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# Inhibitory interneurons in the anterior cingulate and medial prefrontal cortex in prenatally malnourished rats

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SCHOOL OF MEDICINE

Thesis

**INHIBITORY INTERNEURONS IN THE ANTERIOR CINGULATE AND  
MEDIAL PREFRONTAL CORTEX IN PRENATALLY MALNOURISHED RATS**

by

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B.M., Sun Yat-sen University, 2012

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**ABSTRACT**

Prenatal protein malnutrition continues to be a significant problem in the world today. Exposure to prenatal protein malnutrition increases the risk of a number of neuropsychiatric disorders that are associated with inhibitory interneurons, including depression, schizophrenia and attention deficit hyperactivity disorder. Previous studies have found that neurons in anterior cingulate and medial prefrontal regions respond excessively to restraint stress in prenatally malnourished rats. In this study, we investigate if prenatal protein malnutrition affects inhibitory the subpopulation of interneurons in the prefrontal cortex in relationship to the higher initial stress response. This was done using double-labeling immunohistochemistry with c-Fos to mark activated neurons and parvalbumin to mark inhibitory interneurons. Numbers of single and double-labeled neurons were quantified with unbiased stereology. Statistical analysis demonstrated that there was no effect of prenatal malnutrition on the total number of neurons or on the number of parvalbumin neurons. However, prenatal malnutrition was associated with a significant increase in the number of inhibitory parvalbumin positive neurons activated by restraint stress. This suggests that prenatal malnutrition altered the excitability of these inhibitory interneurons either directly or by altering their connectivity.

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## INTRODUCTION

### **Prenatal malnutrition and clinical studies**

The World Health Organization has reported hunger and related malnutrition as the greatest single threat to the world's public health (2008). Nutrients management before and during pregnancy has profound effects on brain development of infants (Georgieff, 2007). Adult survivors of the Dutch and Chinese famines showed an increased morbidity of schizophrenia (Susser and Lin, 1992; Susser et al., 1996), and a higher risk of coronary heart disease (Osmond et al., 2000). In the cohort study conducted by Galler and her colleagues in Barbados since 1990s, children exposed to prenatal protein malnutrition and early childhood malnutrition showed cognitive and behavioral impairments throughout childhood, adolescence, into adulthood (Galler et al., 2012). These impairments included elevated prevalence of depressive symptoms (Galler et al., 2010) and higher risk of attentional deficits (Galler et al., 2012). Recent studies showed the previously malnourished subjects have impaired IQ and academic skills as well as a lower personality trait scores even later in mid-adulthood (Galler et al., 2013 a, b). The changed personality profiles involve heightened anxiety, depression and vulnerability to stress, lowered interpersonal orientation, lowered intellectual curiosity, coupled with withdrawal, distrust, and lowered sense of self-efficacy or competence.

One problem associated with clinical studies is that it is difficult to study the effects of pre- and postnatal malnutrition separately. It is likely that a majority of infants and children who have experienced various degrees of prenatal nutritional deprivation,

and also malnourished after birth of inadequate nursing from their malnourished mothers. Even given a limited degree of fetal brain sparing in utero (Dobbing and Sands, 1979), malnutrition prior to birth can disrupt certain basic developmental and organizational features (Susser and Lin, 1992; Susser et al., 1996).

### **Animal model for prenatal protein malnutrition**

As one of the essential factors in daily diet, understanding the consequences of protein deficiency on brain development and adult brain functions is important for world public health. Here, we introduced a rodent model that has been widely used to study prenatal protein malnutrition (Tonkiss and Galler, 1990; Galler and Tonkiss, 1991; Tonkiss et. al 1993). In this model, pups are exposed to a low protein diet (6% casein) in utero compared to an isocaloric normal protein diet (25% casein) as control. Both malnourished and control pups are cross-fostered with well-nourished (25% casein) dams at birth. Since prenatally malnourished dams tend to spend more time in the nesting area with their litters (Massaro et al., 1997), this model also eliminates the effects of varied maternal behavior. Prenatal protein malnutrition affects adult rat brain function and behavioral development despite diet rehabilitation at birth in various ways.

Neuroanatomical studies have demonstrated that prenatal protein malnutrition changes the total neuron numbers of in specific brain regions. Lister and his colleagues (2005) have shown a reduction of neurons in CA1 subfield of hippocampus in prenatal protein malnutrition rats, accompanied with a loss of volume in CA1 subfield and subiculum. The hippocampus CA1 pyramidal cells project to various cortical and subcortical areas, including the medial prefrontal cortex, (Groen and Wyss, 1990). The

projection to the prefrontal cortex is of interest because it may relate to the attentional deficits (Galler et al., 2012) found in clinical studies that have been attributed with the interactions between hippocampus and the medial prefrontal cortex (Vertes, 2006). A reduction in the granule cells of cerebellum has also been reported (Hillman and Chan, 1981). However, this change has not been related to cognitive or behavioral impairments caused by prenatal protein malnutrition.

Neurophysiological studies have found alterations in the electrophysiological properties of neuronal population and in long-term potentiation among prenatally malnourished subjects. Whole-cell patch clamp recordings have demonstrated increased miniature inhibitory postsynaptic currents generated by CA1 pyramidal cells in vitro rat hippocampal slices a result of prenatal protein malnutrition (Luebke et al., 2000). Long-term potentiation has been widely used to quantitatively assess the efficacy of neuronal transmission in prenatal protein malnutrition model (Galler et al., 1990). Prenatally malnourished rats are unable to transfer enhanced cellular activation (population excitatory postsynaptic potential slope enhancement) into enhanced cellular discharge (population spike attitudes enhancement), which indicates enhancement of synaptic efficacy (Bronzino et al., 1997). Taken together, these results suggest an overall increased inhibitory system and an altered neuroplastic responses within the hippocampal formation in prenatally malnourished subjects.

Changed sensitivity to centrally acting drugs in rats that have experienced prenatal protein malnutrition also has been reported. In the Morris water maze test, prenatally malnourished rats showed less selectivity to the amnestic effects of

benzodiazepines, a positive modulator of GABA<sub>A</sub> receptors that induces spatial learning deficits, by displaying shorter travel distance during acquisition and a more selective search of the target in the probe trial (Tonkiss et al., 2000a, b). These results provide further support for functional changes within the GABAergic system subsequent to prenatal malnutrition.

Another interesting finding is that prenatal protein has been reported to affect sleep as well. Prenatally malnourished rats spend 20% more time in slow wave sleep (SWS) compared to the control rats, and total percentage of time spent in rapid eye movement (REM) sleep has reported to be 61% less (Galler et al., 2000). In addition, when subject to restraint stress, sleep disturbances may become more pronounced in prenatally malnourished rats (Tonkiss et al., 2006). Taken together, prenatal protein malnutrition is sufficient to alter the quality and quantity of adult sleep rats.

Altered cognitive functions, as well as disturbed learning and memory have also been documented in prenatally malnourished rats (Levitsky and Strupp, 1995). In the Morris water maze test, prenatally malnourished rats are slower to learn an efficient search strategy, and are impaired in recalling the former position of the platform on the probe-trial (Lukoyanov and Andrade, 2000), indicating that prenatal protein malnutrition affects spatial working memory and spatial learning. This may be associated with the effects of prenatal protein malnutrition on the hippocampal damage, which is essential for spatial learning and memory (Lister et al., 2005). Since the complex functions of learning and memory are widely distributed, this finding also suggests that other brain areas in

addition to the hippocampal formation are most likely impacted by protein malnutrition as well.

Prenatal protein malnutrition leads to wide array of behavioral impairments in various aspects. It significantly decreases both playful and non-playful social behavior of juvenile rats (Almeida et al., 1996). As a result of impulsivity, prenatally malnourished rats display a greater willingness to enter the open arms in the elevated plus maze test, and show lower latency to enter the open arms when being placed in an enclosed arm of an elevated T-maze (Almeida et al., 1996a,b). Both findings suggest prenatally malnourished subjects have either an increased impulsiveness or a lower level of anxiety in front of an aversive environment. Behavioral alterations have been documented in behavioral flexibility. A recent study has demonstrated that it takes more trials for prenatally malnourished rats before they successfully shift to a new cue in attentional set shifting test compare to control rats, indicating deficits in attentional shifting (McGaughy et al., unpublished).

### **Prenatal protein malnutrition and stress response**

The mechanisms through which prenatal protein malnutrition affects behavior include altering offspring's responses to stress (King et. al, 2004; Kehoe et. al, 2001). Kehoe et al (2001) showed that prenatally malnourished pups have lower stress hormone levels when exposed to acute maternal separation at postnatal day 9 compared to control pups. Even later as adults, prenatally malnourished rats exhibiting a lower level of anxiety in the face of an aversive environment (Almeida et al., 1996a,b). By measuring blood pressure, Tonkiss and his colleagues (1998) have demonstrated that prenatally

malnourished males exhibit higher initial responses to an acute stressor. Given the differential pattern of responses to stress, one would predict that prenatal protein malnutrition affect multiple brain systems that contribute to affective behaviors.

To identify which brain region mediates the higher initial stress response, Rosene and his colleagues (2004) examined the expression of c-Fos 3 h after 20 min restraint stress in a restraint tube. The cellular immediate-early gene c-Fos, which has a transient expression in neurons, responds immediately to extracellular stimuli and helps initiate intracellular cascade to reflect the neuronal activity (Sagar et al., 1988; Chen et al., 1993). Fos immunohistochemistry provides a cellular method to identify cell groups that may be linked into functional circuits in a situation-specific manner (Hoffman et al., 1993). Furthermore, c-Fos mRNA and Fos protein have been readily demonstrated in afferent pathways and implicated as mediating the neuroendocrine responses in the stress paradigm (Chen et al., 1993). Using unbiased stereology, they have found that prenatally malnourished rats showed greater number of c-Fos positive neurons than well-nourished rats in the anterior cingulate and adjacent medial prefrontal regions within stress condition. However, the exact neuronal cell types that were more activated remain unclear.

The prefrontal cortex is known to inhibit hypothalamo-pituitary-adrenocortical (HPA) axis via bysynaptic glutamate-GABA connections (Ziegler and Herman, 2002), and lesion of the medial prefrontal cortex increases corticosterone levels in responses to restraint stress (Viau et al, 1993). There is also considerable evidence that stress affects the prefrontal cortical structure and function (Arnstan, 2009). For example, stress alters

the morphology of dendrites in rat medial prefrontal cortex (Wellman and Cook, 2004; Miller et al., 2006), and stress results in increased dopamine and norepinephrine in rat medial prefrontal cortex (Finlay et al., 1995; Moghaddam, 2002). Given the important inhibitory role of the medial prefrontal cortex in stress response, with the considerable evidence that prenatal protein malnutrition affects inhibitory network in adult brain, we hypothesized that prenatal protein malnutrition increases the activation of inhibitory interneurons in the prefrontal cortex of behavioral stressed rats.

In the current study, we investigated inhibitory interneurons in the anterior cingulate and medial prefrontal cortex in prenatally malnourished rats. After subjecting prenatally malnourished and control rats to 20 min restraint stress and processing their brains for double-labeling immunocytochemistry with c-Fos and parvalbumin, we quantified the labeled neurons using unbiased stereological methods. This study addressed three main questions: (1) Does prenatal protein malnutrition affects the total neuron numbers in the prefrontal cortex; (2) Does prenatal protein malnutrition affects the neuronal numbers of inhibitory interneurons in the prefrontal cortex; and (3) whether stress alters the c-Fos expression in parvalbumin positive interneurons in the prefrontal cortex as a result by prenatal protein malnutrition.

## **METHODS**

### **Subjects and Animal Model**

Subjects were Long-Evans rats (Charles River Laboratories, Kingston, MA). Animals were housed at the Center for Behavioral Development and Mental Retardation at Boston School of Medicine. All procedures followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Boston University. Five weeks before mating, virgin female rats were assigned into one of the two diet conditions: either a diet of adequate protein (25% casein) or an isocaloric diet of deficient protein (6% casein) (Tonkiss and Galler, 1990). One week before mating, male rats were acclimated to the same diets of the mating females. These diets were allowed ad libitum throughout gestation (Teklad Laboratories, Madison, WI). Each male was mated with two female that received the same dietary. After mating, the males and females were housed individually and the females continued to receive the respective diet until parturition. After parturition, litters were culled to 16 pups each and fostered to other well-nourished (25% casein) mothers who had given birth during the same 24-hour period. After weaning all offspring were maintained with same-sex littermates with free access to standard Purina rat chow (Purina Mills, Richmond, IN; Formula 5001). Pups born to mothers provided with the 6% casein diet and cross-fostered to dams given the 25% casein diet were designated as “6/25”. Pups born to mothers provided with the 25% casein diet and cross-fostered to dams given the 25% casein diet were designated as “25/25”.

### **Behavioral Stress and Blood Collection**

On the day of perfusion, each of eight rats were assigned into the four conditions: 6/25 stressed, 6/25 unstressed, 25/25 stressed and 25/25 unstressed. These rats were blind coded to eliminate investigator bias. For the stress condition, rats were brought into the test room individually between 9:00 and 12:30 am. They were placed into the restraint tube for a 20 min period. To obtain their pre-stress corticosterone levels, a blood sample was taken from the tail immediately before they were introduced into the restraint tube. Blood from the same rats was taken immediately after the restraint, providing a measure of post-stress corticosterone levels. Three hours after the onset of stress, rats were removed from the home cage individually and prepared for perfusion. For the unstressed condition, age- and nutrition- matched rats stayed in their home cage until perfusion. All rats were perfused within the same time period between 12:00 and 3:30 pm.

### **Corticosterone Measurements**

Plasma from all rats was measured as previously described (McCormick and Mahoney, 1999) using a radioimmunoassay based on Martin et al. (1977). A highly specific corticosterone antiserum (B3-163, Endocrine Sciences, Tarzana, CA) and [<sup>3</sup>H] corticosterone (88.0 Ci/mmol New England Nuclear, Boston, MA) was used as a tracer. The antiserum cross-reacts slightly with deoxycorticosterone (< 4%), but not with cortisol, aldosterone, and progesterone (< 1%). Intra- and interassay variability are consistently less than 5 and 10%, respectively.

### **Tissue Preparation and Histochemistry**

Rats were anesthetized with sodium pentobarbital and transcardially perfused for 15 min with 200 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS)

solution. Brains were immediately removed and transferred to a cryoprotectant solution consisting of 10% glycerol with 2% dimethylsulfide (DMSO) in 0.1 M PBS for 24 h. They were then transferred to a solution consisting of 20% glycerol with 2% DMSO in 0.2 M PBS for 48h. They were then frozen in isopentane at -75 degrees and stored in -80 degrees before cutting (Rosene et al., 1986). Brains were sectioned at 30µm thickness on a sliding microtome in the transverse plane. Sections were divided into eight interrupted series and stored in a 15% glycerol solution in 0.1M PBS at -80 degrees until processed.

One series was processed for thionin stain. The thionin staining sections were immediately mounted onto gelatin-subbed slides, dried overnight, dehydrated in descending graded alcohols, cleared in xylene and coverslipped with Permount (Fisher Scientific Cat SP-15). This thionin stained series was used for quantification of cortical volume and neuron number, while the remaining series were saved for immunohistochemistry.

### **Immunocytochemistry**

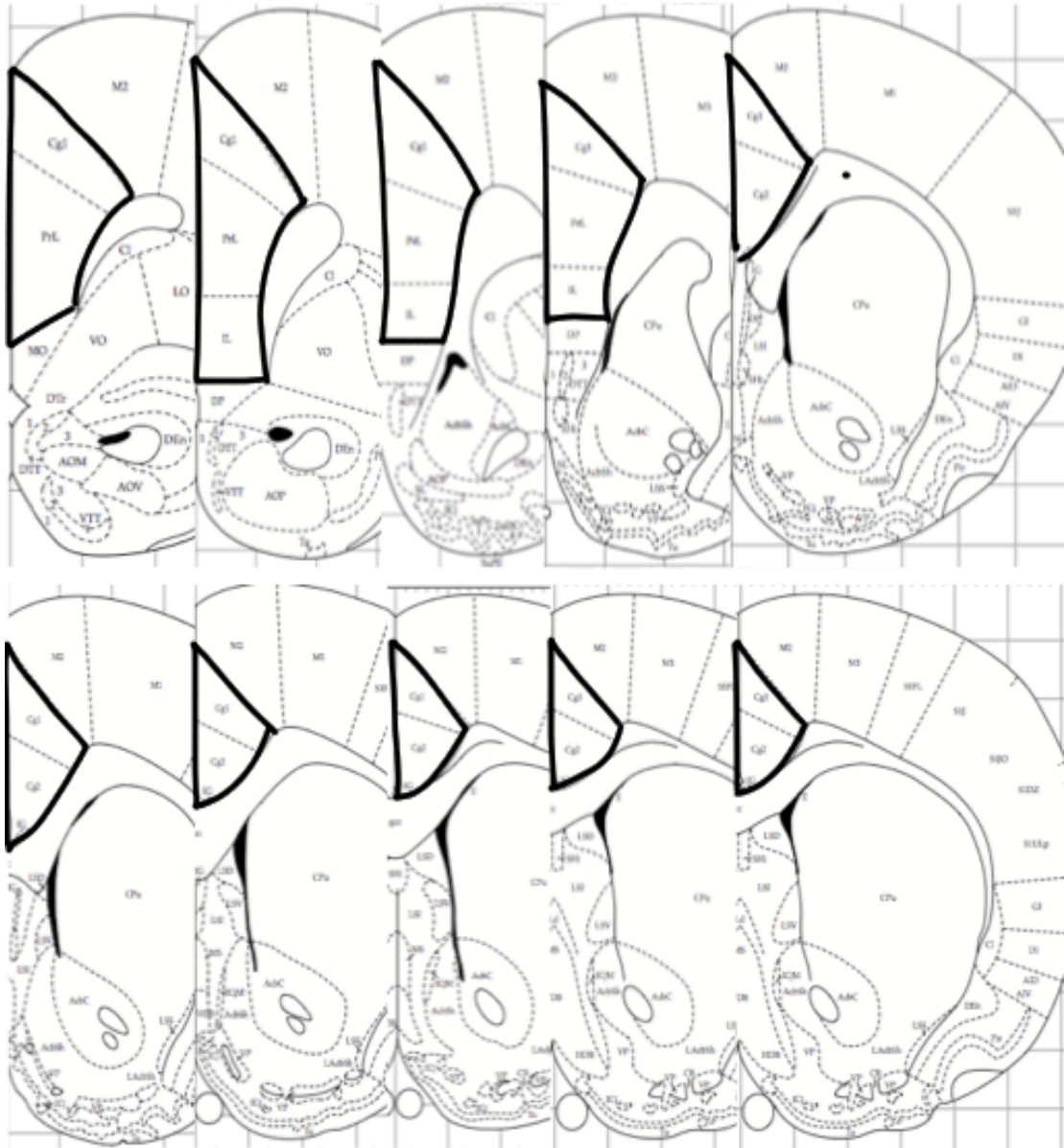
For immunocytochemistry, one series of tissue from all rats were thawed then processed together. All sections were rinsed in 0.05 M tris-buffered saline (TBS) solution three times for 5 min each. Next, sections were incubated for 30 min in a 3% H<sub>2</sub>O<sub>2</sub> solution to quench the endogenous peroxidase activity and rinsed again in 0.05 M TBS three times for 5 min each. Following blocking with 10% normal goat serum (GIBCO BRL, Grand Island, NY) and 0.4% Triton-X in TBS for 2 hours, sections were incubated in anti-c-Fos antibody (polyclonal, Catalog# PC-38, Oncogene Sciences, Cambridge, MA)

at a dilution of 1:10000 for 48 h at 4°C. After rinsing with a block solution (2% normal goat serum with 0.1% Triton-X in TBS) three times for 5 min each, sections were incubated for 2 hrs at room temperature in biotinylated goat secondary antibody (anti-rabbit immunoglobulin, Vector Laboratories, Burlingame, CA) at a dilution of 1:600 in the stock solution. Next, they were washed in the stock solution three times for 5 min each followed by treatment with ABC reagent (Vector Laboratories, Burlingame, CA) for 60 min. Following washing in TBS three times for 5 min each, sections were placed in a Ni-DAB (Sigma, St. Louis, MO) solution (250 mg Nickel (II) sulfate, 2mg diaminobenzidine and 83µl 3% H<sub>2</sub>O<sub>2</sub> in 10 ml 0.175 M sodium acetate) for 6 min. Sections were then washed in sodium acetate three times and followed by 0.05 M TBS three times for 5 min each.

The sections to be double-labeled for parvalbumin were rinsed three times with TBS and quenched with 3% H<sub>2</sub>O<sub>2</sub> as above. They were then incubated in anti-parvalbumin antibody (monoclonal, Lot# 10-11 (F), Switzerland) for 24 hrs at 4°C. The procedures described above were followed except adopting anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) as a secondary antibody. Parvalbumin immunoreactivity was visualized using a DAB (Sigma, St. Louis, MO) solution for 6 min. The sections were then mounted onto gelatin coated glass slides, dried overnight, dehydrated in ascending alcohols, cleared in xylene and coverslipped with Permount (Fisher Scientific Cat SP-15).

## **Stereology**

Unbiased stereology was conducted using StereoInvestigator software (MicroBrightField, Colchester, VT) on a computer coupled to a Nikon light microscope. The region of interest (ROI) for stereology is the most anterior part of the cingulate cortex (ACg) and the adjacent medial prefrontal cortex (mPFC). As shown in Figure 1, the ROI was bounded ventrally by corpus callosum and ventrally by the transition from agranular to granular cortex. Rostrocaudally, the sections were selected between the first appearance of corpus callosum (Bregma 3.70 mm) and the first appearance of anterior commissure (Bregma -0.26 mm). The estimates were conducted using the optical fractionator method (West et al., 1991). Briefly, a grid was selected to overlay the ROI. At each grid intersection, a three-dimension probe (the optical dissector) was placed. Cells were counted only if they reach focus within the dissector frame, which was set with two exclusion lines and exclusion planes at both the top and bottom of each dissector. Sampling grid and frame size was optimized to get a coefficient error lower than the biological variability.



**Figure 1 Overview of ROI at different levels.** Stereological estimates were made between the first appearance of the corpus callosum (Bregma 3.70mm) and the first appearance of anterior commissure (Bregma -0.26mm). The ROI includes anterior cingulate (ACg), the prelimbic area (PrL) and the infralimbic area (IL). Cc, Corpus callosum.

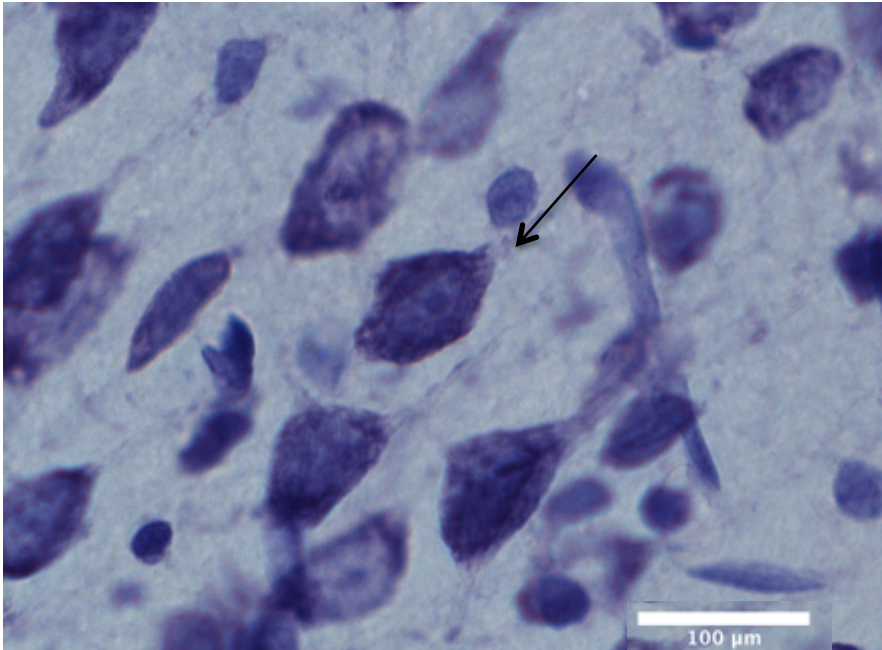
The total neuron number was estimated using a series of thionin staining sections containing the entire ROI. As shown in Figure 2, cells were counted if they met the following criteria: (1) polygonal soma; (2) clearly visible large nucleus surrounded by darkly stained cytoplasm containing Nissl bodies within the inclusion boundaries (Lister et. al, 2005). The ROI was outlined using a 4× objective and counting was performed using a 60×oil objective. A  $500 \times 500\mu\text{m}$  grid and a  $50 \times 50\mu\text{m}$  frame were used for counts. The interrupted series containing every sixteenth sections, so the section sampling fraction (ssf) was 1/16. Since the average thickness of sections shrank to  $\sim 8\mu\text{m}$  after thionin staining, exclusion planes were not used. We focused through the sections during counting, so the height of the optical dissector was the actual section that thickness and the thickness sampling fraction (tsf) was 1.

Counted c-Fos positive nuclei were classified into two categories based on the intensity of staining as determined by visual inspection. The two categories were heavy and light. As shown in Figure 3a, heavily stained cells had a nucleus that was completely filled with dark black reaction product. For light staining cells, the nucleus was at least partially labeled with grey reaction product. Identified neurons classified into one of the two categories were then marked and sampled using a  $400 \times 400\mu\text{m}$  grid and a  $100 \times 100\mu\text{m}$  frame. Counted parvalbumin positive interneurons were counted if they followed the criteria: (1) brown cytoplasm; (2) clearly seen projections, as shown in Figure 3b. The size of dissect grid was  $500 \times 500\mu\text{m}$ , frame was  $100 \times 100\mu\text{m}$ . As shown in Figure 3c, d,

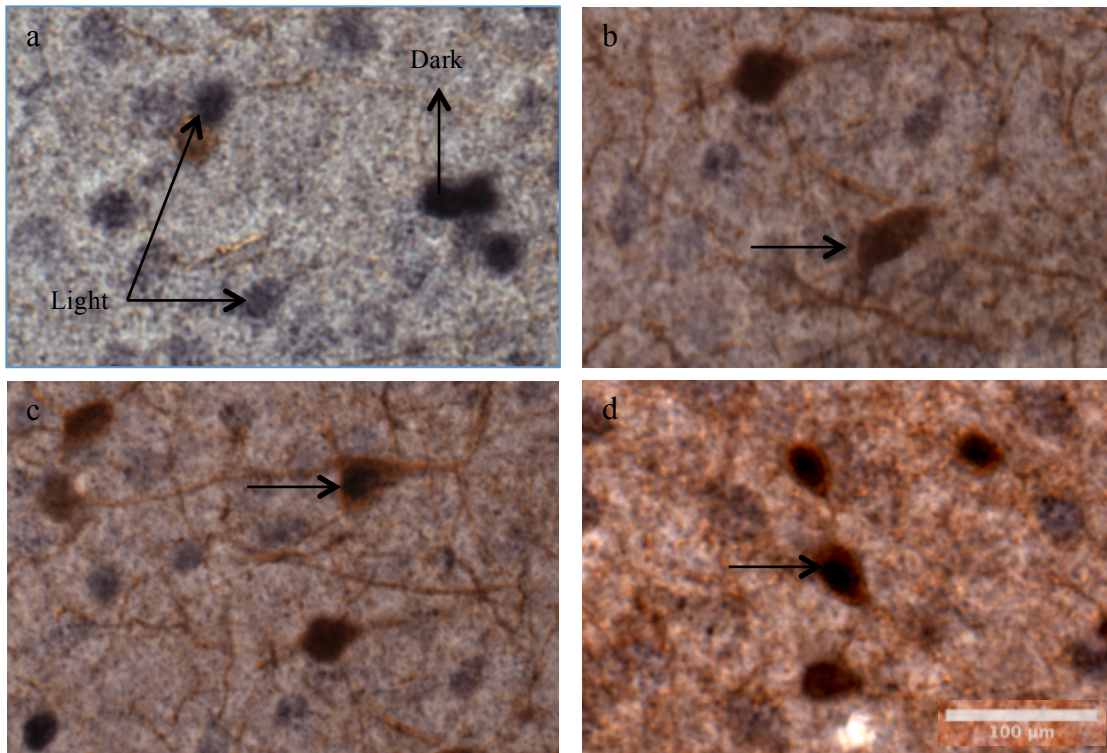
double-labeling neurons with c-Fos and parvalbumin were identified by clearly outlined dark staining nucleus surrounded with brown cytoplasm. Corresponding to the criteria used for classifying c-Fos staining, counted double-labeled neurons were marked into two categories using a  $200 \times 200\mu\text{m}$  grid and a  $150 \times 150\mu\text{m}$  frame. For immunoreactive cells, the ROI was outlined using a 4 $\times$  objective and counted using a 40 $\times$  objective. The interrupted series containing every eighth sections, so the section sampling fraction (ssf) was 1/8. Because of shrinkage we did not use exclusion planes. The height of the optical dissector was the actual section thickness; hence, the thickness sampling fraction (tsf) was 1.

### **Statistical Analysis**

Changes of corticosterone levels before and after stress for rats in stress condition were evaluated using paired t-test. The total neuron numbers, parvalbumin positive neurons, c-Fos positive neurons and double-labeling neurons identified for the two groups in stress condition were analyzed using independent t-test.



**Figure 2 Representative example of thionin staining neurons.** Counted neurons have polygonal soma, clearly seen nucleus surrounded by dark staining cytoplasm.



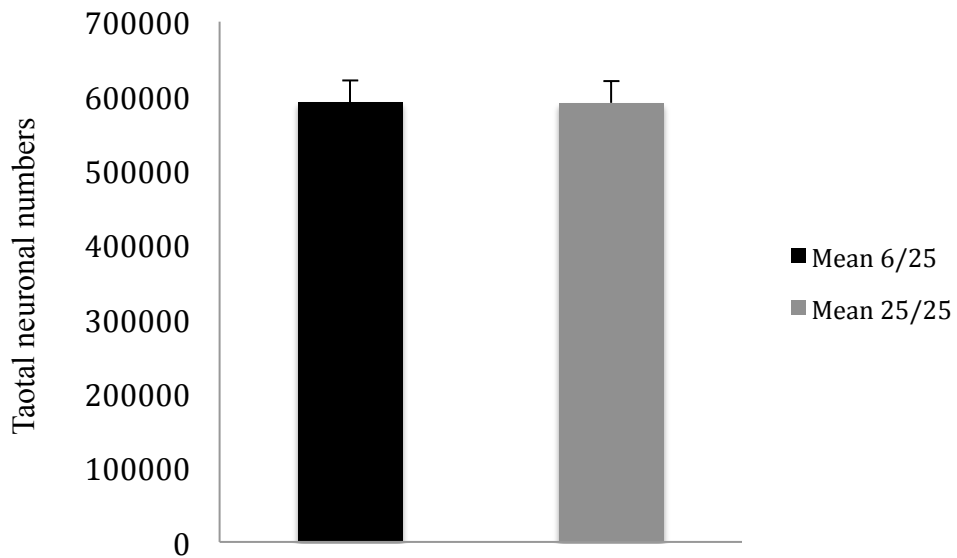
**Figure 3 Representative examples of immunoreactive neurons.** **a**, This illustrates the range of c-Fos positive nuclei, used as a reference of the criteria to differentiate these into the heavily and lightly labeled categories as described in the text. **b**, Parvalbumin positive interneurons have brown cytoplasm with distinguishable projections. **c** and **d**, **c** represents a double-labeling neuron with lightly stained nucleus, while **d** represents a double-labeling neuron with heavily stained nucleus.

## RESULTS

To quantify the total neuron numbers in the ACg-mPFC, thionin staining was performed on one selective series. Neuronal number and morphology were examined using optical fractional methods. Examination of the full extent of the thionin-stained sections did not reveal any discernible differences in cytoarchitecture and anatomical organization. The results of stereological counts are shown in Table 1. An independent sample t-test showed there were no significant differences between two nutrition groups ( $p=0.098$ ), suggesting that prenatal protein malnutrition did not affect the total neuronal number in the ACg-mPFC, as shown in Figure 4.

In order to determine if prenatal protein malnutrition affects the neuronal numbers of inhibitory interneurons in the prefrontal cortex, parvalbumin positive interneurons were quantified within ROI and then summed as shown in Table 2. After standardized by total neuronal numbers, an independent sample t-test showed that there was no significant difference between two nutrition groups ( $p=0.384$ ), indicating that prenatal protein malnutrition did not affect number of inhibitory interneurons in the prefrontal cortex.

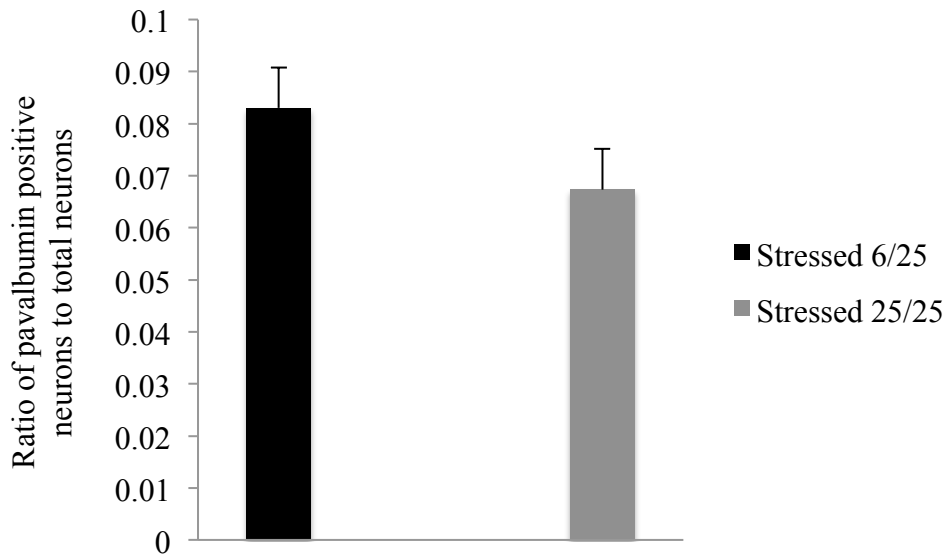
We also counted c-Fos positive neurons and c-Fos/parvalbumin double-labeling neurons within the ACg-mPFC. The results are displayed in Table 3.



**Figure 4 Total neuron numbers in malnourished (6/25) and control (25/25) rats.** This illustrates the total neuronal numbers of both prenatally malnourished (6/25) and control (25/25) groups. There is no significant difference between total neuron numbers in respond to prenatal malnutrition ( $p=0.098$ ).

Malnourished			Nourished		
Group	Neurons	CE	Group	Neurons	CE
(6/25)			(25/25)		
A	416376.91	0.06	B	672384.44	0.05
C	637570.25	0.07	D	735006.06	0.05
E	640244.19	0.05	F	671093.69	0.05
G	629834.06	0.05	H	708724.19	0.05
I	747702.06	0.05	J	657785.06	0.05
K	568620.44	0.06	L	524708.81	0.06
M	440864.81	0.06	N	481443.94	0.06
O	596125.56	0.06	P	581294.50	0.05
Q	700243.50	0.05	R	570137.81	0.05
S	462472.25	0.06	T	507126.44	0.06
U	658192.88	0.06	V	544067.19	0.06
W	620829	0.06	X	509602.06	0.06
Y	563576.31	0.06	Z	440940.66	0.07
AA	699182.69	0.05	BB	722989.44	0.05
CC	488315.13	0.06	DD	529292.88	0.06
Mean/CV	591343.34	0.17		590439.81	0.11
SD	100383.00			95852.21	

**Table 1. Total neuron counts.** Individual estimates of total neuronal number listed by nutritional conditions. Group means, coefficients of error (CE), and coefficients of variance (CV) are listed for each data.



**Figure 5 Parvalbumin positive neurons in malnourished (6/25) and control (25/25) rats.** This graph represents the group means and standard errors for parvalbumin positive interneurons (standardized) of both prenatally malnourished (6/25) and control (25/25) groups. Statistical analysis reveals that there is no significant difference across groups, suggesting that prenatal malnutrition doesn't affect the inhibitory interneurons in the ACg-mPFC ( $p=0.384$ ).

Parvalbumin Positive	
Stressed 6/25	Neurons
A	52838.52
C	63628.68
E	36898.87
I	36068.17
K	32406.99
M	47768.25
Mean/CV	44934.91
SD	12000.81
Parvalbumin Positive	
Stressed 25/25	Neurons
F	57483.20
H*	50693.07
J	30465.44
N	36391.23
P	33482.34
Mean/CV	41703.06
SD	11746.41

**Table 2 Parvalbumin positive interneurons in the ACg-mPFC.** Individual estimates of parvalbumin positive interneurons were listed by nutritional conditions.. Group means, coefficients of error (CE), and coefficients of variance (CV) are listed for each data.

\*In the statistical analysis, Subject H was excluded because it is 2 SD deviated from group mean.

Stressed	c-Fos Positive		Double-labeling	
6/25	Neurons	CE	Neurons	CE
A	41873.42	0.06	1162.22	0.12
C	46885.25	0.06	1052.04	0.14
E	42662.39	0.06	784.84	0.15
I	36862.10	0.07	768.05	0.15
K	39405.89	0.07	827.76	0.15
M	35578.23	0.07	701.14	0.17
Mean/CV	40544.55	0.10	882.68	0.21
SD	4146.73		181.94	
Stressed	c-Fos Positive		Double-labeling	
25/25	Neurons	CE	Neurons	CE
F	20896.96	0.09	429.91	0.21
H*	51851.20	0.06	1502.07	0.11
J	32531.84	0.07	413.83	0.22
N	31248.86	0.08	290.88	0.25
P	30374.27	0.11	624.18	0.17
Mean/CV	33380.63	0.18	652.17	0.31
SD	11306.05		489.86	

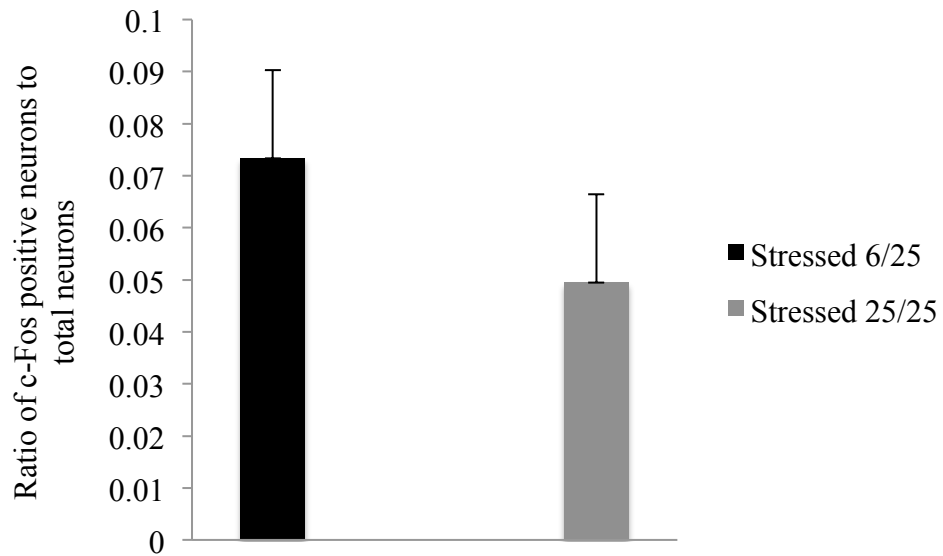
**Table 3 c-Fos positive neurons and c-Fos/parvalbumin double-labeling neurons.**

Individual estimates of c-Fos positive interneurons and double-labeling neurons were listed above. Group means, coefficients of error (CE), and coefficients of variance (CV) are listed for each data.

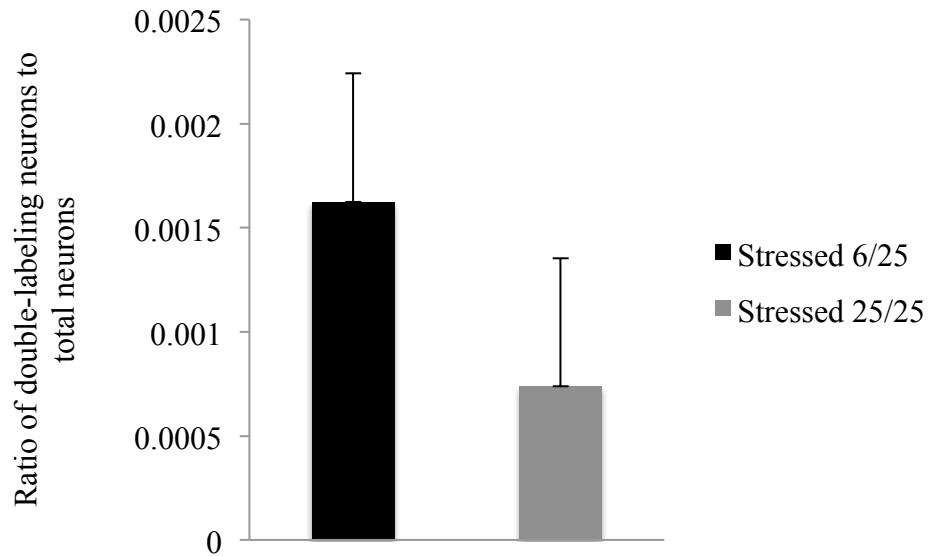
\*In the statistical analysis, Subject H was excluded because it is 2 SD deviated from group mean.

As shown in Figure 6, after standardized by the total neuronal numbers, the number of c-Fos positive cells in the ACg-mPFC cortex was significantly increased in prenatally malnourished group compared to control ( $p < 0.005$ ). In addition, there were significantly more double-labeling neurons in prenatally malnourished rats ( $p < 0.005$ ), as shown in Figure 7.

Additional analyses were run for the two subgroups of c-Fos labeling intensity to determine if the effect was due to greater numbers of heavily stained neurons or lightly labeled neurons. The results are displayed in Table 4, statistic analysis shoed there were no differences between any of the sub categories of labeling intensity.



**Figure 6 c-Fos positive neurons in malnourished (6/25) and control (25/25) rats.** This graph presents the group means and standard errors for c-Fos positive interneurons (Standardized) of both prenatally malnourished (6/25) and control (25/25) groups. An independent t-test reveals that the ACg-mPFC was more activated in prenatally malnourished group compared to control group within stress condition ( $p=0.049$ ).



**Figure 7 Double-labeling neurons in malnourished (6/25) and control (25/25) rats.**

This graph presents the group means and standard errors for c-Fos/parvalbumin positive neurons (Standardized) of both prenatally malnourished (6/25) and control (25/25) groups. Stereological quantification reveals that there is a significant difference between the two nutritional groups ( $p=0.027$ ).

Stressed 6/25	Heavy	Light	Total
A	177.56	984.66	1162.22
C	178.65	873.39	1052.04
E	150.29	634.55	784.84
I	94.19	612.23	706.42
K	125.96	701.80	827.76
M*	19.48	681.66	701.14
Mean	145.33	761.33	906.66
SD	60.60	148.03	191.22
Stressed 25/25	Heavy	Light	Total
F	18.69	411.22	429.91
H*	288.86	1213.21	1502.07
J	39.41	374.42	413.83
N	18.18	272.70	290.88
P	104.03	520.15	624.18
Mean	45.08	394.62	439.70
SD	104.36	343.43	446.65

**Table 4 Subcategories of c-Fos positive neurons.** Heavily stained and lightly stained c-Fos positive neurons were marked separately by the experimenter. Individual estimates of each subcategory were listed by nutritional conditions.

\*In the statistical analysis, Subject H was excluded because the total c-Fos positive neuronal number is 2 SD deviated from group mean. Subject M was excluded because the heavily stained neuronal number is 2 SD deviated from group mean.

## DISCUSSION

This study investigated the inhibitory interneurons in the anterior cingulate and medial prefrontal cortex of prenatally malnourished rats. Analysis of the total neuronal numbers in this region does not show any significant differences between prenatally malnourished rats and controls. While the parvalbumin positive neurons seem to be slightly increased in the prenatally malnourished rats, the increase is not significant. The results reported here also indicate c-Fos positive neurons in the anterior cingulate and medial prefrontal cortices of prenatally malnourished rats to be activated in greater numbers by restrained stress, than they are in normally nourished rats. This may result from an increased activation of parvalbumin positive interneurons, which is indicated by an increased number of c-Fos/parvalbumin double-labeling neurons in the prenatally malnourished rats.

The current study focused on the effects of prenatal protein malnutrition to total neuronal numbers in the prefrontal cortex. The prefrontal cortex plays a central role in cognitive control and emotional regulation (Miller and Cohan, 2011). There is considerable evidence from both clinical and behavioral studies that indicates prenatal protein malnutrition leads to prefrontal cortex dysfunction.

In clinical studies, children that are prenatally malnourished show behavioral changes including attentional problems, increased aggression, hyperactivity, and conduct disorders (Liu et al., 2004). In addition, prenatal malnutrition is a risk of development of psychiatric disorders such as depression (Galler et al., 2012) and schizophrenia (Susser et al., 1998). All of those symptoms are closely associated with the prefrontal cortex.

Given the evidence that prenatal protein malnutrition alters the total neuron numbers of in the CA1 subfield of hippocampus (Lister et al., 2005), where gives pronounced projections to medial prefrontal cortex (Thierry et al., 2000), we expected that prenatal malnutrition reduces the total neurons in the prefrontal cortex. However, the result showed that there are no statistically significant differences between two nutrition groups. This result is consistent with the previous finding in our laboratory (Lister, unpublished) that there are no differences in neuronal numbers of the prefrontal cortex between malnourished pups and controls in Sprague-Dawley rats.

One possible explanation is that prenatal malnutrition is more likely to alter the physiological or neurochemical properties of the prefrontal cortex rather than affect the neurogenesis. For example, behavioral studies in rats have shown that prenatally malnourished rats exhibit higher activities in the prefrontal cortex (Rosene et al., 2004). Galler and her colleagues (2007) demonstrate that prenatal malnutrition leads to structural and neurochemical abnormalities in the prefrontal cortex. Further studies are necessary for us to better understand how prenatal protein malnutrition causes prefrontal dysfunctions that are observed in clinical studies.

In the present study, we also investigated if prenatal protein malnutrition affects the neuronal numbers of inhibitory interneurons in the prefrontal cortex. There is considerable evidence prenatal malnutrition affects the inhibitory network (Leubke et al., 2000; Bronzino et al., 1997). Neurophysiological studies using prenatal protein malnutrition animal model have shown increased miniature inhibitory postsynaptic currents generated by CA1 pyramidal cells in vitro hippocampal slices (Luebke et al.,

2000). And this functional change is related to the altered proportion of parvalbumin positive interneurons in the hippocampus (Lister et al., 2011).

Of particular relevance to the present study is the research examining the inter-relationships between the medial prefrontal cortex and the hippocampus. Many studies have pointed out that there are pronounced projections from the hippocampus to medial prefrontal cortex (Thierry et al., 2000). In rats, the efferent fibers arise from the CA1 and subiculum and terminate in the prelimbic and infralimbic areas of the medial prefrontal cortex (Vertes, 2006). Functionally, Jay and his colleagues (2005) showed that increases in dopamine in the medial prefrontal cortex increase the strength of long-term potentiation at hippocampal-mPFC synapses. Therefore, we expected that prenatal malnutrition alters the inhibitory interneurons in the prefrontal cortex. However, the parvalbumin positive neurons are slightly increased in the prenatally malnourished rats, the change is not significantly different.

Among the possible explanations for this finding are that prenatal malnutrition increases excitability of GABAergic interneurons, or alters GABA reuptake mechanisms, or alters the activity of extra-hippocampal modulatory inputs on GABAergic interneurons (Galler et al., 1990) rather than increases the number of inhibitory interneurons. For example, prenatally malnourished rats are unable to enhance synaptic efficacy (Bronzino et al., 1997), suggesting an enhancement of GABAergic inhibitory modulation of granule cell excitability within hippocampus.

In addition, it is likely that other immunoreactive, such as somatostatin or VIP positive, inhibitory interneurons are altered in the prenatally malnourished rats. Also, our

relatively small sample (Malnourished=6, Control=4) may affect the reliability of the results. There, further studies using multiple inhibitory markers with a larger sample may provide more information about the inhibitory network in the prefrontal cortex in prenatally malnourished subjects.

In the current study, we investigate the effects of prenatal protein malnutrition in the prefrontal cortex in response to restraint stress. Statistical analysis shows significantly greater number of c-Fos positive neurons in the prefrontal cortex of prenatally malnourished rats within stress condition, which is consistent with previous studies (Rosene et al., 2004). A novel finding in the present study is that the c-Fos/parvalbumin double-labeling neurons are significantly increased in prenatally malnourished rats, suggesting the increased c-Fos activity within stress condition may largely come from inhibitory interneurons.

The prefrontal cortex mediates physiologically adaptive changes promoted by stress. Direct evidence comes from lesion studies that damage of the prefrontal cortex altered hypothalamic-pituitary-adrenal (HPA) axis responses to stress (Viau et al, 1993). Diffusion functional MRI study also demonstrated that prefrontal cortex plays a key role in stress responses (Wang et al., 2005). On the other hand, the prefrontal cortex is also the most sensitive to the effects of stress exposure. Animal studies have demonstrated that stress alters the chemical and morphological characteristics of the prefrontal cortex such as a reduction in dendritic spine (Miller et al., 2006). In clinical studies, people who exposed to acute stressor shows impaired working memory and cognitive flexibility, both of which require the prefrontal cortex function (Qin et al., 2009).

More and more evidence have shown that the prefrontal cortex modulates the HPA axis through predominantly inhibitory outputs. For example, lesion of the medial prefrontal cortex increases corticosterone levels in responses to restraint stress (Viau et al, 1993). Moreover, acute restraint-induced cellular activation of periventricular nucleus was exaggerated after lesions of dorsal medial prefrontal cortex (Figueiredo et al., 2005). A recent study (Galler et al., unpublished) demonstrated decreased extracellular dopamine release in the medial prefrontal cortex of prenatally malnourished rats when exposed to restraint stress. Those studies support our result that the increased inhibitory activation in prenatally malnourished rats is associated with the increased initial response to restraint stress.

The estimates that were done using a stereological approach have two technical limitations. First, because our sections shrink extensively in thickness from 30 $\mu$ m down to about 7 $\mu$ m, it was not feasible to set guard volumes above and below the dissector counting box. Since in many places the thickness is only 5 $\mu$ m, setting effective guard volumes would restrict the counting box height as little as 3 $\mu$ m and introduced significant error into estimates of the true fraction sampled. As a result, it can be expected that the estimates were underestimated for two reasons. First, some labeled cells that should have been counted in were probably missed because they were at the cut surface and may have been destroyed during cutting. Second, other labeled cells that should have been counted may have been missed because only a small piece of the labeled nucleus was present but could not be resolved. However, we expected this underestimation is relatively slight

because of our strict inclusion criteria. It is supported by our congruent results, as well as reporters from other investigations (Lister et al., 2005).

The coefficient of effort of the estimates (CE) was calculated for every animal using Matheson's quadratic approximation (Gundersen et al., 1999) and accounting for the nugget effect (West et al., 1999). In the current study, CE of double-labeling cells counts were more than the maximum recommended value of 0.1. In a pilot study, we found that the actual counts of all double-labeling cells in both nutritional groups did not approach an "ideal" CE value. Further study using estimates from the actual counts could increase the reliability of this study.

This study was not prospectively designed to examine the potentially lateralized regulation of stress responses in the prefrontal cortex of prenatally malnourished rats. Thus, conclusions regarding lateralized effects cannot be made. However, previous studies demonstrated that the prefrontal cortex output neurons exhibit a right hemisphere specialization in regulating hormone-related stress response (Viau et al, 1993). Moreover, there are other cortical subcortical regions such as amygdala and hypothalamus that are essential for stress regulation (Herman and Cullinan, 1997). The relationship between prenatal protein malnutrition and brain functions within stress condition will require additional studies.

However, other cortical and subcortical areas are important for stress regulation like the amygdala or the hypothalamus have yet to be examined. In addition, only parvalbumin positive interneurons were investigated in current study. There are a large number of inhibitory interneurons are not parvalbumin immunoreactive. A more

comprehensive analysis of the entire forebrain and even brainstem regions with c-Fos and inhibitory markers may reveal additional mechanism that could be involved in altered stress response in prenatally malnourished subjects.

Nutrition is one of the greatest environmental influences on the fetus, and plays a necessary role in the maturation and functional development of central nervous system. There is little doubt that a large number of the world's populations suffer from prenatal malnutrition. People born around Dutch famines show an increased morbidity of schizophrenia (Susser and Lin, 1992; Susser et al., 1996), and a higher risk of coronary heart disease (Osmond et al., 2000), and they have altered personality. Galler and her colleagues also found the same symptoms in the perinatal malnourished populations in Barbados. Unfortunately, most of these impairments are unlikely to be reversed. Therefore, researches about the relationship between prenatal malnutrition and brain development, as well as adult brain functions are important to improve public health measures and medical interventions to improve the physical well-being of these populations.

The prefrontal cortex plays a central role in cognitive control and emotional regulation. While studies of the hippocampus in this model of prenatal protein malnutrition have identified a reduction of neuronal number (Lister et al., 2005) and an increased inhibitory excitability (Luebke et al., 2000) in the CA1 subfield, in the present study, prenatal protein malnutrition does not affect the total neuronal number, as well as the inhibitory neuronal numbers in the prefrontal cortex.

Complementing its roles in cognitive and affective information processing, the prefrontal cortex is critical for modulating stress-related homeostatic mechanisms, including the hypothalamic–pituitary–adrenal (HPA) axis. This study verifies that the anterior cingulate and adjacent medial prefrontal regions as critical structures for the altered response to stress of prenatally malnourished rats, and demonstrates that the increase of c-Fos positive activation may largely come from inhibitory interneurons. These findings indicate that prenatal protein malnutrition affects stress responses by altering the inhibitory interneurons functional activation.

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**CURRICULUM VITAE**

