150089, Abcam, Cambridge, UK) (1:500), for 1 h at room temperature and rinsed three times in PBS. Finally, the sections were mounted on glass slides, coverslipped with anti-fade mounting medium (H-1000, Vector Laboratories Inc., California, USA), and observed under the Inverted confocal laser scanning microscope, Olympus FV1000.

Western blot analysis

Tissue dissections were performed according to The Rat Brain in Stereotaxic Coordinates. The PFC was defined as Cg1, Cg3, and IL subregions corresponding to the plates 6–9. The prefrontal cortex tissues were immediately dissected out, frozen on dry ice, and stored at -80 ºC until use. For protein preparation, brain tissues were suspended in a lysis buffer composed of 50 mMTris pH 7.4, 150 mM NaCl, 1mM EDTA, 0.5% Na Deoxycholate, 1% SDS, 1 mM PMSF, 1% Triton-X-100 and supplemented with complete

protease and phosphatase inhibitor cocktail set (Calbiochem, Germany), then homogenized for 10 sec and centrifuge at 12,000 rpm at 4 ℃ for 15 min. The supernatant was collected for protein determination by the Bradford method. Twenty-five micrograms of protein samples were denatured in sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 2% mercaptoethanol, and 0.01% bromophenol blue) at 100 ºC for 5 min. Proteins were loaded onto 7-12% SDS-PAGE and electrophoretically transferred to the PVDF membranes (Amersham Bioscience, Piscataway, NJ, USA). The transfer efficiency was checked by Ponceau-S red staining. The membrane was washed with Tris-buffered saline (TBS) for 5 min, then incubated in blocking buffer (3% nonfat milk and 2% bovine serum albumin in TBS containing 0.1% Tween-20, TBST) for 1 h at room temperature and incubated overnight at 4 ºC with rabbit polyclonal anti-reelin antibody (sc-5578, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) (1:1,000), mouse monoclonal anti-VLDLR antibody (sc-18824, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) (1:250), goat polyclonal anti-Dab1 antibody (sc-7827, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) (1:500) or mouse polyclonal anti-β-actin antibody (1:5000) from Chemicon International Inc., Temecula, CA, USA. Then, membranes were washed three times with TBST and incubated in a 1:10,000 dilution of peroxidase-conjugated horseradish secondary antibody for 1 h at room temperature. After that, they were washed three times with TBST and incubate with ECL prime (Amersham Biosciences, Piscataway, NJ, USA) for 5 min and the band density was captured by Azure c400 Visible Fluorescent Western Blot Imaging System (Azure Biosystems, Inc, CA, USA). The immunoblot band densities were quantified using the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

Image analysis

The stained sections were observed under a light and confocal laser microscope and were photographed. The images from both groups were analyzed in the same way. Quantification was based on contrast and color intensity in full-focus projections of images with 20x magnification. Multiple areas were chosen from three cortical regions per hemisphere (medial-, dorsal-, and dorsolateral PFC). The photographs were taken from the middle portion of each layer and three continuous non-overlapping frames, each, in 3 sections. Thus, each mean value represents the mean value of 108 photographs from each cortical layer. Photographs from the selected area were captured by a CCD color camera and transformed into digits. The total areas of each frame were estimated using Adobe Photoshop.

Statistical analysis

Data were expressed as mean ± SEM and statistically analyzed using GraphPad Prism 5 software (GraphPad Software, San

Diego, CA). Statistical comparisons of the data set were performed by Two-way ANOVA followed Bonferroni multiple comparison test for comparison between cortical layers, ages, and groups. For all comparisons, probability values of $p<0.05$ were considered statistically significant.

Results

MS decreases the density of GABAergic interneurons in the PFC of postnatal rat pups

The population of GABAergic interneuron in the deep layer of the prefrontal cortex was investigated and compared between the two groups at P0, P7, and P14 as shown in Figure 1. The immuno-fluorescence signal for GABA was detected in the nucleus and cytoplasm of the neurons. At P0, GABA-positive cells were significantly decreased in the MS pups ($p<0.001$), as compared with the control. Similarly, at P7, GABA-positive cells in the deep layer of the PFC were significantly reduced in the MS pups ($p<0.05$) as compared with the control. At P14, there is no significant difference in the percent of GABA-positive cells when compared between the two groups (Figure 1.).

Maternal stress decreases reelin, VLDLR, and Dab1 expression in the PFC of rat pups

We investigated the level of reelin, VLDLR, and Dab1 in the PFC of rat pups at different postnatal ages and compared between groups. For reelin, the results showed that MS during GD 14-21 caused a significant increase in the level of reelin at P0 ($p<0.001$), but caused a significant decrease at P7 ($p<0.001$) as compared to the control (Figure 2.). However, there was no significant difference in the levels of reelin when observed at P14. There was no significant difference in VLDLR when compared between the two groups at P0 and P7, however, MS-induced a significant decrease of VLDLR at P14 ($p<0.001$) as compared to the control (Figure 3.). For the adaptor protein Dab1 which required for reelin signaling, the results revealed that MS-induced a significant decrease in the amount of Dab1 throughout the neonatal periods from P0 ($p<0.01$), P7 ($p<0.001$), to P14 ($p<0.001$), as compared to the control (Figure 4.). In summary, MS-induced a disturbance of proteins related to reelin signaling in the PFC of rat pups during the first two weeks of life. The overall reduction in the reelin signaling proteins indicates that MS might disturb the function of reelin in the PFC of rat pups.

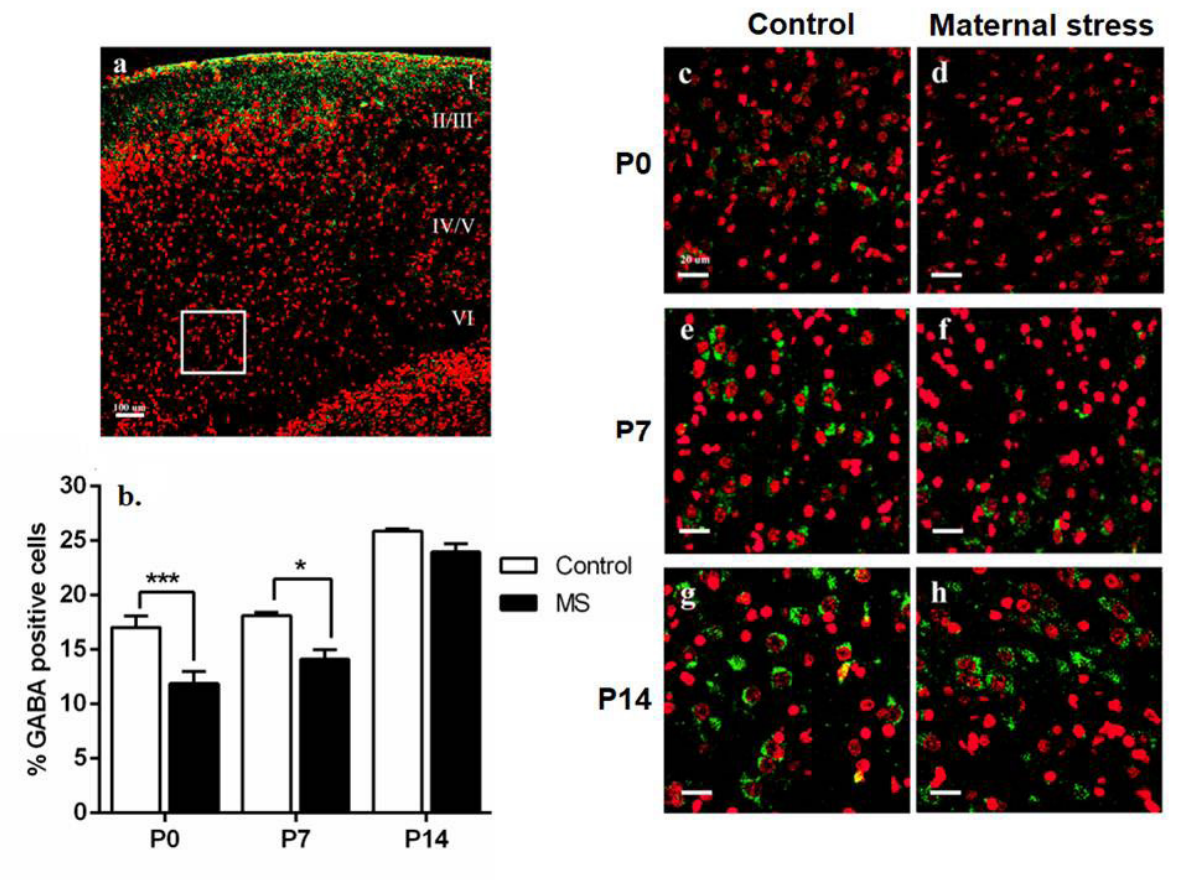

Figure 1. Maternal stress decreases the percent of GABA-immunopositive cells in the PFC of male and female rat pups. (a) Photomicrographs of immunofluorescence staining of GABA (green), and the TO-PRO3 nuclear staining (red). The white frame represents the deep cortical layer that was observed in this study. Bar graph comparing the number of GABAergic interneurons in the deep cortical layer between control and the MS groups (b) at P0 (c, d), P7 (e, f), and P14 (g, h). The values represent the mean ± SEM, n=6 per group. Scale bar, 100 μm (a), and 20 μm (c-h). Significant difference at *p<0.05 and ***p<0.001 compared with the control group. I-VI represents the cortical layer 1-6, respectively.

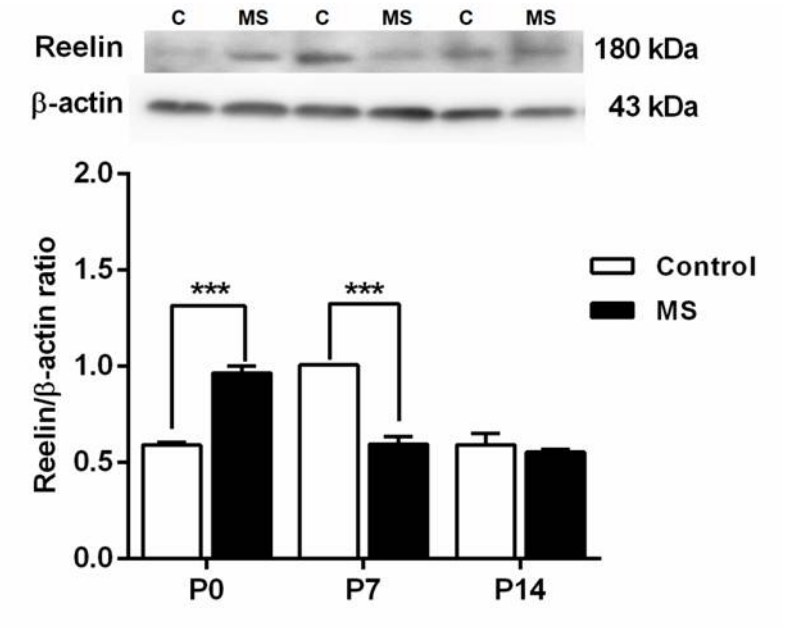

Figure 2. Effects of maternal stress on reelin expression in the prefrontal cortex of rat pups at P0, P7, and P14. Upper panel: Western blot analysis of reelin protein compared between MS and control group at P0-P14. Lower panel: The bar graph displays the quantitative results. The data are expressed as band densities/β-actin ratios. The values represent the mean ± SEM, n=6 per group. ***p<0.001 compared with the control group.

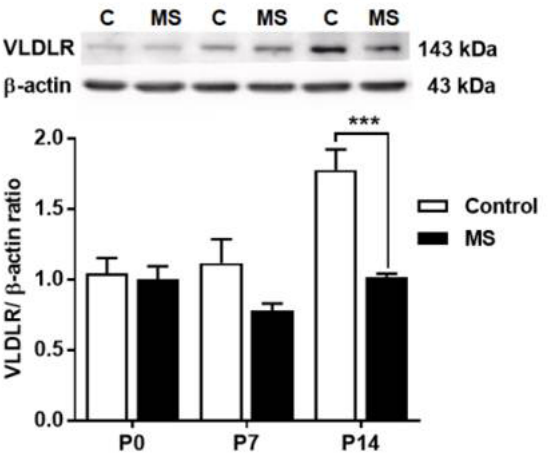

Figure 3. Effects of maternal stress on the VLDLR expression in the prefrontal cortex of rat pups at P0, P7, and P14. Upper panel: Western blot analysis of VLDLR protein compared between MS and control group at P0-P14. Lower panel: The bar graph displays the quantitative results. The data are expressed as band densities/β-actin ratios. The values represent the mean ± SEM, n=6 per group. ***p<0.001 compared to the control group.

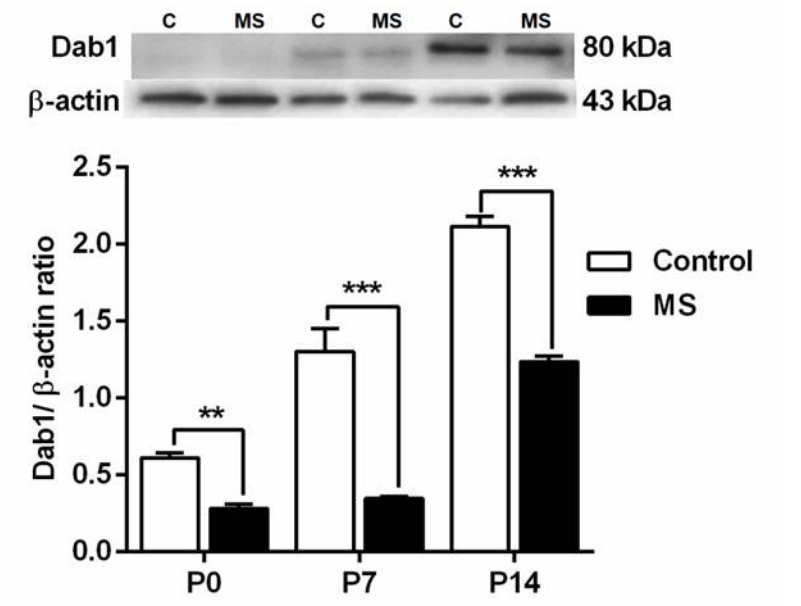

Figure 4. Effects of maternal stress on Dab1 expression in the prefrontal cortex of rat pups at P0, P7, and P14. Upper panel: Western blot analysis of Dab1 protein compared between MS and control group at P0-P14. Lower panel: The bar graph displays the quantitative results. The data are expressed as band densities/β-actin ratios. The values represent the mean ± SEM, n=6 per group. $^{**}p<0.01$ and $^{***}p<0.001$, compared with the control group.

Conclusions and Discussions

Our results showed that MS has long-term consequences on interneurons migration and their positioning in the developing cortex. MS decreases the percentage of GABA-immunopositive cells in the prefrontal cortex in rat pups from P0-P7, especially in the cortical layer IV-V. The results indicated that young GABAergic interneuron is highly vulnerable to the adverse effect of prenatal stress. A decrease in GABA-immunopositive cells in the PFC of rat pups could occur in response to various factors. A decreased in GABA-immunopositive cells in the PFC of rat pups could be explained by various factors. For example, prenatal cocaine exposure decreases the percentage of young interneurons migrating tangentially from the ganglionic eminence into the cerebral wall of the developing mouse brain[26]. Besides, MS could also decrease GABAergic progenitor cells in the medial ganglionic eminence[27]. A previous study reported that MS induces selective loss of parvalbumin (PV) positive GABAergic interneurons in the medial PFC of the mouse offspring[28]. Moreover, MS temporarily decreased the number of GAD67 positive cells in the medial PFC of rat pups at birth, but later increase at the weaning period[29]. Importantly, the adverse effect of MS on interneurons development persists until the late postnatal age, thus, indicates long-

term effects of MS on interneurons development[29]. Our results together with these lines of evidence demonstrate a consistent finding that GABAergic interneurons are more vulnerable to early life stress than previously thought.

Maternal stress has a negative impact on the development of PFC and the cortical GABAergic interneuron is a major target of maternal stress hormone. Abnormal distribution of the GABAergic interneurons is associated with remarkable changes in inhibitory function which underlies the pathology of neurodevelopmental disorders[30,31]. During normal cortical development, the interneurons migrate tangentially from the medial ganglionic eminence into the cortex and then stop for a while before incorporate into specific cortical layers, after that, they begin to switch from the tangential mode toward the radial mode of migration to integrate into the specific cortical layer in the developing cortex[32]. Thus, the abnormal distribution of GABAergic interneurons in the PFC of the MS pups could be due to the disturbance of the switching from the tangential mode toward the radial mode of migration. During the prenatal period, the reelin signaling pathway plays a major role in the radial migration of the projection neuron. Reelin binds to the APOE/VLDLR receptors to trigger the cascade of events by phosphorylating Dab1 protein to activates the Src-tyrosine kinase/Fyn kinase family. The consequences are the activation of several kinase cascades, including the PI3K and PKB/AKT1, and finally, inhibit the GSK3β to promote the microtubule dynamic during the process of neural migration. In the postnatal period, reelin positive cells still present in the layer V of the cortex of postnatal rat pups at P0.5 and in all cortical layers at P7.5 [33]. An in vivo study indicated that reelin not only important for brain growth but also promotes the selective pruning and maturation of the dendritic process in the cortical neurons[35]. A recent study showed that the neural stem/progenitor cells (NSPCs) transplanted into the striatum of the adult hemiplegic mice could also migrate from the striatum to the injured cortex[34]. These NSPCs respond to reelin stimulation and contribute to the functional recovery after neural cell transplantation in the hemiplegic mice. These findings indicated the critical role of reelin in cortical development during the postnatal period.

The finding that MS acutely increases reelin in the PFC of rat pups at birth (P0), but a significant decrease at P7 together with a significant decrease in VLDLR and Dab1 at P7 and P14, indicates that MS causes dysregulation of reelin signaling in the pup's brain. Considering the important role of reelin in modulating the process of interneurons migration, therefore, disruption in reelin signaling may underlie the laminar disorganization in the PFC of rat pups born from maternal stress dam. The decrease of reelin could occur due to the epigenetic change in response to early life stress since a previous study reported that prenatal stress induces hypermethylation of reelin and GAD67, which is associated with the

schizophrenia-like phenotype[36]. Moreover, reduction in the density of reelin-expressing neurons in layer I of the embryonic cortex is associated with the behavioral impairment in the adult[37]. Importantly, the number of reelin expressing interneurons in the PFC and temporal cortex shows a significant decrease in layers I and II (the area of most abundant reelin-positive neurons) of the postmortem brain of Schizophrenia patients[38]. Our findings suggested that reelin dysfunction and abnormal positioning of GABAergic interneurons might be the mechanism that links between early-life stress and the emergence of neuropsychiatric disorders later in life.

In conclusion, GABAergic interneuron is highly vulnerable to the effect of prenatal stress. Prenatal stress decreases GABA-immunopositive cells in the PFC of postnatal rat pups and disturbs the signaling proteins for reelin, which might affect its function in cortical development. Our results suggest that MS alters reelin signaling may contribute to the abnormal distribution of interneurons in the PFC of rat pups. Interfering of the reelin signaling pathway might affect the cortical integration of GABAergic interneurons in the PFC of rat pups.

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