รายงานวิจัยฉบับสมบูรณ์

โครงการ

การตั้งโปรแกรมทารกในครรภ์โดยฮอร์โมนเครียดที่ได้รับจากแม่: แนวทางในการป้องกัน

Fetal Programming of Brain Development by Maternal Glucocorticoids: the Possibility for Reprogramming

โดย

รองศาสตราจารย์ ดร. นวลจันทร์ จุฑาภักดีกุล
สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

31 พฤษภาคม พศ. 2561

สัญญาเลขที่ RSA5780016

รายงานวิจัยฉบับสมบูรณ์

โครงการ

การตั้งโปรแกรมทารกในครรภ์โดยฮอร์โมนเครียดที่ได้รับจากแม่: แนวทางในการป้องกัน

Fetal Programming of Brain Development by Maternal Glucocorticoids: the Possibility for Reprogramming

โดย

รองศาสตราจารย์ ดร. นวลจันทร์ จุฑาภักดีกุล
สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และ มหาวิทยาลัยมหิดล

Acknowledgement

We would like to thank Assistant Prof. Dr. Rapeepun Vanichviriyakit from the Department of Anatomy, Faculty of Sciences, for her kind support on the immunofluorescence staining techniques. Data from this report come from the research thesis of 2 Ph.D. students (Mrs. Pornprom Surakul and Miss Ratirat Kolaka) and 2 M.Sc. students (Mr. Bovorn Verawattananan and Miss Woranan Wongmarsang) from Neuroscience curriculum at Mahidol University. I would like to thanks them all for their contribution. Without them, this work cannot be completed.

This work was supported by Thailand Research Fund (TRF) and Mahidol University (Contract No. RSA-5780016). The authors have no conflicts of interest to declare.

Abstract

Project Code: **RSA5780016**

Project Title: **Fetal Programming of Brain Development by Maternal Glucocorticoids: the Possibility for Reprogramming**

Investigator: **Nuanchan Chutabhakdikul, et al.**

Institute: **Institute of Molecular Biosciences, Mahidol University**

E-mail: **nuanchan.chu@mahidol.edu**

Project Period: 2014-2016

Stress exposure during pregnancy is an important factor for programming the fetal brain and contribute to an emergence of psychiatric symptoms in the offspring later in life. Maternal stress hormone can pass to the fetus directly via placenta and trigger fetal stress with programming consequences. Thus, early life impact of stress hormone on brain development and the possibility to reprogrammed is considered as one of the high priorities for neuroprotective research stemming from the fetal programming hypothesis.

We demonstrated the prenatal programming effects of maternal restraint stress on the following; 1) Prenatal stress (PS) programming the development of GABAergic synapse in the pup's hippocampus. For examples, PS delay maturation of the GABAergic system by altering the expression of NKCC1, KCC2 and GABAA receptor α1 and α5 subunits at adolescence. These changes indicated that dysregulation of inhibitory neurotransmission that might underlie the pathogenesis of psychiatric diseases at adolescence. Moreover, PS also induced biphasic changes in the level of Neuroligin-2, β-Neurexin and Gephyrin in the hippocampus of rat pups, in which the levels increased during the first and the second postnatal week, but decreased during pre-adolescence to adolescence period as compare to control. In contrast, GABAA receptor α1-6 subunits decreased during the third postnatal week, but increased during adolescence period. These results indicate that the synaptic adhesion molecules and scaffolding protein of GABAergic synapse are highly vulnerable to stress hormone when exposed to stress *in utero*. 2) PS programing the neuroimmune response by increase of IL-6 and IL-10, but decrease in the autophagic marker, LC3B-II, in the hippocampus of rat pups from P7-60. The same responses have been observed in the *in vitro* model of CORT-treated SH-SY5Y human neuroblastoma cell lines and the JEG-3 human choriocarcinoma cell lines culture. It is

interesting that when we modulate the placental barrier by using an 11β-HSD1 inhibitor, PFi, it could reverse the effects of CORT on neuroimmune response and the autophagic biomarker in the *in vitro* model of SH-SY5Y and JEG-3 co-culture. Our results indicated that maternal stress induced immune dysregulation in the pup's brain could be mediated by maternal CORT pass through the fetus via the placenta, and subsequently might interrupt autophagy in the developing hippocampus. In summary, our result provided important evidence for the possibility that modulating placental enzyme activity might be a novel target for reprogramming the negative effects of maternal stress on fetal brain development.

Keywords: Prenatal stress, Corticosterone, 11β-HSD1 inhibitors, GABA receptor, neuroinflammation, autophagy, hippocampus

บทคัดย่อ

รหัสโครงการ: RSA5780016
ชื่อโครงการ: การตั้งโปรแกรมทารกในครรภ์โดยฮอร์โมนเครียดที่ได้รับจากแม่:แนวทางในการป้องกัน
ชื่อนักวิจัย: นวลจันทร์ จุฑาภักดีกุล และคณะ
สถาบัน: สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล
E-mail: nuanchan.chu@mahidol.edu
ระยะเวลาโครงการ: กรกฎาคม 2557- มิถุนายน 2559

ความเครียดในช่วงตั้งครรภ์เป็นสาเหตุสำคัญที่ทำให้สมองของทารกในครรภ์ถูกโปรแกรมไปในทางที่นำไปสู่โรคทางจิตเวชในภายหลัง ฮอร์โมนเครียดจากมารดาสามารถส่งผ่านไปยังทารกในครรภ์ได้โดยตรงผ่านทางรกและส่งผลระยะยาวที่เป็นอุปสรรคต่อการพัฒนาสมองของลูก งานวิจัยที่ค้นหาแนวทางในการย้อนโปรแกรมเพื่อหยุดยั้งผลกระทบของฮอร์โมนเครียดในช่วงต้นของชีวิตต่อการพัฒนาสมองจึงเป็นหนึ่งในงานวิจัยด้านประสาทวิทยาศาสตร์ที่มีความสำคัญอย่างมากเพราะจะช่วยให้ค้นพบแนวทางในการลดความเสี่ยงที่เป็นผลจากการตั้งโปรแกรมสมองของทารกในครรภ์โดยฮอร์โมนเครียดที่ได้รับจากแม่

งานวิจัยนี้ ผู้วิจัยได้แสดงให้เห็นผลจากความเครียดในมารดาตั้งครรภ์ต่อการตั้งโปรแกรมพัฒนาการสมองของลูกดังนี้ 1) ความเครียดในแม่ตั้งครรภ์ส่งผลเสียต่อการพัฒนา GABAergic synapse ในสมองส่วน hippocampus ของลูก เช่น ทำให้เกิดความล่าช้าทางพัฒนาการของ GABAergic synapses จากการเปลี่ยนแปลงการแสดงออกของโปรทีนที่ควบคุมการทำหน้าที่ของ GABAA receptor เช่น NKCC1, KCC2 รวมทั้ง GABAA receptor α1และα5 subunits ในสมองลูกหนูช่วงวัยรุ่น การเปลี่ยนแปลงเหล่านี้แสดงให้เห็นว่าการทำหน้าที่ของ GABAergic synapse ขาดความสมดุลย์ในสมองของลูกหนูที่เกิดจากแม่เครียด ซึ่งอาจมีความเกี่ยวข้องกับสาเหตุของการเกิดโรคทางจิตเวชในช่วงวัยรุ่น นอกจากนั้นความเครียดในแม่ตั้งครรภ์ยังทำให้เกิดการเปลี่ยนแปลงการแสดงออกของโปรทีน Neuroligin-2, β-Neurexin และ Gephyrin ในสมองส่วน hippocampusของลูกหนู โปรทีนเหล่านี้เพิ่มขึ้นในช่วงสัปดาห์ที่ 1-2 หลังคลอด แต่ลดลงในช่วงก่อนวัยรุ่นจนถึงวัยรุ่น ในทางตรงกันข้ามตัวรับชนิด GABAA receptor α1-6 ลดลงในช่วงสัปดาห์ที่ 3 หลังคลอด แต่เพิ่มขึ้นในช่วงวัยรุ่นผลการศึกษานี้แสดงให้เห็นว่าโปรทีนที่ทำหน้าที่ยึดเกาะ GABAA receptor รวมทั้ง synaptic โปรตีนที่เกี่ยวข้องในสมองของลูกหนูที่เกิดจากแม่เครียด ถูกโปรแกรมให้แสดงออกลดลงในช่วงที่เป็นวัยรุ่นส่งผลให้เกิดความอ่อนแอของ GABAergic synapses ในสมองของลูกหนูที่เกิดจากแม่เครียด 2) ความเครียดในแม่ตั้งครรภ์ตั้งโปรแกรมการตอบสนองของระบบภูมิคุ้มกันในสมองของลูกโดยเพิ่มการหลั่ง IL-6 และ IL-10 ใน hippocampus ของลูกหนูอายุ 7-60 วัน แต่ลดการแสดงออกของโปรทีนที่เป็นดัชนีชี้วัดทางชีวภาพของกระบวนการ autophagy (หรือ LC3B-II) ซึ่งมีความสำคัญต่อกระบวนการ pruning ในระหว่างการพัฒนาสมอง งานวิจัยนี้พบการตอบสนองแบบเดียวกันด้วยในเซลล์เพาะเลี้ยง SH-SY5Y ที่ได้รับฮอร์โมนเครียดปริมาณสูง

ที่น่าสนใจคือเมื่อศึกษาใน *in vitro* model ที่เลียนแบบสิ่งแวดล้อมในครรภ์โดยการเพาะเลี้ยงเซลล์ร่วม (co-culture) ระหว่างเซลล์ SH-SY5Y human neuroblastoma cell lines และJEG-3 human choriocarcinoma cell lines พบว่าเมื่อให้สารสังเคราะห์ PFi (ทำหน้าที่ยับยั้งเอ็นไซม์ 11β-HSD1) ทำให้เพิ่มการสกัดกั้นฮอร์โมนเครียดในเซลล์รก พบว่าช่วยยับยั้งผลของฮอร์โมนเครียดจากแม่ต่อการตอบสนองของระบบภูมิคุ้มกันและดัชนีชี้วัดทางชีวภาพ LC3B-II ได้อย่างมีนัยสำคัญทางสถิติ ผลจากงานวิจัยนี้ชี้ให้เห็นว่าการตอบสนองของระบบภูมิคุ้มกันในสมองของลูกที่เป็นผลจากความเครียดในมารดาตั้งครรภ์เป็นผลโดยตรงจากฮอร์โมนเครียดของแม่ผ่านทางรกไปสู่ทารกในครรภ์ โดยไปเพิ่มการอับเสบและยับยั้งกระบวนการ autophagy ในสมองส่วน hippocampus ของลูกหนูที่กำลังพัฒนา

ผลการวิจัยนี้เสนอหลักฐานสำคัญที่มีความเป็นไปได้ในการย้อนโปรแกรมที่เป็นผลของฮอร์โมนเครียดในแม่ตั้งครรภ์โดยการยับยั้งเอนไซม์11β-HSD1 ในเซลล์รก งานวิจัยนี้ชี้ให้เห็นว่ายาที่ช่วยสกัดกั้นฮอร์โมนเครียดจากแม่ไปสู่ลูกในครรภ์อาจเป็นเป้าหมายยาตัวใหม่ที่มีประสิทธิภาพในการย้อนโปรแกรมที่เป็นผลจากฮอร์โมนเครียดในแม่ตั้งครรภ์ต่อการพัฒนาสมองของลูกได้

คำหลัก : ความเครียดในแม่ตั้งครรภ์, สารยับยั้งเอ็นไซม์ 11β-HSD1, ตัวรับ GABAA, ระบบประสาทเกี่ยวกับการอักเสบ, การกลืนกินตัวเอง, ฮิปโปแคมปัส

List of Figures

List of Abbreviations

Abbreviations	Scientific term
11β-HSD1	11β-hydroxysteroid dehydrogenase 1 enzyme
11β-HSD2	11β-hydroxysteroid dehydrogenase 2 enzyme
Atg	autophagy related proteins
BDNF	brain a derived neurotrophic factor
CBX	Carbenoxolone, an 11β-HSD2 inhibitor
CCCs	Cation-Chloride Cotransporters
CORT	Corticosterone
EGABA	the reversal potential of the GABAA receptor-mediated current
GCs	glucocorticoids
GD	gestation day
JEG-3	human choriocarcinoma cell lines
KCC2	Potassium-Chloride Cotransporter 2
LC3	1A/1B-light chain 3
LC3B-II	LC3-phosphatidylethanolamine conjugate
LC3-I	a cytosolic form of LC3
LTP	long-term potentiation
NKCC1	Sodium-Potassium Chloride Cotransporter 1
OXSR1	Oxidative stress response kinase1
P0	postnatal day 0
PFi	11β-HSD1 inhibitor
PS	prenatal stress
SH-SY5Y	human neuroblastoma cell lines
TrkB	tyrosine receptor kinase B
ULK-1	Unc-51-like autophagy-activating kinase 1
WNK3	With no K [lysine] protein kinase3

Index

Chapter 1

Maternal Restraint Stress Delays maturation of Cation-Chloride Cotransporters and GABAA Receptor Subunits in the Hippocampus of Rat Pups at Puberty

Abstract:

The GABAergic synapse undergoes structural and functional maturation during early brain development. Maternal stress alters GABAergic synapses in the pup's brain that are associated with the pathophysiology of neuropsychiatric disorders in adults; however, the mechanism for this is still unclear. In this study, we examined the effects of maternal restraint stress on the development of Cation-Chloride Cotransporters (CCCs) and the GABAA receptor α1 and α5 subunits in the hippocampus of rat pups at different postnatal ages. Our results demonstrate that maternal restraint stress induces a transient but significant increase in the level of NKCC1 (Sodium-Potassium Chloride Cotransporter 1) only at P14, followed by a brief, yet significant increase in the level of KCC2 (Potassium-Chloride Cotransporter 2) at P21, which then decreases from P28 until P40. Thus, maternal stress alters NKCC1 and KCC2 ratio in the hippocampus of rat pups, especially during P14 to P28. Maternal restraint stress also caused biphasic changes in the level of GABAA receptor subunits in the pup's hippocampus. GABAA receptor α1 subunit gradually increased at P14 then decreased thereafter. On the contrary, GABAA receptor α5 subunit showed a transient decrease followed by a long-term increase from P21 until P40. Altogether, our study suggested that the maternal restraint stress might delay maturation of the GABAergic system by altering the expression of NKCC1, KCC2 and GABAA receptor α1 and α5 subunits in the hippocampus of rat pups. These changes demonstrate the dysregulation of inhibitory neurotransmission during early life, which may underlie the pathogenesis of psychiatric diseases at adolescence.

Key words:

Prenatal stress, Cation-Chloride Cotransporters, NKCC1, KCC2, GABAA receptor, Hippocampus

Introduction:

Early life stressors that shape the stress response in offspring have profound effects on mood and cognition in adulthood [1]. Chronic exposure to glucocorticoids contribute to the dysfunction of the inhibitory network and impairment of rhythmic oscillations, which are critical for the regulation of brain activity and complex cognitive processes [2]. A dysfunctional GABAergic system is associated with the pathogenesis of neuropsychiatric diseases such as schizophrenia, anxiety and depression [3].

During brain development, GABAergic synapses are formed prior to the formation of glutamatergic synapses and the activation of the GABAA receptor depolarizes immature neurons [4-6]. Excitatory GABA transmission plays important roles in various neurodevelopmental processes including; neuronal migration, cell proliferation, neurite outgrowth and generating synchronized network activity [7]. Cation Chloride Cotransporter (CCC) is the key controlling factor in controlling the switch of the GABAA receptor function. CCCs control the reversal potential of the GABAA receptor-mediated current (EGABA), which is important for the modulation of the GABAA receptor function. There are two main types of CCCs; the outwardly directed Potassium-Chloride Cotransporter 2 (KCC2), and the inwardly directed Sodium-Potassium-Chloride Cotransporter 1 (NKCC1). In immature neurons, NKCC1 increases the chloride reversal potential thus it accumulates Cl- inside the cell. KCC2, on the other hand, reduces the chloride reversal potential thus it extrudes Cl- out of the cell and shifts the actions of the GABA from excitation to inhibition. Although the other chloride regulators channels and transporters also take part in this sequence [8, 9], the expression of KCC2 is thought to initiate the developmental switch of the GABAA receptor function from excitatory to inhibitory transmission [5].

In addition to the expression of Cation-Chloride Cotransporters, the GABAA receptor undergoes postnatal changes in its structure and function by the differential expression of different subunits' composition [10]. The presence of GABAA receptor α1 subunits mediates phasic inhibition by inducing a more rapid decay rate in GABAA-mediated synaptic currents [11]. In contrast, the GABAA receptor α5 subunits mediate tonic inhibition, which can be characterized by a slow decay rate of the synaptic current [10]. The α1 subunits are located at the synaptic sites and mostly found in mature neurons, while the α5 subunits are located at the extrasynaptic sites and found mostly in immature neurons prior to the formation of the inhibitory synapse [10, 12, 13]. Thus, the maturation of the GABAergic function requires the precise expression of specific subunits of the GABAA receptor during postnatal brain development. The early expression of the GABAA receptor α5 subunit is required for the tonic inhibitory function of GABA, while the late expression of the α1 subunit is required for the phasic inhibition that indicates the maturation of the GABAA receptor function.

It is well documented that stress increases glucocorticoid hormones and thereby potentiates excitotoxic damage in hippocampal GABAergic neurons [14, 15]. Early life stress exerts an effect on

the hippocampal neurons and predisposes individuals to psychosis [16-19]. The hippocampus exhibits subtle alterations subsequent to neuropsychiatric diseases such as schizophrenia and mania depressive disorder [20]. Previous studies in postmortem brains from schizophrenia patients have shown a decrease in hippocampal GABAergic activity that could potentiate excitotoxic damage to hippocampal interneurons, consistent with abnormal oscillatory rhythms and increased basal metabolic activity [20]. It is still unclear how prenatal stress affects the development of GABAergic synapses in the hippocampus of the offspring. In this study we hypothesized that prenatal stress may affect the structural and functional maturation of the GABAA receptor in the hippocampus of rat pups. Therefore, the purpose of this study was to examine the effect of maternal restraint stress on the levels of NKCC1 and KCC2, as well as GABAA receptors α1 and α5 subunits in the hippocampus of the offspring to provide insights about the involved mechanisms of maternal stress as a cause of dysregulation of GABAergic synapses that are known to be associated with the pathogenesis of psychiatric diseases at adulthood.

Materials and Methods

Animals:

Pregnant Sprague Dawley rats and their offspring were used in this experiment. Pregnant rats were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Thailand. They were housed in a single housing condition with a temperature and humidity controlled environment and maintained on a 12 hours light/dark cycle with free access to food and water. Each pregnant female rat was weighed on gestation day (GD) 7-21. In the morning of GD 21, each pregnant rat received nesting material, and thereafter, the cage was checked twice daily for the appearance of a litter. The day a litter gets discovered becomes designated as postnatal day 0 (P0), and the length of gestation was noted. All experiments were conducted according to the Guidelines for Care and Use of the Laboratory Animals and 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 Restraint Stress:

Pregnant rats were divided into two groups; 1) control group, 2) maternal restraint stress group (N=4/group). For the restraint stress, each pregnant rat was put into a small Plexiglas cylindrical cage in which the length can be adjusted to accommodate the size of each animal. The restraint stress was performed during GD14-20, at four hours daily intervals during the light phase of the cycle as previously described [21, 22]. The control group was left undisturbed in their home cages. Gestation

days 14-20 were selected because they represent the most sensitive period for the behavioral teratogenic effect of prenatal stress [23].

Tissue Preparation:

Whole hippocampal tissues were collected from rat pups at different postnatal days (P) from P7, P14, P21, P28 and P40, with n=4/group. Brain tissues were then suspended in a lysis buffer composed of 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Sodium Deoxycholate, 1% SDS, 1 mM PMSF, 1% Triton-X-100 and supplemented with a complete protease and phosphatase inhibitor cocktail set (Calbiochem, Germany), then homogenized twice with a sonicator for 10 sec each. The homogenized samples were centrifuged at 14,000 rpm, 4°C for 15 min. The supernatant was collected for protein determination. The protein concentration from each sample was determined by a Bradford protein assay.

Western Blot Analysis:

Cell lysates were mixed with a sodium dodecyl sulfate (SDS) sample buffer and boiled. Equal amounts (20 μg) of extracted protein samples were resolved in 10% SDS-PAGE and electrophoresis at 100 V for 150 mins. The protein bands were then transferred to the PVDF membrane (Amersham, USA) at 100V for 135 min. The membranes were then incubated in a blocking solution containing 3% skimmed milk for NKCC1, KCC2 and GABAA receptor α1 and α5 subunits, and 5% skimmed milk for actin at room temperature for 60 mins. Then the membranes were incubated with the following specific primary antibodies purchased from the available commercial sources. Polyclonal goat anti-NKCC1 (SC-21545; 1:500), polyclonal goat anti-KCC2 (SC-19420; 1:500), polyclonal goat anti-GABAA receptor α1 subunit (SC-7348; 1:500), polyclonal goat anti-GABAA receptor α5 subunit (SC-7357; 1:500), and monoclonal mouse anti-actin (SC-69879; 1:5,000), all antibodies were purchased from Santa Cruz Biotechnology, USA. The membranes were then thoroughly washed 3 times using 0.1% Tween-TBS (TTBS) for 5 mins each, and then incubated with an appropriate HRP-conjugated secondary antibody. After that, the membranes were washed 3 times using 0.1% TTBS for 5 mins each, and then the signals were detected by an Enhanced Chemiluminescence System (ECL Prime, Amersham Biosciences, USA) and film exposure. The films were then scanned and digitally processed using Adobe Photoshop software. The intensities of the band were quantified using densitometry software (Image J, National Institutes of Health, USA). The immunoblot data were corrected for corresponding product of the β-actin extracted from the same tissue which serve as an internal control.

Data and Statistical Analysis:

The data was statistically analyzed using GraphPad Prism software. Quantitative results were expressed as mean ± SEM, calculated from the duplicate experiments. The statistical significance of difference between means was evaluated using Student's t-test (unpaired, unless otherwise stated). Changes produced by prenatal stress were analyzed at different postnatal ages using a two-way ANOVA with the prenatal stress and postnatal ages as independent variables and the protein levels as dependent variables; followed by a Tukey's post hoc multiple comparison test. The probability level of $p \leq .05$ was considered to have a statistically significant difference between the two sets of data.

Results:

Maternal restraint stress alters NKCC1 and KCC2 in the hippocampus of rat pups at puberty.

We examined the effects of maternal restraint stress, during the gestation day (GD) 14-20, on the levels of NKCC1 and KCC2 in the hippocampus of rat pups and compared between the groups at different postnatal ages. The results showed that maternal restraint stress caused a transient but significant increase in the level of NKCC1 in the hippocampus at P14 ($p<0.05$) but no significant difference when observed at the other periods (Figure.1). For KCC2, the results show that maternal restraint stress caused a transient but significant increase in the KCC2 level in the hippocampus of rat pups during the weaning period (P21) ($p<0.01$) and this was followed by a transient but significant decrease during the preadolescence period (P28) ($p<0.05$) (Figure 2). However, there was no difference in the level of KCC2 when compare between groups during the adolescence period (P40).

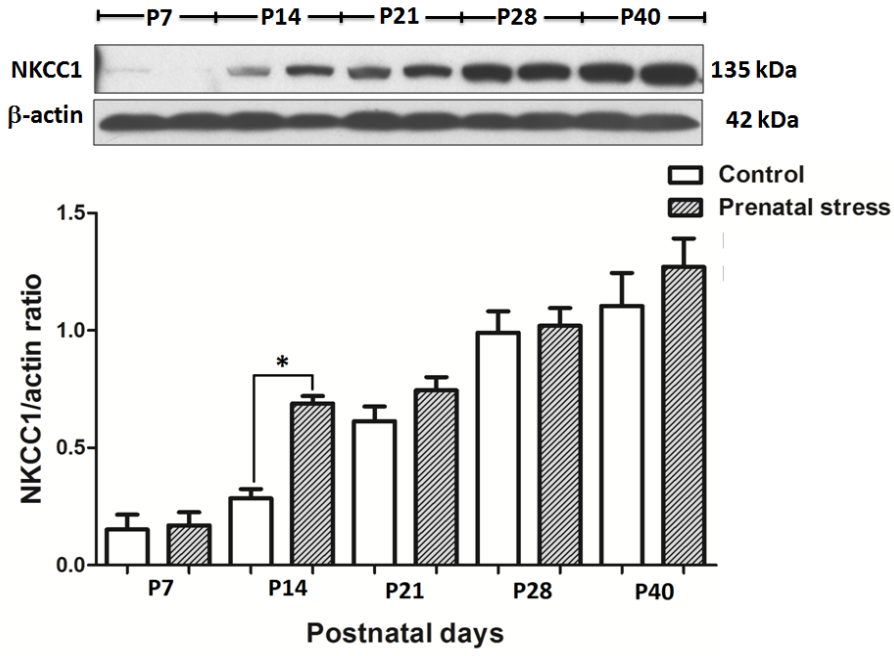


Figure 1. Effects of maternal restraint stress on the level of NKCC1 in the hippocampus of rat pups. The (Upper) western blot analysis of NKCC1 in the hippocampal tissue comparing the prenatal stress groups and the control groups during P7-P40. The (Lower) bar graph displays the results from western blot analysis. Data were expressed as band densities/α-actin ratio; values represent Mean±SEM, N=4. There was a significant difference when compared with control group at *p<0.05.

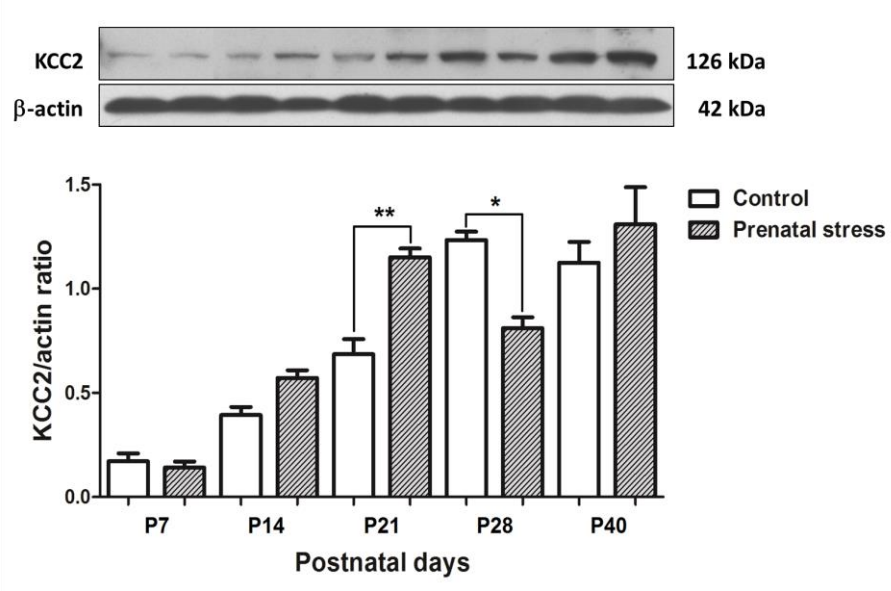


Figure 2. Effects of maternal restraint stress on the levels of KCC2 in the hippocampus of rat pups. The (Upper) western blot analysis of KCC2 in the hippocampal tissue comparing the prenatal stress groups and control groups during P7-P40. The (Lower) bar graph displays the results from the western blot analysis. Data were expressed as band densities/β-actin ratio; values represent Mean±SEM, N=4. There was a significant difference when compared with the control group at **p<0.01 and *p<0.05.

The NKCC1/KCC2 ratios were calculated and compared between groups across the different postnatal ages. We found that maternal restraint stress significantly increases the NKCC1/KCC2 ratio in the pup's hippocampus at P14 and P28 (p<0.01). During this period, the NKCC1/KCC2 ratios in the hippocampus of prenatally stress pups exhibited more fluctuations than those observed in the control group (Figure 3).

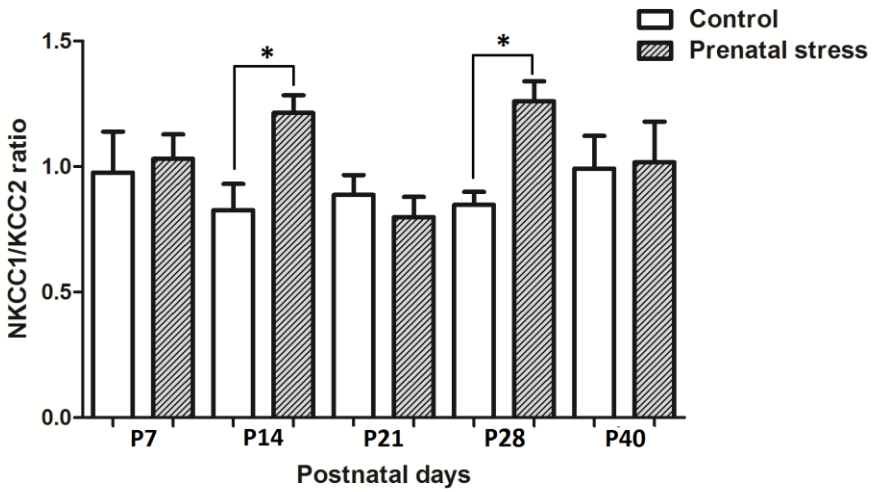


Figure 3. Bar graph comparing the NKCC1/KCC2 ratios between prenatal stress pups and the control pups at different postnatal ages. Data were expressed as Mean±SEM, N=4. There was a significant difference when compared with the control group at *p<0.05.

Maternal restraint stress delays development of the GABAA receptor α1 and α5 subunits in the hippocampus of rat pups

In the control pups, the developmental expressions of GABAA receptor α5 and α1 subunits in the hippocampus appear in the opposite way. The α5 subunit was highly expressed during P7-P14, and then declined during P21-P40 (Figure 4, white bar) while the α1 subunit was expressed at a very low level during P7-P14, then continually increased during P21-P28 (Figure 5, white bar). Maternal restraint stress caused a transient but significant decrease in the level of the GABAA receptor α5 subunit at P14 ($p<0.05$) and followed by a long term increase at P21 ($p<0.01$), P28 ($p<0.05$) and P40 ($p<0.05$) as compared to the control (Figure 4). In contrast, maternal restraint stress caused a transient but significant increase in the level of the GABAA α1 subunit at P14 ($p<0.05$) followed by a significant decrease at P21 ($p<0.01$) and P28 ($p<0.01$) (Figure 5). We found no significant difference in the level of the α1 subunit when comparing between the groups at P40.

When the ratios of the α5/α1 subunit of the GABAA receptor were calculated and compared across the different postnatal ages, we found that maternal restraint stress causes a significant decrease in the ratio of the α5/α1 subunits during P7-P14 ($p<0.01$), but a significant increase in the ratios of the α5/α1 subunits during P21-P28 ($p<0.05$). In fact, we found small increase in the α5/α1 ratios at P40; however, there was no statistically significant difference when compared with the control group (Figure 6).

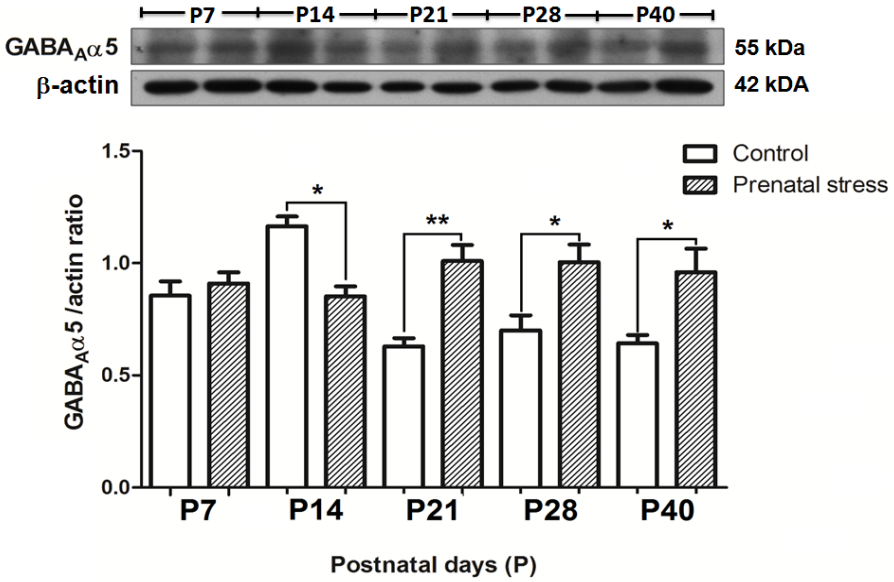


Figure 4. Effects of maternal restraint stress on the levels of the GABAA receptor α5 subunit in the hippocampus of rat pups. The (Upper) western blot analysis of GABAA receptor α5 subunit in the hippocampal tissue comparing the prenatal stress and control groups during P7-P40. The (Lower) bar graph displays the results from the western blot analysis. Data were expressed as a band densities/β-actin ratio; values represent Mean±SEM, N=4. There was a significant difference when compared with the control group at **p<0.01 and *p<0.05.

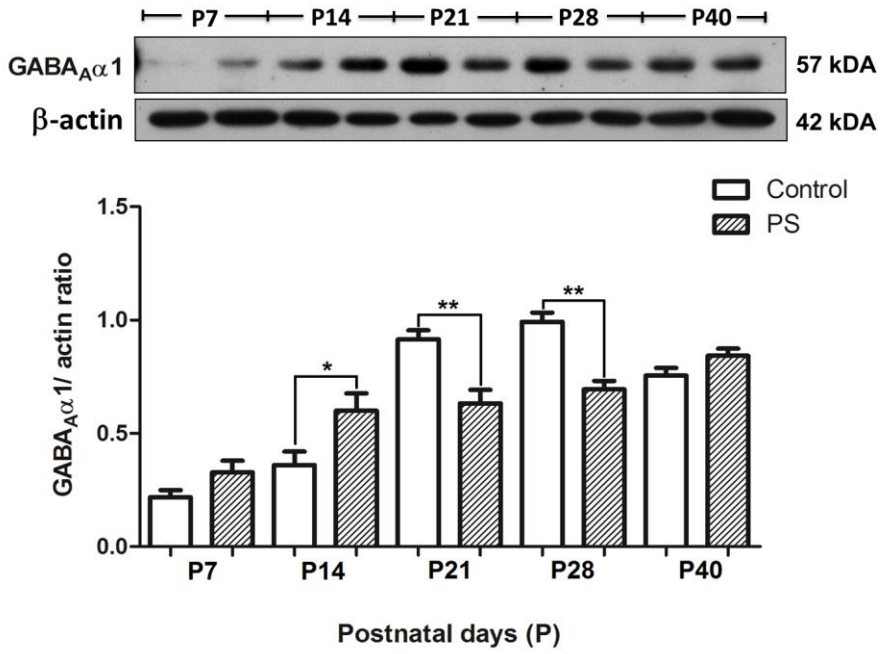


Figure 5. Effects of maternal restraint stress on the levels of the GABAA receptor α1 subunit in the hippocampus of rat pups. The (Upper) western blot analysis of the GABAA receptor α1 subunit in the hippocampal tissue comparing the prenatal stress and control groups during P7-P40. The

(Lower) bar graph displays the results from the western blot analysis. Data were expressed as band densities/β-actin ratio; values represent Mean±SEM, N=3. There was a significant difference when compared with the control group at **p<0.01 and *p<0.05.

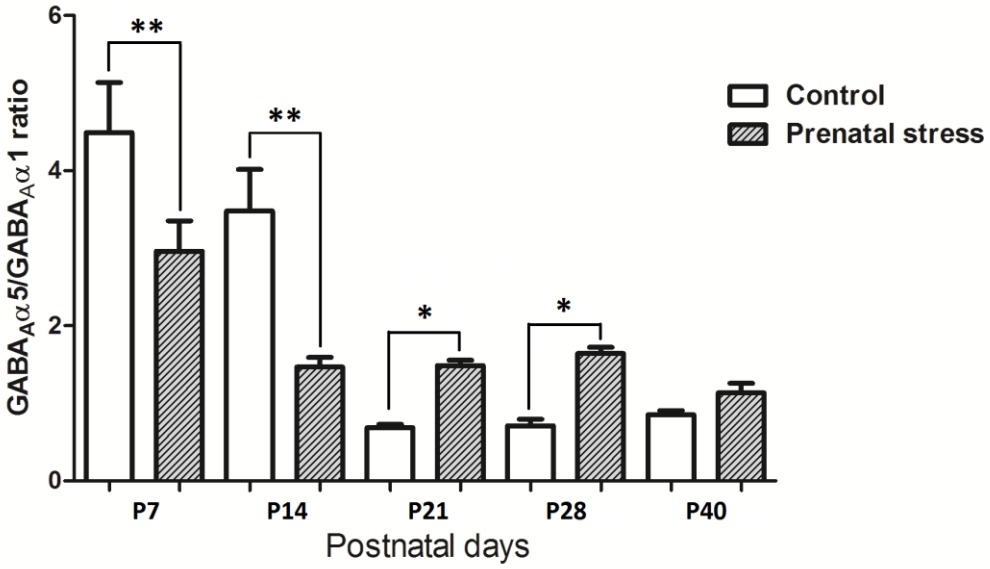


Figure 6. Bar graph comparing the ratios of the α5/α1 subunit of the GABAA receptor between the prenatal stress and the control groups at different postnatal ages. Data were expressed as Mean±SEM, N=4. there was a significant difference when compared with the control group at **p<0.01 and *p<0.05.

Maternal restrain stress alters Neuroligin-2 expression in the hippocampus of rat pups

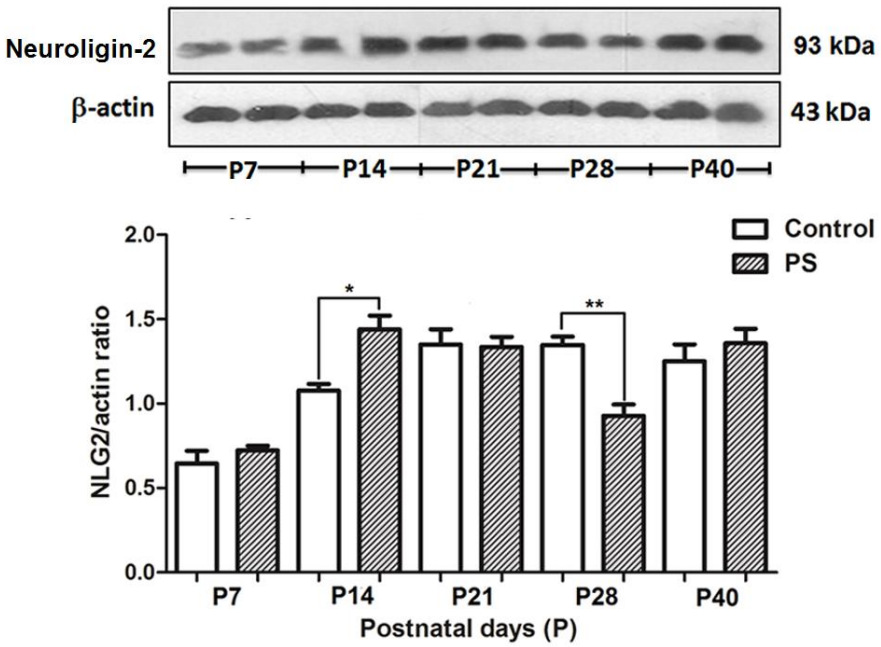


Figure 7. Effects of maternal restraint stress on the levels of the Neuroligin-2 in the hippocampus of rat pups. The (Upper) western blot analysis of Neuroligin-2 in the hippocampal tissue comparing the prenatal stress and control groups during P7-P40. The (Lower) bar graph displays the results from the western blot analysis. Data were expressed as band densities/β-actin ratio; values represent Mean±SEM, N=4. There was a significant difference when compared with the control group at *p<.05 and **p<.01

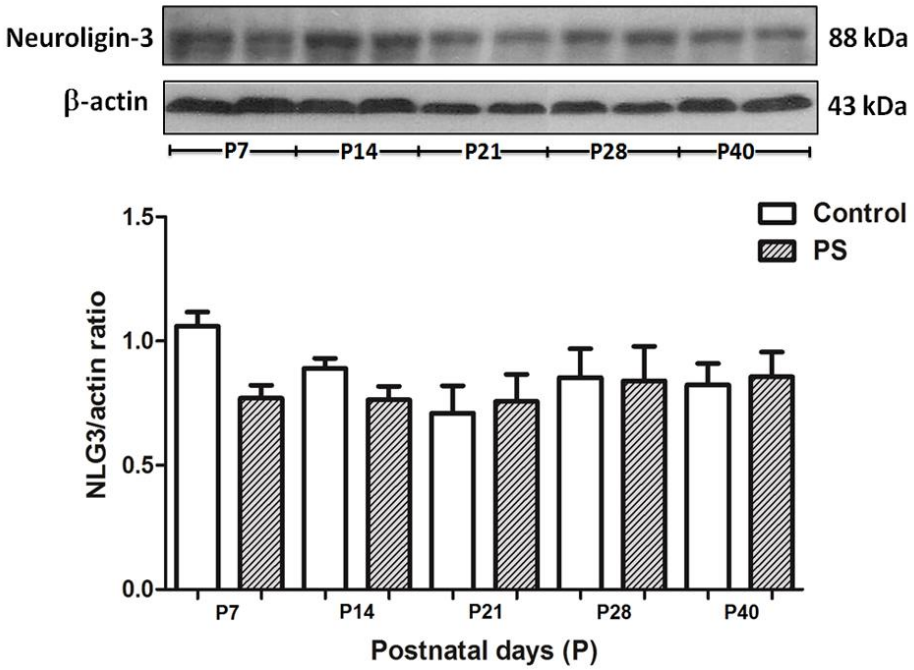


Figure 8. Effects of maternal restraint stress on the levels of the Neuroligin-3 in the hippocampus of rat pups. The (Upper) western blot analysis of Neuroligin-3 in the hippocampal tissue comparing the prenatal stress and control groups during P7-P40. The (Lower) bar graph displays the results from the western blot analysis. Data were expressed as band densities/β-actin ratio; values represent Mean±SEM, N=4.

Maternal restrain stress decrease β-Neurexin in the hippocampus of rat pups at weaning to adolescence

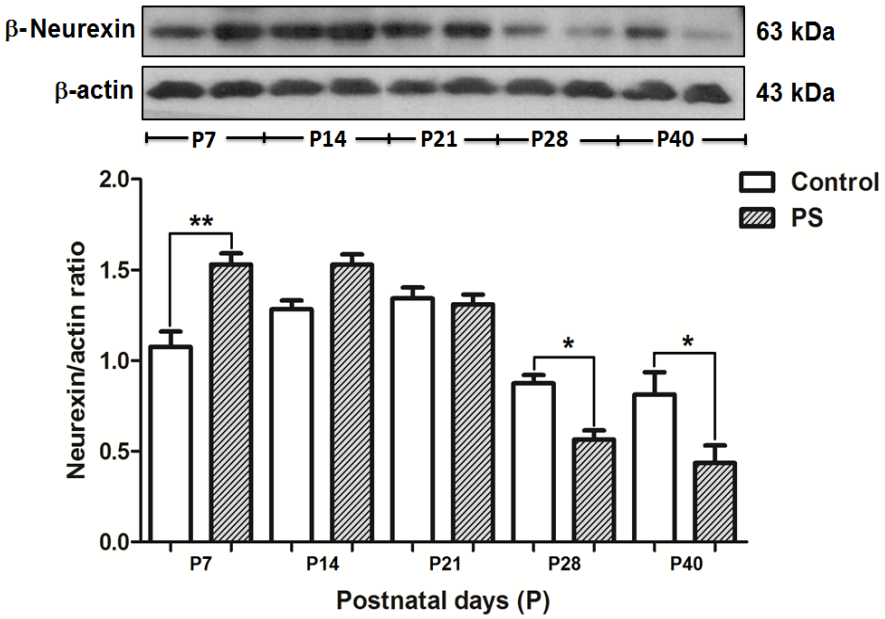


Figure 9. Effects of maternal restraint stress on the levels of the β-Neurexin in the hippocampus of rat pups. The (Upper) western blot analysis of β-Neurexin in the hippocampal tissue comparing the prenatal stress and control groups during P7-P40. The (Lower) bar graph displays the results from the western blot analysis. Data were expressed as band densities/β-actin ratio; values represent Mean±SEM, N=4. There was a significant difference when compared with the control group at *p<.05 and **p<.01

Maternal restrain stress decrease Gephyrin in the hippocampus of rat pups at weaning period

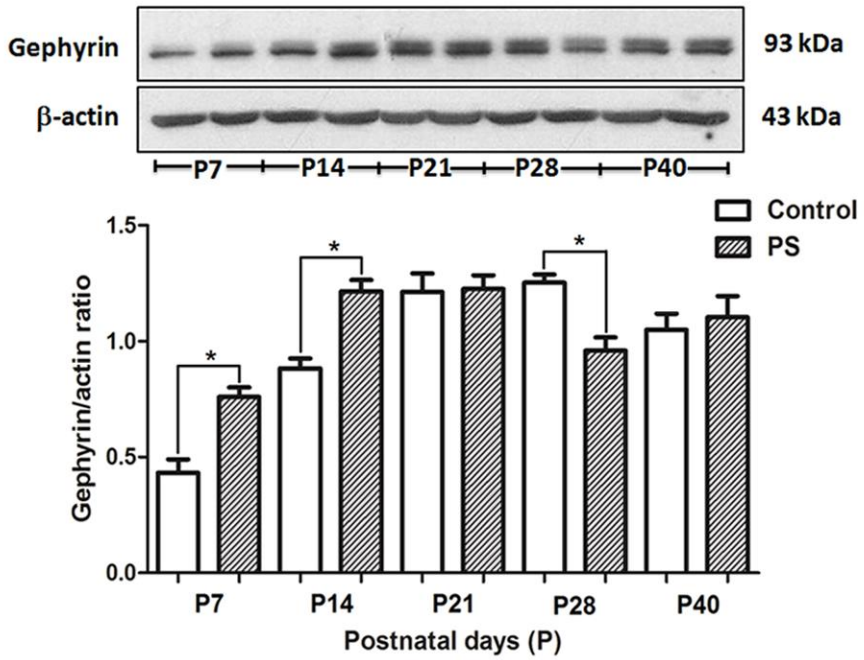


Figure 10. Effects of maternal restraint stress on the levels of the Gephyrin in the hippocampus of rat pups. The (Upper) western blot analysis of Gephyrin in the hippocampal tissue comparing the prenatal stress and control groups during P7-P40. The (Lower) bar graph displays the results from the western blot analysis. Data were expressed as band densities/β-actin ratio; values represent Mean±SEM, N=4. There was a significant difference when compared with the control group at *p<.05

Discussion

NKCC1 induces depolarizing GABA transmission and is mostly active in immature neurons. GABAA receptor depolarization maintained by NKCC1 is important for proper brain development since it is a key factor in the control of several Ca2+-dependent developmental phenomena, including neuronal proliferation, migration and targeting [24]. KCC2, in contrast, shifts the GABAA receptor activity from depolarization to hyperpolarization in mature neurons. In a developing hippocampus, the level of NKCC1 continuously increases starting from P21 to adulthood [25] while the hyperpolarizing GABA is completed by the second postnatal week due to the progressive reduction of NKCC1 activity in parallel with the enhanced activity of KCC2 [24, 26-28]. Previous studies reported that KCC2 expression significantly increases during the second postnatal week, which is the co-incidence time point when the developmental switch of GABAA receptor activity is observed [24, 29] and continually increased until P28 [30]. In the hippocampus, NKCC1 and KCC2 expressions show relatively similar developmental patterns indicating that both are required for the reciprocal regulation of Cl- homeostasis, which is important for the functional maturation of the GABAA receptor.

In this study, maternal restraint stress induced a transient increase in the level of NKCC1 in the hippocampus of rat pups only at P14, while there was a KCC2 increase at P21 and then a decrease at P28. Previous studies reported that the alteration in NKCC1 under a stress response has no effect on the GABAA receptor function. In contrast, the stress-altered KCC2 level has a profound effect on the modulation of intracellular Cl- concentrations [31]. Our finding, that maternal restraint stress increases the KCC2 level in the pup's hippocampus at P21, indicates the protective mechanism that counteracts the higher level of NKCC1 at P14. Maternal restraint stress, which increases KCC2 level at P21 and then decreases it at P28, indicates the dysregulation of intracellular Cl- concentrations and the GABAA receptor mediated current during the preadolescence period. In the mature pyramidal neurons, KCC2 inhibition positively shifts the GABAA receptor reversal potential, thus, GABAA receptor activation causes depolarization [24]. Our results indicate that maternal restraint stress may induce prolonged depolarizing of GABA in the hippocampus of rat pups until the pre-

adolescence period, while in the control pups, hyperpolarizing GABA was completed by the early postnatal week.

Different brain regions may exhibit differential vulnerability to the effects of stress on the level of KCC2 and its activity. For examples, in the rat hypothalamic paraventricular nuclei, acute restraint stress has no effect on the level of KCC2, but attenuates the KCC2 activity [31]. Maternal restraint stress has no effect on the level of KCC2 in the amygdala of male pups at P14 and P22 [32]. In contrast, prenatal stress causes a significant decrease in the KCC2 level and its activity in the hippocampus, as measured by phosphorylation of KCC2 on Ser 940 residue [33]. These findings, together with our results, indicate that maternal restraint stress might alter GABAA transmission in the hippocampus of prenatal stress pups during the post-weaning (P21) to pre-adolescence period (P28) and this mechanism might be due to the alteration in the levels of KCC2.

The underlying mechanism by which prenatal stress alters KCC2 levels remains unclear. Studies have shown that brain a derived neurotrophic factor (BDNF) regulates the expression of KCC2 in both the young and adult brains [34, 35]. BDNF is seen to down-regulate KCC2 expression in the adult hippocampal slices via the activation of tyrosine receptor kinase B (TrkB) [35]. In addition, BDNF promotes KCC2 expression in the developing mice forebrain [34]. Therefore, BDNF regulation of KCC2 expression varies depending on the developmental stages and brain regions. Prenatal stress has been reported to decrease BDNF levels in the rat hippocampus at P21 [36]. Taken together, the results suggest that an alteration in BDNF levels caused by prenatal stress might affect the KCC2 level in the pup's hippocampus. Although it has been noted that BDNF-induced alteration in KCC2 expression was not caused by neuronal excitability and network activity [34], the endogenous action that is regulating the changes is still elusive.

It is assumed that maternal restraint stress altering KCC2 level in the rat pups hippocampus at preadolescence period could affect the excitatory glutamatergic synapses as well. It has been reported that KCC2 has an important role in the modulation of the dendritic spines and AMPA receptor diffusion by interacting with sub-membranous actin cytoskeleton [37], therefore indicating KCC2 is also require for the production of long-term potentiation (LTP) in the hippocampus of young animals.

Prenatal stress has been linked to an increased risk of psychiatric disorders such as schizophrenia and depression [38]. Recent studies show that the KCC2 level is significantly decreased in the hippocampus of schizophrenia patients, while there was no change in the NKCC1 level [39]. Thus, an increase in the NKCC1/KCC2 ratio indicates the delayed maturation of Cation-Chloride Cotransporters in the patient's brain, which may underlie the pathology of neuropsychiatric diseases. Recently, it was demonstrated the significant increase in the level of OXSR1 (Oxidative stress

response kinase1) and WNK3 (With no K [lysine] protein kinase3) in the post-mortem brain of schizophrenia patients [40]. These proteins are kinases that regulate the activities of NKCC1 and KCC2, respectively. Consequently, changes in the level of OXSR1 and WNK3 can shift the balance of chloride transport and leading to an abnormal GABAergic transmission in the prefrontal cortex, thereby contributing to the impaired neural network synchrony and cognitive dysfunction in affected individuals [40].

For the development of GABAA receptor subunits, our results are consistent with those that have been previously reported [41, 42]. We found the α5 subunit is highly expressed during the early postnatal period and declined to the adult level around the 3rd postnatal week, while the α1 subunit was initially expressed at a small level during the 1st and the 2nd postnatal week and gradually increased until reaching its peak around the 3rd postnatal week. Prenatal stress delays the developmental shift of the GABAA receptor α1 and α5 subunits that normally occur around P21 in the control pups. This was clearly observed in the control pups that were seen to have manifested developmental increments in the α1 subunit at P21, but not in the prenatal stress pups, at least until P40. On the contrary, the control pups show a developmental decrease in the α5 subunit at P21, but not in the prenatal stress pups that were seen to maintain the expression of the α5 subunit at least until P40. As a result, the ratio of the α5/α1 subunits in the prenatal stress pups exhibits a significant increase during P7 and P14, but shows a significant decrease when observed at P21 and P28, as compared to the control group.

The underlying mechanism of prenatal stress that induces a prolonged increase of α5 subunits in the rat pup's hippocampus at preadolescence is still elusive. A prolonged increase in the α5 subunit in the pup's brain may create an unpredictable effect on GABA inhibitory transmission; especially during puberty [43]. In the hippocampal CA1 and neocortical pyramidal neurons, the extrasynaptic α5 subunit of the GABAA receptor mediates tonic inhibition and plays an important role in memory and learning [44]. An increased expression of the GABAA receptors' α5 subunit is associated with memory loss [45] while the antagonist of the receptor can enhance learning and memory processes [44, 46]. Thus, our results suggest that prenatal stress induces an increase in the GABAA receptor's α5 subunit expression and the α5/α1 ratios in the hippocampus at the preadolescence period may underlie the long term effects of prenatal stress on learning and memory impairment in the offspring at adulthood. For the GABAA receptors α1 subunit, our findings are consistent with previous report that reveal exposure to stress in juvenile rats can induce biphasic changes in their behavior, including hyperactivity at juveniles which in adulthood becomes hypoactivity accompanied by behavioral anxiety that are associated with the decrease of α1 subunits in the hippocampus and amygdala [47]. Taken together the results are in agreement, the juvenile period is a

sensitive time and is more vulnerable to stress than other periods and this supports the hypothesis that prenatal stress is a predisposing factor for various neuropsychiatric diseases and memory impairment at later life.

In this study, we added further information that the developmental expression of the GABAA receptor α1 subunit is similar to the developmental pattern of the KCC2. Both the GABAA α1 subunit and KCC2 reach their peak around P21-P28 and this indicates that the GABAA α1 subunit and KCC2 might coordinate in enhancing the GABAA receptor mediated synaptic inhibition that occurs around this period. Interestingly, we found that prenatal stress induces changes in the levels of the GABAA α1 subunit and the KCC2 in a similar way. Our results correspond to what has been previously documented in that KCC2 could modulate the expression level of the GABAA receptor α1 subunit via an alteration in intracellular [Cl-] and the decay rate of GABA-mediated inhibitory transmission [48]. Indeed, lower intracellular [Cl-] resulted in a faster decay rate of the GABA transmission [49]. It has been shown that KCC2 can manipulate the expression of GABAA receptor subunits, i.e., overexpression of KCC2 results in the reduction of intracellular [Cl-] and leads to an increase in the level of α1 and α subunits [50]. Taken together, these results are in accordance with the hypothesis that prenatal stress reduces the KCC2 levels, which might lower the intracellular [Cl-], that acts as the intracellular signal and induces a faster decay rate of the GABAA receptor gating and, thus, decreases the expression of the α1 subunit of the GABAA receptor [50]. These changes indicate a delayed maturation of the GABAergic function in the hippocampus of prenatal stress pups, especially during the preadolescence period.

Additionally, it was reported that stress disrupts the GABAergic function in the brain in many ways. Stress induces dysfunction of the inhibitory network and impairs rhythmic oscillations leading to cognitive deficits commonly found in psychiatric disorders [2]. Prenatal stress disturbs the distribution of GABAergic interneurons in the cortical plate, reflecting the changes occurring in tangential migration and radial integration in the developing cortex [51]. Prenatal stress causes a significant decrease in the frequency of spontaneous IPSCs in the immature hippocampal neurons [52] and increasing the vulnerability to stressful situations in the offspring during adulthood accompanied by a reduction of benzodiazepine binding in the hippocampus [53].

In summary, this study has shown that maternal restraint stress has the ability to differentially alter the levels of NKCC1, KCC2, and GABAA receptor α1 and α5 subunits in the hippocampus of rat pups. Consequently, these changes can lead to an imbalance of inhibitory transmission that may delineate the linkage between prenatal stress and neuropsychiatric disorders in later life. Our findings reveal that there is a strong connection between early life stress exposures with an increased risk of developing psychiatric disorders at adulthood. Furthermore, a reduction of KCC2 levels has been

linked to the cause of epilepsy, which is considered as a risk factor for schizophrenia and autism. Moreover, a prolonged increase in the α5 subunits in the hippocampus of rat pups during adolescence indicates a prolongation of the slow decay rate of inhibitory transmission in the pup's hippocampus and predisposes it for neuropsychiatric diseases and memory impairment in adulthood.

Our results revealed that maternal stress induced biphasic changes in the level of Neuroligin-2, β-Neurexin and Gephyrin in the hippocampus of rat pups, in which the levels increased during the first and the second postnatal week, but decreased during the pre-adolescence and the adolescence period, as compare to age match control pups. In contrast, GABAA receptor α1-6 subunits decreased during the third postnatal week, but increased at the adolescence period as compare to control pups of the same age. Our results indicate that the synaptic adhesion molecules and scaffolding protein of GABAergic synapse are more vulnerable to stress hormone especially when animals were exposed to stress in utero. The knowledge from our studied provides a molecular target for the prevention of cognitive deficits in adults with a history of early-life adversity.

Summary

Experiencing adverse events during pregnancy has a negative impact on brain development and may increase vulnerability to developing neurological and psychiatric disorders later in life. The delayed maturation of the GABAergic function has been reported in the schizophrenic brain. As we demonstrated, fetal exposure to maternal stress hormones delays structural and functional development of GABA transmission in the rat pup's hippocampus during the preadolescence period. These changes may lead to the dis-regulation of GABA inhibitory transmission in the developing hippocampus. Similar patterns of changes in the KCC2 and GABAA receptor α1 subunits were observed in response to early life stress, accompanied by supporting evidence that indicates changes in KCC2 levels may underlie the effect of maternal stress on the alterations in the α1 subunit of the GABAA receptors. Moreover, prenatal stress also increases GABAA receptor α5 subunit expression throughout the preadolescence period, which may underlie the learning and memory impairments in the offspring at adulthood. Prenatal stress alter the synaptic adhesion molecules and scaffolding protein of GABAergic synapse indicate that GABAergic synapses are more vulnerable to stress hormone especially when animals were exposed to stress in utero. The knowledge from our studied provides a molecular target for the prevention of cognitive deficits in adults with a history of early-life adversity. In summary, we have provided an explanation of certain prenatal factors mediating structural and functional development of the GABAergic synapse that may be the link between prenatal stress and the emergence of neuro-psychiatric disorders at adulthood.

References

1. Davidson, R.J. and B.S. McEwen, Social influences on neuroplasticity: stress and interventions to promote well-being. Nat Neurosci, 2012. 15(5): p. 689-95.
2. Hu, W., et al., Stress Impairs GABAergic Network Function in the Hippocampus by Activating Nongenomic Glucocorticoid Receptors and Affecting the Integrity of the Parvalbumin-Expressing Neuronal Network. Neuropsychopharmacology, 2010. 35(8): p. 1693-1707.
3. Hines, R.M., et al., Functional regulation of GABAA receptors in nervous system pathologies. Curr Opin Neurobiol, 2012. 22(3): p. 552-8.
4. Ben-Ari, Y., et al., The GABA Excitatory/Inhibitory Shift in Brain Maturation and Neurological Disorders. The Neuroscientist, 2012. 18(5): p. 467-486.
5. Ben-Ari, Y., Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci, 2002. 3(9): p. 728-739.
6. Ben-Ari, Y., et al., GABA: A Pioneer Transmitter That Excites Immature Neurons and Generates Primitive Oscillations. Physiological Reviews, 2007. 87(4): p. 1215-1284.
7. Cherubini, E., et al., The depolarizing action of GABA controls early network activity in the developing hippocampus. Mol Neurobiol., 2011. 43(2): p. 97-106. doi: 10.1007/s12035-010-8147-z. Epub 2010 Nov 3.
8. Blaesse, P., et al., Cation-chloride cotransporters and neuronal function. Neuron., 2009. 61(6): p. 820-38. doi: 10.1016/j.neuron.2009.03.003.
9. Medina, I. and I. Chudotvorova, GABA neurotransmission and neural cation-chloride co-transporters: actions beyond ion transport. Crit Rev Neurobiol, 2006. 18(1-2): p. 105-12.
10. Jacob, T.C., S.J. Moss, and R. Jurd, GABAA receptor trafficking and its role in the dynamic modulation of neuronal inhibition. Nat Rev Neurosci, 2008. 9(5): p. 331-343.
11. Dunning, D.D., et al., GABAA Receptor–Mediated Miniature Postsynaptic Currents and α-Subunit Expression in Developing Cortical Neurons. Journal of Neurophysiology, 1999. 82(6): p. 3286-3297.
12. Farrant, M. and Z. Nusser, Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors. Nat Rev Neurosci, 2005. 6(3): p. 215-229.
13. Owens, D.F. and A.R. Kriegstein, Is there more to gaba than synaptic inhibition? Nat Rev Neurosci, 2002. 3(9): p. 715-727.
14. Elliott, E.M. and R.M. Sapolsky, Corticosterone enhances kainic acid-induced calcium elevation in cultured hippocampal neurons. J Neurochem., 1992. 59(3): p. 1033-40.
15. Stein-Behrens, B.A. and R.M. Sapolsky, Stress, glucocorticoids, and aging. Aging (Milano). 1992. 4(3): p. 197-210.

16. Stumpf, W.E., et al., Dexamethasone and corticosterone receptor sites. Differential topographic distribution in rat hippocampus revealed by high resolution autoradiography. Histochemistry, 1989. 92(3): p. 201-10.
17. Tornello, S., et al., Regulation of glucocorticoid receptors in brain by corticosterone treatment of adrenalectomized rats. Neuroendocrinology., 1982. 35(6): p. 411-7.
18. Zhang, W.Q., et al., Systemic administration of kainic acid increases GABA levels in perfusate from the hippocampus of rats in vivo. Neurotoxicology., 1990. 11(4): p. 593-600.
19. Scheller-Gilkey, G., et al., Relationship of clinical symptoms and substance use in schizophrenia patients on conventional versus atypical antipsychotics. Am J Drug Alcohol Abuse., 2003. 29(3): p. 553-66.
20. Benes, F.M., Evidence for altered trisynaptic circuitry in schizophrenic hippocampus. Biol Psychiatry., 1999. 46(5): p. 589-99.
21. Surakul, P., W. Weerachatyanukul, and N. Chutabhakdikul, Repeated carbenoxolone injections during late pregnancy alter Snk-SPAR and PSD-95 expression in the hippocampus of rat pups. Neurosci Lett., 2011. 494(1): p. 75-9. doi: 10.1016/j.neulet.2011.02.060. Epub 2011 Mar 6.
22. Chutabhakdikul, N. and P. Surakul, Prenatal stress increased Snk Polo-like kinase 2, SCF beta-TrCP ubiquitin ligase and ubiquitination of SPAR in the hippocampus of the offspring at adulthood. Int J Dev Neurosci., 2013. 31(7): p. 560-7. doi: 10.1016/j.ijdevneu.2013.06.011. Epub 2013 Jul 10.
23. Fride, E. and M. Weinstock, The effects of prenatal exposure to predictable or unpredictable stress on early development in the rat. Dev Psychobiol., 1984. 17(6): p. 651-60.
24. Rivera, C., et al., The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature, 1999. 397(6716): p. 251-255.
25. Yan, Y., R.J. Dempsey, and D. Sun, Expression of Na+-K+-Cl⁻ cotransporter in rat brain during development and its localization in mature astrocytes. Brain Research, 2001. 911(1): p. 43-55.
26. Emri, Z., et al., Electrotonic profile and passive propagation of synaptic potentials in three subpopulations of hippocampal CA1 interneurons. Neuroscience, 2001. 104(4): p. 1013-26.
27. Gulyas, A.I., et al., The KCl cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. Eur J Neurosci., 2001. 13(12): p. 2205-17.
28. Stein, V., et al., Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. J Comp Neurol., 2004. 468(1): p. 57-64.

29. Clayton, G.H., et al., Ontogeny of cation–Cl^- cotransporter expression in rat neocortex. Developmental Brain Research, 1998. 109(2): p. 281-292.
30. Lu, J., M. Karadsheh, and E. Delpire, Developmental regulation of the neuronal-specific isoform of K-CL cotransporter KCC2 in postnatal rat brains. Journal of Neurobiology, 1999. 39(4): p. 558-568.
31. Hewitt, S.A., et al., Altered chloride homeostasis removes synaptic inhibitory constraint of the stress axis. Nat Neurosci, 2009. 12(4): p. 438-443.
32. Laloux, C., et al., Anxiety-like behaviour and associated neurochemical and endocrinological alterations in male pups exposed to prenatal stress. Psychoneuroendocrinology, 2012. 37(10): p. 1646-1658.
33. Sarkar, J., et al., Neurosteroidogenesis is required for the physiological response to stress: role of neurosteroid-sensitive GABAA receptors. The Journal of Neuroscience, 2011. 21(50): p. 18198-18210.
34. Aguado, F., et al., BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K+/Cl- co-transporter KCC2. Development, 2003. 130(7): p. 1267-1280.
35. Rivera, C., et al., BDNF-induced TrkB activation down-regulates the K+–Cl^- cotransporter KCC2 and impairs neuronal Cl^- extrusion. The Journal of Cell Biology, 2002. 159(5): p. 747-752.
36. Van den Hove, D.L.A., et al., Prenatal stress and neonatal rat brain development. Neuroscience, 2006. 137(1): p. 145-155.
37. Gauvain, G., et al., The neuronal K-Cl cotransporter KCC2 influences postsynaptic AMPA receptor content and lateral diffusion in dendritic spines. Proceedings of the National Academy of Sciences, 2011. 108(37): p. 15474-15479.
38. Charil, A., et al., Prenatal stress and brain development. Brain Research Reviews, 2010. 65(1): p. 56-79.
39. Hyde, T.M., et al., Expression of GABA Signaling Molecules KCC2, NKCC1, and GAD1 in Cortical Development and Schizophrenia. The Journal of Neuroscience, 2011. 31(30): p. 11088-11095.
40. Arion, D. and D.A. Lewis, Altered expression of regulators of the cortical chloride transporters nkcc1 and kcc2 in schizophrenia. Archives of General Psychiatry, 2011. 68(1): p. 21-31.
41. Ramos, B., et al., Expression of α5 GABAA receptor subunit in developing rat hippocampus. Developmental Brain Research, 2004. 151(1–2): p. 87-98.

42. Laurie, D.J., W. Wisden, and P.H. Seeburg, The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. The Journal of Neuroscience, 1992. 12(11): p. 4151-72.
43. Shen, H., et al., Reversal of neurosteroid effects at alpha4beta2delta GABAA receptors triggers anxiety at puberty. Nat Neurosci., 2007. 10(4): p. 469-77. Epub 2007 Mar 11.
44. Rudolph, U. and H. Mohler, GABAA receptor subtypes: Therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. Annu Rev Pharmacol Toxicol, 2014. 54:483-507. doi: p. 10.1146/annurev-pharmtox-011613-135947. Epub 2013 Oct 23.
45. Wang, D.S., et al., Memory deficits induced by inflammation are regulated by alpha5-subunit-containing GABAA receptors. Cell Rep., 2012. 2(3): p. 488-96.
doi: 10.1016/j.celrep.2012.08.022. Epub 2012 Sep 20.
46. Ling, I., et al., A novel GABA alpha 5 receptor inhibitor with therapeutic potential. Eur J Pharmacol., 2015. 764:497-507. doi: p. 10.1016/j.ejphar.2015.07.005.
47. Jacobson-Pick, S. and G. Richter-Levin, Short- and long-term effects of juvenile stressor exposure on the expression of GABAA receptor subunits in rats. Stress, 2012. 15(4): p. 416-424.
48. Houston, C.M., et al., Intracellular chloride ions regulate the time course of GABA-mediated inhibitory synaptic transmission. The Journal of Neuroscience, 2009. 29(33): p. 10416-10423.
49. Moroni, M., et al., Chloride ions in the pore of glycine and GABA channels shape the time course and voltage dependence of agonist currents. The Journal of Neuroscience, 2011. 31(40): p. 14095-14106.
50. Succol, F., et al., Intracellular chloride concentration influences the GABAA receptor subunit composition. Nat Commun, 2012. 3: p. 738.
51. Stevens, H.E., et al., Prenatal stress delays inhibitory neuron progenitor migration in the developing neocortex. Psychoneuroendocrinology, 2013. 38(4): p. 509-521.
52. Grigoryan, G. and M. Segal, Prenatal Stress Affects Network Properties of Rat Hippocampal Neurons. Biological Psychiatry, 2013. 73(11): p. 1095-1102.
53. Fride, E., et al., Prenatal stress impairs maternal behavior in a conflict situation and reduces hippocampal benzodiazepine receptors. Life Sci., 1985. 36(22): p. 2103-9.

Chapter 2

Roles of Placental Barrier in Modulating the Effect of Maternal Stress on Neuro-inflammation and Autophagic Biomarker in the Developing Brain

Abstract

Corticosterone (CORT) can cause the devastating effects on fetal brain development which might have long term impact at adulthood. The placental 11β-HSD enzyme is a powerful modulator that control the proper level of maternal stress hormone pass through the fetus. Several lines of evidence reported that maternal stress alters immune response in the fetal brain associated with the pathology of neurodevelopmental disorders in the offspring, however, the role of placental barrier in modulating effect of maternal stress on neuroimmune response in the pup brain is still not clear. In this study, we investigated the effects of maternal restraint stress on the level of inflammatory cytokines IL-6, IL-10 and its consequence on the autophagic marker, LC3B-II in the hippocampus of rat pups. The modulatory roles of 11β-HSD1 enzyme inhibitor (PFi) on neuroinflammatory response and autophagic marker were also examined in the in vitro model of the mimic intrauterine milieu (SH-SY5Y cells line co-culture with the JEG-3 Human choriocarcinoma cells line).

Our results revealed that maternal restraint stress during GD 14-21 induce a significant increase of IL-6 and IL-10 level, but a significant decrease in the level of LC3B-II in the hippocampus of rat pups from P7-60. The consistent results were also observed in the in vitro model of CORT-treated SH-SY5Y cells culture. Interestingly, modulating of the placental enzyme activity by using an 11β-HSD1 inhibitor, PFi, could reverse the effects of CORT on neuroimmune response and the autophagic biomarker in the SH-SY5Y and JEG-3 co-culture. Our results indicated that maternal stress induced immune dysregulation in the pup's brain could be mediated by maternal CORT pass through the fetus via the placenta, and subsequently might interrupt autophagy in the developing hippocampus. Our results suggested that modulating placental enzyme activity might be a novel target for reprogramming the negative effects of maternal stress on fetal brain development.

Key words: Stress, Neuroinflammation, Autophagy, 11β-HSD1 inhibitor, Hippocampus

Introduction

Early life stress can cause devastating effects on fetal brain development leading to neurodevelopmental disorders and psychiatric problems later in life. Overexposure of the pregnant rats to stress hormones either by direct exposure to glucocorticoids (GCs) or challenged with stressful condition, could lead to impairment of cognitive function in the rat pups (1-5). Hippocampus is the principle target brain area of stress hormones that undergo structural and functional changes in several animal species in response to chronic stress (2, 6-12).

Increasing evidence postulates that several inflammatory cytokines are produced by various cells in the developing brain. Moreover, recent study has shown that prenatal stress induced the elevation of pro-inflammatory cytokines in the mice hippocampus (13). Interestingly, elevation of pro-inflammatory cytokines i.e., IL-1β, IL-6 and TNF-α have been reported in the brain under neurological conditions (14, 15), parallel to an increase in anti-inflammatory cytokines, IL-10 as well (16). Recently, it has been reported that an inflammatory cytokine IL-6 has been elevated in the autistic postmortem brain. In contrast, the autophagic marker was shown to decreased while the number of immature dendritic spines was increased significantly in the patient's brain as compared to normal control (17).

Autophagy, the natural destructive process of the eukaryotic cells, responses to eliminate the unwanted cellular components under stress condition such as nutrient deprivation, bacterial or viral infection and unfolded protein-induced ER stress. The autophagic signaling activates a cytosolic LC3B-I conjugating phosphatidylethanolamine (PE) to form the LC3B-II which is the principal biomarker of autophagy that can be recruited to the autophagosomal compartment. The double membrane vesicles engulf proteins and invasive pathogens and then were transported to and fuse with the lysosomal membrane for degradation of the whole vesicles (18).

Regarding the overproduction of synapses that occurred at the beginning of life, then followed by the elimination of unnecessary synapses at the puberty. This process is also known as the synaptic pruning which is a very important process for strengthening the newly form synapses during development (16). Several studies reported that an interfering of synaptic pruning process might underlie the pathology of neurodevelopmental disorders (17) and schizophrenia (19, 20). During the developmental pruning, the protein degradation systems such as ubiquitin proteasome system (UPS) and autophagy are required for synaptic refinement (21). Of interest, an *in vitro* study has shown that IL-6 can inhibit autophagy in the U937 human lymphoblast lung cell lines (22). It is still not clear how prenatal stress alters neuroinflammatory response could have the negative impact on pruning process in the developing hippocampus and how the placental barrier can modulate the effect. In this study, we hypothesized that prenatal stress might enhance neuroimmune responses and subsequently have

the negative impact on synaptic pruning in the developing hippocampus via alteration in autophagy system.

Placenta is a unique tissue in all mammalian species. It provides the barrier to protect the fetus from abundant level of maternal stress hormone. For instance, only ten percent of the maternal GCs could be allowed to pass through the fetus via placenta. This is due to the function of 11β-hydroxysteroid dehydrogenase (HSD) enzyme produced by the syncytiotrophoblasts. The 11β-HSD enzyme have two isoforms with distinct functions; 1) the 11β-HSD1, which is a predominant reductase enzyme that activates cortisone to cortisol; and 2) the 11β-HSD2, which is the dehydrogenase enzyme that converts cortisol to inactive cortisone. The 11β-HSD2 is highly express in the placenta during early period of fetal brain development, followed by the dramatically decrease just before birth (23). While the 11β-HSD1 enzyme is highly expressed during late gestation important for the maturation of fetal organs such as alveoli, liver and kidney (24, 25). Disturbance of 11β-HSD enzyme level can have impact on fetal brain development. For example, pregnant rat injected with Carbenoxolone (11β-HSD2 inhibitor) during late gestation could mimic the effects of maternal restraint stress by reduce expression of postsynaptic proteins in the hippocampal of rat pups (26, 27). Maternal stress decrease expression of placental 11β-HSD2 mRNA, thereby increase maternal blood GCs which might interrupt neurogenesis in the pup's brain (28). Moreover, various 11β-HSD1 enzyme inhibitors (i.e., PFi) has been developed for protect the effects of stress-induced metabolic diseases (29, 30, 32).

In this study, we also hypothesized that the PFi could reverse the deterioration effects of maternal stress on neuroimmune response and autophagic biomarker in the hippocampus of rat pups. Thus, we examined the effects of maternal restraint stress on the expression of IL-6, IL-10 and LC3B-II in the SH-SY5Y human neuroblastoma cell lines and in the hippocampus of rat pups. Using an *in vitro* model, we then examined the effects of cortisol (CORT) on the level of IL-6, IL-10, 11β-HSD1 and 11β-HSD2 enzymes in the human choriocarcinoma (JEG-3) cell lines. Finally, the modulatory roles of 11β-HSD1 enzyme inhibitor (PFi) on neuroinflammatory response and autophagic marker were examined in the in vitro model of the mimic intrauterine milieu using SH-SY5Y cells co-culture with the JEG-3 Human choriocarcinoma cells line.

Materials and Methods

Animals

Pregnant Sprague Dawley rats, weighing 270–280 g and their offspring were used in this experiment. Rats were obtained from the National Experimental Animals Center of Mahidol University, Salaya, Thailand and housed in single housing condition in a temperature and humidity controlled

environment and maintained on a 12-h light/dark cycle with free access to food and water. Each pregnant rat was weight on Gestation day (33) 7-21 before any other manipulation. On the morning of GD21, each pregnant rat received nesting material, and thereafter, the cage was checked twice daily for the appearance of a litter. The day a litter was discovered will be designated as postnatal day (PD) 0 and the length of gestation was noted. All experiments conducted according to the Guidelines for Care and Use of the Laboratory Animals and approved by the Experimental Animal Ethics Committee of the Institute. Every effort was taken to minimize the number of animals used and their suffering.

Maternal restraint stress

Pregnant rats were divided into two groups as following; 1) control group, 2) maternal restraint stress (21) group, N=3-5/group. For the PS group, the pregnant rats were induced by restraint stress. Each pregnant rat was put into a small Plexiglas cylindrical cage, in which the diameter and length can be adjusted to accommodate the size of each animal. The restraint stress was performed during GD15-21, four hours daily with same period. The control rats were left undisturbed in their home cages. Gestation days 15-21 were selected because this is the most sensitive period to behavioral teratogenic effects of prenatal stress (3).

Neuronal cell culture

Two kinds of human cell lines were used in this experiment. First, the human neuroblastoma SH-SY5Y cell lines were gifted from Assoc. Prof. Dr. Wipawan Thangnipon. Second, the human choriocarcinoma JEG-3 cell lines were obtained from ATTC (US). Cells were cultivated in a medium containing DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin in 37°C, 95% humidified air with 5% CO2. The medium was changed twice a week and the cells were subpassaged as cells growing to 70-80% of cell culture vessel surface area.

Cell lysates preparation

The cells were harvested by rinse the media and keep the plate on ice for all steps. Iced PBS or iced HBSS were used as washing buffer for SH-SY5Y and JEG-3 cells, respectively. After rinse buffer, each plate was incubated for 5-15 minutes by iced RIPA lysis buffer composed of 50 mM Tris 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). The cells were then scraped by cell scraper or silicone spatula. The cell-suspended lysis buffer was removed to 1.5 ml microcentrifuge tubes, then homogenized for 10 sec and centrifuged at

14,000 RPM at 4°C for 15 min. The supernatant was collected for protein determination. The protein concentration of each sample was determined by Bradford assay.

Tissue lysates preparation

The whole hippocampal tissues were collected from rat pups at different postnatal days from PD7, PD14, PD21, PD28, PD40 and PD60, with n = 3-5/group. Brain tissues were then suspended in a lysis buffer (50 mM Tris 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)), homogenized and collected supernatant for subsequent studies.

Cell viability assay

MTT assay was performed in this experiment to detect the ability of viable by converting the tetrazolium salt to colored formazan. The cells were plated in 96-wells plate as 3 x 104 per well for 5 replicates of each condition. 24 hours after plating, the cells were washed with the appropriate buffer, then overnight cultured in 200 µl of serum-free medium plus 10 µl of cortisol (Sigma Aldrich) for the drug-treated group and equivalent volume of 4% ethanol for the vehicle group. The media was changed to serum-free medium and determined the cell viability by adding MTT reagent to reach a final concentration of 1mg/ml. Following 3 hours incubation at 37°C, the media was removed. The crystals were formed and then dissolved in 100 µl of DMSO. The optical densities of colored formazan were measured by spectrophotometer at 570 nm spectral wavelength. The viable cell value was calculated as percent of control to vehicle group.

Drug treatment

SH-SY5Y cells or JEG-3 cells were seeded on 96-well plates at 3 x 104 cells per well. 24 h After plating, cells were treated with CORT at 25, 50, 100, 200, 250, 300 and 400 µM. Cells were incubated at 37°C, 95% humidified air with 5% CO2 for 24h prior to MTT assay. The drug-pretreated group was administered with PFi (Tocris Bioscience) for 2 h at the concentrations of 0.001, 0.005, 0.01, 0.1 and 1 µM, followed by CORT. The vehicle control group was treated with 0.4% EtOH or 0.01% DMSO in PBS or HBSS.

SH-SY5Y cells and JEG-3 cells line co-culture

JEG3 cells were harvested and re-seeded onto a Transwell at a density of 2.5_104 cells per 100 ml per well. Parallel to SH-SY5Y which harvested and re-seeded onto the lower plate of 6-well plate at a density of 2.5_104 cells per 100 ml per well. 24 h after plating the media was changed to

the free-serum media. By the way, JEG-3 cells were treated with 0.1 μM PFi for 2 h. After drug treatment, the cells were washed with the appropriate buffer. Transwells were moved and hanged on the 6-well plate. 300 μM CORT was treated via the upper chamber for 24 h.

Immunoblot

Extracted protein (20 μg) were mixed in a sodium dodecyl sulfate (SDS) sample buffer and boiled. Samples were resolved in 4-15% SDS-PAGE and electrophoresis at 150 V for 1 hour. The protein bands, then were transferred to PVDF membranes (Amersham, USA) at 90 V for 2 h. The membrane was incubated at 4°C overnight with blocking solution composed of 2% BSA and 3% skimmed milk. Then membrane was incubated in the following specific primary antibodies that have been purchased from the available commercial sources: Polyclonal rabbit anti-11β-HSD2 (1:2,000, ab80317) from Abcam, UK, polyclonal rabbit anti-LC3B (1:1,000, #2775) from Cell Signaling Technology, Inc., USA, polyclonal rabbit anti-11β-HSD1 (1:1,000, sc20175), monoclonal mouse anti-IL-6 (1:12,000, sc57315), monoclonal mouse anti-IL-10 (1:1,000, sc365858) and monoclonal mouse anti-β-actin (1:5,000, sc69879) from Santa Cruz Biotechnology, USA. The membranes were then washed 3 times by 0.1% Tween-TBS for 15 min each, and incubated with appropriate HRP-conjugated secondary antibody. After secondary antibody incubation, membranes were washed 3 times by 0.1% tween TBS for 15 min each, and the signals were detected by using an Enhanced Chemiluminescence System (ECL, Amersham Biosciences, Piscataway, NJ, USA). The immunoblots were quantified by measuring the density of each protein band using densitometry software program (Scion image, National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining

The offspring's brains at PD28 were postfixed overnight in 4% paraformaldehyde at 4°C. The brains were soaked in 30% sucrose in 0.1M PBS overnight at 4°C for adequate cryoprotection. The coronal sections of the hippocampus were embedded on the gelatin-coated slides and washed three times with 0.01 M PBS for 5 min. Hippocampus sections were permeabilized by 0.3% triton X-100 for 20 min, followed by 30 min blocking with 10% donkey serum and 1% BSA at room temperature. The sections were overnight incubated with rabbit polyclonal anti-LC3B (1:200, #2775) at 4°C. After washing, the hippocampus sections were incubated with anti-rabbit IgG conjugated with FITC (1:200) for 2 h at room temperature. The sections were washed, mounted with anti-fade mounting medium (H-1000, Vector Laboratories Inc., California, USA) and visualized by FluoView 300 confocal laser scanning microscopy (Olympus, Tokyo, Japan).

Statistical analysis

The data were statistically analyzed using GraphPad Prism software. Quantitative results were expressed as mean ± SEM, calculated from the triplicate experiments. The statistical significance of difference between the means was evaluated using Student's t-test (unpaired, unless otherwise stated). The changes produced by prenatal stress were analyzed at different postnatal ages using a two-way ANOVA with the prenatal stress and postnatal ages as independent variables and the protein levels as dependent variables; followed by a Tukey's post hoc multiple comparison test. The probability level of $p \leq 0.05$ was considered to have a statistically significant difference between the two sets of data.

Results

Effects of CORT on cell viability in SH-SY5Y cells

SH-SY5Y cells were treated with cortisol for 24 h, at the concentrations of 25-400 μM. The proliferation and cytotoxicity of the cells were determined using MTT assay. The data were presented as a percentage of the control cell number (veh). Cortisol could reduce the cell viability in a dose-dependent manner (*p<0.05, **p<0.01 and ***p<0.0; Figure 11), among these dosages, 300 μM could reduce the percentage of cell viability to 56.16%. Therefore, the concentration was chosen for subsequent studies.

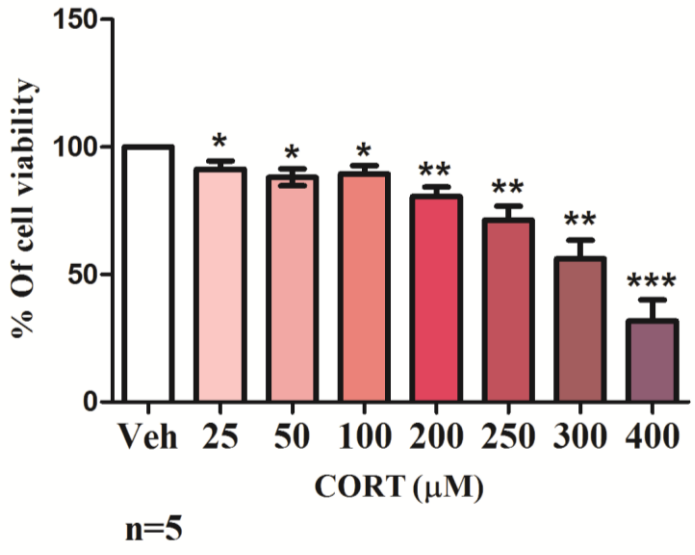


Figure 11. Effects of corticosterone (CORT) on cell viability in SH-SY5Y cells culture. Bar graph displays the results from MTT assay, values represent means ± SEM, n=4. Significant difference when compared with the vehicle group (veh) at *p<0.05, **p<0.01 and ***p<0.001.

CORT induced pro- and anti-inflammatory cytokines in the SH-SY5Y cells culture

We examined the effects of cortisol on the expression of pro-inflammatory cytokine, IL-6, and anti-inflammatory cytokine, IL-10, in SH-SY5Y cells. After incubating the cells with 300 μM CORT for 24 h, the level of cytokines was measured by immunoblot, which heeded to IL-6 and IL-10. IL-6 was significantly increased in the cortisol-treated cells (**p<0.01; Figure 12A). The same results were remarking on the expression of IL-10 (*p<0.05; Figure 12B).

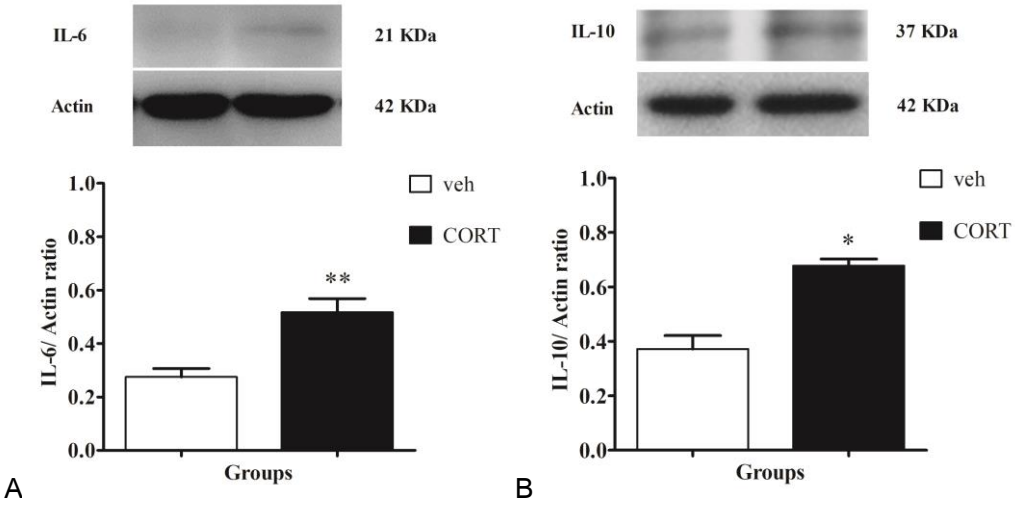


Figure 12. Effects of cortisol on the expression of IL-6 and IL-10 in SH-SY5Y cells. Immunoblot of IL-6 (A) and IL-10 (B) in SH-SY5Y compared between veh and CORT. The bar graphs display the results from the immunoblot. Data were expressed as protein band densities normalized to β-actin serve as internal control. The values represent means ± SEM, n=4. Significant difference when compared with the untreated group (control) at *p<0.05, **p<0.01 and ***p<0.001.

CORT decreased LC3B-II expression in the SH-SY5Y cells culture

We then examined the expression of LC3B-II (14 KDa), which served as a certain indicator for autophagy system. Immunoblot was performed and compared between vehicle group and CORT group. The significant reduction of LC3B-II was rendered in the CORT group (***p<0.001; Figure 13).

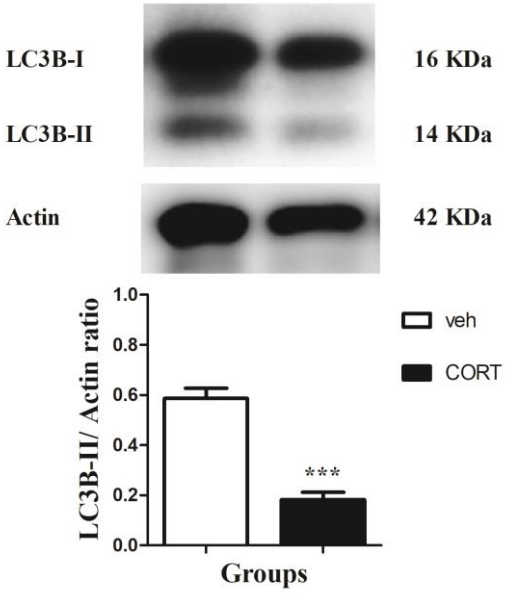


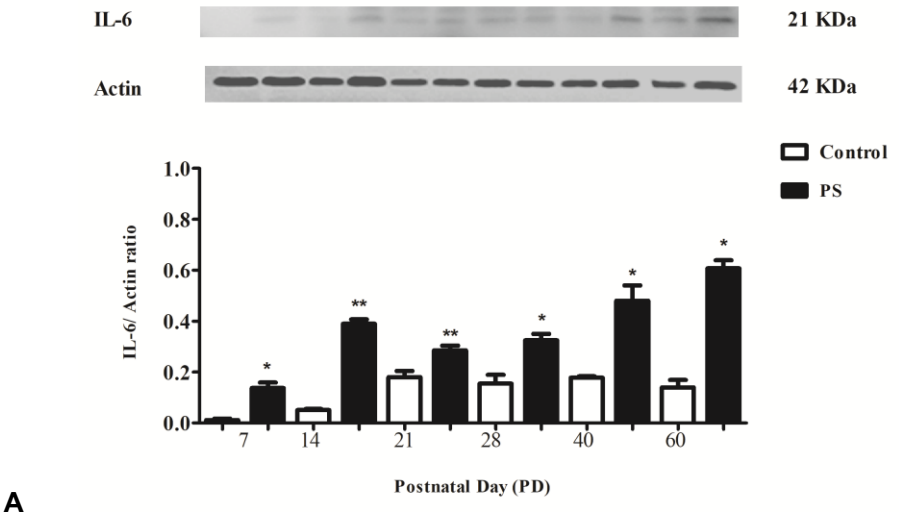
Figure 13. Effects of cortisol on the expression of LC3B-II in SH-SY5Y cells. Immunoblot of LC3B-II in SY-SY5Y compared between control group and CORT group. Data were expressed as protein band densities normalized to β-actin serve as internal control. The values represent means ± SEM, n=4. Significant difference when compared with the veh group at *p<0.05, **p<0.01 and ***p<0.001.

Maternal restraint stress induces IL-6 and IL-10 in the hippocampus of rat pups

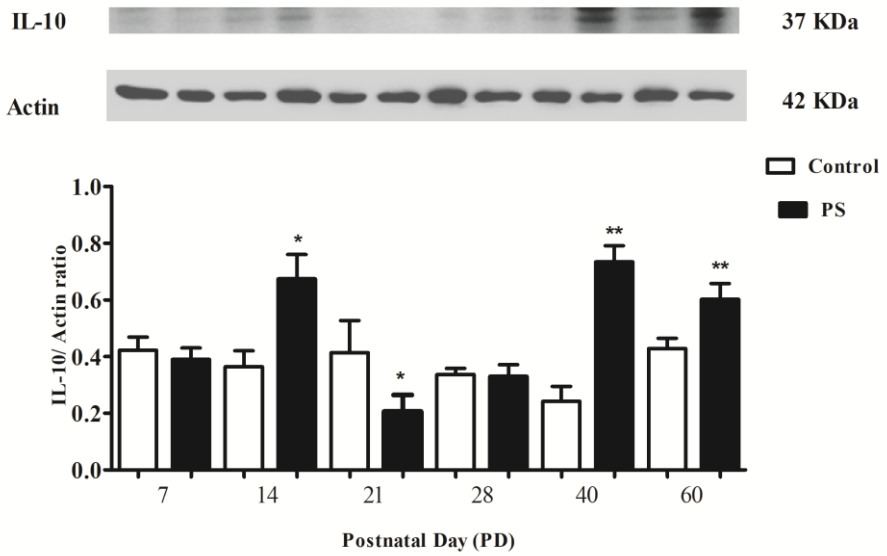
To further examine the effects of prenatal stress, we induced maternal restraint stress in the pregnant rats during late gestation period (GD15-21). The expression of IL-6 and IL-10 in the hippocampus of the rat pups, were explored by immunoblot. The results showed that prenatal stress could significantly increase the level of IL-6 from PD7-PD60 (*p<0.05 and **p<0.01; Figure 14A), when compared to the control group. While, IL-10 could significantly enhance at PD14 (*p<0.05), PD40 and PD60 (**p<0.01), and was noted for the significant decrease in IL-10 at PD21 (*p<0.05: Figure 14B).

Maternal restraint stress decreased autophagic biomarker in the hippocampus of the rat pups.

We investigated the effects of prenatal stress on the expression of LC3B-II, the principal autophagic biomarker. Immunoblot was performed and compared between control group and PS group. The data were presented as a percentage of the control group, and showed the significant decrease in LC3B-II in the hippocampus of the rat pups from PD7-PD60 (*p<0.05 and **p<0.01; Figure 15). The immunohistochemistry was performed to verify the changes in LC3B in immunoblot. The green color displays LC3B-positive immunostaining in the hippocampal CA1 (Figure 51A) and CA3 (Figure 15B), while the nuclear staining was presented in the red color. We found the little staining of LC3B in either CA1 or CA3 areas of the PS group as compared to the control group.



A



B

Figure 14. Effects of maternal restraint-induced prenatal stress on the expression of IL-6 and IL-10 in the hippocampus of the rat pups. Immunoblot of IL-6 (A) and IL-10 (B) in the hippocampus of the rat pups compared between control group and prenatal stress (PS) group from PD7-60. Data were expressed as protein band densities normalized to β-actin serve as internal control. The values represent means ± SEM, n=4. Significant difference when compared with the control group at *p<0.05, **p<0.01 and ***p<0.001.

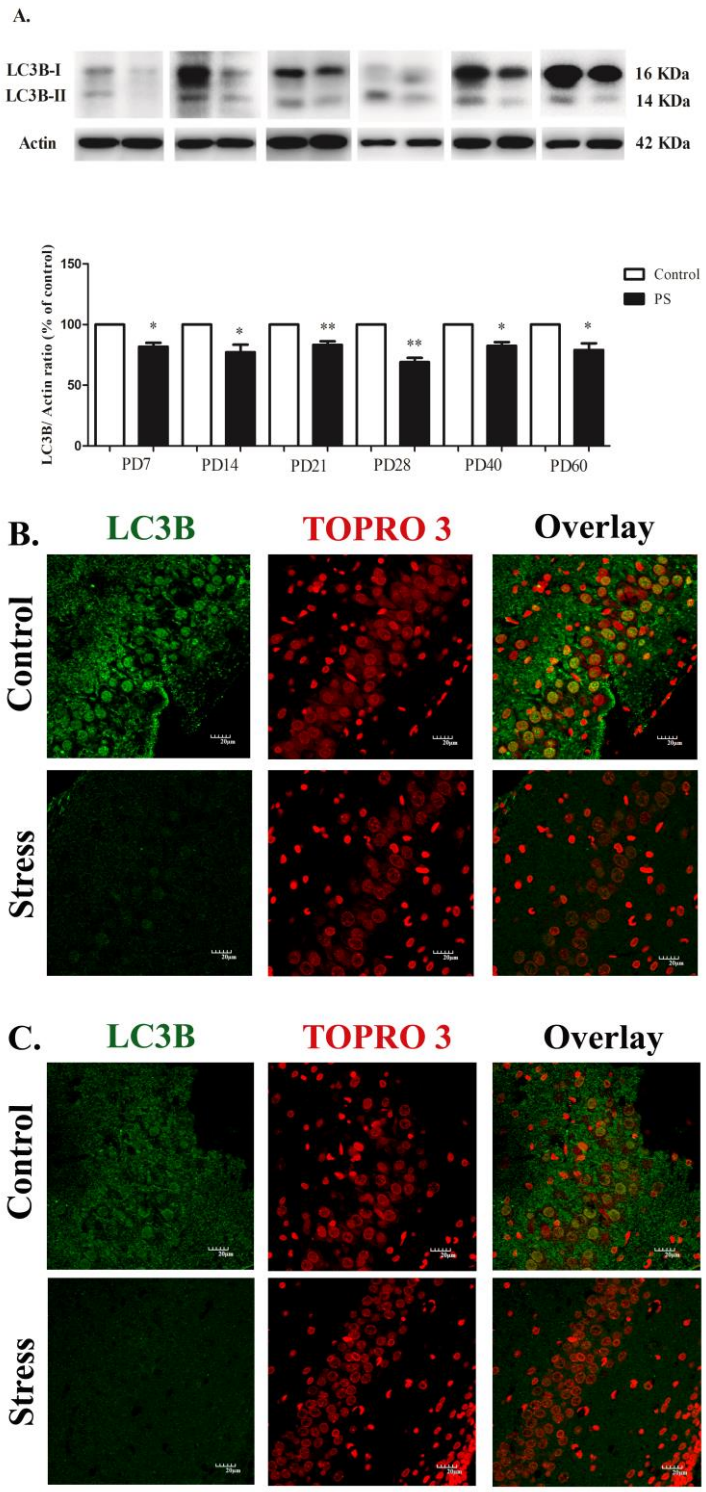


Figure 15. Effects of prenatal stress on the expression of autophagy in the hippocampus of the rat pups. (A) Immunoblot of LC3B-II in the hippocampus of the rat pups compared between control group and PS group from PD7-60. Data were expressed as protein band densities normalized to β-actin serve as internal control. The values represent means ± SEM, n=4. Significant difference when compared with the control group at *p<0.05, **p<0.01 and ***p<0.001. Confocal images displaying the expression of LCB3 in hippocampal CA1 (B) and CA3 (C) areas, the green color displays LC3B using

goat anti-rabbit IgG conjugated with FITC and the red color is TOPRO-3, a nuclear marker. Scale bar=20 μm.

Effect of CORT on cell viability of JEG-3 cells culture

We examined a protective effect of placenta against CORT passing through the fetus. The human choriocarcinoma cell lines, JEG-3, were purchased from ATCC and used in this experiment. The cells were treated with cortisol for 24 h, at the concentrations of 25-400 μM. The proliferation and cytotoxicity of the cells were determined by MTT assay. The data were presented as a percentage of the veh group. Cortisol could significantly reduce the cell viability in a dose-dependent manner (*p<0.05, **p<0.01 and ***p<0.001; Figure 16), among which, 100 μM could reduce the percentage of cell viability to 58.69%, so the concentration was chosen for subsequent studies.

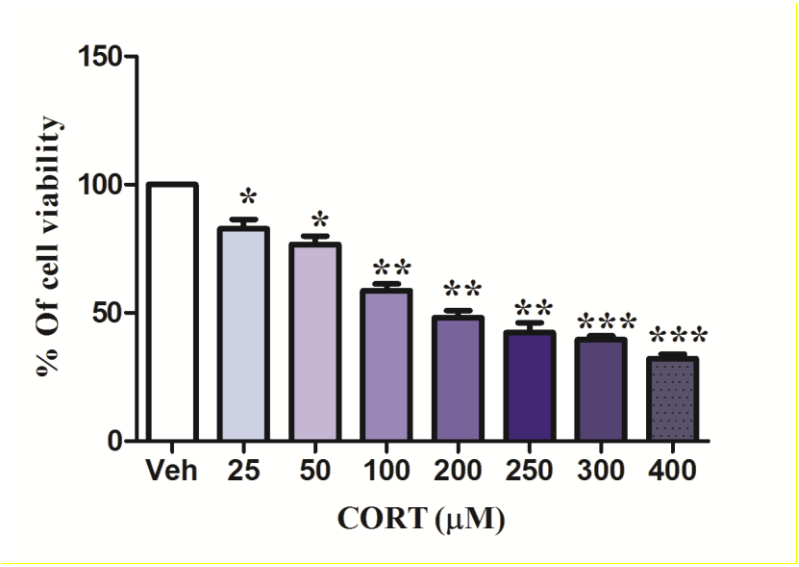


Figure 16. Effects of cortisol (CORT) on cell viability in JEG-3 cells. Bar graph displays the results from MTT assay, values represent means ± SEM, n=4. Significant difference when compared with the vehicle group (veh) at *p<0.05, **p<0.01 and ***p<0.001.

Effects of 11β-HSD1 inhibitor on cell viability in CORT-treated JEG-3 cells culture

We firstly determined the impacts of 11β-HSD1 inhibitor (PFi) on JEG-3 cells by measurement of the cell proliferation and cytotoxicity. MTT assay was performed, which followed by calculation of relatively of cell viability. The data were presented as a percentage of the veh group. The results showed that no significant difference between the groups at the concentrations of 0.001-100 μM (Figure 17A). We chose the concentration of PFi at 0.001, 0.1 and 1 μM for pre-treatment JEG-3 cells. After incubating for 2 h, the cells were washed and incubated with 100 μM CORT for 24 h before MTT assay. The results showed that 0.1 μM PFi and 1 μM PFi could prevent JEG-3 cells lost when compared to CORT-treated group ($^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001; Figure 17B).

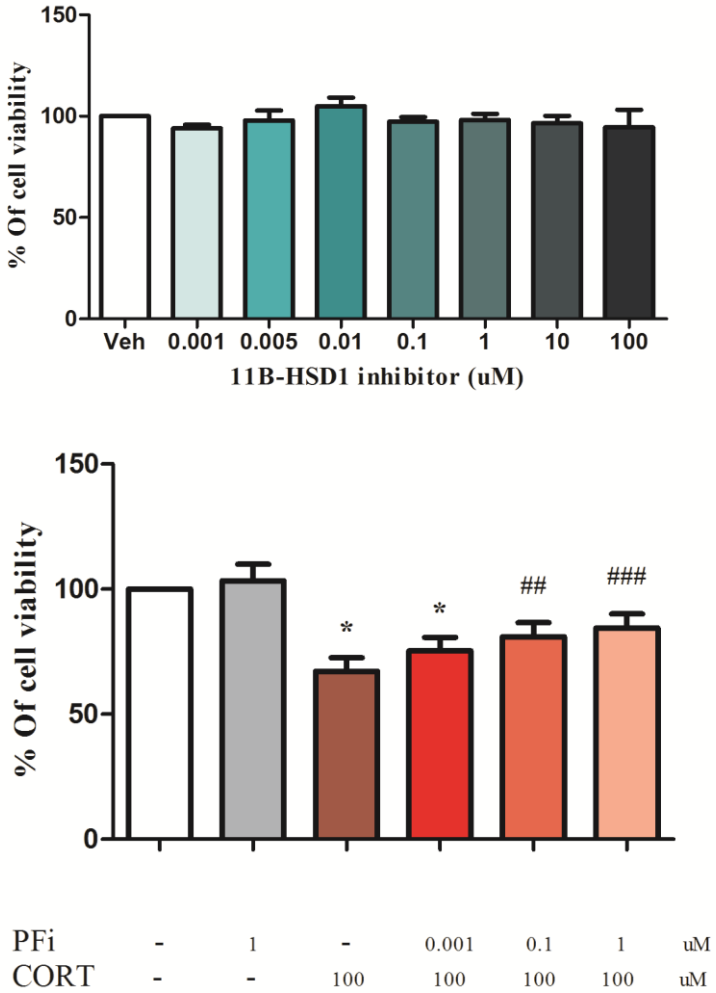


Figure 17. (A) Effects of 11β-HSD1 inhibitor (PFi) on cell viability in JEG-3 cells. (B) The effects of PFi pretreatment against to CORT on cell viability in JEG-3 cells. Bar graph displays the results from MTT assay, values represent means ± SEM, n=3. Significant difference when compared with the veh group at *p<0.05, **p<0.01 and ***p<0.001. Significant difference when compared with the CORT treated group at $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001.

Protective effect of PFi, an 11β-HSD1 inhibitor, in cortisol-treated JEG-3 cells culture

We examined the effects of CORT on the expression of cytokines, IL-6 and IL-10. JEG-3 cells were incubated with 100 μM CORT for 24 h, the protein levels were explored by immunoblot analysis. The results showed that CORT could significantly increase IL-6 in JEG-3 (**p<0.01; Figure 18A), as well as the level of IL-10 (*p<0.05; Figure 18B). Next, the effects of CORT on the expression of placental enzymes, 11β-HSD1 and 11β-HSD2, were analyzed by immunoblot, as well. The data showed that CORT could significantly enhance 11β-HSD1 in JEG-3 cells (*p<0.05; Figure 19A). Meanwhile, 11β-HSD2 was significantly reduced in CORT-treated group when compared to the non-treated group (**p<0.01; Figure 19B).

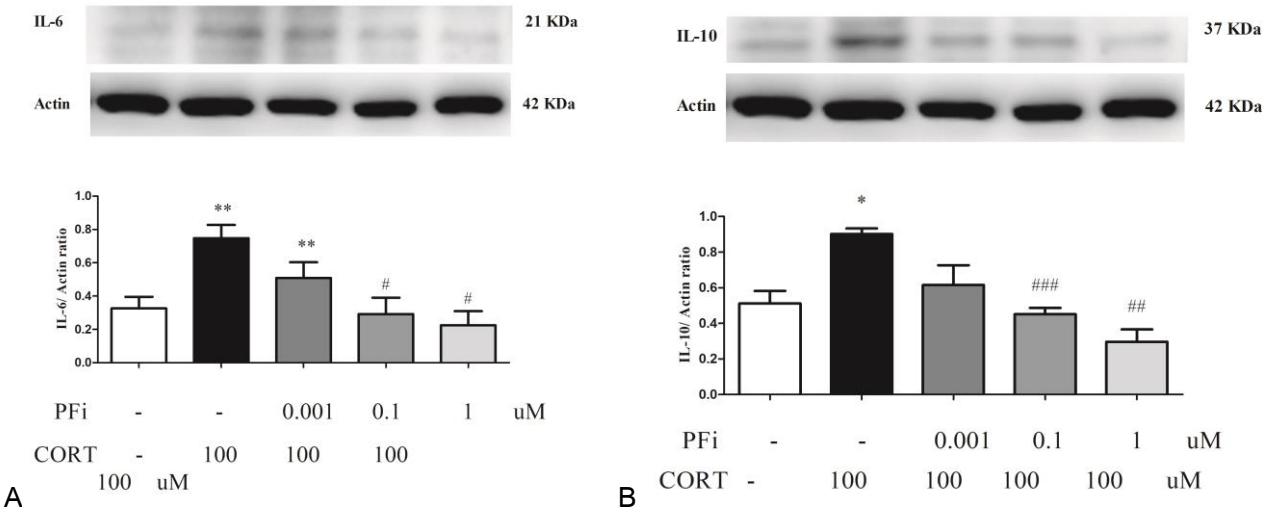
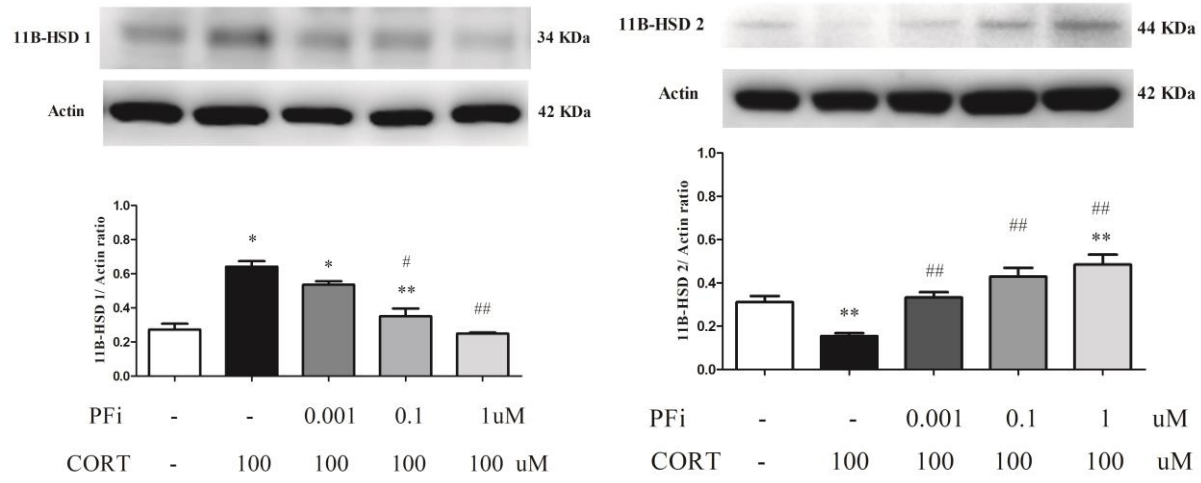


Figure 18. Effects of 11β-HSD1 inhibitor on the expression of IL-6, IL-10 in cortisol-treated JEG-3 cells. Upper panel show an immunoblot of IL-6 (A), IL-10 (B) in the CORT-treated JEG-3 cells. Bar graphs display the results from the immunoblot. Data were expressed as protein band densities normalized to β-actin serve as internal control. Values represent means ±SEM, n=4. Significant difference when compared with the veh group at *p<0.05, **p<0.01. Significant difference when compared with the CORT group at $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001.

The protective effects of PFi on the expression of IL-6, IL-10, 11β-HSD1 and 11β-HSD2 was further investigated. JEG-3 cells were pretreated with PFi at the concentrations of 0.001, 0.1 and 1 μM for 2 h. After washing, the cells were incubated with 100 μM CORT for 24 h. The immunoblot data of PFi-pretreated groups were compared to CORT group. The results showed that PFi pretreatment could significantly attenuate IL-6, IL-10 and 11β-HSD1 at the concentration of 0.1 and 1 μM ($^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$; Figure 19 A, B). Meanwhile, the same doses of PFi could prevent the reduction of 11β-HSD2 enzymes in JEG-3 cells ($^{\#}p<0.05$; Figure 19B) as compared to CORT group.



A B

Figure 19. Effects of 11β-HSD1 inhibitor on the expression of 11β-HSD1 and 11β-HSD2 in cortisol-treated JEG-3 cells. Immunoblot of 11β-HSD1 (A) and 11β-HSD2 (B) in the CORT-treated JEG-3 cells culture. Bar graphs display the results from the immunoblot. Data were expressed as protein band densities normalized to β-actin serve as internal control. Values represent means ±SEM, n=4. Significant difference when compared with the veh group at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. Significant difference when compared with the CORT group at $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$.

Protective effect of PFi, an 11β-HSD1 inhibitor, in cortisol-treated SH-SY5Y cells and JEG-3 cells co-culture

The protective effects of PFi on the expression of IL-6, IL-10 and LC3B-II in CORT-treated SH-SY5Y cells and JEG-3 cells co-culture were investigated by immunoblot. The non-contact co-culture technique was performed for cultivation two different cells. JEG-3 cells were seeded onto the upper layer of Transwell while SH-SY5Y cells were plated on the lower plate. Culture condition were divided into 6 groups as follows:

Group	Treatment
I	SH-SY5Y cells were seeded on the lower plate without JEG-3 cells on the upper plate. The buffer without drug was put in the upper chamber.
II	SH-SY5Y cells were seeded on the lower plate without JEG-3 cells on the upper plate. 300 μM CORT was put in the upper chamber.
III	SH-SY5Y cells were seeded on the lower plate and JEG-3 cells were on the upper plate. The buffer without drug was put in the upper chamber.
VI	SH-SY5Y cells were seeded on the lower plate and JEG-3 cells were on the upper plate. 300 μM CORT was put in the upper chamber.
V	SH-SY5Y cells were seeded on the lower plate. JEG-3 cells were on the upper plate which pretreated with 0.1 μM for 2 h. The buffer was put in the upper chamber.
VI	SH-SY5Y cells were seeded on the lower plate. JEG-3 cells were on the upper plate which pretreated with 0.1 μM for 2 h. 300 μM CORT was put in the upper chamber.

After drug treatment for 24 h, SH-SY5Y cells were harvested for immunoblot. The results showed the non-significant difference in the level of IL-6, IL-10 and LC3B-II among group I, III and V (Figure 20 A-C). CORT significantly increased IL-6 and IL-10 levels in group II, IV and VI as compared to group I, III and V, respectively (*p<0.05, **p<0.01 and ***p<0.001; Figure 20 A,B). JEG-3 could significantly reduce the expression of IL-6 in group IV, as compared to group II (*p<0.005, Figure 9A). JEG-3 pretreated with PFi significantly decreased IL-6 and IL-10 levels in group VI, as compared to group II (*p<0.005, Figure 20 A, B).

LC3B-II showed the non-significant difference among the group I, III and V (Figure 20 C). CORT significantly decreased the expression of LC3B-II in group II, IV and VI as compared to group I, III and V, respectively (*p<0.05 and ***p<0.001; Figure 20 C.). JEG-3 pretreated with PFi significantly increased the levels of LC3B-II in group VI, as compared to group II (*p<0.005, Figure 20 C).

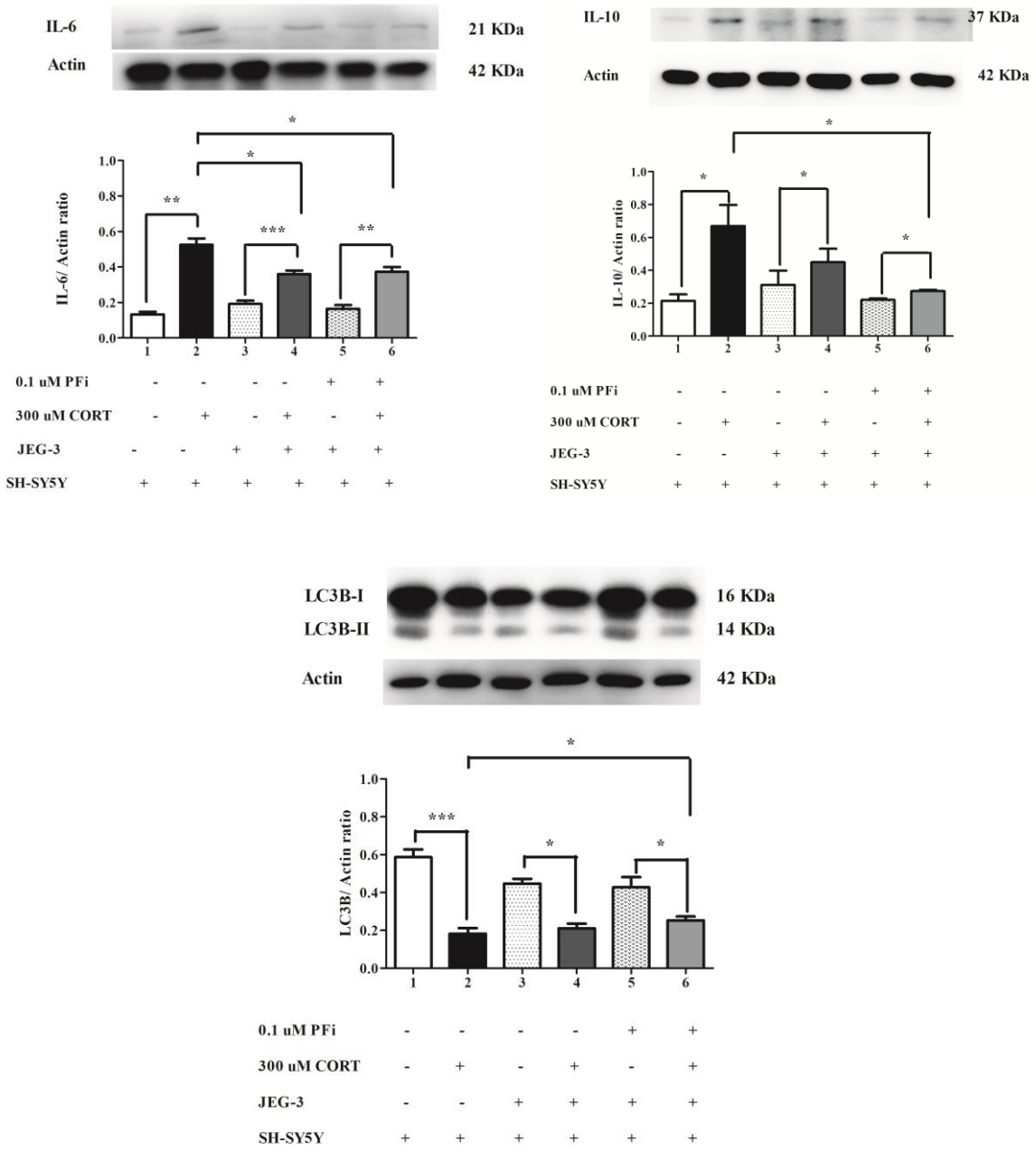


Figure 20. The protective effects of PFi against the expression of IL-6, IL-10 and LC3B-II in CORT treated SH-SY5Y cells and JEG-3 cells co-culture. Immunoblot of IL-6 (A), IL-10 (B) and LC3B-II (C) in SH-SY5Y cells. The bar graphs display the results from the immunoblot. Data were expressed as protein band densities normalized to β-actin serve as internal control. The values represent means ± SEM, n=4. Significant difference when compared with the referent group at *p<0.05, **p<0.01 and ***p<0.001.

Discussion

It is well-known that stress affects bodily physiology and behavior via stress hormones, GCs. Hippocampus, the part of the neuroendocrine system, negatively regulates HPA-axis and GCs secretion. However, prolonged stress exposure causes the alteration to the hippocampus that leads HPA-axis shifting and dysfunction. Maternal stress increases maternal blood GCs (34, 35), consequently alters the expression of inflammatory cytokines in hippocampus of the offspring (36). Likewise, our results have shown that maternal restraint stress and CORT enhance the expression of IL-6 in the hippocampus of the PS group and CORT-treated SH-SY5Y, respectively. In the normal circumstance, GCs play a role of immunosuppressive effect by negatively regulating the expression of pro-inflammatory cytokines, including IL-6, TNF-α and IL-1β. Stress hormones act on adrenergic and glucocorticoid receptors, resulting in an inhibition of NF-κB pathway. Meanwhile, GCs promote the differentiation of Th2, which in turn elevate the expression of anti-inflammatory cytokines. IL-10 negatively regulates the expression of pro-inflammatory cytokines. Chronic stress can induce these cells resisting to GCs which causes flux of IL-6 and decrease in the expression of IL-10. It is also known that IL-6 has a contrary function to IL-10. However, the previous study by Slusarczyk J. and colleagues (37) has shown the elevation of hippocampal IL-6 levels in response to prenatal stress. According to the restraint stress exposure enhances extracellular glutamate concentration in the rat hippocampus (38). Of interest, the overexposure of neuron to glutamate or NMDA has been shown the effect of neurotoxicity in either primary cortical neurons or SH-SY5Y that alleviated by the expression of IL-6 (39). JAK/STAT pathway, activated by IL-6, consequently increases in the expression of SOCS3 which negatively regulating IL-6 and STAT3 (40). The expression of STAT3 is the key regulator to protect neuron from glutamate-induced cell death by increased in anti-apoptotic factor, Bcl-xL (39). Furthermore, we found that IL-10, the anti-inflammatory cytokine, elevated in either the hippocampus of the PS group or CORT-treated SH-SY5Y, as well. Several studies have elucidated the increased in IL-10 in parallel with IL-6 in several kinds of insult (41, 42). IL-6 induces the expression of IL-10 in mice T cell via STAT3 (43). This may imply the co-operation of IL-6 and IL-10, which may play a role of neuroprotection for hippocampal neuron survival.

Autophagy impairment contributes to the neurodegenerative disorders, including Alzheimer's disease, Huntington's disease and Parkinson's disease (33, 44). A recent study has shown the relationship of immune response and autophagy that LPS elevates IL-6, while decrease LC3B-II in the hippocampus of adult mice (45). Herein, our current study has indicated that stress hormones decrease the expression of LC3B-II protein in either the hippocampus of the PS group or CORT-treated SH-SY5Y. LC3B-II is also known as the principle biomarker of autophagy. Correspondingly, the immunofluorescence has rendered that prenatal stress induces the decrease in LC3B staining in

CA1 and CA3 of the pup hippocampus. Stress causes excessive glutamate released, which leads Ca^{2+} influx, followed by mitochondria dysfunction, and released production of reactive oxygen species (17) (46). ROS-induced neuronal injury or death is a critical factor of neurodegenerative diseases and neurodevelopmental disorders, as well. The waste products of cell injury activate the protein degradation system, including UPS, ERAD and autophagy, to eliminate the unwanted proteins. Perturbations of autophagy during the critical period causes the neurodevelopmental disorders and psychiatric problems in later life. This idea is supported by the previous work of Tang and colleagues, who shows the decrease in autophagy while the increase in IL-6 in the autistic postmortem brain (17). IL-6 promotes cell survival, which has been described in the human lymphoblast lung cell lines by activating STAT3/Bcl-2 pathway, followed by the elevation of Bcl-2/Beclin-1 complex (22). This inhibits Beclin-1 to dissociate out and turn on autophagy. By the way, IL-10 promotes the expression of AKT in the human hypertrophic scar fibroblasts (47). Nutrition-rich circumstance induces the translocation of mTOR close to AKT and p70S6 kinase on lysosomal membrane. mTOR is phosphorylated at S2448, which in turn phosphorylates and inhibits the downstream proteins, Unc-51-like autophagy-activating kinase 1 (ULK-1) at S757, and autophagy related proteins (Atg) 13. This process inhibits autophagy by prevention ULK-1-Atg13-FIP200 complex adding the phosphate groups to Beclin-1 at S14 (48). The present data has demonstrated that maternal restraint stress-induced prenatal stress interrupts neuroendocrine immune system of the offspring. Stress hormones increase the expression of IL-6 and IL-10 which may play a novel role to protect neuronal cell death in the offspring. This may imply the capability of the offspring adaptation itself against to the intrauterine milieu changes. However, cytokine imbalance inhibits autophagy which involves in the developmental pruning of the offspring. Thereby, the disturbance of the neuroendocrine immune system during the critical period exists in later life. These findings offer the additional information according to the crosstalk between neuroendocrine immune system and the protein degradation system.

We further investigate the role of placental barrier enzymes, which have a critical function to regulate the permeability of maternal blood cortisol to the fetus. We selectively examined in JEG-3, human choriocarcinoma cell lines, which expresses the 11β-HSD isozymes (49). Our results have shown the increase in 11β-HSD1 while decrease in 11β-HSD2 in the cortisol-treated JEG-3 cells. A previous study has shown that prenatal stress downregulates the mRNA levels of 11β-HSD2 in the placenta (28). 11β-HSD2, the NAD-dependent hydrogenase enzyme, converts the active cortisol to the inert cortisone, that cannot pass the placenta. The dysregulation of placental 11β-HSD2 may imply the mechanism of stress hormones influx into the placenta by inhibiting the placenta barrier. Moreover, we have shown that stress hormones induced the expression of IL-6 and IL-10 in the human choriocarcinoma cell lines, as well. The alteration of the 11β-HSD isozymes in JEG-3 is

regulated by the variable expression of the inflammatory cytokines. IL-1β and TNFα have been applied to JEG-3 cells in the previous study of Johnstone and colleagues in 2005. They have shown that the pro-inflammatory cytokines have increased the protein levels of 11β-HSD1, compatible with the increase in 11β-HSD1 expression in the placenta with chorioamnionitis (49). 11β-HSD2 is contrary to 11β-HSD1, which is negatively regulated by the pro-inflammatory cytokines. Like we demonstrated the inverse relationship between IL-6 and 11β-HSD2 in CORT-treated JEG-3 cells. The previous study has shown that IL-1β and TNFα reduce either the enzymes activity or the expression of proteins and mRNA levels of 11β-HSD 2 in JEG-3 cells (50). Of interest, we could show the increase in IL-10 in the CORT-treated group, as well. The expression of IL-10 in JEG-3 cells might be regulated by the downstream signaling of IL-6. A line of evidence has suggested that SOCS-3 overexpression enhances IL-10 levels in JEG-3 cells (51). SOCS-3 and IL-10 negatively regulates the pro-inflammatory cytokines, including IL-6. However, S. Typhimurium-infected JEG-3 cells have increased IL-10, which promotes the proliferation of bacteria in the cells (52). Here, we are able to elucidate that maternal stress or stress hormones have induced the dysregulation of inflammatory cytokines. This in turn interrupts the placental barrier and allows maternal CORT to influx to the fetus. Obviously, CORT causes the devastating effects to the fetal brain development. As we can show the increases in IL-6 and IL-10, but decrease in LC3B-II in the hippocampus of the offspring. Furthermore, we would like to alleviate the effects of maternal restraint-induced stress hormones on the fetal brain development. PF915275 has inhibited 11β-HSD1 activity (53), which in turn increase the restriction of placental permeable to CORT. Our results have rendered that 11β-HSD1 inhibitor has a powerful effect to alleviate the effects of CORT-induced cytokine alteration in JEG-3 cells. The drug treatment promotes the expression of 11β-HSD2 in JEG-3 cells, as well. We further demonstrate the integrated effects of 11β-HSD1 inhibitor and JEG-3 cells, which play a role as the barrier against CORT effects on neuronal cells. The co-culture of two distinct cells was performed in this study. We have shown that JEG-3 cells can partly protect SH-SY5Y cells by downregulating in CORT-induced IL-6 expression. PFi-pretreated JEG-3 has more potent effect against to CORT-induced immune dysregulation and upregulates LC3B-II in SH-SY5Y cells.

To summarize, our study showed the crosstalk among the neuroendocrine and the neuro-immune system. Stress hormones induced dysregulation of the neuroinflammatory cytokines, including IL-6 and IL-10, which consequently interrupt the autophagic biomarker in developing neurons. We could show that stress hormones also disturbed the placental barrier in the trophoblast cells that contains the 11β-HSD isozymes. This in turn upregulated the levels of IL-6 and IL-10 in the JEG-3 cells, as well. Furthermore, we could demonstrate in the co-culture of two distinct cells type that mimic the intrauterine environment. We found that JEG-3 cells have a critical role of placental barrier to

protect the adverse effect of CORT on developing neurons. However, the placental trophoblasts can partly protect the developing neurons from harmful effect of stress hormones. In addition, we could demonstrate the powerful effects of 11β-HSD1 inhibitor to ameliorate the adverse effects of stress during pregnancy. 11β-HSD1 inhibitor might be the novel drug against the negative effect of gestation stress on developing brain. Further study should be done the test the effect of PFi drug *in vivo* in which the effects of PFi drug on metabolism of mother and the offspring should be considered.

References

1. Jonson KM, Lyle JG, Edwards MJ, Penny RH. Effect of prenatal heat stress on brain growth and serial discrimination reversal learning in the guinea pig. Brain Res Bull. 1976; 1(1): 133-50.
2. Loi M, Mossink JC, Meerhoff GF, Den Blaauwen JL, Lucassen PJ, Joels M. Effects of early-life stress on cognitive function and hippocampal structure in female rodents. Neuroscience. 2015.
3. Fride E, Weinstock M. The effects of prenatal exposure to predictable or unpredictable stress on early development in the rat. Dev Psychobiol. 1984; 17(6): 651-60.
4. Schneider ML, Roughton EC, Koehler AJ, Lubach GR. Growth and development following prenatal stress exposure in primates: an examination of ontogenetic vulnerability. Child Dev. 1999; 70(2): 263-74.
5. Zeng Y, Brydges NM, Wood ER, Drake AJ, Hall J. Prenatal glucocorticoid exposure in rats: programming effects on stress reactivity and cognition in adult offspring. Stress. 2015; 18(3): 353-61.
6. Veerawatananan B, Surakul P, Chutabhakdikul N. Maternal restraint stress delays maturation of cation-chloride cotransporters and GABAA receptor subunits in the hippocampus of rat pups at puberty. Neurobiol Stress. 2016; 3: 1-7.
7. Son GH, Geum D, Chung S, Kim EJ, Jo JH, Kim CM, et al. Maternal stress produces learning deficits associated with impairment of NMDA receptor-mediated synaptic plasticity. J Neurosci. 2006; 26(12): 3309-18.
8. Coe CL, Kramer M, Czeh B, Gould E, Reeves AJ, Kirschbaum C, et al. Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. Biol Psychiatry. 2003; 54(10): 1025-34.
9. Lui CC, Wang JY, Tain YL, Chen YC, Chang KA, Lai MC, et al. Prenatal stress in rat causes long-term spatial memory deficit and hippocampus MRI abnormality: differential effects of postweaning enriched environment. Neurochem Int. 2011; 58(3): 434-41.

10. Hosseini-Sharifabad M, Hadinedoushan H. Prenatal stress induces learning deficits and is associated with a decrease in granules and CA3 cell dendritic tree size in rat hippocampus. Anat Sci Int. 2007; 82(4): 211-7.
11. Yang J, Han H, Cao J, Li L, Xu L. Prenatal stress modifies hippocampal synaptic plasticity and spatial learning in young rat offspring. Hippocampus. 2006; 16(5): 431-6.
12. Sun H, Guan L, Zhu Z, Li H. Reduced levels of NR1 and NR2A with depression-like behavior in different brain regions in prenatally stressed juvenile offspring. PLoS One. 2013; 8(11): e81775.
13. Zhao Q, Peng C, Wu X, Chen Y, Wang C, You Z. Maternal sleep deprivation inhibits hippocampal neurogenesis associated with inflammatory response in young offspring rats. Neurobiol Dis. 2014; 68: 57-65.
14. Dursun E, Gezen-Ak D, Hanagasi H, Bilgic B, Lohmann E, Ertan S, et al. The interleukin 1 alpha, interleukin 1 beta, interleukin 6 and alpha-2-macroglobulin serum levels in patients with early or late onset Alzheimer's disease, mild cognitive impairment or Parkinson's disease. J Neuroimmunol. 2015; 283: 50-7.
15. Kim YS, Joh TH. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. Exp Mol Med. 2006; 38(4): 333-47.
16. Stoeck K, Bodemer M, Ciesielczyk B, Meissner B, Bartl M, Heinemann U, et al. Interleukin 4 and interleukin 10 levels are elevated in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. Arch Neurol. 2005; 62(10): 1591-4.
17. Tang G, Gudsnuk K, Kuo SH, Cotrina ML, Rosoklija G, Sosunov A, et al. Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. Neuron. 2014; 83(5): 1131-43.
18. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009; 43: 67-93.
19. Glausier JR, Lewis DA. Dendritic spine pathology in schizophrenia. Neuroscience. 2013; 251: 90-107.
20. Lewis DA, Levitt P. Schizophrenia as a disorder of neurodevelopment. Annu Rev Neurosci. 2002; 25: 409-32.
21. Marx MC, Billups D, Billups B. Maintaining the presynaptic glutamate supply for excitatory neurotransmission. J Neurosci Res. 2015; 93(7): 1031-44.
22. Qin B, Zhou Z, He J, Yan C, Ding S. IL-6 Inhibits Starvation-induced Autophagy via the STAT3/Bcl-2 Signaling Pathway. Sci Rep. 2015; 5: 15701.

23. Schoof E, Girstl M, Frobenius W, Kirschbaum M, Repp R, Knerr I, et al. Course of placental 11β-hydroxysteroid dehydrogenase type 2and 15-hydroxyprostaglandin dehydrogenase mRNA expression during human gestation. Eur J Endocrinol. 2001; 145(2): 187-92.
24. Murphy VE, Clifton VL. Alterations in human placental 11beta-hydroxysteroid dehydrogenase type 1 and 2 with gestational age and labour. Placenta. 2003; 24(7): 739-44.
25. Fowden AL, Li J, Forhead AJ. Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? Proc Nutr Soc. 1998; 57(1): 113-22.
26. Surakul P, Weerachatyanukul W, Chutabhakdikul N. Repeated carbenoxolone injections during late pregnancy alter Snk-SPAR and PSD-95 expression in the hippocampus of rat pups. Neurosci Lett. 2011; 494(1): 75-9.
27. Chutabhakdikul N, Surakul P. Prenatal stress increased Snk Polo-like kinase 2, SCF beta-TrCP ubiquitin ligase and ubiquitination of SPAR in the hippocampus of the offspring at adulthood. Int J Dev Neurosci. 2013; 31(7): 560-7.
28. Jensen Pena C, Monk C, Champagne FA. Epigenetic effects of prenatal stress on 11beta-hydroxysteroid dehydrogenase-2 in the placenta and fetal brain. PLoS One. 2012; 7(6): e39791
29. Fotsch C, Wang M. Blockade of glucocorticoid excess at the tissue level: inhibitors of 11beta-hydroxysteroid dehydrogenase type 1 as a therapy for type 2 diabetes. J Med Chem. 2008; 51(16): 4851-7.
30. Tomlinson JW, Stewart PM. Mechanisms of disease: Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 as a novel treatment for the metabolic syndrome. Nat Clin Pract Endocrinol Metab. 2005; 1(2): 92-9.
31. Bencsik N, Sziber Z, Liliom H, Tarnok K, Borbely S, Gulyas M, et al. Protein kinase D promotes plasticity-induced F-actin stabilization in dendritic spines and regulates memory formation. J Cell Biol. 2015; 210(5): 771-83.
32. Courtney R, Stewart PM, Toh M, Ndongo MN, Calle RA, Hirshberg B. Modulation of 11beta-hydroxysteroid dehydrogenase (11β-HSD) activity biomarkers and pharmacokinetics of PF-00915275, a selective 11β-HSD1 inhibitor. J Clin Endocrinol Metab. 2008; 93(2): 550-6.
33. Cherra SJ, 3rd, Dagda RK, Chu CT. Review: autophagy and neurodegeneration: survival at a cost? Neuropathol Appl Neurobiol. 2010; 36(2): 125-32.
34. Amugongo SK, Hlusko LJ. Impact of maternal prenatal stress on growth of the offspring. Aging Dis. 2014; 5(1): 1-16.

35. Lingas R, Dean F, Matthews SG. Maternal nutrient restriction modifies brain corticosteroid receptor expression and endocrine function in the fetal guinea pig. Brain Res. 1999; 846(2): 236-42.
36. Diz-Chaves Y, Astiz M, Bellini MJ, Garcia-Segura LM. Prenatal stress increases the expression of proinflammatory cytokines and exacerbates the inflammatory response to LPS in the hippocampal formation of adult male mice. Brain Behav Immun. 2013; 28: 196-206.
37. Slusarczyk J, Trojan E, Glombik K, Budziszewska B, Kubera M, Lason W, et al. Prenatal stress is a vulnerability factor for altered morphology and biological activity of microglia cells. Front Cell Neurosci. 2015; 9: 82.
38. Lowy MT, Wittenberg L, Yamamoto BK. Effect of acute stress on hippocampal glutamate levels and spectrin proteolysis in young and aged rats. J Neurochem. 1995; 65(1): 268-74.
39. Park KW, Nozell SE, Benveniste EN. Protective role of STAT3 in NMDA and glutamate-induced neuronal death:negative regulatory effect of SOCS3. PLoS One. 2012; 7(11): e50874.
40. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol. 2008; 19(4): 414-22.
41. Ropelle ER, Flores MB, Cintra DE, Rocha GZ, Pauli JR, Morari J, et al. IL-6 and IL-10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKbeta and ER stress inhibition. PLoS Biol. 2010; 8(8).
42. Fayad L, Keating MJ, Reuben JM, O'Brien S, Lee BN, Lerner S, et al. Interleukin-6 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with phenotypic characteristics and outcome. Blood. 2001; 97(1): 256-63.
43. Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. Nat Immunol. 2007; 8(12): 1363-71.
44. Rami A. Review: autophagy in neurodegeneration: firefighter and/or incendiarist? Neuropathol Appl Neurobiol. 2009; 35(5): 449-61.
45. Francois A, Terro F, Quellard N, Fernandez B, Chassaing D, Janet T, et al. Impairment of autophagy in the central nervous system during lipopolysaccharide-induced inflammatory stress in mice. Mol Brain. 2014; 7:56.
46. Vyas S, Rodrigues AJ, Silva JM, Tronche F, Almeida OF, Sousa N, et al. Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration. Neural Plast. 2016. 2016: p. 6391686.

47. Shi J, Wang H, Guan H, Shi S, Li Y, Wu X, et al. IL10 inhibits starvation-induced autophagy in hypertrophic scar fibroblasts via cross talk between the IL10-IL10R-STAT3 and IL10-AKT-mTOR pathways. Cell Death Dis. 2016; 7: e2133.
48. Randall-Demllo S, Chieppa M, Eri R. Intestinal epithelium and autophagy: partners in gut homeostasis. Front Immunol. 2013; 4: 301.
49. Johnstone JF, Bocking AD, Unlugedik E, Challis JR. The effects of chorioamnionitis and betamethasone on 11beta hydroxysteroid dehydrogenase types 1 and 2 and the glucocorticoid receptor in preterm human placenta. J Soc Gynecol Investig. 2005; 12(4): 238-45.
50. Chisaka H, Johnstone JF, Premyslova M, Manduch Z, Challis JR. Effect of pro-inflammatory cytokines on expression and activity of 11beta-hydroxysteroid dehydrogenase type 2 in cultured human term placental trophoblast and human choriocarcinoma JEG-3 cells. J Soc Gynecol Investig. 2005; 12(5): 303-9.
51. Dong Q, Fan R, Zhao S, Wang Y. Over-expression of SOCS-3 gene promotes IL-10 production by JEG-3 trophoblast cells. Placenta. 2009; 30(1): 11-4.
52. Nguyen T, Robinson N, Allison SE, Coombes BK, Sad S, Krishnan L. IL-10 produced by trophoblast cells inhibits phagosome maturation leading to profound intracellular proliferation of Salmonella enterica Typhimurium. Placenta. 2013; 34(9): 765-74.
53. Bhat BG, Hosea N, Fanjul A, Herrera J, Chapman J, Thalacker F, et al. Demonstration of proof of mechanism and pharmacokinetics and pharmacodynamic relationship with 4'-cyano-biphenyl-4-sulfonic acid (6-amino-pyridin-2-yl)-amide (PF-915275), an inhibitor of 11 -hydroxysteroid dehydrogenase type 1, in cynomolgus monkeys. J Pharmacol Exp Ther. 2008; 324(1): 299-305.

output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

โครงการวิจัยนี้ทำให้เกิดผลงานตีพิมพ์ในวารสารวิชาการต่างประเทศที่มี impact factors จำนวน 2 เรื่องคือ

1. Veerawatananan B, Surakul P, Chutabhakdikul N.* (2016) Maternal restraint stress delays maturation of cation-chloride cotransporters and GABAA receptor subunits in the hippocampus of rat pups at puberty. Neurobiology of Stress, Vol. 3: page 1-7.
2. Surakul P, Vanichviriyakit R, Chutabhakdikul N.* Protective effects of 11β-HSD1 inhibitor on corticosterone induced neuroinflammatory response and autophagic biomarker in SH-SY5Y cells and JEG-3 cell line co-culture. (manuscript being submit to Developmental Neuroscience Journal)

Appendix