EFFECTS OF MATERNAL STRESS ON THE EXPRESSION OF GROWTH-ASSOCIATED PROTEIN-43 (GAP-43) IN PREFRONTAL CORTEX OF NEONATAL RAT

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ABSTRACT

Prenatal stress may be a risk factor for psychopathology later in life. In the offspring of prenatally stressed animals, overactivity and impaired negative feedback regulation of the hypothalamic-pituitary-adrenal axis are a consistent finding. Growth-Associated Protein-43 (GAP-43) is a presynaptic membrane phosphoprotein whose expression increases during developmental events such as axonal growth or remodeling and axon regeneration. Phosphorylation of GAP-43 by PKC has been reported to correlate with enhanced axonal growth and transmitter release. In adult animals, increase of GAP-43 has been reported to correlate with monoaminergic deficit in neuropsycho-pathology. The present study examines the effects of maternal restraint stress during gestation day (GD) 14-21 on the level of GAP-43 in different layers of neonatal rat prefrontal cortex (PFC). From postnatal day (PND) 7-21, pups born from a stressed mother show a significant increase in density of GAP-43 immunostaining in PFC compared to control. Increase of GAP-43 was observed in specific cortical layers (i.e., layer II, IV, V and VI) but not different in layer III. These changes indicate the direct effects of elevated maternal stress hormone, since maternal injection of corticosteroid (CORT, 40 mg/kg) during GD 14-21, also gave the same results. The results suggest that maternal stress may be harmful to the developing brain and upregulation of GAP-43 may serve as a protective mechanism against the toxicity of maternal stress hormone. Increase of GAP-43 may alter the pattern of axonal growth and formation of synapses in prefrontal cortex of rat pups because PND 7-14 has been reported to correlate with the peak period of synaptogenesis in this brain area.

KEY WORDS: PRENATAL STRESS /GROWTH-ASSOCIATED PROTEIN-43 (GAP-43) /PREFRONTAL CORTEX
120 P.

การศึกษาผลของความเครียดขณะตั้งครรภ์ต่อการเปลี่ยนแปลงระดับโปรตีน GAP-43 ในสมองส่วนหน้าของลูกหนู (EFFECTS OF MATERNAL STRESS ON THE EXPRESSION OF GROWTH-ASSOCIATED PROTEIN-43 (GAP-43) IN PREFRONTAL CORTEX OF NEONATAL RAT)

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บทคัดย่อ

ความเครียดในระหว่างตั้งครรภ์เป็นปัจจัยเสี่ยงที่อาจเป็นสาเหตุหนึ่งของการเกิดพยาธิสภาพของจิตประสาทในภายหลัง มารดาตั้งครรภ์ที่มีความเครียดสูงจะส่งผลโดยตรงต่อทารกในครรภ์ทำให้ระบบประสาทที่ควบคุมการตอบสนองต่อความเครียด (HPA axis) ทำงานมากกว่าปกติ และประสิทธิภาพในการยับยั้งการทำงานของ HPA axis ลดลง การได้รับฮอร์โมนเครียดปริมาณสูงเป็นเวลานานย่อมส่งผลกระทบระยะยาวต่อขั้นตอนต่างๆในการพัฒนาของสมอง โปรทีน GAP-43 เป็นโปรทีนที่มีความสำคัญต่อการพัฒนาของสมอง จะพบมากในช่วงที่มีการงอกของปลายประสาท และการเกิดจุดเชื่อมต่อสัญญาณประสาท นอกจากนั้นยังเป็นกลไกสำคัญที่เกี่ยวข้องในการหลั่งสารสื่อประสาทบริเวณ synapse งานวิจัยชิ้นนี้มีวัตถุประสงค์ในการศึกษาผลของความเครียดในแม่หนูตั้งครรภ์ ต่อระดับของโปรทีน GAP-43 ในสมองส่วนหน้าของลูกหนู ผลจากการทดลองพบว่า ลูกหนูที่เกิดจากแม่หนูกลุ่มที่ถูกทำให้เครียดโดยวิธีจำกัดการเคลื่อนไหว 4 ชั่วโมง/วัน ในช่วงอายุครรภ์ 14-21 วัน มีระดับโปรทีน GAP-43 เพิ่มขึ้นในสมองส่วน prefrontal cortex อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับลูกหนูในกลุ่มควบคุม การเปลี่ยนแปลงดังกล่าวเห็นได้ชัดเจนเมื่อลูกหนูมีอายุ 7-21 วันและพบเฉพาะในเปลือกสมองชั้นที่ 2, 4, 5 และ 6 ส่วนในชั้นที่ 3 นั้นไม่พบว่ามีการเปลี่ยนแปลงอย่างมีนัยสำคัญ จากการศึกษาโดยการฉีดฮอร์โมน Coricosteriod (40 mg/kg) ให้กับแม่หนูตั้งครรภ์ ในช่วงอายุครรภ์เดียวกัน พบว่ามีผลทำให้เพิ่มระดับโปรทีน GAP-43 ในสมองส่วนหน้าของลูกหนูได้เช่นเดียวกับการทำให้แม่หนูเครียด ผลการทดลองบ่งชี้ว่าฮอร์โมนเครียดในปริมาณสูงที่เกิดจากความเครียดในระหว่างตั้งครรภ์ส่งผลระยะยาวต่อการงอกของปลายประสาทและการสร้างวงจรประสาทในเปลือกสมองส่วนหน้า ซึ่งอาจทำให้เกิดความไม่สมดุลของวงจรประสาทและสารสื่อประสาท และอาจเป็นสาเหตุหนึ่งของการเกิดโรคทางจิตประสาทในภายหลัง

120 หน้า.

CONTENTS

CONTENTS (Cont.)

CONTENTS (Cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF FIGURES (Cont.)

LIST OF ABBREVIATIONS

11β-HSD	11 β-hydroxysteroid hydrogenase
5-HT	Serotonin
5UTR	5’ untranslated region
A	Adrenaline
AA	Arachidonic acid
AC	Anterior cingulated gyrus
ACd	Dorsal anterior cingulated area
ACTH	Adrenocorticotropic hormone
Agm	Agranular medial cortex
AMPH	Amphetamine
ANS	Autonomic Nervous System
APV	amino-5-phosphonovalerate
BDNF	Brain-derived neurotrophic factor
CA	Cornus Ammon
CaM	Calmodulin
CaMK II	Calmodulin kinase type II
CBG	Corticosteroid binding globulin
CNS	Central Nervous System
CORT	Corticosterone
CP	Core promoter sequence
CRH	Corticotrophic-releasing hormone
DA	Dopamine
DAG lipase	Diacylgycerol lipase
DAG	Diacyglycerol
FEF	Frontal eye field
Fr2	Frontal area 2
GAP-43	Growth associated protein-43

LIST OF ABBREVIATIONS (Cont.)

GD	Gestation day
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
IL	Infralimbic
LC	Locus coeruleus
LC-NA	Locus Ceruleus-Noradrenergic
LHPA	Limbic-hypothalamic-pituitary-adrenal
MB	Membrane-binding
mPVN	Medial parvocellular part of PVN
MR	Mineralocorticoid
NA	Noradrinaline
NAc	Nucleus accumben
NCAM	Neural cell adhesion molecule
NE	Norepinepherine
NLC	Nucleus locus ceroruleus
PBS	Phosphate buffer saline
PFC	Prefrontal cortex
PKC	Protein Kinase C
PL	Prelimbic
PLC	Phospholipase C
PLCG	Phospholipase Cg
PND	Postnatal day
PrCm	Precental medial area
PS	Prenatal stress
PTSD	Post-traumatic stress disorder
SAGE	Serial analysis of gene expression
SAN	Sympatho-adrenergic-noradrenergic
Ser41	Serine 41

LIST OF ABBREVIATIONS (Cont.)

SMA	Supplementary motor area
VA	Ventral anterior of thalamus
VGCC	Voltage-gated calcium channels
VL	Ventrolateral nucleus
VLa	anterior ventrolateral nucleus
VLO	Ventrolateral obital area
VLp	posterior ventrolateral nucleus
VM	Ventromedial nuclei
VMp	posterior ventromedial of thalamus
VP	Vasopressin
VTA	Ventral tegmental area
β-FGF	basic fibroblast growth factor

CHAPTER 1
INTRODUCTION

There is increasing evidence that variations in prenatal environment can influence on the brain and behavioral development of newborn. It can be speculated that prenatal environment can act on the mother and the fetus, to alter the differentiate functions of an organ or tissue system to prepare the unborn animal optimally for the environmental conditions during gestation. However, in extreme conditions like stress, offspring of stressed mothers displayed short and long-term physiological and behavioral abnormalities such as increase anxiety and the cognitive retardation, (Meijer 1985, Schell 1981, Stott 1973). Prenatal environment has an influence on individual's development profoundly, inducing changes lasting into adulthood (Weinstock 1997). These intriguing findings have spawned the fetal origins hypothesis of adult disease (Barker 1995).

In adult, stress and elevated stress hormone levels are known to alter cognition, learning, memory and emotional responses. Three weeks after chronic stress or high level of glucocorticoids exposure is reported to decrease the neurogenesis in hippocampal dentate gyrus and alter neuronal morphology in the prefrontal cortex, hippocampus and amygdala. During development, the brain is very sensitive to glucocorticoids since this hormone have a powerful brain-programming properties (Seckl 1998). One of the most intensively systems studied is the hypothalamic-pituitary–adrenal (HPA) axis. Substantial evidence suggests that prenatal stress programs the HPA axis, and that plasticity of developing brain monoamine systems underlies these changes. A high level of glucocorticoids has become an obvious candidate for the role of programming factor in the prenatal stress paradigm.

Growth association protein-43 (GAP-43) is a presynaptic membrane phospho-protein whose expression increases during developmental events such as axonal growth or during brain remodeling and plasticity such as learning and regeneration of

an injured axon. GAP-43 is the major substrates for protein kinase C (PKC) in the presynaptic nerve terminals. It is phosphorylated by PKC at Ser 41 and this phosphorylation has been reported to correlated with enhanced neurotransmitter release (Dekker et al 1989). Distribution of GAP-43 in the fetal rat brain has been described by *in situ* hybridization studied. The signal for GAP-43 mRNA is very abundant in almost all regions of the fetal brain at mid-gestation (Neve et al 1987). The nervous system of mice lacking the GAP-43 gene is grossly normal, although the mice rarely survive weaning (Strittmatter et al 1995). Analysis of GAP-43 (-/-) null mutation mice revealed that GAP-43 has a functional role in axon guidance (Kruger et al 1998, Sretavan & Kruger 1998, Strittmatter et al 1995).

In the present study, we hypothesized that prenatal stress may alter the level of GAP-43 in neonatal rat brain. The prefrontal cortex was chosen because this area has important roles in control the behavior such as mood expression, cognition and learning memory. Moreover, disorders of prefrontal cortex have been paralleled by both childhood- and adult- onset cognitive and mental health disorders of developmental etiology. To our knowledge, the effects of prenatal stress on GAP-43 expression in the neonatal rat brain have not yet examined. Thus, the present study aims to examine the effect of prenatal stress on brain development, particularly the axonal growth in the prefrontal cortex of postnatal rat.

CHAPTER 2
OBJECTIVES

GAP-43 mRNA is very abundant in almost all regions of the fetal rat brain at mid-gestation period as described by *in situ* hybridization studied (Neve et al 1987). A recent study also reveals the expression of GAP-43 protein in human during gestation period (Strittmatter et al 1995). This finding supports the most likely role of GAP-43 in the developing brain as the modulation of synaptic transitions, and axonal path-finding. In the present study, we hypothesized that prenatal stress may alter the levels of GAP-43 in neonatal rat brain. Thus, the specific aims of the present study are as following;

1. To identify and localize the GAP-43 immunoreactivity in the prefrontal cortex of postnatal rats.
2. To examine developmental pattern of GAP-43 immunoreactivity in the prefrontal cortex at different stage of postnatal rats.
3. To examine the effect of prenatal stress on GAP-43 immunoreactivity in the prefrontal cortex of postnatal rats.
4. To compare the density of GAP-43 immunoreactivity in specific cortical layer of prefrontal cortex between control and prenatal stress pups.
5. To examine the effect of prenatal glucocorticoid injection on GAP-43 immunoreactivity in prefrontal cortex of postnatal rats.

CHAPTER 3

LITERATURE REVIEW

3.1 Stress and neural response to stress

A stress is defined as a physical or psychological stimulation that disrupts an organism's homeostatic equilibrium. Physical stressors pose a material threat to an organism's survival (e.g., hemorrhage, hypoglycemia, high, or low of extremes environmental temperature, physiological injury, etc). Physiological stressors, on the other hand, are stimuli such as fearful thoughts, processes regulated by central nervous system (CNS) and the periphery that serve to reestablish homeostasis. This consists of an immediate neuroendrocrine response to an acutely stressful stimulus. If animals still exposure to repeated stress, however, one of two outcomes ensues: the organism either adapts to its stressful environment or, if it is unable to adapt, develops pathophysiological response. Stress can be divided into two classes;

Acute stress is a short term, positive stress that is not detrimental to the individual but rather often gives that extra burst of energy that is needed during the stressful period. As it is a short lived stress it is not considered a danger to one's health, unlike the chronic stress. The acute stress can be used to the advantage of the individual. However, every individual should make a point managing their stress levels whether they are short lived daily, weekly or monthly and avoid getting into the position of chronic stress.

Chronic stress is a long term, negative stress that has become detrimental to the individual. It can produce stress-related illnesses such as digestive disorders, cardiovascular problems, or musculoskeletal problem. It is often the result of ignored warning signs and has built up over a period of time. There are both physical and psychological consequences of chronic stress. Some examples are psychological i.e., panic attacks, aggressive behavior, feelings of being unable to cope, poor

concentration, etc., and some are physical i.e., digestive disorders, muscle tensions, increase blood pressure, decrease immune functioning.

The principal components of the adaptive response to stress are the sympatho-adrenergic-noradrenergic (SAN) and the limbic-hypothalamic-pituitary-adrenal (L-HPA) systems. The SAN system implies the biosynthesis and release of adrenaline (A) and noradrenaline (NA), regulated respectively by the sympathetic division of the autonomic nervous system (ANS), and the nucleus locus ceoruleus (NLC) in the CNS. The L-HPA system involves limbic structures, such as the amygdala and the hippocampus, in association with the HPA axis, and their respective interconnections. The SAN and L-HPA systems also participate in their mutual positive regulation, so that activation of one of them involves the activation of the other as well (Chrousos et al., 1998).

The sensitivity of the individual organism to stressor is determined by the interplay of genetic diatheses, biological development, and environmental conditions. L-HPA axis and the locus ceruleus–noradrenergic (LC–NA) system (Figure.1) receive and integrate sensory as well as blood-borne, limbic, and cortical information (Barker 1995). The LC–NA system originates in the brainstem. Activation of this system leads to release of norepinephrine from axons that project to neurons throughout the brain. In addition, there is release of norepinephrine by sympathetic neurons that innervate many organs as well as release of norepinephrine and epinephrine into the circulation by the adrenal medulla. One effect of release of norepinephrine into the brain is to improve alertness. Activation of systemic receptors produces a number of well-described effects, including increased cardiac output (Barker 1995, Clarke et al 1994). Other participants are the adrenal medulla, which produces noradrenaline and adrenaline, and the sympathetic nervous system which modulates physiologic functions through neurotransmitters.

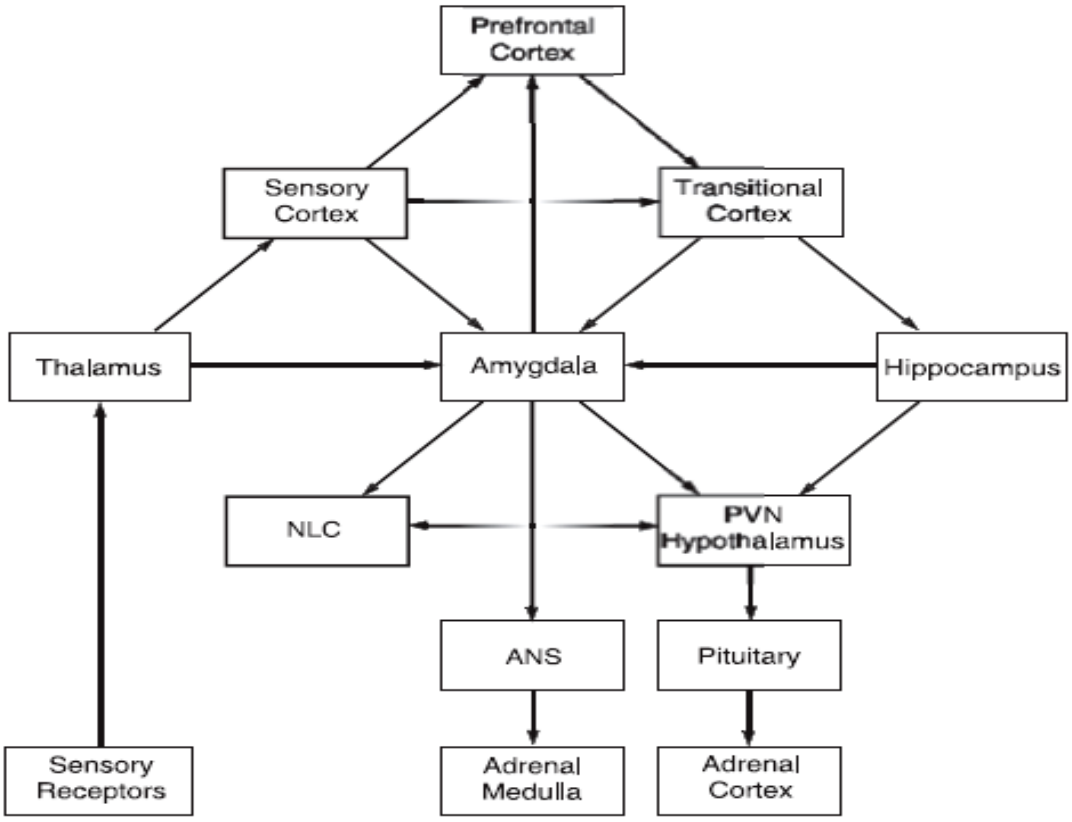

Figure 3.1. Neural structures involved in the adaptive response to stress. The neuroendocrine component involves the activation of the PVN of the hypothalamus, the anterior pituitary, and the adrenal cortex, with the consequent release of CRH, ACTH and cortisol. (LeDoux et al., 1998)

In the HPA system, Corticotrophin-releasing hormone containing neurons in the hypothalamic nucleus project to the hypothalamo- hypophysial portal vascular system. When activated, these neurons release a 41 amino-acid peptide, so called the Corticotrophin-Releasing Hormone (CRH). The region with highest expression of CRH is the medial parvocellular part of the PVN (mPVN). CRH neurons in the mPVN project to the external median eminence, where peptides are secreted into the portal bloodstream, through which they are transported to the anterior pituitary. In addition to CRH, the same neurons in the PVN also express and release vasopressin (VP), although most of the VP is expressed in neighboring magnocellular elements of the PVN that project to the posterior pituitary. Corticotropes in the anterior pituitary express receptors for CRH and VP. CRH binds to specific receptors in the anterior pituitary, causing release of adrenocorticotropin (ACTH) and β-endorphin. ACTH through the systemic circulation binds and activates its receptors on the surface of cells of the adrenal cortex. In response to receptor activation, adrenocortical cells synthesize and release of glucocorticoids (GC) into the blood circulation (Clarke et al 1994). The major GC in rodents is corticosterone (CORT), and in humans is cortisol.

Basal activity of the brain-pituitary-adrenal axis oscillates. CRH is released in a pulsatile manner from terminals in the median eminence, but the system is activated under emergency conditions through neural input. In the hypothalamus, the PVN appears to sum and integrate input from numerous loci. Input to PVN is divided into several broad classes, like brainstem (catecholaminergic fibers via the NTS convey viscerosensory information); hypothalamic or limbic inputs (amygdala, septum, hippocampus, prefrontal cortex reach PVN primarily via the bed nucleus of the stria terminalis). Blood-borne signals through neural projection from SFO and OVLT apparently also reach stress-related PVN parvocellular neurons (Clarke et al 1994).

Concentration of circulating glucocorticoids and ACTH also show a circadian rhythm. In humans, glucocorticoid levels peak around 7 AM and decline steadily throughout the day. The nadir is reached in the late evening at 7 PM to midnight after which glucocorticoid levels begin to rise. The phase of the daily ACTH rhythm precedes that of the glucocorticoids by about 1-2 hrs. CRH mRNA levels precedes the increase in glucocorticoid release by several hours. A similar pattern of glucocorticoid release is seen in rats, with highest levels when animals are awakening (in the case of

rats, this is in the evening) and lowest level in the morning. SCN lesion abolishes the CRH rhythm.

Cortisol binds glucocorticoid receptors found in many brain areas and the immune system, producing a variety of organ-specific changes. Glucocorticoid receptors are members of a superfamily of receptors that act as ligand-regulated transacting receptors. The receptor protein resides in the cytoplasm in a complex containing heat-shock proteins, which fold the receptor into the appropriate configuration for recognizing corticosteroid ligands. Upon steroid binding, the receptor moves to the nucleus of the cell and interacts with specific hormone recognition (or response) elements on the DNA, thereby changing transcription rate (Gispen et al 1985).

Short-term activation of the HPA axis allows for rapid mobilization of energy stores, where as in the long run, suppression of anabolic processes, depletion of energy store and suppression of immune system can be devastating to organism (Barbazanges et al 1996). CRH neurons of the PVN contain steroid receptors, and glucocorticoids inhibit transcription of CRH and VP genes through the genomic feedback. The feedback of glucocorticoids onto brain and pituitary negatively regulates the synthesis of CRH and ACTH. Removal of the adrenal, and hence of glucocorticoids, removes the negative feedback effects of these steroids. In this situation, concentrations of CRH and VP mRNA in the mPVN is increased (Clarke et al 1994).

In addition to the primary hormonal feedback loops within the HPA axis, the stress response is regulated by negative feedback from high affinity mineralocorticoid (MR) and low affinity glucocorticoid receptors (GR) in the hippocampus (Dekker et al 1989), anterior cingulate cortex and prefrontal prefrontal cortex (Diorio et al 1993). For examples, electrical stimulation (Weidenfeld et al 1985b) or lesions (Diorio et al 1993) of the PFC or glucocorticoids placed locally within the PFC (Diorio et al 1993), hippocampus, or hypothalamus (Kovacs & Makara 1988) can alter the plasma GC levels. Under a stress situation, hippocampal GRs appear to be sensitive to elevated glucocorticoid levels (Reul & de Kloet 1985), and the GR proteins or their mRNA are down-regulated by chronic stress (Herman et al 1995, Kitraki et al 1999). Such regulatory mechanisms of GRs in the feedback sites of the brain due to chronic stress may cause the attenuation of the glucocorticoid negative feedback to DEX. Although

glucocorticoids secretion is negatively regulated by glucocorticoids at the level of the anterior pituitary gland (Kalin et al 1982), several regions of the brain, such as the hypothalamus, hippocampus, and prefrontal cortex (PFC), that have abundant GRs are also involved (Diorio et al 1993, Kovacs & Makara 1988, Magarinos et al 1987, Weidenfeld et al 1985b). GRs are thought to be sensitive to stress-induced elevated levels of CORT. Positive feedback from GRs in amygdala can enhance the stress response in fear-conditioned learning and fear potentiated startle. The presence of GRs in the rat hippocampus, hypothalamus and pituitary has been shown from gestational day 13 (Chapman & Stern 1979b).

Evidence from studies in rats suggests that the HPA axis is functional in the fetus in late pregnancy, suppressed in early postnatal period, and functional at an adult level by about 15 days (Drago et al 1999b). Notably, most of the hormonal and behavioral stress effects reported in rodents involve stress during the last week (trimester) of gestation (Elsworth et al 2001). During the latter part of gestation, the negative feedback from cortisol to pituitary is suppressed. It is thought that upregulation of circulating cortisol binding globulin (CBG) which reduces levels of free cortisol, down-regulation of hypothalamic GRs and increased 11β-hydroxysteroid hydrogenase (11β-HSD), which metabolizes cortisol, contribute to the suppression of the negative feedback. In umbilical cord blood samples from healthy fetuses subsequently born at term, fetal ACTH and cortisol, but not CRH levels, increased with gestational age between 18 and 40 weeks (Dhabhar et al 2000). Thus, while mechanisms exist to protect the fetus from maternal cortisol, it is likely that overproduction of cortisol following stress could have adverse effects on the developing fetal HPA axis at different stages in gestation, and that cortisol could even precipitate premature delivery and suppress fetal growth (Dhabhar et al 2000).

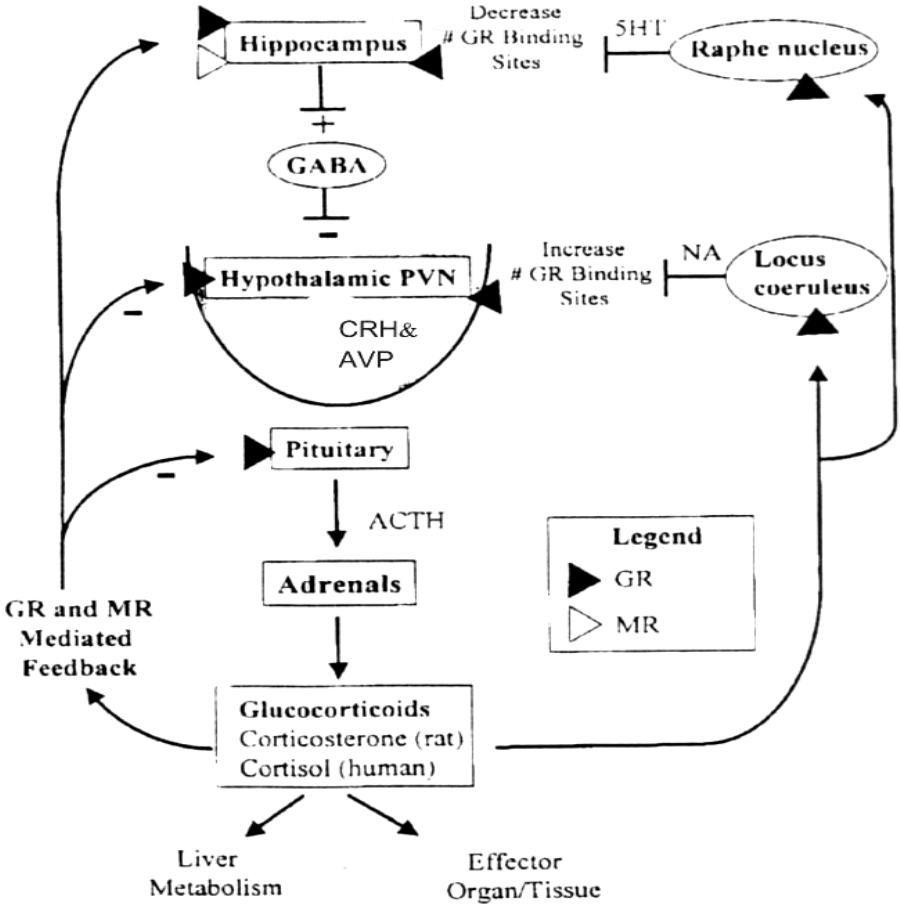

Figure 3.2 Negative feedback of HPA axis in an animal's response to stressful stimuli (LeDoux et al., 1998).

3.2 Prenatal stress and brain development

In pregnant animals, induced stress adversely affects behavioral adaptation, motor and mental development of the offspring. Stress in early or mid gestation in nonhuman primates resulted in impaired motor development, declined attention, and delayed cognitive development of offspring in the first year of life. The greatest sensitivity of the developing fetus to the stresses appeared to be during the second trimester of pregnancy. Interestingly, it is in this period that a number of brain regions are developing (Bayer et al 1993). Though these findings have not yet been replicated in humans, there is increasing appreciation of the relevance of the prenatal stress paradigm for the human situation (Brown et al 1996).

Exposure of pregnant female rats to stress has been used as a means to alter the behavior of the offspring later in life (Weinstock 2001, Welberg & Seckl 2001). There are abundant literatures on the effects of prenatal stress on the hypothalamic–pituitary–adrenal (HPA) axis. In general, these studies show that exposure of pregnant female rats to a variety of stressors during the final week of gestation reprograms the fetal HPA stress axis. The most commonly found change in HPA axis function is a prolongation of the glucocorticoid response to an acute stress in the adult prenatally stressed rats (Barbazanges et al 1996, Henry et al 1994, Kinnunen et al 2003, Koehl et al 1999, Szuran et al 2000, Takahashi et al 1992) but some studies also found a shift in basal levels of HPA axis activity (Fride et al 1986, Ward et al 2000). These changes in HPA axis function appear to arise as a consequence of a selective diminution in the expression of glucocorticoid receptors in the hippocampus (Barbazanges et al 1996, Henry et al 1994, Koehl et al 1999). The HPA axis changes can be ameliorated by clamping maternal glucocorticoid levels during pregnancy using corticosterone pellets (Barbazanges et al 1996). Additionally, because of the feed-forward effects of glucocorticoids on amygdala corticotropin-releasing hormone (CRH) neurons, CRH levels in the amygdala are increased, leading to a propensity for the animals exposed to stress during gestation to display increased anxiety, despair and impaired coping behaviors as adults (Fride et al 1986, Ward et al 2000, Weinstock 2001).

3.2.1 Effect of prenatal stress on behavior of rodent offspring

Aspects of development that could be influenced by prenatal stress in rodents were the early physical and motor development, exploration in a novel environment, disturbance behavior under stressful conditions, and social and sexual behaviors. Prenatal stress was associated with lower birth weights of the pups (Weinstock et al 1988) and compromised early motor development (DeSantis & Schmaltz 1984, Grimm 1987). Prenatal stress further affected the behavior of rodent offspring in a novel situation, with prenatally stressed offspring showing a decreased exploration and more defecation in an open field (Grimm 1987). Other studies, however, revealed a reverse trend, with shorter latencies to explore in the prenatally stressed rodents and more active behavior in a novel situation (Deminiere et al 1992) or found no influence of prenatal stress on these behavioral measures (Chapman & Stern 1979a). Genetic factors might contribute to these differing results, since it was found that prenatal stress caused different offspring activity levels depending on the characteristics of their breed. For instance, two inbred strains of mice, which showed either high or low activity levels, were used to study the influence of genetic factors on the offspring response to prenatal stress. The male offspring of a low-activity strain of prenatally stressed mothers were more active than control males, whereas prenatally stressed male offspring of a high activity strain were less active. Female offspring of both strains were less active (Stohr et al 1998). Thus, both sex effects and genetic effects seem relevant to explain different results following the exposure to prenatal stress. Environmental variables also proved to be relevant, since exploratory activity in reaction to novelty was significantly less in a bright light but not in dim light conditions (Poltyrev et al 1996).

3.2.2 Effect of prenatal stress on the modification of limbic structures

Limbic structures involved in regulation of the stress response are particularly vulnerable to stress in adults, as evidenced by the reduction in hippocampal volume in patients with post-traumatic stress disorder (PTSD) (Bremner et al 1995) and in monkeys who had been exposed to severe social stress and drastic weight loss (Uno et al 1989). Although changes in hippocampal volume in primates following PS have not been reported, there was some reported that prenatal administration of dexamethasone

to pregnant rhesus monkeys 72 h before delivery resulted in reduced density of the pyramidal neurons and reduced thickness and circumference of the Cornus Ammon (CA) and dentate gyrus regions of the hippocampus (Uno et al 1990a). In animals whose mothers were treated for 30 days with dexamethasone, the entire hippocampal formation was smaller than that of controls, with a dose-related loss of neurons. In rodents, prenatal stress induced significant alterations to the hippocampus and dentate gyrus. Restraint stress three times daily in the last trimester of pregnancy in rats resulted in reduced cell proliferation in the dentate gyrus, which was evident throughout the lifespan. In particular, there was a decrease in the number of granule neurons between 3 and 22 months of age and a marked absence of hippocampal neurogenesis in prenatal stress rats compared to control rats as a result of learning a spatial task (Lemaire et al 2000a). Prenatal stress reduced the density of nitric-oxide producing neurons in the dentate and part of the hippocampus (Vaid et al 1997). Severe restraint stress during the last week of gestation did not affect the volume of the sexually dimorphic medial amygdala, but mild prenatal stress (saline injection) enlarged the basolateral nucleus of the amygdala in adult offspring which, may be related to the changes in emotional behavior following prenatal stress (Drago et al 1999a, Lehmann et al 2000, Nishio et al 2001, Schneider 1992, Weinstock et al 1988).

3.2.3 Effect of prenatal stress on learning abilities of the offspring

Studies on learning abilities of the offspring of stressed mothers have revealed impairments on a number of tasks. Early work has adduced evidence for impairments of discrimination learning (Mednick et al 1994), reversal of a learning set on a T-maze and acquisition of an operant response (Imamura et al 1999) in the offspring of prenatally stressed rats. The results of two recent studies, however, are conflicting. Using crowding combined with one daily painful experience as stressors in Wistar rats; learning acquisition in a water-maze at day 30 did not differ between the prenatal stress and control conditions. In the reversal task, however, prenatally stressed rats spent more time than control animals searching for the platform. After the application of restraint stress in Sprague–Dawley rats in the last week of pregnancy, the cognitive performance of the adult offspring (age 120 days) was tested in the water-maze and using a two-trial memory test in a Y-maze with progressive inter-trial intervals.

Though in this design, the animals showed problems in coping with novelty expressed as an increased escape behavior, spatial learning or memory performance proved not to be affected by prenatal stress. These findings suggest that learning impairments due to prenatal stress may be presented at young rather than at older age in rats, although also other design variance of the studies may explain the discrepancies.

3.2.4 Effects of prenatal stress on modulatory of neurotransmitters

The levels or function of monoamines in the brain have been shown to be affected by mild to severe prenatal stress. Regionally specific alterations in norepinepherine (NE) and dopamine (DA) turnover were observed after PS, including reduced levels of NE in the cortex and locus coeruleus (LC), reduced levels of DA in the LC, but not in the cortex, reduced DA turnover in the striatum and nucleus accumbens and increased DA turnover in the prefrontal cortex (Diorio et al 1993). Dopaminergic function is a frequently investigated endpoint for studies on the effects of prenatal stress exposure. Prenatal stress and early exposure to glucocorticoids have been shown to enhance brain dopaminergic activity and dopamine release (Diaz et al 1995, Henry et al 1995, Piazza et al 1996, Takahashi et al 1992). Prenatal stress exposure also significantly increased dopamine D2 receptor binding, while decreasing D3 dopamine receptor binding in both the shell and core of the rat nucleus accumbens (Henry et al 1995). In addition, a report from the same laboratory revealed that prenatal stress exposure can potentiated sensitization of amphetamine (AMPH) induced locomotor activity in adult male rats (Alonso et al 1994). Prenatal stress has also been reported to abolish the lateralization of the dopaminergic system and this loss of specific left/right distinctions in the brain may have implications in neuropsychiatric diseases, such as schizophrenia (Weinstock 2001).

Administration of the CORT analogue dexamethasone on days 17–19 of gestation, elicited regionally specific changes in levels of monoamines and their metabolites in male offspring (DiPietro et al 1996). Hippocampal and cortical NE were reduced at different time points. DA was reduced in the hypothalamus, but elevated in the striatum. PS induced by crowding and daily saline injections increased plasma tryptophan in the mothers, and increased serotonin (5-HT) in fetal brains and postnatally in the cortex (DiPietro et al 1996). PS also increased the behavioural

‘serotonin syndrome’ response to a 5-HT agonist as late as age 60–70 days (Drago et al 1999a). Since serotonin is implicated in affective and mood disorders in humans, PS-induced modification of brain serotonin may predispose offspring to be more sensitive to stress.

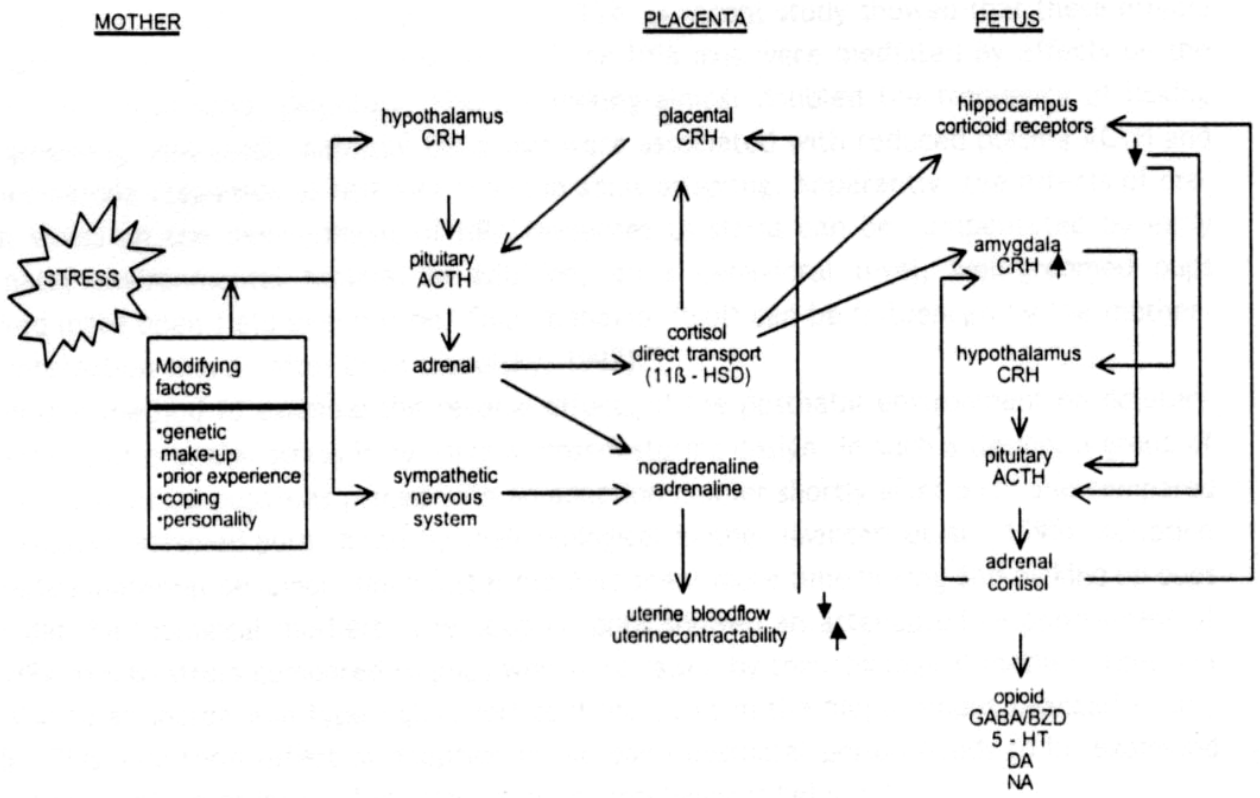

Figure 3.3 Schematic diagram shows how maternal stress hormone can affect the fetal brain development (Weinstock, 1997).

3.3 Prefrontal cortex

The prefrontal cortex (PFC) is the anterior part of the frontal lobes of the brain, lying in front of the motor and premotor areas. Cytoarchitectonically, it is defined by the presence of an internal granular layer IV (in contrast to the agranular premotor cortex). Thus, the prefrontal cortex was considered unique to the primate species and called the 'frontal granular cortex' (Lee et al 1991). It can be divided into the lateral, orbitofrontal and medial prefrontal areas; this brain region has been implicated in planning complex cognitive behaviors, personality expression and moderating correct social behavior. The basic activity of this brain region is considered to be orchestration of thoughts and actions in accordance with internal goals. The most typical neurologic term for functions carried out by the prefrontal cortex area is executive function. Executive function relates to abilities to differentiate among conflicting thoughts, determine good and bad, better and best, same and different, future consequences of current activities, working toward a defined goal, prediction of outcomes, expectation based on actions, and social "control" (the ability to suppress urges that, if not suppressed, could lead to socially unacceptable outcomes). Many authors have indicated an integral link between a person's personality and the functions of the prefrontal cortex. Generally, the cerebral cortex is characterized as having six distinct layers. After migration, neurons form efferents and receive afferent connections characteristic of their layer (Creutzfeldt, 1995). It is interesting to note that, during development, the inner layers are formed before the outer layers. The characteristic of each cortical layer are as follows:

1. The molecular layer, or layer I, contains few scattered neurons and consists mainly of extensions of apical dendrites and horizontally oriented axons, and some Cajal-Retzius and spiny stellate neurons can be found.
2. The external granular layer, or layer II, contains small pyramidal neurons and numerous stellate neurons.
3. he external pyramidal layer, or layer III, contains predominantly small and medium sized pyramidal neurons, as well as non-pyramidal neurons with vertically-oriented intracortical axons. Layer I to III are the main target of

interhemispheric corticocortical afferents, and layer III is the principal source of corticocortical efferents.

4. The internal granular layer, or layer IV, contains different types of stellate and pyramidal neurons, and is the main target of thalamocortical afferents as well as intra-hemispheric corticocortical afferents.
5. The internal pyramidal layer, or layer V, contains large pyramidal neurons (as Betz cells in the primary motor cortex), as well as interneurons, and it is the principal source of efferent for all the motor-related subcortical structures.
6. The multiform layer, or layer VI, contains few large pyramidal neurons and many small spindle-like pyramidal and multiform neurons. The layer VI sends efferent fibers to the thalamus establishing a very precise reciprocal interconnection between the cortex and the thalamus.

The definition of prefrontal cortex at that time was based upon the cytoarchitectonic criterion of having a granular layer IV and a location rostral to the agranular (pre)motor areas. However, comparing different cortical areas in more distantly related species solely on the basis of cytoarchitectonic criteria appeared to be untenable. For example, the primary motor cortex in rats is considered to be homologous to the one in monkeys (Northcutt & Kaas 1995) but this cortical area is agranular in mature primates and granular in rats. (Barbas & Pandya 1989) consider limbic cortices in primate brains, which are agranular and dysgranular (i.e. layer IV is not and is not easily discernible, respectively) as part of the prefrontal cortex. These are just two examples to emphasize why cytoarchitectonic criteria have been replaced by other criteria in seeking homologies between different brain areas in more distantly related species. It is now generally accepted that the following criteria have to be taken into account when discussing homologies between cortical areas in different species: (1) the pattern of specific connections and the relative density of these connections; (2) the functional (i.e. electrophysiological and behavioral) properties; (3) the presence and specific distribution of different neuroactive substances and neurotransmitter receptors; (4) the embryological development; and (5) only for closely related species, the cytoarchitectonic characteristics. The greater the similarities between the characteristics, the more likely it is that brain regions are homologous. In the following

account we will employ the first three criteria for comparing prefrontal cortical areas in primates and rats. These three criteria also were applied (Preuss 1998). From this perspective we will consider the connections of the rat and primate prefrontal cortices with thalamic, basal ganglia, cortical, limbic and monoaminergic structures, with respect to both pattern and density.

3.3.1 Neuronal networks involving the prefrontal cortex

3.3.1.1 Connections between prefrontal cortex and thalamus

Thalamocortical connections are important for cortical differentiation and specialization. The reciprocal connections of the major thalamic nuclei are therefore used to define cerebral cortical areas. At the time of, the mediodorsal nucleus of the thalamus was assumed to be the only nucleus with thalamocortical projections to the prefrontal cortex, and was therefore viewed as the 'defining' nucleus. However, with the advent of more refined anterograde and retrograde tracing techniques, it became apparent that the (prefrontal) cortical areas that receive mediodorsal thalamic input also are connected with other thalamic nuclei. Thalamic nuclei other than the mediodorsal nucleus that reach the prefrontal cortex include the intralaminar and midline nuclei, the anterior medial nucleus and the rostral parts of the ventral complex (Groenewegen et al 1997). In addition, thalamic mediodorsal nucleus projections appear to reach some cortical areas outside the prefrontal cortex, such as the premotor, motor, temporal and parietal cortices, as has been demonstrated in, for example, macaque monkeys, cats, sheep, and dogs (Akert & Hartmann-von Monakow 1980). Among others, (Leonard 1969) regarded the *reciprocity* of the cortical projections of the thalamic mediodorsal nucleus as an important criterion for defining the prefrontal cortex. However, this definition also does not lead to an unambiguous delineation of the prefrontal cortex. Therefore, (Uylings & van Eden 1990) suggested inclusion of only those cortical areas in the prefrontal cortex for which the reciprocal connections with the mediodorsal nucleus (MD) are stronger (i.e. in terms of a higher number of projecting neurons and a higher density of terminals) than the reciprocal connections with other thalamic nuclei. This feature, together with the pattern of cortico-cortical connections, has led us to include the primate and rat anterior cingulate cortex in the prefrontal cortex (Uylings & van Eden 1990). This approach in defining the prefrontal

cortex is strengthened by the recent analysis demonstrated the special, predominant position of the mediodorsal nucleus for the macaque prefrontal cortex on basis of multidimensional scaling analysis of the thalamo-prefrontal-cortical projections. In addition, in rhesus monkey that the majority of the thalamic neurons projecting to the prefrontal cortex (Barbas & Blatt 1995), as it has been defined before, are located in the mediodorsal nucleus. This also goes for the rat prefrontal cortex (Figure. 4) (Groenewegen et al 1997) reported that area 25 in macaques (which is considered to be a prefrontal cortical area receives more thalamic afferents from the thalamic ventral anterior nucleus than from the mediodorsal nucleus. When this is also true for the efferent corticothalamic projections from area 25, then macaque area 25 should probably not be included in the prefrontal cortex (Barbas et al 2002b). A similar situation holds likely for the ventrolateral orbital cortical area (VLO) in the rat (Groenewegen et al 1997).

For a further comparison of monkey and rat thalamic data it is important to know that in non-human primates thalamic ventral nuclei have a differential cortical projection pattern for the prefrontal, premotor and motor cortex, respectively (Jones & Leavitt 1974). The thalamic ventral anterior (VA) and posterior ventromedial (VMp) nuclei project quite diffusely and spread out over an extensive region of neocortex (i.e. premotor, supplementary motor area (SMA), supplementary eye field, anterior cingulate (AC) and posterior parietal cortex), but these projections have a relatively higher concentration in the prefrontal cortex. In contrast, the anterior ventrolateral (VLa) cortical projections are more concise and concentrated in premotor and SMA areas, with additional lesser projections also to the motor area. In addition, the posterior ventrolateral nucleus (VLp) is the principal source of projections to primary motor area 4, but projects also to premotor, SMA, and some to posterior parietal cortex (Jones & Leavitt 1974). In rats the topography and the pattern of connectivity of the mediodorsal nucleus is rather comparable with the one in monkeys (Groenewegen 1988). The VA in rats, however, is usually included in the VL due to its difficult cytoarchitectonic delineation (Uylings & van Eden 1990). In the last decade the inclusion of the rat frontal area 2 (Fr2) and the dorsal anterior cingulate area (ACd) (Figure. 4) in the prefrontal cortex has been disputed (Conde et al 1990, Preuss 1998). The results show that retrograde tracer studies on thalamic afferents (Conde et

al 1990) and cortico-cortical afferents to rat medial frontal cortex were decisive for namely that the rat Fr2 (also called the precentral medial area (PrCm) or agranular medial cortex (Agm),) and the rat Acd are premotor areas and do not belong to the prefrontal cortex. As a consequence they denied the presence of dorsolateral-like prefrontal cortical features in rats described in their retrograde tracer study that only a few neurons from the mediodorsal thalamic nucleus project to Fr2 and ACd and that a higher number of thalamic projecting neurons are positioned in the intralaminar, the ventrolateral (VL) and ventromedial (VM) nuclei (Conde et al 1990) . In this respect, there is conspicuous for illustrating a case with a relatively high number of MD neurons projecting to ACd. Furthermore, several groups have observed in extensive anterograde and retrograde tracing studies that the rat Fr2 and ACd (Figure. 4) have a very dense reciprocal connection with the paralamellar or ventrolateral segment of the thalamic mediodorsal nucleus(Ghashghaei & Barbas 2002). This can also be concluded from the anterograde and retrograde tracer (Reep & Corwin 1999), which were directed especially to the Fr2 (their AGm). Distinguished caudal AGm from mid and rostral AGm, and showed that rostral and mid AGm receives thalamic afferents from a higher number of neurons in the mediodorsal nucleus. It appeared that only the caudal AGm has afferents from cells mainly located in VL and only a few neurons in the mediodorsal nucleus. However, this part of AGm or Fr2 (Zaborszky et al 1997) is not included in the rat prefrontal cortex as defined (Groenewegen 1988, Krettek & Price 1977, Uylings & van Eden 1990), because it is caudal to about －1mm from bregma (see also note below). In addition, (Vertes 2002)] showed that both ACd and Fr2 have strong projections particularly to the MD. Therefore, we conclude that ACd and Fr2 have a more prefrontal than premotor type of thalamic connections. On basis of both thalamic and subcortical and cortical connections we suppose also that the caudal Fr2 and (supragenual) parts of Acd incorporates a zone homologous to the macaque frontal eye field (FEF) (Uylings & van Eden 1990). This is corroborated by electrophysiological studies (Guandalini 2001, Neafsey et al 1986) and by unilateral lesion studies causing multimodal neglect (King & Corwin 1990, Mesulam 1999). Even with the use of modern neuroanatomical tracing techniques, it is rather difficult to determine unequivocally for those cortical regions showing considerable overlap of connections from various thalamic nuclei, which of these thalamic nuclei provides the

strongest connections. For example, comparison of numbers of different types of neurons projecting to a particular cortical area with retrograde tracing implies the assumption that the extent of these axonal terminal fields is similar in the different regions that have been injected with a particular tracer (Uylings 2002). This is not always the case, however It is therefore important to consider in addition the quality of connections (e.g. ‘driver’ and ‘modulator’ type of thalamic connections (Sherman 2001), the cortico-cortical and subcortical neuronal networks and functional properties in which different prefrontal cortical areas have a particular, different position.

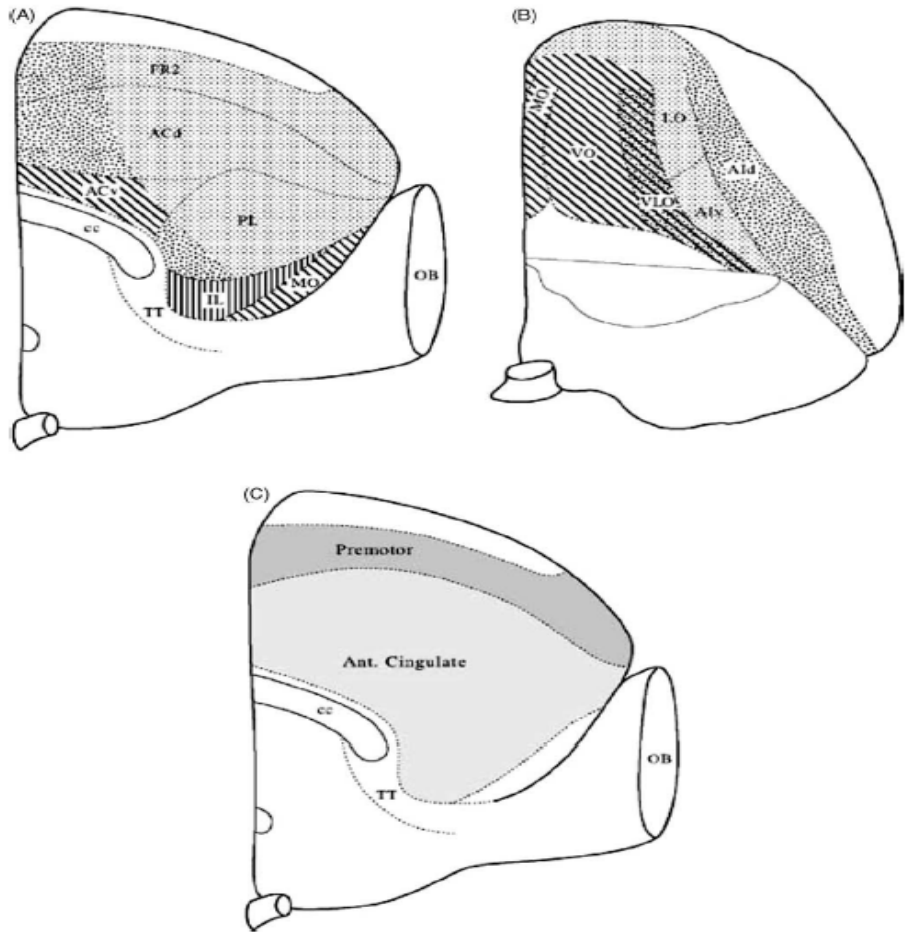

Figure 3.4 The extent of rat's prefrontal cortex. (A, B). The increase in what is considered to be prefrontal cortex due to improvements in anatomical techniques. Fine dots indicate the area described (Leonard 1969), large dots the extension described (Krettek & Price 1977), oblique lines the extension proposed by (Groenewegen 1988) and vertical lines the extension proposed in (Uylings & van Eden 1990). The nomenclature has been followed with the exception of the neutral term frontal area 2 (Fr2), in text. MO and VO are the medial and ventral orbital areas and AI the rat anterior insular area. (C) The view illustrated (Preuss et al 1999), modified (Preuss 1998), in which ACd is now incorporated in the anterior cingulate cortex. In the view of (C) the anterior cingulate (AC), the prelimbic (PL) and the infralimbic (IL) areas of (A) form the prefrontal cortex, while the orbital and lateral prefrontal cortex are conspicuously lacking.

3.3.1.2 Relationships between prefrontal cortex and basal ganglia

The frontal lobe as a whole has a special relationship with the basal ganglia in that it is the part of the cerebral cortex that receives the main input from the basal ganglia, through a relay in the thalamus (Middleton & Strick 2000). Like the frontal cortex, the parietal, occipital and temporal cortices all project to the basal ganglia; in particular the striatum, but these posteriorly located cortices do not receive information back from the basal ganglia (Middleton & Strick 1996). A particular subset of thalamic nuclei (i.e. the mediodorsal, ventromedial, ventral anterior and, to a lesser degree, the ventral lateral nuclei) form the essential link between the pallidum and substantia nigra pars reticulata, as the output structures of the basal ganglia (Alexander et al 1990) and the prefrontal cortex. It is also relevant to emphasize the high degree of topographical organization in the frontal cortical–basal ganglia connections and in the basal ganglia–thalamic projections. This topographical organization forms the basis for the existence of a number of parallel, largely functionally segregated basal ganglia–thalamocortical circuits that have been identified in primates and rats (Alexander et al 1990). In rats, similar circuits have been described, as illustrated in Figure. 5. Further important aspects of the circuitry in which the prefrontal cortex and the basal ganglia are involved concern the specific relationships of the projections from the midline/intralaminar thalamic complex as well as the amygdala and, to a lesser extent the hippocampus, to the cortical and striatal relay stations in the basal ganglia–thalamocortical circuits (Barbas & Blatt 1995). In both rats and primates, the circuits that involve the prefrontal cortical areas are characterized by amygdaloid inputs. However, in rats the dorsal anterior cingulate (ACd) and Fr2 areas receive the least amygdaloid fibers of the prefrontal areas (Groenewegen et al 1997), while also in nonhuman primates the dorsolateral prefrontal cortex has the weakest amygdaloid input in the prefrontal cortex (Amaral & Price 1984). All these connectional aspects point to similarities between prefrontal cortex–basal ganglia–thalamocortical circuits of primates and rats.

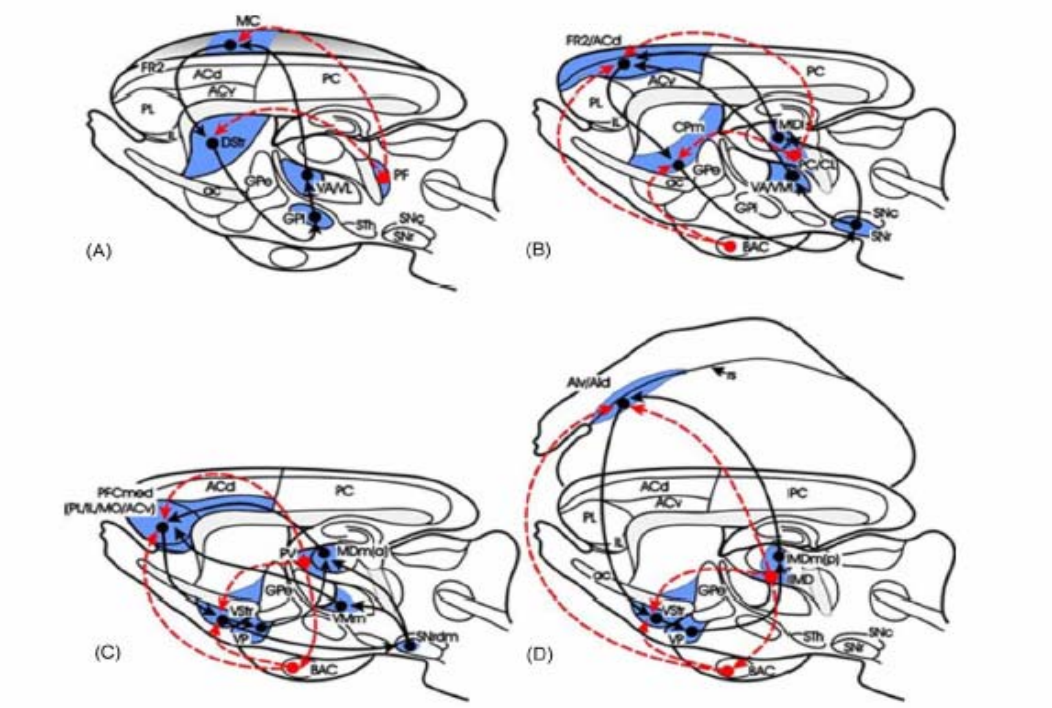

Figure 3.5 The involvement of different parts of the prefrontal cortex in various basal ganglia–thalamocortical circuits and amygdaloid and midline/intralaminar thalamic circuits in the rats. Four basal ganglia–thalamocortical circuits are depicted with similar organizational characteristics. All four circuits involve different parts of the prefrontal cortex, the dorsal or ventral striatum, the pallidal and nigral complex and the ventral and medial thalamic nuclei. (A) 'Motor circuit': note the projections from the parafascicular thalamic nucleus to the motor cortex and the dorsal striatum, together with the absence of the amygdala connections. (B) 'Dorsal shoulder prefrontal' circuit (Fr2 and ACd): the cortical and striatal way stations of this circuit receive specific projections from the intralaminar thalamic complex (PC/CL) and the anterior part of the basal amygdaloid complex. (C) 'Medial prefrontal/anterior cingulate' circuit (PL/IL/MO/ACv): the paraventricular thalamic nucleus and the posterior part of the basal amygdaloid complex are strongly involved in this particular circuit. (D) 'Lateral prefrontal/agranular insular' circuit (AIv/AId): the intermediodorsal thalamic nucleus and the anterior part of the basal amygdaloid complex project to the cortical and striatal way stations of this circuit.

3.3.1.3 Cortico-cortical networks

Both the thalamocortical and the cortico-cortical connections with in the rat and primate prefrontal cortex are predominantly ipsilateral (Akert & Hartmann-von Monakow 1980). The majority of terminal axons in the prefrontal cortex are cortical afferent (Pandya & Yeterian 1990). In both rats and primates, the prefrontal cortex is extensively connected with different cortical areas such as premotor, somatosensory, auditory, visual, olfactory, gustatory and limbic cortical area (Pandya & Yeterian 1990). In macaques, the connections between the prefrontal and other cortical areas are reciprocal and that such connections exist preferentially between cortical areas with a similar level of cytoarchitectonic differentiation. In addition, the cytoarchitectonically less differentiated prefrontal regions have fewer specific cortico-cortical connections (Barbas et al 2002a). They receive projections from two or more cortical areas representing different sensory modalities together with a substantial input from limbic cortices. The cytoarchitectonically more highly differentiated (thus eulaminate or strongly granular) prefrontal areas receive more specific projections representing only one or two different modalities, and relatively few projections from limbic cortices. In primates, in particular the macaque, the dorsolateral prefrontal cortex has extensive cortico-cortical connections. The rat data currently available do not contradict this "hierarchical" theory, more information is required before definitive conclusions can be drawn. As mentioned above the retrograde tracer study on cortico-cortical afferents has strengthened that the rat Fr2 and ACd is largely a premotor cortex and do not belong to the prefrontal cortex (Conde et al 1995). By excluding this 'dorsal shoulder' region from the rat prefrontal cortex, they arrived to their opinion that the prefrontal cortex in rats lack features of the primate dorsolateral prefrontal cortex. A typical feature of the macaque prefrontal cortical areas is, as noted above, however, the property of a multimodal association area in a hierarchical organized cortex and the feature of a nodal station in several distributed parallel networks (Cavada & Goldman-Rakic 1989). Like the macaque prefrontal cortex, the rat prefrontal cortex receives multimodal cortico-cortical projections from motor, somatosensory, visual, auditory, gustatory, and limbic cortices in such a way that the rat prefrontal cortex appears to be a nodal station embedded in several parallel

networks (Van Eden & Uylings 1985). Moreover, Fr2 and ACd received more projections from somatosensory and associational visual cortices than from the primary motor cortex. The macaque prefrontal cortex has the strongest connections with posterior parietal and temporal association areas (Cordova et al 2002a, Cordova et al 2002b). Rat equivalents are not well described for these parietal and temporal association areas (Van Eden & Uylings 1985). The data available indicate extensive, reciprocal connections with premotor, motor, somatosensory, visual, auditory and paralimbic cortical regions. The reciprocal cortico-cortical connections reveal at least three subfields in the rat medial prefrontal cortex, i.e. a 'dorsal shoulder' region (Fr2 and ACd); a rostral part of the medial prefrontal cortex; and the prelimbic and infralimbic cortices. The architectonically less differentiated areas in the rat, the infralimbic and prelimbic cortices and the lateral prefrontal cortex (i.e. agranular insular cortices), have reciprocal connections with the perirhinal and entorhinal cortex, and with the CA1 field and subiculum of the hippocampal formation (Groenewegen et al 1997). The rostral part of the rat medial prefrontal cortex has reciprocal connections with motor, mixed somatosensory-motor, and somatosensory association cortices. Thus, premotor characteristics appear to coincide here with prefrontal characteristics (Uylings & van Eden 1990). The 'dorsal shoulder' region has reciprocal connections mainly with visual cortices and the retrosplenial cortex. As mentioned above the caudal part of the dorsal shoulder region appears to incorporate features of the frontal eye field. It is of interest to note that views the interactions of prefrontal with other cortices in the context of 'perception–action cycle' (Fuster 1997). This is a hierarchical concept too. The prefrontal cortex and other cortices are functionally connected for as long as the behavior contains novelty, uncertainty or ambiguity, and has to span time intervals with short-term or working memory. These functional connections disappear or weaken when the action becomes automatic. The action is then integrated in lower brain structures.

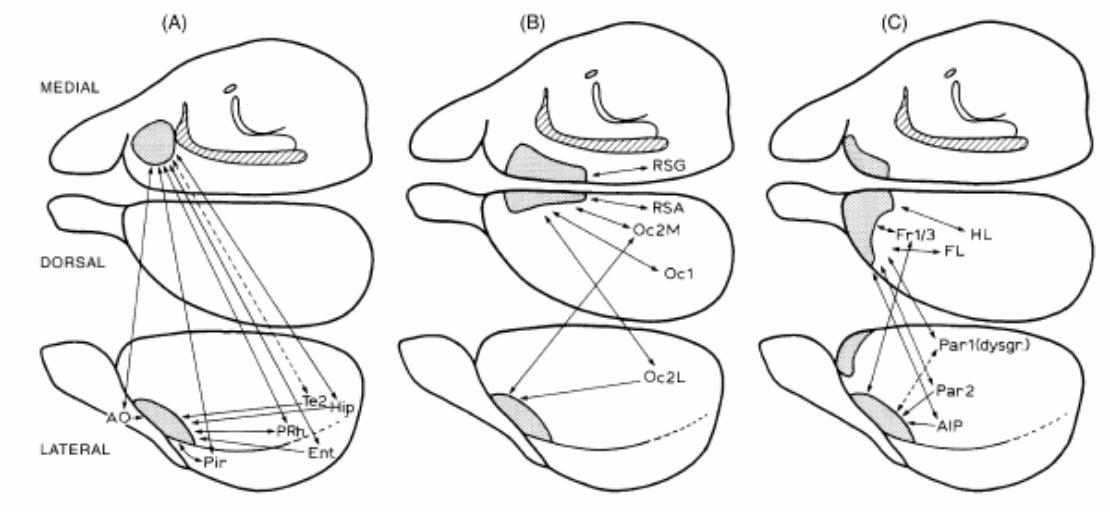

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Figure 3.6 A diagram show the cortico-cortical connections in the rat prefrontal cortex (modified from Uylings & van Eden 1990).

3.3.1.4 Prefrontal gating of cholinergic and monoaminergic systems

Data on the cholinergic and monoaminergic transmitter systems together with prefrontal cortico-cortical connections in primates and rats also show the unique gating position of the prefrontal cortex. In both rats and primates the entire neocortex receives cholinergic innervation from the basal forebrain nuclei. The prefrontal cortex, in addition, gets cholinergic fibers from the laterodorsal tegmental nucleus. Likewise in both species, the prefrontal cortex (in primates mainly the orbital and medial PFC and in rats mainly the medial PFC) is the only cortical region that has direct projections back to these basal forebrain and brainstem nuclei (Gaykema et al 1991). The noradrenergic fibers from the locus coeruleus and the serotonergic fibers from the dorsal and median raphe nuclei are widely distributed over almost the entire neocortex. Also with respect to these transmitter systems in both rats and primates certain prefrontal areas are the only cortical areas that project back to the locus coeruleus and to the dorsal and median raphe nuclei. In primates the cortical projections to the noradrenergic locus coeruleus and the serotonergic raphe nuclei derive from the dorsolateral PFC (Arnsten & Goldman-Rakic 1984). In rats the cortical projections to the locus coeruleus derive from the medial PFC and the agranular insular PFC areas, while the cortical projections to the dorsal and median raphe nuclei are from the medial PFC, especially its ventral part (Hajos et al 1998). In rats, the cortical terminal fields of dopaminergic fibers are mainly restricted to the prefrontal areas and the entorhinal cortex, whereas only the prefrontal cortex (both medial and agranular insular PFC) projects back to the dopaminergic neurons in ventral tegmental area (VTA or A10) (Carr & Sesack 2000) and the dopaminergic pars compacta of the substantia nigra (A9). The rat prefrontal cortex receives distinct parallel dopaminergic inputs. The supragenual anterior cingulated cortex receives dopaminergic fibers in the superficial layers from the pars compacta of the substantia nigra and in the deeper layers from the lateral part of the ventral tegmental area (Lewis 1997). The ventral tegmental area is the origin of the major dopaminergic input in the rat prefrontal cortex and different parts of the ventral tegmental area appear to project to different prefrontal areas (Kalsbeek et al 1987). Particularly in primates, cortical dopaminergic fibers appear not to be restricted to the prefrontal cortex. As suggested

this appears to be caused mainly by extension of dopaminergic midbrain cellular groups such as the retrorubral areas A8 and A9 (Williams & Goldman-Rakic 1998). Direct recurrent projections from the primate dorsolateral prefrontal cortex to the medial substantia nigra pars compacta (A9) have been reported, but they can also be expected to project to the ventral tegmental area on the basis of the above (Lewis 1997).

3.3.2. Functional characteristics of prefrontal areas

In rats, prefrontal cortex the prelimbic, infralimbic, and dorsal anterior cingulated areas are the major subdivisions of the PFC (Groenewegen 1988, Krettek & Price 1977). Functional studies suggest that the PFC, via its integration into the neural network of the basal ganglia, participates in the organization and planning of goal-directed tasks (Fuster 1997). A key mechanism involved is a form of short-term memory called the working memory, which serves as a workspace for holding momentarily an item of information and using it subsequently to guide correct responses (Fuster 1997). Excitatory recurrent neural circuits in the PFC are believed to form the cellular basis for the working memory (Alexander & Crutcher 1990). As expected, dysfunction of the PFC has been implicated in several mental illnesses, particularly schizophrenia. Deficiency in the working memory process in the PFC has been associated with the symptoms and cognitive deficits that are prominent of schizophrenia (Weinberger 1987). Although the causes for such malfunction may be complex, many studies suggest abnormalities that occur during early postnatal development (Jones & Leavitt 1974, Lewis 1997). Electrical activities play important roles in developmental processes including neuronal differentiation, cell migration, formation, and refinement of synaptic connections (Lemaire et al 2000b). Two broad mechanisms control the activity of any given neuron. The first is intrinsic membrane properties of the neuron, which are determined by its morphology and the level and distribution of various ion channels (Conde et al 1990). The second type of mechanisms consists of interactions with other cells, in particular synaptic transmission between neurons.

The major afferent innervations in prefrontal cortex mostly from thalamus and its project to the middle layer of prefrontal cortex and other innervations are dopaminergic system, serotonergic system and from the inner layer of cortex. The distribution of cortical DA fibers has been described in rodents as well as in human and nonhuman primates,using several histochemical techniques (Berger et al 2002, Lewis 1997). At the ultrastructural level, DA terminals synapse on the dendritic tree of both pyramidal cells and local circuit neurons (Gorelova & Yang 1997). Recent evidence suggests that DA terminals selectively synapse on specific classes of local circuit neurons, as categorized by their content of calcium-binding proteins (Silva-Gomez et al 2003). However, the subpopulations of pyramidal cells that are synaptically innervated by DA have not been determined. One of the principal efferent of the PFC is its projection to the nucleus accumbens (NAc) (Silva-Gomez et al 2003). The PFC accumbens projection is part of a multisynaptic processing loop that also involves the ventral pallidum and mediodorsal thalamus (Alexander & Crutcher 1990, Groenewegen 1988). This circuit plays a role in motivated and cognitive behaviors and has been implicated in the etiology of schizophrenia and other neuropsychiatric disorders (Weinberger 1987). Anatomical and electrophysiological studies have demonstrated that PFC input to spiny projection neurons in the NAc is subject to modulation by mesolimbic DA afferents (Harvey et al 2004). Whether DA afferents also modulate PFC-accumbens transmission by synapsing on PFC neurons that project to the NAc is not known. However several lines of evidence suggest that such synapses may occur. The projection from the PFC to the Nac arises from pyramidal cells located primarily in layer V (Piazza et al 1991), which, along with layer VI, contains the highest concentration of DA terminals in the rat PFC (Lindefors et al 1997). Electrophysiological studies also show that PFC pyramidal cells projecting to the NAc respond to applied DA or stimulation of the ventral tegmental area (VTA) (Glenthoj et al 1999). Furthermore, mRNA for D1 and D2 DA receptors has been localized in pyramidal neurons retrogradely labeled from the striatum (Gaspar et al 1992). However, DA may exert at least some of its actions via local circuit neurons or through extrasynaptic mechanisms making it unclear whether PFC-accumbens neurons receive direct synaptic contacts from DA terminals (Singh et al 1997).

XH-8-OH-DPAT binding sites are abundant in the cerebral cortex which is innervated by two 5-HT projection systems (Meijer et al 1997). In the prefrontal cortex, the overall distribution of binding sites matches that of sH-5-HT binding sites in rhesus monkeys (Jang et al 2004). In the posterior cingulate cortex, a prominent 'H-8-OH-DPAT labeling is observed in layers V and VI, which contain relatively low numbers of serotonergic fibers (Meijer et al 1997). The labeling extends into the retrosplenial cortex thus covering isocortical and allocortical. Also in the striate and the whole occipital cortex, layers V and VI are labeled (Zilles et al 1985).

3.4 Development of the Cerebral Cortex

The structural organization of the fetal brain differs substantially from the mature brain and it is continuously changing. The fetal brain displays conspicuous zones that are unique to the developing brain and have no direct counterparts in the adult. During fetal development, the cerebral gallium shows a typical pattern of lamination consisting of the following zones (from ventricle to gallium): ventricular zone, sub ventricular zone (second prolifertive zone), intermediate zone (future white matter), subplate, cortical plate and marginal zone (Lewis 1997). The fetal layers reflect the transient arrangements of fibers, synapses, and nerve cells. The content of each zone is permanently changing. The entire fetal development represents a period of great dynamics (Lewis 1997).

Migration of recently proliferated cells from the ventricular zone and other germinal layers occurs radially in the medial/dorsal neocortex and tangentially in other regions of the forebrain such as the olfactory bulb and lateral neocortex (Divac 1971). In the neocortex, for example, the mature cortical plate has histologically distinct laminae, classified as layers I through VI, and a subplate (layer VII). Layer VI is the deepest layer of the cortical plate; i.e., it is closest to the geometric center of the brain. Layer I is the most superficial layer; it is the outward-most subpial layer. The formation of layers VII and I is distinct from the cell migrations that populate layers VI through II (Marin-Padilla 1971). Cells from the ventricular zone populate layers VI-II in an inside-out fashion, meaning that the deeper layers form first (Ignacio et al 1995). On GD14, a four-layered cortex appeared in the dorsal region but not in the lateral cortex. By GD 16 and 17 in the rat, the first cells are arriving in the area that

will ultimately form the laminae of the cortical plate. Through the remainder of gestation, the cortical plate gets thicker as more cells migrate from the ventricular zone. In the days before parturition, layer VI is histologically distinct from the remainder of the maturing cortical plate. On postnatal day (PND) 5 of the rat, layers VI and V are readily distinguishable, but layers IV-II are not as clearly defined. These superficial layers (IV-II) are more distinct in adulthood but never constitutes more than one-third of the cortical width. The remaining two-thirds of the cortical width are split approximately equally between layers VI and V, depending on the neocortical region of study (Ignacio et al 1995). The first phase (from day 1 until day 18) is dominated by differentiation of the neurons within the cortical plate and by the formation of the cortical layers. At day 1, regional differences are observed in the cytoarchitecture of the cortical plate which correspond to the future subareas of the prefrontal cortex. The formation of layer IV occurs in the dosolateral cortex around PND 6, and from this age the agranular prefrontal cortex is well demarcated from the other pert of the prefrontal cortex. Between PND 6 and PND 10, the cortical plate has disappeared and all cortical layers can be recognized in the prefrontal cortex. In addition to the migration of neurons to the cortical plate, other factors modify the lamination of the neocortex including cell packing density, cell size, extra cellular matrix, gliogenesis, myelination, and synaptogenesis of cortical afferents/efferents, all of which contribute to the final differentiation of this neural structure. In general, these cortical areas are homologous between species, with notable differences in the relative size of structures, cell number, and extent of extra cellular neuropil. It is important to note that when proliferation is disrupted, migration is often also affected.

There are various factors that involved in brain development such as vimintin, laminins and GAP-43. One of the molecules that has been implicated in brain development is the growth-associated protein, GAP-43. We are interested GAP-43 because it is important to define marker molecules whose expression could correlate with particular developmental events. The diversity of experimental contexts in which GAP-43 was discovered offers the first clue that this protein might provide a link between events that occured during neural development and activity-dependent changes in the mature brain (Ehrlich et al 1974). This protein was subsequently shown

to be a major presynaptic substrate of PKC (Aloyo et al 1983) and to undergo a persistent change in phosphorylation during long-term potentiation. The involvement of GAP-43 in PKC casade and phosphorylation plays a role to understand the mechanisms that regulated the axonal growth in prefrontal cortex during development.

3.5 Growth associated proteins-43

The growth-associated proteins (GAPs) are thought to play important roles in axon growth. It is well accepted that one membrane phosphoprotein, GAP-43 (also referred to as B-50, F-l, pp46, GAP-48 or neuromodulin) is the best characterized GAP. The many different designations of this protein may reflect the potentially diverse functions within the nervous system, including neuronal development and regeneration (Skene 1989), synaptic modulation, and long term potentiation. GAP-43 is expressed at high levels in developing neurons, transported anterogradely along the axon, and accumulated in growth cones. Accumulating evidence suggests that the role of GAP-43 is not limited to the peripheral nervous system as was originally proposed (Benowitz et al 1989), but is also involved in sophisticated crucial roles in the central nervous system. Intracellularly, it associates with the plasma membrane through two palmitate residues covalently attached to cysteines 3 and 4. Thus, most GAP-43 is extracted in membrane-enriched fractions, although smaller amounts can be extracted in soluble form or associated with the actin-rich membrane skeleton. GAP-43 is a multiphosphorylated protein. Among its several phosphorylation sites, Ser41 is the best characterized. It is phosphorylated by protein kinase C (PKC) and is located in the IQ domain, a region of the molecule involved in GAP-43 interaction with calmodulin (CaM). This interaction is prevented by Ser41 phosphorylation and stabilized at submicromolar calcium concentrations.

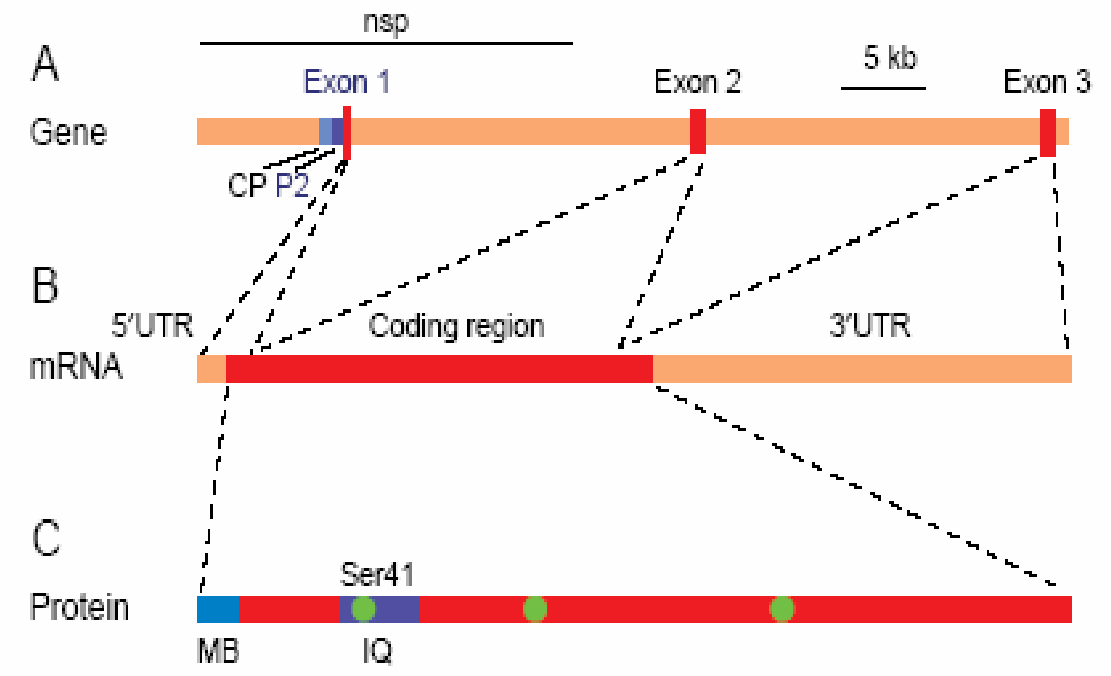

Figure 3.7 The GAP-43 gene, mRNA and protein.

(A) The GAP-43 gene spans at least 50 kb. The three exons that encode the mature mRNA are shown in red. Regions implicated in directing neuron-specific expression include P2, a 386 bp 'core promoter' (CP), and the more extensive region designated as the neuron-specific promoter, nsp, which includes sequences within the first intron. Potential CAATT and TATAA boxes, along with consensus sequences at which several known transcription factors might bind, lie upstream of CP. **(B)** Most GAP-43 mRNA transcripts contain a 5' untranslated region (5 UTR) of 50–55 nucleotides, a coding region of 681 bases (red), and a 600–700 nucleotide 3'UTR. Longer transcripts with several hundred more bases in the 5 UTR also exist. **(C)** The rat protein is 226 amino acids in length and contains an N-terminus membrane-binding (MB) region, the calmodulin-binding IQ domain, and several phosphorylation sites, including serine 41 (Ser41), whose phosphorylation by protein kinase C regulates calmodulin binding. (Fukura et al 1996)

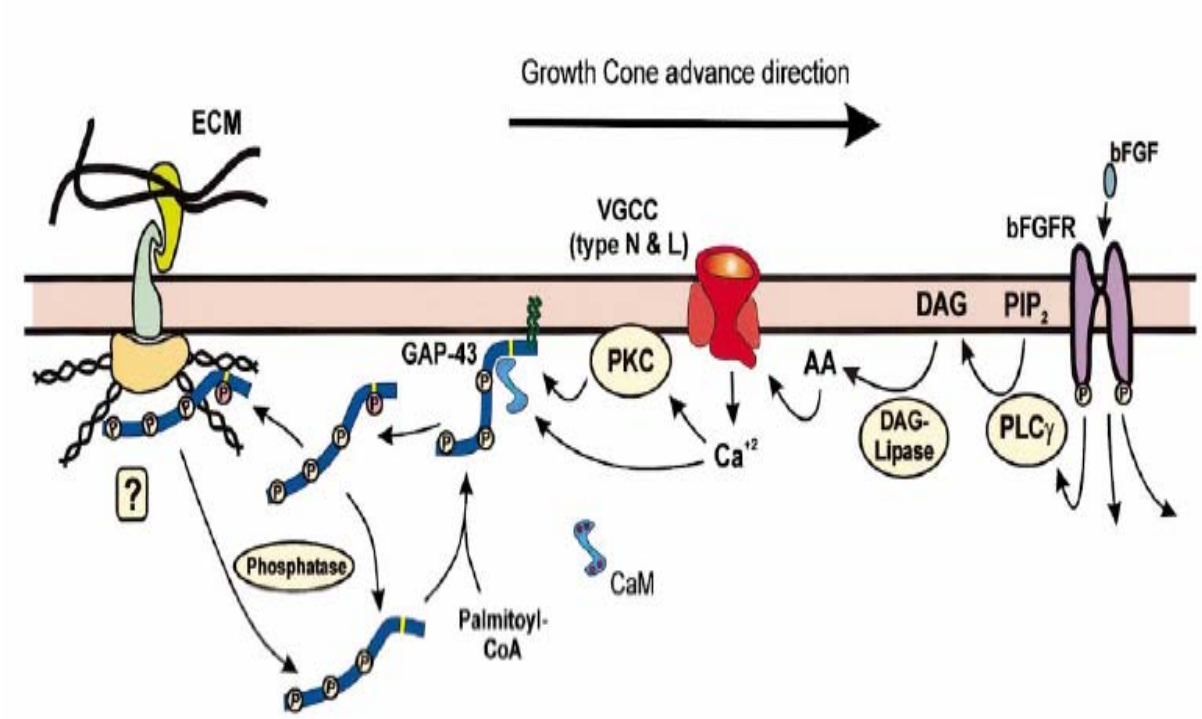

Figure 3.8 Model proposed for GAP-43 functionality in bFGF-induced neurite outgrowth. GAP-43 associates with the plasma membrane through two acylated palmitic residues and also its nonphosphorylated IQ domain. FGF-R activation triggers the sequential activation of phospholipase Cg (PLCg) and diacylglycerol lipase (DAG lipase) and the release of arachidonic acid (AA), which, in turn, activates voltage-gated calcium channels (VGCC). Local increases in submembrane calcium concentrations promote the release of calmodulin (CaM) from GAP-43 and contribute to stimulation of PKC activity, which phosphorylates membrane-associated GAP-43 at Ser41. Phosphorylation at the IQ domain diminishes GAP-43 affinity for membranes and facilitates its translocation to the cytosol or cytoskeleton. In the cytosol, GAP-43 is quickly dephosphorylated and returned to the plasma membrane. However, in the cytoskeleton, GAP-43 remains phosphorylated and are trapped in newly formed adhesion complexes. Association of phosphorylated GAP-43 to such complexes contributes to its stabilization and facilitates neurite outgrowth (ECM, extracellular matrix; PIP2, phosphatidylinositol 4, 5-bisphosphate.) (Widmer & Caroni 1993).

3.5.1 GAP-43 and membrane linkage and targeting

GAP-43 is tightly attached to the cytoplasmic face of the nerve-terminal membrane (LaBate & Skene 1989) through a hydrophobic sequence in its N-terminus (Liu et al 1991, Strittmatter et al 1991). Cysteines 3 and 4 are crucial for the membrane association. These cysteines can undergo reversible, covalent addition of palmitic acid (Skene 1989), and this has been proposed as being essential for targeting GAP-43 to the growth cone membrane (Sudo et al 1992). However, other studies have found little palmitate incorporation, and a mass-spectroscopic study has suggested that the two cysteines are linked to each other through a disulfide bond. A further argument against acylation being the mechanism for membrane linkage comes from the observation that GAP-43 can be dissociated from the membrane using reducing agents at concentrations insufficient to disrupt thioester linkages. GAP-43 has also been reported to undergo ADP ribosylation (Paudel et al 1993). GAP-43 first becomes associated with the membrane compartment of the neuron in the *trans*-Golgi (Palacios et al 1994), and is then transported rapidly down the axon on membranous vesicles at a rate of 400 mm/day (Skene 1989). Initially, this transport occurs into each process of the cell, but with the establishment of cellular polarity, the protein becomes transported selectively down the axon (Goslin et al 1990, Van Lookeren Campagne et al 1992). *In vivo*, GAP-43 is found almost exclusively in presynaptic terminals (Benowitz et al 1989), with only very low levels observed occasionally in dendrites.

Several studies suggest that GAP-43 might influence the growth state of the presynaptic terminal through an association with the membrane skeleton, the network of proteins on the inner surface of the plasma membrane that governs its shape, motility and pathway guidance. In growth cones, a sizable portion of the total GAP-43 pool remains insoluble in the presence of anionic detergents, and is associated with the cytoskeletal proteins actin, □-actinin, talin and fodrin (brain spectrin) (Benowitz et al 1989). *In vitro* binding studies likewise point to an association between GAP-43 and actin and with brain spectrin. Further evidence for such an interaction comes from studies showing that the changes in cell shape that result from transfection of an exogenous *GAP-43* gene are associated with the formation of F-actin, with which GAP-43 co-localizes.

3.5.2 Phosphorylation and dephosphorylation of GAP-43

The trimeric G proteins Go and Gi are abundant in growth-cone membranes (Strittmatter et al 1991), and stimulation of these by neurotransmitters, cell–cell contact and pharmacological agents affects growth-cone motility (Igarashi et al 1995). *In vitro*, GAP-43 stimulates GDP–GTP exchange in the alpha subunits of Go and Gi, and this effect can be mimicked by a GAP-43 N-terminus peptide (Strittmatter et al 1991). In addition, several studies have suggested that GAP-43 or peptides representing fragments of its N-terminus can influence G-protein activity *in vivo* (Strittmatter et al 1994). GAP-43 has been proposed to modulate the set point of G proteins, influencing their responsiveness to other intra- and extracellular signals (Strittmatter et al 1991). However, activation of G proteins generally inhibits growth-cone motility (Strittmatter et al 1994), raising the question of how stimulation by GAP-43 could enhance growth. Palmitoylation of GAP-43 on cysteines 3 and 4 blocks G-protein stimulation (Sudo et al 1992), and one proposal has been that most of the GAP-43 at the nerve terminal is inactive by virtue of this post-translational modification, with only a small deacylated pool being available to modulate G-protein activity and dampen growth at any given moment. However, the fact that increasing levels of GAP-43 clearly augment growth and that most of the protein is deacylated, raise problems for this model.

Although GAP-43 has long been known to be one of the principal substrates of PKC in neurons, the relationship of its phosphorylation state to growth is only now becoming apparent. PKC phosphorylates GAP-43 on Ser 41, and recent studies have examined the consequences of mutating this site, substituting either a charged amino acid for ser 41 to mimic constitutive phosphorylation, or an uncharged amino acid to mimic the dephosphorylated state of the protein. Transgenic mice that overexpressing the pseudo-phosphorylated protein show the same exuberant projections in the hippocampus and enhanced sprouting at the neuromuscular junction; by contrast, transgenic animals overexpressing the dephosphosphorylated form of the protein show much less sprouting. Parallel results have been obtained in non-neuronal cells transfected with similar GAP-43 mutations (Benowitz et al 1989). Transfecting the pseudo-phosphorylated form of the protein induced striking changes in filopodial

outgrowth, adhesion and actin reorganization, whereas transfecting the Ala 41 mutant had less-dramatic effects. Hence, phosphorylating GAP-43 on Ser 41 appears to put cells in a state associated with growth and sprouting. In addition to Ser 41, there might be sites in GAP-43 that are phosphorylated by other kinases. After radiolabeling cells in culture, one study, using phosphopeptide analysis, reported that Ser96 and Thr172 were phosphorylated independently of PKC activation. However, a tandem mass-spectrometry study indicated that Thr87 and Ser152, but not the former two sites, are phosphorylated *in vivo*. Thr87, along with Ser192, can be phosphorylated by casein kinase II *in vitro*, although the significance of phosphorylation at these other sites is presently unknown. Equally important for understanding the regulation of GAP-43 activity are the pathways involved in its dephosphorylation. Ser 41 can be dephosphorylated by Ca^{2+} independent phosphatases that are associated with the membrane (types 1, 2A) and by the soluble, Ca^{2+} and calmodulin-dependent phosphatase, calcineurin. *In vivo*, there might be complex interactions among these phosphatases. The activity of calcineurin is inhibited by the immunophilins, a group of proteins that mediate many of the actions of immunosuppressant drugs and which are abundant in the nervous system. In neurons, the immunosuppressant drug, FK506, strongly enhances the phosphorylation of GAP-43 and augments axonal outgrowth, though it remains to be established whether these two observations are causally linked.

3.5.3 Roles of GAP-43 in activity-dependent synaptic plasticity

Activation or inhibition of PKC has profound effects on synaptic function, including activity dependent changes in synaptic efficacy (Benowitz et al 1989). The earliest indication that GAP-43 might be one of the PKC substrates mediating these effects came from studies showing that its phosphorylation increased with the induction of LTP in the rat hippocampus and correlated with the magnitude and duration of LTP induced plasticity. The relationship between phosphorylation of GAP-43 and LTP was strengthened by the finding that NMDA-receptor antagonist, D -2-amino-5-phosphonovalerate (APV), which blocks postsynaptic events that trigger LTP, prevented the increase in phosphorylation. However, these studies were based upon *in vitro* phosphorylation assays carried out after LTP had been induced *in vivo*,

leaving the interpretation of the results uncertain. This problem has since been addressed using two different methods to evaluate changes in protein phosphorylation *in vivo*. In one of these studies, inorganic $^{[32p]}$ PO_4 was used to pre-label intracellular ATP pools in hippocampal slices prior to inducing LTP. The levels of $^{[32p]}$ PO_4 incorporation into GAP-43 were found to correlate strongly with the degree of synaptic enhancement; increased phosphorylation became apparent ten minutes after induction, the earliest time point examined, and persisted for about one hour. A similar conclusion was reached in another study, which detected an increase in *in vivo* GAP-43 phosphorylation after LTP, but not after control stimulation, using a monoclonal antibody that reacts selectively with the phosphorylated form of GAP-43 (by virtue of recognizing an epitope that includes phosphoserine 41). As the antibody recognizes only the region of the protein phosphorylated by PKC, these findings provide further evidence that PKC is the phosphotransferase involved. Moreover, in both approaches, the increase in GAP-43 phosphorylation was blocked by applying APV prior to LTP induction. Thus, these studies reinforce the idea that the changes in GAP-43 phosphorylation depend upon an initial postsynaptic induction step that requires NMDA-receptor activation and, by inference, the subsequent release of a retrograde signal across the synapse to regulate presynaptic GAP-43.

Increases in GAP-43 phosphorylation might affect neurotransmitter release. Membrane depolarization and other manipulations that induce transmitter release are accompanied by changes in the phosphorylation of GAP-43; in addition, anti-GAP-43 antibodies that block phosphorylation at Ser 41 inhibit transmitter release in permeabilized synaptosomes. Because phosphorylation of GAP-43 is also associated with the growth state of the nerve terminal, it is possible that the phosphorylation changes that occur with LTP might help bring about both short-lived changes in transmitter release and long-lasting changes in synaptic structure. Of the multiple PKC isoforms found in the nervous system, PKC β shows a pattern of activation and expression consistent with phosphorylating GAP-43 *in vivo.* Oleic and arachidonic acids activate the beta and gamma isoforms of PKC selectively and stimulate LTP. In synaptic membranes, these *cis* unsaturated fatty acids stimulate the phosphorylation of GAP-43 selectively. *In vitro*, the βII isoform of PKC optimally phosphorylates GAP-

43 and colocalizes with it in the presynaptic terminal; PKC γ is found primarily in postsynaptic elements and phosphorylates GAP-43 poorly. Nevertheless, LTP in Schaffer collaterals also correlates with a translocation of PKC γ , and LTP induction in the perforant pathway induces parallel change in gene expression for GAP-43 and PKC γ. Other evidence has implicated the epsilon105 and zeta106 isoforms of PKC in LTP, presumably by regulating the phosphorylation of pre- and postsynaptic substrates other than GAP-43. Finally, PKC ε is also capable of phosphorylating GAP-43 *in vitro*; however, it is present in high concentrations in the granule cells of the dentate gyrus, where GAP-43 is not expressed, and is at low levels in CA3 neurons, where GAP-43 is abundant.

The evidence reviewed here shows that GAP-43 undergoes PKC-mediated phosphorylation in response to various signals impinging on the nerve ending and, in this state, influences the pattern of nerve-terminal outgrowth during development and in select sites of the mature brain (Benowitz et al 1989). Phosphorylation is normally transient in response to signals that depolarize the membrane, but in the case of LTP, a signal generated in the postsynaptic cell traverses the synapse, perhaps to synergize with other effectors (for example, DAG or Ca^{2+}) to bring about a more persistent phosphorylation. Phosphorylation on Ser 41 in turn causes CaM to dissociate from the protein, perhaps allowing GAP-43 to influence the organization of the cytoskeleton (Figure. 10) Phosphorylation does not appear to influence the interaction of GAP-43 with G proteins, another route through which it might regulate the nerve ending.

3.5.4 Roles of GAP-43 in axonal growth and regeneration

Although neurons in the PNS and CNS differ greatly in their capacity to regenerate axons after injury, the relationship of GAP-43 expression to the neuron's growth state is essentially similar in the two. In the rat PNS, *GAP-43* mRNA becomes detectable in the principal sensory neurons of dorsal-root ganglia after their final cell division around embryonic day 10 and within a day or so, high concentrations of the protein appear along the entire length of the axons and their terminals. High levels persist until PND 4 or so, through which time GAP-43 immunohistochemistry allows for a detailed visualization of axonal outgrowth and branching in the periphery. Levels

of the mRNA and protein then decline rapidly and become undetectable in the large neurons of the mature ganglion, though modest levels persist in small nociceptive neurons. The upregulation of GAP-43 seen during early development is recapitulated during the regrowth of injured peripheral nerves (Benowitz et al 1989). As regeneration proceeds, the principal sensory neurons transport GAP-43 down both the peripheral nerve and the dorsal roots. Transport down the dorsal roots causes high levels of GAP-43 to appear in newly developed growth cones in the dorsal horn of the spinal cord, and as a consequence of this transition to the growth mode, central projections become reorganized. This is not likely to be beneficial, however, because fibers that normally convey tactile information now form synapses in laminae devoted to pain perception. This mechanism might contribute to the neuropathic pain suffered by people who have had peripheral-nerve injury. In the CNS, GAP-43 is likewise first detected after neurons have undergone their final cell division. In the rat neocortex and hippocampal formation highest levels of expression occur during the first two postnatal weeks, coincident with the period of axon terminal branching, synapse formation and, in some instances, the known peak of LTP-induced plasticity. In the cat striate cortex, highest levels occur during the critical period for activity-dependent plasticity in synaptic organization (Skene 1989). Although levels of the mRNA and protein decline sharply after birth in most regions of the brain, certain populations of neurons continue to express high levels constitutively. Regional variations in GAP-43 levels are particularly striking in the adult neocortex of both human and non-human primates, where the mRNA and protein are abundant in limbic and associative regions, but present at only low levels in primary sensory and motor areas. This distribution pattern suggests a role for GAP-43 in allowing specific neuronal populations to continue to undergo functional or structural changes related to information storage. Although neurons of the mature CNS are normally unable to regenerate damaged axons over long distances, this can occur if neurons are presented with the permissive environment of a peripheral-nerve graft and are accompanied by a massive upregulation of GAP-43 expression. In addition, local reorganization of synaptic connections can occur spontaneously in the CNS after injury. Lesions of the perforant pathway stimulate intact neurons to sprout collateral branches that reinnervate the

newly available target regions, and this sprouting is accompanied by increased levels of GAP-43. A similar induction of GAP-43 is produced with kainate treatment in parallel with the sprouting of mossy-fiber terminals. Blockade of seizures by the NMDA receptor antagonist MK801, blocks both the induction of *GAP-43* mRNA and sprouting, thus strengthening the link between the two.

The neurons that continue to express high levels of GAP-43 in the adult brain retain a capacity to undergo synaptic reorganization in response to impingent patterns of physiological activity; this hypothesis receives strong support from the observation that GAP-43 can itself stimulate exuberant growth in axon terminals (Figure.11). It is of interest that in the brain of humans and other primates, highest levels of GAP-43 expression occur in just those regions known from behavioral neurology to be sites of higher-level associations. Although establishing a structural basis in the CNS of higher vertebrates for the long-term storage of transitory experiences has been elusive, the presence of a GAP-43 protein in those areas of the brain related to associative processes lends support to a morphological basis for long-term memory.

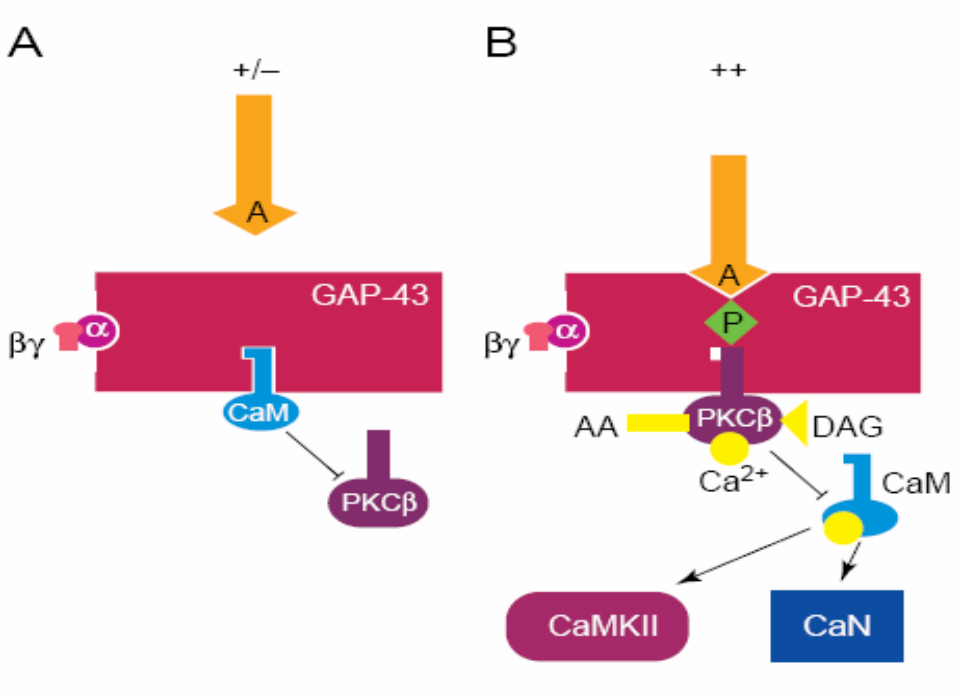

Figure 3.9 Regulation of GAP-43 Activity by Calmodulin. (A) At low concentrations (+/–) of intracellular Ca^{2+} and other second messengers [arachidonic acid (AA) or diacylglycerol (DAG)], calmodulin (CaM) binds to the IQ motif of GAP-43 (Alexander et al., 1987; Chapman et al., 1991) and inhibits protein kinase C (PKC) from phosphorylating serine 41. In this state, GAP-43 does not interact with proteins that alter the motility of the nerve terminal [for example, actin or other cytoskeletal elements]. The interaction between GAP-43 and the subunits of G proteins Go and Gi is unaffected by phosphorylation. (B) With increasing levels (++) of Ca^{2+}, AA or DAG, PKC becomes sufficiently activated to overcome the CaM block and phosphorylate Ser 41 .This allows GAP-43 to interact with elements of the cytoskeleton to alter the motility of the nerve ending. Phosphorylation of Ser 41 inhibits CaM from re-associating with GAP-43, allowing GAP-43 to remain in the activated state. At the same time, CaM becomes available to activate other target proteins, for example, the phosphatase calcineurin (CaN) or Ca^{2+} Calmodulin kinase type II (CaMKII). Activation of CaN is part of a feedback loop that ultimately leads to GAP-43 dephosphorylation. (Aloyo et al., 1983; Lovinger et al., 1986).

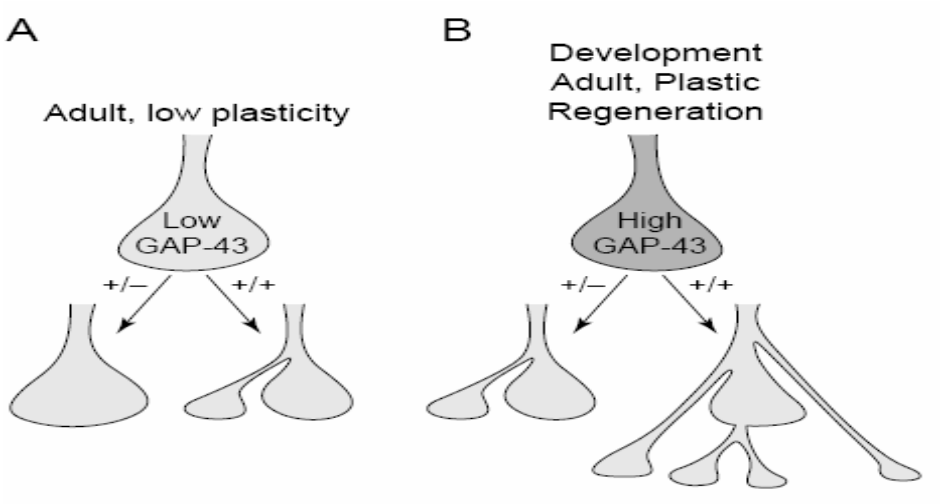

Figure 3.10 GAP-43 is an intrinsic determinant of synaptic growth and plasticity. In those nerve terminals that express little or no GAP-43 (left), weak stimuli (+/–) elicit little or no growth reaction, whereas stronger stimuli (++) (for example, differentiation) elicit a limited sprouting. In nerve terminals that contain substantial levels of GAP-43 in the adult (for example, in the limbic system and the associative neocortex), reactivity to local growth stimuli is potentiated (right), and leads to synaptic remodeling (Aigner et al 1995).

3.5.5 Roles of GAP-43 in neural development and structural plasticity

Convergent evidences indicate that GAP-43 plays a key role in guiding the growth of axons and modulating the formation of new connections. One clear indication of this comes from a recent study in transgenic mice, where animals overexpressing high levels of an exogenous gene encoding GAP-43 showed a spontaneous formation of aberrant connections. In the hippocampal formation, mossy fibers that arise from dentate granule cells normally project upon a restricted zone in the stratum lucidum of the CA3 region, but in mice overexpressing the GAP-43 transgene, these fibers projected beyond their normal territory into the stratum oriens. At the neuromuscular junction, extrajunctional contacts appeared spontaneously and increased greatly in number after local blockade of synaptic transmission with botulinum toxin or after peripheral-nerve injury. In a related study, overexpression of GAP-43 in primary olfactory neurons resulted in enlarged nerve endings in the olfactory bulb. Thus, even in the absence of additional trophic factors, GAP-43 enables neurons to sprout new terminals, and can therefore be considered an intrinsic determinant of the neuron's growth state (Benowitz et al 1989). Consistent with this view, earlier cell-culture studies found that expression of a GAP-43 transgene augmented neurite outgrowth in PC12 or neuroblastoma cells stimulated with NGF or cAMP, respectively (Strittmatter et al 1991). Even in non-neuronal cells, expression of a GAP-43 transgene has been found to induce extensive process outgrowth that is accompanied by a reorganization of the membrane cytoskeleton.

Conversely, suppressing GAP-43 expression has adverse effects on axon outgrowth. Primary sensory neurons from embryonic chick, when grown on poly-L-lysine, failed to extend axons when treated with antisense oligonucleotides complementary to portions of GAP-43 mRNA. When antisense-treated cells were grown on a more permissive laminin substrate, they extended thin processes with severely atrophied growth cones that interacted poorly with extrinsic cues in the environment. Similarly, antibodies to GAP-43, introduced into neuroblastoma cells using a lipid carrier, prevented cAMP-induced neurite outgrowth on certain substrates, while allowing cells to extend processes with attenuated growth cones on others. Studies in PC12 cells have yielded ambiguous results (Strittmatter et al 1991).

CHAPTER 4
MATERIALS AND METHODS

4.1 Experimental animals and conditions.

Virgin female, Sprague- Dawley rats (10-12 weeks old) and their offspring were used as experimental animals in the present study. The rats were obtained from the National Experimental Animals Center of Mahidol University, Salaya Campus, Thailand. Animal were given 2 weeks to habituate to a reversed 12 h light/dark cycle (light off at 07.00 AM) and single housing conditions in stainless steel cages with woodchip bedding material. The females were timed to mate by placing them overnight with sexually active males. The vaginal smear was examined on the next morning and the day on which the smear was sperm-positive was designated as gestation day 0 (GD0).

Each pregnant female was weight on GD7 through GD21 before any other manipulation. On the morning of GD 21, each pregnant female was received nesting material, and there after the cage was checked twice daily for the appearance of a litter. The day a litter was discovered was designated as postnatal day 0 (PND 0) and the length of gestation was noted. Food and water were available ad lib except during stress session in prenatal stress group. All procedures were carried out in accordance to Guideline for Care and Use of Laboratory Animals of The Institute of Science and Technology for Research and Development, Mahidol University, Thailand.

4.2 Restraint stress

Pregnant females were randomly assigned to either a prenatal stress (PS) condition (n=3) or non stress (NS) condition (n=3). For restraint stress, each pregnant rat was put inside a small Plexiglas cylindrical cage with the diameter of 7x18 cm with air hole for breathing. It is closed at one end, and its length can be adjusted to accommodate the size of animals. The restraint stressed was performed during the dark phase of the reverse light/dark cycle during GD14 to 21. Days 14-21 were

selected because this period has been show to be sensitive to behavioral teratogenic effects of prenatal stress (Fride & Weinstock 1984). The rats in the restraint cage were kept in their home cages daily for 4 hours, while the control pregnant rats remain in their home cages. To prevent habituation of animals to the daily procedure, the started periods were randomly shifted within certain time periods (8.00 am – 12.00 am). Within the first 12 h after birth, the number of pubs born, their sex and body weight were recorded and each litter was culled to eight pups. At PND 21, all offspring were weaned and housed four per cage with the same sex. Rat pups at 0, 7, 14 and 21 days old were used for immunohistochemical study of GAP-43 in prefrontal cortex.

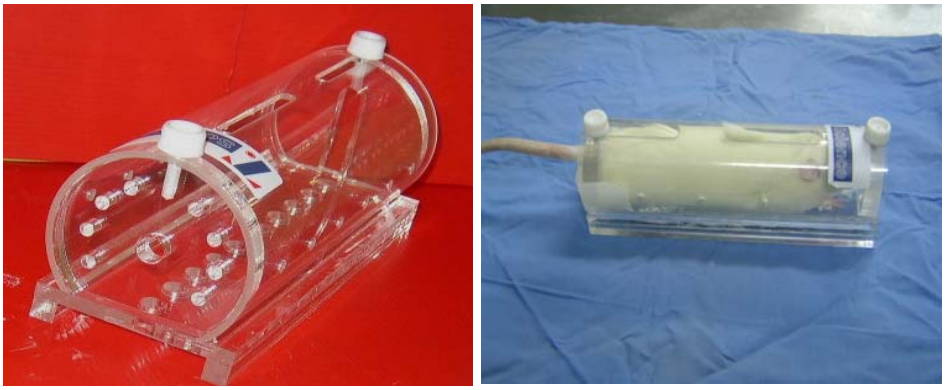

Figure 4.1 Equipment of Restraint Stress Treatment. Pregnant rat was put in a small Plexiglas cylindrical cage with a diameter of 7.5-8.5 cm and 18 cm in length, with air hole for breathing. Prenatal stress was performed during the dark phase of the cycle from GD 14-21, 4 hours daily.

4.3 Maternal corticosterone injection

Pregnant rats were randomly divided to two groups: control and corticosterone treatment group (n=3 for each group**).** Corticosterone solution was freshly prepared by suspension of corticosteroid (Sigma) in sesame oil. For the corticosterone treatment group, pregnant rats were received daily, intrasubcutaneous injection of corticosterone, 40 mg/kg, during GD14-21. The injections were done at the beginning of dark phase of the cycle. For control group, pregnant rats were received intrasubcutaneous injection of equivalent volume of vehicle (sesame oil). Rat pups at PND 7 and 14 from

both group (n=8 for each group) were used for study of GAP-43 immunoreactivity (IR) in the prefrontal cortex.

4.4 Immunohistochemistry procedures

4.4.1 Tissue preparation.

Rat pups were deeply anesthetized with sodium pentobarbital (30 mg/kg; i.p.) and perfused trancardially with 0.1 M phosphate buffer saline (PBS, pH 7.4) and followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the brain tissues were removed and posted fixed with the same fixative at 4°C overnight, then they were rinsed 3 times with 0.1 M PBS. The prefrontal cortex was dissected out and incubated overnight at 4°C in 30% sucrose in 0.1 M PBS. The brain tissues were cut with cryostat into coronal sectioned, the olfactory bulb was removed from the prefrontal cortex and the rest of prefrontal cortex was section into 40 µm thickness until reaching the corpus callosum according to the rat brain alat (Paxinos & Watson 1982). The sections were kept in 0.1 M PBS at 4°C and ready for the immunohistochemistry study.

4.4.2 Method for immuno-peroxidase staining of GAP-43

The immuno-peroxidase staining was performed on free floating prefrontal cortex sections with monoclonal antibody for GAP-43 (G 9264, Sigma-Aldrich Inc., USA). First, the sections were rinsed 2 times, 5 min each, in 0.1 M PBS, then, pre-treated with 1% H_2O_2 for 10 min and rinsed for 5 min with 0.1 M PBS containing 1% BSA and 0.3% Triton X-100 (PBS-B). Then the sections were incubated in 5% normal horse serum diluted in PBS containing 0.25% BSA and 0.1% Triton X-100 (PBS-A) for 30 min at room temperature. Subsequently, they were incubated for 1 hour at 4°C with the primary antibody (1:12000) and washing for 3x5 min in PBS-B. Afterward, they were exposed for 30 min at room temperature to biotinylated antimouse IgG (1:200) in PBS-B. After washing in PBS-B 2x5 min and PBS 1x5 min, the sections were incubated with ABC horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Lab, Burlingame, USA) diluted 1:50 in PBS, for 30 min at room temperature. Then, the sections were washed sequentially in 2x5 min in PBS and 10 min in 0.05 M Tris-HCl buffer (pH 7.6). After that, they were reacted for peroxidase activity in

0.025% DAB solution containing 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min. After rinsing in distilled water for 2x5 min, the sections were placed in gelatinize coated slide, dried and cover slipped with permount. The immunoperoxidase activity was visualized under the light microscope, Nikon Eclips E400, Nikon, Tokyo, Japan. Some series of prefrontal section were counterstained with Cresyl's Violet in order to demonstrate the staining characteristic of GAP-43 IR in the prefrontal cortex.

4.5 Data analysis

The stained sections were observed under a light microscope. The layers in the prefrontal cortex were identified and the photographs were taken from the middle of each layer. In both groups, the areas selected for photographs were treated with the same procedures (Figure.13). In all cases, three continuous and non-overlapping frames were photographed in each layer from each section, Total 10 sections per rat (n=8 rats for each group). Thus, each mean value was represented the mean value of 30 photographs from each layer in each rat. Photographs from the selected area of prefrontal cortex were captured by CCD color camera and were transformed into digits. Total areas of each frame were estimated using Adobe Photoshop 7.0 (Figure.14) and the percent density of GAP-43 IR were measured from each picture using Image tools software (University of Texus Health of Science Center). Density of GAP-43 IR in different brain areas were analyses using Instat software. Statistical comparisons of data set were performed by one-way ANOVA, followed by Tukey's Post hoc multiple comparison test. Data was presented as mean± SEM. The stained sections were observed under a light microscope and the cortical layers in the prefrontal cortex were identified. The photographs were taken from the middle of each layer. In all sections, three continuous and non-overlapping frames were photographed in each layer of prefrontal cortex section.

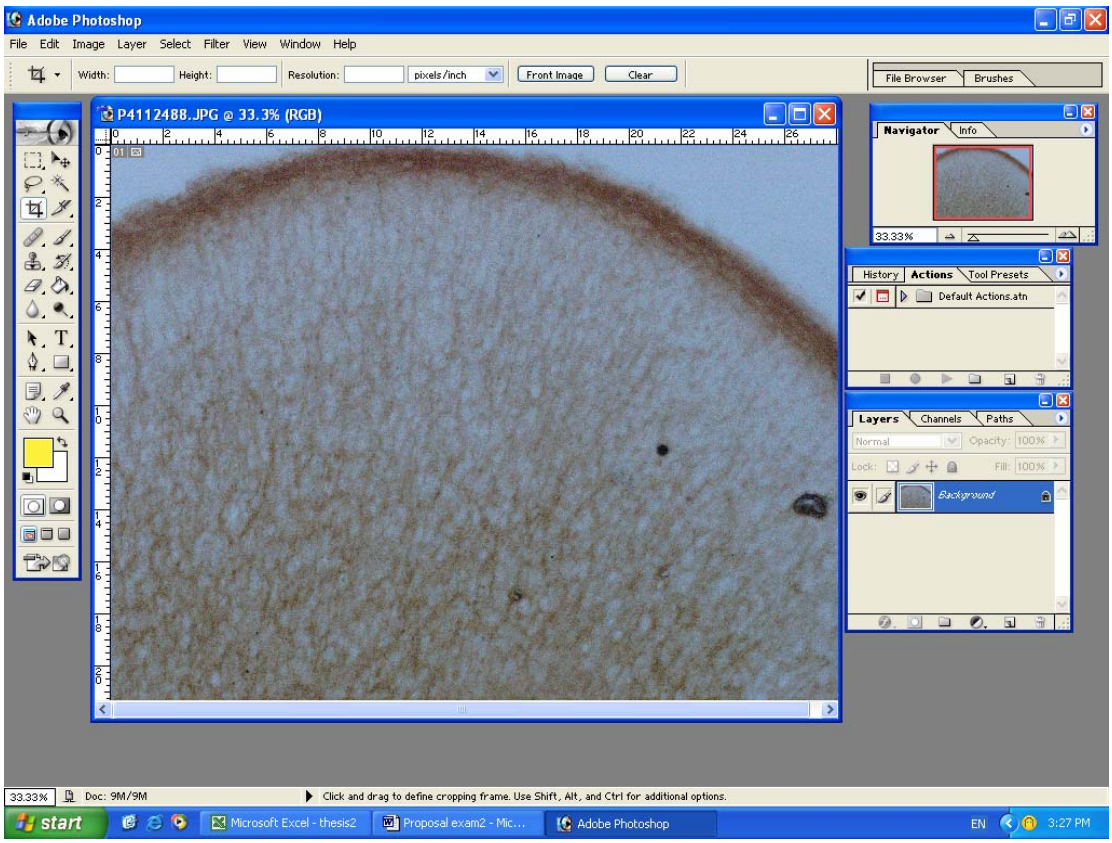

Figure 4.2 The total area of each frame was measurement by Adobe Photoshop 7.0 program.

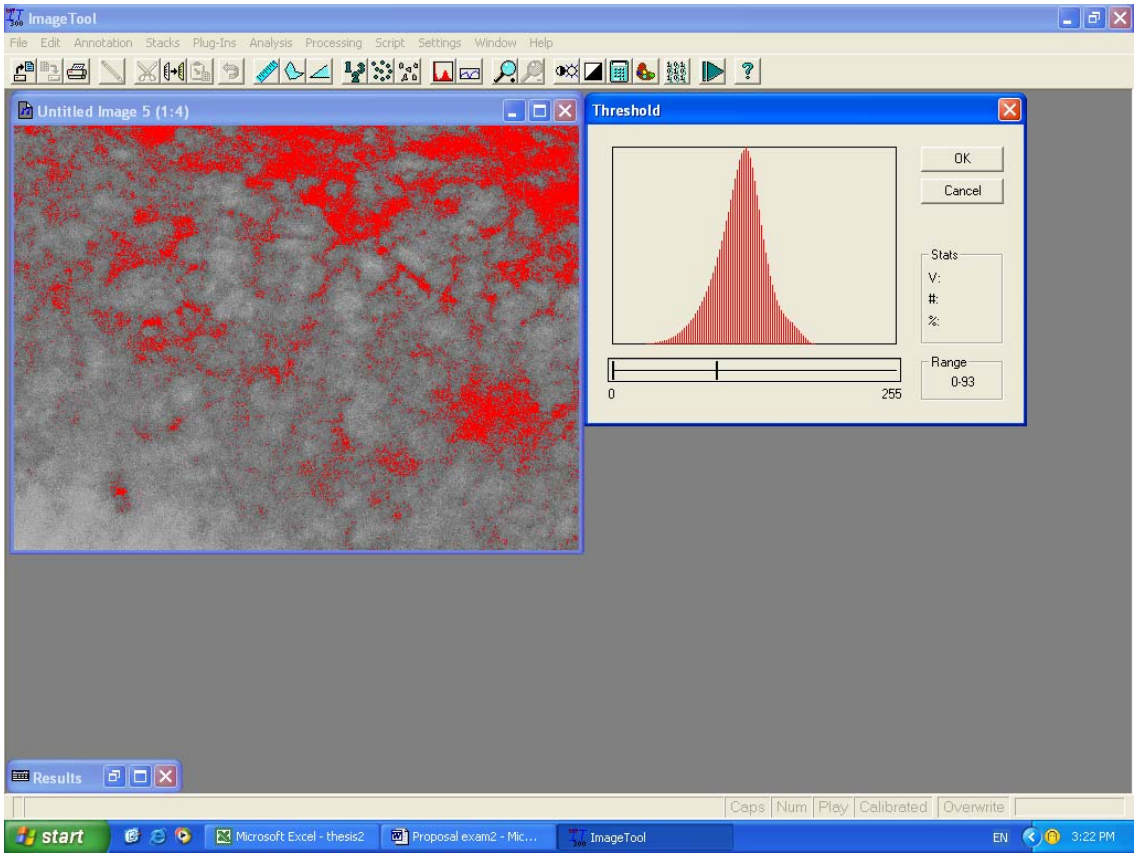

Figure 4.3 Measurement of the percent density of GAP-43 IR by using Image tool software**.**

CHAPTER 5
RESULTS

5.1 Immunoperoxidase staining of GAP-43 in prefrontal cortex

5.1.1 The characteristics of GAP-43 immunoreactivity in prefrontal cortex

In prefrontal cortex of neonatal rat, GAP-43 IR was found in the nerve fibers (Figure 5.1A). GAP-43 IR was not observed in the cell body of all neuronal cell types. In layer II-VI of prefrontal cortex, GAP-43 IR was observed in neuronal processes of all cell type both pyramidal cells and non-pyramidal cells. The immunoreactivity of GAP-43 was abolished in the section that omit the anti GAP-43 (Figure 5.1B).

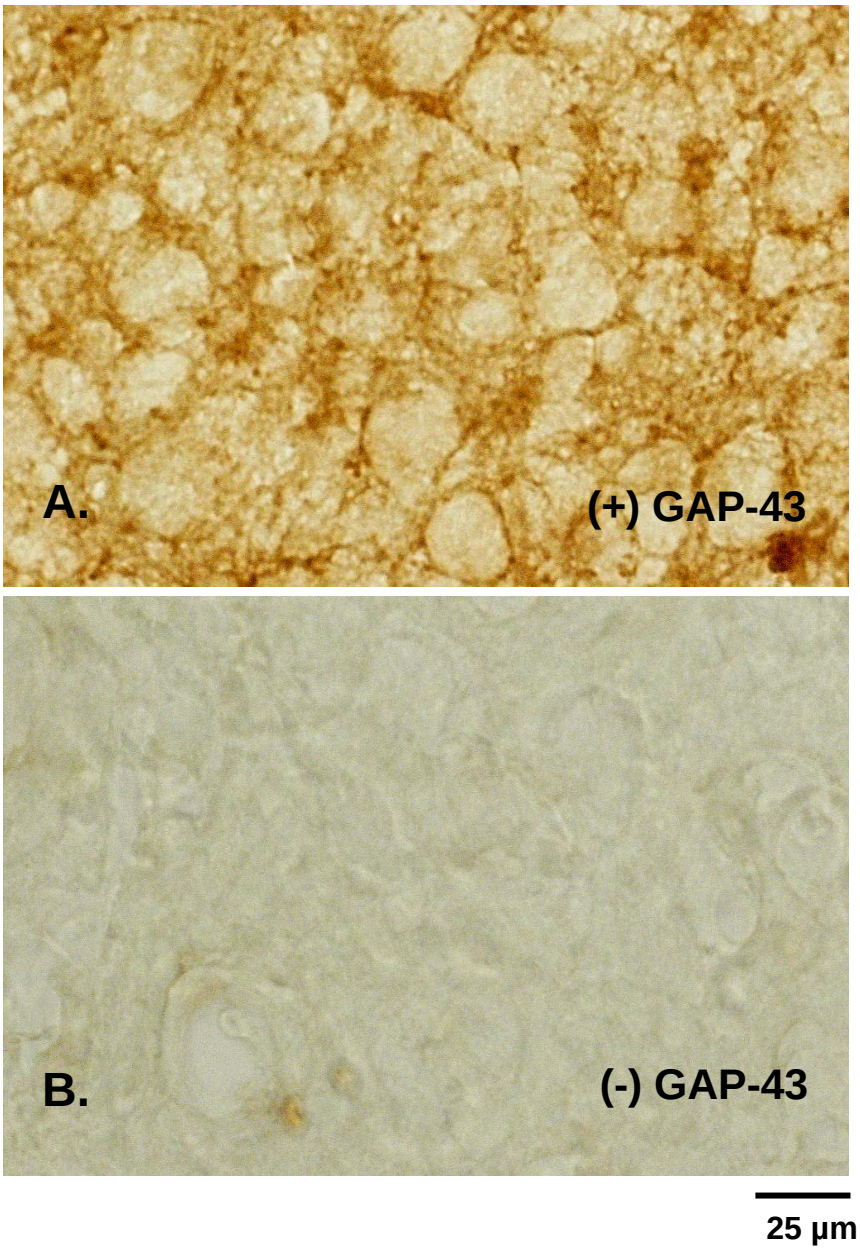

Figure 5.1 A) Photomicrograph shows the characteristics of GAP-43 immunoperoxidase staining (1:12000) in the prefrontal cortex of rat pups at PND 7. GAP-43 IR was not observed in the section that was processed without the primary antibody (B).

5.1.2 Classification of cortical layer in prefrontal cortex of neonatal rat

After immunohistochemical staining of GAP 43, the sections were counter-stained with cresyl violet in order to distinguish specific cortical layer in the prefrontal cortex (Figure 5.2 and 5.3). Section of prefrontal cortex can be classified in to six different layers. Layer I, the most outer layer, is thin to medium and contain fibers from the deep cortical layer. Layer II is also thin but prominent and easily identifiable. It contains small granular and pyramidal cells that have a medium to dark staining. Layer III is the widest layer in the frontal pole and have a medium to dark staining. Pyramidal cells in Layer III have a small, but gradual change in size; the pyramidal cells close to layer II are smaller than those close to layer IV. Layer IV is also thin but continuous with pale to medium stained granular and pyramidal cells. The borders between layer III and layer IV are very clear and regular. Layer V is wide and includes large pyramidal cells. Their sizes are only minimally larger than those of the pyramidal cells of layer III. Layer VI includes dark pyramidal and fusiform cells. The borders between layer VI and the white matter are regular.

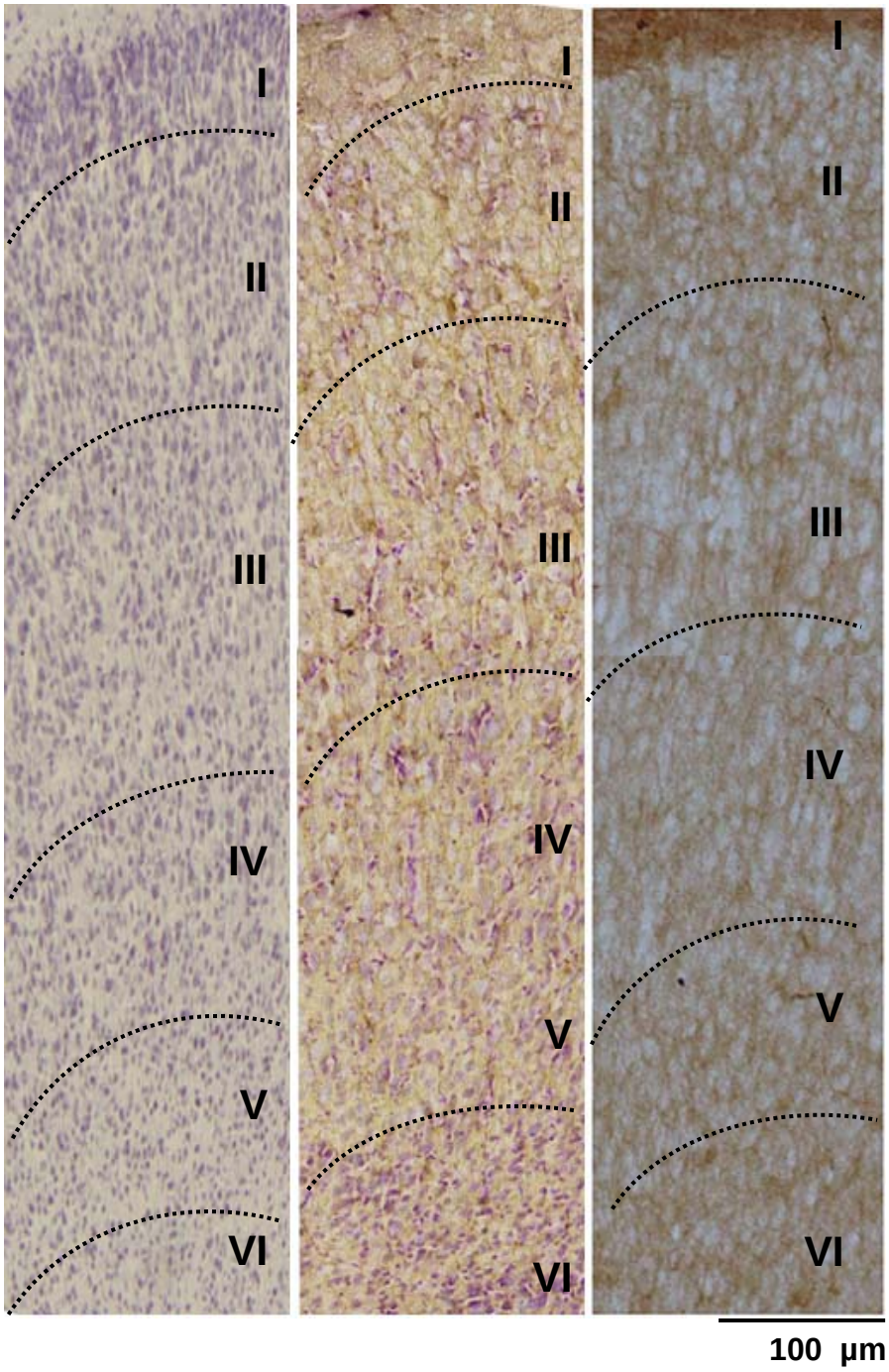

Figure 5.2 Photomicrographs of prefrontal cortex section that was stained with Cresyl Violet (left), GAP-43 counter stained with Cresyl Violet (middle) and GAP-43 alone (right). Classification of the cortical layer was performed as described in the text. Dash lines represents border between each cortical layer. I-VI represent the cortical layer 1-6, respectively.

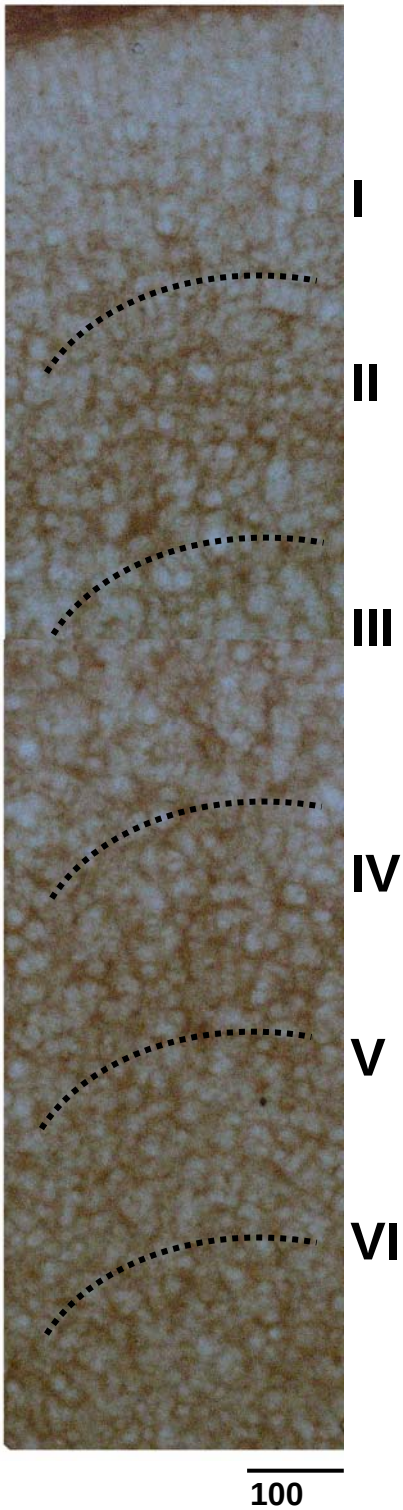

Figure 5.3 Photomicrograph showed the staining pattern of GAP-43 IR in different layer of prefrontal cortex. Classification of the cortical layer was performed as described in the text. Dash lines represents border between each cortical layer. I-VI represent the cortical layer 1-6, respectively.

5.2 Accuracy of the methods

The percent density of GAP-43 IR in different layer of prefrontal cortex was analyzed by Image tools software (University of Texas Health of Science Center). In order to confirm the accuracy of the technique for measurement of the percent density of GAP-43 IR, reproducibility were performed by analyzed 5 different photographs with the same procedure but performed in different time for 10 times. The datasets were then analyzed for the constancy of the technique. The results show that percent density of GAP-43 IR of the same picture from 10 independent measurements did not show a significant different and percent change between each measurement was not larger than 8% as shown in Table 5.1. The result suggested that the method use for measurement of the percent density of GAP-43 IR in the present study is highly accurate and acceptable for further analyzed of the developmental change of GAP-43 IR and the effect of prenatal stress on GAP-43 IR in the prefrontal cortex of postnatal rat.

Table 5.1 The accuracy of technique for measurement of the percent density of GAP-43 IR. Reproducibility of the technique was done by analyzed 5 different photographs with Image tool software using the same procedure but performed in different time manner for 10 times. Data from 10 independent measurements, did not show a significant difference. The percent change between each measurement was no larger than 8%.

Fig.	1	2	3	4	5
Exp. 1	22.24	19.64	13.92	14.72	24.57
Exp. 2	22.24	19.21	12.97	14.72	24.53
Exp. 3	22.24	18.78	12.88	14.72	23.94
Exp. 4	22.23	18.36	12.49	14.72	23.85
Exp. 5	22.42	18.36	12.49	14.16	23.17
Exp. 6	22.41	17.62	12.44	14.16	23.13
Exp. 7	22.31	17.27	11.56	14.16	22.50
Exp. 8	22.27	17.20	11.56	12.70	22.45
Exp. 9	21.48	17.18	11.56	12.64	21.91
Exp. 10	21.48	16.77	11.56	12.58	21.91
Mean ±SEM	22.13 ± 0.11	18.04 ± 0.31	12.34 ± 0.25	13.93 ± 0.29	23.19 ± 0.31
%change	4	7	1	8	6

5.3 Development of GAP-43 IR in prefrontal cortex of postnatal rat pup.

In prefrontal cortex of postnatal rat, GAP-43 IR was observed throughout the cortical layers. GAP-43 IR was localized in neuronal processes of all neuronal cell types both pyramidal cells and non-pyramidal cells. During development of the rat prefrontal cortex, GAP-43 IR was changed throughout the postnatal periods (Figure 5.3). At birth, GAP-43 IR was already presented at low level (Figure 5.4A) and progressively increased during the first and the second postnatal weeks. It appeared that the density of GAP-43 IR was reaching the peak at PND 7 (Figure 5.4B). At PND 14, the density of GAP-43 IR was gradually deceased compared with PND 7 but still high compared with PND 0. After that, the density of GAP-43 IR was reduced throughout the cortical layer until saturated around PND 21 (Figure 5.4C and 5.4D).

The distribution pattern of GAP-43 IR in each cortical layer of the prefrontal cortex of postnatal rat was observed in this study. The results show that distribution pattern of GAP-43 IR was identical among all periods (PND 0, 7, 14 and 21) observed in this study (Figure 5.5). In prefrontal cortex, the density of GAP-43 IR was lowest in the layer III when compared with another cortical layer. Moderate level of GAP-43 was observed in the cortical layer II and VI and highest density was observed in layer IV and V compared with the other cortical layers in most cases. In summary, the density of GAP-43 IR was highest in layer IV and V, moderately in layer II and VI, and rarely observed in layer III of prefrontal cortex of postnatal rat. This is because cortical layer IV and V mainly contain the neuronal fibers but cortical layer III usually contains the pyramidal cell body and a few of neuronal processes.

Developmental changes in the percent density of GAP-43 IR in each cortical layer of prefrontal cortex of neonatal rat were shown in Figure 5.6. At PND0, GAP-43 IR was found in the axons terminal of the neuron in all layers of the prefrontal cortex, however, the percent density of GAP-43 IR was very weak. The percent densities of GAP-43 IR were equal in all cortical layers (II, IV-VI) and lowest in layer III. At PND7, entire cortical layers were highly stained with GAP-43. The percent density of GAP-43 IR in prefrontal cortex became mostly intense, and clearly labeled in all cortical layers, especially in the layer IV and V. At this period, the percent density of GAP-43 IR reach its peak and showed significantly increase compared to those observed at PND0 ($P<0.0001$). The cortical layer that shown marked increase in GAP-

43 IR are layer IV and V. The other cortical layers also show a significant increase in the percent density of GAP-43 IR but the increased was small when compared to those of PND0. At PND14, the density of GAP-43 in some cortical layers (layer IV, V and VI) of prefrontal cortex was still highly stained with GAP-43, but in some cortical layers (layer II, III) showed slightly decrease from PND7. At PND21, the percent density of GAP-43 IR in all cortical layer showed further decrease from PND14 until reach the same level as observed at PND0. In summary, the percent density of GAP-43 IR was found in all layers of prefrontal cortex at birth, while the highest density were found at PND7 especially in layer IV and layer V. The percent density of GAP-43 IR in all cortical layers showed slightly decrease from PND14 until reaches the adult level at PND21.

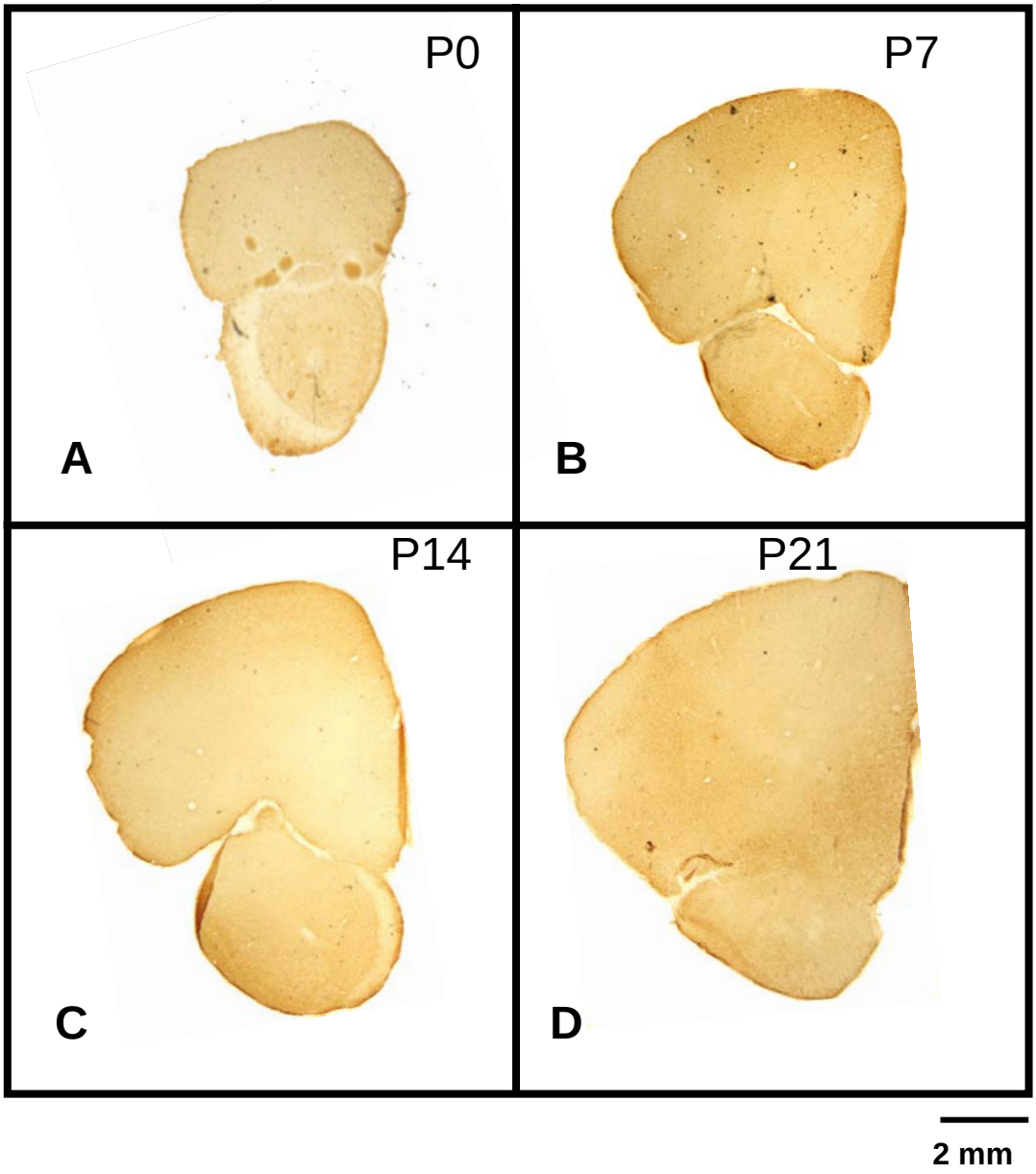

Figure 5.4 Photomicrographs showed the developmental changes of GAP-43 IR in the prefrontal cortex section of rat pups at different postnatal periods from PND 0 (A), 7 (B), 14 (C) and 21 (D). At birth, GAP-43 IR was already presented at low level and progressively increased during the first and the second postnatal weeks. At PND 14, the density of GAP-43 IR was decreased but still high and then saturated around PND 21

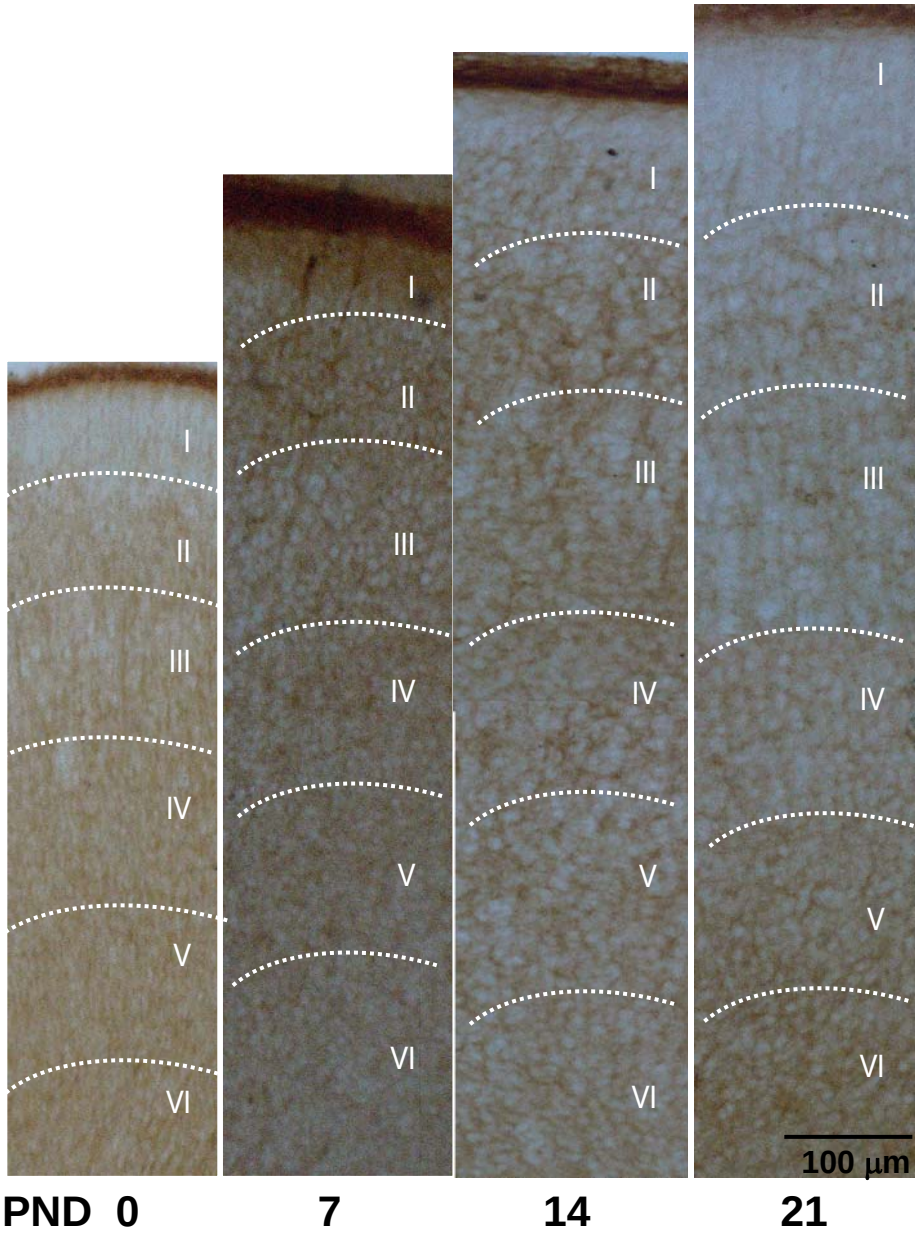

Figure 5.5 Photomicrographs illustrate the developmental changes in GAP-43 IR in the prefrontal cortex section at different postnatal periods from PND 0, 7, 14 and 21. Classification of the cortical layer was performed as described in the text. Dash lines represents border between each cortical layer. I-VI represent the cortical layer 1-6, respectively.

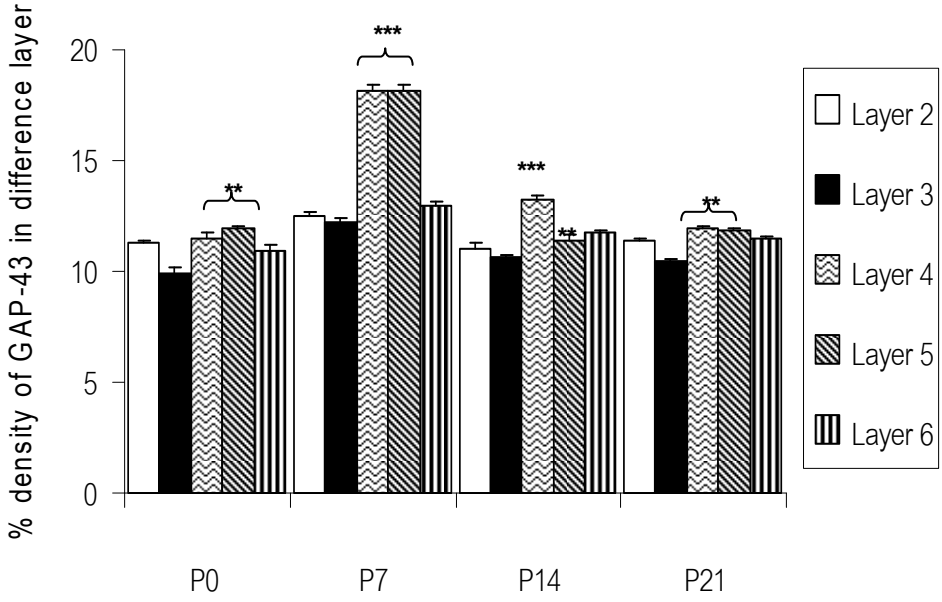

Figure 5.6 Bar graph showed the percent density of GAP-43 IR in different layers of rat prefrontal cortex at different postnatal periods The percent density of GAP-43 IR were measured using Image Tools software as described under the experimental methods. Each value represents mean ± SEM. Each mean value represents the value measured from 30 photographs in each rat (n=8). **p<0.001, ***p<0.0001 compared with the other layers.

5.4 Effects of prenatal stress on GAP-43 IR in prefrontal cortex of postnatal rat.

In the present study, we hypothesized that prenatal stress may alter the level of GAP-43 in neonatal rat brain, especially in the prefrontal cortex. In order to investigate the hypothesis, immunohistochemical staining of GAP-43 in prefrontal cortex of rat pups from both groups were performed at different postnatal period from PND0-21. After that, the percent density of GAP-43 IR was compared between PS and control group. Maternal stress was performed during the period of neurogenesis of pyramidal and non-pyramidal cells in the cortex of the offspring. (GD14-21). The result shown that, prenatal stress can induce the increase GAP-43 IR throughout the prefrontal cortex of rat pups as shown in figure 5.7-5.14.

The developmental pattern of GAP-43 IR in prefrontal cortex of PS group was comparable, to those observed in control group. However, GAP-43 IR in prefrontal cortex of pups from PS group was significantly higher than control group in all periods observed in this study. At birth, there was small increase the density of GAP-43 IR in prefrontal cortex of pups born from PS group compared to pups born from control group (Figure 5.8). At PND7, the density of GAP-43 IR was increase markedly in prefrontal cortex of PS group compared to the control group (Figure 5.9A-C). At this stage, GAP-43 IR in prefrontal cortex usually reaches its peak in control group; however, the increase in density of GAP-43 IR was augmented in the prefrontal cortex of pups born from stressed group (Figure 5.11A-C). At PND14, the density of GAP-43 IR in prefrontal cortex of PS group still shows small increase when compared to control group. At PND21, the density of GAP-43 in prefrontal cortex looks quite similar in prefrontal cortex of both groups (Figure 5.13A-C). At this stage, GAP-43 IR in prefrontal cortex of PS group was declined to the baseline and saturated, as found control group.

The percent density of GAP-43 IR in each layer of prefrontal cortex was measured in order to examine the effect of prenatal stress on GAP-43 IR in prefrontal cortex of rat pups. At PND0, the distribution pattern of GAP-43 IR in different layers appear to be the same as those found in control group, that is, low in layer III and high in layer II, IV, V, and VI as shown in figure 5.8. Prenatal stress induces significant increase in a percent density of GAP-43 IR in all cortical layer of prefrontal cortex,

except layer III. At birth, GAP-43 IR in layer II, IV, V, and VI in the prefrontal cortex of PS group was significant higher than in control group ($p<0.01$).

The effects of prenatal stress on the density of GAP-43 IR were clearly observed throughout the prefrontal cortex at PND7 (Figure 5.9A-C). Generally, at PND7, the density of GAP-43 IR in prefrontal cortex of PS pups were most intensed throughout the prefrontal cortex than other periods. The data shown that the percent density of GAP-43 IR in prefrontal cortex of PS pups was increased markedly in layer IV, V and VI ($p<0.001$) and did not show any significantly different in layer II and III when compared to control pups figure 5.10..

At PND 14 (Figure 5.11A-C), the density of GAP-43 IR in prefrontal cortex of PS pups still show some intensely stained compared to control. The percent density of GAP-43 IR still increase, specifically in layer IV, V and VI ($P<0.001$) compared to control pups while no change were observed in layer II and III (Figure 5.12). At PND21 (Figure 5.13A-C), the percent density of GAP-43 IR in prefrontal cortex of PS pups still increase in layer IV and V ($P<0.001$), and layer VI ($P<0.01$) with no significant change in layer II and III figure 5.14.

In summary, effect of prenatal stress on the percent density of GAP-43 IR was evidenced at birth with higher GAP-43 IR in PS pups compared to control. The effect was increased markedly during PND7, 14 and slowed down at PND21. Mostly, the increase in GAP-43 IR was observed in layer IV, V and VI but not observed in layer II and III.

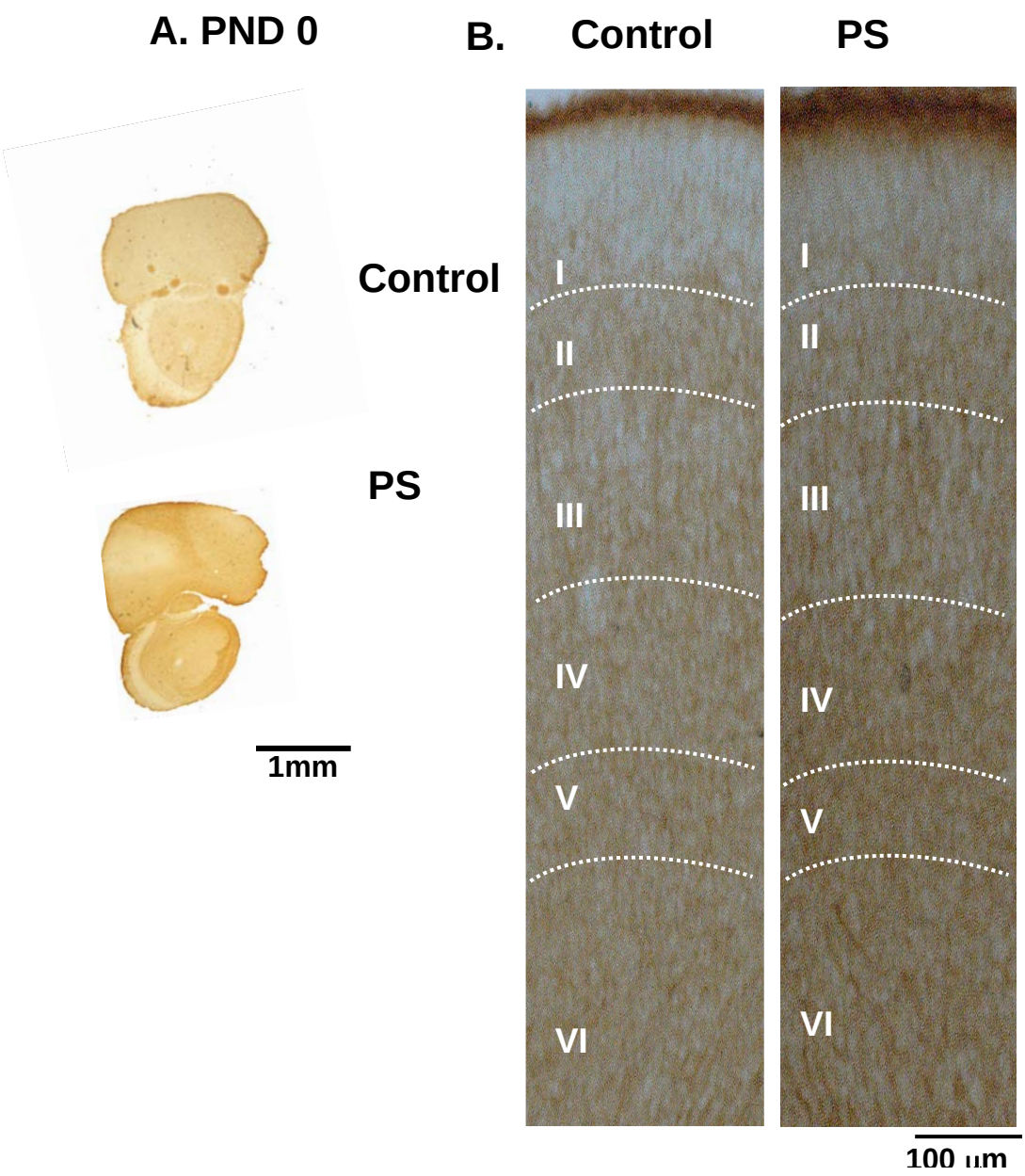

Figure 5.7 A. Photomicrographs showed the GAP-43 IR in the prefrontal cortex section of rat pups at PND 0 compared between control and prenatal stress group, respectively. B. Photomicrographs showed the GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 0 compared between control and prenatal stress group, respectively. C. Photomicrographs showed the GAP-43 IR at PND 0 in prefrontal cortex of rat pups compared between control (A, C, E, G and I) and prenatal stress group (B, D, F, H and J), respectively.

PND0

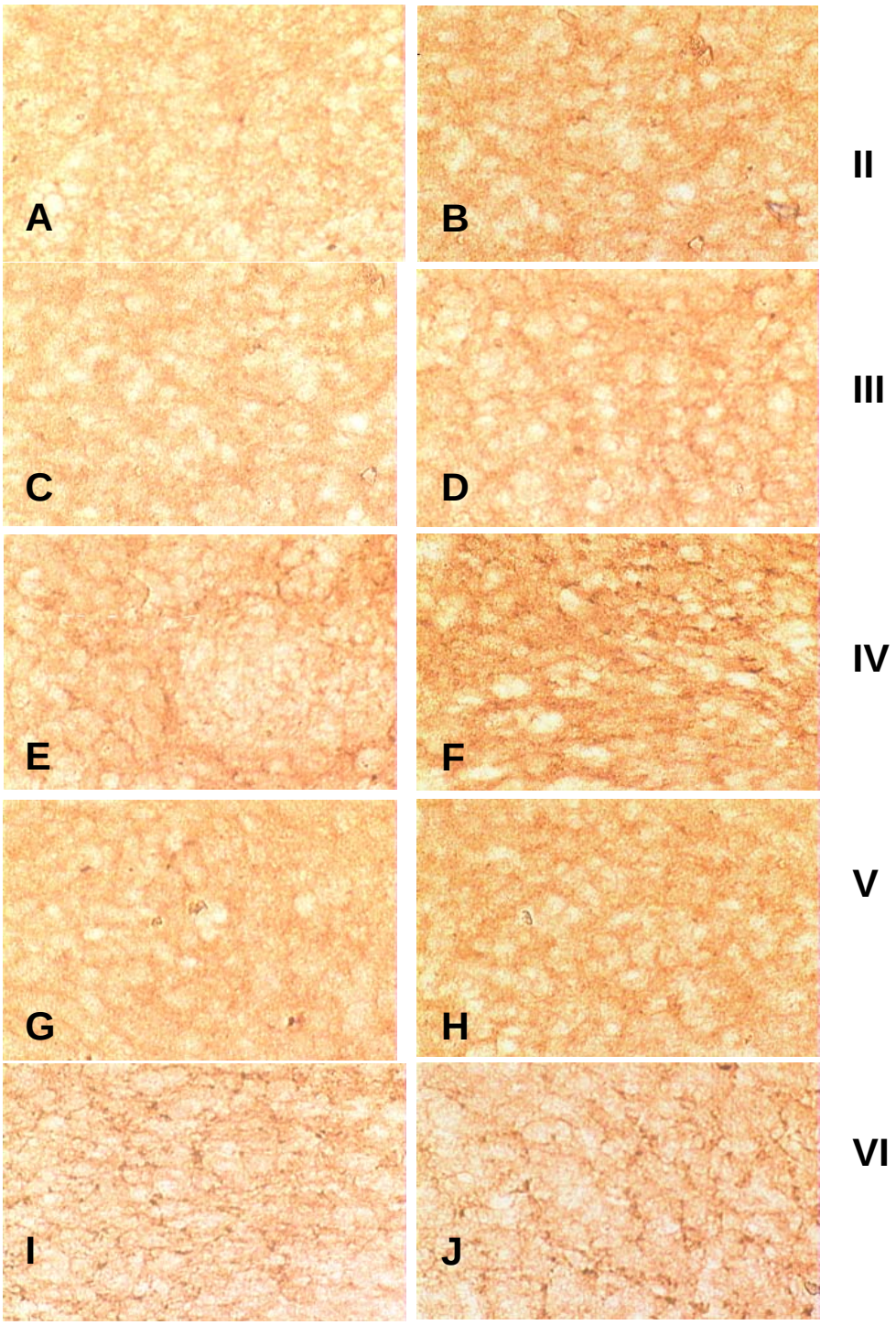

Fig. 5.7 C

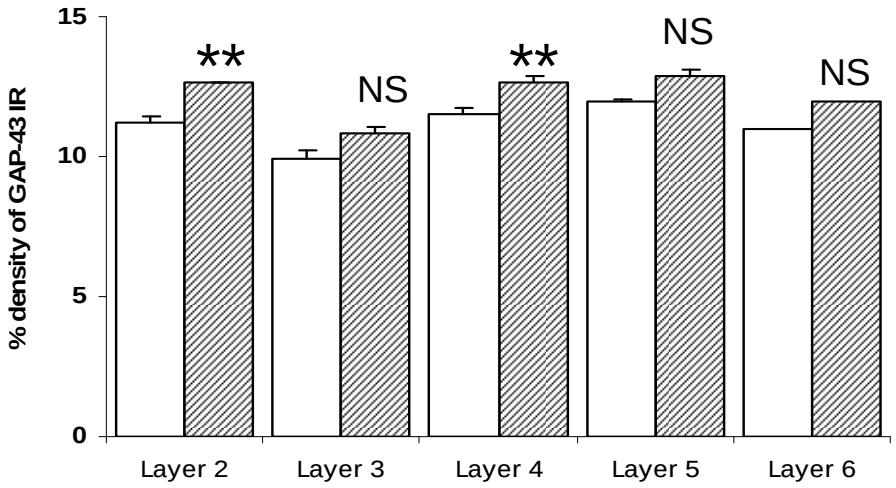

Figure 5.8 Bar graph showed the percent density of GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 0. The percent density of GAP-43 IR was measured using Image Tools software as described under the experimental methods. Each value represents mean ± SEM. Each mean value represents the value measured from 30 photographs in each rat (n=8). **p<0.001, ***p<0.0001 compared with the other layers.

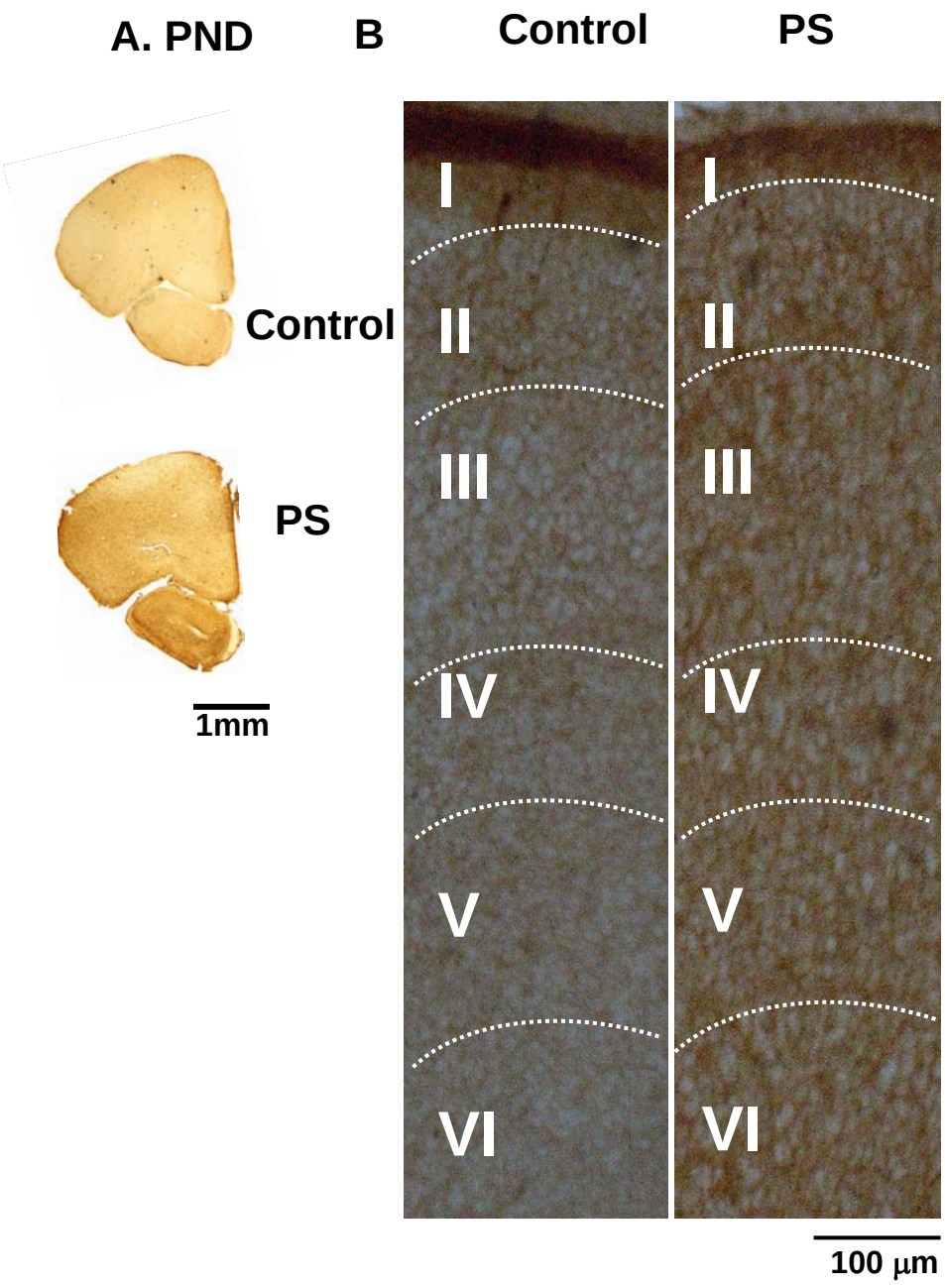

Figure 5.9 A. Photomicrographs showed the GAP-43 IR in the prefrontal cortex section of rat pups at PND 7 compared between control and prenatal stress group, respectively. B. Photomicrographs showed the GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 7 compared between control and prenatal stress group, respectively. C. Photomicrographs showed the GAP-43 IR at PND 7 in prefrontal cortex of rat pups compared between control (A, C, E, G and I) and prenatal stress group (B, D, F, H and J), respectively.

PND 7

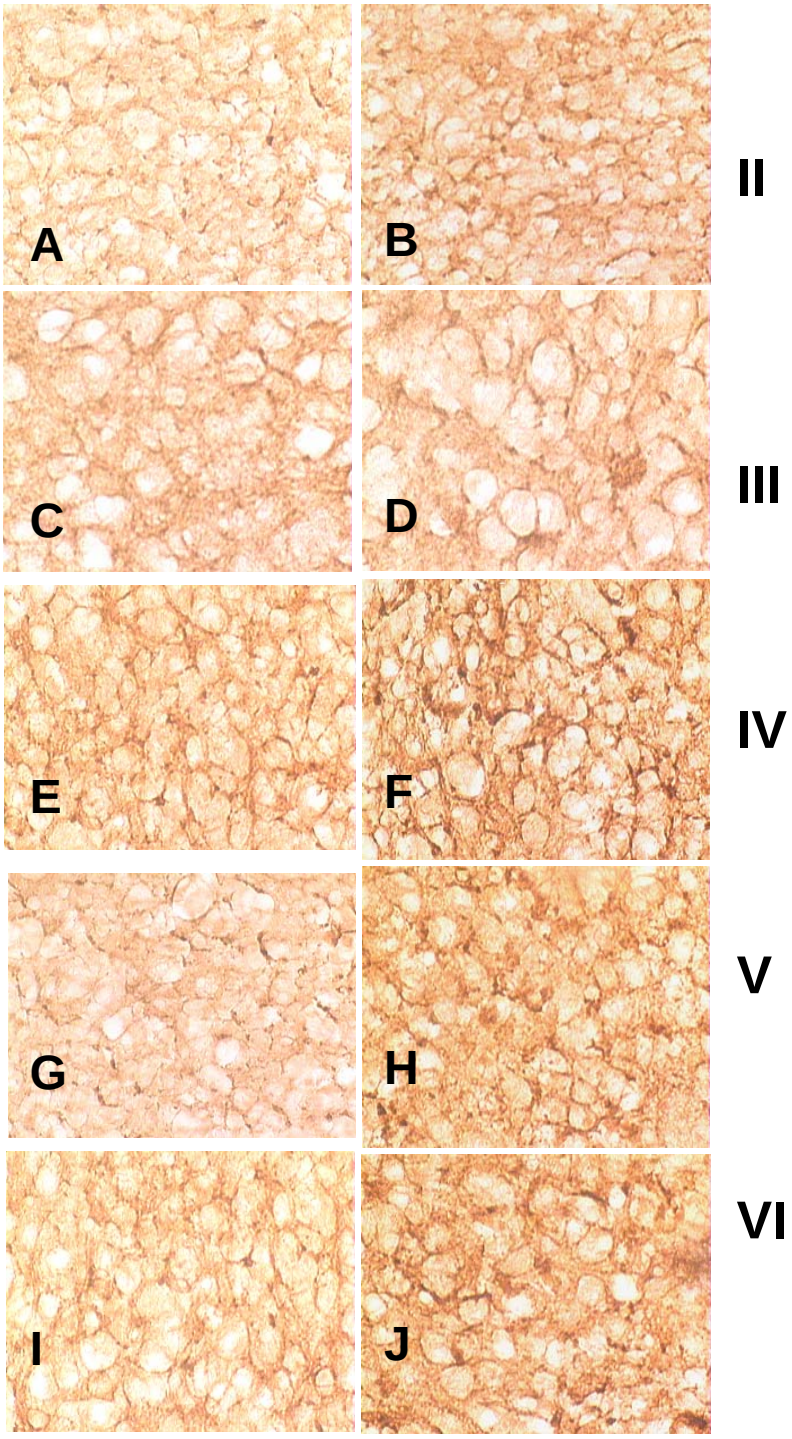

Fig. 5.9 C

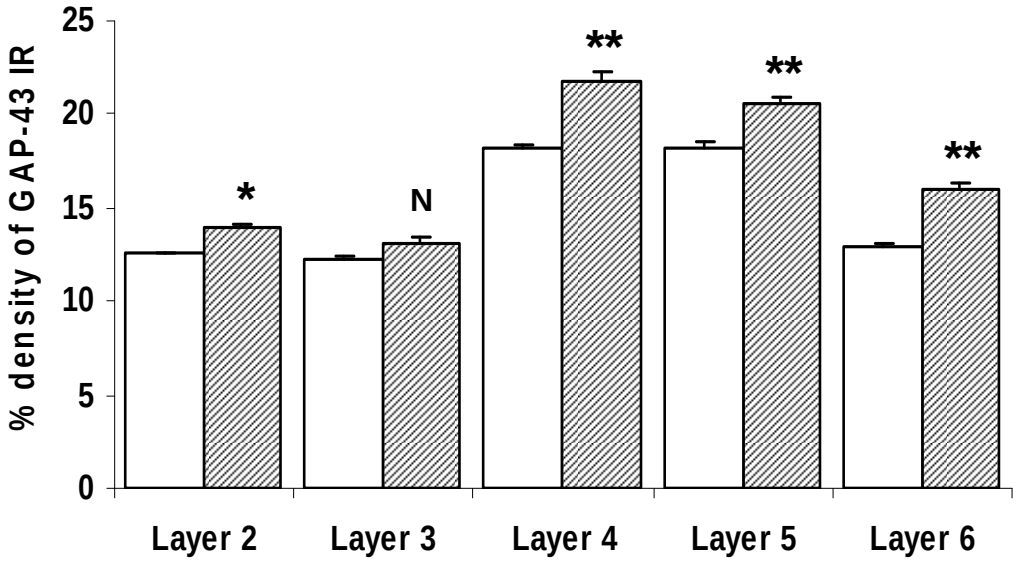

Figure 5.10 Bar graph showed the percent density of GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 7. The percent density of GAP-43 IR were measured using Image Tools software as described under the experimental methods. Each value represents mean ± SEM. Each mean value represents the value measured from 30 photographs in each rat (n=8). **p<0.001, ***P<0.0001 compared with the other layers.

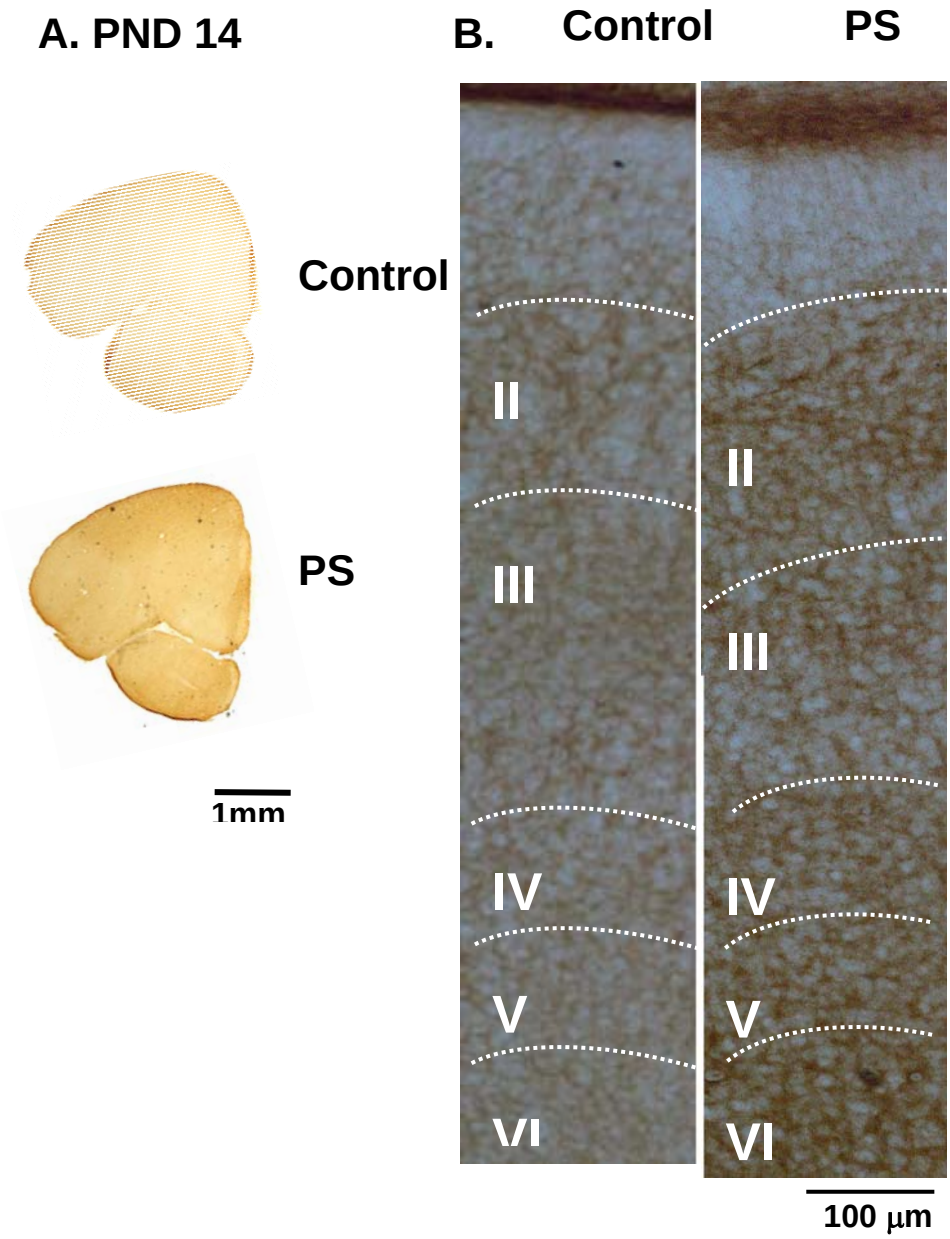

Figure 5.11 A. Photomicrographs showed the GAP-43 IR in the prefrontal cortex section of rat pups at PND 14 compared between control and prenatal stress group, respectively. B. Photomicrographs showed the GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 7 compared between control and prenatal stress group, respectively. C. Photomicrographs showed the GAP-43 IR at PND in prefrontal cortex of rat pups compared between control (A, C, E, G and I) and prenatal stress group (B, D, F, H and J), respectively.

PND 14

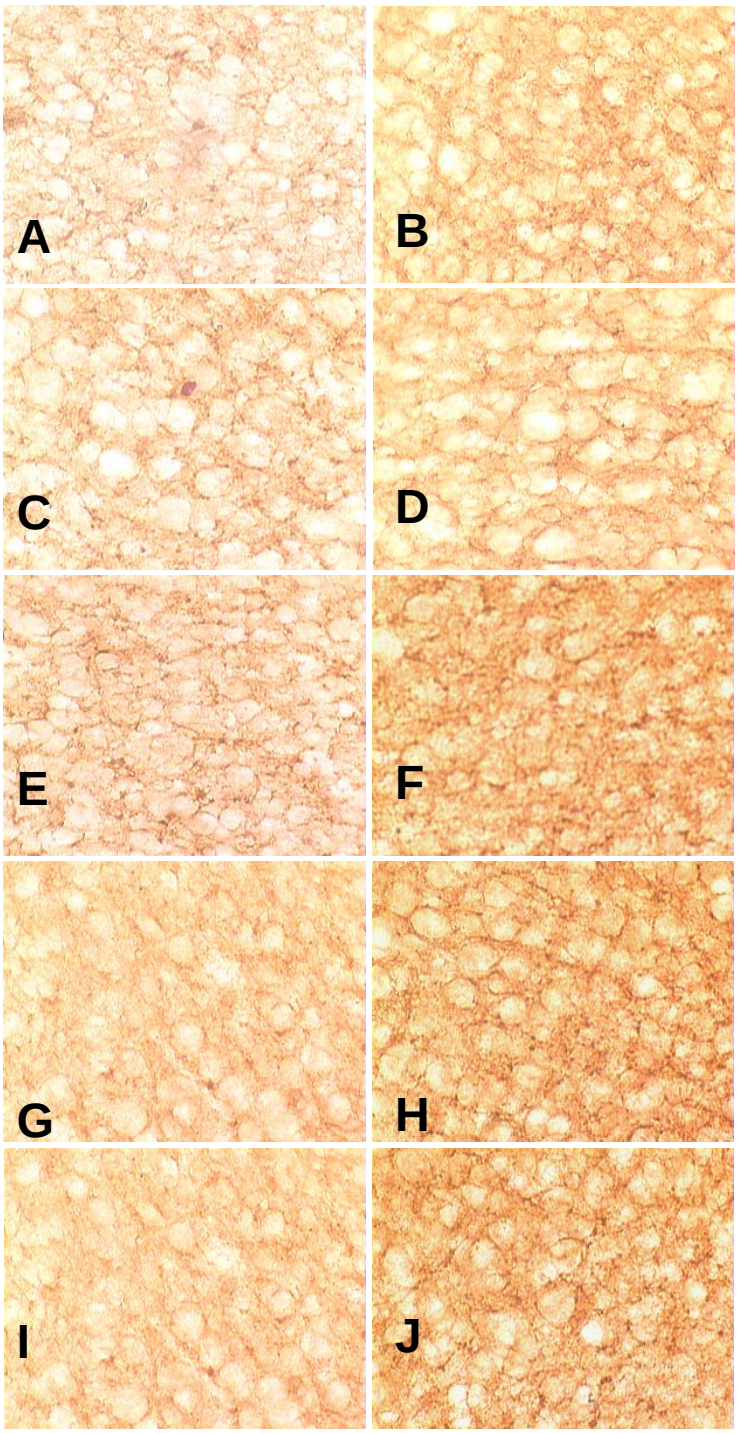

Fig. 5.11 C

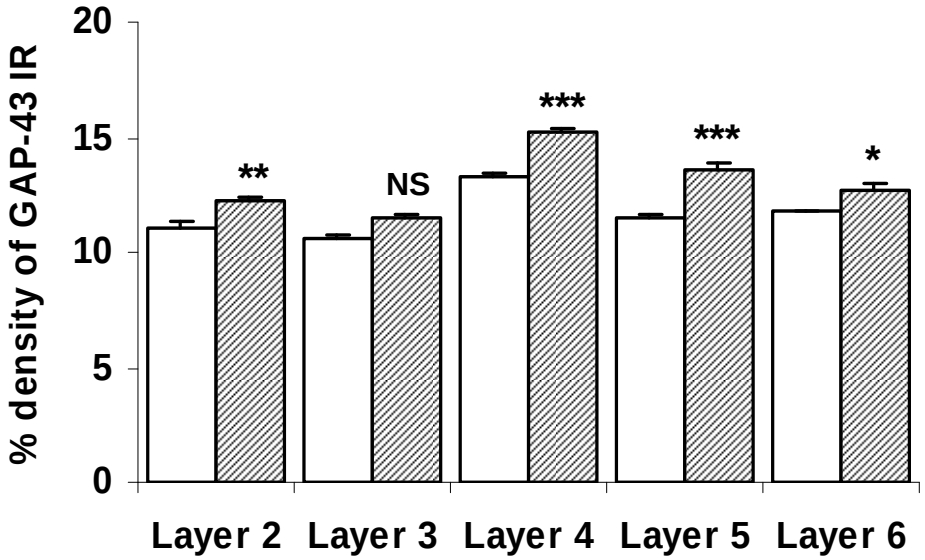

Figure 5.12 Bar graph showed the percent density of GAP-43 IR in different layers of prefrontal cortex of rat pups at PND14. The percent density of GAP-43 IR were measured using Image Tools software as described under the experimental methods. Each value represents mean ± SEM. Each mean value represents the value measured from 30 photographs in each rat (n=8). **p<0.001, ***P<0.0001 compared with the other layers.

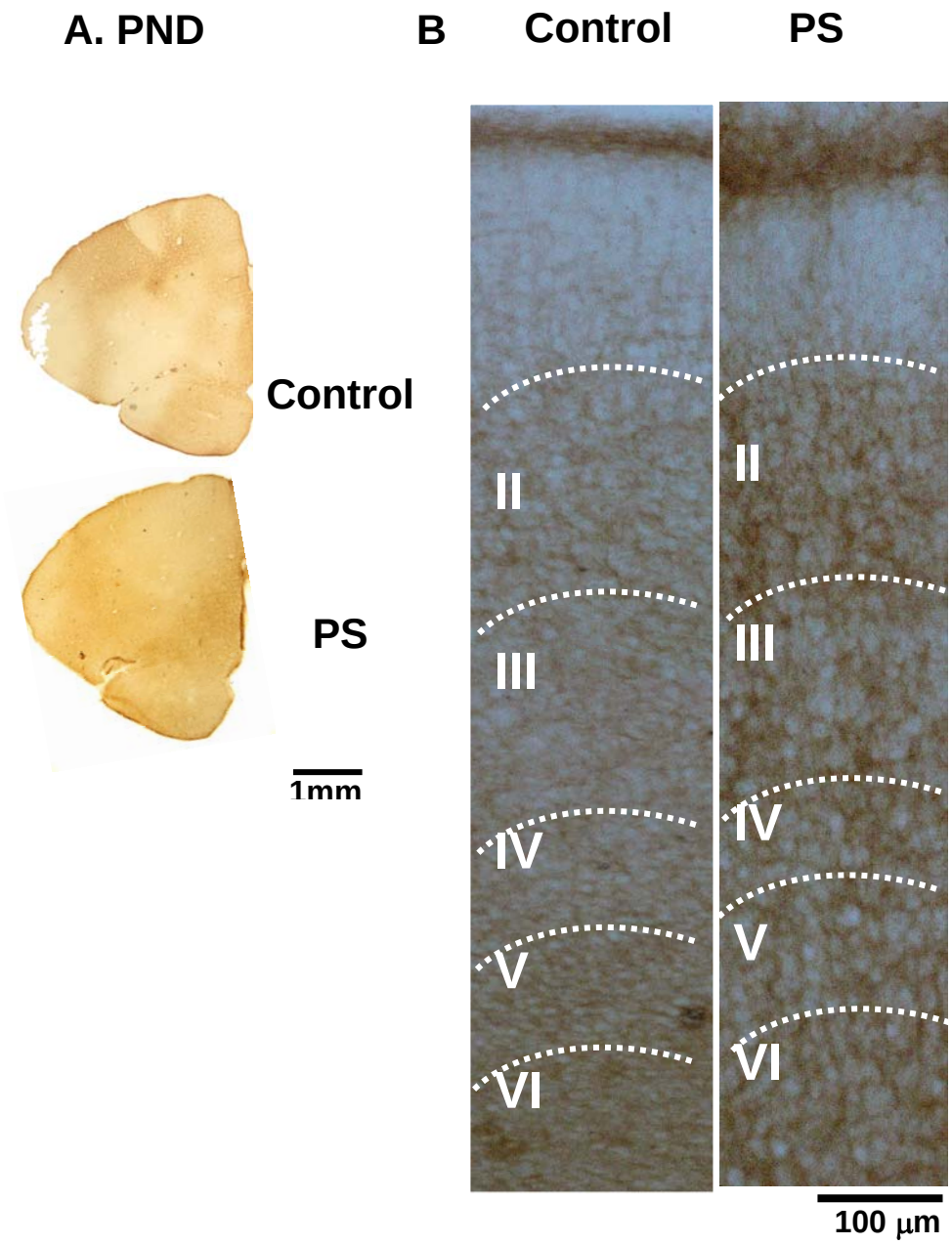

Figure 5.13 A. Photomicrographs showed the GAP-43 IR in the prefrontal cortex section of rat pups at PND 21 compared between control and prenatal stress group, respectively. B. Photomicrographs showed the GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 21 compared between control and prenatal stress group, respectively. C. Photomicrographs showed the GAP-43 IR at PND 21 in prefrontal cortex of rat pups compared between control (A, C, E, G and I) and prenatal stress group (B, D, F, H and J), respectively.

PND21

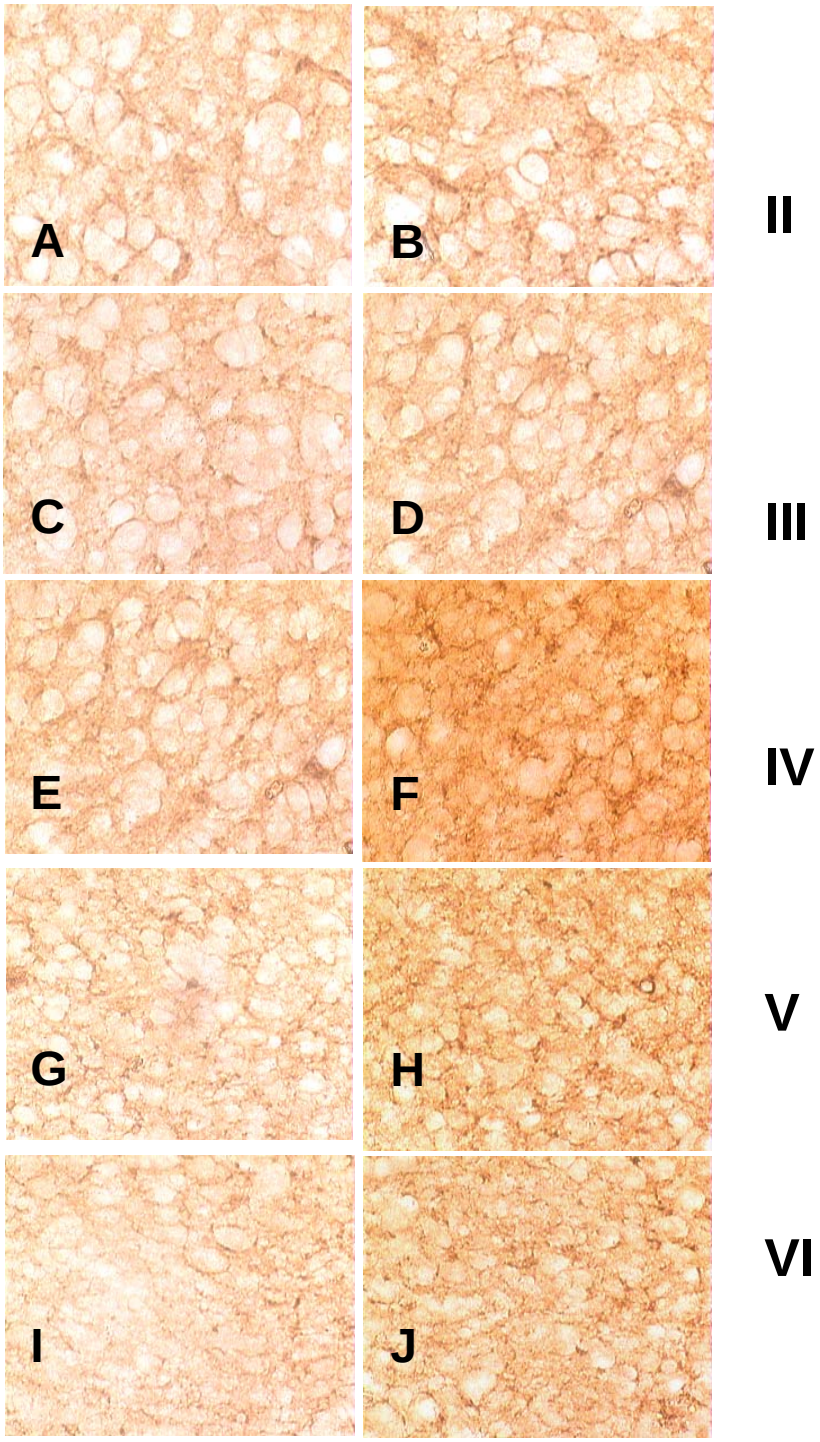

Fig. 5.13 C

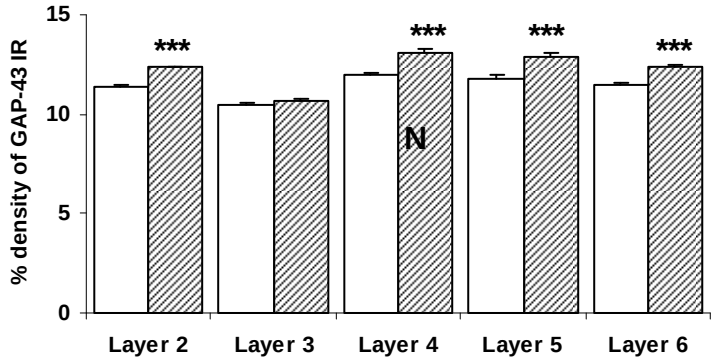

Figure 5.14. Bar graph showed the percent density of GAP-43 IR in different layers of prefrontal cortex of rat pups at PND 21. The percent density of GAP-43 IR were measured using Image Tools software as described under the experimental methods. Each value represents mean ± SEM. Each mean value represents the value measured from 30 photographs in each rat (n=8). **p<0.001, ***P<0.0001 compared with the other layers.

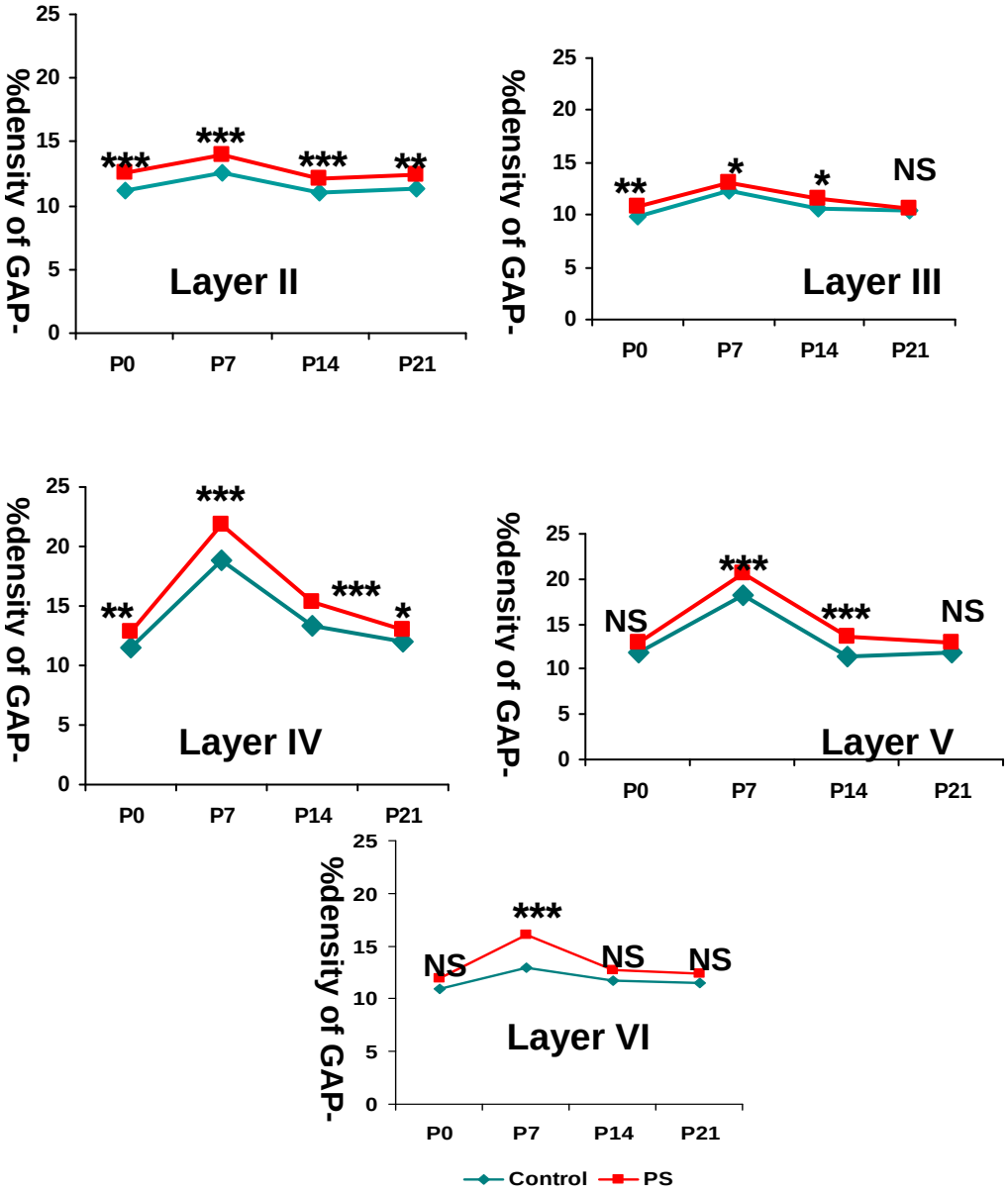

Figure 5.15 Graphs show the density of GAP-43 IR in different layer of prefrontal cortex compared between control and PS group at different postnatal period. Data represent Mean ± SEM (N=8 for each group). ***p<0.0001, **p<0.001, *p<0.01 and NS, not significantly difference, compare to control group.

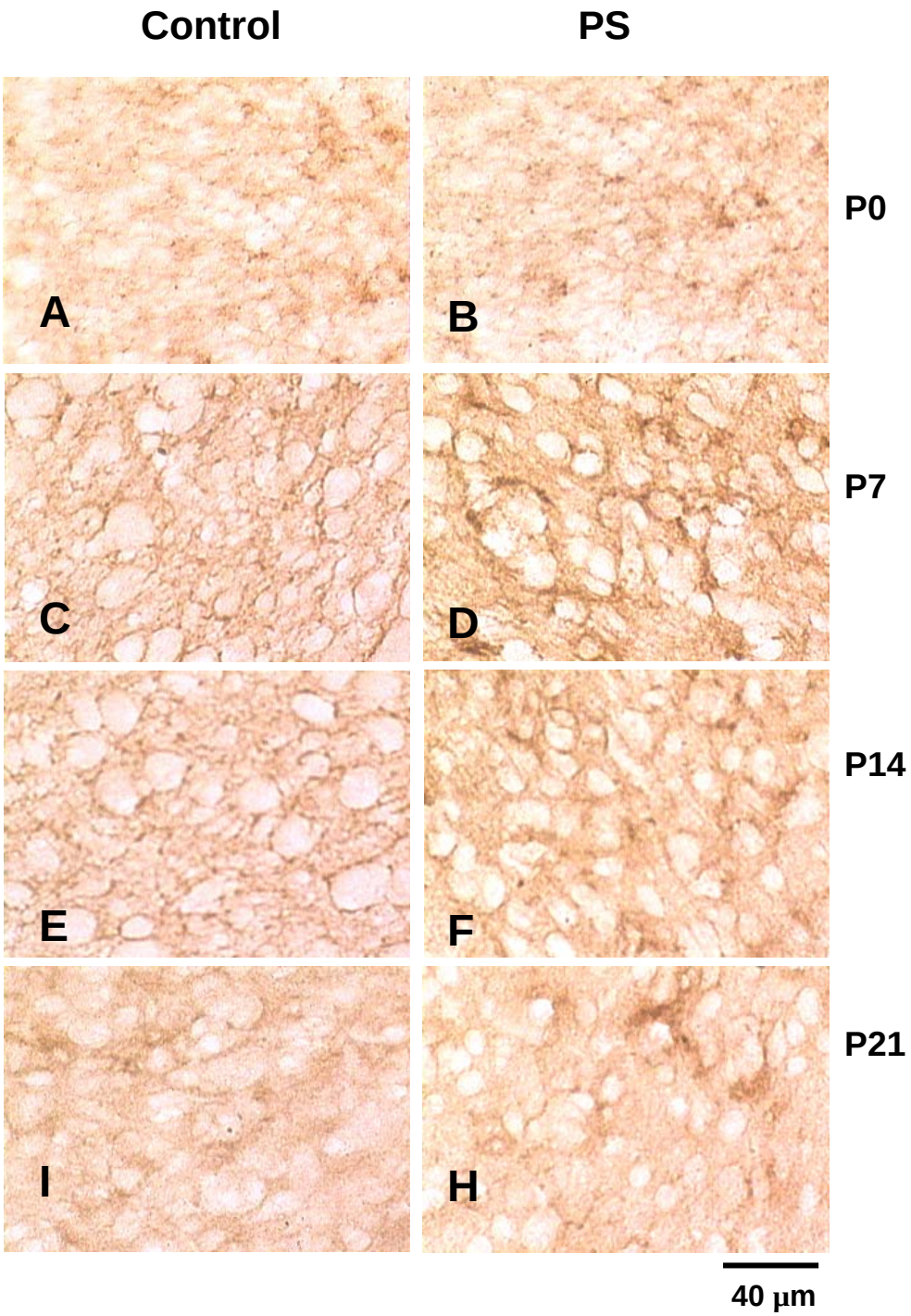

Figure 5.16 Photomicrographs showed the GAP-43 IR in the layer IV of prefrontal cortex of rat pups compare between control (A, C, E and G) and prenatal stress group (B, D, F and H) at different postnatal stages from PND 0, 7, 14, and 21, respectively.

Table 5.2 Data showed the % density of GAP-43 IR in prefrontal cortex compared between control and prenatal stress group at different postnatal stages from PND 0, 7, 14, and 21. Data represent Mean ± SEM (N=8 for each group). ***p<0.0001, **p<0.001, *p<0.01 and NS, not significant difference, compare to control group.

% density of GAP-43 in prefrontal cortex				
	P0	**P7**	**P14**	**P21**
Control				
layer2	11.25 ± 0.17	12.51 ± 0.13***	11.02 ± 0.10	11.40 ± 0.10
layer3	9.91 ± 0.32	12.25 ± 0.12*	10.64 ± 0.13	10.48 ± 0.05
layer4	11.52 ± 0.24	18.14 ± 0.26***	13.27 ± 0.26	11.97 ± 0.08
layer5	11.94 ± 0.14	18.16 ± 0.30***	11.42 ± 0.15	11.82 ± 0.13
layer6	10.97 ± 0.26	12.96 ± 0.22***	11.75 ± 0.12	11.50 ± 0.07
PS				
layer2	12.62 ± 0.68**	13.93 ± 0.14*	12.17 ± 0.21**	12.35 ± 0.05***
layer3	10.85 ± 0.22NS	13.09 ± 0.33NS	11.47 ± 0.06NS	10.70 ± 0.12NS
layer4	12.91 ± 0.22**	21.84 ± 0.40***	15.28 ± 0.13***	13.08 ± 0.19***
layer5	12.65 ± 0.26NS	20.59 ± 0.39***	13.58 ± 0.28***	12.86 ± 0.19***
layer6	11.97 ± 0.26NS	16.06 ± 0.34***	12.74 ± 0.28***	12.40 ± 0.10***

5.5 Effects of maternal corticosterone injection on GAP-43 IR in prefrontal cortex

In this experiment, we proposed that prenatal stress exerts its effects via an increase in the levels of stress hormone. Therefore, this experiment was designed to examine whether matwernal corticosterone CORT injection during GD14-21 could affect the density of GAP-43 IR in prefrontal cortex of rat pups. The CORT animals were divided into 2 groups; 1) CORT injection and 2) vehicle group. Pregnant rats in CORT injection group received daily intrasubcutaneous injection of CORT (40 mg/kg) whereas pregnant in vehicle group received seasame oil injection at the same volume. The result shown that CORT injection induce increased in the percent density of GAP-43 IR in all cortical layers of prefrontal cortex compared to vehicle injection group ($p<0.0001$). The result of CORT injection were comparable to those found in PS group as shown in figure 5.17-5.19.

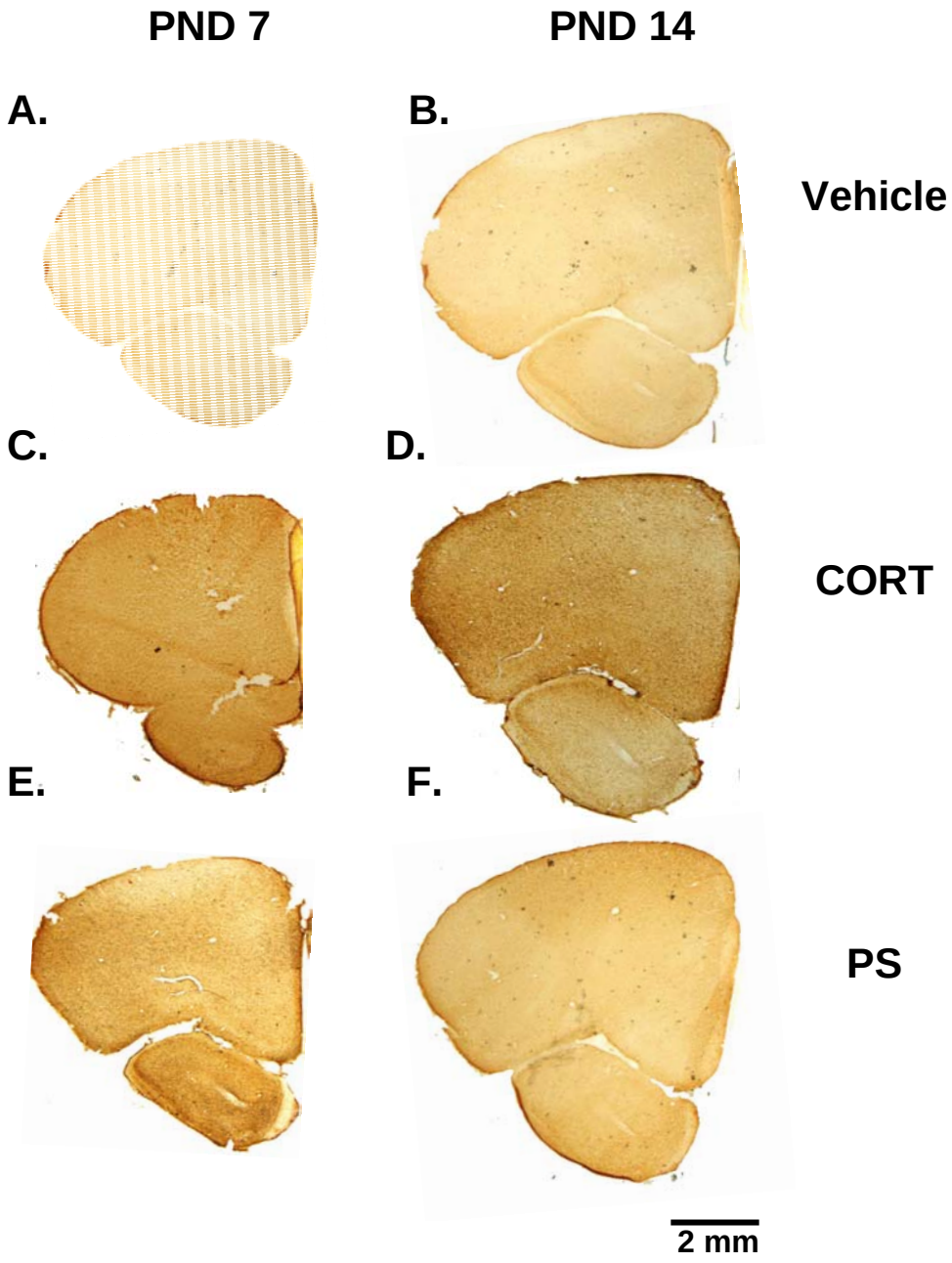

Figure 5.17 Photomicrographs showing the density of GAP-43 IR in the prefrontal cortex section of rat pups compared between vehicle (A, B), CORT injection group (C, D) and prenatal stress group (E, F).

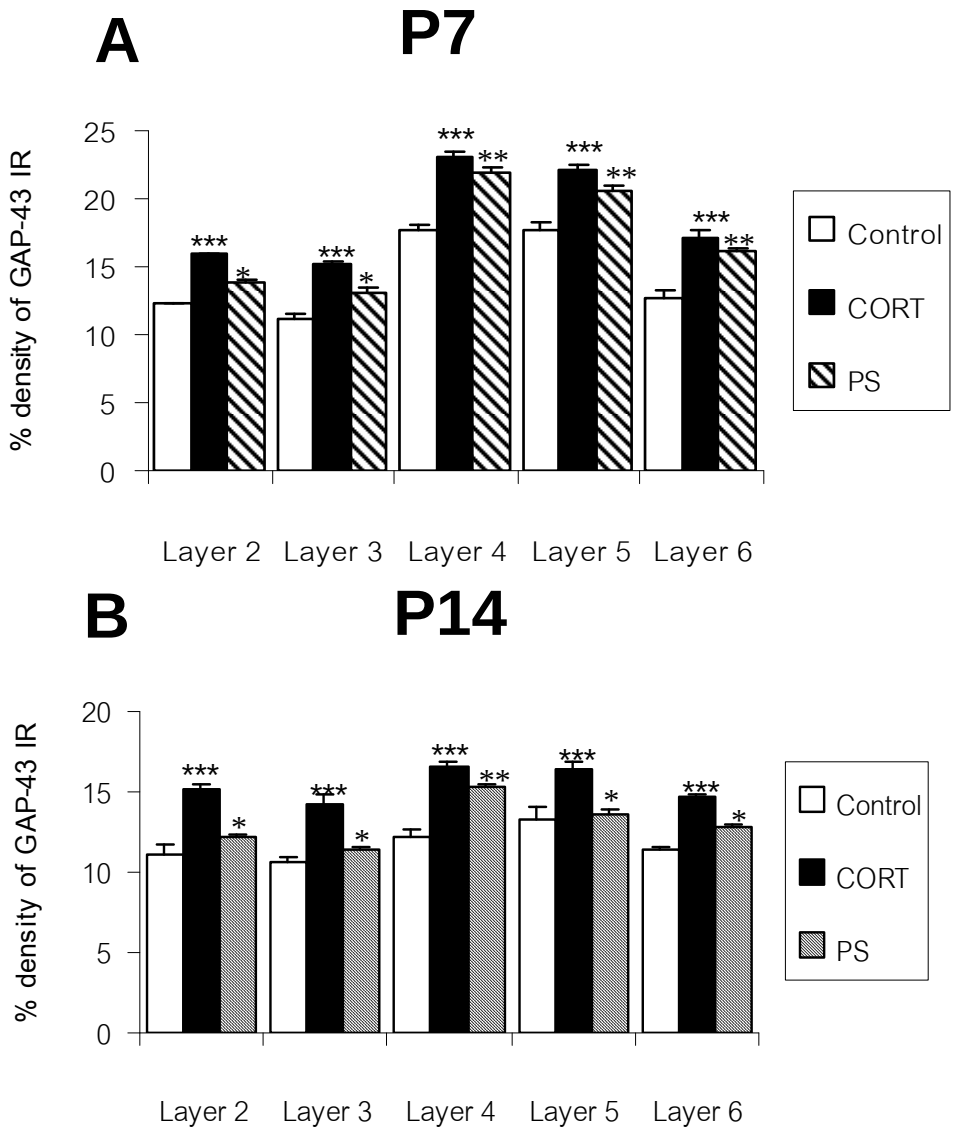

Figure 5.18 Bar graphs show the percent density of GAP-43 IR in different layers of prefrontal cortex of rat pups compared between control group, CORT injection and prenatal stress (n=8 for each group) at PND 7 (upper panel) and PND 14 (lower panel). The percent density of GAP-43 IR was measured using Image tools software as described under the experimental methods. Each value represents Mean ± SEM. Mean value from each layer represents the mean value from 30 photographs from each rat (N=8 for each group). ***P<0.0001, **P<0.001, *P<0.01 and NS, not significant difference, compare to control group.

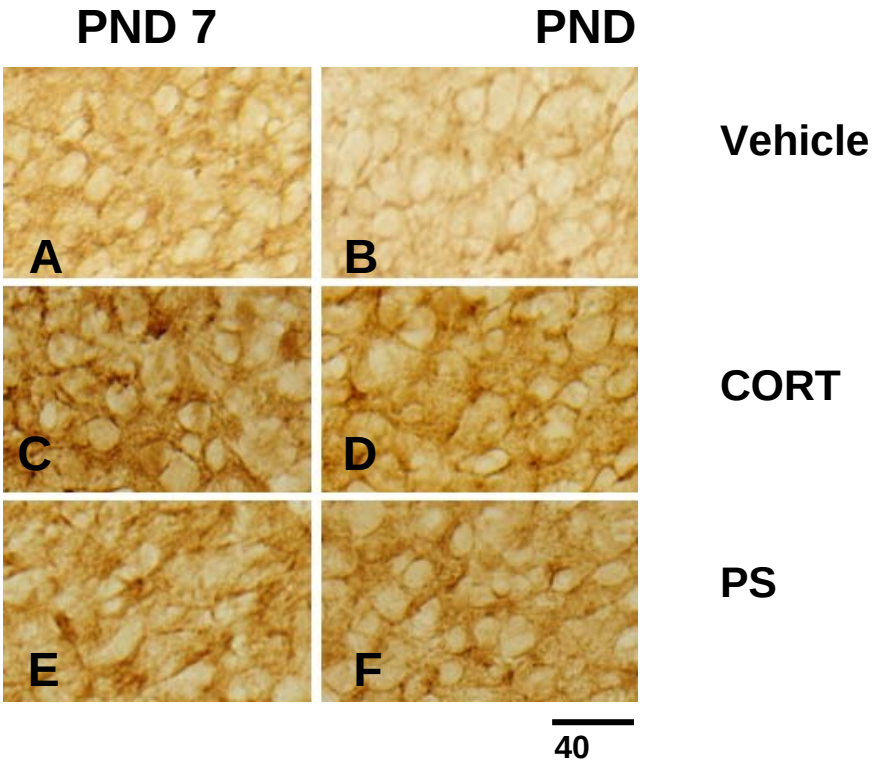

Figure 5.19 Photomicrographs show the GAP-43 IR in layer IV of the prefrontal cortex of rat pups Compared between vehicle injection (A,B), CORT injection (C,D) and prenatal stress (E,F) at PND 7 and PND 14, respectively

Table 3. Data showed the % density of GAP-43 IR in prefrontal cortex compared between sham control, prenatal stress and CORT injection group, at different PND 7 and P14. Data represent Mean ± SEM (N=8 for each group). ***p<0.0001, **p<0.001, *p<0.01, compare to control group.

% density of GAP-43 IR in prefrontal cortex		
	P7	**P14**
Sham Control		
Layer2	12.64 ± 0.37	11.01 ± 0.71
Layer3	11.17 ± 0.39	10.63 ± 0.37
Layer4	17.73 ± 0.73	12.24 ± 0.35
Layer5	17.76 ± 0.83	12.27 ± 0.75
Layer6	12.66 ± 0.61	11.46 ± 0.17
Prenatal stress		
Layer2	13.93 ± 0.14**	12.17 ± 0.21**
Layer3	13.09 ± 0.33**	11.47 ± 0.06**
Layer4	21.84 ± 0.40***	15.28 ± 0.13***
Layer5	20.59 ± 0.39***	13.58 ± 0.28**
Layer6	16.06 ± 0.34***	12.74 ± 0.28**
CORT injection		
Layer2	15.92 ± 0.09***	15.11 ± 0.37***
Layer3	15.12 ± 0.35***	14.29 ± 0.49***
Layer4	23.24 ± 0.33***	16.57 ± 0.26***
Layer5	22.24 ± 0.47***	16.46 ± 0.48***
Layer6	16.96 ± 0.58***	14.66 ± 0.24***

CHAPTER 6
DISCUSSION

6.1 Development of GAP-43 in prefrontal cortex of rat pups

In the present study the developmental change of GAP-43 IR in prefrontal cortex of neonatal rat were examined by using immunohistochemical technique. At birth, GAP-43 was already present at low level in all layers of prefrontal cortex. GAP-43 IR increased over time until it reached the highest densities at PND 7. Mostly GAP-43 IR was found in the nerve fiber around the cell body of neuron, in the proximal part of dendrite and in the axon terminal of all neuronal cells especially in the layer II, IV, V and VI. Expression of GAP-43 was observed shortly after the onset of neurogenesis (Enderlin et al 1987) and the period that density of GAP-43 IR reaches the highest level at PND 7 corresponds to the peak period of synaptogenesis in the prefrontal cortex (Blaesing et al 2001). The results suggested that GAP-43 might have some important roles during early postnatal period. The developmental pattern of GAP-43 IR found in this study was corresponded to previously report in other animal species.

In the neocortex, the mature cortical plate has histologically distinct laminae, classified as layers I through VI, and a subplate (layer VII). Layer VI is the deepest layer of the cortical plate; i.e., it is closest to the geometric center of the brain. Layer I is the most superficial layer; it is the outward-most subpial layer. The formation of layers VII and I is distinct from the cell migrations that populate layers VI through II (Marin-Padilla 1971). Cells from the ventricular zone populate layers VI-II in an inside-out fashion, meaning that the deeper layers form first (Miale & Sidman 1961). By GD 16 and 17 in the rat, the first cells are arriving in the area that will ultimately form the laminae of the cortical plate. Through the remainder of gestation, the cortical plate gets thicker as more cells migrate from the ventricular zone. In the days before parturition, layer VI is histologically distinct from the remainder of the maturing cortical plate.

During postnatal development, the cortical layers still continue to develop. The first phase (PND 1-18) is dominated by differentiation of the neurons within the cortical plate and by the formation of the cortical layers. At PND 1, regional differences are observed in the cytoarchitecture of the cortical plate which correspond to the future subareas of the prefrontal cortex. The formation of layer IV occurs in the dosolateral cortex around PND 6, and from this age agranular prefrontal cortex is well demarcated from the other pert of the prefrontal cortex. Between PND 6 and 10, the cortical plate has disappeared and all cortical layers can be recognized in the prefrontal cortex. On PND 5 of the rat, layers VI and V are readily distinguishable, but layers IV-II are not as clearly defined. These superficial layers (IV-II) are more distinct in adulthood but never constitute more than one-third of the cortical widths. The remaining two-thirds of the cortical width are split approximately equally between layers VI and V, depending on the neocortical region of study (Ignacio et al 1995). In addition to the migration of neurons to the cortical plate, other factors modify the lamination of the neocortex including cell packing density, cell size, extra cellular matrix, gliogenesis, myelination, and synaptogenesis of cortical afferents/ efferents, all of which contribute to the final differentiation of this neural structure. In general, these cortical areas are homologous between species, with notable differences in the relative size of structures, cell number, and extent of extra cellular neuropil.

The expression of neuronal specific growth-associated genes such as α-tubulin, MAP-2 and GAP-43, have been proposed as putative markers of neurite outgrowth in the cultured neurons (Kim et al 1999a, Kim et al 1999b, Li et al 1999). GAP-43 is a neuron-specific protein which exhibits elevated synthesis and fast transport during development and nerve regeneration. Correlation of increased GAP-43 expression with axonal growth has been demonstrated in the CNS of fish (Grinvald et al 1986) amphibians, and mammals (Skene and Kalil, 1984; Freeman et al., 1986). Similar increases relative to normal mature nerves are seen in the developing of mammalian CNS (Skene & Willard 1981a) and in the regenerating PNS (Skene & Willard 1981b). Increased expression is also observed in cultured neurons when maintained under conditions which promote neurite outgrowth, in contrast, it was not observed following injury in CNS of higher vertebrates where axonal regeneration does not occur (Skene & Willard 1981b). Spatiotemporal changes in GAP-43 content and

localization in the brain are also positively correlated with neuronal cell growth. The levels and rates of synthesis of GAP-43 in rat cerebellum and cerebral cortex are about 10 folds greater in neonates compared with adults (Jacobson et al 1986). During development, GAP-43 is localized to growing neuronal processes, while in the mature brain it is present in most neuropil areas and is especially rich in structures exhibiting synaptic plasticity (McGuire et al 1988). Consistent with this latter observation, elevated levels of GAP-43 transcripts are observed in cell bodies of neurons synapse in plastic areas of the adult brain (Neve et al 1987, Rosenthal et al 1987). These observations suggest that the growth- and plasticity associated changes in GAP-43 synthesis rate and steady-state levels are mediated through changes in its mRNA levels. Identification of GAP-43 with the independently studied of its phosphoproteins suggests that its function in synaptic plasticity may be dependent on post-translational modification, as well as its level of expression (Basi et al 1987, Karns et al 1987, Nielander et al 1993, Snipes et al 1987). Induction and maintenance of LTP in the hippocampus is correlated specifically with increased of GAP-43 phosphorylation (Routtenberg 1995) suggests that it might play a direct role in the processes underlying synaptic plasticity.

The time course of GAP-43 synthesis and steady-state abundance was studied in detail in both cerebral cortex and cerebellum. In the cerebral cortex, synthesis of GAP-43 is relatively high at birth, and increases about 30-40% in the first week. There after, the synthesis declines sharply during the second week. Over the next 4 weeks, the level of synthesis declines to the adult level, approximately 20-fold lower than the 7 days level. A similar pattern is seen in the cerebellum over the same time periods, except that the maximum relative synthesis of GAP-43 appears to occur at or before the time of birth. GAP-43 synthesis in relative to total protein synthesis is approximately 10 times greater in a newborn rat cerebellum than in adults. The steady-state abundance, shown by Coomassie staining of GAP-43 also declines with increasing age. However, the abundance of the protein remains relatively constant for several weeks after GAP-43 synthesis begins to decline, indicating that degradation of GAP-43 in the older animals is slow, with a half-life on the order of weeks. The results from the present study suggest that the peak of the axonal growth in prefrontal cortex

occur during the 1st postnatal week and GAP-43 is important for the development of the cortical layer of the prefrontal cortex of neonatal rat.

6.2 Prenatal stress induce increase in GAP-43 level in prefrontal cortex

Prenatal stress animals provide an important model to investigate how early environment can influence fetal brain development. Previous studies reported that pregnant rat exposed to restraint stress produced significant elevations in the maternal levels of plasma CORT which may directly influence the development of offspring after crossing the placenta and entering into the fetal circulatory system and brain. The stressors such as exposure of rats to a novel environment resulted in a rapid and reversible impairment of plasticity in the prefrontal cortex and this effect involve the actions of glucocorticoids. In the present study, effect of prenatal stress on GAP-43 IR in prefrontal cortex of neonatal rat was examined. The results show that nerve fiber containing GAP-43 in prefrontal cortex of rat pups were increased following challenges with stress in mother. Since the expression of GAP-43 was observed shortly after the onset of neurogenesis (Enderlin et al 1987) and the period that the density of GAP-43 IR reaches the highest level at PND 7 corresponds to the peak period of synaptogenesis in the prefrontal cortex (Blaesing et al 2001), thus, the result suggests that maternal stress during the 3rd week of gestation may disrupt the process of axonal growth and synaptic formation in the prefrontal cortex of rat pups. An intermediate level of GAP-43 has been reported for the optimal survival of neuron in layer IV. When level of GAP-43 was higher than normal, neuron in layer IV was more compensated to the effect of prenatal stress (Kandel et al., 2000). At the same time, completely lack of GAP-43 has no further increase resistance of neuron in layer IV, but rather increased vulnerability. Thus, increase of GAP-43 may alter the pattern of axonal growth and formation of synapse because postnatal day 7-14 has been reported to correlate with the peak period of synaptogenesis in the rat prefrontal cortex.

In transgenic mice with reduced or no GAP-43 (GAP43 +/- and GAP43-/-), the outgrowth of 5-HT projections from raphe nuclei innervate several brain regions was disrupted (Donovan et al., 2002). In wild-type mice, at PND 7, 5-HT axons densely innervate layer IV of primary somatosensory cortex and hippocampus is clearly visible. In contrast, the absence of GAP-43 causes a persistent and severe disruption in

the pattern of 5-HT axon ingrowths, for examples, some brain areas shows markedly devoid of 5-HT axons, whereas other regions are hyperinnervated. These differential effects on 5-HT axon ingrowths may have relevance to developmental disorders that involve regionally specific changes in monoamine expression, such as autism and schizophrenia. Based upon the observations that 5-HT innervated in cortex and hippocampus are decreased in GAP-43 -/- mice and immunohistochemical analysis proposed that lack of GAP-43 results in increased activation of calcium-sensitive potassium channels (Donovan et al., 2002). Perhaps the moderate decrease in GAP-43 level might allow for activation of such potassium channels as well as for calcium buffering. Studies of developing neurons in culture have shown that there is a narrow range of intracellular free calcium levels that is optimal for promoting cell survival and neurite outgrowth (Mattson 1992, Mattson et al 1991). It may therefore be the case that an intermediate density of GAP-43 results in intracellular calcium concentration that is within the optimal range. A study of the relationship between cellular expression of GAP-43 and neuronal vulnerability to transient global forebrain ischemia revealed a strong inverse correlation in immature animals.

These results support the role of GAP-43 in the formation of cortico-cortical connection (Barbas & Rempel-Clower 1997). In the present study, the percent density of GAP-43 IR in prefrontal cortex of pups from prenatal stress group was increased, especially in layer IV and V, when compared to control group. Prenatal stress induce small increase of GAP-43 IR in prefrontal cortex of pups when observe at birth, but the effect was clearly observed during PND 7-21. Our findings show the significant alterations in density of GAP-43 IR in prefrontal cortex in response to the prenatal exposure to stress hormone. The prefrontal cortex is a particularly sensitive and vulnerable brain region that responds to stress and stress hormones. This brain area appears to be differentially susceptible to the toxic effects of prenatal stress and selective vulnerability of different layer of prefrontal cortex seems to be correlated with GAP-43 content within the axon terminal. A previous study demonstrated that in area 10 in prefrontal cortex of patients with schizophrenia, expressions of GAP-43 IR resistant to neurotoxicity induced either by glutamate or calcium ionophore (Jacques et al 1993). In the present study, we provide evidence that prenatal stress induced significantly long lasting increase in the density of GAP-43IR in several layers of

prefrontal cortex of neonatal rat pups when compared to control pups. However, it is interested that, layer III is insensitive to the effect of prenatal stress since prenatal stress induced no change in GAP-43 IR in layer III of prefrontal cortex.

Several studies reported the modulatory roles of GAP-43 on stress induced injury to prefrontal cortex neurons (Alonso et al 1994). GAP-43 has been reported in the rat prefrontal cortex during postnatal development where the densities of GAP-43 are stimulated by glucocorticoid (Aleksandrov et al 1999). In this experiment, we proposed that prenatal stress might exert its effects via increase level of stress hormone. In order to test this hypothesis, effects of CORT or vehicle injection during pregnancy period on the density of GAP-43 IR in prefrontal cortex were studied. CORT injection (40 mg/kg) in to the pregnant rat during GD14-21 resulted in an increase in the density of GAP-43 IR in all prefrontal cortex layers compared with vehicle injection group. The results suggest that effects of prenatal stress on GAP-43 IR might be mediated via an increase level of corticosteroid hormone.

CORT have a profound effect on brain function. In the hippocampus, CORT can mediate activation or repression of target genes via coordinated manner by mineralocorticoid (MR) and glucocorticoid receptors (GR). CORT-responsive hippocampal genes regulated via MR and/or GR were studied and compared under different conditions of CORT exposure by using SAGE (Serial analysis of gene expression) (Nicole et al 2001). This study had identified a number of genes already known to be CORT-responsive gene. An example of a known CORT-responsive gene is GAP-43, of which it was already documented that mRNA levels are increased by ADX in the hippocampus and this increase was prevented by administration of corticosterone to ADX animals (McEwen 1994). It is possible that GAP-43 gene in prefrontal cortex could also be regulated by CORT. In contrast, other known corticosteroid-responsive genes with lower expression levels by ADX are; the 5-HT1a receptor (Chalmers et al 1993, De Kloet et al 1986, Meijer et al 1997), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (β-FGF) and FGF receptors. The putative CORT-responsive genes identified provide insight into the molecular mechanisms underlying the differential and sometimes opposing effects of MR and GR on neuronal excitability, memory formation and behaviour, as well as their role in neuronal protection and damage (Nicole et al 2001).

Prefrontal cortex is a target for glucocorticoids involved in the stress response (Meaney MJ, Aitken DH. 1985). [3H]dexamethasone binds to receptors in frontal and prefrontal cortex at about 75% of the concentration found in hippocampus. In addition, [3H]dexamethasone binding in frontal cortex is altered by both corticosterone treatment and adrenalectomy, indicating the presence of endogenously regulated corticosterone receptors (Meaney et al 1985). Furthermore, prefrontal cortex is also involved in many of the tasks that are influenced by chronic elevations of circulating glucocorticoids. For instance, lesions of prefrontal cortex in rats impair spontaneous alternation, radial maze performance, and passive avoidance. Likewise, lesions of prefrontal cortex in primates impair inhibition of the line-of-sight response (Dias et al 1997). Thus, potential alterations in prefrontal cortex may mediate some corticosterone-induced behavioral changes. In addition, chronic elevations of corticosterone result in decreased expression of the neural cell adhesion molecule (NCAM) (Sandi & Loscertales 1999), a cell surface macromolecule involved in regulating aspects of synapse stabilization, which suggests the possibility of structural changes as a result of chronic stress levels of corticosterone.

The effects of chronic corticosterone administration on dendritic morphology of pyramidal neurons in layer II-III of medial prefrontal cortex have been studied. Glucocorticoid administration has been reported to induce morphological changes in the medial prefrontal cortex. In corticosterone treated animals, redistribution of apical dendrites has been reported (Arnsten 1997). The amount of dendritic material proximal to the soma was increased relative to intact rats, while distal dendritic material was decreased relative to intact animals. Thus, chronic glucocorticoid administration dramatically reorganized apical arbors in medial prefrontal cortex. These reorganizations likely reflect the functional changes and may contribute to stress-induced changes in cognition. Since glucocorticoid receptors are plentiful in medial prefrontal cortex (Meaney et al 1985), the reorganization of apical dendrites could be a direct effect of corticosterone in prefrontal cortex. Alternatively, it is possible that the atrophy of distal dendrites of layer II-III pyramidal neurons is the result of loss of input from CA3 pyramidal neurons. In addition to produce regressive dendritic changes in CA3 pyramidal neurons (Woolley et al 1990), both administration of corticosterone and chronic stress can alter hippocampal excitability (De Kloet et al

1986, Foy et al 1987). Hippocampal areas CA1 and CA3 both project directly to medial prefrontal cortex, and the projection from CA3 to cingulated cortex terminates exclusively in layer I (Swanson 1977). Electrophysiological data suggest that excitatory synapses on proximal apical dendrites of prefrontal neurons serve to amplify EPSPs generated in distal apical dendrites (Seamans et al 1997). The increase in proximal dendrites could be a compensatory response to distal atrophy an attempt to maintain the excitation provided by reduced distal inputs. Chronic corticosterone administration can produces serotonergic alterations in prefrontal cortex (Crayton et al 1996, Inoue et al 1996, Luine et al 1993, Takao et al 1997), while stress alters glutamate release in prefrontal cortex (Moghaddam 1993, Moghaddam 1994, Moghaddam et al 1997). These findings suggest that similar neurochemical mechanisms could mediate the dendritic alterations observed in hippocampus and prefrontal cortex as a result of chronic elevations of corticosteroid hormone.

The corticosterone-induced reorganization of apical dendrites reflects important functional changes in medial prefrontal cortex. Pyramidal neurons segregate their inputs in the piriform cortex, in which, the distal part of the apical dendrites of layer III pyramidal neurons receives extrinsic inputs, while more proximal portions of the apical dendrite as well as the basilar dendrites, receive intrinsic inputs (Albano et al 1973). While the segregation of inputs to pyramidal neurons in neocortex is not as straightforward, pyramidal neurons in medial prefrontal cortex nonetheless tend to segregate inputs, with extracortical afferents (for instance, from the mediodorsal nucleus of the thalamus and hippocampal area CA3) tended to cluster on distal dendrites, i.e., in layer I (Groenewegen 1988, Swanson 1977) and synapses of local cortical circuits tended to cluster on proximal portions of the apical and basilar arbor (Scheibel & Scheibel 1970). Thus, the corticosterone induced reorganization of the apical dendrites of layer II-III pyramidal neurons in medial prefrontal cortex likely results in a shift in emphasis from subcortical to intracortical information. This functional reorganization of individual neurons has important implications for the functioning of medial prefrontal cortex and behaviors. Thus, the corticosterone-induced changes in dendritic morphology of medial prefrontal cortex may contribute to stress-induced cognitive changes.

In this study, we provide the evidence that prenatal stress alters the density of GAP-43 containing in axon terminal in prefrontal cortex of rat pups. Prenatal stress induced neurotoxicity in prefrontal cortex may be mediated by elevating levels of excitatory amino acid and subsequent calcium influx into neurons that are associated with HPA axis. The disturbance of Ca^{2+} homeostasis has been proposed as a common step in the development of cell dysfunction in the CNS. Differences from the rapid and transient changes occurring in physiological condition, as a sustained increase in cytosolic Ca^{2+} concentration is highly associated with neuronal damage (Nicotera et al 1992, Orrenius et al 1992). An increase in the intracellular Ca^{2+} concentration appears to mediate the toxicity of several neurotoxic agents, which induce either alterations in the physical integrity of the plasma membrane or mitochondrial impairment and consequent ATP depletions. It has been reported that GAP-43 gene expression is subject to both positively control by nerve growth factor and negative control by glucocorticoids in PC12 cells, which are believed to be resemble to the precursor cells of adrenomedullary lineage (Howard et al 1988). Both effects are direct and do not requiring new protein synthesis. Interestingly, cycloheximide can further augment the dexamethasone suppression of GAP- 43 mRNA perhaps due to inhibition of synthesis of an mRNA stabilizing protein. In vivo, the GAP-43 gene is highly regulated in neuron. Peak levels in the animal are achieved at the time of neurite growth, relating either to normal development or to regeneration. In the present study, an increase in the density of GAP-43 containing in axons terminal in prefrontal cortex of rats pups suggest that elevated level of CORT during gestation period may be harmful to the developing brain and GAP-43 containing in axon terminal were upregulated in order to protect neuron against the toxicity effect of stress hormone. These changes may lead to an alteration in the density and types of synapses in prefrontal cortex since PND 7-14 is the peak period of synaptogenesis in this brain area.

6.3 Possible mechanism underlying prenatal stress induce increase in GAP-43

The possible mechanism of prenatal stress induces GAP-43 in prefrontal cortex of neonatal rat can be either direct or indirect effect of maternal CORT. For the direct effect, maternal CORT can directly affect the development of fetal brain when crossing the placenta and entering into the fetal blood circulation. A number of genes

have already known to be CORT-responsive; an example of a known CORT-responsive gene is GAP-43, of which it was already documented that mRNA levels are increased by ADX in the hippocampus and that this increase was prevented by administration of corticosterone to ADX animals (McEwen 1994). For the indirect effect, adrenal steroids may exert their effects on neuronal structure and function through the regulation of expression of neurotrophic factors. In the hippocampus, adrenalectomy not only resulted in the decreases the level of basic fibroblast growth factor and neurotrophin-3 mRNA but also induced increases the level of brain-derived neurotrophic factor mRNA levels (McEwen 1994). Early maternal separation was found to increase NGF and BDNF expression in the dentate gyrus and hilus of hippocampus, amygdala, thalamus and other brain areas that project axons to prefrontal cortex (Cirulli et al 1998). It has been reported that BDNF can led to *de novo* upregulation (a threefold increase) of GAP-43 and L1 mRNA in axotomized RGCs (Sandi & Loscertales 1999). Moreover , NGF can directly increases expression of several other genes, such as c-fos, NGFIA, NGFIB, β–actin, and intermediate filaments (Milbrandt 1987). There are several remarkable differences between the NGF regulation of these genes and NGF regulation of GAP-43. For some, such as c-fos, NGFIA, and NGFIB, NGF induction is rapid, exerted within minutes, and declines after several hours. This is in contrast to the NGF effect on GAP-43 expression which is slower in the onset and persistent. The delayed response of GAP-43 to NGF suggests that it may fall into a different class of NGF-regulated genes than do c-fos, NGFIA, NGFIB, etc., and may play a role in longer term adaptation rather than in immediate responses. GAP-43 transcription is suppressed by corticosteroids, and the concomitant presence of NGF does not prevent this repression (Howard et al 1988). Thus, GAP-43 is dually regulated by NGF and corticosteroids in a manner at least compatible with the known divergent effects of these modulators of cell fate (Ignacio et al 1995).

Cell adhesion molecules such as N-CAM and L1 have been shown to stimulate axonal growth through the activation of fibroblast growth factor receptor (FGF-R) (Williams & Goldman-Rakic 1998). Moreover, GAP-43 has been shown to be required for N-CAM-stimulated neurite outgrowth (Meiri et al 1986). Consequently, bFGF may triggers intracellular events that lead to a translocation of GAP-43 present

in the cytosol to peripheral complexes that are firmly attached to the substrate and stabilized by the actin cytoskeleton. Moreover, it has been shown that cerebellar neurons from GAP-43 knock-out mice are unable to extend neurites over N-CAM-expressing fibroblasts (Meiri et al 1986). This work has also demonstrated that GAP-43 is phosphorylated at Ser41 in isolated growth cones exposed to bFGF or N-CAM soluble chimeras, thus establishing an association between FGF-R activation, GAP-43 phosphorylation, and neurite outgrowth.

During development, the numerical balance between excitatory (glutamatergic) neurons, inhibitory (GABAergic) interneuron, serotonergic, dopaminergic and thalamic projection to the prefrontal cortex is relatively conserved across mammalian species. Although the data from the present study do not reveal the identity of the nerve fibers, synapses and types of neurotransmitters, other studies have shown that prenatal stress can alters the release of noradrenaline, dopamine, serotonin and glutamate in the pup's brain (Weinstock 2001; Berger et al. 2002). Therefore, the disruption of axonal growth during development is likely to have functional consequences. Even minor disturbances in the balance of excitation and inhibition may be profound. Interneuron not only regulate the degree of excitation in the prefrontal cortex but also essential for the fine-tuning of neuronal circuits and determining the quality of information processing in prefrontal cortex, the area playing an important role in learning, behavioral, cognition and memory. Because GAP-43 regulates growth of axons and modulates the formation of new connections, our findings suggest that prenatal stress may have an effect on axonal growth in the prefrontal cortex of the offspring. Consistent with this, the results suggest that prenatal stress during the vulnerable period might cause the long-term effects on density of projection and might interfere with the balance between excitatory and inhibitory processing in the prefrontal cortex.

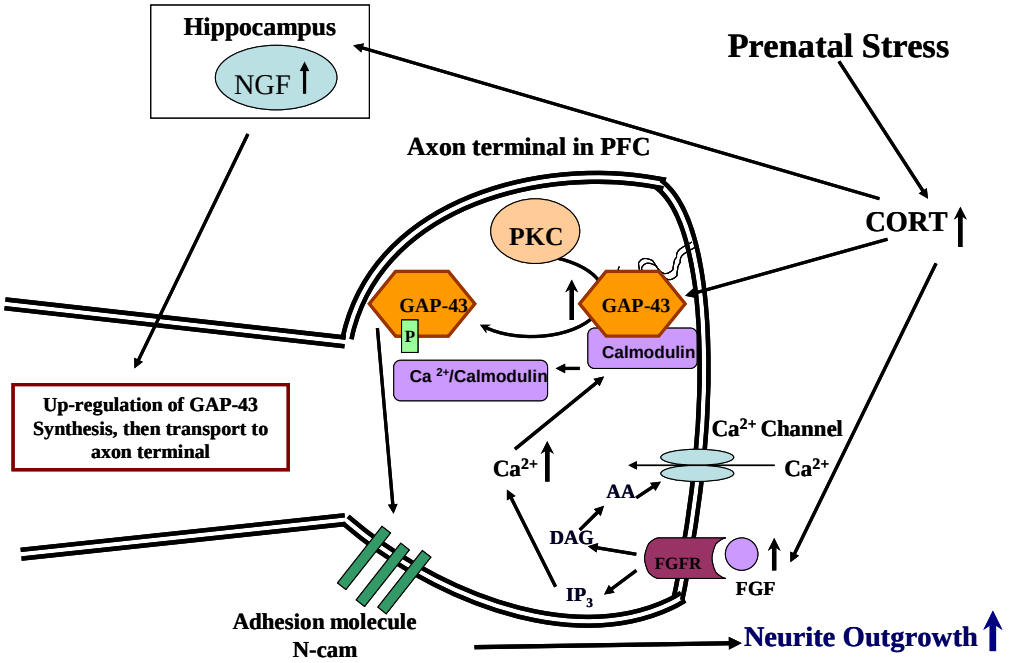

Figure 6.1 The simplified proposed mechanism of the prenatal stress effects on the level of CORT. Exposure of prenatal stress can induce the increase of CORT. The schematic diagram illustrated CORT signaling and the mechanism of NGF receptor in the intracellular space. NGF unregulated GAP-43 level. The increase of CORT level might dedicate to direct and indirect increase of GAP-43 under stress condition. According to Cirulli and his colleaguse in 1998, the increase of CORT can induce the volume of NGF in the hippocampus, which might correspond with Howard and Meiri's studies in 1986 and 1988, that the increase of NGF can upregulate GAP-43 in the prefrontal cortex. Alternative direct pathway , concluded by McEwen in 1994, the increase of CORT under stress condition can directly stimulate and increase the density of GAP-43in the prefrontal cortex.

CHAPTER 7
CONCLUSION

The developmental changes of GAP-43 IR in prefrontal cortex of neonatal rat were examined by using immunohistochemical technique. At birth, GAP-43 was already present at low density in all layers and the density increased over the time until it reached the highest density at PND7 and then slightly decline to saturate level at PND21. The effects of prenatal stress on GAP-43 IR in neonatal rat prefrontal cortex were examined in detail in this study. Maternal stress during GD14-21 induces increase in the percent density of GAP-43 IR in prefrontal cortex of rat pups. The effect was found at PND7 than the other periods. In prenatal stress pups the percent density of GAP-43 IR was increased especially in layer II, IV and V. At PND14 and PND21, pups from prenatal stress dam still exhibit a significant higher percent density of GAP-43 IR when compared to control group. The result suggest that prenatal stress might have long term effect for at least 21 days on the density of GAP-43 containing in prefrontal cortex of rat pups. Prenatal stress induced neuronal toxicity in prefrontal cortex may mediates by elevating level of excitatory amino acids that associated with HPA axis. The upregulation of GAP-43 may serve as a protective mechanism against the toxicity effect of maternal stress or CORT hormone. The finding of the present study support evidence for a neuroregulatory role of maternal corticosteriod on development prefrontal cortex. GAP-43 plays an important role in axonal growth and synaptogenesis, thus change in the density of GAP-43 IR may effect the processing of their roles and subsequence function of prefrontal cortex in later life. On the other hand, change in GAP-43 density during critical period may affect several steps of brain development such as a formation of neuronal circuit. The present study suggest that developing brain is vulnerable to environmental factors such as prenatal stress, which can cause long term effect on it structure and function in later life.

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