TREATMENT INTERVENTIONS FOLLOWING PRENATAL STRESS AND NEONATAL CORTICAL INJURY

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A Thesis
Submitted to the School of Graduate Studies Council
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

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ABSTRACT

Treatment Interventions Following Prenatal Stress and Neonatal Cortical Injury.

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This thesis explores the potential for treatment interventions of an enriched environment and a vitamin/mineral supplemented diet toward the recovery of gestational stress and/or early cortical injury in rats. Two different intensities of prenatal stress (moderate and mild) were employed. The results showed sexually dimorphic behavioural and anatomical results that varied with the intensity of the stress. The enriched environment reversed some of the behavioural deficits related to stress and lesion. Both mild prenatal stress and the vitamin -supplemented diet reversed all frontal lesion behavioural deficits.

Acknowledgements

First and foremost I would like to give many thanks to my co-supervisor Dr. Bryan Kolb for the opportunity to enroll into graduate school with funding. Also, Dr. Robbin Gibb my supervisor, for the guidance necessary to complete this project. Dr. Igor Kovalchuk as a committee member with only little knowledge of the brain showed me valuable mentoring outside of our department. I thank Dr. Bonnie Kaplan for agreeing to be an external examiner and making the trip from Calgary. She also has a different and valuable style of mentorship from my supervisors. All of the staff at the CCBN has been valuable in providing me with all of the tools and guidance necessary for the completion of my experiments. I must thank CIHR for the funding to complete these experiments. My family has given me an endless understanding with blessed love and support through three degrees and my friends in Vancouver have waited 10 years for my return. True Hope has always been available and willing to supply the vitamin/mineral supplement (EMPower+) as a rat chow for me to accomplish two graduate research degrees. Finally, last but not least, I thank my rats for helping me better understand animal behaviour and anatomy.

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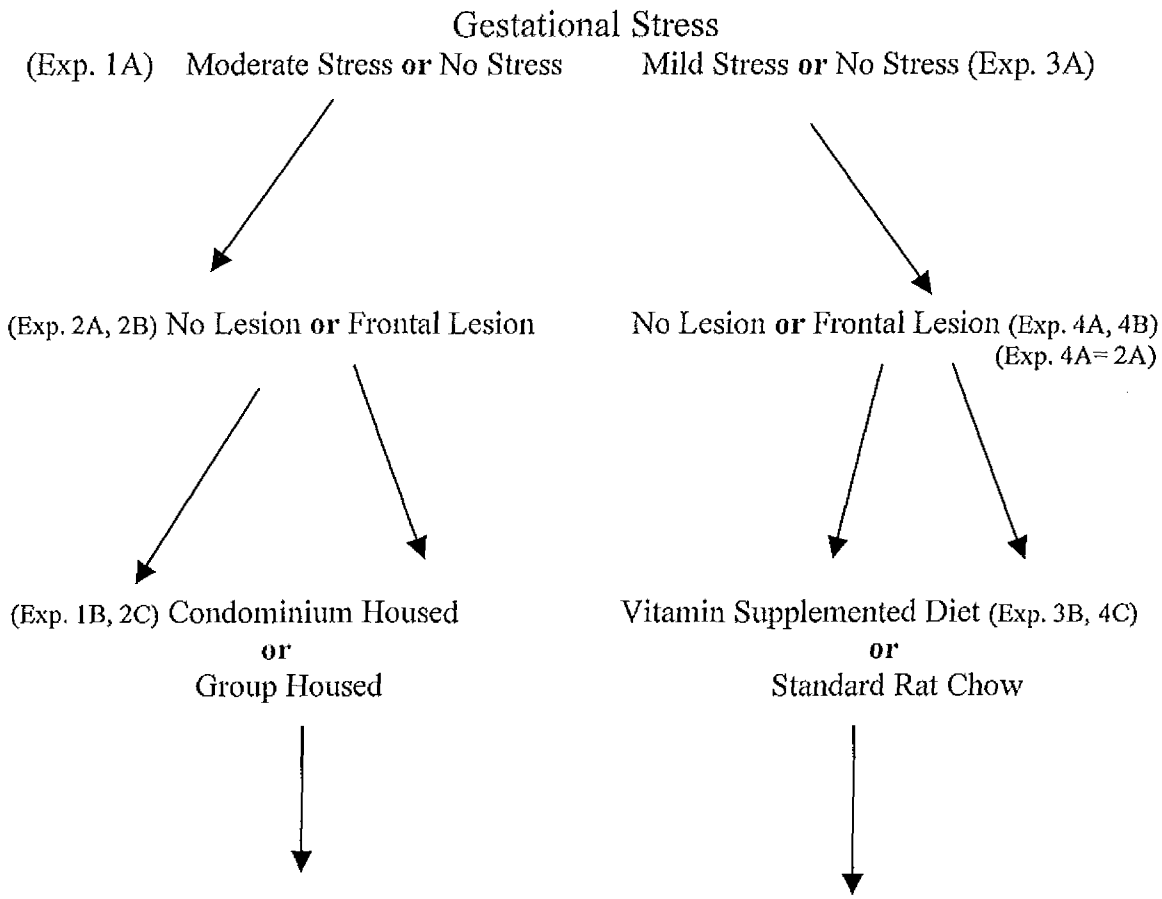
Abbreviations

11β- HSD1	11β- hydroxysteroid dehydrogenase type I
11β- HSD2	11β- hydroxysteroid dehydrogenase type II
5-HT	Serotonergic
ACTH	Adrenocorticotropin hormone
ADHD	Attention deficit hyperactivity disorder
CRH	Corticosterone releasing hormone
CRH-BP	Corticosterone releasing hormone- binding protein
CORT	Corticosterone
DA	Dopamine
DHEA	Dehydoepiandrosterone
GABA	γ-aminobutyric acid
GR	Glucocorticoid receptors
HG-ABN	High licking grooming and arched-back nursing
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
IGF-I and IGF-II	Insulin-like growth factors
IUGR	Intrauterine growth restriction
LG-ABN	Low licking grooming and arched-back nursing
MFP	Maternal-placental-fetal
MR	Mineralocorticoid receptor
NA	Noradrenalin
NL	No Lesion
SGA	Small for gestational age

Experimental Design



Chapter One: General Introduction

Producing and maintaining the health of a fetus during pregnancy and the newborn during lactation initially led many nutrition researchers to formulate a number of philosophies toward the necessity of maintaining homeostatic regulation of both mother and fetus. Changes in diet alone can "re-program" the internal milieu to which the fetus must adapt and become a part of, in addition to internal signals conveyed through the mother's responses to her environment. The ideal pregnancy event revolves around the mother's maintenance of balances in homeostatic control for healthy infants; free of complications from malnutrition, disease, and excess environmental stressors. Once the infant is born, the environment it is exposed to must provide the adequate necessities for proper growth and nourishment in order for it to maturate into a long living and well-functioning individual.

'The fetal origins of adult disease hypothesis' or the 'Barker hypothesis,' (1995) proposed that a strong relationship exists between the environment encountered by the fetus and infant in early life and risk of non-communicable disease in later life (Langley-Evans, 2006; Barker, 1995). The causal relationship was proposed to be a response of adaptation to an adverse environment causing inappropriate nutritional supply to the fetus/infant during development that disturbs fetal and infant homeostasis (Barker, 1995).

Homeostatic control mechanisms have long been known to maintain an organism's internal temperature and appetite at regulatory "set points". If such events of under-, or malnutrition were to occur during the perinatal period of infant development, permanent responses are considered factors of 'nutritional or metabolic programming',

and 'fetal programming' from low birth weight (Langley-Evans, 2006; Burge, Lillycrop, and Jackson, 2009; Barker, 1995).

Type II adult onset (non-insulin dependent) diabetes, and cardiovascular diseases were the first known disorders considered to be caused by dysregulation of metabolism, both of which have been found to have a relationship with fetal events that produce low birth weights in infants (Taylor and Francis, 2006; Langley-Evans, 2006). Ecological studies of retrospective cohorts born in the first third of the twentieth century suggested a relationship between the environment of nutritional availability and low birth weight with various disease factors (Barker, 1995; Langley-Evans, 2006). More recently, endocrine cancer, hypertension, depression and cognitive disorders, among others are presently included in 'at risk' developmental profiles (Wadhwa and Federenko, 2006).

More recent reports of maternal anxiety during pregnancy have also resulted in low birth weight infants (Mennes, Stiers, Lagae, and Van den Bergh, 2006). Observations in children born from antenatal anxiety include behavioural and emotional disturbances measured by various developmental scales, such as attention deficit hyperactivity disorder (ADHD); disturbances that follow through adolescence (Mennes, Stiers, Lagae, and Van den Bergh, 2006).

Wadhwa and Federenko (2006), suggest that the observed effects of hypertension, depression, and the above aforementioned variety of disorders that stem from low birth weight or small for gestational age (SGA) infants are actually applicable across the normal distribution of all birth phenotypes. Instead, their approach toward developmental outcomes are contingent on two evolutionary forces that have determined and shaped the development of all living organisms; 1) nutrition; the availability and utilisation of energy

substrates, and 2) the adaptation to stress; physical or psychological challenges of the homeostatic control of organisms. Their final argument is that the resultant reproductive outcome of premature birth and low weight birth are characterized by large disparities that continue to extend across those that reflect poor socioeconomic status, including racial and minority populations. Overall, maternal stress is a significant risk factor for low birth weight, SGA, and preterm born infants (Wadhwa and Federenko, 2006; Lundberg, 2005).

I. Pregnancy

The health of a newborn infant, as in all mammals, can be determined by its size, which reflects the maternal investment and length of gestation (Murphy, Smith, Giles, and Clifton, 2006). The pregnant mother is required to supply all of the essential nutrients, hormones, and oxygen to the fetus through the placenta. An adequate trophoblast invasion of the endometrium wall with progressive increases in uteroplacental blood flow is critical to prevent growth restriction in the fetus. Within the endometrium the placenta develops along with the transformation of uterine spiral arteries into low resistance blood vessels. The placenta is where nutrients are exchanged from the mother and transferred to the fetus, while fetal waste products are transferred to the mother for elimination. Adequate growth and function of the placenta is necessary for healthy birth weights and size in newborn infants (Murphy, Smith, Giles, and Clifton, 2006).

Hormone secretion by the placenta into both the mother and fetus is necessary to mobilize the nutrients from the mother to be passed onto the fetus in addition to supplying the nutrients necessary for growth of the placenta (Murphy, Smith, Giles, and

Clifton, 2006). Nutrients the fetus requires are mobilized through maternal metabolic processes and thus, maternal nutritive status prior to and during pregnancy is critical. There is however, greater concern that the fetus is able to receive and utilize nutrients without any restriction (Murphy, Smith, Giles, and Clifton, 2006). Limitations in the fetus's ability to receive required nutrients exist in cases of maternal hypertension, placental vascular disorders, and placental disruption resulting in small birth sizes (Harding, Bloomfield, and Oliver, 2006).

Regulation of embryonic and subsequent fetal growth requires the maternal secretion of insulin-like growth factors (IGF-I and IGF-II), which in turn induces somatic cell proliferation and cell growth. Teen pregnancies are commonly at risk for fetal undernutrition because nutrients ingested would be partitioned between the growing teen mother and the growing fetus. Any decrease in IGF growth factors will alter the amount of glucose and amino acids supplied to the fetus; thereby decreasing cell growth and proliferation (Murphy, Smith, Giles, and Clifton, 2006). During the embryonic stage up to the first 16 weeks gestation, cell number increases maximally, forming a fetus. Fetal growth thereafter from 16 to 32 weeks gestation is the fetal stage when increases in cell size correspond with fetal growth. Maturation of fetal organs and tissues prior to birth requires maternal glucocorticoids through placental metabolic processes (Murphy, Smith, Giles, and Clifton, 2006).

Infants born with low birth weights are associated with increased risk of mortality up to 15 years of age (Murphy, Smith, Giles, and Clifton, 2006). Full-term infants with less concern for long-term health risk weigh 3000 to 3499 grams. Infants of low birth weight at 2500 to 2999 grams are twice as likely as the former for increased risk of

mortality, and infants weighing 2000 to 2499 grams are four times likely for neonatal morbidity. Infants are considered small for gestational age if they have birth weights less than the 10^{th} percentile of a normal reference population for their age (Murphy, Smith, Giles, and Clifton, 2006).

Nutrition in pregnancy

Nutrient transport to the fetus consists of amino acid transporters located within the fetal (basal) and maternal microvilli membranes of the placenta (Murphy, Smith, Giles, and Clifton, 2006). Various amino acids can also be metabolised by the placenta to enable amino acids of different properties (positively or negatively charged, polar, aromatic or uncharged) for effective transport to the fetus. Some non-essential and essential amino acids have been found reduced in SGA infants and are thought to be due to reduced activity in sodium-dependent transporters. In hypoxic infants, the ratio of nonessential to essential amino acids increase with respect to increases in umbilical vein pressure of oxygen (ppO_2) (Murphy, Smith, Giles, and Clifton, 2006; Groff, Groper, and Hunt, 1995).

Glucose transporters (GLUT1) are found on mother and fetal membranes of the trophoblast and operate via a concentration gradient (Murphy, Smith, Giles, and Clifton, 2006). Some studies indicate the mother's glucose concentrations are similar in either normal or growth-restricted pregnancies, but a reduction in fetal glucose concentration is commonly found in growth-restricted pregnancies. Interestingly, some studies have found no difference in GLUT1 transporter density in intrauterine growth restriction (IUGR), or

glucose transfer to the fetus, while others indicate an increased glucose concentration in the mother to fetus concentration gradient (Murphy, Smith, Giles, and Clifton, 2006).

Fatty acids also cross the placenta to provide an energy source, encourage cell signaling, and to become essential components of myelin sheath and plasma membranes as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (Murphy, Smith, Giles, and Clifton, 2006; Groff, Groper, and Hunt, 1995; Jumpsen and Clanidin, 1995). Free fatty acids are able to pass through the placenta from mother to fetus relatively easily. Phosphoglycerides and triacylglycerols cannot however and thus, require interconversion among placental fatty acids to become biologically available for fetal transport (Jumpsen and Clanidin, 1995).

Metabolic imprinting

In addition to vascular and placental function complications, dietary components are also an issue of fetal nutrition. The balance of macronutrients (carbohydrate, protein, and fat) mothers ingest during pregnancy can change the development of the infant's metabolic imprinting (Harding, Bloomfield, and Oliver, 2006). In undernourished populations, mothers that were provided food supplements with a lower percent of calories from protein (higher fat and/or carbohydrate) tended to have infants with larger birth weights than infants from unsupplemented women. Conversely, women supplemented with high percent protein (lower fat and/or carbohydrate) had infants with lower birth weights than infants from unsupplemented women (Harding, Bloomfield, and Oliver, 2006). The timing of imbalances in maternal nutrition has also provided insight into the nutritional need of the developing fetus. Ideally, the caloric intake, especially

from protein, should gradually increase throughout gestation to reflect the rate of the growing fetus.

Under- or malnutrition during pregnancy is also considered a factor that contributes to a functionally altered hypothalamic-pituitary-adrenal (HPA) axis. Maternal protein restriction has been found to reduce placental 11β- hydroxysteroid dehydrogenase type II (11β- HSD2), resulting in an increased fetal glucocorticoid load (Nyirenda and Seckle, 2006). Undernutrition also alters pulsatile, circadian and ultradian patterns of glucocorticoid release resulting in increased plasma glucocorticoid levels (Dauncy, et al., 2001). Thyroid hormone, which is necessary for differentiation, growth and metabolism for many cells, is also sensitive to low energy intake. The nutritional influence of thyroid hormone is to regulate hormone synthesis, metabolism and hormone receptor and regulation. The coordination of thyroid hormone, glucocorticoids, and insulin-like growth hormone is central to fetal development and maturation. Thyroid deficiency during perinatal development has resulted in severe mental and physical retardation (Dauncy, et al., 2001). The activity of the HPA axis also affects activity of the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary gonadal (HPG) axis; thereby altering metabolic imprinting and dysregulating the rate and maturation of many developmental interactions (see 'Stress Response'). The resulting metabolic alteration "imprints" various physiological functions, which if mild could promote a shift in the infant's homeostatic mechanisms, or if severe could produce a series of events underlying maladaptive responses in an attempt to gain homeostasis.

Animal studies have been successful in determining the various consequences of maternal undernutrition, or of imbalanced nutrient intakes in relation to infant birth

weight, cell size, DNA count, and cell cycle arrest (Harding, Bloomfield, and Oliver, 2006; Lewis, 1990, Heuther, 1990). A primary finding concerned with alterations in the developmental trajectory of the fetus is in the alteration of the cell cycle phases. These alterations have resulted in decreased levels of growth hormones and overall cell production with morphologically degenerate postmitotic cells in the brain (Lewis, 1990).

Fetal protection from stress

The maternal-placental-fetal (MFP) neuroendocrine system represents the components involved in gestational stress; the maternal and fetal hypothalamic-pituitary-adrenal (HPA) axes, and the placenta (Wadhwa and Federenko, 2006). All communication between mother and fetus is done through activities and responses of the placenta. Direct neural or hormonal connections do not exist from mother to fetus. Any stress the mother might experience and express is mediated by the MFP neuroendocrine system that regulates fetal growth and parturition.

Placental 11β- hydroxysteroid dehydrogenase type I (11β- HSD1) has a high affinity for cortisone to convert it to its active form, cortisol (Murphy, Smith, Giles, and Clifton, 2006). The increase in (cortisol) and glucocorticoids increases glucocorticoid receptor (GR) availability, and is involved in prostaglandin biosynthesis and metabolism necessary for fetal maturation. Type II (11β- HSD2) of this enzyme, also with a high affinity, converts cortisol to its inactive form, cortisone, and provides the growing fetus protection from excess exposure to maternal glucocorticoids (Murphy, Smith, Giles, and Clifton, 2006).

During pregnancy, the placenta is also required to regulate a natural secretion of glucocorticoids that promotes fetal cortisol and dehydoepiandrosterone (DHEA) production from the adrenal cortex of fetal adrenal glands. These hormones are returned to the placenta to promote further secretion of corticosterone releasing hormone (CRH) and DHEA in feed-forward fashion. Dehydoepiandrosterone is the precursor for estriol (E_3) production to influence uterine contractility towards the end of gestation and the promotion of events leading to labor. The first trimester establishes placental CRH positive feedback, which increases exponentially corresponding with fetal maturation and delivery. Placental CRH levels thereby provide some indication for gestational timing of outcomes for preterm, term and post-term delivery (Wadhwa and Federenko, 2006) (see Fig.1).

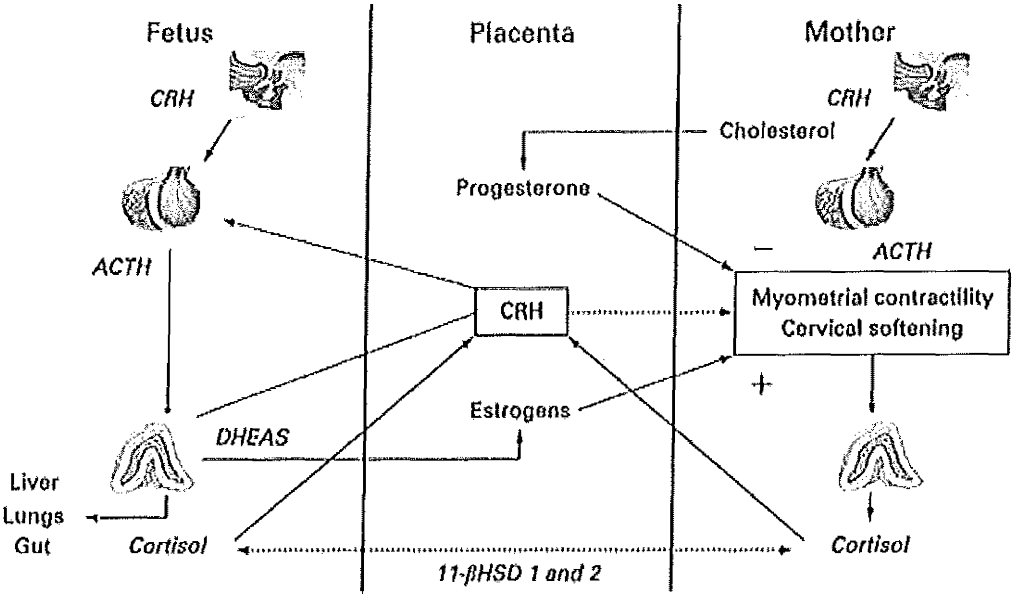


Figure 1. A schematic of the maternal-fetal-placental neuroendocrine system (Adapted from Wadhwa and Federenko, 2006). The hypothalamus of the fetus and the mother secretes CRH to signal the pituitary gland to secrete ACTH, which is received by the adrenal glands.

Placental CRH activity is however stress sensitive particularly in pregnancies that have been complicated by pre-eclampsia, reduced uteroplacental perfusion, intrauterine infection, and elective preterm delivery (Wadhwa and Federenko, 2006). Some studies have reported direct associations between maternal psychosocial stress and placental CRH, with support from in vivo studies correlating maternal adrenocorticotropin hormone (ACTH) and cortisol levels with placental CRH activity. Further confirming the effects of maternal stress on the fetus are studies using cultured human placental tissue that report a dose-response increase in the production of CRH in response to the addition of cortisol, catecholamines, and proinflammatory cytokines (Wadhwa and Federenko, 2006).

The stress response

The stress response is a critical fight-or-flight response for the survival of an organism when confronted with life-threatening situations. Short-term activation is adaptive for an organism to escape danger; however, long-term activation from over-exposure to a hostile environment becomes maladaptive exposing individuals to susceptibility of pathological conditions.

Physiologically, the stress response primarily incorporates the neuroendocrine system which employs both central and peripheral divisions of the autonomic nervous system. The autonomic nervous system regulates the activity of cardiac muscle, smooth muscle, and the glands of the body as involuntary activity (Spence and Mason, 1992). It is subdivided into two systems; the sympathetic nervous system and the parasympathetic nervous system (see Appendix A for a brief outline).

The neuroendocrine system consists of: 1) the endocrine system with hormone-secreting and receiving glands (pituitary, thyroid/parathyroid, adrenal glands, pancreas, and gonads), 2) the vascular system for the transport of hormonal messages, and 3) the nervous system to receive and interpret signals prior to issuing a response (Groff, Groper, and Hunt, 1995; Spence and Mason, 1992).

Glucocorticoids (primarily cortisol and corticosterone) as well as mineralocorticoids (primarily aldosterone) are all secreted from the adrenal cortex (Spence and Mason, 1992). The adrenal medulla secretes two catecholamine hormones, epinephrine (adrenalin) and norepinephrine (noradrenalin) (Spence and Mason, 1992).

Prenatal stress

The stress response can be briefly defined as the activation of the HPA axis in an individual due to perceived emotional threat, and/or inflammatory, or physical stressors of malnutrition and substance abuse within the maternal environment that interacts with the fetus. Inhibition of the maternal stress response occurs primarily through the occupation of glucocorticoid receptors (GR) in various central locations in the brain and by circulating stress hormones (glucocorticoids, cortisol/corticosterone) to produce a negative feedback response.

The mediation of stress during pregnancy is in part, dependent on the maternal threshold to the perception of stress and the integrity of the feto-placental unit; although, there are a series of biological changes throughout pregnancy that generally render pregnant women less vulnerable to stress (Wadhwa and Federenko, 2006). Hormonal changes toward the completion of full-term pregnancy promote a reduction in feedback

sensitivity and attenuation of the HPA axis, altering the stress responsiveness in pregnant women. Women who do not exhibit this late stage HPA suppression, and continue to experience uncontrollable stressors become susceptible to experiencing adverse birth outcomes. For example, studies of maternal psychosocial stress, particularly high job strain with high demand and low latitude, found significant associations with risk of hypertensive disorders. Maternal anxiety and psychosocial stress are significantly related to uteroplacental and fetal hemodynamics (Wadhwa, and Federenko, 2006; Lundberg, 2005).

Maternal plasma CRH levels increase seven-fold during gestation, which is usually bound by CRH binding protein (CRH-BP) and unbound CRH remains as free CRH (Weinstock, 2006). At the placenta, about eighty percent of cortisol is converted to its inactive form, cortisone, preventing the fetus from hormone exposure. A rise of ten to twenty percent in maternal plasma cortisol however, could cause significant increases of cortisol in fetal blood, thus exposing the developing brain to glucocorticoids. In the placenta, cortisol stimulates the release of CRH into fetal blood, which has the potential to release adrenal hormones by mid-gestation and establish a positive placental-adrenal feedback loop (Weinstock, 2006).

The sensitivity of placental tissue to glucocorticoids has been found to further stimulate CRH gene expression through the activation of cyclic adenosine monophosphate (cAMP) response site on the CRH promoter (Weinstock, 2006). The difference between feto-placental CRH and maternal CRH expression is due to the expression of different transcription factors, co-activators, and co-repressors between the placenta and the maternal hypothalamus (Wadhwa and Federenko, 2006).

A fetal response to the stress of blood sampling through the maternal abdominal wall around mid-gestation has been reported to increase circulating levels of ACTH, cortisol, and β-endorphin (Weinstock, 2006). Increases in fetal heart rate and induction of motor hyperactivity are also reported to be result of maternal psychological stress, confirming the effects of perceived stress during gestation (Weinstock, 2006).

Prenatal stress in animal studies

Measuring stress in animals has been very beneficial to overcome some of the bias and methodological shortcomings observed in earlier human research. Animal models also are used to determine some of the molecular and morphological changes that correspond to behavioural measures. A primary finding in research of adult humans, rhesus monkeys, and rats after exposure to stress during the gestation period, is the increased propensity for anxiety-like reactive behaviour that correspond to increased circulating plasma cortisol and corticosterone, β-endorphin, and ACTH of HPA function (Maccari and Morley-Fletcher, 2007; Arborelius, et al., 1999). Sex differences in behavioural and neurochemical measures are reported in most studies using both sexes, although the majority of research has been studied in males (Zuena, et al., 2008; Weinstock, 2007). The placental barrier enzyme 11-β-dehydroxysteroid dehydrogenase type 2 is often reduced resulting in a depression of fetal body weight as well as decreased weights in fetal adrenal glands, pancreas, and testes measured at the embryonic age of day 21 (Mairesse, Lesage, Breton, and Viltart, et al., 2007).

Behavioural measures of anxiety from rat tests in novel mazes, such as the elevated plus maze and open field are generally used. An increase in stress reactivity and

impairments in habituation to a novel environment are common behavioural reports from prenatally stressed animals (Maccari and Morley-Fletcher, 2007; Weinstock, 2007). In conjunction with markers of chronic HPA axis function are changes in glucocorticoid (GR) and mineralocorticoid receptor (MR) densities and function in the hippocampus and prefrontal cortex (Vazquez, 1998; Gratton, and Sullivan, 2005). The glucocorticoid receptor is required to quench the initial rapid onset and surge of glucocorticoids after activation of the stress response to restore basal levels via feedback mechanism. A general reduction of GR density is commonly found in prenatally stressed rats rendering the animal over-exposed to stress hormones. Chronic exposure to circulating glucocorticoids has been found to develop abnormal regulation of the HPA axis and subject inter-related axes to abnormal rhythms (Weinstock, 2001; Koel, et al., 1999).

Anxiogenic behaviour is also apparent in tests of locomotion, and social interaction and is often accompanied with escape attempts and freezing, or immobility if unable to escape (Sandmann, Wadwa, Chicz-DeMet, Porto, and Garite, 1999; Weinstock, 2001). In addition to an increased reactivity to stress that prenatally stressed rats exhibit is the possibility of sensitization of brain areas to CRH progressively enhancing the stimulation of stress neurons in the brain. For example, locus ceruleus neurons of the brain stem are innervated by many and various CRH neurons in the brain, thereby further increasing CRH drive by noradrenalin (NA). Limbic regions of the brain containing CRH neurons, such as the prefrontal cortex, striatum, amygdalae, and hippocampus are integral in the processing and storage of emotional memories (see Fig. 2).

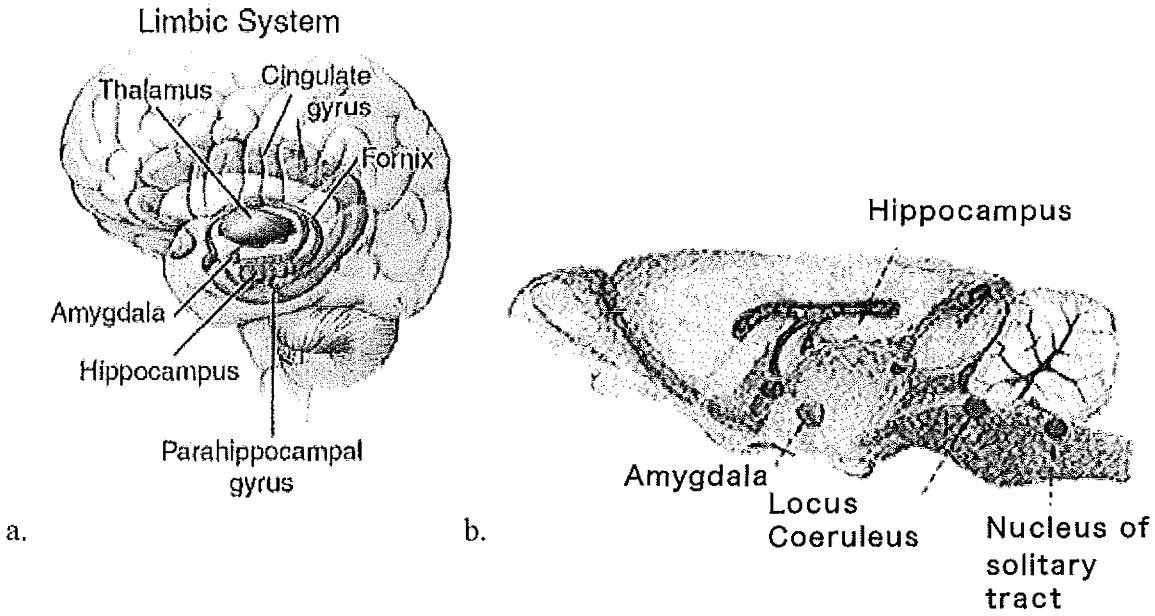


Figure 2. The limbic system of a) the human brain (Adapted from ahaf.org) and b) the rat brain (Adapted from rat brain labels-faculty.virginia.edu).

Sensitization to perceived stressful stimuli is thought to lower the threshold for those stimuli that activate the stress response. A higher turnover rate of NA in the brainstem and cortex has been reported in prenatally stressed animals as well as, an increased release of dopamine (DA) in the prefrontal cortex (Gratton and Sullivan, 2005; Fride and Weinstock, 1998). Prenatally stressed rats have also indicated a reduction of benzodiazepine receptors in the cortex and hippocampus contributing to an over-activated stress response through a NA drive from brainstem nuclei (Gratton and Sullivan, 2005; Fride and Weinstock, 1998).

A phase shift in circadian rhythm, temperature, and baseline cortisol is suggested to disrupt normal sleep patterns in people with depression (Goldwin, 1982; Kupfer, 1995). The phase shift is considered as evidence of abnormal regulation of the HPA axis

in prenatal stress research in primates and rats (Clark, Wittwer, Abbott, and Schneider, 1994; Koel, et al, 1999) with a shift of corticosterone secretion to the end of the light cycle rather than the beginning of the light cycle (Koel, 1999). Alterations in serotonergic systems and in metabotropic glutamate receptor-1 are also apparent with both conditions having differential effects on the sexes (Maccari, 2004). Some aspects of human depression can be modeled in rats, such as anhedonia, circadian rhythm of activity and corticosterone, and learned helplessness from experimental prenatal stress treatment (Arborelius, 1999; Weinstock, 2001).

Monkeys have been found to have a higher GR binding of glucocorticoids in the prefrontal cortex than in the hippocampus, leading to suspicions that this may be true for humans as well; whereas in rodents, the prefrontal cortex has been estimated to contain approximately 75 to 80 percent GR sites to that found in the hippocampus (Gratton, and Sullivan, 2005).

Anatomically, changes in dendritic arbor from prenatal stress in rats include reductions in dendritic arborization in the orbitofrontal cortex with lower spine densities in both apical and basilar portions of layer II/III pyramidal cells (Mirmu, Salomon, Biala, Weinstock, Braun, and Bock, 2006). Prenatal stress has also been found to alter the cell volume of the sexual dimorphic nucleus of the preoptic region of the hypothalamus, and the size of the anterior commissure in males to resemble those in females (Weinstock, 2007). Males exposed to stress during gestation also had larger amygdalae than control males (Weinstock, 2007).

In rats, there is a normal surge of testosterone during days 18 and 19 of gestation; a time when many of the hypothalamic nuclei are developing a normal masculine brain

for males' sexual activity, but is suppressed to below normal levels from gestational stress (Weinstock, 2001; Weinstock, 2007).

Gestational stress has also been reported to decrease aromatase activity in the hypothalamus and amygdalae in the male, but not female fetus during days 18 and 19 of gestation, thereby interfering with normal development of these nuclei (Murase, 1994). Aromatase is a critical enzyme necessary for the conversion of testosterone to estradiol and the subsequent masculinization of male brain and behaviour (Weinstock, 2001). Dalhöf et al, (1978) found a decrease in testes weights in prenatally stressed rats with a reduced activity of δ-3- β-hydroxysteroid dehydrogenase in Leydig cells during critical periods of gestation (Dalhöf, Hard, and Larsson, 1978).

This testosterone surge has been found to be lower in prenatally stressed rats; however, administration of testosterone to the stressed pregnant rats during the last days of gestation (~ 18 to 21) prevented the abnormalities in sexual behaviours in male offspring (Dalhöf, Hard, and Larsson, 1978). The changes in aromatase activity with the changes in hypothalamic nuclei might imprint the developing male brain to become more effeminate, contributing to homosexuality in some males (Dalhöf, Hard, and Larsson, 1978; Weinstock, 2001).

Adrenal gland hypertrophy, and in some cases an enlarged pituitary gland, has been reported in patients during depressive episodes as a result of chronic stimulation of basal cortisol, which subsided with treatment (Van Den Eede, Van Broeckhoven, and Claes, 2005). Animal studies of prenatally stressed rats have also reported hyper- or hypotrophy of the adrenal glands, some of which have abberant cell morphology in the

cortex and some with irregular vascularization in the medulla (Fameli, Kitraki, and Stylianopoulou, 1994).

Glucocorticoid exposure to the developing brain is also critical for the normal development and function of neurotransmitter systems; including receptor function, neurotransmitter synthesis and metabolism, and protein expression (Leret, et al., 2004). Dams that were adrenalectomized during the onset of pregnancy gave birth to rat pups with changes in maturation of the serotonergic system in the hippocampus. During normal developmental timescales, levels of serotonin (5-HT) usually increase after the stress hyporesponsive period, or after the first two postnatal weeks in rats. Without maternal corticosterone, male pups have significantly lower levels of 5-HT by postnatal day 22. Alterations in maturation of GABA and glutamate systems were also pronounced in males, both of which are functionally related to sexual differentiation of the male brain. In addition to maternal glucocorticoids, perinatal androgens also have organizational effects on the developing HPA axis and thus, perinatal programming (McCormick, et al., 1998; Leret, et al., 2004).

Rationale

The objectives for the following set of experiments were to determine the effects of prenatal stress on the developing brain and behaviour in addition to the effects of prenatal stress in the recovery from early cortical injury. Injuries might occur from difficult deliveries, shaken baby syndrome, or accidental falls in the early years of the developing infant, which could change in symptomatology with respect to the magnitude of gestational stress experienced by the fetus.

In addition to the effects of prenatal stress with early cortical injury are two treatment interventions to determine the capacity for correction of some adverse perinatal events. Because the brain continues to develop and is plastic through infancy, childhood, and adolescence, the treatment interventions were administered during the postnatal period to the experimental rats. Both treatments are also currently available and presently used in clinical studies for various disorders. The intervention used in the first set of experiments was an enriched environment with the inclusion of the opportunity to exercise based on the size of the condominium housing. The intervention used for the second set of experiments was the incorporation of a multivitamin/micronutrient formula (EM Power+) into a rat chow that was prepared locally.

The purpose for examining the effects of prenatal stress underlies two fundamental conditions commonly found in modern society. Situations of gestational stress include those that might involve uncontrollable environmental and/or emotional conflict experienced by the expectant mother. Another situation is of premature births requiring the aid of a synthetic corticosteroid, such as dexamethasone for the maturation of very young organs in the infant, or for their prophylactic use to prevent preterm deliveries.

II. Maternal care

Numerous studies concerning the proper development of children to become healthy and well-socialized adults have indicated a number of factors that have become increasingly important. The overall health of the mother prior to and during the perinatal period is of importance to produce healthy offspring. What has become of primary

interest among many researchers is the quality of care given by their mother and the environment that will contribute to the development of the newborn. Factors such as nutritive status, family support, education, socioeconomic status, teenage pregnancies, drug or alcohol abuse, and demographic changes with cultural differences can all determine the quality of care received by the newborn. Many external factors, along with any inherent disorders, all contribute to the levels of stress that a mother might experience during the perinatal period. The quality of maternal care an infant receives has been shown integral to the growing infants' ability to regulate emotional and psychological stressors. As adults, the capability of regulating various stressors and maintaining relationships usually determines the success of integrating into society as a well-adjusted individual and continues into following generations.

Among primate studies of socioemotional development in the young, rhesus monkeys (*Macaca mulatta*) have been noted as a highly successful species that live in large social groups defined by kinship relationships and social hierarchies (Suomi, 2002). During the postnatal period of a newborn, it is very typical that mother's will keep their young in close and constant physical contact during their first month of life to ensure proper emotional and psychological development. Thereafter, infants are known to become more adventurous, leaving their mother's side temporarily to explore with peers and knowing she is near for protection against predators. Into adolescence, monkeys who had experienced the necessary nurturing from their mothers are considered as socioemotionally stable, have the ability to moderate emotions of fear and anger, and are known to grow into well-adjusted members of their troops. Some infants however, who have not received the same quality in maternal care, develop into individuals whom are

unable to properly regulate their emotions and usually display exaggerated responses of fear or aggression, potentially disrupting the social harmony established in their troops. Similar to human societies, individuals who are not well integrated into their community are generally marginalized from their own and also experience difficulty with integration into other communities. The monkey's ability to regulate emotional and psychological stressors determines their integration and adaptation into new communities as adults. This adaptability has been directed towards the quality of their maternal care (Suomi, 2002).

Quality maternal care in the respect of nurturing a newborn has been defined similarly among interested researchers and appears to be applied across the mammalian species of humans, primates, and rats (Ladd et al, 2000; Champagne and Meaney, 2001). The care an infant receives is considered to contribute to its 'environmental programming' of neural circuitry and neuroendocrine function during the development and maturation of the central nervous system. Programming occurs as an effect of the interactions between the inherited biological genome of the newborn and the environment that influences various epigenetic mechanisms, including the biological responses from the maternal care the newborn receives.

Champagne and Meaney (2001) have identified natural variations of maternal care in rat studies of high licking grooming and arched-back nursing (HG-ABN) and low licking grooming and arched-back nursing (LG-ABN). Pups reared from attentive dams that provided a high level of licking, grooming, anogenital stimulation, and arched-back nursing were much better regulated emotionally and psychologically to various environmental stressors into adulthood. Conversely, those pups that were receiving a lower level of attention from their mothers matured to perceive and react with

exaggerated responses to the same stressors when tested in adulthood (Champagne and Meaney, 2000).

Experimentally, these authors have demonstrated that separation, and isolation, (maternal separation) of the pups from its mother for a period 180 minutes per day from postnatal days two to 14 resulted in aberrant maternal care towards her pups. This consequently resulted in a permanent expression of premature and inflated stress responses by the pups in the presence of environmental stressors. If on the other hand, the pups were separated for only 15 minutes per day from postnatal days two to 14 (handling), an increase in the frequency of dams licking, grooming, stimulating, and nursing of pups was observed. This increase in attentiveness provided a significant effect in the pups' ability to regulate emotional and psychological stressors. Furthermore, the cross fostering of pups from LG-ABN dams to HG-ABN dams and vise-verse blunted the effects of the rearing differences between the two groups. This effect has implicated changes in regulatory mechanisms to environmental stressors, which has been 'programmed' through a non-genetic mechanism of maternal care upon reunion of mother and her pups (Champagne and Meaney, 2000).

The effects of maternal separation in rat pups as adults have revealed the same biological effect to that of anxiety and depressive disorder. Primarily, the changes 'programmed' are premature perceptions of stress and fear, and impairment in feedback mechanisms of the stress response, resulting in repeated and prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is dysregulated due to inefficient feedback mechanisms (Champagne and Meaney, 2000).

Handled rats show the opposite effects in reactivity to stressors in comparison to their separated counterparts (Champagne and Meaney, 2000). They readily adapt to various environmental stimuli, do not show premature and inflated activation of the stress response (HPA axis), and thereby appear better able to regulate emotional and psychological stressors (Champagne and Meaney, 2000). Additional research of handling in rats revealed an increase in infant heart rate during suckling, and that maternal licking stimulated the secretion of growth hormone and increased enzymatic (ornithine decarboxylase) activity for progression of the replicative cell cycle (Ladd et al, 2000). Progression of the cell cycle in turn is necessary for the generation of the many and varied cells during development.

Effects of stress from isolation (neglect) or poor maternal care

Some of the noted changes in HPA axis activity in animals that experienced isolation are; 1) altered functions of neurotransmitters, primarily serotonin, (Kehoe et al, 2001), noradrenalin and dopamine, (Kehoe et al, 2001; McCormick et al, 2002; Champagne and Meaney, 2001) Downregulation in noradrenergic α-2 receptor density (Champagne and Meaney, 2001), 3) increases in corticotropin releasing hormone (CRH) secretion, 4) with subsequent increases in adrenocorticotropin hormone (ACTH), and activation of glucocorticoid secretion (corticosterone in rats), 5) downregulation of glucocorticoid receptors in the hippocampus and frontal cortex (Kehoe et al, 2001; Ladd et al, 2001; Champagne and Meaney, 2001; McCormick et al, 2002), 6) downregulation of $GABA_A$-benzodiazepine receptors (Champagne and Meaney, 2001), 7) cytokine induction (Simmons and Broederick, 2005), 8) suppression of neurogenesis in adulthood

(Mirescu, Peters and Gould, 2004), and 9) altered patterns of DNA methylation, histone acetylation and transcription factor binding to the GR (glucocorticoid receptor) promotor (Weaver et al, 2004), of which are noted as epigenetic changes as a result of behavioural programming through maternal care.

Effects of maternal care from stress during pregnancy

The natural variations of high and low grooming characteristics revealed with maternal care in rats have shown to have extended into intergeneration effects of care for future offspring. This phenomenon has also been extrapolated to that in primates and humans (Champagne and Meaney, 2006). Child abuse and neglect have been long associated with poor developmental outcomes in mental wellness, the ability to cope with stressors, and learning capacities both scholastically and socially. Interestingly, socioeconomic status has not been considered a factor in poor development when parental care is factored out statistically; there are no significant findings of adverse effects of low income on cognitive development in children (Champagne and Meaney, 2006).

To test this hypothesis in rats, Champagne and Meaney (2006) induced restraint stress in pregnant dams that were already established with respect to licking and grooming behaviour towards their offspring. Results found that when HG-ABN and LG-ABN dams were stressed during gestation, significant effects in maternal care were found in HG-ABN dams toward their second litter. In fact, the HG-ABN stressed dams' level of maternal care was similar to that found in LG-ABN non-stressed and LG-ABN stressed dams' maternal care, indicating that stress during gestation interferes with the quality of

maternal care and strongly contributes to the effects of anxiety observed in the offspring. The quality of maternal care from gestational stress in LG-ABN dams was not different to non-stressed LG-ABN dams. The same results were replicated in a third pregnancy with the same dams that underwent stress during their second pregnancy; however, stress was not administered with the third pregnancy. Maternal care of the third pregnancy was consistent with the second; LG-ABN dams with stress, LG-ABN dams without stress and HG-ABN dams with stress, delivered similar levels of maternal care among each other and between the second and third pregnancies in response to exposure to prenatal stress. Furthermore, the female offspring (generation three and four) of the second and third pregnancies showed significantly reduced maternal care levels from LG-ABN (stressed, non-stressed) dams and reduced quality in maternal care in HG-ABN females. The time spent with their pups did not differ among dams, but oxytocin binding in the medial preoptic area of the hypothalamus did vary between dams of HG-ABN and LG-ABN and was found decreased in HG-ABN dams that were stressed during gestation. The oxytocin receptor has been associated with the individual differences in maternal care. High LG dams have a higher receptor density than low LG dams and these differences in receptor density are seen in the female offspring. Female offspring of high LG dams have a high receptor density and those from low LG dams a lower receptor density. When an oxytocin receptor antagonist was applied to high LG dams, their maternal care was similar to low LG dams (Champagne and Meaney, 2006). Maternal care during the lactation stages of rat pup development has been considered a critical factor in the functional and behavioural outcome in the offspring of rats and could explain some of the effects of stress during gestation.

III. Brain Plasticity

The experiences that an individual will endure throughout a lifetime are many and varied and can be presumed to affect the brain, although likely in different ways. As a result such various exposures have been proposed to sufficiently enable an individual to adapt to new changes in their environment. The ability to make appropriate behavioral changes in response to a changing environment is considered to be a form of learning and learning has been proposed to be accompanied by structural and chemical changes in the brain (Kolb, 1999). If an individual has at some time during their life suffered from injury to the brain the behavioral outcome generally changes over time, presumably in association with changes in brain structure and/or activity. These subsequent changes in the brain are known to depend on factors such as locus of lesion, age at injury, extent of injury, gonadal hormones, growth factors, pre- and postinjury experience, and so on (Kolb, 1995).

Neuroplasticity, as it is presently known, encompasses a multitude of processes. Each process involves molecular changes, is different through stages of the lifespan, and has specific requirements including limitations that are incorporated into the remodeling of a group of synapses.

The early pioneers of plasticity, Tanzi, and Ramón y Cajal began constructing theories of plasticity in 1893 based on learning primarily from the various external signals that must be processed on a daily basis (Greenough & Chang, 1988). The experiences would be normal experiences people encounter, but might not pay much attention to. Experiences include learning an alternative route to work, meeting a new neighbor, or trying a new restaurant. Thereafter, a series of research psychologists began

to form various theories of learning, memory, and plasticity (Greenough & Chang, 1988; Cooper, 2005). Cajal, as an anatomist worked with stained postmortem tissue and was able to study the ontogenesis of the developing mammalian cerebral cortex through its various stages. In collaboration with Camillo Golgi, and his histological technique of impregnating nerve cells with silver, Ramón y Cajal won the Nobel Prize in Physiology or Medicine in 1906, which he shared with Golgi for his work on the nervous system. Cajal eventually developed a theory of the neuron: neurogenesis, chemotaxis and dynamic polarization. By analyzing properties and characteristics of growth cones on newly developing and migrating neurons in the chick spinal cord, Cajal suggested that there were physio-chemical signals along their pathway that secreted and directed growth cones to their final destinations. Not only were there positive chemicals, or attractants, there were also negative, or inhibitory chemicals to aid in guiding growth cones to their proper destination. This became Cajal's chemotactic or 'neurotropic hypothesis' (de Castro, Lopez-Mascaraque, & De Carlos, 2007). These theories have become further developed and are currently considered the template to provide guidance into knowledge of the brain's operations and characteristics, but primarily to understand the mechanisms involved under situations of brain injury, treatment effects, and psychological disorders.

In 1928, Cajal proposed the notion that the learning process might be accompanied by the establishment of neural connections in the brain that become efficiently functional. Donald Hebb (1949) suggested the principle site of these functional neural changes would be at the synapse. Hebb also included that if the activity between neurons was sufficiently strong enough the changes would become permanent and this idea became known as *the Hebbian synapse* (Cooper, 2005; Kolb, 1995). Hebb

extrapolated his theories of plasticity by refining the current classical and instrumental learning theories into the 'Hebbian' learning rule. His rule that neurons that fire together wire together was proposed as a general rule that defines the conditions required for the establishment and maintenance of the neural connections that enable learning while changing the structural morphology of the synapse (Cooper, 2005).

Early experimental procedures to demonstrate brain and behavioural plasticity in animals began in Donald Hebb's (1947) kitchen where he allowed a group of young laboratory rats to live freely until adulthood (Kolb and Whishaw, 1998). The control rats remained at McGill University and were compared to the "kitchen-enriched" rats in a series of cognitive tests at maturity. The enriched rats' performances were superior to the control rats suggesting a positive environmental factor during development (Kolb and Whishaw, 2001). Thereafter, various paradigms were employed to demonstrate changes developmental factors, such as visual deprivation or imprinting, and social interaction (Greenough & Chang, 1988). Rozenweig and colleagues extended their behavioural findings to changes in the rat's brain as a result of enrichment. The dorsal regions of the cortex were heavier and thicker in enriched animals compared to the isolated animals with the largest emphasis observed in the occipital cortex. The neurons were also larger and were accompanied with more glial cells (Greenough & Chang, 1988).

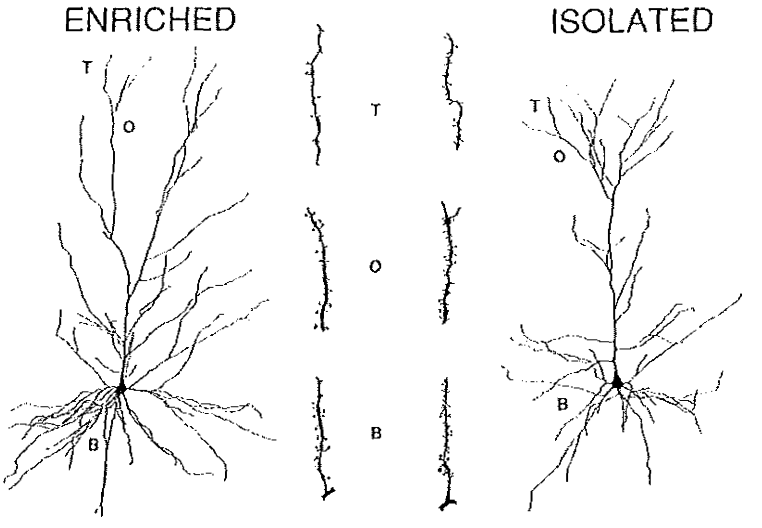


Illustration of representative layer III parietal pyramidal neurons from a rat placed in an enriched environment at weaning versus a littermate that was housed in standard laboratory housing. The dendritic branches down the midline are expanded view of terminal (T), oblique (O), and basilar (B) portions, illustrating the dendritic spines. Note that the spine density varies with location on the dendritic tree. Enriched housing at this age produced an increase in branching but a decrease in spine density

Figure 3. Illustration of dendrites, axons, and spines in neural cells enriched from condominium housing (From Kolb, 1999 with permission).

The idea that brain plasticity might vary with age at injury arose from the observation of the effects of early childhood brain injury (Kolb, 1995). Broca noted that his young patients with damage to the language areas of the brain rarely developed aphasia later in life. This led Broca to suspect that there was something different about the effects of brain injury in the infant compared to effects seen in the adult. Kennard supported this hypothesis with her research on the effect of brain lesions in monkeys. Infant monkeys with motor cortex lesions showed improved behavioral recovery as opposed to similar injury in adulthood. The argument posed by Kennard (1942), namely that brain injury during childhood has a more favorable behavioral outcome than similar

injury later in life, was presumed to be due to the fact that the brain is still developing during these early years. Responses to injury in the early years could be different, possibly by some form of reorganization of neural circuitry. It was therefore postulated that "earlier is better" for recovery from brain injury (Kolb, 1995). Contrary to Kennard's views, Hebb (1949) later suggested that there may be certain periods during development of the cortex that would result in more deleterious effects than that produced by similar injury later in life. Hebb studied children with damage to the frontal lobes and noted that if this damage occurred at certain periods during development, there were severe cognitive and behavioral consequences in adulthood (Hebb, 1949; Kolb, 1995).

To understand these cognitive and behavioral consequences of brain injury, Kolb and colleagues have performed extensive research in rats to determine these critical periods for recovery and plasticity in the mammalian brain and to investigate the effects of various treatment interventions. Results of cortical plasticity after brain injury can be quite significant, quantified with measurements taken for changes in axon and dendrite characteristics as well as changes in glial cells. From these data, Kolb and colleagues have constructed charts to identify growth periods during cortical development, as well as time dependent periods of maximal and minimal periods of cortical plasticity throughout the lifetime of a rat (see Fig.4).

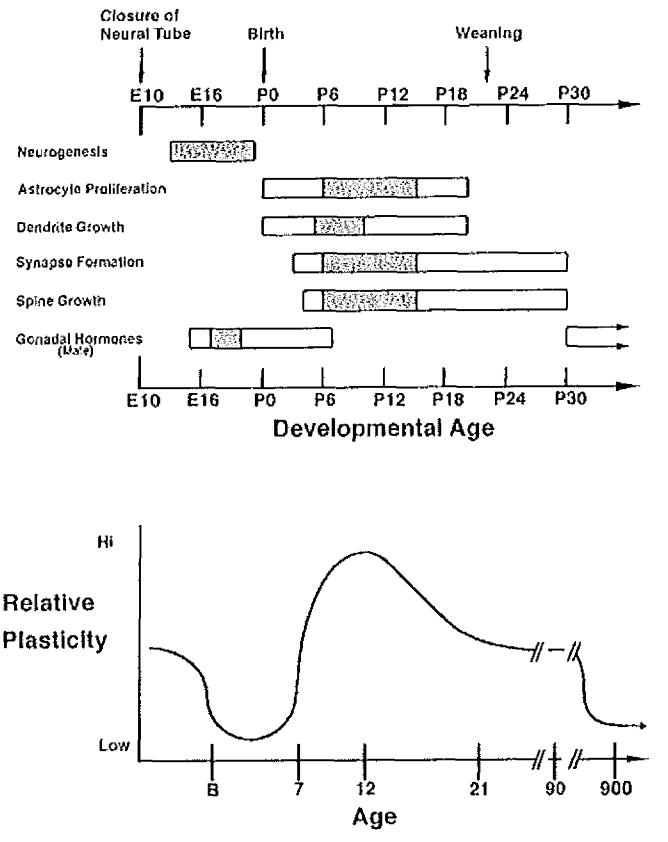


Figure 4. Cortical plasticity of the developing rat brain. Top- approximate time period of the main cellular events. Bottom- Summary of the time dependent processes of plastic cortical periods during the life of the rat (From Kolb, 1999 with permission).

IV. The Frontal Cortex: Functions in Humans and in Rats

One of the simplest ways to describe the frontal lobe and its function would probably be as the anterior region of the brain that temporally integrates neuronal input from all regions of the brain, records it into memory, organizes it, and prepares the individual for the execution of a behavioral response. The frontal lobe as a whole consists of the association areas of the prefrontal cortex, the premotor cortex, and the primary motor cortex. The prefrontal cortex in humans is considered the region of highest intellect

of the mammalian species. The complexity in connectivity and function gives this area of the brain a recognized characteristic of executive function, which implies that all complicated decisions regarding appropriate behavioral outcome are assessed in the prefrontal cortex. Another section of the prefrontal cortex, the orbital cortex (orbitofrontal), adjacent and anterior to Broca's speech area, is dominant for social awareness and behavior as well as inhibition of distractions (see Fig. 5). The premotor cortex prepares the motor cortex for action which is contingent on the decision-making processes from the prefrontal cortex during complex and novel experience.

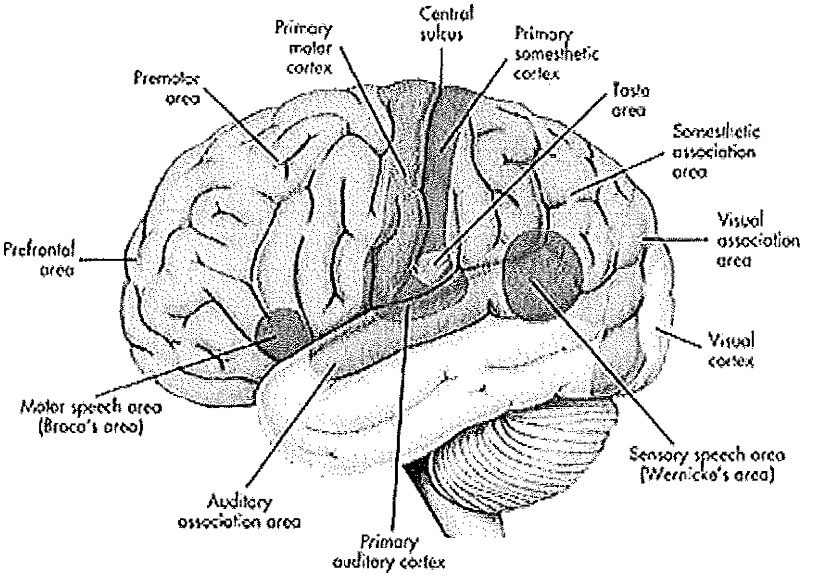


Figure 5. Anatomical areas of the human brain (From http://universe.review.ca/110-80-prefrontal.jpg).

Injury to the motor cortex in humans is known to cause difficulties in fine finger movements and losses in speed and strength (Kolb & Whishaw, 2003). The premotor cortex provides adequate preparation of voluntary motor movements. It does this as it

simultaneously receives and sends signals to the parietal and temporal association areas. An injury to this area of the brain often results in disturbances of voluntary eye gaze, and speech, as well as disturbances to messages forwarded to the posterior areas to accommodate for planned movements (Kolb & Whishaw, 2003). Injuries to the orbitofrontal region (ventral prefrontal area) have revealed impulsive and disinhibited behaviors as a result of poor response inhibition, some of which develop into social and sexual disorders (Kolb & Whishaw, 2001; Cummings, 1995). The orbitofrontal cortex has also been proposed to contribute to the attention processes by filtering out unnecessary information prior to entering the executive function processes (dorsal prefrontal area). Hence, dysfunction of the orbital or ventral prefrontal cortex leads to the execution of unwanted and unfavorable responses and behaviour (Fuster, 2002; Cummings, 1995). This area is also part of a frontoparietal network as part of the attentional processes (Corbetta & Shulman, 2002). During in vivo neuroimaging, neural activity occurred when subjects were required to redirect, or shift their attention to locations of salient or unexpected stimuli (Corbetta & Shulman, 2002). The parietal connection enables the ventral frontal areas to make spatial shifts of attention. Although the ventral (orbital) cortical areas are not specialized to focus attention spatially, it is suggested that this cortical area is differentially engaged by task-relevant stimuli and determines their behavioral valence (Corbetta & Shulman, 2002).

Prefrontal cortex injuries to the dorsolateral region are known to cause executive dysfunction. Because this set of cognitive function is very complex, impairments generally result in an array of behavioral deficits, namely, large deficits in the supervision of attentional control (Fuster, 2002).

The human dorsolateral prefrontal cortex selects and recruits sensory information from posterior and temporal cortical regions necessary to integrate and process information sufficiently. Attention is required to select the appropriate or relevant features and inhibit irrelevant features of a task and uses working memory (short-term memory) to keep information "on line". These processes are necessary for an individual to focus their attention and to allow the prefrontal cortex to organize and manipulate the information to prepare for an appropriate behavioral response according to the task requirements. Impairments with these attention processes generally result in a disorganized plan and inappropriate preparation and execution of actions. Furthermore, these processes all require accurate monitoring and upgrading of ongoing cognitive processes and executed behaviour to continue to produce favorable responses and filter out any inappropriate urges (Fuster, 2002). These are some of the hallmarks of executive functions. Fuster's (2002) model of executive function, or supervision of attention, is defined by four contingencies of established prefrontal cortical functions: 1) attention; 2) working memory; 3) prepatory set; and, 4) monitoring. These processes however do not function in a linear form from the first to the last process, but rather operate in an overlapping manner (Fuster, 2002). Fuster's model of executive function operates within long-term memory processes, he calls executive memory. Previously performed actions necessitate past memories for reference, which would progress as an updated version with the present action, and returned back into long-term memory. The updated version of newly acquired behavior requires processes of rehearsal using working memory, filtering out unnecessary distractions prior to consolidating the updated information into long-term memory.

Other hallmarks of executive function are the cognitive and behavioral inflexibility commonly found in prefrontal cortex injuries. These individuals also have difficulty in shifting attention set. Inflexibility can be seen when an individual has achieved an attention set with a particular rule for a task, but then is unable to shift to another rule. For example, some people who are accustomed to cooking a meal one particular way, with the same seasonings and methods, struggle to try a new recipe with new flavor combinations. They may want to try a new recipe and understand what is required to go through with a new plan of cooking, but cannot actively shift to a new method. Rules can also be considered as plans necessary to adapt to a new desired behavior.

Injury to the frontal cortex in rats indicates some similarities to that of humans (Kolb, 1984). Brain lesions including the motor cortex impair the serial ordering of digit and forelimb movements and the execution of a chain of voluntary movements. Effects of damage can also be seen in tasks requiring strength or speed.

Injury of the orbital regions, or the ventral portions of the rat prefrontal cortex, produces abnormal social interactions, hyperactivity, and an inability to initiate spontaneous behaviors such as in generating new strategies for task solutions. Defective odor discriminations are also characteristic of orbitofrontal lesions, and Kolb (2004) has proposed that orbital lesions interfere with processes that integrate olfactory information into working memory. Maintaining stimuli "on line" is a medial prefrontal characteristic and is necessary to make the appropriate associations among stimuli to attach the appropriate meanings to the perceived stimuli. This type of deficit could provide numerous complications for rats, as olfaction is one of their primary sensory modalities

necessary for orientation within their environment and among peers (Kolb, 1984). Lesions to the orbitofrontal cortex in rats have also revealed impairments in delayed response of low reinforcement tasks that measures bar-pressing extinction (Kolb, Nonneman, & Singh, 1974). Deficits in extinction behaviors have been considered as impairments in shifting response strategies. The rat is required to extinguish a previously rewarded response that was no longer rewarded on subsequent trials. Extinction behavior also requires the inhibition of recently rewarded discriminations to shift to and adopt new discriminations. Spatial reversal tasks have also been problematic for rats with orbital lesions, but the impairment is actually one of perseverative tendencies to the previously rewarded arm, and not one of solving spatial problems. Although lesions of the medial frontal regions of the prefrontal cortex reveal spatial problem solving deficits and deficits in delayed-response type solving tasks, bar-pressing extinction tasks are not affected (Kolb, Nonneman, & Singh, 1974).

The serial ordering, or temporal integration, of movement has been determined to be a function of the medial frontal cortical areas in the rat (Kolb, 1984). Measures associated with the serial organization of behavior have been determined in delay-response type tasks that determine capabilities in working memory, or in spatial aspects of that task (Kolb, 1984). In consideration of the possibility that rats might possess executive functions, many researchers have devised various experiments in attempts to isolate a specific executive function with particular regions of the prefrontal cortex. Kolb (1984) has defined impairments in behavior of temporal ordering of movements, response inhibition, spatial processing, and habituation as a result of prefrontal cortical damage.

Although these impairments have not been labeled as "executive dysfunction", the resulting behavior may be comparable.

Researchers who have attempted to delineate the separate effects of executive processes in rats have isolated certain aspects of these functions through brain lesion experiments. A "supervisory attention system" residing in the prefrontal cortex of rats has been proposed by Delatour and Gisuet-Verrier (2000). Attention, behavioral flexibility or attention shift, spatial attention and working memory were examined. By using chemically induced excitotoxic lesions of the prelimbic-infralimbic region, they were able to determine behavioral deficits of these executive functions defined by attention and working memory abilities. One of the paradigms tested for flexibility was navigation on a circular arena. Starting positions changed with each trial to assess the ability of rats to shift with each new starting position (Delatour & Gisuet-Verrier, 2000), similar to the strategies required to learn the water maze task. Birrell and Brown (2000) maintain that executive function of attention shift does exist in the rat, and resides in the prelimbic-infralimbic region of the prefrontal cortex (see Fig. 6). This was demonstrated using excitotoxic lesions and a behavioral task that did not require spatial shifts of attention, but was intended to measure only a shift of attention using the inhibition of previous responses (Birrell and Brown, 2000).

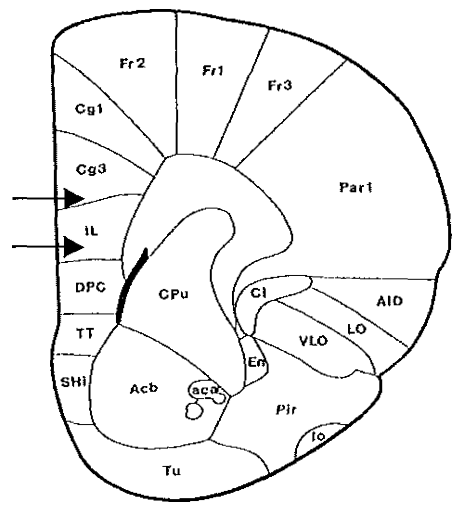


Figure 6. Illustration of rat brain hemisphere displaying the infralimbic (IL) and prelimbic (PL) regions of the prefrontal cortex. IL and PL are Paxinos and Watson's (1997) nomenclature which corresponds to Zille's (1985) IL and DPC (Dorsal Peduncular Cortex).

Behavioral tasks measuring spatial attention and working memory have been indicated as part of the prelimbic functions, as determined by Fritts (Fritts, Asbury, Horton & Isaac, 1998). Fritts, performed a study of cortical lesions from surgical removal of the medial frontal cortical region, some or which included the prelimbic areas, some did not. The results of this experiment suggests that the anterior cingulate, infralimbic and medial precentral (premotor) areas are required for attention with working memory, and temporal sequencing of information in rats (Fritts, Asbury, Horton & Isaac, 1998).

More recent research surrounding executive function in the prefrontal cortex has gone beyond cognition to include the emotional and motivational aspects of behaviour governed by the orbitofrontal cortex. Although the two regions of medial and dorsal prefrontal cortex have some distinct differences in function from the orbital or ventral

prefrontal cortex, both regions are part of a frontostriatal circuit connecting to the amygdala, hippocampus and other limbic areas (Happaney, Zelazo, and Stuss, 2004). In the extradimensional shift task demonstrated by Birrell and Brown, the shift requires the inhibition of attending to the previously rewarded discrimination and shifting to the next discrimination of the task. The inhibition is a property of the orbitofrontal cortex that flexibly enables the medial and dorsal areas of the prefrontal cortex to shift attention to a new behavioural selection.

Table1. Summary of the effects of medial frontal and orbital frontal lesions in rats.

Brain Region	Behavioral Impairment	Basic Reference
Medial Frontal: (including motor)	Serial ordering of digit and forelimb movements, Execution of a chain of voluntary movements,	Kolb et al., 1984
	Decreases in strength and speed.	
	Delay-response type solving tasks, Working memory, Habituation.	Kolb, Nonneman & Singh, 1974
	Spatial attention, attention shift, or behavioral flexibility.	Delatour & Gisett-Verrier, 2000
	Non-spatial attention shift.	Birrell & Brown, 2000
	Attention with working memory, Temporal sequencing of information.	Fritts, Ashbury, Horton & Isaac, 1998

Brain Region	Behavioral Impairment	Basic Reference
Orbital Frontal:	Social interaction, Hyperactivity, Response inhibition, Inability to initiate spontaneous behavior, Defective odor discriminations	Kolb, 2004
	Extinction, Perseveration.	Kolb, Nonneman & Singh, 1974

Organization of the Thesis

This thesis addresses several questions:

1) What are the effects of stress on the brain and in behavioural development? We predicted that stress would have a negative effect.

2) What is the effect of perinatal frontal cortical injury on brain and behavioural development? We have consistently found mild behavioural impairments with some sex differences.

3) How does prenatal stress influence the recovery from perinatal injury? We assumed that stress would have a negative effect on the already existing lesion deficits.

4) Is there an effect of stress intensity on either the intact or injured brain? We predict negative effects increase with the intensity of the stress.

5) Can the effects of stress and injury be influenced by either dietary or environmental manipulations? We predicted that treatments would ameliorate the effects of stress or a lesion.

6) Do any of the treatments affect play behaviour? We predicted that all treatments would be positive for play behaviour.

Owing to the complexity of the answers to these questions, the results will be presented in pieces in order to facilitate reading. Because the methods are constant across experiments, they are presented first as one omnibus methods section.

Prenatal Stress Experiments

Methods and Materials

I. Prenatal Stress Protocols

Experiments 1 and 2

Ten virgin female rats were introduced to breeding males and were considered in heat with potential impregnation when a temperature sensitive probe inserted indicated a temperature above 3.5 and a subsequent vaginal smear indicated the presence of sperm. Five females were assigned to a non-stressed control group; the other five to the experimental stress paradigm. On the 12th day of gestation stress procedures began twice daily and continued until day 18 of gestation. Restraint stress in 7.6 cm inner diameter Plexiglas tubes for small pregnant females and 10.2cm inner diameter tubes for larger pregnant females for 20 minutes followed by a 5 minute forced swim in a rubber tank (30 cm*w* x 53 cm*l* x 86 cm*h*) containing tepid water of approximately 20°C (see Fig. 7). The water was deep enough to prevent pregnant rats from being able to touch the bottom with their tails.

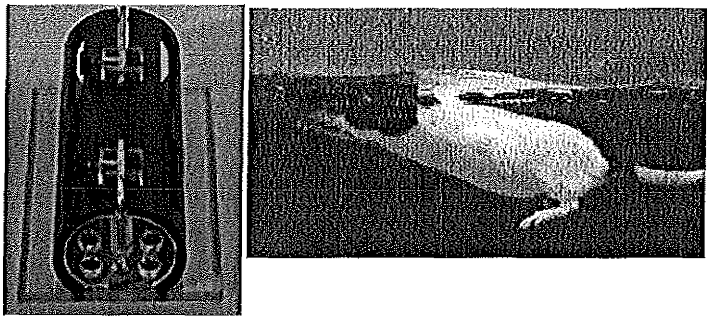


Figure 7. Left: a picture of a restraint tube used for restraint stress. Right: a picture of a rat swimming in the tank.

Subjects

A total of 237 rats (120 females, 117 males); 78 rats were control (39 females, 39 males); 64 were prenatally stressed (32 females, 32 males); 16 were non-stressed control and condominium housed (8 females, 8 males); and, 27 rats were prenatally stressed (10 females, 17 males); and a small set of supplemented diet (3 females, 4 males) animals were used in experiments 1 and 2.

Of the total number of rats, subsets had frontal lesions; control frontal lesion (11 females, 7 males), stress frontal lesion (17 females, 10 males), non-stressed condo-housed frontal lesion (2 females, 6 males); and, stressed condo-housed frontal lesion (5 females, 10 males). Two females (1 control frontal and 1 stressed frontal) died in adulthood prior to perfusion.

Experiments 3 and 4

All procedures follow as above with the exception of: 1) restraint stress was induced for 15 minutes, 2) water temperature was increased to approximately 32°C, 3) the water tank had dimensions of 120cm*l* x 43cm*w* x 50cm*h*, 4) all stressed and non-stressed females remained housed with their male partners until the birth of the pups, 5) all ten mating pairs received snacks (granola bars, Froot Loops™, bread) after the stress bout and throughout the stress period to blunt the stress response in the stressed females of the experimental group, and 6) blood samples were not taken with this group.

Subjects

A total of 204 rats (102 females, 102 males); 78 rats were control (39 females, 39 males); 71 were prenatally stressed (34 females, 37 males). Of these two groups: 29 were

non-stressed control with supplemented diet (14 females, 15 males); and, 33 rats were prenatally stressed with supplemented diet (14 females, 19 males); were used in experiments 3 and 4.

Of the total number of rats, subsets had frontal lesions; control frontal lesion (15 females, 11 males), stress frontal lesion (15 females, 11 males), non-stressed supplemented diet frontal lesion (2 females, 6 males); and, stressed supplemented diet with frontal lesion (7 females, 7 males). Two stressed males died prior to perfusion; one after surgery, the other in adulthood.

Cameras for maternal care

Cameras were infrared (model E200WP, Taiwan), which filmed the dams and litters on a 24-hour moderately movement sensitive program. Camera adjustments were made with MSI- TVR computer program. Data was collected from Eyecopia™ computer program in the form of short-segment movies. Movies were scored for analysis.

Litters

Pups from stressed dams were born gestation day 20, or 22 in both experiments. Dams and pups were undisturbed until approximately postnatal day three when cages underwent a regular cleaning schedule every three days, which increased in frequency with the growing pups. All litters (except in the condo treatment group) were group housed in large Plexiglas tubs 40cm*w* x 58cm*l* x 20cm*h* containing four to eight rats per tub. Animals were housed with a 12 hour light/ 12 hour dark cycle beginning at 7:30am/7:30pm.

Prenatally stressed pups in experiments one and two did not deviate in body weight after birth, whereas pups from stressed litters in the 3rd and 4th experiments weighed more than their control counterparts. Weights were taken approximately postnatal day 10.

II. Treatment Interventions

Experiments 1 and 2: Condominium housing was provided for rats to live in throughout their lives beginning day 21 or 22 after weaning (see Fig. 8). The condo and toys were cleaned well once a week and rats would receive a bath prior to returning. Upon their return, toys would change to introduce some novelty.

Figure 8. A picture of a condominium the rats were reared in.

Experiments 3 and 4: The vitamin/mineral supplement EMPower+ was incorporated into rat feed (see Fig. 23). Rat dams began this diet on the day of birth until their pups were weaned. Thereafter, rat pups ate this diet for the remainder of their lives.

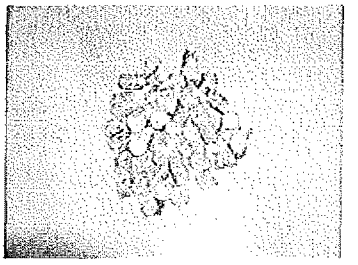


Figure 9. Picture of the rat chow with the incorporated supplement EMPower+.

III. Behavioural Tasks

Animals of all five experiments were tested on four behavioral tests; 1) activity (locomotor activity); 2) the Morris water maze (a spatial learning task); 3) tray reaching (a skilled forelimb motor task); and, 4) the elevated plus maze (measure of anxiety).

Subsets of rats from experiments one and two were tested in 1) circadian rhythm; 2) running wheel activity; 3) sucrose consumption; and, 4) maternal care. These behavioural measures were not tested in experiments three and four because the circadian and wheel running cages were not available and the startle foot-shock was not included in the updated protocol for those experiments.

In experiment five, subsets of animals from all experiments participated in social play-fighting.

<u>Social play</u>

Play-fighting behaviour in rats was filmed in the dark (lights off) with a Sony 8mm camcorder using night-shot for 10-minute intervals for each pair of animals. The testing area consisted of a wooden box with 50cm*l* x 50cm*w* x 50cm*h* dimensions (see Fig. 10). The top of the box was open to transport animals in and out, the back wall was a mirror to reflect the activity filmed, and the frontal panel was clear Plexiglas for viewing

through. The bottom of the play box was filled with corncob bedding to a height of two to three cm. Prior to filming animals are habituated to the play arena for one-half to one full hour for three consecutive days. The day before test day (day four) the animals are isolated from having contact with each other; a process considered to be effective for the induction of play behaviour in rats (Pellis and Pellis, 1990). They were all separately put into retaining containers with bedding and food and water provided. On the fourth day, the animals have their tails marked with livestock grade markers (control-black, experimental-yellow) to distinguish between rats during scoring of the film. Thereafter, the animal pairs are introduced to each other in the play box, the camera is set to record, lights are turned off, and a timer set for ten minutes is started. Following the ten-minute play period, animals are returned to their cage-mates and eventually all returned to their home cages. This process occurred during the week when animals were 40 to 45 days of age as well as during the week of 60 to 65 days of age.

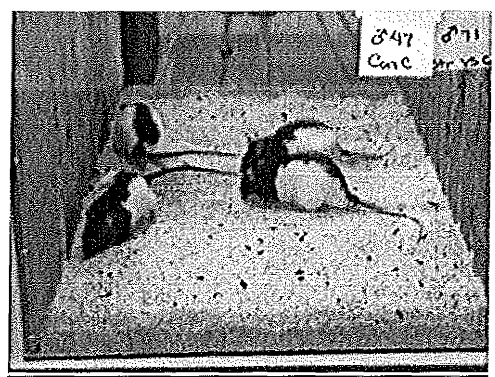


Figure 10. A picture of the play box arena that rats were filmed during social play-fighting.

Locomotor activity

The activity apparatus is commonly used to measure a variety of locomotor movement in rodents (see Fig. 11). Each box is made of plexiglas and measures 42cmw

x 42cm*l* x 30cm *h*. Sensors surround each box measured the rat's activity with Versamax Animal Activity Monitor (Accuscan Instruments Inc., USA). The data is recorded into Versamax 400 computer program. The activity type reported in the present experiment includes the distance of activity and the number of movements recorded from each rats. All animals were tested for a 10-minute period.

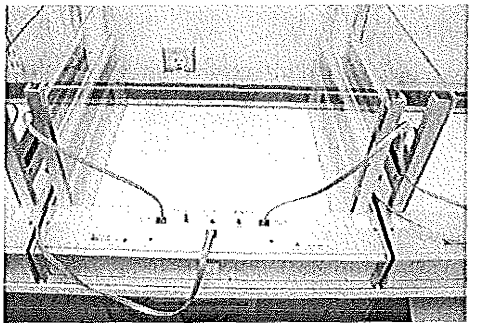


Figure 11. The Activity box to measure locomotor activity in rats.

Morris water maze task

The Morris water maze requires rats to form cognitive representations of their world by navigating within their local environment (see Fig. 12). The maze is a circular pool with a diameter of 1.5m and a height of 0.5m deep. During place learning, a hidden platform (11×12 cm.) was submerged under water with approximately 1.5 centimeter below the waters surface. The temperature of the water was 22°C each day and rendered opaque against the pool's white walls using a non-toxic white paint. Training of rats used a procedure of place learning, as described by Sutherland (1993). Rats were placed into the pool facing toward the sides of the pool, from four random locations; north, south, east, and west. Rats swam four trials per day for five days, with each trial allowing 90 seconds to find the hidden platform. Rats that did not find the platform during the 90 second period were placed on the platform to learn of its existence. The platform was

centered in the northeast quadrant (quadrant 1). A twenty second inter-trial interval enabled rats on the platform to observe their surroundings. The latency for rats to find the platform was used to measure learning deficits. All swim trials were recorded by means of a video camera mounted from the ceiling directly above the pool. Data recorded from the camera were analyzed by a computer program (Water 2020) and expressed as latency for five-day acquisition, and total sums latency.

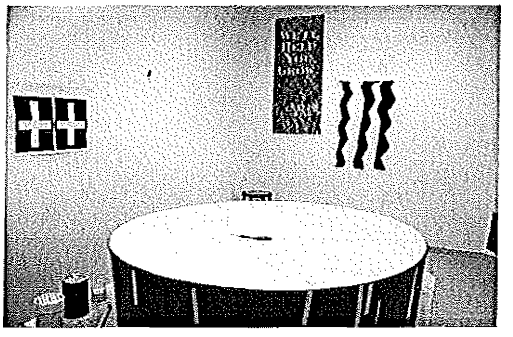


Figure 12. The Morris water maze to measure cognition in rats.

Tray reaching

The tray-reaching task devised from Whishaw's skilled reaching task (Whishaw, O'Connor, and Dunnet, 1986) is useful to assess limb and grasp function of motor control (see Fig. 13). The test cages consist of clear Plexiglas walls with a closable lid (20× 28× 26 cm). Mounted in front of each set of cages was a stainless steel tray, which held small pellets of chicken feed at a distance of about 5cm from the face of the cage. Rats are required to reach through the steel bars on the face of the cage to the tray of food pellets, grasp a pellet, and bring it back to their mouths to eat. Once they are trained to their normal capacity, each rat is filmed individually for 10 minutes. Rats were food deprived daily for six days by limiting food intake to 15g for each female and 20g for each male. On the seventh day rats were fed ad libitum. These food portions have been determined to

slowly reduce body weight, which should not exceed a loss of 85% body weight. Rats were weighed at the end of both weeks to ensure minimal weight loss. Rats were filmed by a video camera individually for 10 minutes and scored. Successful reaches were a 'hit', unsuccessful, a 'miss'. Scores were represented as percent success; the number of hits was calculated as a percent of total attempts (hits and misses combined).

Figure 13. The tray reaching cages to measure skilled reaching in rats.

Elevated plus maze

A very common and reliable measure for anxiety in rodents is the elevated plus maze (see Fig. 14). The maze is in the shape of a '+' sign with two opposing arms having high walls for shelter and the other two opposing arms without walls. The maze is made of wood, painted gray in color, and stands 88cm high, with each arm at 113 cm in length and 10 cm in width. All rats were timed in the maze for a total of 5 minutes and filmed from a high angle with a Canon digital video camcorder (800x digital zoom) ZR 500. The experimenters waited outside the testing room until the 5-minute time limit. Scoring consisted of timing the amount of time that each rat spent venturing out onto the open arms. Rats were not considered into the open area until all four paws crossed over the

center into the open arm. In the present experiments, the rats had already undergone four behavioural tasks and were not novel to handlers, tasks, or mazes.

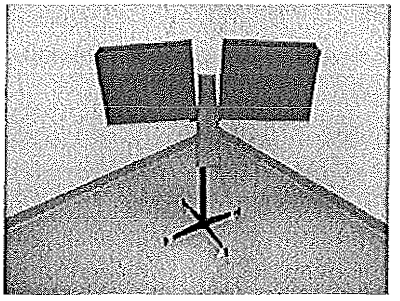


Figure 14. The elevated plus maze to measure behaviours of anxiety in rats.

Circadian activity

Rats were housed for approximately 36 hours in cages (~ 35cm*l* x 25cm*w* x 15cm*h*) that had detectors on the front of the cages to measure activity. One detector was placed on the left side of the cage, the other on the right. Food and water was provided. Computer software for circadian activity measured and recorded activity for each hour. The last 24 hour period was analyzed to determine any differences in circadian activity. The period prior to the last 24-hour period was used to habituate rats to a new environment.

Wheel running activity

The circadian cages were not available for some of the rat groups, therefore wheel running cages were used to get an indication of activity levels during day and evening hours.

Rats were placed individually into Plexiglas cages 27(*w*) x 47(l) x 20(h) cm, which had a wire bottom with a tray underneath. A stainless steel cover included an

attached wheel with 38cm perimeter. An activity counter (Hamlin) was hooked up to the wheel and would read a digital recording of the number of complete rotations of the wheel.

Rats were placed into the cages for 24 hours beginning at 5:00pm and left overnight until 5:00pm the next day. The number of running activity was taken at 7:00am in the morning (overnight), 12:00 noon (am), and again at 5:00pm (pm).

Maternal Care

Maternal care for rat pups was filmed using infrared cameras, which were programmed to activate with movement at moderately high sensitivity. Scoring of movie clips on DVD consisted of recording the frequency of movements designated as 1) licking and grooming pups, 2) arched-back nursing (active nursing), 3) passive nursing (blanket, or side), 4) contact, and 5) no contact. Measures are reported as percent time.

IV. Anatomical Procedures

Intracardiac perfusion

At the completion of behavioral testing, rats were given an overdose of sodium pentobarbital (0.5ml-0.7ml) until they reached a comatose state. At this point, they were perfused intracardially, using 0.9 % saline solution. Rat brains destined for Cresyl Violet staining, continued perfusion with of 4% phosphate buffered paraformaldehyde. Removal of the brains were done using Rongeurs and weighed immediately following removal from the skull.

For Cresyl Violet preparations, the brains were placed in small bottles containing 4% phosphate buffered paraformaldehyde for 24 hours and replaced with 30% buffered sucrose solution.

For Golgi preparations, brains were weighed at the end of saline perfusions and placed in small bottles containing Golgi solution. Golgi-Cox prepared brains were sliced on a Vibratome at 200μm thickness, saving all slices on 2% gelatin coated slides. These brains were processed following procedures devised by Gibb and Kolb (1998).

The brains for Cresyl Violet staining were sliced on a Microm Cryostat HM 560 which maintains tissue at -20°C. Every 7th slice of each brain was taken at 40 μm thickness and placed on slides fixed with a film consisting of 1.0% gel and 0.2% chromium potassium sulfate dodecahydrate. Once the brains were dried to the slides, the slides were placed in slide trays, which hang on a Fisher Histomatic Slide Stainer (model 172). This apparatus runs through a programmed series of solutions required to complete Cresyl Violet staining.

Brain Removal and weight (at sacrifice)

The measures of brain weight are obtained by weighing each brain directly after removal from the skull of the perfused rat. The spinal cord was cut along the caudal edge of the cerebellum, the cerebellar paraflocculi were removed, the optic nerve was cut 1-2 mm anterior to the optic chiasm, and remaining dura was stripped off the brain's surface. At the front end of the brain, the olfactory bulbs were cut at 1.5-2.5 mm from the frontal cortex.

Adrenal gland removal and weight (at sacrifice)

Adrenal glands were located embedded in fatty tissue caudal and dorsal to the kidney on both sides of the body. Using a pair of tweezers, the glands were extracted and trimmed of excess fat prior to weighing. Glands were then place into centrifuge tubes containing 4% paraformaldehyde. Prior to slicing, the glands were placed into 30% sucrose solution until they had sunk to the bottom of the cuvette (indicating full permeation). Glands were sliced on Microm Cryostat HM 560 and placed onto slides coated with 1% gelatin, .2% chromium potassium sulfate dodecahydrate. All slides were stained with Cresyl Violet, using the same procedure as the brains.

Testes removal (at sacrifice)

Testes were located in the scrotal sac connected to the epididymis and embedded in fatty tissue. The testes were shaped like a bean and were both extracted and weighed after perfusion then discarded.

Dendritic arbor and length measurements

Pyramidal cells of layer III in the agranular insular dorsal (AID), layer III in the posterior parietal area I (experiment 1), and layer V of the anterior cingulate (Cg3) of the prefrontal cortex was drawn using camera lucida technique at 200X magnification. Criterion for cells inclusion: 1) cells must be well impregnated with the Golgi-Cox stain; 2) not obscured by blood vessels, astrocytes, or clusters of dendrites from other cells; and, 3) the apical and/or basilar regions of the cells were visibly intact (see Fig. 15).

Quantification of cells used two procedures: 1) dendritic branch order (from Coleman and Reisen, 1968) that counted each branch bifurcation beginning at the cell body (basilar) and the primary apical bifurcation (apical) beginning at the primary apical bifurcation, and 2) Sholl analysis (Sholl, 1981) that quantifies the number of rings that dendrites intersect when a transparency of concentric rings spaced 20 μm apart is placed overtop the cell drawings. Dendritic length (μm) was estimated by multiplying the number of intersections crossed by 20.

Five cells were drawn in each areas AID and Cg3 of each hemisphere (see Fig. 15).

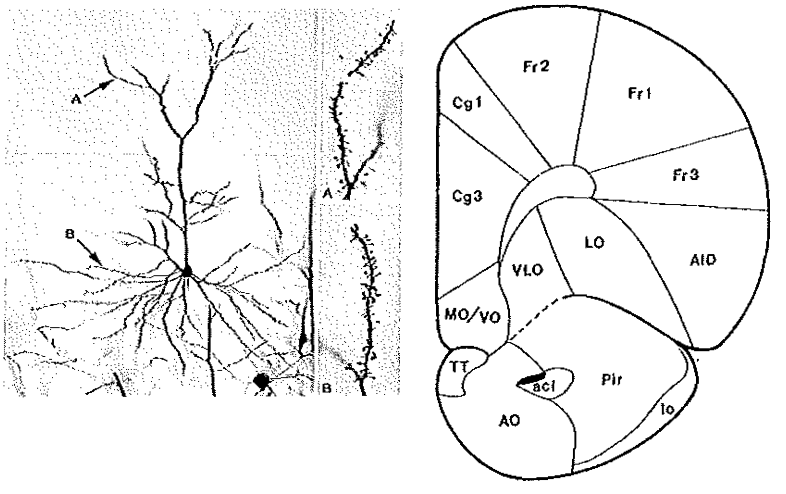


Figure 15. Left: Illustration of Golgi-Cox stained pyramidal cell. A: apical dendrites and B: basilar dendrites on the left side of the illustration. Dendritic spines on right side of illustration (Courtesy of Grazyna Gorny). Right: Illustration of prefrontal areas the Cingulate cortex area 3 (Cg3) and the agranular insular dorsal cortex (AID) in the rat brain.

Adrenal gland areal measures

The slides containing Cresyl violet- stained adrenal glands were photographed with a Canon Powershot A640 Digital camera. Each slide was increased in size (~1.8x digital) prior to photographing. The pictures of slides of adrenal glands were increased to 100% for areal measures using a tracer with Image J computer software (see Fig. 16). The number of pixels per square centimeter was determined for areal measures of adrenal glands. An estimated center-most region of the gland was measured in addition to ± five measures for each gland pair (bean and triangle) giving a total of 11 areal measures.

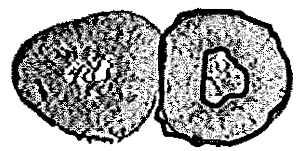


Figure 16. Example of tracing around adrenal gland cortex and medulla for areal measures.

Cortical thickness measures

After slide preparation, the brains were viewed and assessed at a magnification of 17.5 × with a Zeiss DL 2 POL petrograph projector. Cortical measurements were made using a clear plastic ruler. Three different cortical measurements (lateral, medial, and central) for each hemisphere were determined at five planes (see Fig. 17). A standard technique was followed according to Zille's (1995) anatomical markers and described by Stuart and Kolb, (1988). Briefly, three measures were taken at; 1) medial, 2) central, and 3) lateral points on each hemisphere throughout five planes of the brain. Plane 1) (a) consists of AID, Par I, and Fr2 (at first caudate); plane 2) (b) Par2, Par1, and Fr1 (anterior commissure); plane 3) (c) GI/DI, Par1, and Fr1 (first hippocampus); plane 4) (d) Te1,

Oc2L, and RSA (posterior commissure); and, plane 5) (e) Te1, O1B, and Oc2ML (end of hippocampus).

Figure 17. The five planes (a-e) measured to determine cortical thickness in rat brains.

Prelimbic and Infralimbic measures of the medial prefrontal cortex

Measures were taken similar to the cortex measures, with the exception of measuring four planes at the beginning of the prefrontal cortex across both hemispheres (see Fig. 18). Planes one to four to measure the Prelimbic cortex were determined by bregma: plane 1) bregma 3.7; plane 2) bregma 3.2; plane 3) bregma 2.7; and, plane 4) bregma 2.2. The Infralimbic cortex measured at plane1) bregma3.2; plane 2) bregma 2.7; and, plane 3) bregma 2.2.

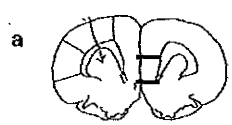


Figure 18. Example of measures for the prelimbic measures (top) and infralimbic measures (bottom).

Thalamic width measures

Thalamic width was measured at two planes based upon previous studies by Kolb and Whishaw (1981). The first was the anterior plane, at the point of a predominant MD nucleus, and when the hippocampus CA fields and dentate gyrus had become full. The second plane was at the last slice that had a full thalamus, which is at the beginning of the posterior commissure (see Fig. 19). Degeneration of thalamic nuclei is commonly seen as

a result of cortical injury. Degeneration is generally characterized by darker stained cells in thalamic nuclei, shrunken nuclear areas, as well as abnormally shaped cells.

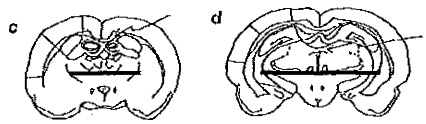


Figure 19. Example of thalamic measures. Left: anterior measure. Right: posterior measure.

Statistics

The statistics used for the current set of experiments was SPSS14. The general linear model (GLM) with a univariate analysis was most commonly used to test the analysis of variance for the independent variable of interest by factors, such as stress, sex, treatment, and/or lesion. GLM repeated measures analysis was used for multiple measures of a single independent variable followed by a multivariate analysis to determine any significant differences among each repeated measure. The large groups however, were excluded from the thesis due to the large numbers of subjects involved.

Chapter Three: Results

I. Experiment 1A: The effects of moderate prenatal stress on brain and behavioural development

This set of experiments used the first protocol for stress; 20 minutes of restraint stress altered with 5 minutes of forced swim, twice daily from gestation days 12 to 18.

The following section compares the effects of prenatal stress (Stress NL) to non-stressed animals (Control NL).

Behavioural Results

Locomotor Activity

Distance

Females showed greater activity than males in the activity box, but was reduced from prenatal stress (see Fig. 20).

Univariate analysis for distance indicated a main effect of sex ($F(1,76)= 10.075$, $p= 0.002$), but not of stress ($F(1,76)= 2.379$, $p= 0.127$), nor for the interaction between stress and sex ($F(1,76)= 1.834$, $p= 0.180$).

Number of movements

Both sexes that were prenatally stressed performed a greater number of movements in the activity box (see Fig. 20).

Univariate analysis for movement frequency indicated a main effect of stress ($F(1,76)= 6.452$, $p=0.013$), but not of sex ($F(1,76)= 0.292$, $p=0.591$), nor for the interaction ($F(1,76)= 0.173$, $p=0.679$).

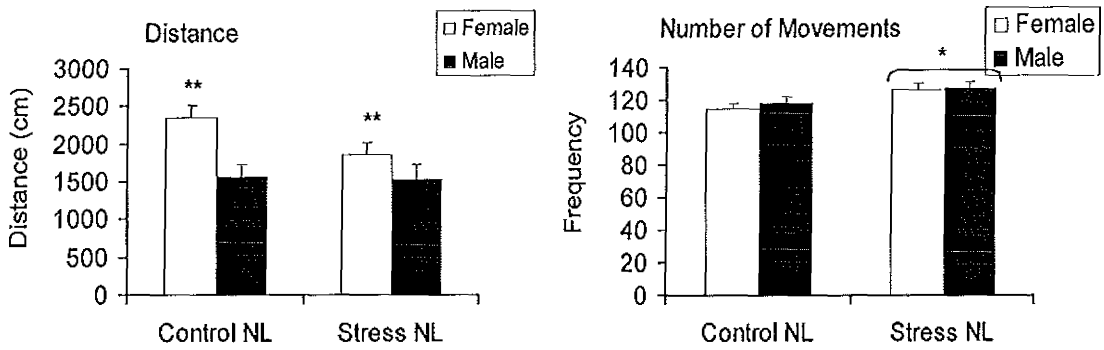


Figure 20. Left: Sums of mean distance (cm) of exploration in the activity box in prenatally stressed animals. Right: Sums of mean movement frequency in prenatally stressed animals. ** Denotes significantly different between the sexes ($p<.05$). * Denotes statistically different from non-stressed (Control NL) animals ($p<.05$). Bars indicate mean ± SEM.

Morris water maze

The stress treatment did not significantly affect performance in the water maze (see Fig. 21).

Univariate analysis for latency sums did not reveal any statistically significant effects of stress in latency to find the hidden platform $F(1,141)= 1.566$, $p= 0.213$), or of sex ($F(1,141)= 0.043$, $p= 0.835$), nor for the interaction between Stress X Sex ($F(1,141)= 2.75,6$ $p= 0.099$).

A multivariate analysis for day found day two significantly different among groups ($p<.05$) (see Fig. 21).

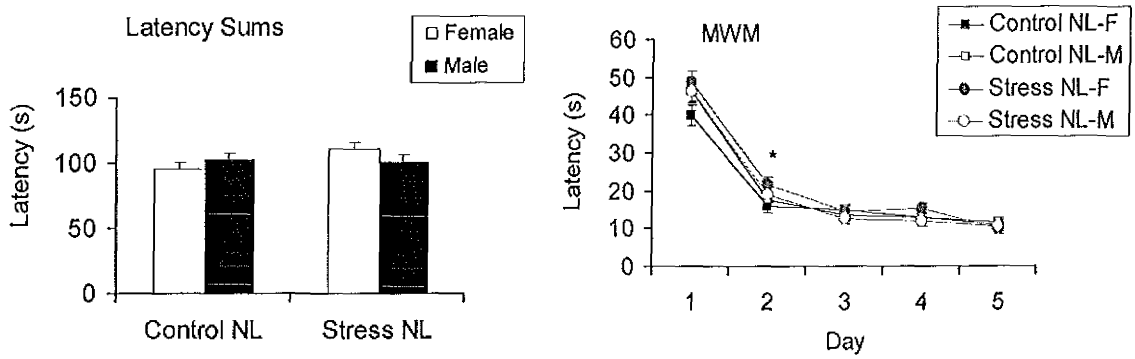


Figure 21. Left: Sums of mean five-day latency in prenatally stressed animals. Right: Mean latencies during five-day acquisition. * Denotes the significant day among groups ($p<.05$). Bars indicate mean ± SEM.

Morris water maze probe

There were no differences between groups during the probe test (see Fig. 22).

Univariate analysis for percent time spent swimming in the correct quadrant (Q1) did not indicate a main effect of stress ($F(1,82)= 0.029$, $p= 0.866$), of sex ($F(1,82)= 0.002$, $p= 0.965$), nor for the interaction ($F(1,82)= 0.085$, $p= 0.771$).

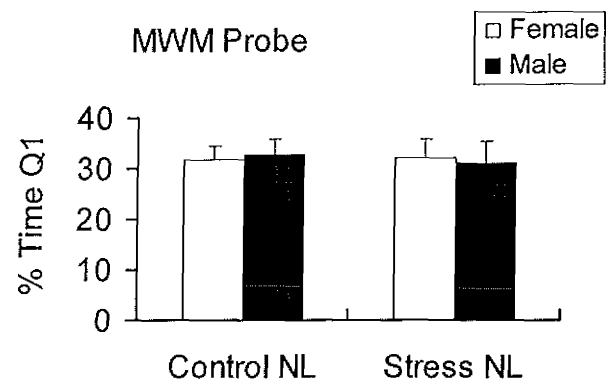


Figure 22. Percent time spent in quadrant one during the Morris water maze probe test for memory of the hidden platform. Bars indicate mean ± SEM.

Tray Reaching

Prenatal stress treatment impaired skilled reaching in both males and females (see Fig. 23). Although most animals show a strong bias to use one paw, six out of 43 animals that were prenatally stressed used both paws during reaching compared with three of 34 non-stressed control animals that used both paws. Regardless of the degree of bi-paw use in skilled reaching, stressed animals were reaching impaired.

Univariate analysis revealed a main effect of stress ($F(1,96)= 11.247$, $p= 0.001$), but no main effect of sex ($F(1,96)= 0.924$, $p= 0.339$) and no significant interaction between stress and sex ($F(1,96)= 3.585$, $p= 0.061$).

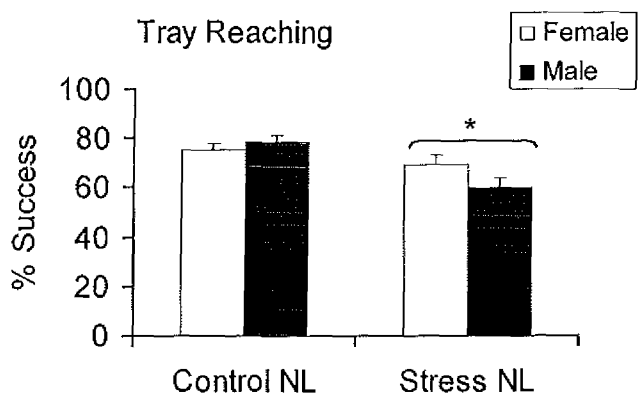


Figure 23. Mean percent success for skilled reaching in prenatally stressed animals. * Denotes the significant effects of prenatal stress ($p<.05$). Bars indicate mean ± SEM.

Elevated Plus Maze

Normal rats typically wander about the maze, but overall, 21 animals (7 females, 14 males) in the stressed group did not enter the open arms (scores = zero), resulting in a high degree of variance. Prenatal stress had a significant effect in exploration, especially in males (see fig. 24).

Univariate analysis for emotionality in the elevated plus maze found a main effect of stress ($F(1,78)= 24.319$, $p< 0.0001$), and of sex ($F(1,78)= 4.349$, $p= 0.040$), but not for the interaction ($F(1,78)= 0.020$, $p= 0.889$).

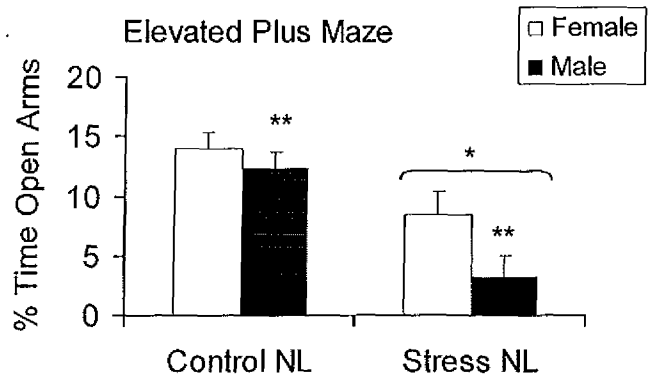


Figure 24. Mean percent time spent exploring open arms in the elevated plus maze. * Denotes statistically significant from non-stressed control animals ($p<.05$). ** Denotes significantly different between the sexes ($p<.05$). Bars indicate mean ± SEM.

Circadian Rhythm

Prenatally stressed females showed a large increase in activity during the night hours peaking at approximately 10:00 pm (see Fig. 25).

Repeated measures analysis for circadian activity in prenatally stressed rats revealed an interaction between stress and sex ($F(1,43)= 4.910$, $p= 0.032$), but not for main effects of stress ($F(1,43)= 2.549$, $p= 0.118$), or of sex ($F(1,43)= 3.312$, $p= 0.084$).

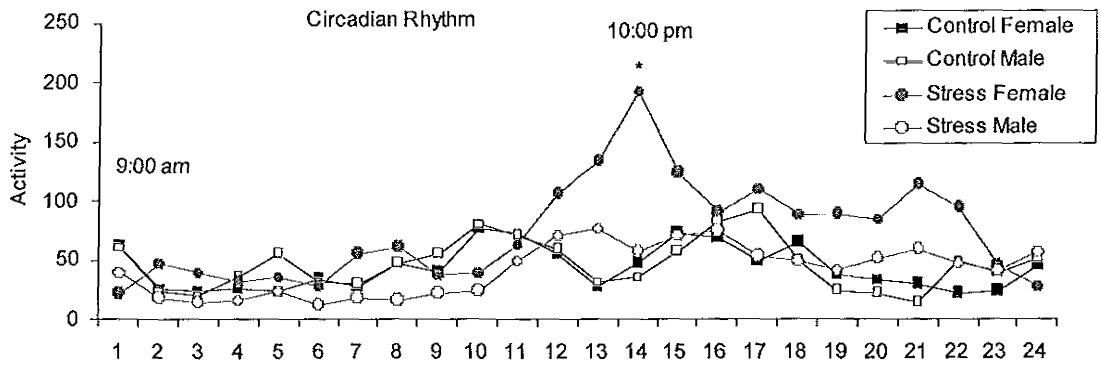


Figure 25. Measures of circadian rhythm activity for a 24-hour period in prenatally stressed rats. * Denotes significantly different effect in prenatally stressed females ($p<.05$). Bars indicate mean ± SEM.

Wheel Running

Wheel running activity was substituted for the circadian cages that were not available for use. Wheel running can indicate a peak activity time through day and evening hours.

The activity cages suggested changes in diurnal activity in prenatally stressed females, as was suggested in the circadian cages noted above. Thus whereas males showed no effect of stress on activity, females showed a marked suppression in the morning hours, but showed the highest amount of activity in the afternoon period of the day, although the latter was not significant (see Fig. 26).

Univariate analysis for wheel running during the morning hours revealed an interaction between stress and sex ($F(1,23)= 4.194$, $p= 0.054$), but no main effects of stress ($F(1,23)= 3.428$, $p= 0.079$), or of sex ($F(1,23)= 0.178$, $p= 0.678$).

Univariate analysis for PM activity in the running wheel did not indicate significant effects of stress ($F(1,23)= 0.621$, $p= 0.440$), or of sex ($F(1,23)= 1.555$, $p= 0.227$), nor for the interaction ($F(1,23)= 0.088$, $p= 0.770$).

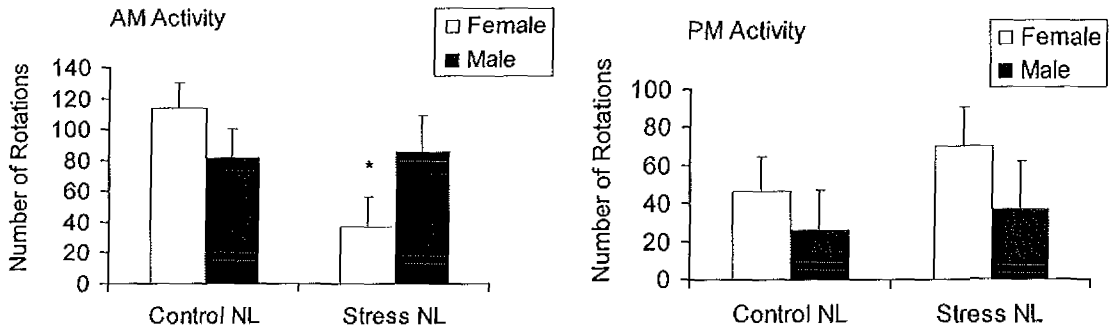


Figure 26. Left: Mean activity during the morning hours (7:00am – 12:00 noon) in prenatally stressed animals. Right: activity during the afternoon hours (12:00 noon – 5:00pm) * Denotes the significant effects of prenatal stress in females ($p<.05$). Bars indicate mean ± SEM.

Anatomical Results

Golgi-Cox brain and saline-perfused adrenal gland measures

The brains and adrenal glands were taken from animals that were prepared with only saline perfusion prior to placing the brains in the Golgi-Cox solutions. These tissues are not comparable to animals prepared with formalin fixation and are thus analyzed separately.

Brain weight

Male brains were significantly heavier than female brains, but there were no effects of prenatal stress (see Table 2).

Univariate analysis for measures of brain weight indicated a main effect of sex ($F(1,82)= 80.233$, $p<.0001$), but not of stress ($F(1,82)= 0.000$, $p= 0.996$), nor for the interaction ($F(1,82)= 0.286$, $p=0.594$).

Table 2. Mean brain weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control NL	1.857 ± .021 (n= 30)	2.101 ± .025 (n= 22)*
	Prenatal Stress	
Stress NL	1.866 ± .028 (n= 17)	2.087 ± .031 (n=14)*

Brains are measured in grams mean ± SEM. * Denotes statistically different between sexes ($p<.05$).

Dendritic basilar arbor and length

Cingulate cortex (Cg3)

Basilar fields

Prenatally stressed rats of both sexes had increased dendritic basilar arbor in Cg3, but had different effects between the sexes in dendritic length pyramidal cells in Cg3 (see Fig. 27).

Univariate analysis for basilar dendritic branch arbor of the cingulate cortex indicated a main effect of stress ($F(1,19)= 9.594$, $p= 0.007$), but not of sex ($F(1,19)= 0.004$, $p= 0.951$), nor for a significant interaction between stress and sex ($F(1,19)= 0.183$, $p= 0.674$).

Univariate analysis for Sholl measures of dendritic length in Cg3 found a main effects for sex ($F(1,25)= 8.604$, $p= 0.008$), as well as a significant interaction between stress and sex ($F(1,25)= 4.919$, $p= 0.037$), but not for a main effect of stress ($F(1,25)= 1.066$, $p= 0.313$).

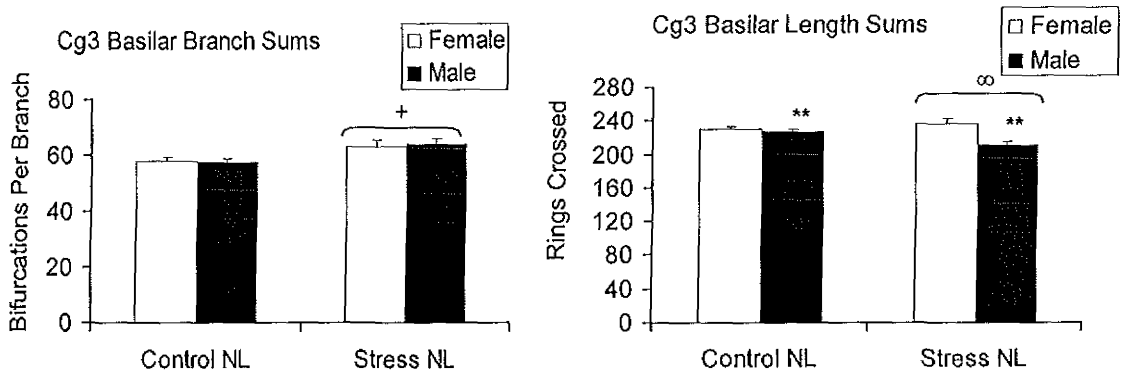


Figure 27. Left: Sums of mean dendritic basilar branch in Cg3 pyramidal cells of prenatally stressed animals. Right: Sums of mean dendritic length from Sholl analysis. + Denotes statistical significance from non-stressed control animals ($p<.05$). ** Denotes

statistical significance between the sexes ($p<.05$). ∞ Denotes the significant effects of stress between the sexes ($p<.05$). Bars indicate mean ± SEM.

Agranular Insular Dorsal (AID) cortex

Basilar fields

Males of both groups had more basilar dendritic arbor than females, with a reduction of arbor density with prenatal stress in both sexes. Sholl analysis revealed prenatally stressed animals to have a reduction in dendritic length of basilar field (see Fig. 28).

Univariate analysis for dendritic branch of the AID region revealed a main effect of stress ($F(1,27)= 7.483$, $p= 0.012$), and of sex ($F(1,27)= 9.677$, $p= 0.005$), but not for an interaction ($F(1,27)= 0.005$, $p= 0.945$).

Univariate analysis of dendritic length in AID indicated a main effect of stress ($F(1,27)= 7.004$, $p= 0.014$), but not of sex ($F(1,27)= 1.324$, $p= 0.261$), nor for an interaction ($F(1,27)= 0.010$, $p= 0.923$).

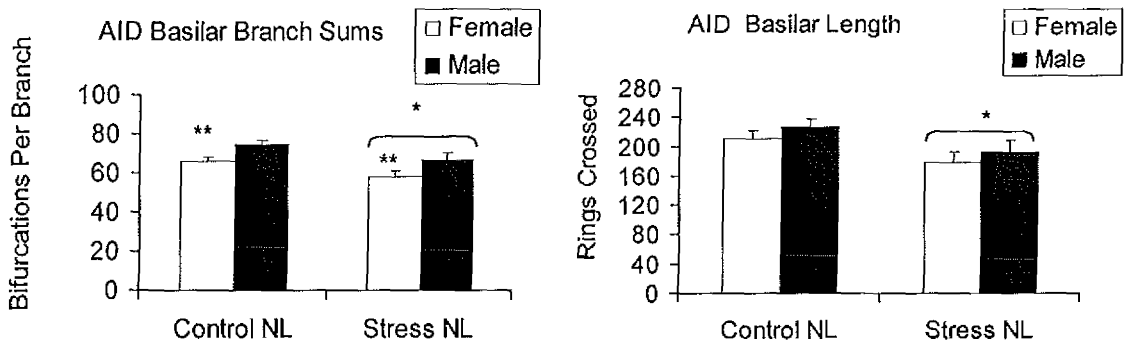


Figure 28. Left: Sums of mean basilar branch arbor and Right: Sums of mean dendritic length of pyramidal cells in the AID region of the cortex. * Denotes significantly

different from non-stressed control animals ($p<.05$). ** Denotes statistical differences between the sexes ($p<.05$). Bars indicate mean ± SEM.

Saline-perfused adrenal measures

Adrenal gland weight

Prenatal stress significantly increased adrenal weight. Females had heavier glands than males (see Table 3).

Univariate analysis indicated main effects of stress ($F(1,51)= 6.982$, $p= 0.011$), and sex ($F(1,51)= 8.601$, $p= 0.005$), but not for the interaction ($F(1,51)= 0.308$, $p= 0.581$).

Table 3. Mean adrenal gland weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control NL	0.081 ± .005 (n= 17)	0.068 ± .005 (n= 14)*
	Prenatal Stress	
Stress NL	0.099 ± .006 (n= 11)	0.079 ± .006 (n= 10)*+

Adrenal glands are measured in grams mean ± SEM. * Denotes significantly different between the sexes ($p<.05$). + Denotes group statistically significant from non-stressed control animals ($p<.05$).

Adrenal gland areal measures

Cortex & Medulla

Both sexes that were prenatally stressed had thinner adrenal cortices and smaller medulla areal measures (see Fig. 29). Although the adrenal cortex measures suggest the cortex is thinner in the stressed animals than in non-stressed control animals, the glands' weight is heavier and thus they must have a denser cell mass. The weight reflects the higher amount of activity (ie. glucocorticoid secretion) in the stressed animals.

Univariate analysis for areal measures of adrenal glands indicated a main effect of stress ($F(1,31)= 8.482$, $p= 0.007$), but not of sex ($F(1,31)= 0.079$, $p= .780$), nor for the interaction ($F(1,31)= 0.884$, $p = 0.355$).

Univariate analysis of adrenal gland medulla areal measures revealed a main effect of stress ($F(1,31)= 17.851$, $p< .0001$), but not of sex ($F(1,31)= 0.063$, $p= 0.804$), nor for the interaction ($F(1,31)= 0.055$, $p=0.816$).

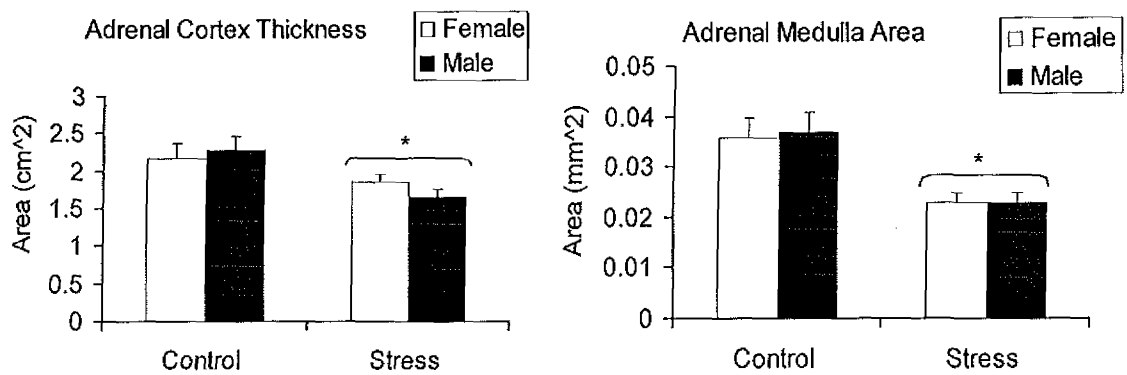


Figure 29. Left: adrenal cortex thickness from adrenal gland cortex areal measures in animals that were prenatally stressed. Right: Adrenal gland areal measures for medulla in prenatally stressed animals.* Denotes significantly different from non-stressed control animals ($p<.05$). Bars indicate mean ± SEM.

Testes weight

The stress treatment did not significantly alter testes weight that could reflect the effects of prenatal stress on testosterone secretion (see Fig. 30) ($F(1,9)= 1.591$, p 0.243).

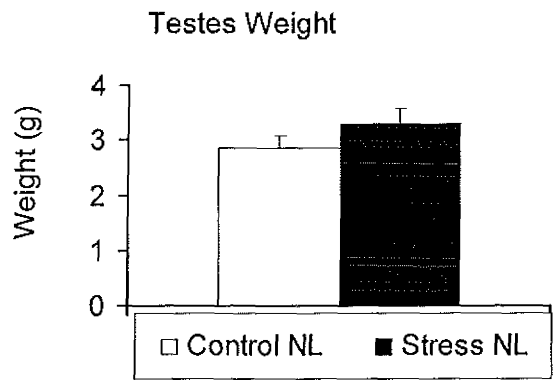


Figure 30. Mean testes weight in prenatally stressed males. Bars indicate ± mean SEM.

Brain measures from formalin-fix perfusion

Prefrontal cortex prelimbic and infralimbic measures

The results revealed a large reduction in cortical thickness in the subregions of the medial prefrontal cortex of prenatally stressed animals (see Table 4). These measures have been analyzed separately from posterior cortical thickness measures to represent the rostral region of the prefrontal cortex that is innervated by ventral and subcortical brain regions.

Univariate analysis for stress treatment in the *prelimbic* cortex indicated a main effect of stress ($F(1,24)= 110.169$, $p< 0.0001$), but not of sex ($F(1,24)= 3.163$, $p= 0.090$), nor for the interaction ($F(1,24)= 0.274$, $p= 0.606$).

Univariate analysis for the *infralimbic* region also revealed a main effect of stress ($F(1,24)= 149.907$, $p< 0.0001$), but not of sex ($F(1,24)= 1.522$, $p= 0.231$), nor an interaction ($F(1,24)= 0.029$, $p= 0.868$).

Thalamus

Prenatally stressed animals had decreased thalamic width in both anterior and posterior regions of the brain (see Table 4).

Univariate for the anterior region of the thalamus found a main effect of stress ($F(1,33)= 33.007$, $p< .0001$) and of sex ($F(1,33)= 6.473$, $p= 0.016$), but not for an interaction ($F(1,33)= 0.790$, $p= 0.390$).

Univariate analysis for the posterior region of the thalamus also found a main effect of stress ($F(1,30)= 79.484$, $p< .0001$), and of sex ($F(1,30)= 8.950$, $p= 0.006$), but not for an interaction ($F(1,30)= 0.894$, $p= 0.353$).

Table 4. Mean sums of measures from formalin-fixed brains and adrenal glands.

Area Measured	**Females**	**N**	**Males**	**N**
	Non- Stressed Control			
[1]Prefrontal Cortex				
Infralimbic	0.660 ± .020	7	0.690 ± .020	7
Prelimbic	1.159 ± .033	7	1.239 ± .033	7
[2]Thalamic Width: anterior	0.859 ± .010	11	0.873 ± .010	11
posterior	0.953 ± .008	11	0.969 ± .008	11

	Prenatal Stress			
[1]Prefrontal Cortex				
Infralimbic	0.400 ± .024	5	0.423 ± .022∞	6
Prelimbic	0.813 ± .038	5	0.856 ± .035∞	6
[2]Thalamic Width: anterior	0.801 ± .008	5	0.831 ± .008*∞	6
posterior	0.879 ± .007	5	0.909 ± .007*∞	6

1. Prefrontal Cortex: prelimbic and infralimbic regions are measured in mean cm. sums ± SEM. ∞ Denotes significantly different from non-stressed control animals ($p<.05$).

2. Thalamic width: anterior and posterior measures of thalamic width are measured in mean cm. sums ± SEM. * Denotes significantly different between the sexes ($p<.05$). ∞ Denotes statistically significant from non-stressed control animals ($p<.05$).

Cortical thickness

Both sexes with prenatal stress treatment had a significantly thinner cortical mantle compared with that of non-stressed control animals (see Fig. 31). Cortical thickness measures were analyzed without the midline cortical regions of the prefrontal cortex (prelimbic, infralimbic) to represent a global and dorsal measure of the cortex.

Univariate analysis for mean thickness across the five planes measured indicated a main effect of stress ($F(1,35)= 280.678$, $p< .0001$), but not of sex ($F(1,35)= 1.469$, $p= 0.234$), nor for the interaction ($F(1,35)= 0.151$, $p= 0.700$).

A multivariate analysis found each plane to be significantly different among groups for the five planes measured ($p< .0001$) (see Fig. 31).

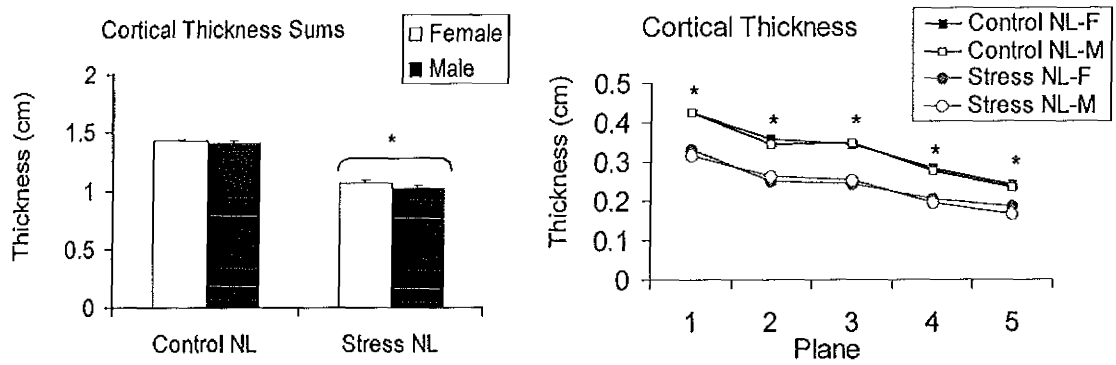


Figure 31. Left: Sums of mean cortical thickness across the five planes measured in animals with prenatal stress. * Denotes statistical significance from the Control NL group ($p<.05$). Right: Mean cortical thickness measures of five planes. * Denotes statistically significant differences among groups ($p<.0001$). F= Female, M= Male. Bars indicate mean ± SEM.

Moderate prenatal stress experiment

Summary

Table 5. Significant findings for experiment 1a.

Behavioural Task	Females	Males
Activity: Distance	Sex: Females more active	Sex: Males less active
Number of Movements	Stress increased activity	Stress increased activity
Tray Reaching:	Impairments with stress	Impairments with stress
Elevated Plus Maze:	Decreased exploration with stress	Decreased exploration with stress
Circadian Rhythm:	Increased activity in pm	No changes

Anatomy (Golgi-Cox preparation)		
Brain Weight:	Sex: Females had lighter weights	Sex: Males had heavier weights
Basilar and Apical Branch Arbor/Length:	Stress increased arbor in Cg3, increased arbor but decreased length in AID	Stress increased arbor in Cg3, decreased arbor and length in AID
Adrenal Gland Weight:	Sex: heavier weights than males; heavier with stress	Sex: lighter weights than females; heavier with stress
Adrenal Cortex Thickness:	Reduced with stress	Reduced with stress
Adrenal Medulla Area:	Reduced with stress	Reduced with stress
Anatomy (Formalin preparation)		
Prelimbic cortex:	Stress had thinner cortex	Stress had thinner cortex
Infralimbic cortex:	Stress had thinner cortex	Stress had thinner cortex
Anterior Thalamus:	Sex: Smaller than males, smaller area from stress	Smaller area from stress
Posterior Thalamus:	Sex: Smaller than males, smaller area from stress	Smaller area from stress
Cortical Thickness:	Thinner cortex from stress	Thinner cortex from stress

II. Experiment 1B: The effects of condominium housing or a micronutrient supplemented diet treatment on moderate prenatal stress

The following section includes prenatally stressed animals (Control NL and Stress NL) with the addition of condominium rearing (Condo Control NL and Condo Stress NL) and vitamin supplemented (VS) diet treatments (Stress VS NL) compared to untreated control (Control NL) animals.

Behavioural Results

Locomotor Activity

Distance

Overall, the condominium group and the supplemented diet group were less active than control animals in the activity box. Females were more active, except for those in the Condo Control NL group (see Fig. 32).

Univariate analysis for distance in the activity box found a main effect of treatment ($F(2,103)= 8.539$, $p< .0001$), and significant differences for a three-way interaction between Stress X Treatment X Sex ($F(1,103)= 3.707$, $p= 0.057$), but not for main effects of stress ($F(1,103)= 0.356$, $p= 0.552$), or of sex ($F(1,103)= 0.760$, $p= 0.385$), nor for the Treatment X Sex interaction ($F(4,103)= 1.776$, $p= 0.140$), and the Stress X Sex interaction ($F(1,103)= 0.463$, $p= 0.498$)

Pairwise LSD comparisons for treatment found condo housing significant ($p= 0.001$) as well as the VS diet treatment ($p< 0.0001$).

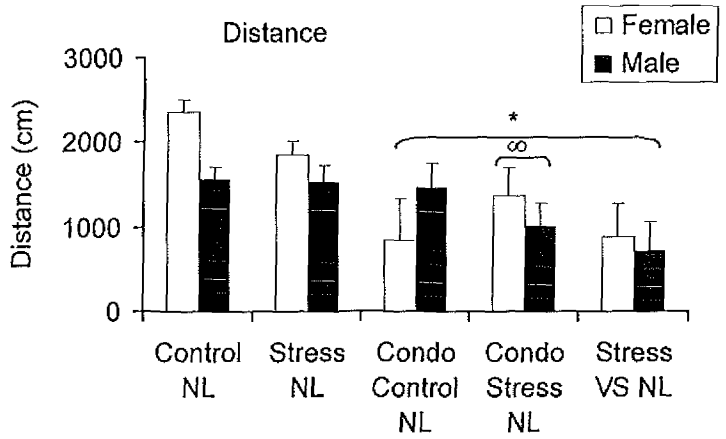


Figure 32. Sums of mean distance explored in the activity box. * Denotes the significant effects of condominium housing and the supplemented diet ($p<.05$). ∞ Denotes the significant effects of prenatal stress with condominium housing on the sexes ($p<.05$). Bars indicate mean ± SEM.

Number of movements

The condominium housed and supplemented diet animals displayed the least number of movements in the activity box (see Fig. 33).

Univariate analysis for number of movements revealed a main effect of stress ($F(1,103)= 8.521$, $p= 0.004$), and of treatment ($F(1,103)= 59.716$, $p< .0001$), but not of sex ($F(1,103)= 0.108$, $p= 0.744$). There were also no significant interactions between Stress X Treatment ($F(1,103)= 0.636$, $p= 0.427$), Stress X Sex ($F(1,103)= 2.387$, $p=0.126$), Treatment X Sex ($F(1,103)= 0.049$, $p= 0.952$), or for Stress X Treatment X Sex ($F(1,103)= 1.435$, $p= 0.234$).

Pairwise LSD comparison for treatment found both the condo housing and the supplemented diet significant ($p< 0.0001$).

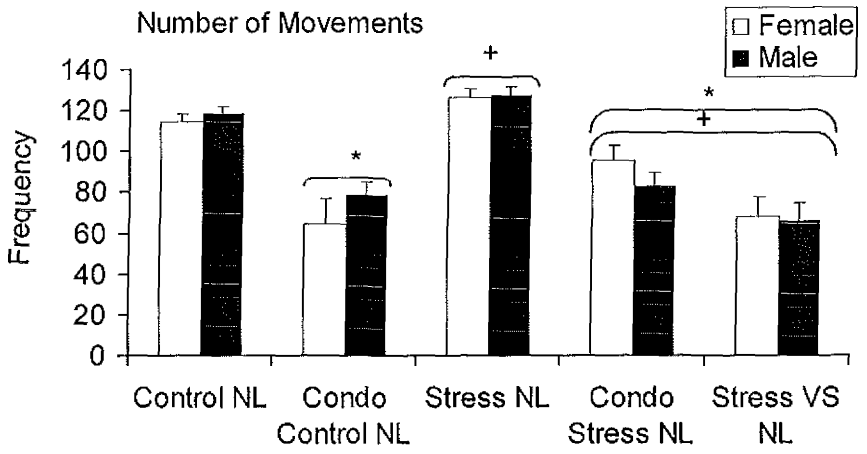


Figure 33. Sums of mean frequency of movement in the activity box. * Denotes statistically significant from the Control NL and Stress NL groups ($p<.05$). + Denotes significant differences in prenatally stressed animals ($p<.05$). Bars indicate mean ± SEM.

Morris water maze

Latency

Males of the condo and diet treatment groups took the least amount of time to find the platform (see Fig. 34).

Univariate analysis for latency sums indicated a main effect of sex ($F(1,168)= 4.344$, $p= 0.039$), but not of stress ($F(1,168)= 1.396$, $p= 0.239$), or of treatment ($F(2,168)= 0.824$, $p= 0.440$). There were no significant interactions between Stress X Treatment ($F(1,168)= 0.128$, $p= 0.721$), Stress X Sex ($F(1,168)= 0.385$, $p= 0.536$), Treatment X Sex ($F(2,168)= 2.728$, $p= 0.068$), nor for Stress X Treatment X Sex ($F(1,168)= 0.222$, $p= 0.638$).

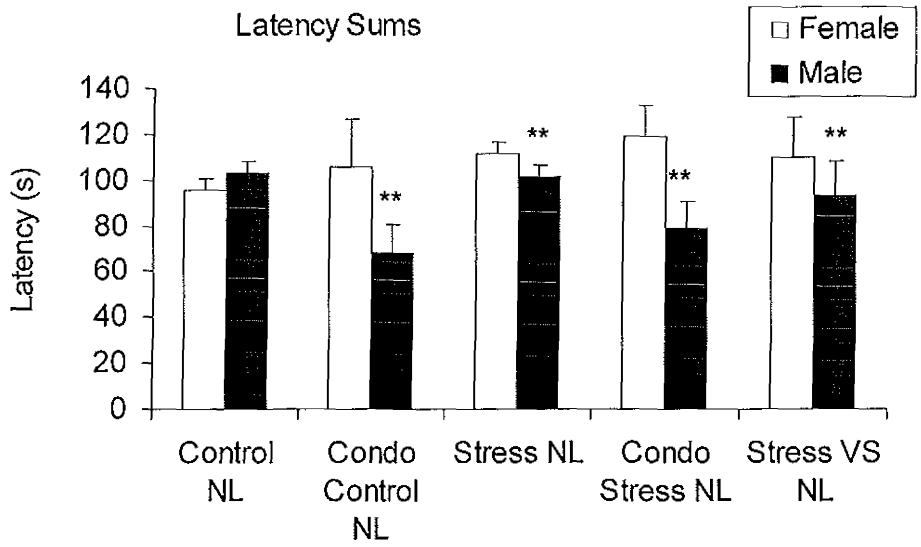


Figure 34. Latency sums for measures of performance in the Morris water maze. ** Denotes statistical significance between the sexes ($p<.05$). Bars indicate ± mean SEM.

Morris water maze probe

The results during the probe test suggest that all groups spent a similar amount of time in the correct quadrant (see Fig. 35).

Univariate analysis for the probe test for memory of the hidden platform of the water maze did not indicate any significant main effects of stress ($F(1,108)= 0.010$, $p= 0.922$), treatment ($F(2,108)= 0.015$, $p= 0.986$), or of sex ($F(1,108)= 0.928$, $p= 0.338$). There were also no significant interactions between Stress X Treatment ($F(1,108)= 0.054$, $p= 0.817$), Stress X Sex ($F(1,108)= 1.657$, $p=0.201$), Treatment X Sex ($F(2,108)= 0.492$, $p=0.613$), or for Stress X Treatment X Sex ($F(2.308$, $p= 0.132$).

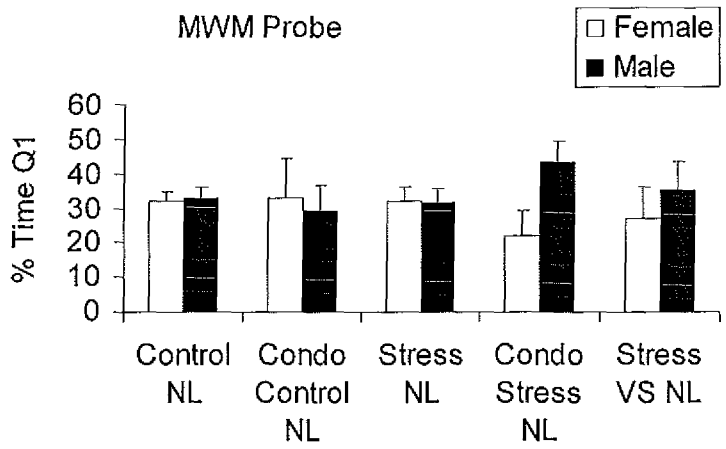


Figure 35. Mean percent time animals spent swimming in the previously correct quadrant. Bars indicate mean ± SEM.

Tray reaching

Both prenatal stress and condominium stress animals had reaching deficits compared to control animals. The supplemented diet appears to have reversed the deficits in both sexes of the prenatally stressed animals, but was not the case for condo-housed animals (see Fig. 36).

Univariate analysis for reaching success indicated a main effect of stress ($F(1,121)= 9.951$, $p= 0.002$), but not of treatment ($F(2,121)= 2.731$, $p= 0.070$), or of sex ($F(1,121)= 0.055$, $p= 0.816$). There were also no significant interactions: for Stress X Treatment ($F(1,121)= 0.059$, $p= 0.809$), Stress X Sex ($F(1,121)= 2.265$, $p= 0.135$), Treatment X Sex ($F(2,121)= 0.470$, $p= 626$), or for Stress X Treatment X Sex ($F(1,121)= 0.000$, $p= 0.997$).

Pairwise comparisons LSD indicated the Condo treatment group significant ($p= 0.010$).

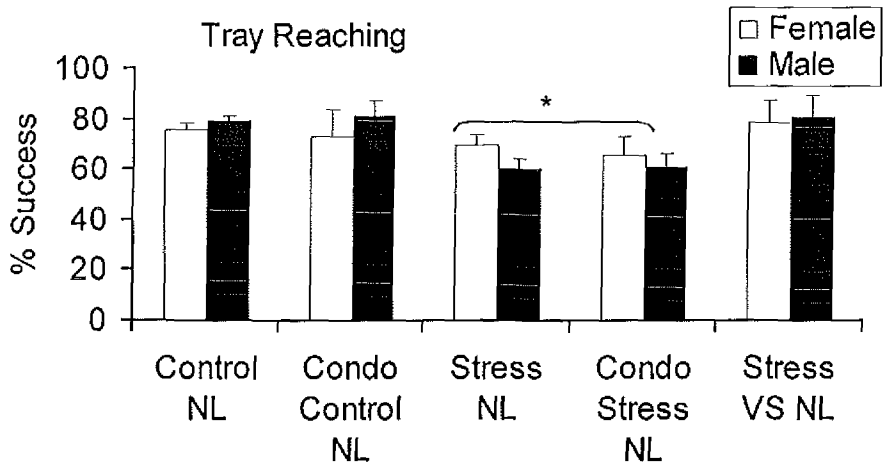


Figure 36. Mean percent success of skilled reaching among treatment groups compared to untreated control animals. * Denotes statistically different from Control NL, Condo Control NL, and Stress VS NL groups ($p<.05$). Bars indicate mean ± SEM.

Elevated plus maze

Both sexes exposed to stress and the two condo-housed groups spent less time out on the open arms with the largest effect in the males (see Fig. 37).

Univariate analysis for emotionality among groups did not reveal main effects of stress ($F(1,128)= 3.263$, $p= 0.073$), of treatment ($F(2,128)= 1.032$, $p= 0.359$), of sex ($F(1,128)= 0.744$, $p= 0.390$). There were also no significant interactions: Stress X Treatment ($F(2,128)= 3.078$, $p= 0.082$), Stress X Sex ($F(1,128)= 0.493$, $p= 0.484$), Treatment X Sex ($F(2,128)= 1.068$, $p= 0.347$), or for Stress X Treatment X Sex ($F(1,128)= 0.024$, $p= 0.876$).

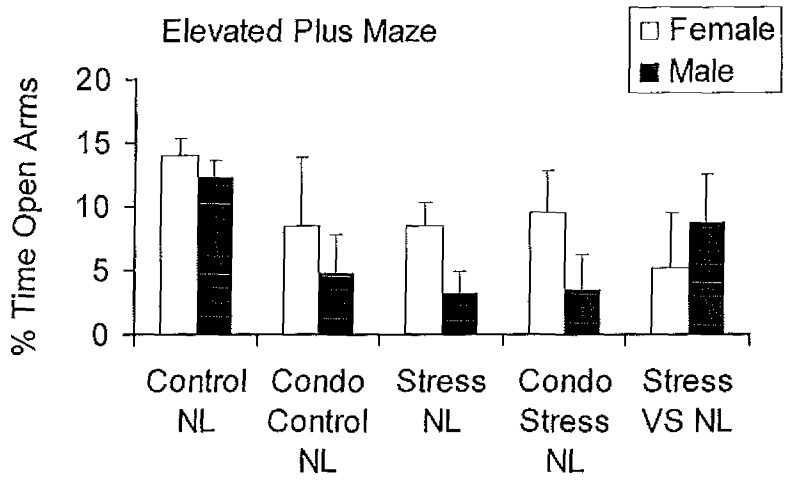


Figure 37. Mean percent amount of time spent exploring the open arms of the elevated plus maze. Bars indicate mean ± SEM.

Circadian rhythm

Females that were prenatally stressed continued to showed peak activity in the evening hours (see Fig. 38).

A univariate analysis found the effect of stress approaching significance ($F(1,63)= 3.440$, $p= 0.069$), but no main effects of treatment ($F(1,68)= 0.552$, $p= 0.461$), or of sex ($F(1,68)= 0.963$, $p= 0.331$). There were no significant interactions: between Stress X Treatment ($F(1,68)= 0.227$, $p= 0.636$), Stress X Sex ($F(1,68)= 2.560$, $p= 0.115$), Treatment X Sex ($F(1,68)= 0.299$, $p= 0.587$), or for Stress X Treatment X Sex ($F(1,68)= 0.098$, $p= 0.755$). The cages were not available for animals treated with the supplemented diet therefore there are no data for the VS group.

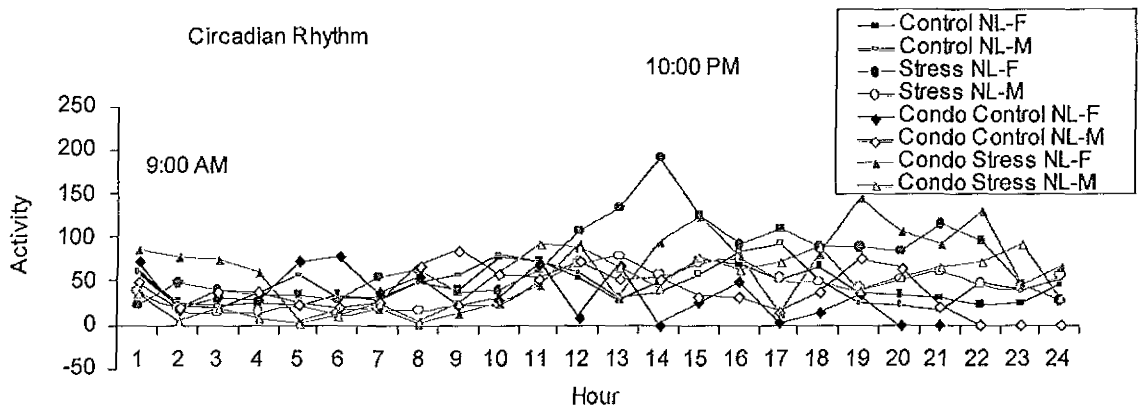


Figure 38. Circadian rhythm measures of activity during a recorded 24-hour period. Animals were treated with prenatal stress, and/or condominium housed rearing.

Wheel running

The circadian cages were not available for use, therefore a subset of animals were put into wheel running cages to get an idea of day and evening activity. There were no apparent differences among males in activity levels during the morning hours (see Fig. 39).

A univariate analysis for circadian activity during the morning hours in wheel running found a significant interaction of Stress X Sex ($F(1,30)= 4.312$, $p= 0.048$), but no main effects of stress ($F(1,30)= 3.524$, $p= 0.072$), of treatment ($F(1,30)= 0.177$, $p= 0.677$), or of sex ($(1,30)= 0.173$, $p= 0.681$). There were no significant interactions between: Treatment X Sex ($F(1,30)= 0.226$, $p= 0.638$).

A comparison of the same above groups in wheel running activity in the afternoon hours did not reveal significant differences (see Fig. 39).

Univariate analysis of afternoon activity levels found no main effects of stress ($F(1,30)= 0.732$, $p= 0.400$), of treatment ($F(1,30)= 2.114$, $p= 0.158$), or of sex ($F(1,30)=$

0.637, p= 0.432). There were also no interaction between groups: Stress X Sex (F(1,30)= 0.104, p= 0.750) or between Treatment X Sex (F(1,30)= 0.492, p= 0.490).

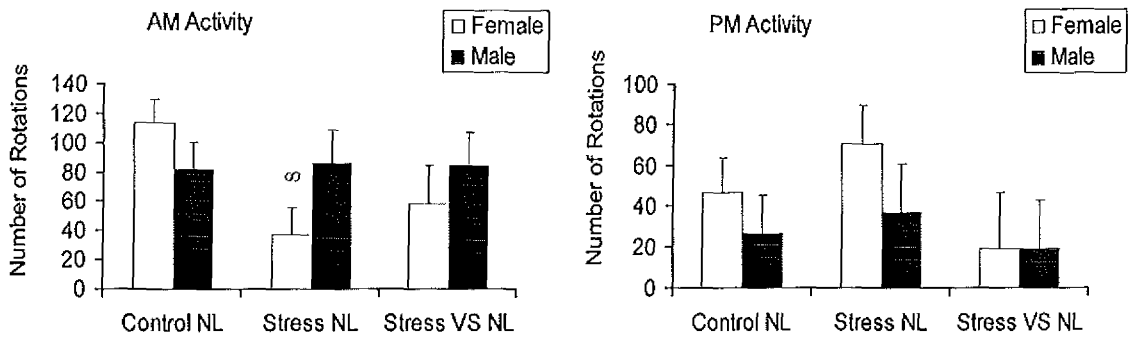


Figure 39. Left: Morning (7:00-12:00 am) activity levels indicated by wheel running activity in prenatally stressed animals treated with a supplemented diet. Right: Afternoon (12:00-5:00 pm) activity levels. ∞ Denotes the significant effects of prenatal stress in females ($p<.05$). Bars indicate mean ± SEM.

Overall, analysis of overnight (not presented), morning, and afternoon activity levels indicated the prenatally stressed animals with the supplemented diet to show the lowest amount of activity and the overnight means of the untreated control animals were very similar to the prenatally stressed animals.

Anatomical Results

Golgi-Cox brain and adrenal gland measures

Brain weight

Brain weights of males were heavier than females; however both sexes in the condominium groups showed increased brain weight, whereas the supplemented diet group showed decreased brain weight (see Table 6).

A univariate analysis for brain weight among treatment groups indicated main effects of treatment ($F(2,107)= 7.362$, $p= 0.001$), and of sex ($F(1,107)= 47.386$, $p< 0.0001$), but not of stress ($F(1,107)= 0.001$, $p= 0.971$). There were also no significant interactions between variables: Stress X Treatment ($F(1,107)= 0.004$, $p= 0.949$), Stress X Sex ($F(1,107)= 0.006$, $p= 0.938$), Treatment X Sex ($F(1,107)= 0.006$, $p= 0.994$), or Stress X Treatment X Sex ($F(1,107)= 0.299$, $p= 0.586$).

Pairwise LSD comparisons for treatment found both Condo-housed groups ($p< .0001$) and the supplemented diet group ($p=0.022$) significantly different from the untreated control group.

Table 6. Mean brain weights for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control NL	1.850 ± .021 (n= 30)	2.101 ± .024 (n= 22)*
Condo Control NL	1.936 ± .081 (n=2)	2.151 ± .047 (n= 6)*∞
	Prenatal Stress	
Stress NL	1.866 ± .028 (n=17)	2.087 ± .030 (n=14)*
Condo Stress NL	1.913 ± .057 (n= 4)	2.168 ± .043 (n= 7)*∞
Stress VS NL	1.726 ± .066 (n= 3)	1.955 ± .057 (n= 4)* ∞

Brain weights are measured in grams mean ± SEM. * Denotes statistically different between the sexes ($p<.05$). ∞ Denotes statistically different from untreated control and stress animals ($p<.05$).

Dendritic basilar arbor and length

Agranular Insular Dorsal (AID) cortex

Basilar fields

The untreated prenatally stressed animals had a significant reduction in dendritic arbor in pyramidal cells. The condominium treatment and the supplemented diet treatment for prenatal stress reversed the effects of stress in both sexes (see Fig. 40).

Univariate analysis for basilar dendritic arbor indicated main effects of stress ($F(1,40)= 8.436$, $p= 0.007$), of treatment ($F(2,40)= 7.568$, $p= 0.002$), and of sex

(F(1,40)= 4.353, p= 0.045). There were no significant interaction between variables: Stress X Sex (F(1,40)= 0.005, p= 0.942) or for Treatment X Sex (F(1,40)= 1.205, p= 0.313).

Pairwise LSD for treatment found the supplemented diet (VS) group (p= 0.012) significantly different from untreated control animals.

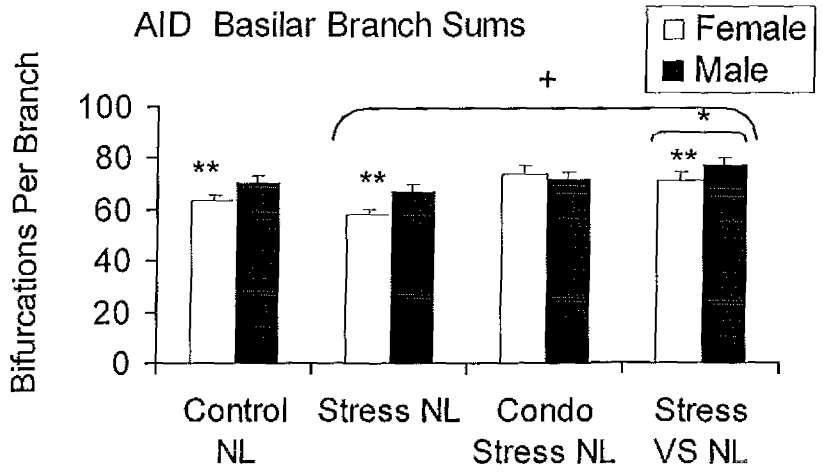


Figure 40. Sums of mean dendritic basilar branch in AID in rats treated with prenatal stress, condominium housing, and the supplemented diet. + Denotes statistically different from non-stressed control animals ($p<.05$). * Denotes the significant effects of the supplemented diet with prenatal stress ($p<.05$). ** Denotes significantly different between the sexes ($p<.05$). Bars indicate mean ± SEM.

Prenatal stress decreased dendritic length, which was not recovered with either treatment (see Fig. 41).

Univariate analysis for basilar dendritic length found a main effect of stress (F(1,40)= 8.511, p= 0.006), but not of treatment (F(2,40)= 1.044, p= 0.364), or of sex

(F(1,40)= 1.364, p= 0.251). There were no interactions between: Stress X Sex groups (F(1,40)= 0. 012, p= 0.915) or Treatment X Sex (F(1,40)= 0.031, p= 0.970.

Pairwise LSD comparisons did not indicate any significant differences in the treatment groups from untreated control animals.

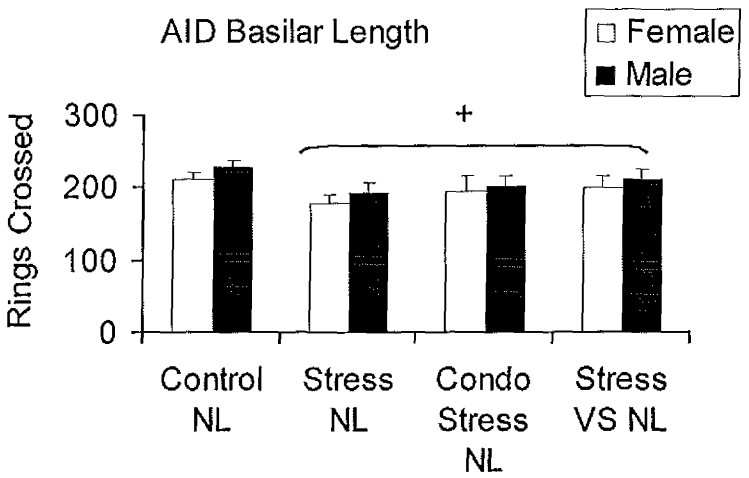


Figure 41. Sums of mean dendritic basilar length of pyramidal cells in AID brain regions. + Denotes statistically different effects of prenatal stress ($p<.05$). Bars indicate mean ± SEM.

The Golgi staining was not clear enough to draw cells confidently for the condo-housed control animals, therefore cortical thickness measurements were made in the brains of both condo-housed treatment animals (control and stress), in addition to the prenatally stressed animals to get an idea of what contribution condominium housing had anatomically.

Results showed that prenatal stress reduced cortical thickness in males which was not alleviated with condominium housing (see Fig. 42).

A univariate analysis for cortical thickness found significant effects of stress ($F(1,40)= 4.637$, $p= 0.039$), but not of treatment ($F(1,40)= 0.880$, $p= 0.355$), or of sex ($F(1,40)= 0.084$, $p= 0.774$). There were no significant interaction between groups: Stress X Treatment ($F(1,40)= 0.002$, $p= 0.962$), Stress X Sex ($F(1,40)= 2.220$, $p= 0.146$), Treatment X Sex ($F(1,40)= 1.002$, $p= 0.324$), or for Stress X Treatment X Sex ($F(1,40)= 0.325$, $p= 0.573$).

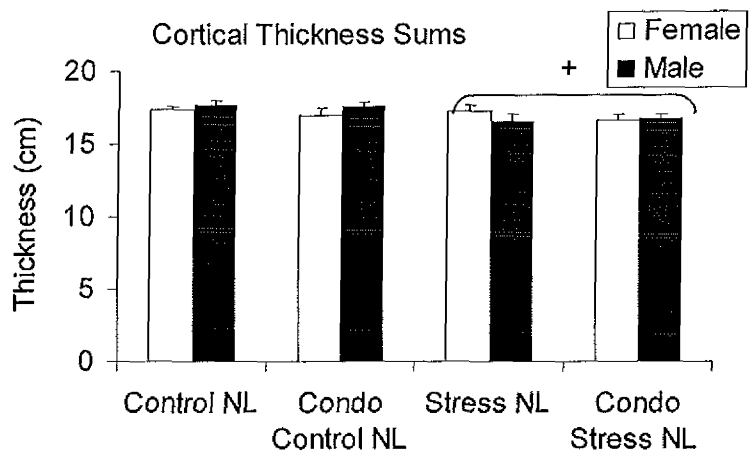


Figure 42. Sums of mean cortical thickness measures taken from Golgi-Cox stained brains in prenatally stressed animals housed in condominiums. + Denotes statistically different from non-stressed control animals ($p<.05$). Bars indicate mean ± SEM.

Adrenal gland weight

Overall, the adrenal weights of all females were heavier than males with the exception of the Condo Control animals. Both sexes in the condominium groups showed decreased adrenal weight, while the supplemented diet group showed an increase (see Table 7).

A univariate analysis for adrenal weight among treatment groups indicated a main effect of treatment ($F(2,106)= 20.002$, $p< 0.0001$), but not of stress ($F(1,106)= 1.521$, $p=$

0.220), or of sex (F(1,106)= 1.483, p= 0.226). There were also no significant interactions between groups: Stress X Treatment (F(1.738, p= 0.190), Stress X Sex (F(1,106)= 0.173, p= 0.678), Treatment X Sex (F(1,106)= 0.209, p= 0812), or for Stress X Treatment X Sex (F(1,106)= 1.542, p= 0.217).

Pairwise LSD comparisons for treatment found both condominium housing (p< .0001) and the supplemented diet (p< .0001) significantly different from untreated control animals.

Table 7. Mean adrenal gland weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control NL	0.105 ± .005 (n= 30)	0.087 ± .006 (n= 22)
Condo Control NL	0.050 ± .019 (n=2)	0.060 ± .011 (n= 6)∞
	Prenatal Stress	
Stress NL	0.098 ± .007 (n=17)	0.093 ± .007 (n=14)
Condo Stress NL	0.082 ± .014 (n= 4)	0.067 ± .010 (n= 7) ∞
Stress VS NL	0.158 ± .016 (n= 3)	0.138 ± .014 (n= 4)∞

Adrenal gland weights are measured in grams mean ± SEM. ∞ Denotes statistically different from Control NL and Stress NL animals (p<.05).

Adrenal gland areal measures

Cortex

The prenatally stressed females that were reared in condominiums had the thickest adrenal cortex (see Fig. 43).

A univariate analysis for adrenal cortex thickness found a significant interaction between Stress X Treatment ($F(1,45)= 24.945$, $p< 0.0001$) and between Stress X Sex ($F(1,45)= 4.144$, $p= 0.049$), but didn't find any main effects of stress ($F(1,45)= 2.412$, $p= 0.129$), of treatment ($F(2,45)= 2.207$, $p= 0.125$), or of sex ($F(1,45)= 1.226$, $p= 0.276$). There were also no significant interactions between Treatment X Sex ($F(1,45)= 1.195$, $p= 0.314$), or for Stress X Treatment X Sex ($F(1,45)= 1.934$, $p= 0.245$).

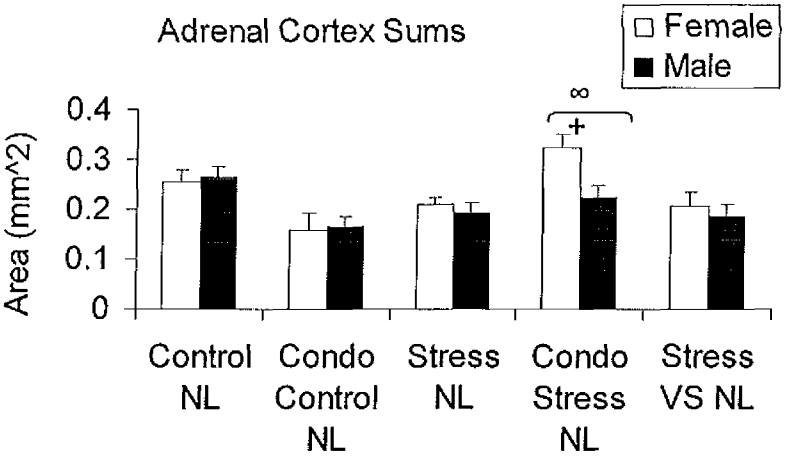


Figure 43. Mean sums of areal measures of adrenal cortexes in prenatally stressed animals. ∞ Denotes the significant effects of condo housing with prenatal stress ($p<.05$). + Denotes the significant effects of stress in females ($p<.05$). Bars indicate mean ± SEM.

Medulla

Prenatal stress decreased medulla areal measures, which was reversed in animals supplemented with the diet. Condo-housed unstressed females had decreased medulla area (see Fig. 44).

Univariate analysis for the adrenal medulla area indicated a main effect of treatment ($F(2,45)= 5.536$, $p= 0.008$), but not of stress ($F(1,45)= 0.861$, $p= 0.360$), or of sex ($F(1,45)= 1.777$, $p=0.191$). There were also no significant interactions between: Stress X Treatment ($F(1,45)= 0.923$, $p= 0.343$), Stress X Sex ($F(1,45)= 0.531$, $p= 0.471$), Treatment X Sex ($F(1,45)= 1.855$, $p= 0.171$), or for Stress X Treatment X Sex ($F(1,45)= 0.461$, $p= 0.502$).

Pairwise LSD comparison found the Stress VS diet group to be significantly different ($p= 0.005$) from untreated control group and from the condo-housed group ($p= 0.022$).

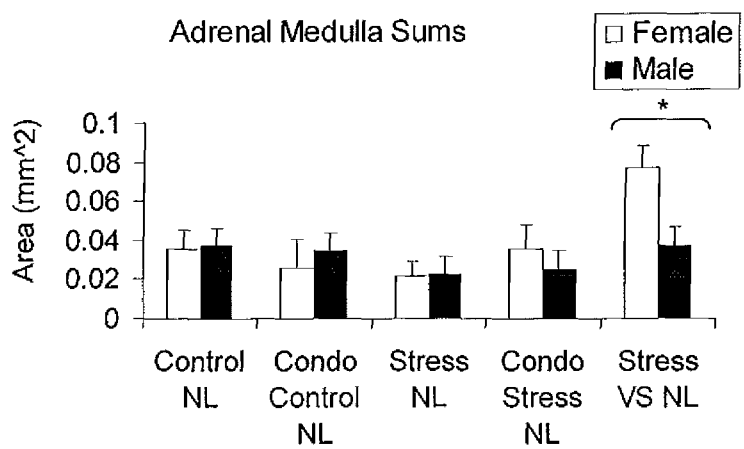


Figure 44. Mean sums for adrenal medulla area in prenatally stressed animals. * Denotes statistically different from all other groups ($p<.05$). Bars indicate mean ± SEM.

Prenatal stress did significantly affect adrenal gland weight and the glands' appearance as illustrated in Figure 45. The adrenal glands that were exposed to stress have a dorsal appearance of shrinkage and were less rounded than the control or treatment adrenal glands.

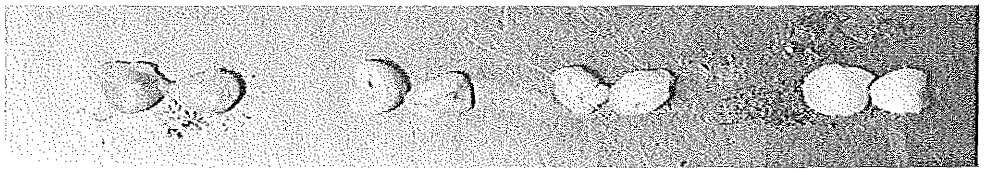


Figure 45. Dorsal view of adrenal gland pairs from the females representative of their groups. Far left: untreated control; second from left: prenatal stress; second from right: condo-housed prenatal stress; and, far right: supplemented diet and prenatal stress.

Testes weight

Testes weights increased with the combination of diet and prenatal stress (see Fig. 46). Unfortunately we do not have weights for the condo-housed animals.

Univariate analysis for testes weight in prenatally stressed males indicated a main effect of diet treatment ($F(1,13)= 14.314$, $p= 0.003$), but not of stress ($F(1,13)= 1.723$, $p= 0.216$).

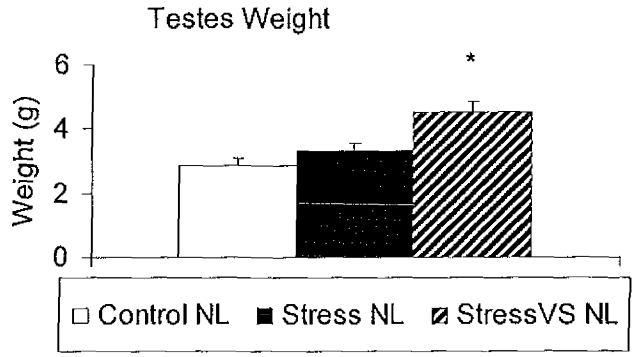


Figure 46. Mean testes weight in prenatally stressed males reared with the supplemented diet. * Denotes statistically different from Control NL and Stress NL animals ($p<.05$). Bars indicate mean ± SEM.

Moderate prenatal stress and treatments of condominium housing or micronutrient supplemented diet

Summary

Table 8. Significant findings for experiment 1b.

Behavioural Task	Females	Males
Activity: Distance Number of Movements	Condo housing and diet decreased activity	Condo housing and diet decreased activity
Tray Reaching:	Impairments with stress with & without condo treatment, reversed with VS diet	Impairments with stress with & without condo treatment, reversed with VS diet

Anatomy (Golgi-Cox and saline-perfused preparation)		
Brain Weight:	Females lighter than males, Increased with condo treatment, decreased with diet	Males heavier than females, Increased with condo treatment, decreased with diet
Basilar and Apical Branch Arbor/Length:	Stress decreased arbor in AID; partially recovered with diet and condo housing	Stress decreased in arbor in AID; partially recovered with diet and condo housing
Cortical Thickness	Increased with stress, and in stress with condo	Increased with stress, and in stress with condo
Adrenal Gland Weight:	Females heavier than males, Condo housing decreased weight; diet increased wt.	Males lighter than females, Condo housing decreased weight; diet increased wt.
Adrenal Cortex Thickness:	Increased with condo and stress	Slightly increased with condo and stress
Adrenal Medulla Area:	Increased with stress and diet	Increased with stress and diet
Testes Weight:	N/A	Slightly increased with stress; significantly increased with stress and diet

The summaries in tables 5 and 8 are displayed as itemized lists of what was statistically significant in each of the previous experiments. The summary to follow is a general overview of the three experiments with frontal lesions that will be discussed in the discussion.

Moderate prenatal stress and treatments of condominium housing or micronutrient supplemented diet

Discussion

The main findings from prenatal stress are: 1) the changes in behaviour depend on the tasks employed; 2) some of the anatomical changes are specific to brain regions; 3) condominium housing did not alleviate the behavioural deficits; and, 4) the supplemented diet improved behavioural deficits.

Behavioural changes from prenatal stress

Skilled reaching

Various studies examining the effects of prenatal stress have focused on impairments in behavioural tasks of cognition (Lemaire, Koel, LeMoal, and Abrous, 2000) and anxiety (Weinstock, 1997; Fumagalli, et al, 2007) that corresponded with changes in hormone secretions from the stress axis. Tests of motor skills in prenatally stressed animals are limited and in the current study, we have found that moderate gestational stress has changed the brain enough to produce reaching deficits.

What makes this deficit interesting is that is has been commonly found in animals with frontal lesions that have damage to either the medial prefrontal region or to the forelimb area (Whishaw, Pellis, and Gorney, 1992). It appears that animals exposed to stress during gestation must have developed one of these brain regions differently enough to affect skilled movements.

Deficits in skilled reaching from gestational stress induced by repeated injections of dexamethasone (DEX) in both early and late stages of pregnancy have been found in Marmoset monkeys (Hauser, et al, 2008). Early DEX treatment indicated impairments in the success of both the reach attempt as well as in the grasp. Late DEX treatment resulted in some motor type of impulsive behaviour in reaching attempts in females, characterized by pushing the food reward away from them during their reach. Hauser and colleagues discussed these results as deficits in the development of the mediation of motor movements because the remainder of the animal's motor repertoire of movement was intact, including those in social play. Support for their theory came from lesion studies of the cerebellum that is dependent upon the cerebello-thalamo-cortical pathway as well as the basal ganglia and its thalamo-cortical loop (Hauser, et al, 2008).

During reaching movements in rats, neural activity recordings of rostral and caudal forelimb motor areas during reaching movements have indicated modulations between excitatory and inhibitory input begin (Hyland, 1997). The proportions of inhibitory to excitatory input from neurons differed within each epoch of the reach. There was an excess of inhibitory activity that occurred during the pre-reach period followed by excitatory activity just after reach onset (Hyland, 1997). Alterations in the balance of excitatory to inhibitory activity have the potential change the resulting pattern in reach attempts.

There could be a number of mechanisms that contribute to reaching deficits in the prenatally stressed animals, from changes in dopaminergic activity in the striatum (Weinstock, 2001) to changes in the development of γ- aminobutyric acid (GABA)ergic (inhibitory) and glutamatergic (excitatory) neurotransmitter systems in the motor cortex,

prefrontal cortex, or striatum (Leret, et al, 2004) sufficient enough to alter the synchronous activity necessary for successful reaches and grasps.

Elevated plus maze

The elevated plus maze is a popular behavioural test used to test anxiety in rodents because it purports to demonstrate an inner conflict in rats for the need to explore the novelty of an environment, but is risky from a safety standpoint with the high elevation and open unprotected arms. Inhibitory avoidance is a common measure used that measures the latency for animals to come out of the secure closed arm to explore an open arm. Escape can be measured by the animal's escape from an unsecured open arm into a secure closed arm (Estanislau and Morato, 2005). All animals in the current study were placed in the center facing a closed arm. Most would enter the closed arm however some went directly onto the open arm. On the other hand, many prenatally stressed animals did not even enter the open arms. Their exploration was limited to an escape to the opposing closed arm. It has been suggested that prenatally stressed rats have reductions in the expression of 5-HT_1 (serotonergic) binding sites in the hippocampus contributing to increased emotionality (Weinstock, 1997; Fumagalli, et al, 2007).

Circadian activity

Previous studies on the effects of prenatal stress have found a phase shift in circadian rhythm of corticosterone secretion with the pregnant dams having higher activity at the end of the day's hours (Koel, et al., 1999; Leonhardt, et al., 2006) This shift in maternal rhythm has been proposed responsible for rhythm changes in both male and female off spring, but without changing basal levels of corticosterone or ACTH

(Weinstock, 2001; Koel, et al., 1999). The circadian rhythm in behavioural activity and wheel running activity in the current study showed prenatally stressed females to have experienced a phase shift in activity to peak in the evening hours. This shift presumably would have been reflected in altered circadian corticosterone activity, whereas males were unaffected.

Anatomical changes from prenatal stress

Brain measures

The measures of branch arbor and dendritic length from prenatal stress in the current study showed reductions in dendritic *length* in AID (layer III) and Cg3 (layer V), but an increase in dendritic branch *arbor* in Cg3. Reductions in dendritic length would likely reduce the synaptic space, but this was not measured directly.

Mirmu and colleagues (2006) reported decreased dendritic arbor and length of layer II/III in the anterior cingulate cortex (Cg3) and layer II/III of the orbitofrontal cortex (OFC) in the offspring males of gestationally stressed dams. Females did not exhibit significant differences in dendritic characteristics. There was a sex difference in the non-stressed animals however, as males showed greater dendritic length and complexity in Cg3 and this was not found in the prenatally stressed animals. Dendritic spine density also followed a similar pattern (Mirmu, Salomon, Biala, Weinstock, Braun, and Bock, 2006). The differences from the current study and that of Mirmu (2006) is their stress procedure and timeline of one daily stressor from gestation days 15 to 20, which changed twice thereby exposing dams to three different forms of stressors during this period (G15 to G20) to prevent habituation.

Adrenal measures

Some previous studies of prenatal stress have found hypertrophy, and some, hypotrophy of the adrenal glands indicative of the adrenal activity from the stress response. An increase in adrenal weight has also been considered a biomarker of overuse from the chronic secretion of glucocorticoids (Lemaire, Koel, LeMoal, and Abrous, 2000; Fameli, Kitraki, and Stylianopoulou, 1994).

In the current study, adrenal gland weights were heavier in females than in males and prenatal stress further increased adrenal weight. The adrenal cortex thickness however, was thinnest in prenatally stressed animals which seems paradoxical to their heavier weight. The cortex of prenatally stressed animals is likely denser in cell number (unquantified) than in control animals, which would explain the increased weight.

Condominium housing and vitamin supplemented diet

Skilled reaching

Animals that were prenatally stressed and reared in condominium housing also displayed deficits in the skilled reaching task with the deficit being larger in females. The enriched environment has been purported to increase many neurotrophic factors (Will et al, 2004) and because much of the brain had developed prior to the condominium-housing treatment (day 21), the prenatal stress appears to have exerted some permanent effects in the organization of the brain. The animals in the supplemented diet did not show any reaching impairments and began their treatment on the day of birth. Although both treatments have more global effects, there are differences between them with respect

to skilled reaching tasks. The timing of treatment may also be a factor with respect to the developing brain.

Elevated plus maze

Condominium-housed animals showed a decline in exploratory activity in the elevated plus maze regardless of prenatal treatment. In contrast to the current findings in the elevated plus maze, Peña, et al., (2009) reported an increased exploration of the open arms and in number of entries with a decrease in stretch-attend postures from the closed arms toward the open arms in non-stressed enriched rats. These results indicated a reduction in anxious behavioural responses from control animals housed in enriched environments, an effect that was not observed in the present study. The animals in Peña's study were condo-housed during the same time frame as those in the current study however, the rat strain was a different strain (Wistar) than those used in the current study (Long-Evans). Environmental enrichment has been considered to have a differential biochemical impact that is dependent on the animal's history and susceptibility to stressors and may apply to some animals in the current study (Fox, Merali, and Harrison, 2006). The authors reported a decrease in plasma corticosterone in enriched animals that were prenatally stressed, but not apparent in non-stressed control animals (Fox, Merali, and Harrison, 2006). It is possible that the prenatally stressed animals in the present study were more sensitive to sensory stimuli, as seen in the Activity box, which became apparent in novel situations.

Males supplemented with the diet showed a partial reversal of the effects of prenatal stress on the elevated plus maze, but was not the case in females. The effects from the supplement did increase males' testes weight and might be related to a reduction

in anxiety from Weinberg's (1998) perspective of androgen's inhibitory effects on the HPA axis.

Circadian activity

The supplemented diet animals did not show an activity shift and the condo-housed females exhibited slightly higher circadian levels in the evening hours, but not as large as the untreated stressed females. Plasma corticosterone and ACTH have been shown to change in envrionmentally enriched animals from low morning levels to increased levels in the evening hours, especially in females (Peña, Prunell, Rotllant, Armario, and Eschorihuela, 2009), which appears to have occurred in the current study.

Brain and adrenal measures

Condominium housing increased brain weights in all animals in the current study but this was not the case in animals reared on the supplemented diet. The increased weight has been considered from the contribution of numerous cortical changes, such as increased cortical thickness, spine density, synapses per neuron, increases in glial cells and complexity and vascularization (Greenough, and Chang, 1988; Svieraag, and Greenough, 1988). Some of the above mentioned measures have not been examined with the supplemented diet, but like the enriched housing, it would also have a more global effect of the brain.

In the current study, both condominium housing and the supplemented diet reversed the effects of prenatal stress in AID basilar dendritic branch arbor and length measures toward normal control values. Unfortunately, dendritic measures for Cg3 were not available for the brains of animals given condominium housing, so we cannot

comment on treatment-related changes in these animals. However, the medial region of the prefrontal cortex has not previously shown much change in response to long-term condo housing when animals are placed in condominiums during the peri-adolescent period (Halliwell, 2009).

Adrenal gland weights were heaviest in the prenatally stressed animals supplemented with the diet and the condominium-housed animals had decreased adrenal weights. The adrenal cortex thickness however, was thinnest in non-stressed condo animals. Conversely the prenatally stressed condo-housed animals had significantly thicker cortices, especially in females suggesting the continuance of a release in stress hormones.

The diet-supplemented animals had thinner adrenal cortexes and an enhancement in adrenal medulla area. The alternative to using the adrenal cortical hormones would be the use of the medullary hormone adrenalin. The adrenal medulla was larger in prenatally stressed females supplemented with the diet. Perhaps the medulla will become more active if there is some limitation in use of adrenal cortex hormones during the stress response.

In sum, the results found in the current experiment shows that moderate prenatal stress does alter brain and behavioural development. Many previous studies have used more intense forms of stress and thus, the current results appear to be somewhat attenuated relative to those studies. The postnatal interventions of condominium housing and the supplemented diet did partially attenuate the effects of the prenatal stress although there were some unexpected stress-treatment interactions such as the increase in emotionality in the elevated plus maze.

III. Experiment 2A: The effects of perinatal frontal cortical injury on brain and behavioural development

The following sets of comparisons are with control animals with No Lesions (Control NL) with frontal lesions (Control Frontal)

Behavioural Results

Locomotor Activity

Distance

Females explored more distance than males in the activity box, but frontal lesions had no effect activity levels of both sexes (see Fig. 47).

A univariate analysis indicated a main effect of sex ($F(1,58)= 11.573$, $p=.001$), but not of lesion ($F(1,58)= 0.236$, $p=.629$), nor the interaction ($F(1,58)= .087$, $p= .770$).

Number of movements

Females with frontal lesions increased movement frequency whereas males decreased movement (see Fig. 47).

A univariate analysis of the number of movements between lesion and sex groups found a significant interaction ($F(1,58)= 4.069$, $p= .049$), but not for main effects of lesion ($F(1,58)= 0.028$, $p=.868$), or of sex ($F(1,78)= 2.099$, $p= 0.153$).

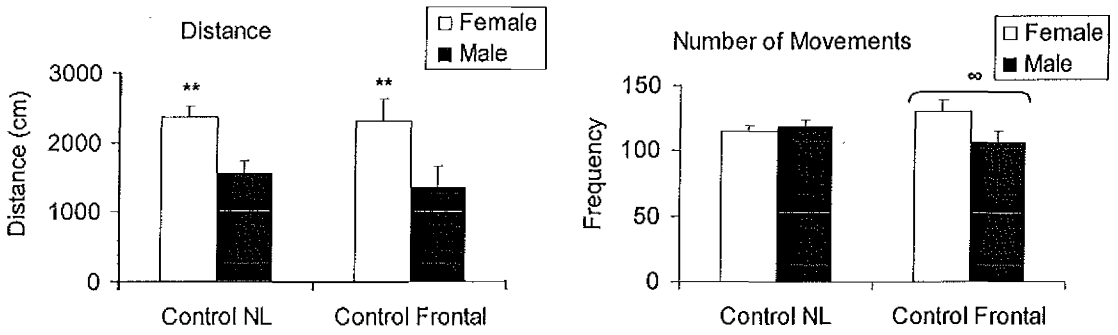


Figure 47. Left: Sums of mean locomotor distance for animals with frontal lesions. Right: Sums of mean number of movement frequency. ** Denotes significantly different between sexes ($p<.05$). ∞ Denotes the significant interaction of frontal lesions between the sexes ($p<.05$).Bars indicate mean ± SEM.

Morris water maze

Latency

Frontal lesions produced a deficit in females but not in males (see Fig.48).

Univariate analysis revealed a main effect of lesion ($F(1,95)= 11.636$, $p= 0.001$), and of sex ($F(1,95)= 6.235$, $p= 0.014$), as well as a significant interaction ($F(1,95)= 11.516$, $p= 0.001$).

A multivariate analysis for day revealed days' one and two significantly different among groups ($p<.05$) (see Fig.48).

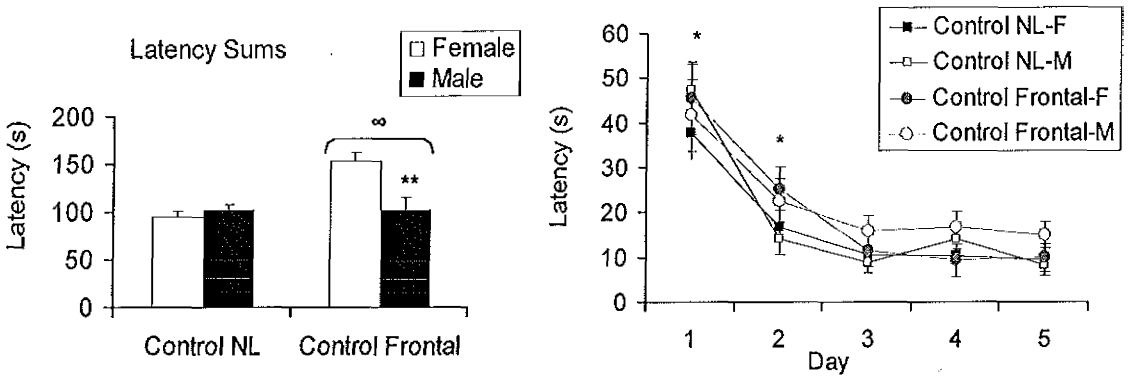


Figure 48. Left: Sums of mean latency (seconds) for animals with frontal lesions. ** Denotes significantly different between the sexes ($p<.05$). ∞ Denotes significant effects of lesion in females ($p<.05$). Right: Mean latencies across the five-day acquisition period. * Denotes the significantly different days among groups ($p<.05$). F= Female, M= Male. Bars indicate mean ± SEM.

Morris water maze probe

All groups spent more time in the previously correct quadrant than any other quadrant and the frontal males spent nearly 40% of their time there (see Fig. 49).

Univariate analysis for the correct quadrant (Q1) containing the hidden platform did not indicate any main effects of lesion ($F(1,60)= 0.587$, $p= 0.447$), or of sex ($F(1,60)= 0.404$, $p= 0.527$), or an interaction between lesion and sex groups ($F(1,60)= 0.179$, $p= 0.674$).

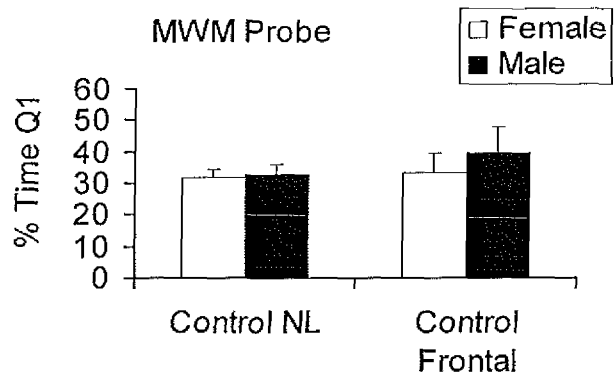


Figure 49. Percent amount of time spent in the previously correct quadrant during a probe test in animals with frontal lesions ($p<.05$). Bars indicate mean ± SEM.

Tray Reaching

Rats with frontal lesions had a large deficit in skilled reaching as they performed at about 50% of the accuracy of the controls (see Fig. 50).

Univariate analysis indicated a significant main effect of lesion for skilled reaching ($F(1,77)= 45.458$, $p<.0001$), but not of sex ($F(1,77)= 2.243$, $p= 0.138$), nor for the interaction ($F(1,77)= 0.627$, $p= 0.431$).

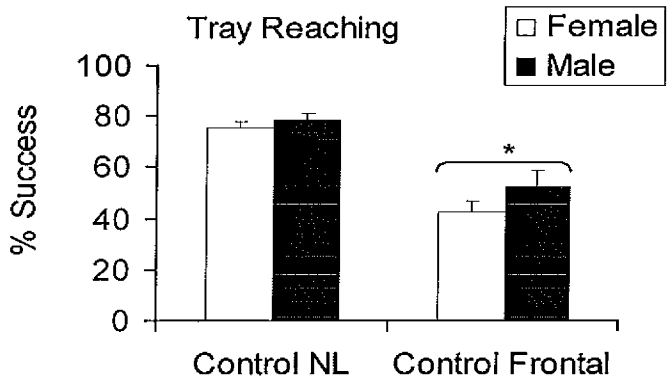


Figure 50. Mean percent success of skilled reaching in animals with frontal lesions. * Denotes significantly different from intact control animals ($p<.05$). Bars indicate mean ± SEM.

Elevated Plus Maze

The frontal lesions did not increase anxiety and in fact, the frontal males were more likely to enter the open arms than the other groups. Males with a frontal lesion spent the most time exploring the open arms (see Fig. 51).

Univariate analysis for measures of emotionality in the elevated plus maze found a significant interaction ($F(1,81)= 5.108$, $p=0.027$), but did not indicate main effects of lesion ($F(1,81)= 1.964$, $p= 0.165$), or of sex ($F(1,81)= 2.662$, $p= 0.107$).

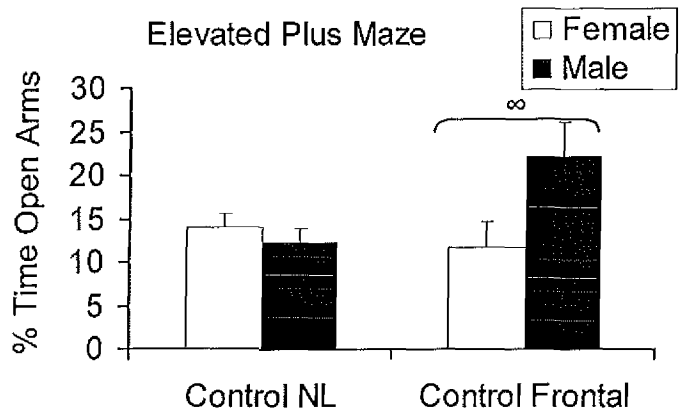


Figure 51. Exploration time in the elevated plus maze in animals with frontal lesions ($p<.05$). ∞ Denotes the significant effects of the lesion between the sexes ($p<.05$). Bars indicate mean ± SEM.

Anatomical Results

Frontal lesion

The intended removals were the anterior portions of Cg1 and Cg3 along with the adjacent regions of Fr2 (see Fig. 52). Because medial frontal lesions at this age often show complete or partial filling of the lesion cavity with re-grown tissue (Kolb et al., 1998), the lesion extent varied across animals.

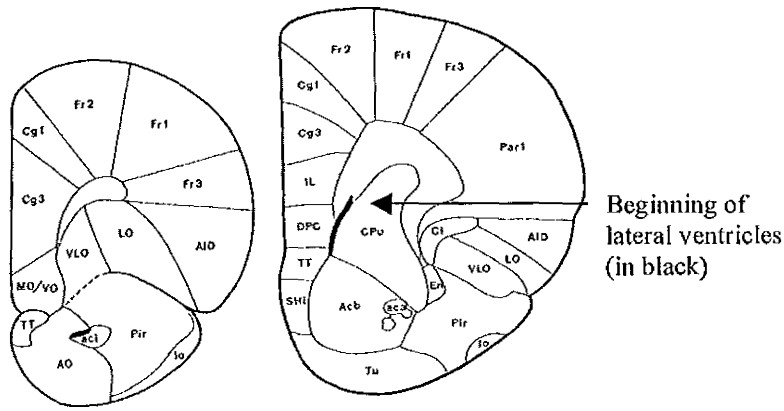
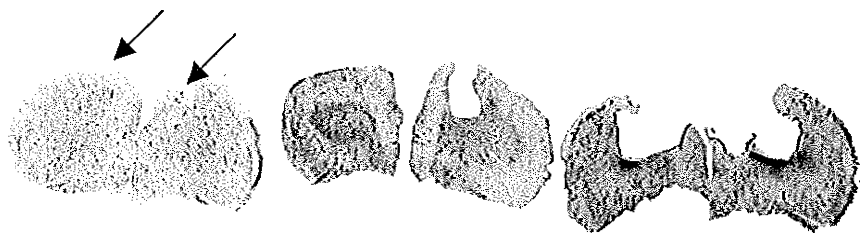


Figure 52. Illustration of the rat brain demarcating areas Cg3, Cg1, and Fr2 of the prefrontal cortex. Stereotaxic drawings of coordinates left: Bregma 3.7; and, right: Bregma 2.2 (Adapted from Karl Zilles, 1985). Cg1, Cg3= Cingulate cortex 1 and 3 respectively; Fr2= Frontal cortex area 2.

Four of fifteen brains filled in with tissue; three brains had filled in tissue with small portions of Cg1 and Fr2 missing; three brains had one hemisphere filled in with the other hemisphere missing portions of Cg1 and Fr2; three brains showed partial filling in with portions of Cg1, Fr2, and small amounts of Cg3 missing; and two brains had large lesions remaining with portions of Cg3, Cg1, and Fr2 missing (see Fig. 53). The lateral ventricles became larger with the size of the lesion reducing the size of the caudate nucleus.



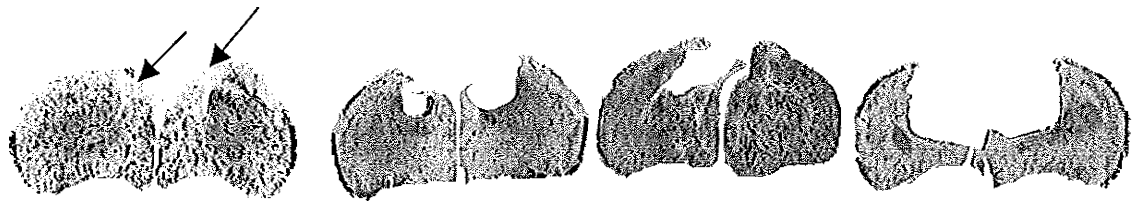


Figure 53. Examples of postnatal day 10 lesions in adult brains. Left: top and bottom; brains that had nearly completely filled in. Arrows indicate filled in tissue at the lesion site. Second from left: top and bottom; brains that showed one hemisphere of filling in (top), or both hemispheres partially filled in with tissue (bottom). Right: brains show very little filling in and are missing cortical areas with some caudate nucleus.

Golgi-Cox brain and saline-perfused adrenal gland measures

Brain weight

Male brains were heavier than female brains in both sexes of animals with frontal lesions had decreased brain weight (see Table 9).

Univariate analysis found a main effect of lesion ($F(1,60)= 4.890$, $p= 0.031$), and of sex ($F(1,60)= 24.294$, $p< .0001$), but not for the interaction ($F(1,60)= 1.787$, $p= 0.187$).

Table 9. Mean brain weight for the Golgi-Cox prepared group.

Group	Female (g)	Male (g)
	No Lesion	
Control NL	1.927 ± .023 (n= 25)	2.138 ± .026 (n= 20) *
	Frontal Lesion	
Control Frontal	1.897 ± .038 (n= 9)	2.018 ± .043 (n= 7)* ∞

Brain weights are measured in grams ± SEM. * Denotes statistically different between sexes ($p<.05$). ∞ Denotes statistically significant from intact non-lesion animals ($p<.05$).

Dendritic basilar and apical arbor and length

Cingulate cortex (Cg3)

Cells could only be drawn in animals with partial or complete regeneration of the tissue but there were enough animals with filling to make the analysis possible.

Basilar fields

Both sexes, particularly females with frontal lesions showed increased basilar dendritic branch arbor in Cg3, particularly females (see Fig. 54).

Univariate analysis for basilar dendritic branch arbor in the cingulate cortex found a main effect of lesion ($F(1,25)= 19.007$, $p< .0001$), and an effect of sex ($F(1,25)= 4.971$, $p= 0.040$), as well as a significant interaction between groups ($F(1,25)= 4.576$, $p= 0.044$).

In contrast to the increase in dendritic branching in Cg3, dendritic length in the basilar region of Cg3 cells was significantly shorter with frontal lesions (see Fig. 54).

A univariate analysis for dendritic length in Cg3 indicated a main effect of lesion ($F(1,32)= 6.431$, $p= 0.017$), but not of sex ($F(1,32)= 0.670$, $p= 0.420$), nor for an interaction ($F(1,32)= 0.129$, $p= 0.722$).

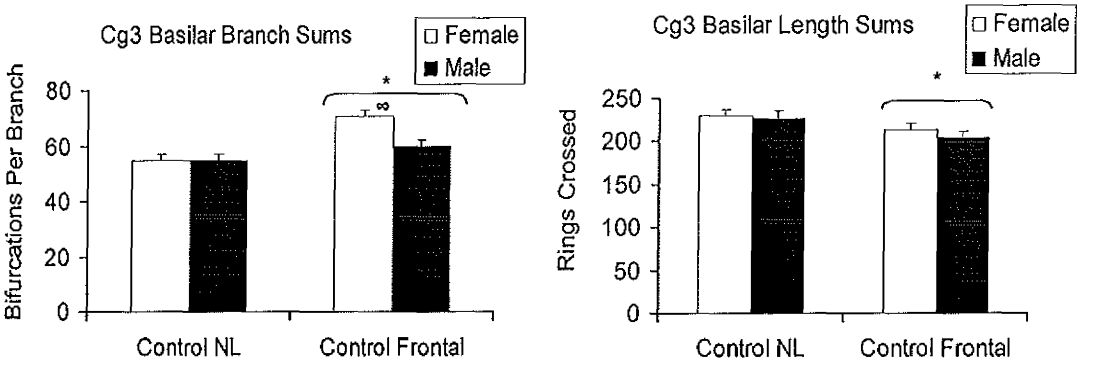


Figure 54. Left: Sums of mean dendritic branch arbor of the Cg3 region in animals with frontal lesions. Right: Sums of mean dendritic length in Cg3. ∞ Denotes the statistically different effects of lesion on the sexes ($p<.05$). * Denotes the statistical differences in frontal lesion animals ($p<.05$). Bars indicate mean ± SEM.

Apical fields

Animals with frontal lesions had increased dendritic arbor in the apical portion of the cells, but no significant changes in dendritic length (see Fig. 55).

Univariate analysis for the apical dendritic branch arbor in Cg3 found a main effect of lesion ($F(1,13)= 8.094$, $p= 0.017$), but not for sex ($F(1,13)= 0.712$, $p= 0.419$), or for an interaction ($F(1,13)= 0.012$, $p= 0.916$).

A univariate analysis of dendritic length in the apical field of Cg3 did not find any significant effects of lesion ($F(1,15)= 0.287$, $p= 0.602$), or of sex ($F(1,15)= 0.167$, $p= 0.690$), nor for an interaction between groups ($F(1,15)= 0.476$, $p= 0.504$).

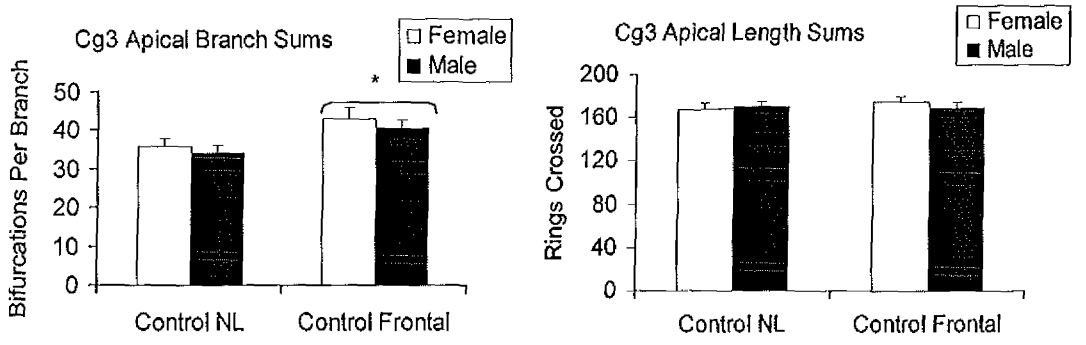


Figure 55. Left: Sums of mean dendritic branch arbor in the apical Cg3 in animals with frontal lesions. Right: Sums of mean dendritic length in apical Cg3. * Denotes statistically significant from intact control animals ($p<.05$). Bars indicate mean ± SEM.

Agranular Insular Dorsal (AID) cortex

Basilar fields

Cells in AID showed decreased arbor in males with frontal lesions, but without any significant differences in dendritic length in either sex (see Fig. 56).

Univariate analysis of the basilar branch arbor of the AID region indicated a significant interaction between treatment and sex ($F(1,34)= 4.418$, $p= 0.044$), but not for main effects of treatment ($F(1,34)= 1.687$, $p= 0.204$), or of sex ($F(1,34)= 0.972$, $p= 0.332$).

Univariate analysis of dendritic length in pyramidal cells of AID did not indicate any significantly different effects of lesion ($F(1,34)= 1.840$, $p= 0.185$), of sex ($F(1,34)= 0.629$, $p= 0.434$), or for an interaction between groups ($F(1,34)= 0.459$, $p= 0.503$).

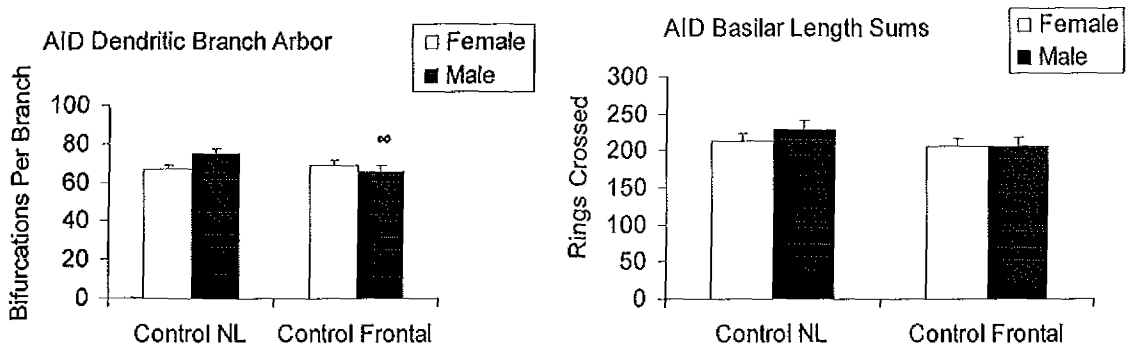


Figure 56. Left: Sums of mean dendritic branch arbor of AID in animals with frontal lesions. Right: Sums of mean dendritic length in AID. ∞ Denotes the significant effects of frontal lesions in males ($p<.05$). Bars indicate mean ± SEM.

Apical fields

There were no lesion- or sex-related effects on the apical field in AID (see Fig. 57).

Univariate analysis for apical branch arbor in AID did not indicate any significant effect of lesion ($F(1,12)= 0.817$, $p= 0.384$), or of sex ($F(1,12)= 0.138$, $p= 0.716$), nor for an interaction ($F(1,12)= 1.495$, $p= 0.245$).

Analysis of the dendritic length in apical areas of cells also did not find significant effects of lesion ($F(1,15)= 0.057$, $p= 0.815$), of sex ($F(1,15)= 0.021$, $p= 0.888$), nor for an interaction ($F(1,15)= 2.309$, $p= 0.155$).

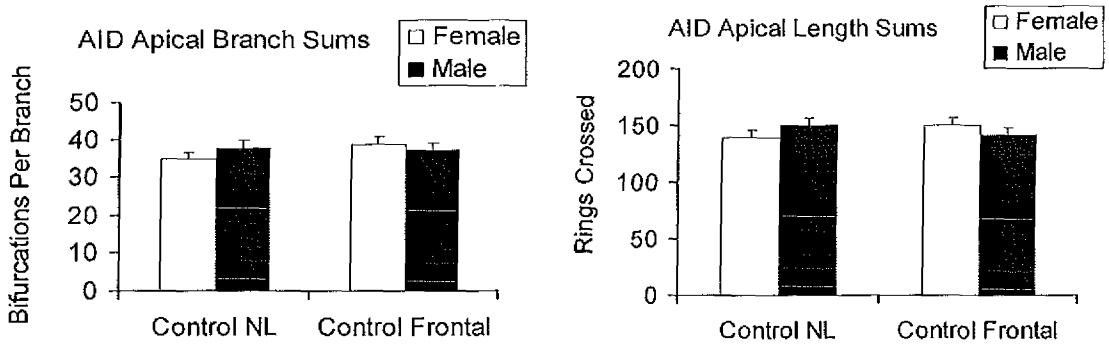


Figure 57. Left: Sums of mean dendritic apical branch arbor of AID, and Right: Sums of mean dendritic length in animals with frontal lesions. Bars indicate mean ± SEM.

Adrenal gland weight

Females had heavier adrenal weights than males, but both sexes were not affected by frontal lesions (see Table 10).

Univariate analysis for adrenal weight among lesion and sex groups found a main of sex ($F(1,60)= 10.918$, $p= 0.002$), but not of lesion ($F(1,60)= 0.207$, $p= 0.650$), and not for the interaction ($F(1,60)= 3.054$, $p= 0.086$).

Table 10. Mean adrenal gland weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Lesion	
Control NL	0.083 ± .004 (n= 25)	0.074 ± .004 (n= 20)*
	Frontal Lesion	
Control Frontal	0.089 ± .006 (n=9)	0.063 ± .007 (n=7)*

Adrenal glands are measured in grams mean ± SEM. * Denotes statistically different between sexes ($p<.05$).

Adrenal gland areal measures

Cortex

Frontal lesions did not affect adrenal gland cortex thickness measures in either sex (see Fig. 58).

Univariate analysis for cortex thickness of adrenal glands did not indicate any significant effects of lesion ($F(1,15)= 0.038$, $p= 0.848$), or of sex ($F(1,15)= 0.001$, $p= 0.970$), or for the interaction ($F(1,15)= 0.081$, $p= 0.781$).

Medulla

Although males with frontal lesions had an average of approximately 22% larger medulla areal measures, there were no statistical differences (see Fig. 58).

Univariate analysis for adrenal medulla did not find any significant effects of lesion ($F(1,15)= 0.636$, $p= 0.441$), of sex ($F(1,15)= 0.270$, $p= 1.337$), or for an interaction between groups ($F(1,15)= 0.802$, $p= 0.388$).

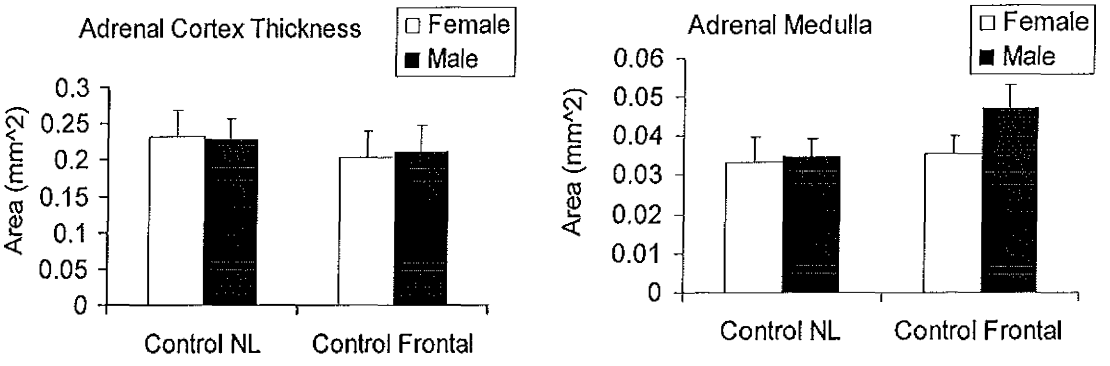


Figure 58. Left: Sums of mean adrenal cortex measures of adrenal glands in animals with frontal lesions. Bars indicate ± mean SEM.

Formalin-fixed brain and adrenal gland measures

Prefrontal cortex prelimbic and infralimbic measures

The lesion primarily affected the more dorsal portions of the midline prefrontal cortex, leaving ventral portions relatively intact (see Table 11). The frontal lesion led to a reduced thickness in the prelimbic, but not the infralimbic cortex.

A univariate analysis for the prefrontal *prelimbic* cortex indicated a main effect of lesion ($F(1,29)= 35.350$, $p < 0.0001$), but not of sex ($F(1,29)= 0.620$, $p= 0.438$), nor for an interaction between groups ($F(1,29)= 0.010$, $p= 0.922$).

Univariate analysis for the *infralimbic* cortex did not indicate significant effects of lesion ($F(1,29)= 3.035$, $p= 0.093$), sex ($F(1,29)= 0.789$, $p= 0.380$), nor for the interaction ($F(1,29)= 1.611$, $p= 0.216$).

Thalamus

Both sexes with frontal lesions had smaller thalamic measures in the anterior portion, the effect being larger in males (see Table 11).

A univariate analysis for the anterior thalamus indicated a main effect of lesion ($F(1,29)= 24.357$, $p< .0001$), and an interaction between lesion and sex ($F(1,29)= 4.774$, $p= 0.038$), but no main effect of sex ($F(1,29)= 1.094$, $p= 0.305$).

Univariate analysis for the posterior measures did not reveal any significant findings for lesion ($F(1,29)= 0.023$, $p= 0.879$), or sex ($F(1,29)= 2.368$, $p= 0.136$), or an interaction ($F(1,29)= 0.046$, $p= 0.832$).

Table 11. Mean brain measures from formalin-fixed brains.

Area Measured	**Females**	**N**	**Males**	**N**
	Non- Lesion Control			
[1]Prefrontal Cortex				
Infralimbic	0.590 ± .029	11	0.607 ± .029	11
Prelimbic	1.065 ± .053	11	1.115 ± .053	11
[2]Thalamic Width: anterior	0.085 ± .001	11	0.087 ± .001	11
posterior	0.094 ± .001	11	0.095 ± .001	11

	Control		Frontal Lesion	
[1]Prefrontal Cortex				
Infralimbic	0.569 ± .055	4	0.470 ± .055	4
Prelimbic	0.627 ± .088	4	0.691 ± .088∞	4
[3]Thalamic Width: anterior	0.081 ± .002	4	0.077 ± .002 ∞*	4
posterior	0.093 ± .001	4	0.095 ± .001	4

1. Prefrontal cortex: prelimbic and infralimbic regions are measured in cm ± SEM. ∞ Denotes statistically significant from non-lesion control animals in prelimbic cortex measures ($p<.05$).

3. Thalamic measures for both regions are measured in cm ± SEM. ∞ Denotes significant differences from non-lesion control animals ($p<.05$). * Denotes significantly different effects between the sexes in the anterior thalamus ($p<.05$).

Cortical thickness

Animals with frontal lesions had thinner cerebral cortices (see Fig. 59).

Univariate analysis for cortical thickness measures averaged across five planes found a main effect of lesion ($F(1,53)= 31.713$, $p<.0001$), but not of sex ($F(1,53)= 0.563$, $p= 0.456$), nor for an interaction between groups ($F(1,53)= 0.087$, $p= 0.769$).

A multivariate analysis found planes one, two, and five significant among groups ($p<.05$) (see Fig. 59).

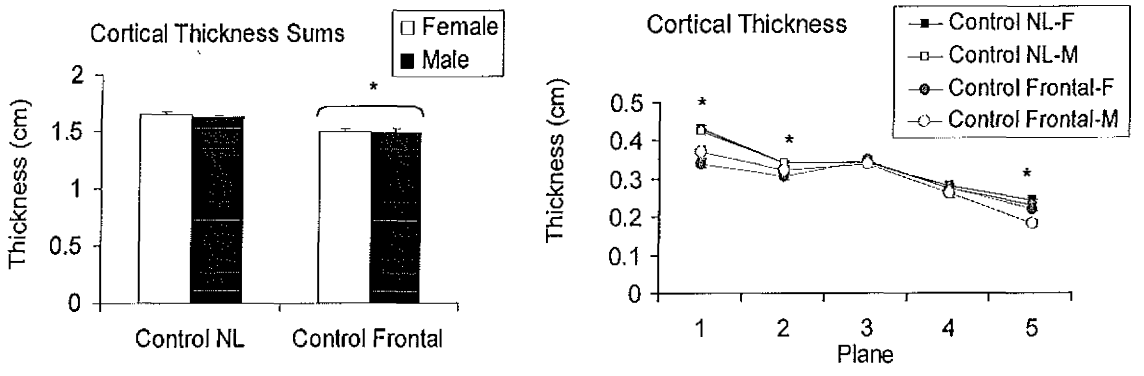


Figure 59. Left: Sums of mean thickness measures in animals with frontal lesions. * Denotes statistical significance from intact control animals ($p<.05$). Right: Mean thickness of five planes measured across the cortex. * Denotes significantly different among groups ($p<.05$). F= Female, M= Male. Bars indicate mean ± SEM.

The effects of perinatal frontal cortical injury on brain and behavioural development

Summary

Table 12. Significant findings for experiment 2a.

Behavioural Task	Females	Males
Activity: Distance	Sex: Females more active	Sex: Males less active
No. Movements	Increased movement	Decreased movement
Water Maze:	Impairments with lesion	Little difference with lesion
Tray Reaching:	Impairments with lesion	Impairments with lesion
Elevated Plus Maze	Decreased activity	Increased activity

Anatomy (Golgi-Cox and saline-perfused preparation)		
Brain Weight:	Sex: Females lighter weight Lighter weights with lesion	Sex: Males heavier weight Lighter weights with lesion
Basilar and Apical Branch Arbor:	Increased arbor in Cg3 with lesion, decrease length, Increase arbor in AID, decrease length with lesion	Increased arbor in Cg3 with lesion, decrease length, decrease arbor and length in AID with lesion
Adrenal Gland Weight:	Sex: Females had heavier weights	Sex: Males had lighter weights
Anatomy (Formalin preparation)		
Prelimbic cortex:	Thinner cortex with lesion	Thinner cortex with lesion
Anterior Thalamus:	Smaller area with lesion	Smaller area with lesion
Cortical Thickness:	Thinner cortex with lesion	Thinner cortex with lesion

The summary above in table 12 is displayed as an itemized list of what was statistically significant in the previous experiment. Following Experiment 2B is a general overview of the two experiments that will be discussed in the discussion.

IV. Experiment 2B: The effects of moderate prenatal stress on early cortical injury

The following section compares frontal lesion (Control Frontal) animals with and without prenatal stress treatment (Stress Frontal).

Behavioural Results

Locomotor Activity

Distance

Females exhibited a greater distance of exploration than males of either group in the activity box, but there was no effect of stress (see Fig. 60).

Univariate analysis of distance of exploration in the locomotor activity test indicated a main effect of sex ($F(1,30)= 17.244$, $p< 0.0001$), but not of stress ($F(1,30)= 0.444$, $p= 0.511$), nor for the interaction between Stress X Sex ($F(1,30)= 0.259$, $p= 0.615$).

Number of movements

There were no significant differences between groups in movement frequency (see Fig. 60).

Univariate analysis revealed no significant effects of stress ($F(1,30)= 0.818$, $p= 0.374$), of sex ($F(1,30)= 1.198$, $p= 0.283$), nor for the interaction ($F(1,30)= 2.256$, $p= 0.145$).

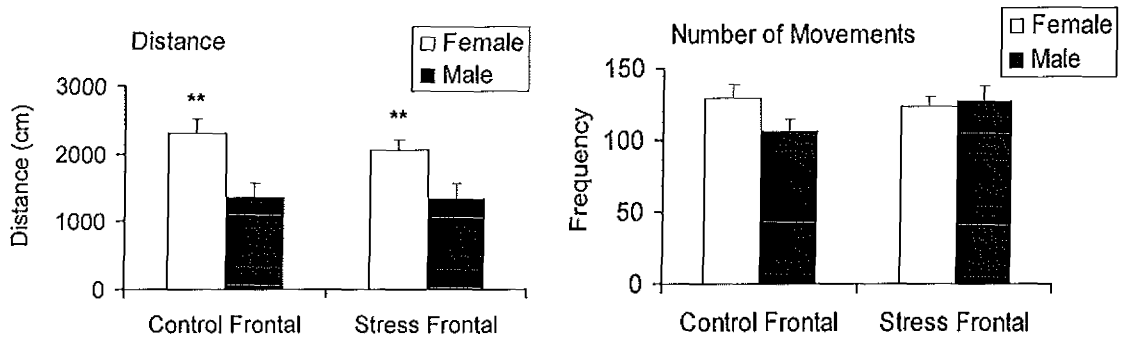


Figure 60. Left: Sums of mean distance of exploratory activity in prenatally stressed animals with frontal lesions. Right: Sums of movement frequency. ** Denotes significant differences between the sexes ($p<.05$). Bars indicate mean ± SEM.

Morris water maze

Latency

Prenatally stressed females with frontal lesions were significantly faster at finding the hidden platform, thus reversing the lesion effect, whereas stressed males performed the same as the non-stressed males (see Fig. 61).

Univariate analysis for latency sums indicated an interaction between stress and sex ($F(1,44)= 5.835$, $p= 0.020$), but no main effects of stress ($F(1,44)= 0.765$, $p= 0.387$), nor of sex ($F(1,44)= 1.964$, $p= 0.169$).

A multivariate analysis for day found days' one and two significantly different among groups ($p<.05$) (see Fig. 61).

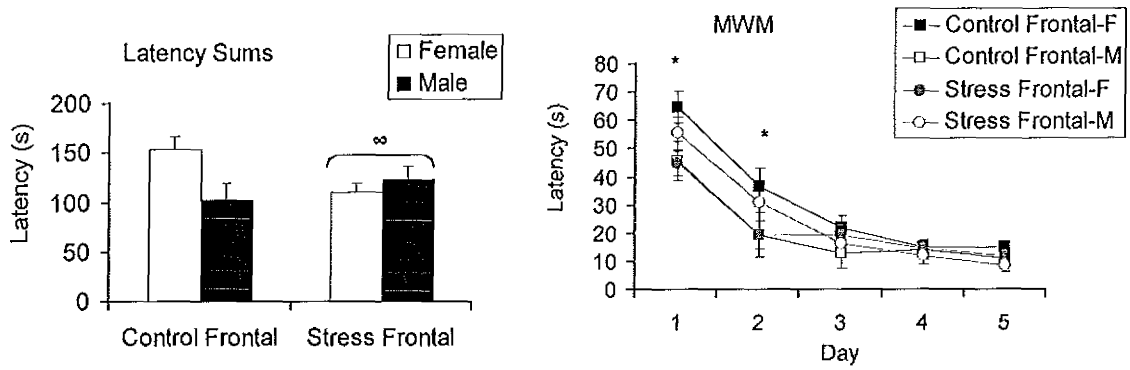


Figure 61. Left: Sums of mean latencies for the five-day acquisition trials in the water maze. ∞ Denotes the significant effects of prenatal stress between the sexes ($p<.05$). Right: Mean latencies during the five-day acquisition. * Denotes significantly different among groups ($p<.05$). F= Females, M= Males. Bars indicate mean ± SEM.

Morris water maze probe

All groups showed that they had learned the location of the platform as they spent more time in the previously correct quadrant than any other quadrant (see Fig. 62)

No significant effects were found with a univariate analysis for the water maze probe test for stress ($F(1,26)= 0.068$, $p= 0.796$), a main effect of sex ($F(1,26)= 0.191$, $p= 0.666$), nor for an interaction between stress and sex ($F(1,26)= 0.024$, $p= 0.878$).

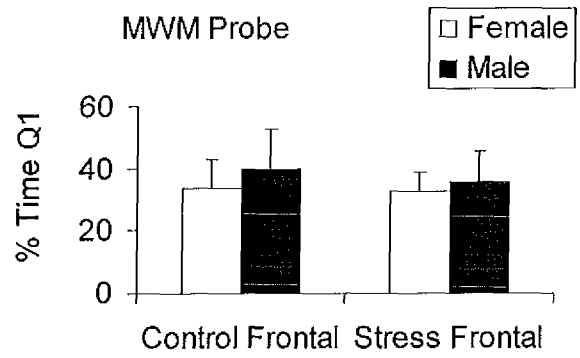


Figure 62. Probe test for the water maze in Control Frontal and Stress Frontal groups. Bars indicate mean ± SEM.

Tray Reaching

The prenatal stress treatment had little effect on the deficit in skilled reaching (see Fig. 63).

Univariate analysis for skilled reaching in frontal lesion animals did not indicate a main effect of stress ($F(1,38)= 0.471$, $p= .497$), or of sex ($F(1,38)= 1.319$, $p= 0.259$), nor for the interaction ($F(1,38)= 0.036$, $p= 0.851$).

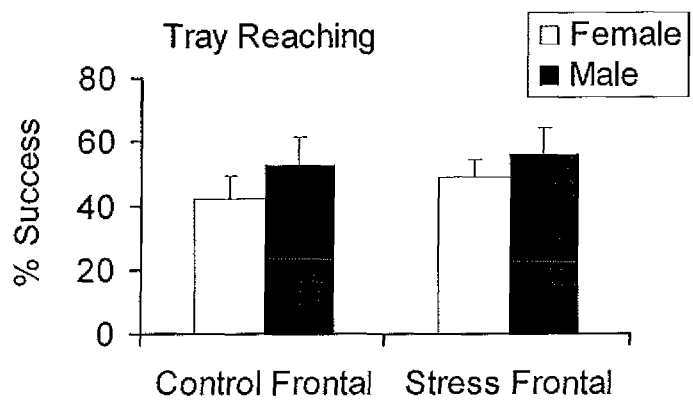


Figure 63. Mean percent success of skilled reaching in prenatally stressed animals with frontal lesions. Bars indicate mean ± SEM.

Elevated Plus Maze

Prenatal stress decreased exploratory activity in frontal lesion animals (see Fig. 64).

Univariate analysis revealed a significant effect of stress ($F(1,26)= 9.425$, $p= 0.005$) and a main effect of sex ($F(1,26)= 8.610$, $p= 0.007$), and a significant interaction between Stress X Sex ($F(1,26)= 5.357$, $p= 0.030$).

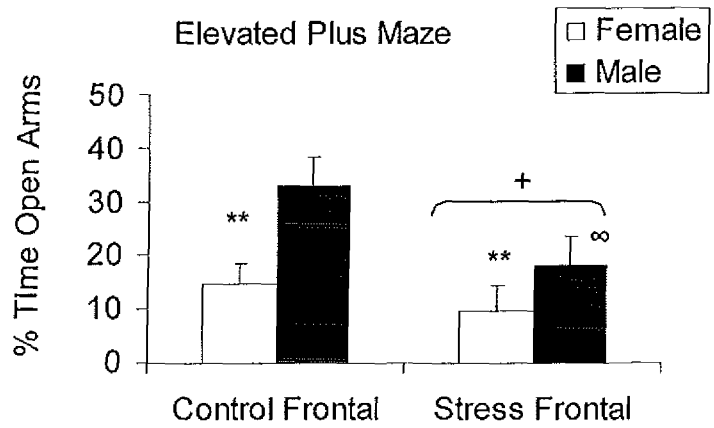


Figure 64. Percent time exploring the open arms in prenatally stressed animals with frontal lesions. $^{+}$ Denotes statistical difference from the non-stressed (Control Frontal) animals ($p=.057$). ** Denotes significant differences between the sexes ($p<.05$). ∞ Denotes the significant effects of stress in males ($p<.05$). Bars indicate mean ± SEM.

Anatomical Results

Frontal lesion

Four of twenty-one brains from animals exposed to moderate prenatal stress showed filling in with thinning, or small portions missing in Fr2; three brains had one hemisphere filled in with Cg3 missing from the other hemisphere; seven brains had partial filling in both hemispheres with small amounts of Cg1, and Cg3, or Fr2 missing; five brains showed partial filling in one hemisphere with the other missing larger portions of Cg3, Cg1, and Fr2; and, two had a large portions of Cg3, Cg1, with some of Fr2 missing (see Fig. 65).

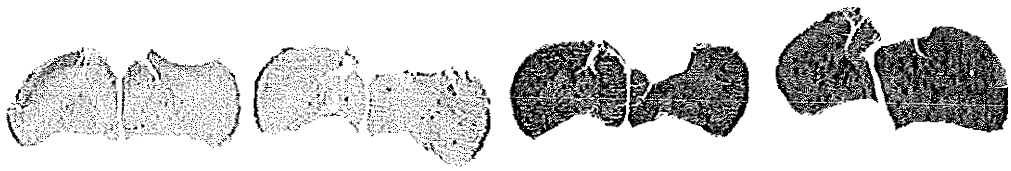


Figure 65. Examples of frontal lesions in prenatally stressed animals. Left: Brain with filled in of tissue at the lesion site with a flat dorsal Fr2 area in one hemisphere. Second from left and second from right: brains with partial filling of tissue in both hemispheres with various amounts of cortex missing. Right: Both hemispheres show some filling in, but are morphologically very different.

Golgi-Cox brain and adrenal gland measures

Brain weight

Brains from male animals were significantly heavier than female brains, but there was no effect of prenatal stress (see Table 13).

Univariate analysis for brain weight indicated a main effect of sex ($F(1,33)= 20.508$, $p< .0001$) but no main effect of stress ($F(1,33)= 2.219$, $p= 0.147$), or for a significant interaction ($F(1,33)= 0.198$, $p= 0.660$).

Table 13. Mean brain weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control Frontal	1.897 ± .027 (n=9)	2.018 ± .031 (n= 7)*
	Prenatal Stress	
Stress Frontal	1.840 ± .023 (n=13)	1.987 ± .036 (n=5)*

Brains are measured in grams as mean ± SEM. * Denotes statistically significant between sexes ($p<.05$).

Dendritic basilar arbor and length

Cingulate cortex (Cg3)

Basilar fields

Unfortunately the apical fields were not stained well enough to draw in the cingulate cortex of the stressed animals.

There were no effects of prenatal stress on the dendritic branching or length (see Figs. 66).

Univariate analysis for basilar dendritic arbor in Cg3 did not indicate any significant effects of stress ($F(1,15)= 0.425$, $p= 0.527$), or of sex ($F(1,15)= 1.696$, $p= 0.217$), or for an interaction between groups ($F(1,15)= 0.093$, $p= 0.765$).

Univariate analysis for dendritic length in the basilar region of pyramidal Cg3 cells did not find significant effects of stress ($F(1,15)= 0.425$, $p= 0.527$), or of sex ($F(1,15)= 1.696$, $p= 0.217$), or for an interaction ($F(1,15)= 0.093$, $p= 0.765$).

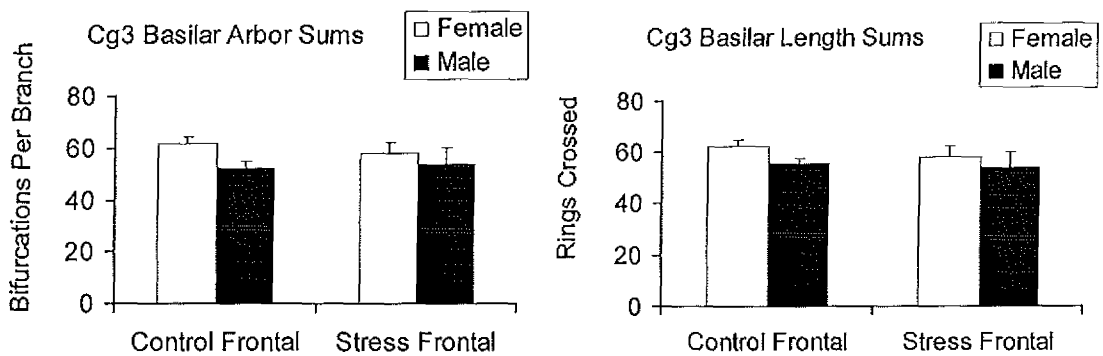


Figure 66. Left: Sums of mean dendritic basilar arbor, and Right: Sums of mean dendritic length in area Cg3 in prenatally stressed animals with frontal lesions. Bars indicate mean ± SEM.

Agranular Insular Dorsal (AID) cortex

Basilar fields

Unfortunately the apical fields were not clear enough to draw in the stressed frontal brains in AID.

Prenatal stress did not change basilar dendritic arbor density in AID frontal lesion animals, but reduced dendritic length in males and females with frontal lesions (see Fig. 67).

Univariate analysis for basilar dendritic arbor in the AID region did not indicate any main effects of stress ($F(1,19)= 0.068$, $p= 0.798$), or of sex ($F(1,19)= 0.555$, $p= 0.467$), nor for an interaction between groups ($F(1,19)= 0.002$, $p= 0.965$).

Univariate analysis for dendritic length in AID found a main effect of stress ($F(1,20)= 6.523$, $p= 0.021$), but not of sex ($F(1,20)= 0.007$, $p= 0.934$), nor for the interaction ($F(1,20)= 0.060$, $p= 0.809$).

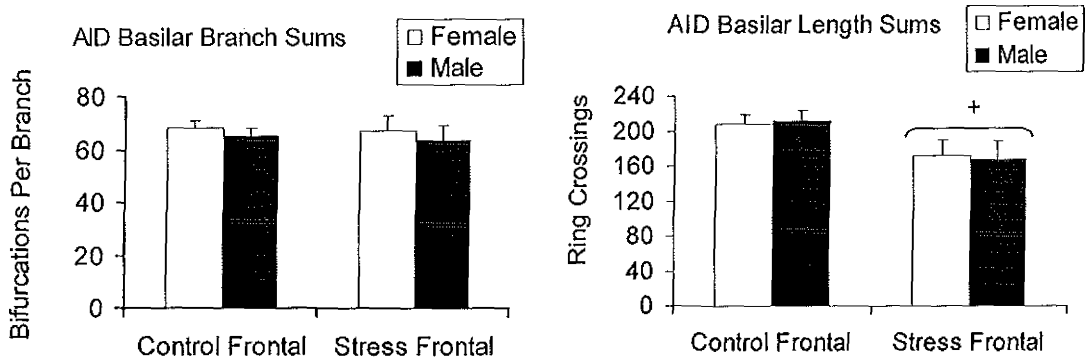


Figure 67. Left: Sums of mean dendritic branch arbor in AID in prenatally stressed animals with frontal lesions. Right: Sums of mean dendritic length in prenatally stressed animals with frontal lesions. + Denotes the significant effects of prenatal stress ($p<.05$). Bars indicate mean ± SEM.

Adrenal gland weight

The adrenal weight for both sexes was significantly greater in the stressed group than the Control Frontal group (see Table 14).

Univariate analysis for adrenal gland weight found a main effect of stress ($F(1,33)= 50.941$, $p< 0.0001$), and a main effect of sex ($F(1,33)= 10.634$, $p= 0.003$), but no significant interaction between Stress and Sex ($F(1,33)= 0.371$, $p= 0.547$).

14. Mean adrenal gland weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control Frontal	0.089 ± .006 (n=9)	0.063 ± .007 (n= 7)
	Prenatal Stress	
Stress Frontal	0.134 ± .005 (n=13)	0.116 ± .011 (n=5) ∞

Adrenal glands are measured in grams as mean ± SEM. ∞ Denotes the significant effects of prenatal stress ($p<.05$).

Adrenal gland areal measures

Cortex

Prenatally stressed males had a 35% reduction in mean adrenal cortex thickness, but there were no significant differences (see Fig. 68).

Univariate analysis did not find any significant effects of stress ($F(1,12)= 0.480$, $p= 0.506$), or of sex ($F(1,12)= 1.345$, $p= 0.276$), or an interaction ($F(1,12)= 0.799$, $p= 0.395$).

Medulla

As with the cortex measures, prenatally-stress males with lesions had about a 40% decrease in the medulla area, but again without significant effects (see Fig. 68).

Univariate analysis for adrenal medulla area did not indicate main effects of stress ($F(1,12)= 2.145$, $p= 0.177$), or of sex ($F(1,12)= 0.454$, $p= 0.518$), or an interaction between groups ($F(1,12)= 1.841$, $p= 0.208$).

An independent sample T-Test medulla area and males did not produce significant results likely to the small numbers ($t = 2.224$, $p= 0.080$).

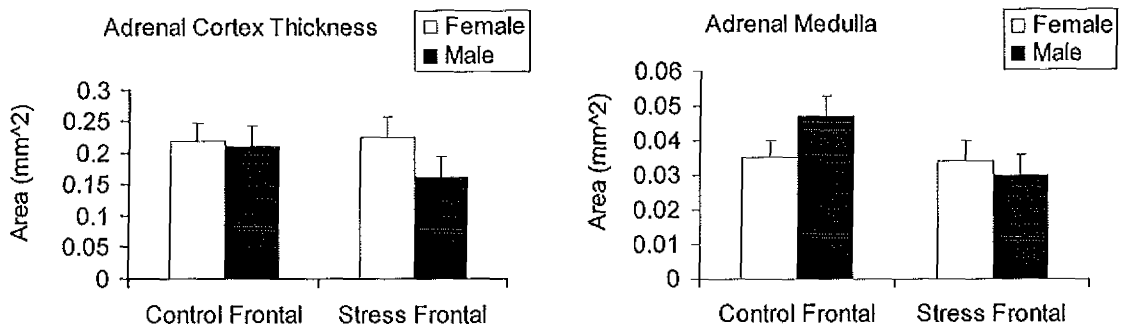


Figure 68. Left: Sums of mean adrenal gland cortex thickness and Right: Sums of mean adrenal gland medulla area in prenatally stressed animals with frontal lesions. Bars indicate mean ± SEM.

Table 15. Significant findings for experiment 2b.

Behavioural Task	Females	Males
Activity: Distance	Sex: Females more active	Sex: Males less active
Water Maze:	Stress reversed impairments from lesion	Partial impairments with stress
Elevated Plus Maze:	Decreased exploration with stress	Decreased exploration with stress
Anatomy (Golgi-Cox and saline-perfused preparation)		
Brain Weight:	Females lighter than males, Decreased with stress	Males heavier than females, Decreased with stress
Basilar and Apical Branch Arbor/Length:	Decreased length in AID with stress	Decreased length in AID with stress
Adrenal Gland Weight:	Heavier than males, Increased with stress	Lighter than females, Increased with stress

V. Experiment 2C: The effects of condominium housing on moderate prenatal stress and early frontal cortical injury

This section continues with the comparison of untreated animals with frontal lesions (Control Frontal) to that of prenatal stress treatment (Stress Frontal) and the addition of condominium housing treatments, all with frontal lesions; (Condo Control Frontal and Condo Stress Frontal)

Behavioural Results

Locomotor Activity

Distance

Overall, females covered more distance in the activity box and the stressed condominium- housed animals exhibited much less activity (see Fig. 69).

Univariate analysis for distance traveled in the activity box revealed a main effect of treatment ($F(1,53)= 9.428$, $p= 0.011$), and of sex ($F(1,53)= 12.076$, $p= 0.001$), but not of stress ($F(1,53)= 0.904$, $p= 0.347$). There were also no significant interactions between groups: Stress X Treatment ($F(1,53)= 0.009$, $p= 0.924$), Stress X Sex ($F(1,53)= 0.316$, $p= 0.577$), Treatment X Sex ($F(1,53)= 3.439$, $p= 0.070$, nor for Stress X Treatment X Sex ($F(1,53)= 0.008$, $p= 0.927$).

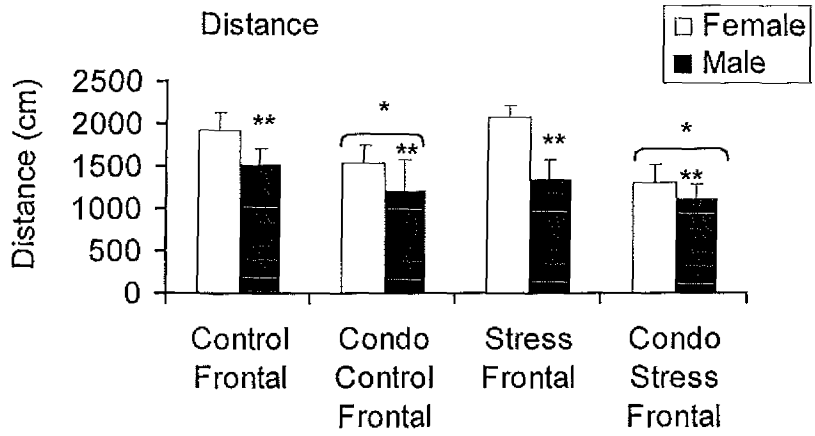


Figure 69. Sums of mean distance explored in the activity box in frontal-lesion animals. * Denotes statistically significant from Control Frontal and Stress Frontal groups ($p<.05$). ** Denotes statistical significance between the sexes ($p<.05$). Bars indicate mean ± SEM.

Number of movements

Both sexes of the condominium reared treatment groups (control and stress) exhibited a marked decrease in overall activity (see Fig.70).

Univariate analysis for number of movements in the activity box indicated a main effect of treatment ($F(1,53)= 33.187$, $p< .0001$), but not of stress ($F(1,53)= 1.372$, $p= 0.248$), or of sex ($F(1,53)= 0.037$, $p= 0.849$). There were no significant interaction between: Stress X Treatment ($F(1,53)= 0.000$, $p= 0.996$), Stress X Sex ($F(1,53)= 0.001$, $p= 0.970$), Treatment X Sex ($F(1,53)= 2.609$, $p= 0.113$), or for Stress X Treatment X Sex ($F(1,53)= 3.674$, $p= 0.061$).

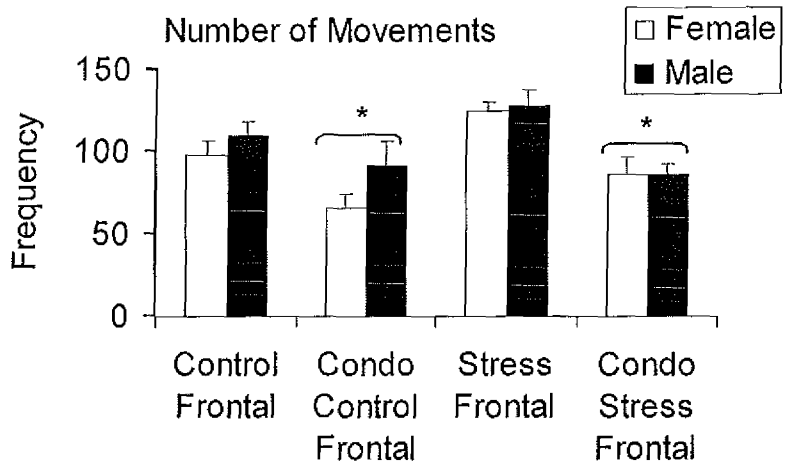


Figure 70. Sums of mean number of movements in the activity box. * Denotes statistically significant from Control Frontal, and Stress Frontal groups ($p<.05$). Bars indicate mean ± SEM.

Morris water maze

Latency

Condominium housing improved the performance of the animals with frontal lesions, both with and without prenatal stress (see Fig.71).

Univariate analysis for latency to find the platform found a main effect of treatment ($F(1,67)= 11.865$, $p= 0.001$), but not for main effect of stress ($F(1,67)= 0.349$, $p= 0.557$), or of sex ($F(1,67)= 1.280$, $p= 0.262$). There were also no significant interactions between Stress X Treatment ($F(1,67)= 2.733$, $p= 0.104$), Stress X Sex ($F(1,67)= 1.193$, $p= 0.279$), Treatment X Sex ($F(1,67)= 0.327$, $p= 0.570$), or for Stress X Treatment X Sex ($F(1,67)= 3.398$, $p= 0.070$).

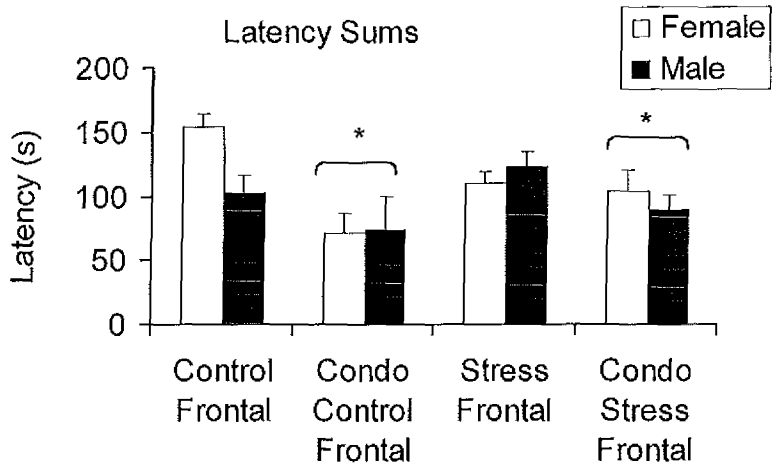


Figure 71. Mean latency sums for animals with frontal lesions to find the hidden platform in the water maze. * Denotes significantly different from Control Frontal and Stress Frontal groups ($p<.05$). Bars indicate mean ± SEM.

Morris water maze probe

There were no effects of condominium rearing on the performance on the probe trial (see Fig. 72).

Univariate analysis for the probe test in frontal lesion animals did not find main effects of stress ($F(1,50)= 0.663$, $p= 0.420$), of treatment ($F(1,50)= 0.119$, $p= 0.732$), or of sex ($F(1,50)= 0.512$, $p= 0.478$). There were also no significant interactions: Stress X Treatment ($F(1,50)= 0.139$, $p= 0.711$), Stress X Sex ($F(1,50)= 0.289$, $p= 0.594$), Treatment X Sex ($F(1,50)= 0.000$, $p= 0.983$), or for Stress X Treatment X Sex ($F(1,50)= 0.076$, $p= 0.785$).

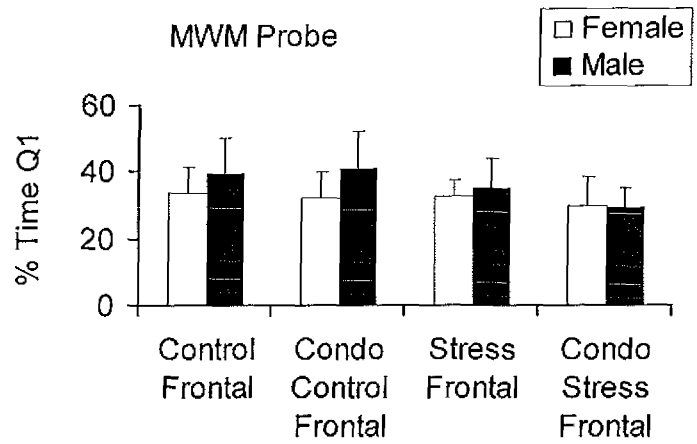


Figure 72. Percent amount of time spent swimming in the correct quadrant during the probe test in the water maze. Bars indicate mean ± SEM.

Tray Reaching

Condominium- housing completely reversed the effects of the frontal lesion on skilled reaching in the unstressed group but had no benefit in the stressed group (see Fig. 73).

Univariate analysis for skilled reaching in frontal lesion animals indicated a significant Stress X Treatment interaction ($F(1,61)= 7.103$, $p= 0.010$), but no main effect of stress ($F(1,61)= 3.206$, $p= 0.079$), of treatment ($F(1,61)= 3.161$, $p= 0.081$), or of sex ($F(1,61)= 1.258$, $p= 0.267$). There were also no interactions between: Stress X Sex ($F(1,61)= 0.000$, $p= 0.991$), Treatment X Sex ($F(1,61)= 0.117$, $p= 0.734$), or for Stress X Treatment X Sex ($F(0.053$, $p= 0.819$).

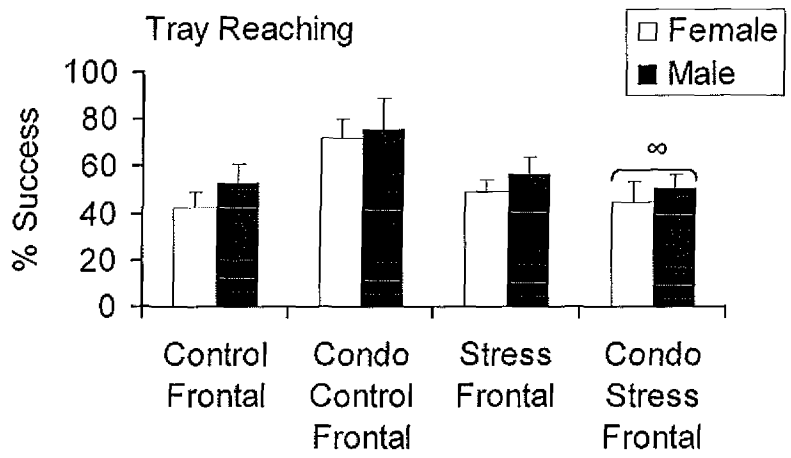


Figure 73. Percent success in skilled reaching in frontal lesion animals treated with prenatal stress, condominium housing, or a combination of treatments. ∞ Denotes statistically significant effects of stress with condo housing ($p<.05$). Bars indicate mean ± SEM.

Elevated Plus Maze

The prenatally stressed animals spent much less time exploring the open arms, which was further decreased with condominium housing. Males in the stress and treatment groups exhibited a significant suppression of exploration on the open arms (see Fig. 74).

Univariate analysis for emotionality in the plus maze indicated a main effect of treatment ($F(1,55)= 12.733$, $p= 0.001$), but not of stress ($F(1,55)= 0.734$, $p= 0.396$), or of sex ($F(1,55)= 0.770$, $p= 0.384$). There were also no significant interactions between: Stress X Treatment ($F(1,55)= 0.451$, $p= 0.505$), Stress X Sex ($F(1,55)= 0.941$, $p= 0.337$), Treatment X Sex ($F(1,55)= 2.963$, $p= 0.092$) or for Stress X Treatment X Sex ($F(1,55)= 0.557$, $p= 0.495$).

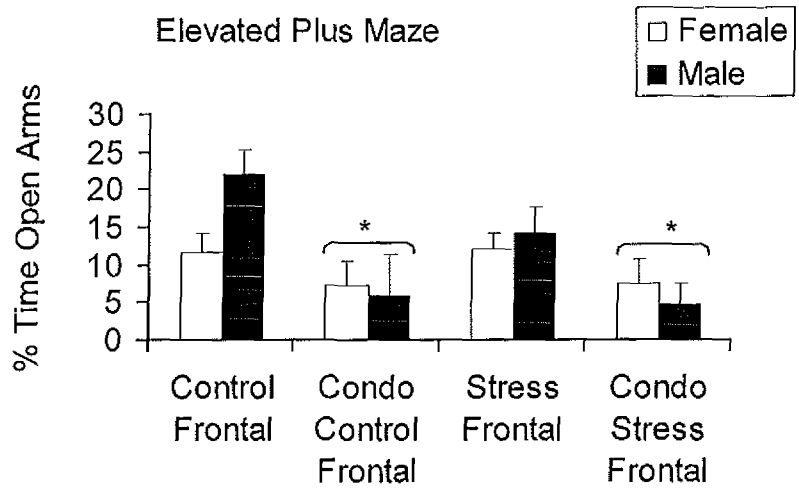


Figure 74. Percent amount of time frontal lesion animals explored the open arms of the elevated plus maze. * Denotes statistical difference from untreated control animals ($p<.05$). Bars indicate mean ± SEM.

Anatomical Results

Frontal lesion

Condo Control: two of eight brains with frontal lesions showed filling in; two brains showed filling in of one hemisphere with missing dorsal portions of Cg1 and Fr2 in the other hemisphere; two brains showed partial filling in both hemispheres; one brain had one hemisphere partially filled in with the other missing large portions of Cg1 and Fr2; and one had very little filling in with large portions of Cg3, Cg1, and Fr2 missing in both hemispheres (see Fig. 75).

Condo Stress: Eight of fifteen brains with frontal lesions showed filling in; some with very small portions of Cg1 and Fr2 missing and although one brain had filling in of

the anterior areas, the lateral ventricles were large. Three brains had filling in of tissue in one hemisphere and the other hemisphere missing dorsal portions of Cg1 and Fr2. Two brains had partial filling in one hemisphere with the other hemisphere missing large portions of Cg3, Cg1, and Fr2. Finally, one brain had very little filling in, missing portions of Cg3, Cg1, and Fr2.

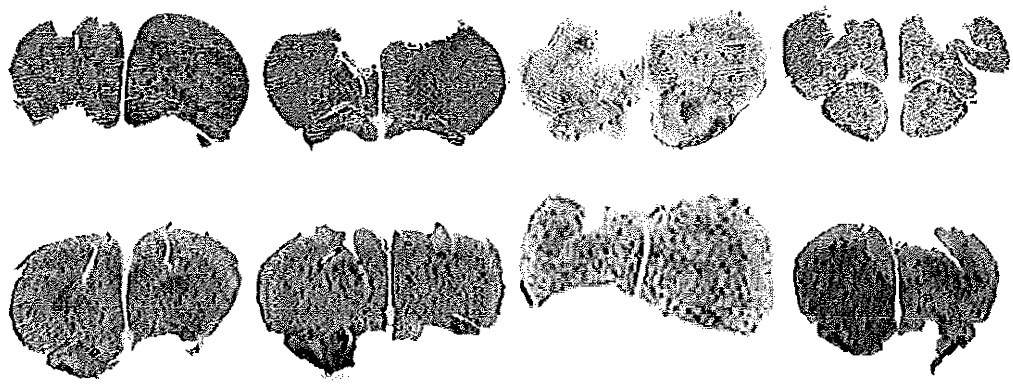


Figure 75. Examples of filling in at the frontal lesion site in condo-housed animals. Top: Non-stressed condo-housed animals with frontal lesions with various degrees of filled-in tissue at the lesion site. Bottom: Prenatally stressed condo-housed animals with frontal lesions and varying amounts of filled in, or partially filled in tissue.

Golgi-Cox brain and saline-perfused adrenal gland measures

Brain weight

Males had heavier brains than females in all groups, but there was no effect of condominium housing on brain weight (see Table 16).

Univariate analysis for brain weight in frontal lesion animals found a main effect of sex ($F(1,56)= 36.728$, $p < .0001$), but not of stress ($F(1,56)= 0.719$, $p= 0.401$), or of treatment ($F(1,56)= 0.154$, $p= 0.697$). There were also no significant interactions between variables: Stress X Treatment ($F(1,56)= 0.602$, $p= 0.441$), Stress X Sex ($F(1,56)= 0.006$,

p= 0.940), Treatment X Sex (F(1,56)= 1.263, p= 0.267), or for Stress X Treatment X Sex (F(1,56)= 0.314, p= 0.578).

Table 16. Mean brain weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control Frontal	1.897 ± .030 (n= 9)	2.018± .034 (n= 7)*
Condo Control Frontal	1.820 ± .036 (n= 6)	2.032 ± .060 (n= 2)*
	Prenatal Stress	
Stress Frontal	1.840 ± .028 (n= 13)	1.987 ± .040 (n= 5)*
Condo Stress Frontal	1.835 ± .040 (n= 5)	2.013 ± .028 (n=10)*

Brain weights are measured in grams mean ± SEM. * Denotes statistical significance between the sexes ($p<.05$).

Dendritic basilar arbor and length

The Golgi-Cox staining in the condominium-reared groups proved to be unreliable and only the AID cells could be reliably drawn. These cells are reported followed by a measure of cortical thickness, which could be made in these brains.

Agranular Insular Dorsal (AID) cortex

Basilar fields

There was no effect of condominium housing on basilar dendritic branching in AID (see Fig. 76).

A univariate analysis for basilar dendritic arbor in AID did not find any significant effects of stress ($F(1,25)= 0.077$, p 0.784), of treatment ($F(1,25)= 1.102$, $p= 0.306$), or of sex ($F(1,25)= 0.373$, $p= 0.548$). There were also no significant interactions between groups: Stress X Sex ($F(1,25)= 0.002$, $p= 0.962$), or Treatment X Sex ($F(1,25)= 0.128$, $p= 0.724$).

Condominium housing partially reversed the effects of stress on dendritic length in AID (see Fig. 76).

Univariate analysis for dendritic branch arbor in AID found a main effect of stress ($F(1,26)= 7.327$, $p= 0.013$), but not of treatment ($F(1,26)= 1.925$, $p= 0.180$), nor of sex ($F(1,26)= 0.000$, $p=0.985$) There were also no significant interactions between groups: Stress X Sex ($F(1,26)= 0.068$, $p= 0.797$) or Treatment X Sex ($F(1,26)= 0.028$, $p= 0.869$).

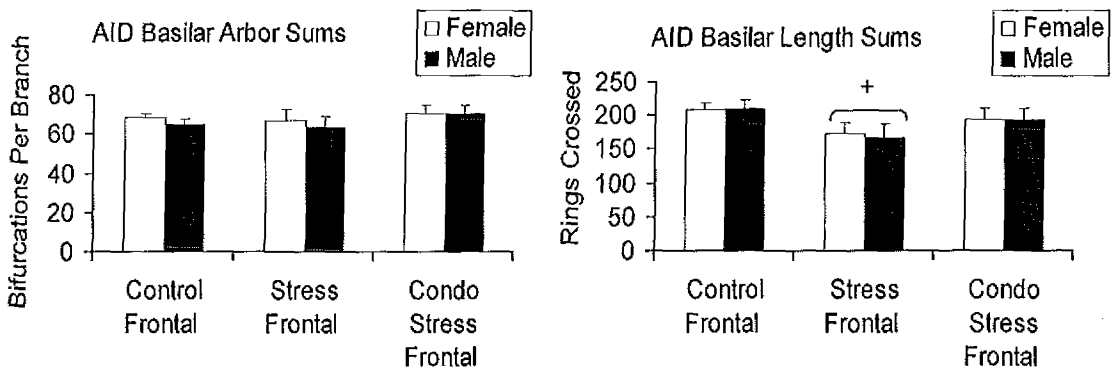


Figure 76. Left: Sums of mean dendritic arbor in the basilar area of cells in AID. Right: Sums of mean dendritic length in AID. $^{+}$ Denotes statistical significance from non-

stressed control animals ($p<.05$). Animals were prenatally stressed, housed in condos and had frontal lesions. Bars indicate mean ± SEM.

Cortical Thickness

Males that were prenatally stressed and reared in condominiums had a reduction in thickness of the cortical mantle (see Fig. 77).

Univariate analysis for cortical thickness indicated a main effect of sex ($F(1,42)= 3.925$, $p= 0.055$) and a significant interaction between Stress X Treatment ($F(1,42)= 7.570$, $p= 0.009$). There were no main effects of stress ($F(1,42)= 0.529$, $p= 0.472$), or of treatment ($F(1,42)= 0.098$, $p= 0.756$). There were also no further significant interactions between Stress X Sex ($F(1,42)= 0.173$, $p= 0.680$), Treatment X Sex ($F(1,42)= 2.493$, $p= 0.123$), or for Stress X Treatment X Sex ($F(1,42)= 0.640$, $p= 0.429$).

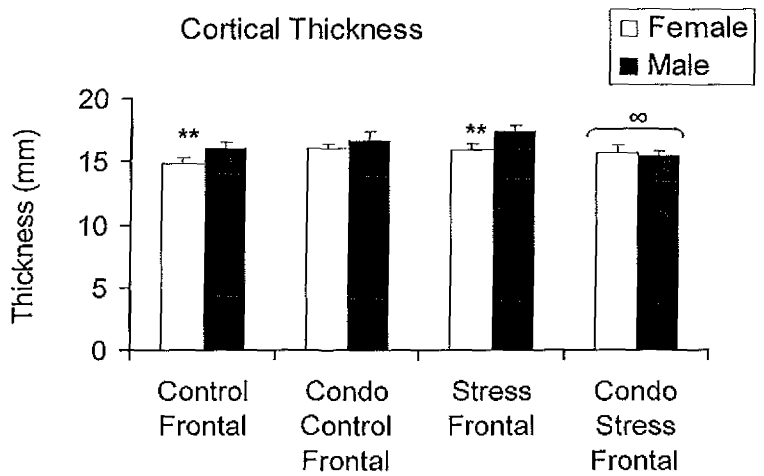


Figure 77. Sums of mean cortical thickness measurements in prenatally stressed animals and in condo housed animals with frontal lesions. ** Denotes the significant differences between sexes ($p<.05$). ∞ Denotes the significant effects of prenatal stress with condominium housing ($p<.05$). Bars indicate mean ± SEM.

Adrenal gland weight

The prenatal stress treatment greatly increased adrenal gland weight in both sexes, which was reversed with condominium treatment (see Table 17).

Univariate analysis for adrenal weight found a main effect of stress ($F(1,56)= 37.147$, $p< 0.0001$), and of treatment ($F(1,56)= 28.607$, $p< 0.0001$), and of sex ($F(1,56)= 11.726$, $p= 0.001$). There is also a significant interaction between Stress X Treatment ($F(1,56)= 13.663$, $p= 0.001$) and Stress X Treatment X Sex ($F(1,56)= 4.781$, $p= 0.034$). There was no significant interactions between Stress X Sex ($F(1,56)= 1.824$, $p= 0.183$) or Treatment X Sex ($F(1,56)= 1.101$, $p= 0.299$).

Table 17. Mean adrenal gland weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control Frontal	0.089 ± .005 (n=9)	0.063 ± .006 (n= 7)*
Condo Control Frontal	0.065 ± .00 (n=6)	0.071 ± .012 (n= 2)*
	Prenatal Stress	
Stress Frontal	0.134 ± .005 (n=13)	0.116 ± .010 (n=5) +
Condo Stress Frontal	0.095 ± .007 (n=5)	0.065 ± .005 (n=10)*∞

Brain weights are measured in grams mean ± SEM. * Denotes statistically significant between sexes ($p < .05$). ∞ Denotes statistically significant effect of condo housing with prenatal stress ($p < .05$). $^{+}$ Denotes the effects of prenatal stress ($p < .05$).

Adrenal gland areal measures

Cortex

Condominium housing increased adrenal cortex thickness in stressed animals, especially in females (see Fig. 78).

Univariate analysis for adrenal cortex thickness in condo housed rats indicated a main effect of stress ($F(1,27) = 8.128$, $p = 0.010$), and of sex ($F(1,27) = 10.916$, $p = 0.004$). There were also significant interactions between Stress X Treatment ($F(1,27) = 21.827$, $p < 0.0001$) and Stress X Sex ($F(1,27) = 4.895$, $p = 0.039$). There was no main effect of treatment ($F(1,27) = 2.097$, $p = 0.163$), nor for interactions of Treatment X Sex ($F(1,27) = 1.895$, $p = 0.184$) or for Stress X Treatment X Sex ($F(1,27) = 0.003$, $p = 0.958$).

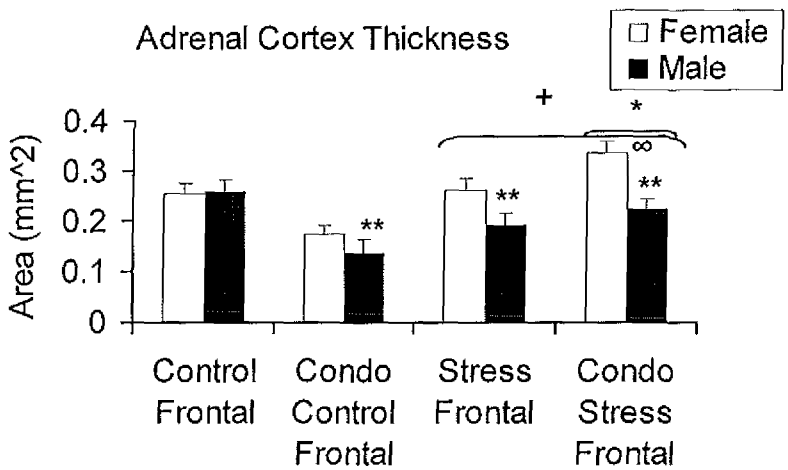


Figure 78. Sums of mean adrenal cortex thickness in prenatally stressed and condo housed animals with frontal lesions. * Denotes statistical different effects of condo

housing in prenatally stressed animals ($p<.05$). ** Denotes statistically significant between the sexes. + Denotes significant differences from non-stressed animals ($p<.05$). ∞ Denotes the significant effects of stress in females ($p<.05$). Bars indicate mean ± SEM.

Medulla

Condominium housing decreased medulla areal measures regardless of prenatal experience (see Fig. 79).

Univariate analysis for adrenal medulla indicated a main effect of treatment ($F(1,27)= 9.613$, $p= 0.006$), but not stress ($F(1,27)= 2.423$, $p= 0.135$), or of sex ($F(1,27)= 0.060$, $p= 0.809$). There were no significant interactions between groups: Stress X Treatment ($F(1,27)= 1.712$, $p= 0.206$), Stress X Sex ($F(1,27)= 3.215$, $p= 0.088$), Treatment X Sex ($F(1,27)= 1.151$, $p= 0.296$), or for Stress X Treatment X Sex ($F(1,27)= 0.741$, $p= 0.399$).

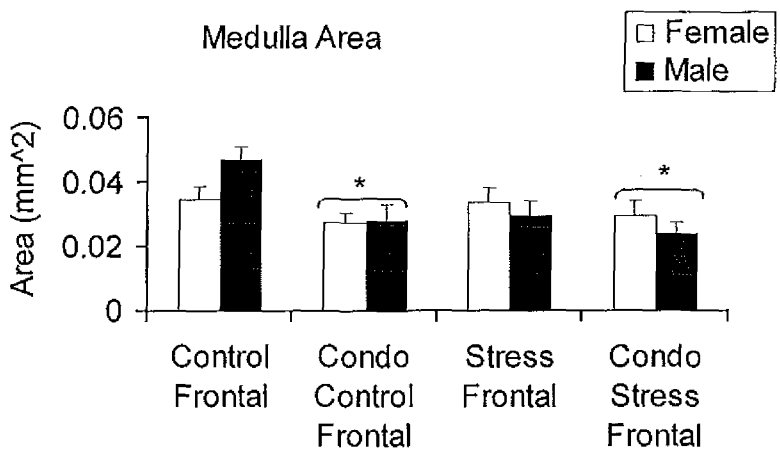


Figure 79. Sums of mean adrenal medulla area in prenatally stressed and condo housed animals with frontal lesions. * Denotes the significantly effects of condominium housing ($p<.05$). Bars indicate mean ± SEM.

The effects of condominium housing on moderate prenatal stress and early frontal cortical injury and brain and behavioural development

Summary

Table 18. Significant findings for experiment 2c.

Behavioural Task	Females	Males
Activity: Distance Number of Movements	Females more active, Condo housing decreased activity	Males less active, Condo housing decreased activity
Water Maze:	Condo housing decreased latency	Condo housing decreased latency
Tray Reaching:	Condo housing with stress impaired reaching, without stress reversed impairments	Condo housing with stress impaired reaching, without stress reversed impairments
Elevated Plus Maze:	Decreased exploration with condo treatment	Decreased exploration with condo treatment
Anatomy (Golgi-Cox and saline-perfused preparation)		
Brain Weight:	Sex: Females had lighter weights	Sex: Males had heavier weights
Basilar and Apical Branch Arbor/Length:	decreased length in AID; partially reversed with condo	decreased length in AID; partially reversed with condo

	housing	housing
Cortical Thickness	Increased with condo and stress	Decreased with condo and stress
Adrenal Gland Weight:	Heavier in females decreased with condo housing	Lighter in males; decreased with condo housing
Adrenal Cortex Thickness:	Decreased with condo housing, increased with stress and condo	Decreased with condo housing, increased with stress and condo
Adrenal Medulla Area:	Decreased with condo housing	Decreased with condo housing

The summaries in tables 12, 15 and 18 are displayed as itemized lists of what was statistically significant in each of the previous experiments. The summary to follow is a general overview of the three experiments with frontal lesions that will be discussed in the discussion.

Discussion

Medial frontal lesions

The primary findings for animals with frontal lesions were (same as in experiment 2A: Control No Lesion and Control Frontal Lesion): 1) behavioral impairments affected both sexes differentially depending on the task; 2) anatomical results were sexually dimorphic.

Medial frontal lesions with prenatal stress

The main findings in animals with frontal lesions and exposed to prenatal stress were: 1) behavioral impairments affected both sexes differentially depending on the task; 2) stress induced anxiogenic behaviours in both sexes in the elevated plus maze; 3) prenatal stress produced sex differences in some anatomical measures; and, 4) adrenal gland weights significantly increased in prenatally stressed animals.

Medial frontal lesions with prenatal stress treated with condominium housing

The main findings in animals treated with prenatal stress, condominium housing, or a combination of stress with condo housing were: 1) there were significant behavioural differences between the stressed and non-stressed animals housed in condominiums; 2) condominium housing did not alleviate the anxious behaviour in the elevated plus maze regardless of prenatal treatment; 3) condominium housing restored some of the anatomical measures toward control values; and, 4) condominium housing decreased adrenal gland weights.

Behavioural measures

Morris water maze task

Males are superior at place navigation that requires the coordination of remembering the distal cues and their association with the hidden platform. Rats with lesions incurred during the first week of life are very impaired at place learning, as are rats with lesions after two weeks of age, or in adulthood (Kolb, Brown, Witt- Lajeunesse, and Gibb, 2001). Rats with lesions induced during days 7 to 10 however, do not show the same impairments, although there is often a sex difference with females showing a small residual impairment (Kolb and Stewart, 1991). Males and females also use different navigation techniques potentially contributing to the differences in navigation (Kolb and Cioe, 1996).

Both treatments of prenatal stress and condominium housing (stressed and non-stressed) animals were beneficial for navigation success in frontal lesion animals in the water maze. Prenatal stress completely reversed the frontal deficit found in non-stressed control females and condominium housing was beneficial for all animals with frontal lesions. This could be due in part to an increase in neurotrophic factors from enrichment in condominium housing (Will et al, 2004). The effect of prenatal stress in the females with frontal lesions is interesting because it appears to have improved navigation. One explanation is that the early stress acted to masculinize the female brain as some studies have found prenatally stressed female guinea pigs to show masculinization in behavioural and neuroendocrine profiles (Kaiser, Kruijver, Swaab, and Sachser, 2003). An upregulation of androgen and estrogen-α receptors were found in the arcuate nucleus of

the hypothalamus, the MPOA, the PVN of the thalamus, and in CA1 region of the hippocampus, all of which corresponded with increased serum testosterone concentration and adrenal gland tyrosine hydroxylase concentration (Kaiser, Kruijver, Swaab, and Sachser, 2003) If masculinization of the female brain occurred during the perinatal stages of brain development, it might have enhanced their spatial ability to solve problems such as place learning in the water maze task.

Skilled reaching

Prenatal stress had no obvious effect in reaching accuracy when compared to non-stressed control frontal animals. With the introduction of the condominium-housed animals however, it appears that prenatal stress might have contributed to some of the reaching deficits because the enrichment effect was only seen in animals that were not prenatally stressed.

Elevated plus maze

The frontal lesion males were more adventurous on the open arms of the elevated plus maze. The sharp decline in exploration in animals treated with prenatal stress, condominium housing, and the combination of stress and housing is indicative of an enhancement in anxiogenic behaviour and a reversal of the lesion effect. One explanation might be that the frontal lesions altered limbic activity and this was reversed by the treatments, although the reasons for either effect are unknown.

Thus, both treatments were beneficial for performance in frontal animals on the cognitive tests, but not so in tests of emotionality.

Brain and adrenal anatomical measures

Brain measures

The lesions were intended to remove the anterior portions of Cg1, Cg2, and Cg3, with the prelimbic cortex, and the most medial regions of Fr2. Previous studies of rats with similar lesions on day 10 found partial to complete filling in of the lesion cavities that was related to spontaneous neurogenesis (eg. Kolb, et al., 1998). The same result was seen in the current study.

Kolb and colleagues (2007) have correlated many of their anatomical findings with the corresponding behavioural results. For example, a nearly complete restoration of function would correlate with an increased thickness in cortical measures, relative to animals with less functional recovery, which would further correspond to an increase in dendritic arbor. Such findings have been noted with brain injury from any cerebral region, and thus appears to be a general phenomenon.

A general finding in animals with postnatal injury incurred during the developmental time of days 7 to 10, show complete or partial recovery in behavioural measures and should therefore show corresponding brain measures. Lighter brain weights and cortical thickness were still apparent, but the cortex was thicker than that found in animals with earlier lesions of day three or four operates (Kolb and Gibb, 2007).

The increase in dendritic arbor in Cg3 might have been a result of spontaneous neurogenesis and increased branching (sprouting) in dendrites. There was also an effect of lesion in the dendritic arbor of AID with decreased arbor in both sexes.

Prenatal stress decreased dendritic length in Cg3 in females and dendritic length in AID in both sexes, which was partially reversed with condominium housing. Unfortunately, we do not have data for the Cg3 area in condo-housed brains.

Adrenal measures

Prenatal stress significantly increased adrenal gland weight, which suggests a higher adrenal cortical activity from over-exposure to stress hormones of the HPA axis. The treatment of condominium housing differentially affected all measures of the adrenal gland. The increase in adrenal weight from prenatal stress was reversed from condo housing in males, but changed little in females.

The adrenal cortex thickness remained larger in prenatally stressed animals, which was emphasized in condo housed females, suggesting a greater responsiveness to stress in females.

VII. Experiment 3B: The effects of a micronutrient supplemented diet on mild prenatal stress and brain and behavioural development

The following section compares unstressed control (Control NL) and prenatally stressed (Stress NL) animals with the introduction of a treatment of a vitamin/mineral (VS) supplemented diet to each group (Control VS NL and Stress VS NL).

Behavioural Results

Locomotor Activity

Distance

Overall, females are more active than males (see Fig. 92).

Univariate analysis for distance in the activity box found a main effect of sex ($F(1,106)= 23.239$, $p< 0.0001$), but not of stress ($F(1,106)= 0.543$, $p= 0.463$), nor treatment ($F(1,106)= 2.997$, $p= 0.087$). There were no interactions between variables: Stress X Treatment ($F(1,106)= 0.081$, $p= 0.777$), Stress X Sex ($F(1,106)= 2.235$, $p= 0.138$), Treatment X Sex ($F(1,106)= 0.612$, $p= 0.436$), or for Stress X Treatment X Sex ($F(1,106)= 0.001$, $p= 0.980$).

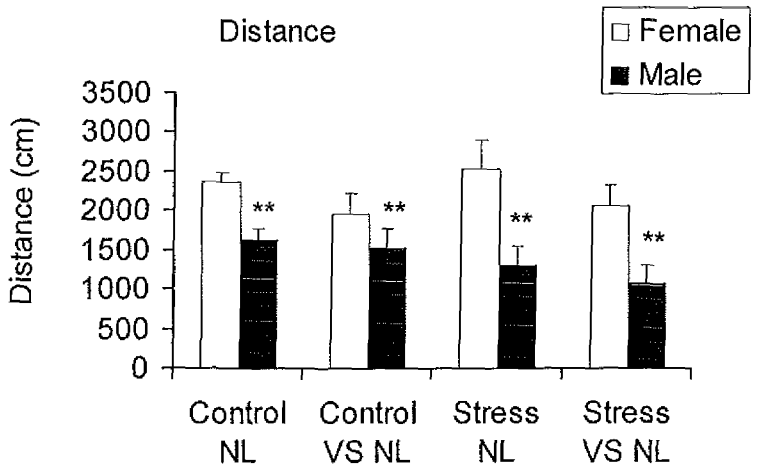


Figure 92. Sums of mean distance explored in the activity box. ** Denotes significant differences between the sexes ($p < .05$). Bars indicate mean ± SEM.

Number of movements

Prenatally stressed females continued to show the greatest number of movements in the activity box, which was reversed with the supplemented diet (see Fig. 93).

Univariate analysis for number of movements revealed a main effect of stress ($F(1,106) = 8.417$, $p = 0.005$), of treatment ($F(1,106) = 20.142$, $p < .000$), and of sex ($F(1,106) = 21.862$, $p < .0001$). There were also significant interactions between variables: Stress X Treatment ($F(1,106) = 20.329$, $p < .0001$), Stress X Sex ($F(1,106) = 20.175$, $p < .0001$), Treatment X Sex ($F(1,106) = 13.881$, $p < .0001$), and for Stress X Treatment X Sex ($F(1,106) = 15.396$, $p < .0001$).

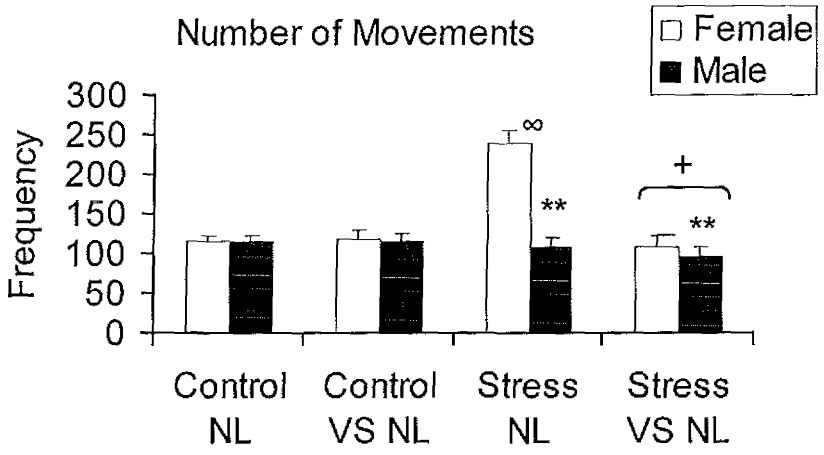


Figure 93. Sums of mean number of movements in the activity box. ∞ Denotes the significant effects of prenatal stress in females ($p<.05$). + Denotes the significant effects of prenatal stress with diet treatment ($p<.05$). ♦ Denotes the significant effects of stress with diet treatment in females ($p<.05$). ** Denotes the significant differences between the sexes ($p<.05$). Bars indicate mean ± SEM.

Morris Water Maze

Latency

The supplemented diet reduced the latency to find the hidden platform during acquisition (see Fig. 94).

Univariate analysis for latency sums indicated a main effect of treatment ($F(1,125)= 5.850$, $p= 0.017$), as well as for the interaction between Stress X Treatment ($F(1,125)= 7.160$, $p= 0.009$). There were no main effects of stress ($F(1,125)= 2.261$, $p= 0.135$), or of sex ($F(1,125)= 0.540$, $p= 0.464$), or for interactions between other variables: Stress X Sex ($F(1,125)= 1.129$, $p= 0.270$), Treatment X Sex ($F(1,125)= 0.427$, $p= 0.514$), or for Stress X Treatment X Sex ($F(1,125)= 1.016$, $p= 0.315$).

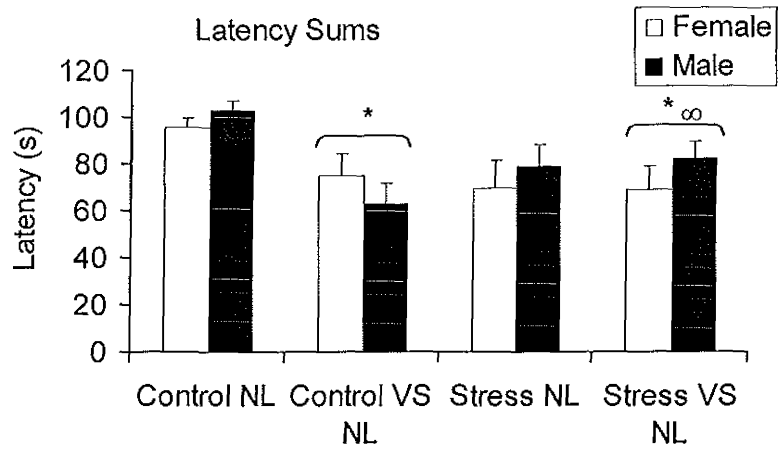


Figure 94. Sums of mean latency for measures of cognition in the Morris water maze. * Denotes significant effects of the VS diet treatment ($p<.05$). ∞ Denotes the significant effects of the VS diet with prenatal stress ($p<.05$). Bars indicate mean ± SEM.

Morris water maze probe

The results showed that all groups spent a similar amount of time in the correct quadrant (see Fig. 95).

Univariate analysis for the probe test in quadrant one of the water maze did not find any main effects of stress ($F(1,114)= 0.133$, $p= 0.716$), of treatment ($F(1,114)= 1.804$, $p= 0.182$), or of sex ($F(1,114)= 0.255$, $p= 0.637$). There were also no significant interactions between groups: Stress X Treatment ($F(1,114)= 0.928$, $p= 0.337$), Stress X Sex ($F(1,114)= 0.574$, $p= 0.450$), Treatment X Sex ($F(1,114)= 2.034$, $p= 0.157$) or for Stress X Treatment X Sex ($F(1,114)= 0.420$, $p= 0.518$).

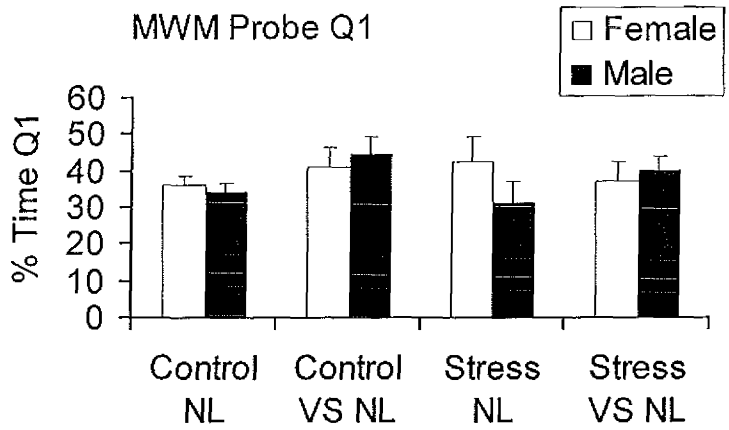


Figure 95. Sums of mean percent time animals spent swimming in the correct quadrant. Bars indicate mean ± SEM.

Tray Reaching

The results indicate that neither treatment of prenatal stress, or diet supplementation influenced skilled reaching results (see Fig. 96).

Univariate analysis for reaching success did not find any significant differences between groups for a main effect of stress ($F(1,102)= 0.017$, $P= 0.898$), of treatment ($F(1,102)= 0.992$, $p= 0.322$), or of sex ($F(1,102)= 0.104$, $p= 0.748$). There were also no significant interactions between groups: Stress X Treatment ($F(1,102)= 0.069$, $p= 0.793$), Stress X Sex ($F(1,102)= 2.027$, $p= 0.158$), Treatment X Sex ($F(1,102)= 1.072$, $p= 0.303$), or for Stress X Treatment X Sex ($F(1,102)= 0.035$, $p= 0.851$).

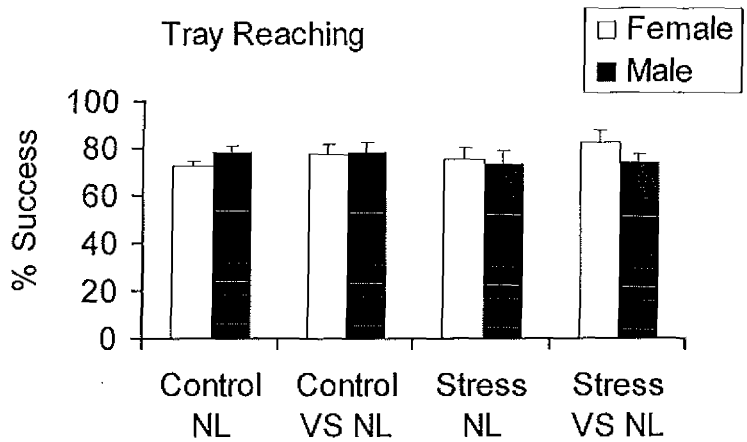


Figure 96. Sums of percent success skilled reaching among treatment groups. Bars indicate mean ± SEM.

Elevated Plus Maze

There were no significant differences in time on the open arms among groups (see Fig. 97).

Univariate analysis for emotionality among groups did not find any main effects of stress ($F(1,113)= 0.164$, $p= 0.686$), of treatment ($F(1,113)= 0.795$, $p= 0.375$), or of sex ($F(1,113)= 1.298$, $p= 0.257$). There were also no significant interactions between groups: Stress X Treatment ($F(1,113)= 0.883$, $p= 0.349$), Stress X Sex ($F(1,113)= 0.320$, $p= 0.573$), Treatment X Sex ($F(1,113)= 0.215$, $p= 0.815$), or for Stress X Treatment X Sex ($F(1,113)= 0.055$, $p= 0.815$).

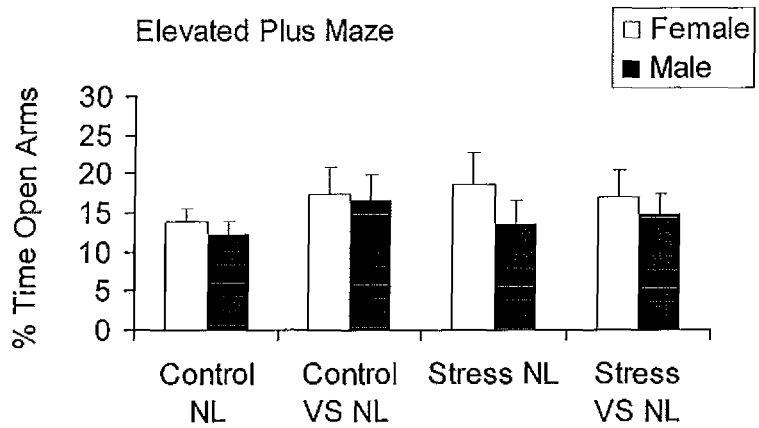


Figure 97. Mean percent amount of time spent exploring the open arms of the elevated plus maze. Bars indicate mean ± SEM.

Anatomical Results

Golgi-Cox brain and adrenal gland measures

Brain Weight

The addition of the supplemented diet to prenatally stressed animals did not make any difference in brain weights (see Table 23).

A univariate analysis for brain weight among treatment groups indicated a main effect of sex ($F(1,70)=25.370$, $p<.0001$), but not of stress ($F(1,70)= 0.402$, $p= 0.528$), or of treatment ($F(1,70)= 0.424$, $p= 0.518$). There are also no significant interactions between groups: Stress X Treatment ($F(1,70)= 0.431$, $p= 0.514$), Stress X Sex ($F(1,70)= 1.012$, $p= 0.318$), Treatment X Sex ($F(1,70)= 1.446$, $p= 0.234$), or for Stress X Treatment X Sex ($F(1,70)= 0.977$, $p= 0.327$).

Table 23. Mean brain weight from Golgi-Cox prepared brains.

Group	Female (g)	Male (g)
	No Stress	
Control NL	1.934 ± .024 (n= 21)	2.140 ± .025 (n= 19)*
Control VS NL	1.963 ± .062 (n= 3)	2.024 ± .054 (n= 4) *
	Prenatal Stress	
Stress NL	1.932 ± .034 (n= 10)	2.140 ± .041 (n= 7)*
Stress VS NL	1.940 ± .054 (n= 4)	2.133 ± .062 (n= 3)*

Brain weights are measured in grams mean ± SEM. * Denotes statistically significant between sexes ($p<.05$).

Dendritic Basilar and Apical Arbor and Length

Cingulate cortex (Cg3)

Basilar fields

All animals among groups had similar amounts of dendritic arborization in the Cg3 area (see Fig. 98).

Univariate analysis for dendritic branch arbor in the cingulate cortex did not indicate any significant findings with the addition of the supplemented diet to some of the animals. There were no main effects of stress ($F(1,34)= 1.378$, $p= 0.251$), of treatment ($F(1,34)= 0.417$, $p= 0.524$), or of sex ($1,34)= 1.980$, $p= 0.171$). There were also no significant interactions between groups: Stress X Treatment ($F(1,34)= 0.632$, $p= 0.434$),

Stress X Sex (F(1,34)= 0.430, p= 0.517), Treatment X Sex (F(1,34)= 0.626, p= 0.436), or for Stress X Treatment X Sex (F(1,34)= 2.153, p= 0.154).

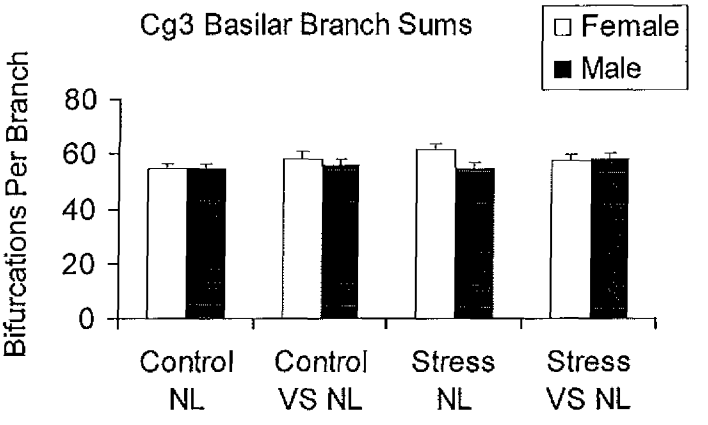


Figure 98. Sums of mean branch arbor of the Cg3 area in prenatally stressed and/or supplemented diet animals. Bars indicate mean ± SEM.

Dendritic length in the basilar area of Cg3 cells were longer in the supplemented diet treated animals (see Fig. 99).

Univariate analysis of basilar length found a main effect of treatment (F(1,39)= 7.130, p= 0.012) and a marginally significant interaction between Treatment X Sex (F(1,39)= 3.911, p= 0.057). There were no main effects of stress (F(1,39)= 0.797, p= 0.379), or of sex (F(1,39)= 0.008, p= 0.931), or any other significant interactions between groups: Stress X Treatment (F(1,39)= 0.032, p= 0.859), Stress X Sex (F(1,39)= 0.214, p= 0.647), or for Stress X Treatment X Sex (F(1,39)= 0.757, p= 0.391)

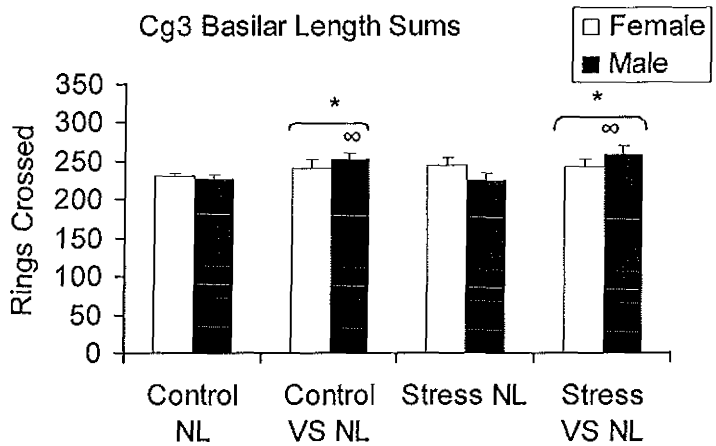


Figure 99. Sums of mean dendritic length in the basilar region of pyramidal cells of Cg3. * Denotes the significant effects of the supplemented diet ($p<.05$). ∞ Denotes the significant effects of diet between the sexes ($p= .057$). Bars indicate mean ± SEM.

Apical fields

There were no differences among groups in apical branch measures of Cg3 (see Fig 100).

Univariate analysis for dendritic apical branch arbor in Cg3 did not indicate any main effects of stress ($F(1,29)= 0.144$, $p= 0.708$), of treatment ($F(1,29)= 0.420$, $p= 0.524$), or of sex ($F(1,29)= 0.797$, $p= 0.382$). There were no interactions between groups: Stress X Treatment ($F(1,29)= 0.023$, $p= 0.880$), Stress X Sex ($F(1,29)= 0.393$, $p= 0.537$), Treatment X Sex ($F(1,29)= 1.722$, $p= 0.203$), or for Stress X Treatment X Sex ($F(1,29)= 0.010$, $p= 0.923$).

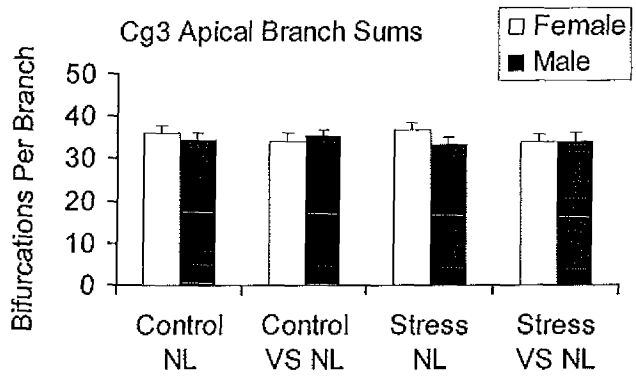


Figure 100. Sums of mean branch dendritic arbor of the Cg3 area in prenatally stressed and/or supplemented diet animals. Bars indicate mean ± SEM.

There were no significant differences among groups in apical branch arbor or in dendritic length (see Fig. 101).

A univariate analysis did not find any main effects of stress ($F(1,29)= 0.260$, $p= 0.615$), of treatment ($F(1,29)= 0.091$, $p= 0.766$), or of sex ($F(1,29)= 0.004$, $p= 0.952$). There were also no significant interactions between groups: Stress X Treatment ($F(1,29)= 0.000$, $p= 0.999$), Stress X Sex ($F(0.920$, $p= 0.348$), Treatment X Sex ($F(1,29)= 0.901$, $p= 0.353$), or for Stress X Treatment X Sex ($F(1,29)= 0.087$, $p= 0.771$).

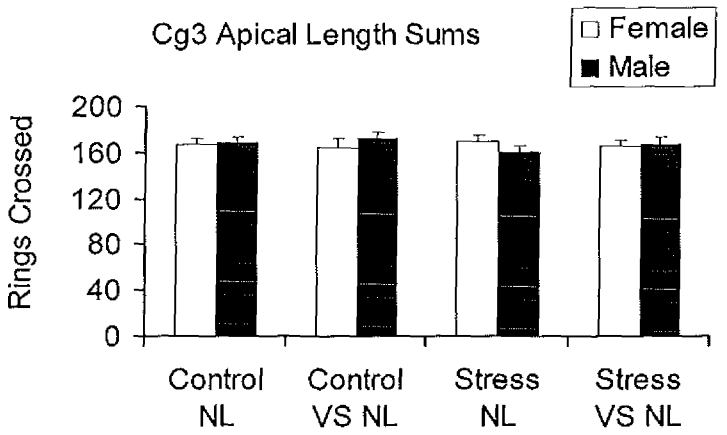


Figure 101. Sums of mean apical dendritic length Cg3 in prenatally stressed animals and supplemented diet animals. Bars indicate mean ± SEM.

Agranular Insular Dorsal (AID) cortex

Basilar fields

The supplemented diet reversed the effects of stress on dendritic arbor in AID returning their developmental pattern toward control values (see Fig. 102).

Univariate analysis for the AID basilar branch arbor found a significant three-way interaction between Stress X Treatment X Sex ($F(1,40)= 12.641$, $p= 0.001$). There were no main effects of stress ($F(1,40)= 1.700$, $p= 0.201$), of treatment ($F(1,40)= 0.335$, $p= 0.566$), or of sex ($F(1,40)= 0.006$, $p= 0.940$). There were also no further significant interactions between groups: Stress X Treatment ($F(1,40)= 0.054$, $p= 0.818$), Stress X Sex ($F(1,40)= 3.133$, $p= 0.086$), or for Treatment X Sex ($F(1,40)= 2.396$, p 0.131).

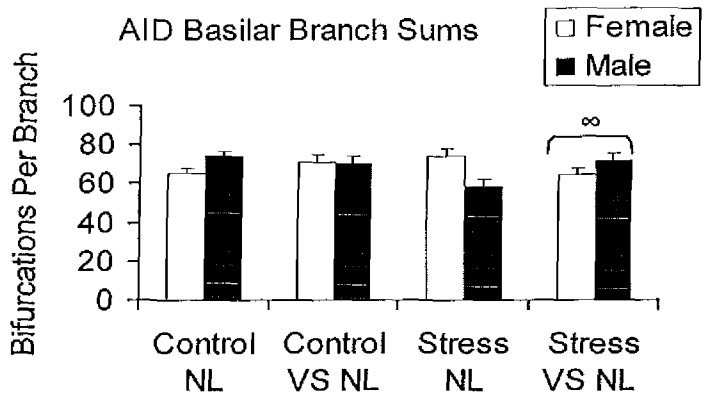


Figure 102. Sums of mean basilar dendritic branch arbor of the AID region in prenatally stressed and/or supplemented diet treated animals. $^{\infty}$ Denotes significantly different effects of stress with diet between the sexes ($p<.05$). Bars indicate mean ± SEM.

Basilar fields

Prenatal stress increased dendritic length in females, while decreasing it in males, reversing the normal growth patterns. The supplemented diet restored dendritic length towards normal in stressed females and increased length in stressed males (see Fig. 103).

Univariate analysis for the AID basilar branch arbor found a significant three-way interaction between Stress X Treatment X Sex ($F(1,40)= 5.296$, $p= 0.028$). There were no main effects of stress ($F(1,40)= 0.032$, $p= 0.859$), of treatment ($F(1,40)= 0.119$, $p= 0.732$), or of sex ($F(1,40)= 0.026$, $p= 0.872$). There were also no other significant interactions between groups: Stress X Treatment ($F(1,40)= 0.221$, $p= 0.642$), Stress X Sex ($F(1,40)= 1,440$, $p= 0.239$), or for Treatment X Sex ($F(1,40)= 2,733$, $p= 0.108$).

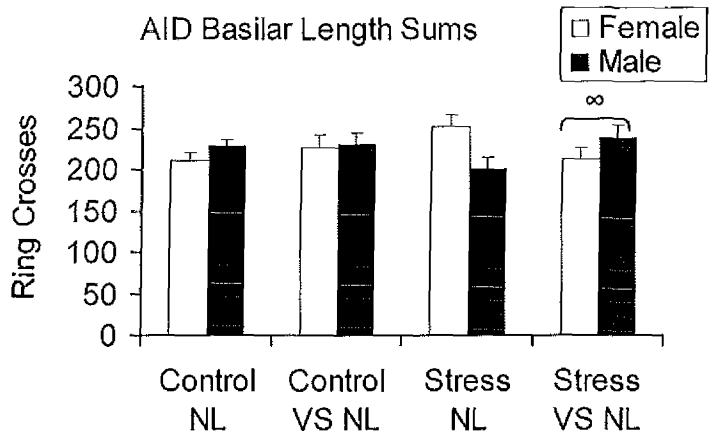


Figure 103. Sums of mean dendritic length in the apical area of pyramidal cells in AID. $^{\infty}$ Denotes the significant effects of prenatal stress with the supplemented diet treatment between the sexes ($p<.05$). Bars indicate mean ± SEM.

Apical fields

There were no significant differences in branch density in AID (see Fig. 104).

Univariate analysis for dendritic apical branch arbor in AID did not find any main effects of stress ($F(1,29)= 0.016$, $p= 0.902$), of treatment ($F(1,29)= 0.946$, $p= 0.341$), or of sex ($F(1,29)= 1.404$, $p= 0.249$). There are also no significant interactions among groups: Stress X Treatment ($F(1,29)= 0.005$, $p= 0.946$), Stress X Sex ($F(1,29)= 0.288$, $p= 0.597$), Treatment X Sex ($F(1,29)= 1.423$, $p= 0.246$), or for Stress X Treatment X Sex ($F(1,29)= 1.929$, $p= 0.179$).

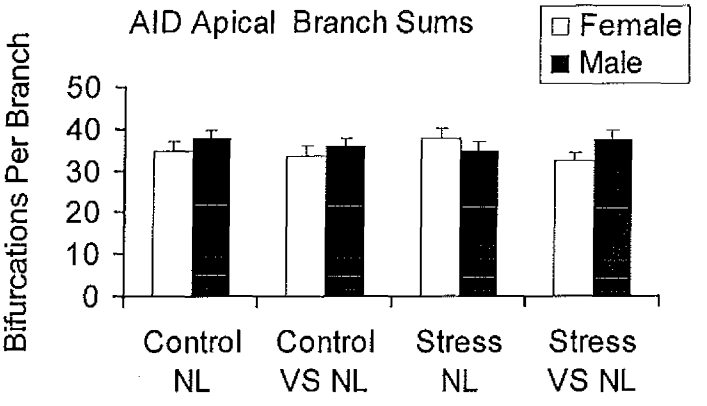


Figure 104. Sums of mean dendritic apical branch arbor in AID region in prenatally stressed and/or supplemented diet treated animals. Bars indicate mean ± SEM.

The supplemented diet reversed the increase in dendritic length shown in prenatally stressed in females shown toward control values (see Fig. 105).

A univariate analysis for dendritic length in the apical area of AID cells found a main effect of VS treatment ($F(1,29)= 4.427$, $p= 0.047$), and a significant interaction between Treatment X Sex ($F(1,29)= 4.588$, $p= 0.044$), but no main effects of stress ($F(1,29)= 1.797$, $p= 0.194$), or of sex ($F(1,29)= 3.827$, $p= 0.063$). There were no other significant interactions between groups: Stress X Treatment ($F(1,29)= 0.298$, $p= 0.590$), Stress X Sex ($F(1,29)= 1.009$, $p= 0.326$), or for Stress X Treatment X Sex ($F(1,29)= 2.502$, $p= 0.128$).

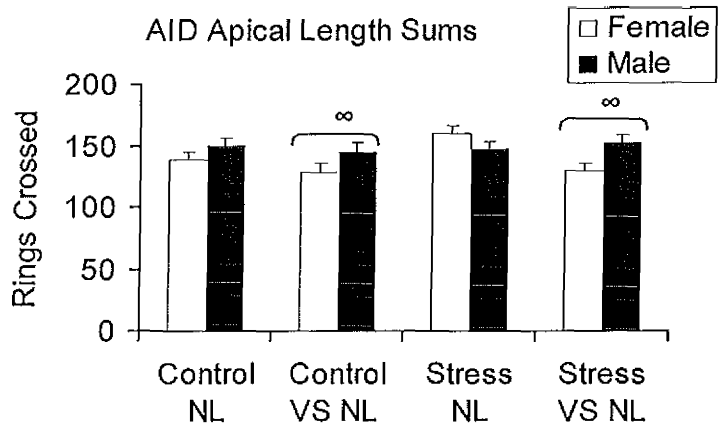


Figure 105. Sums of mean dendritic length in the apical area of cells in AID. $^{\infty}$ Denotes statistically different effects of the supplemented diet between the sexes ($p<.05$). Bars indicate mean ± SEM.

Adrenal gland weight

The females had heavier gland weights than males and the supplemented diet groups decreased adrenal weight in males (see Table 24).

Univariate analysis for adrenal weight indicated a main effect of sex ($F(1,60)= 19.280$, $p< 0.0001$), and a significant interaction of Treatment X Sex ($F(1,60)= 4.905$, $p= 0.031$). There were no main effects of stress ($F(1,60)= 0.175$, $p= 0.678$), or of treatment ($F(1,60)= 2.026$, $p= 0.160$). There were also no other significant interactions between variables: Stress X Treatment ($F(1,60)= 0.588$, $p= 0.446$), Stress X Sex ($F(0.303$, $p= 0.585$), or for Stress X Treatment X Sex ($F(1,60)= 0.197$, $p= 0.659$).

Table 24. Mean adrenal gland weight from Golgi-Cox prepared glands.

Group	Female (g)	Male (g)
	No Stress	
Control NL	0.081 ± .003 (n= 21)	0.069 ± .004 (n= 18)*
Control VS NL	0.083 ± .009 (n= 3)	0.044 ± .008 (n= 4)*∞
	Prenatal Stress	
Stress NL	0.074 ± .008 (n=4)	0.063 ± .008 (n=4)
Stress VS NL	0.080 ± .008 (n= 4)	0.051 ± .009 (n= 3)* ∞

Adrenal gland weights are measured in grams mean ± SEM. * Denotes statistically different between the sexes ($p<.05$). ∞ Denotes the effect of the diet treatment between the sexes ($p<.05$).

Adrenal gland areal measures

Cortex

Prenatally stressed animals treated with the supplemented diet had the thinnest adrenal cortex (see Fig. 106).

Univariate analysis for areal measures of adrenal gland medulla found a main effect of treatment ($F(1,26)= 8.468$, $p= 0.009$), but not of stress ($F(1,26)= 1.222$, $p= 0.283$), or of sex ($F(1,26)= 0.000$, $p= 0.998$). There were no significant interactions between groups: Stress X Treatment ($F(1,26)= 0.266$, $p= 0.612$), Stress X Sex ($F(1,26)= 0.002$, $p= 0.962$), Treatment X Sex ($F(1,26)= 0.026$, $p= 0.873$), or for Stress X Treatment X Sex ($F(1,26)= 0.038$, $p= 0.848$).

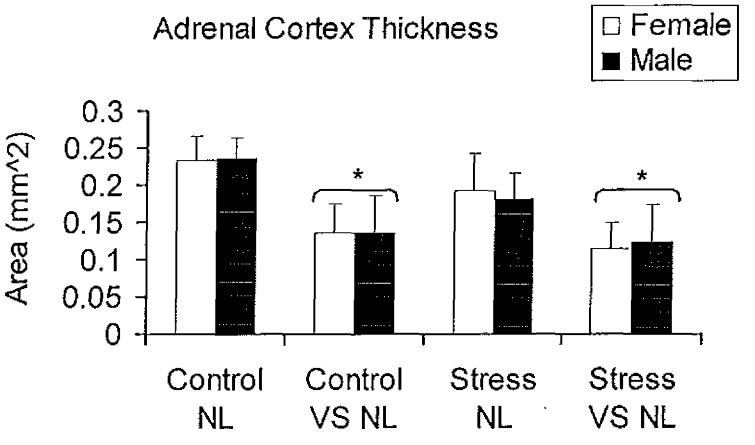


Figure 106. Sums of mean areal measures of adrenal gland cortex in animals treated with prenatal stress, supplemented diet, and combination. * Denotes statistically different effects of the supplemented diet ($p<.05$). Bars indicate mean ± SEM.

Medulla

Overall, the supplemented diet animals had smaller medulla areas measured (see Fig. 107).

Univariate analysis for areal measures of adrenal gland medulla found a main effect of treatment ($F(1,26)= 4.421$, $p= 0.049$), but not of stress ($F(1,26)= 1.102$, $p= 0.307$), or of sex ($F(1,26)= 1.283$, $p= 0.272$). There were no significant interactions between groups: Stress X Treatment ($F(1,26)= 0.007$, $p= 0.936$), Stress X Sex ($F(1,26)= 0.184$, $p= 0.673$), Treatment X Sex ($F(1,26)= 0.277$, $p= 0.605$), or for Stress X Treatment X Sex ($F(1,26)= 0.408$, $p= 0.531$).

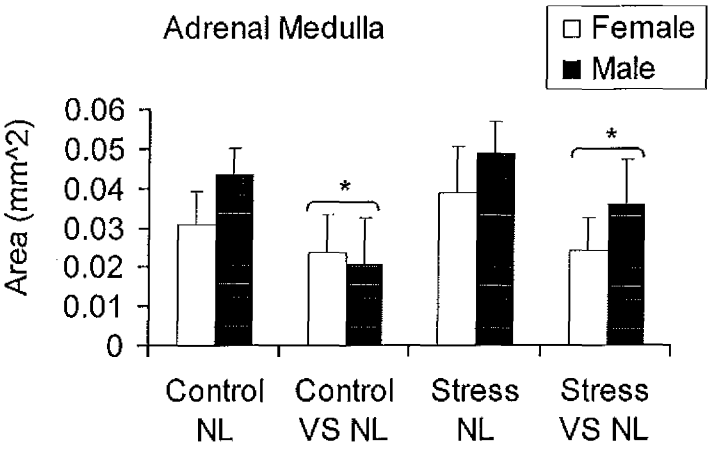


Figure 107. Sums of mean areal measures of the adrenal medulla in animals treated with prenatal stress, supplemented diet, or both. * Denotes the significant effects of the diet treatment ($p<.05$). Bars indicate mean ± SEM.

Mild prenatal stress and the supplemented diet (VS) resulted in thinner adrenal cortexes in females. The medulla area was larger in prenatally stressed animals than in the supplemented diet animals (see Fig. 108). Overall, the adrenal glands in diet supplemented animals were smaller.

Figure 108. Examples of adrenal glands in representative females of each group. Left: untreated control; second from left, mild prenatal stress; second from right, non-stressed VS diet; and right, Stress VS diet combination.

Testes weight

Testes weights did not vary significantly among treatment groups (see Fig. 109).

Univariate analysis for testes weight did not find any main effects of stress ($F(1,14)= 3.173$, $p= 0.102$), or of treatment ($F(1,14)=1.860$, $p=0.200$), or for the interaction ($F(1,14)= 0.349$, $p= 0.566$).

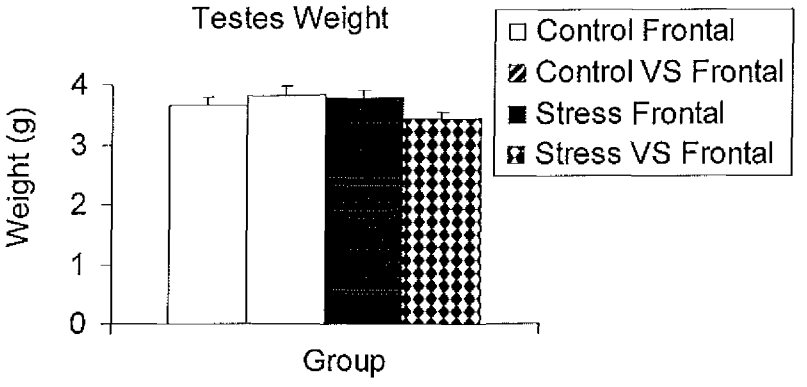


Figure 109. Mean testes weights in prenatally stressed males treated with the supplemented diet. Bars indicate mean ± SEM.

Formalin fixed brain measures

Various measures were made with the formalin-fixed brains with the means demonstrated in Table 25. The data displayed only pertains to the addition of the vitamin/mineral supplemented diet (see Table 21 for previous data).

Prefrontal cortex prelimbic and infralimbic thickness

Prenatal stress resulted in larger thickness measures in both the infralimbic and prelimbic prefrontal regions and in addition to stressed animals reared on the

supplemented diet. Non-stressed animals reared on the supplemented diet had smaller prefrontal cortex measures (see Table 25).

Univariate analysis for *prelimbic cortex* thickness indicated a main effect of stress ($F(1,55)= 3.923$, $p= 0.053$), and of treatment ($F(1,55)= 5.113$, $p= 0.028$), but not of sex ($F(1,55)= 0.306$, $p= 0.583$). There were no significant interactions between groups: Stress X Treatment ($F(1,55)= 0.343$, $p= 0.561$), Stress X Sex ($F(1,55)= 0.580$, $p= 0.450$), Treatment X Sex ($F(1,55)= 0.069$, $p= 0.794$), or for ($F(1,55)= 0.131$, $p= 0.719$).

Univariate analysis for *infralimbic cortex* thickness found main effects of stress ($F(1,55)= 13.413$, $p= 0.001$) and of treatment ($F(1,55)= 6.031$, $p= 0.018$), but not of sex ($F(1,55)= 0.001$, $p= 0.972$). There were no significant interactions between groups: Stress X Treatment ($F(1,55)= 0.006$, $p= 0.941$), Stress X Sex ($F(1,55)= 0.588$, $p= 0.447$), Treatment X Sex ($F(1,55)= 0.107$, $p= 0.745$), or for Stress X Treatment X Sex ($F(1,55)= 0.057$, $p= 0.813$).

Thalamus

The animals treated with the supplemented diet had smaller thalamic measures. The anterior thalamic region was very small in the prenatally stressed animals, which was partially reversed in the Stress VS NL animals (see Table 25).

Univariate analysis for anterior thalamic measures indicated a main effect of stress ($F(1,55)= 15.354$, $p<.0001$), and a marginally significant effect of sex ($F(1,55)= 3.740$, $p= 0.059$), There was also a significant interaction of Stress X Treatment ($F(1,55)= 25.861$, $p< 0.0001$). There were no main effects of treatment ($F(1,55)= 2.986$, $p= 0.090$) of for interactions Stress X Sex ($F(1,55)= 0.204$, $p= 0.654$), Treatment X Sex

(F(1,55)= 2.247, p= 0.140), or for Stress X Treatment X Sex (F(1,55)= 1.456, p= 0.234).

Univariate analysis for posterior measures also indicated a main effect of treatment (F(1,55)= 15.936, p< 0.0001) and a marginally significant effect of stress (F(1,55)= 3.751, p= 0.059), but not of sex (F(1,55)= 1.122, p= 0.295). There were no significant interactions between groups: Stress X Treatment (F(1,55)= 0.076, p= 0.784), Stress X Sex (F(1,55)= 0.143, p= 0.707), Treatment X Sex (F(1,55)= 0.042, p= 0.838), or for Stress X Treatment X Sex (F(1,55)= 0.049, p= 0.825).

Table 25. Mean measures from formalin-fixed brains.

Area Measured	**Females**	**N**	**Males**	**N**
Non-stressed Control + Vitamin Supplemented Diet				
[1]Prefrontal Cortex:				
Infralimbic	0.527 ± .042	4	0.549 ± .038	5
Prelimbic	0.969 ± .062	4	1.011 ± .055	5
[2]Thalamic Width: anterior	0.824 ± .016	4	0.835 ± .015	5
posterior	0.897 ± .017	4	0.911 ± .015∞	5
Prenatal Stress + Vitamin Supplemented Diet				
[1]Prefrontal Cortex:				
Infralimbic	0.630 ± .038	5	0.626 ± .030∞+	8
Prelimbic	1.088 ± .055	5	1.073 ± .044∞+	8
[2]Thalamic Width: anterior	0.842 ± .015	5	0.839 ± .012∞+	8
posterior	0.920 ± .015	5	0.931 ± .012	8

1. Prefrontal cortex: prelimbic and infralimbic regions are measured in cm. sums ± SEM. ∞ Denotes significant effects of the VS diet with and without stress ($p<.05$). + Denotes the significant effects of stress ($p<.05$).

2. Thalamic measures for both regions are measured in cm. sums ± SEM. ∞ Denotes significant effects of the VS diet with stress ($p<.05$) + Denotes the significant effects of stress ($p<.05$).

Cortical thickness

Prenatal stress and the diet treatment resulted in thinner cortices (see Fig. 110).

Univariate analysis for cortical thickness measures found a main effects of stress ($F(1,69)= 9.897$, $p= 0.003$), of treatment (($F(3,69)= 79.955$, $p< .0001$) and of sex ($F(1,69)= 23.413$, $p< .0001$). There were also significant interactions of Stress X Treatment ($F(1,69)= 22.072$, $p< .0001$), Stress X Sex ($F(1,69)= 9.322$, $p= 0.003$), and for Stress X Treatment X Sex ($F(1,69)= 3.778$, $p= 0.056$). There was no significant interaction of Treatment X Sex ($F(1,69)= 1.461$, $p= 0.231$).

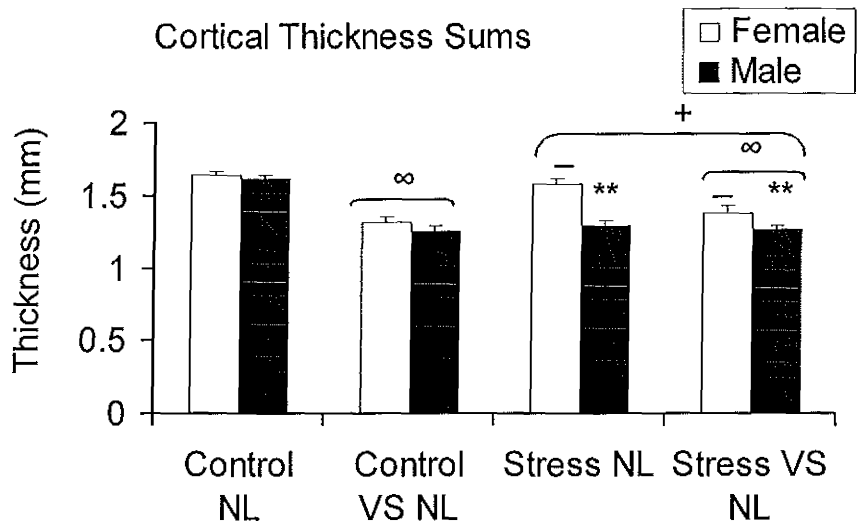


Figure 110. Sums of mean cortical thickness measures in prenatally stressed, supplemented diet, and prenatally stressed plus supplemented diet animals. + Denotes significant differences from non-stressed control animals ($p<.05$). ** Denotes statistically different between sexes ($p<.05$). ∞ Denotes significant effects of the VS diet between the sexes ($p<.05$). ♦ Denotes the significant effects of stress in females ($p<.05$). Bars indicate mean ± SEM.

The effects of a micronutrient supplemented diet on mild prenatal stress and brain and behavioural development

Summary

Table 26. Significant findings for experiment 3b.

Behavioural Task	Females	Males
Activity: Distance	Sex: Females more active	Sex: Males less active
Number of Mvmnts	Diet reversed stress effects	No differences among groups
Morris Water Maze:	Diet and stress/diet combo decreased latency	Diet and stress/diet combo decreased latency
	Anatomy (Golgi-Cox and saline-perfused preparation)	
Brain Weight:	Sex: Females had lighter weights	Sex: Males had heavier weights
Dendritic Arbor and Length:	Diet reversed the stress effects toward control in AID, diet increased length in Cg3	Diet reversed the stress effects toward control in AID, diet increased length in Cg3
Adrenal Gland Weight:	Sex: Females had heavier weights with VS diet	Sex: Males had lighter weights with VS diet
Adrenal Cortex Thickness:	Decreased with diet and combination diet with stress	Decreased with diet and combination diet with stress

Anatomy (formalin preparation)		
Anterior Thalamus:	Smallest with diet, increased with stress	Smallest with diet, increased with stress
Posterior Thalamus:	Smallest with diet, increased with stress	Smallest with diet, increased with stress
Cortical Thickness:	Diet decreased cortical thickness	Diet decreased cortical thickness

The summaries in tables 22 and 26 are displayed as itemized lists of what was statistically significant in each of the previous experiments. The summary to follow is a general overview of the three experiments with frontal lesions that will be discussed in the discussion.

Discussion

The main behavioural and anatomical findings for animals exposed to mild prenatal stress were: 1) the effects of stress in behavioural and anatomical measures affected the sexes differently; and, 2) the supplemented diet ameliorated some of the effects of stress.

Behavioural measures

Exploratory activity in the activity box and elevated plus maze

The current experimental stress paradigm has shown a much greater increase in behavioural activity, with an emphasis in females. This was observed in measures of locomotor activity and exploration in the elevated plus maze (compared to stressed animals).

An increase in activity in prenatally stressed animals has previously been attributed to changes in dopaminergic influences in locomotion by Son (2007) in addition to the responsiveness of the HPA axis to stress; a response that has been measured to be higher in females and lower in males (McCormick, 1998). Estrogen has been found to have excitatory effects on the HPA axis, whereas androgens are inhibitory with differences in measures along the HPA axis checkpoints: CRH from the PVN and ACTH from the pituitary (McCormick, 1998).

Alterations in catecholamine metabolism have been measured in the offspring of gestationally stressed dams with the potential to target specific brain regions affecting the mesolimbic and nigrostriatal systems (Son, et al, 2007). Hyper- or hypodopaminergia has

been proposed responsible for the alterations in motor activity that corresponds with increased extracellular DA in dopamine transporter knockout mice. This behaviour of hyperactivity was completely reversed by the blockade of dopamine receptors, or by the inhibition of tyrosine hydroxylase; the enzyme required for conversion to dopamine (Son, et al, 2007).

In addition to increased locomotor activity, both sexes experienced an increased propensity to explore the open arms of the elevated plus maze. This display of anxiolytic behaviour to novel environments suggests positive effects of mild prenatal stress.

Morris water maze task

Prenatal stress significantly facilitated place learning in the water maze task in both sexes. Memory retention has been reported to be enhanced in rats exposed to a paradigm of chronic variable stress (CVS) during gestation (Mueller, and Bale, 2007). A modified version of the Barnes circular maze was used to test prenatally stressed mice in order to ascertain their coping strategies immediately following exposure to fox odor. Prenatally stressed females were much faster than control females to solve the task, and when assessed for memory retention six weeks following maze exposure, stressed females again were much quicker than control animals at maze problem solving (Mueller, and Bale, 2007).

Fujioka and colleagues (2001) found mild prenatal stress to enhance spatial learning in the eight-arm radial arm maze and enhanced active avoidance requiring alert responses. Plasma corticosterone levels did not differ from control animals during active avoidance and *Fos* expression for neural activity was actually lower in prenatally stressed

animals in hypothalamic PVN, the amygdala, and in the hippocampus (Fujioka, et al, 2001).

Brain and adrenal anatomical measurements

Brain measures

The effects of mild prenatal stress from Golgi-prepared brains of the current study resulted in some very interesting anatomical differences between males and females in different areas of the brain. Although the brain weights did not differ from non-stressed control animals, females showed increased basilar dendritic arbor and dendritic length in AID with the apical arbor and length showing similar growth patters. Conversely, the males showed decreased dendritic measures in both basilar and apical areas of pyramidal cells in AID.

A study comparing mild prenatal stress to more severe chronic prenatal stress found that mild and short-lasting prenatal stress increased dendritic length in the hippocampus with a higher number of BrdU-ir (labeled for proliferation) cells and NeuN-colabeled (proliferating neurons) cells (Fujioka, Fujioka, Ishida, Maekawa, and Nakamura, 2006). Because the prefrontal cortex also contains many glucocorticoid receptors, the results in the cortex in the current study might reflect that seen in the hippocampal dendritic fields of CA3, having reciprocal connections with the ventral prefrontal areas.

Increased thickness in the infralimbic and prelimbic cortical areas in prenatally stressed animals could have contributed to behavioural flexibility in social and cognitive measures. Recall, the infralimbic and prelimbic cortex areas have been proposed to be involved in behavioural flexibility and attentional processes (Delatour, and Gisquet-Verrier, 2000; Birrell and Brown, 2000).

Males were also predominantly affected from prenatal stress from decreased cortical thickness measures, whereas females were very close to non-stressed control values. This measure does not however correspond with the increased brain weights in the stressed males. Cells in the cerebral cortex might be denser in some brain regions.

Adrenal measures

Contrary to what was found with the more intense stress paradigm, the adrenal gland weights and adrenal cortex thickness with mild stress were actually lower than the untreated control animals suggesting less exposure to ACTH and thus, lower secretions of glucocorticoids. This phenomenon suggests that animals exposed to mild prenatal stress might have experienced less stress during their behavioural tasks, as this was also suggested from the results of 24-hr. isolation prior to social play.

The vitamin/mineral supplemented diet

Behavioural measures

The diet treatment in non-stressed and stressed animals restored the high levels of locomotor activity in females and increased exploration, although not significant, in both sexes on the elevated plus maze. Whether this is from the suppression of dopamine (DA)

or from an anxiogenic response to novelty is uncertain, although this response was not revealed in the plus maze. Clinical trials using the vitamin supplement for treatment intervention for adults diagnosed with ADHD and severe mood dysregulation have reported moderations in hyperactivity and improved mood; the precise mechanisms however have not been described.

The vitamin-supplemented diet examined in the current experiment also enhanced performance in the Morris water maze in both sexes, especially in the non-stressed animals. Previous findings of performance in VS supplemented animals in the Morris water maze have also shown very good navigation abilities (Halliwell, 2004).

Anatomical measures

Animals that were supplemented with the diet and prenatally stressed also had increased basilar dendritic length in Cg3. Dendritic measures in AID were not altered with the addition of the supplemented diet, but the diet did restore growth patterns toward normal control in the prenatally stressed rats.

Brain weights and corresponding anatomical measures in the supplemented diet group were all lower than their control counterparts. This is surprising considering previous findings indicated a thicker cortical mantle in animals supplemented with the diet (Halliwell, 2004). It is possible that the formula mixture had become more refined than previous years (D. Hardy, in discussion, 2006). The company that compressed the supplemented food powder was also different for this set of experiments than for the original set. These factors can affect the absorption and utilization of the vitamins and minerals changing the effects on body and brain. The supplemented diet was introduced

on the day of birth and affected weight gain in postnatal life. Their dendritic profile however is similar to control animals and behavioural measures were favorable. It is possible that the cells are more compact reducing the neuropil space, but because the supplemented diet was introduced during the developmental time frame of cell migration and astrocyte proliferation, it might have altered the rate of the cell cycle, thus delaying the maturation processes of brain development.

The supplemented diet further decreased adrenal weights and adrenal cortex thickness in both sexes suggesting less glucocorticoid secretion. The means for testes weight in the stressed males were increased slightly (4.142g vs. 3.815g), whereas those for the diet treated males did not differ from control values.

The addition of the vitamin supplemented diet enhanced exploration and decreased behaviours of anxiety as measured by the amount of time spent on the open arms. The primary and initial finding in the various clinical trials using the same vitamin and mineral supplement is of improvement in mood and decreased anxiety that accompany various disorders. [Disorders of ADHD, Asperger's, Oppositional Defiant Disorder (ODD), generalized anxiety disorder (GAD), Bipolar Disorder, rage, and Prader-Willi Syndrome (Kaplan, Fischer, Crawford, and Kolb, 2004; Rucklidge and Harrison, 2010; Rucklidge and Whitehead, 2010), in addition to the aforementioned studies that included OCD (Rucklidge, 2009), Autism (Mehl-Madrona, et al, 2010), and Explosive Rage (Kaplan, Fischer, Crawford, Field, and Kolb, 2004)].

VIII. Experiment 4B: The effects of mild prenatal stress on early frontal cortical injury

The following section compares control animals with frontal lesions (Control Frontal) to prenatally stressed animals with frontal lesions (Stress Frontal).

For referral to Experiment 4A (Control Frontal) results see Experiment 2A.

Behavioural Results

Activity

Distance

Females exhibited greater distance explored than males in the activity box. Overall, prenatally stressed animals exhibited higher activity levels than non-stressed control animals (see Fig. 111).

Univariate analysis of distance explored found a main effect of stress ($F(1,31)= 5.154$, $p= 0.031$), and of sex ($F(1,31)= 22.311$, $p< 0.0001$), but not for the interaction between stress and sex ($F(1,31)= .642$, $p= 0.430$).

Number of movements

Prenatally stressed females with frontal lesions exhibited the greatest number of movements in the activity box (see Fig. 111).

Univariate analysis revealed a significant main effect of stress ($F(1,31)= 12.295$, $p= 0.002$), and of sex ($F(1,31)= 9.568$, $p= 0.004$), as well as for the interaction ($F(1,31)= 5.964$, $p= 0.021$).

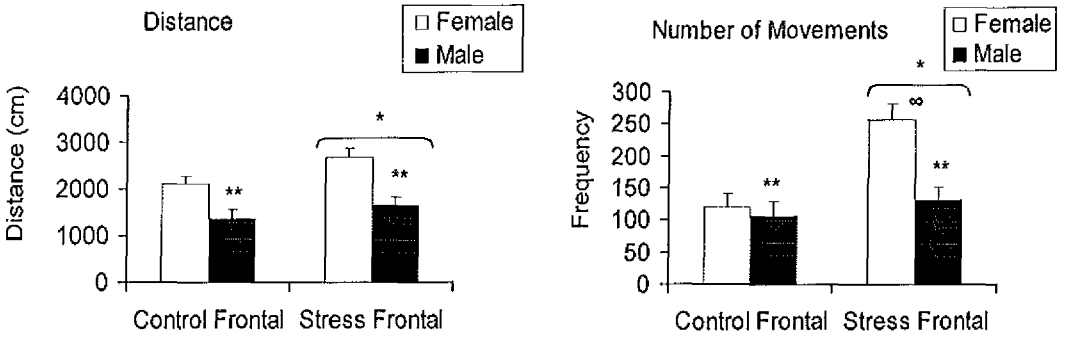


Figure 111. Left: Sums of mean distance traveled, and right: sums of mean number of movements in prenatally stressed animals with frontal lesions. * Denotes the significant effects of prenatal stress ($p<.05$). ** Denotes significantly different between the sexes ($p<.05$). ∞ Denotes the effect of prenatal stress in females ($p<.05$). Bars indicate mean ± SEM.

Morris Water Maze

Latency

Prenatally stressed animals with frontal lesions were faster at finding the hidden platform- particularly males (see Fig. 112).

Univariate analysis for latency sums indicated a main effect of stress ($F(1,40)= 6.519$, $p= 0.015$), and of sex ($F(1,40)= 10.246$, $p= 0.003$), but not for an interaction between stress and sex ($F(1,40)= 0.169$, $p= 0.683$).

A multivariate analysis for day found days' one and two significantly different among the groups (p's<.05) (see Fig. 111).

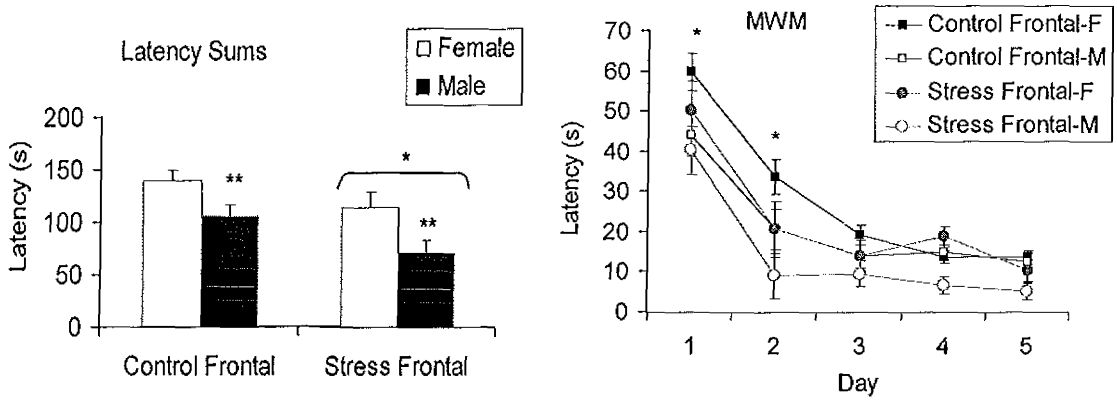


Figure 112. Left: Latency sums for the five-day acquisition trials in the water maze. * Denotes significantly different from the Control Frontal group ($p<.05$). ** Denotes statistically different between sexes ($p<.05$). Right: Mean five-day latencies during learning acquisition. * Denotes statistically different among groups ($p<.05$). F= Females, M= Males. Bars indicate mean ± SEM.

Morris water maze probe

It appeared that prenatally stressed animals spent more time in the correct quadrant, although the difference was not statistically significant (see Fig. 113).

No significant effects were found with univariate analysis for the probe test for prenatal stress ($F(1,31)= 1.764$, $p= 0.195$), or sex ($F(1,31)= 2.799$, $p= 0.105$), or for the interaction between stress and sex ($F(1,31)= 0.072$, $p= 0.791$).

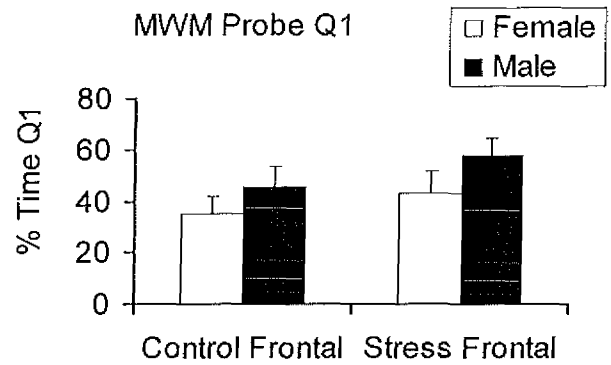


Figure 113. Probe test for Morris water maze in Control Frontal and Stress Frontal groups. Bars indicate mean ± SEM.

Tray Reaching

The results suggest the stress treatment was beneficial for animals and reversed the frontal lesion deficit (see Fig. 114).

Univariate analysis for skilled reaching in frontal lesion animals indicated a main effect of stress ($F(1,28)= 22.620$, $p< .0001$), but not of sex ($F(1,28)= 0.181$, $p= 0.674$), nor for the interaction ($F(1,28)= 0.057$, $p= 0.814$).

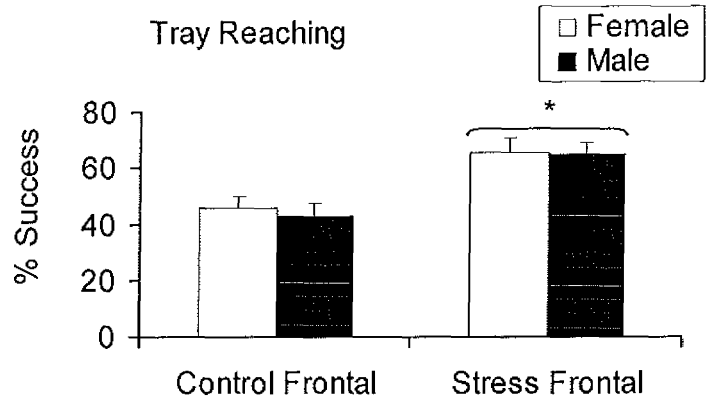


Figure 114. Percent success in skilled reaching in animals with frontal lesions and prenatal stress treatment. * Denotes statistically significant from the Control Frontal group ($p<.05$). Bars indicate mean ± SEM.

Elevated Plus Maze

Prenatally stressed females with frontal lesions spent more time exploring the open arms on the elevated plus maze, whereas males spent less (see Fig. 115).

Univariate analysis for measures of anxiety in the elevated plus maze revealed a significant interaction between Stress and Sex ($F(1,28)= 8.017$, $p= 0.009$), but not for a main effect of stress ($F(1,28)= 0.796$, $p= 0.381$, or of sex ($F(1,28)= 0.016$, $p= 0.900$).

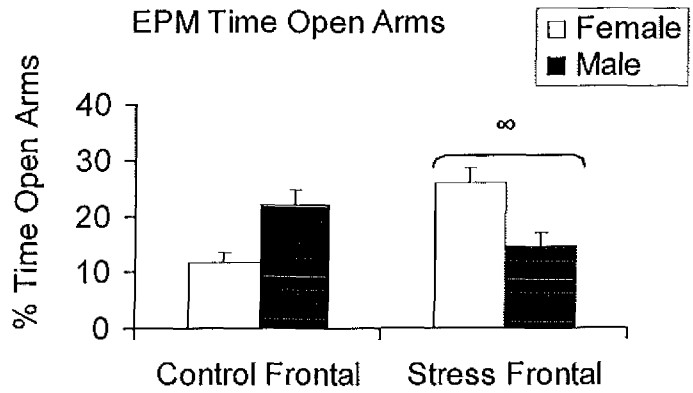


Figure 115. Animals with prenatal stress and frontal lesions in the elevated plus maze. ∞ Denotes the significantly different effect of prenatal stress in animals with frontal lesions ($p<.05$). Bars indicate mean ± SEM.

Anatomical Results

Frontal lesion

Four of twelve brains from prenatally stressed animals with frontal lesions, showed filling in of tissue at the lesion site, some with very small portions of Cg1 and Fr2 missing. Four brains showed one hemisphere filled in with the other having varying amounts of Cg1, Cg3, and Fr2 missing. Three brains had partial filling in one hemisphere with the other hemisphere missing large portions of Cg3, Cg1, and Fr2. One brain showed little filling in with large lesion sites in both hemispheres (see Fig. 116).

Figure 116. Examples of frontal lesions in animals exposed to mild prenatal stress. Left: Example of filled in tissue with morphological alterations. Second from left: One hemisphere filled in at the lesion site with potions of Cg3, Cg1, and Fr2 missing in the other hemisphere. Second from right: Partial filling in one hemisphere and missing most of Cg1 and Fr2 with a hole in the other hemisphere that gradually developed into a very large ventricle. Right: One hemisphere shows very little filling in with the other showing partially filling in of tissue at the lesion site.

Golgi-Cox brain and saline-perfused adrenal gland measures

Brain weight

Males had heavier brain weights than females, which were lighter in animals that were prenatally stressed (see Table 27).

Univariate analysis for brain weight in frontal lesion animals found a marginally significant effect of stress ($F(1,23)= 4.261$, $p= 0.052$), and a main effect of sex ($F(1,23)= 25.745$, $p< 0.0001$), but no significant interaction between groups ($F(1,23)= 1.053$, $p= 0.317$).

Table 27. Mean brain weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control Frontal	1.897 ± .023 (n= 9)	2.018 ± .026 (n= 7)*
	Prenatal Stress	
Stress Frontal	1.805 ± .034 (n= 4)	1.987 ± .034 (n= 4)* ∞

Brains are measured in grams as mean ± SEM. * Denotes statistically significant between sexes ($p<.05$). ∞ Denotes statistically different from Control Frontal animals ($p=.052$).

Dendritic Basilar and Apical Arbor and Length

Cingulate cortex (Cg3)

As in the previous studies, only a subset of frontal animals had identifiable Cg3 containing cells. In addition, the apical fields were clear enough to draw only in a smaller subset.

Basilar fields

Both sexes that were prenatally stressed had less dendritic arbor in the basilar field, with no effects of stress in dendritic length (see Fig. 117).

Univariate analysis of basilar dendritic branch arbor in Cg3 found a main effect of stress ($F(1,20)= 10.729$, $p= 0.004$), but no effects of sex ($F(1,20)= 3.604$, $p= 0.075$), nor for an interaction between groups ($F(1,20)= 0.946$, $p= 0.344$).

There were no differences between groups in dendritic length for basilar Cg3 (see Fig. 117).

Univariate analysis for dendritic length in Cg3 did not find any main effects of stress ($F(1,22)= 0.005$, $p= 0.943$), or of sex ($F(1,22)= 0.320$, $p= 0.578$), nor an interaction ($F(1,22)= 0.009$, $p= 0.926$).

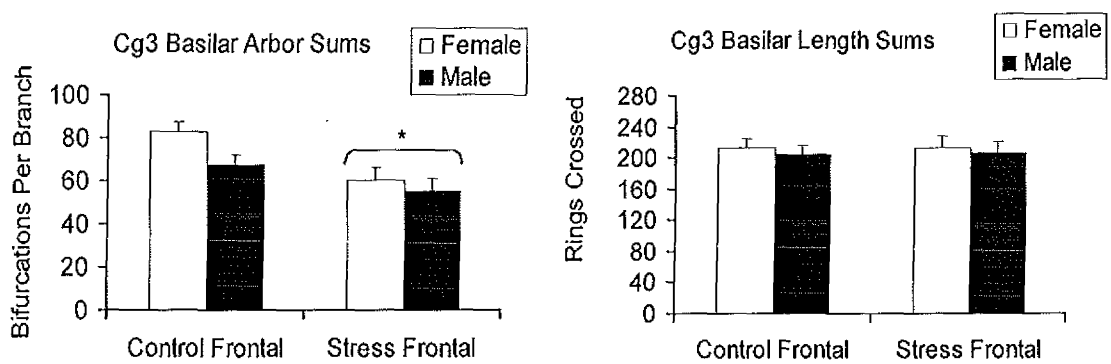


Figure 117. Left: Sums of mean basilar dendritic arbor, and right: Sums of mean dendritic length in Cg3 in prenatally stressed animals with frontal lesions. * Denotes statistically significant from non-stressed animals ($p<.05$). Bars indicate mean ± SEM.

Apical fields

There were no significant effects of stress in apical dendritic measures (see Fig. 118).

Univariate analysis for apical arbor in Cg3 did not indicate any significant effects of stress ($F(1,11)= 3.293$, $p= 0.107$), or of sex ($F(1,11)= 0.483$, $p= 0.507$), or for an interaction ($F(1,11)= 0.024$, $p= 0.880$).

Univariate analysis for dendritic length in Cg3 did not find any significant effects of stress ($F(1,15)= 0.001$, $p= 0.977$), of sex ($F(1,15)= 0.210$, $p= 0.655$), or for an interaction between groups ($F(1,15)= 1.600$, $p= 0.230$).

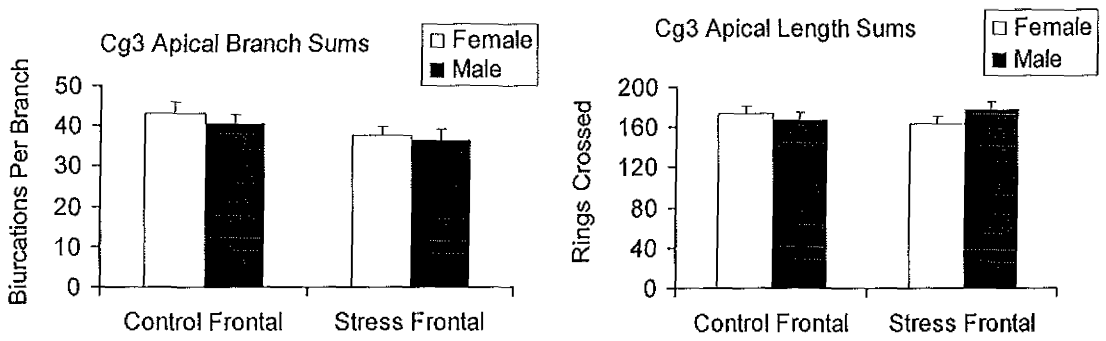


Figure 118. Left: Sums of mean dendritic branch arbor, and right: sums of mean dendritic length in the apical area of Cg3 in animals with prenatal stress and frontal lesions. Bars indicate mean ± SEM.

Agranular Insular Dorsal (AID) cortex

Basilar fields

There were no significant differences between groups in dendritic branching or length (see Fig. 119).

Univariate analysis for basilar dendritic arbor in AID did not indicate any significant differences between groups. There were no main effects of stress ($F(1,23)= 2.790$, $p= 0.110$), or of sex ($F(1,23)= 1.309$, $p= 0.266$), nor for an interaction ($F(1,23)= 0.081$, $p= 0.778$).

Univariate analysis of dendritic length in the basilar area of AID pyramidal cells did not indicate main effects of stress ($F(1,23)= 0.432$, $p= 0.519$), or of sex ($F(1,23)= 0.140$, $p= 0.712$), nor an interaction ($F(1,23)= 0.071$, $p= 0.792$).

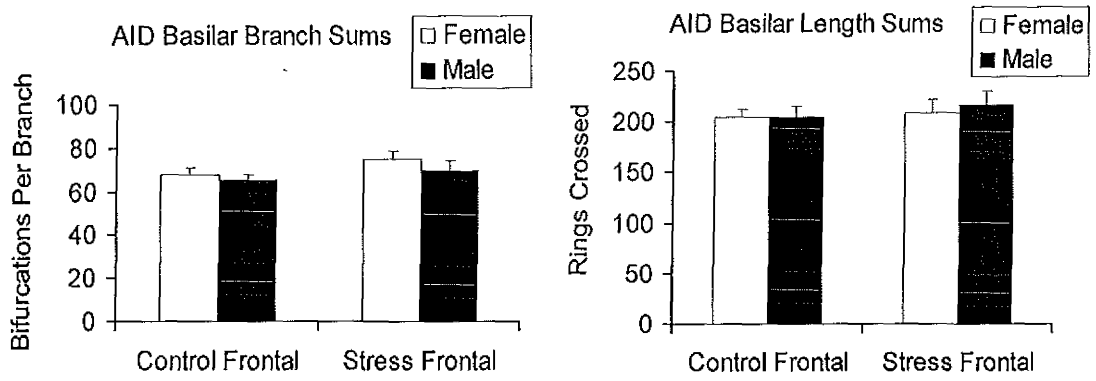


Figure 119. Left: Sums of mean dendritic branch arbor in AID in prenatally stressed animals with frontal lesions. Right: Sums of mean dendritic length in AID. Bars indicate mean ± SEM.

Apical fields

There were no effects of stress in apical branching or length (see Fig. 120).

Univariate analysis for dendritic arbor in apical AID cells did not indicate main effects of stress ($F(1,15)= 0.279$, $p= 0.607$), of sex ($F(1,15)= 3.279$, $p= 0.095$), or for an interaction ($F(1,15)= 1.469$, $p= 0.249$).

There were no effects of stress treatment on apical dendritic length in AID (see Fig. 119). Univariate analysis of dendritic length in apical areas in pyramidal AID cells did not find significant effects of stress ($F(1,15)= 0.788$, $p= 0.392$), of sex ($F(1,15)= 0.834$, $p= 0.379$), or for an interaction between groups ($F(1,15)= 0.010$, $p= 0.921$).

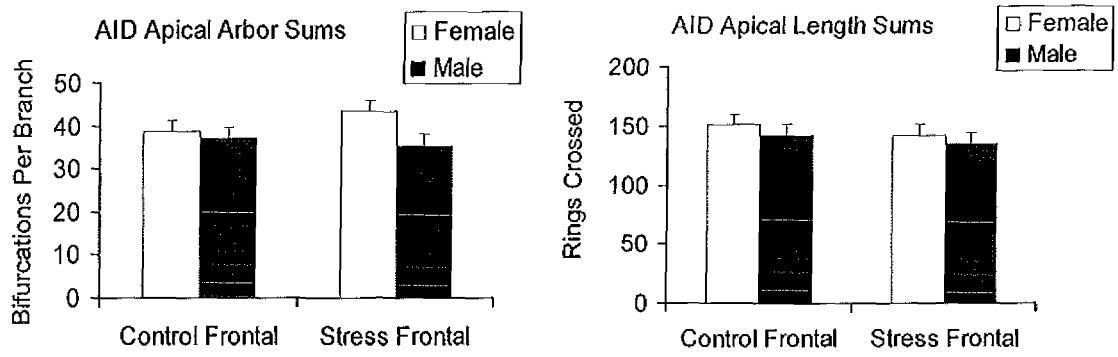


Figure 120. Left: Sums of mean dendritic branch apical arbor in AID in animals with prenatal stress and frontal lesions. Right: Sums of mean dendritic length in AID. Bars indicate mean ± SEM.

Adrenal gland weight

The adrenal weights in female animals were significantly greater than male adrenal gland weights (see Table 28).

Univariate analysis of adrenal gland weight found a main effect of stress ($F(1,23)= 4.677$, $p= 0.043$), and of sex ($F(1,23)= 21.688$, $p< 0.0001$), but not for the interaction ($F(1,23)= 0.317$, $p= 0.580$).

Table 28. Mean adrenal gland weight for Golgi-Cox preparation.

Group	Female (g)	Male (g)
	No Stress	
Control Frontal	0.089 ± .004 (n=9)	0.063 ± .004 (n= 7)*
	Prenatal Stress	
Stress Frontal	0.076 ± .006 (n=4)	0.055 ± .006 (n=4)* ∞

Adrenal glands are measured in grams as mean ± SEM. * Denotes statistically significant between sexes ($p<.05$). ∞ Denotes significantly different from Control Frontal animals ($p<.05$).

Adrenal gland areal measures

Cortex

Prenatal stress increased cortex thickness in females, but decreased thickness in males (see Fig. 121).

Univariate analysis for adrenal cortex found a significant interaction between stress and sex ($F(1,17)= 8.335$, $p= 0.012$), but no main effect of stress ($F(1,17)= 3.561$, $p= 0.080$), or of sex ($F(1,17)= 2.652$, $p= 0.126$).

Medulla

As in the cortex, prenatally stressed females with frontal lesions had an increased medulla area and males a decreased medulla area (see Fig. 121).

Univariate analysis for adrenal medulla found a significant interaction between Stress and Sex ($F(1,17)= 15.676$, $p= 0.001$), but no main effect of stress ($F(0.780$, $p= 0.392$), or of sex ($F(2.809$, $p= 0.116$).

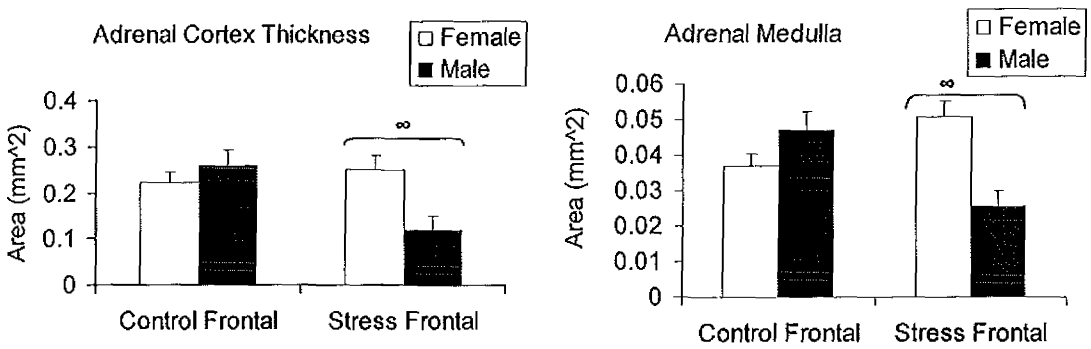


Figure 121. Left: sums of mean adrenal cortex thickness in animals with prenatal stress and frontal lesions. Right: sums of mean adrenal gland medulla areal measures. ∞ Denotes the significantly different effect of prenatal stress with frontal lesions ($p<.05$). Bars indicate mean ± SEM.

Testes Weight

A univariate analysis did not find differences in mean testes weight in animals that were prenatally stressed ($F(1,5)= 0.045$, $p= 0.843$) (see Fig. 122).

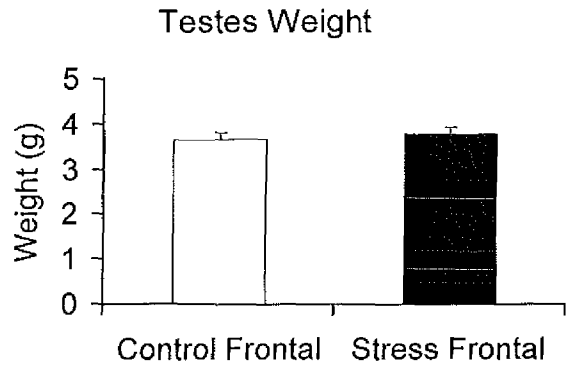


Figure 122. Sums of mean testes weight in prenatally stresses males with frontal lesions. Bars indicate mean ± SEM.

Formalin-fixed brain measures

Prefrontal cortex prelimbic and infralimbic measures

There were no significant differences in either prelimbic or infralimbic cortical thickness (see Table 29).

Univariate analysis for prefrontal measures of the prelimbic cortex did not reveal any significant differences with prenatal stress treatment ($F(1,19)= 0.043$, $p= 0.839$), or a main effect of sex ($F(1,19)= 0.885$, $p= 0.361$), nor for an interaction ($F(1,19)= 0.156$, $p= 0.698$).

Univariate analysis for infralimbic cortex measures did not indicate significant effects of stress ($F(1,19)= 0.434$, $p= 0.519$), or of sex ($F(1,19)= 0.700$, $p= 0.415$), nor for an interaction between groups ($F(1,19)= 1.589$, $p= 0.226$).

Thalamus

Prenatal stress decreased posterior thalamic measures in both sexes, particularly in males (see Table 29), but there was no effect on the anterior thalamus.

A univariate analysis for anterior thalamic measures did not reveal any significant effects of stress ($F(1,19)= 0.777$, $p= 0.391$), of sex ($F(1,19)= 0.008$, $p= 0.929$), or an interaction ($F(1,19)= 2.436$, $p= 0.138$).

Univariate analysis found a main effect of stress in the posterior thalamic measures ($F(1,19)= 8.184$, $p= 0.011$), but not of sex ($F(1,19)= 0.012$, $p= 0.913$), nor for an interaction between groups ($F(1,19)= 1.237$, $p= 0.282$).

Table 29. Mean measures from formalin-fixed brains.

Area Measured	Females	N	Males	N
Non-Stress Control + Frontal Lesion				
[1]Prefrontal Cortex:				
Infralimbic	0.569 ± .051	4	0.470 ± .051	4
Prelimbic	0.627 ± .128	4	0.691 ± .128	4
[2]Thalamic Width: anterior	0.813 ± .031	4	0.766 ± .031	4
posterior	0.934 ± .017	4	0.953 ± .017	4
Prenatal Stress + Frontal Lesion				
[1]Prefrontal Cortex:				
Infralimbic	0.478 ± .039	7	0.498 ± .046	5
Prelimbic	0.605 ± .097	7	0.762 ± .115	5
[2]Thalamic Width: anterior	0.793 ± .024	7	0.835 ± .028	5
posterior	0.908 ± .012	7	0.893 ± .015∞	5

1. Prefrontal cortex: prelimbic and infralimbic regions are measured in cm. sums ± SEM.

2. Thalamic measures for both regions are measured in mean cm. sums ± SEM. ∞ Denotes the significantly different effects of prenatal stress in the posterior thalamic region ($p<.05$).

Cortical thickness

Prenatally stressed animals had much thinner cortexes than non-stressed animals (see Fig. 123).

Univariate analysis for cortical thickness found a main effect of stress ($F(1,24)= 19.356$, $p<.0001$), but not of sex ($F(1,24)= 2.057$, $p= 0.166$), or an interaction ($F(1,24)= 1.513$, $p= 0.232$).

A multivariate analysis for mean cortical thickness measures across five planes found planes' one to four significantly different among groups ($p<.05$) (see Fig. 120).

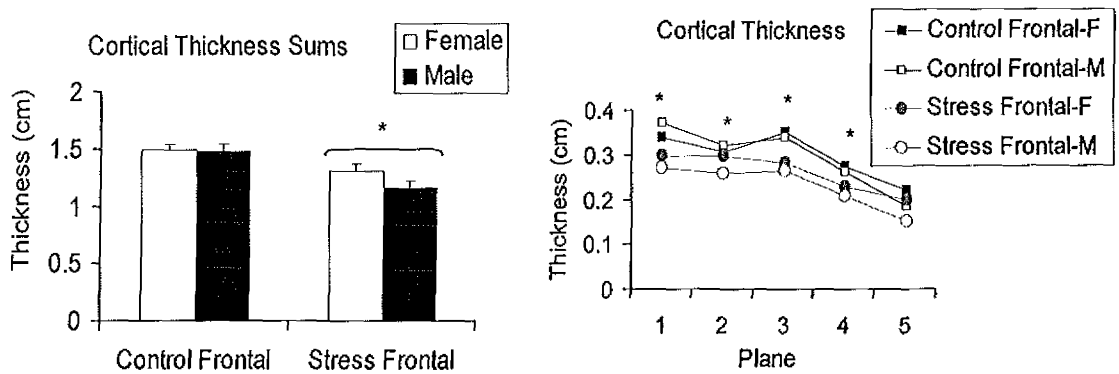


Figure 123. Left: Sums of mean cortical thickness in non-stressed and prenatally stressed animal with frontal lesions. * Denotes significantly different from the Control Frontal group ($p<.05$). Right: Mean cortical thickness across five planes. * Denotes statistically different among groups ($p<.05$). F= Females, M= Males. Bars indicate mean ± SEM.

The effects of mild prenatal stress on early frontal cortical injury

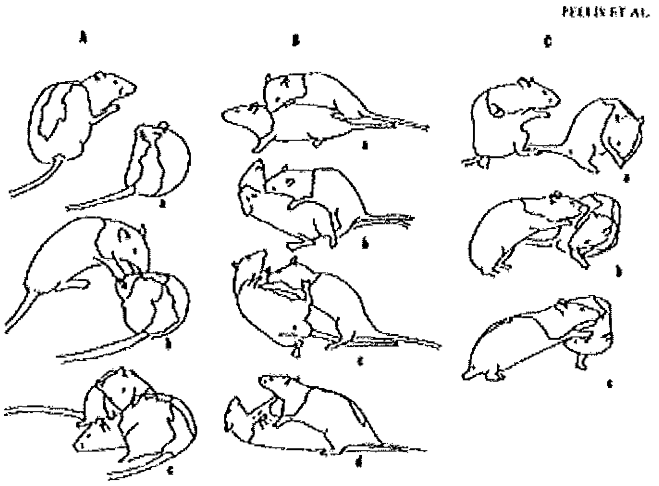
Summary

Table 30. Significant findings for experiment 4b.

Behavioural Task	Females	Males
Activity: Distance Number of movements	Sex: Females more active; Stress increased activity	Sex: Males less active; Stress increased activity
Water Maze:	Stress facilitated acquisition	Stress facilitated acquisition
Tray Reaching:	Stress improved reaching	Stress improved reaching
Elevated Plus Maze:	Stress increased exploration	Stress decreased exploration
Anatomy (Golgi-Cox and saline-perfused preparation)		
Brain Weight:	Females lighter than males Lighter weights with stress	Males heavier than females Lighter weights with stress
Basilar and Apical Branch Arbor:	Decreased arbor in Cg3 of stress females	Decreased arbor in Cg3 of stress males
Adrenal Gland Weight:	Sex: Females had heavier weights, stress decreased wt.	Sex: Males had lighter weights, stress decreased wt.
Adrenal Cortex/ Medulla:	Stress increased cortex thickness, & medulla area	Stress decreased cortex thickness, & medulla area
Anatomy (Formalin preparation)		
Posterior Thalamus:	Smaller area with stress	Smaller area with stress
Cortical Thickness:	Thinner cortex with stress	Thinner cortex with stress

Chapter Four: Experiment V. Social Play:

Play-Fighting in Rats



Play behaviour has been observed in various animal species and identified as being as critical to the social development of an animal as it is in humans (Pellis, 1988). In rodents, Pellis (1997) has suggested that playing is actually a juvenile form of play fighting in that rodents learn the social signals of other rodents, which ultimately forms the hierarchical social structure of the animal colony. It is the repeated coordination and recombination of sequences of behaviour during play-fighting that has been considered a requirement for the development of physical, cognitive and social capacities with the ability to use them flexibly (Trezza and Vanderschun, 2008).

If rats are deprived of rough-and-tumble play-fighting during their juvenile days, they show long-term cognitive, behavioural and socio-emotional deficits (Pellis, Pellis,

and Bell 2010) with notable differences in the synaptic organization of both the medial and orbital prefrontal cortex (Bell, Pellis, and Kolb, 2010).

Play fighting has been observed to occur more frequently in male rats than in females by approximately 25 to 33 percent (Pellis, Field, Smith, and Pellis, 1997). Males will initiate play bouts in the form of an attack to the nape of the neck and will also respond using various defense tactics more often than females. An attack to the nape of the neck is considered a playful attack in rodents, whereas an attack to the rump is an aggressive attack. Contact with the nape is the essence of play such that it behaviourally defines an invitation to a play bout. Pellis has defined a number of defense tactics known to occur during play-fighting. The type and frequency of some defense tactics following a playful attack are also considered to change from a juvenile form into a mature adult form within the family and community of rats, particularly in males (Pellis, Field, Smith, and Pellis, 1997).

For example, juvenile males will often use a complete rotation defense tactic (see Figure 143) (b) that matures into a partial defense tactic (c) into adulthood. The partial defense is seen primarily in dominant males to gain an advantage over its partner. Subordinate males tend to continue using a complete rotation tactic (b) when playing with dominant males, but will use partial tactics (c) with other subordinate males, as well as with females. Another defense tactic used by post-pubertal males is a standing defense, or a push against its partner face-to-face. During the formation of dominance relationships with the onset of puberty, dominant males generally exhibit an adult pattern of play, whereas subordinate males vary their play patterns according to the identity (dominant, subordinate, female) of their partner (Pellis and Pellis, 1991). In adulthood,

from about 70 to 90 days of age, the dominant male weighs more and is known to receive more attacks than it initiates and is less likely to rotate, or supine (c) as subordinates do. Declines in play initiation as well as in defense tactics occur with increasing age (Pellis, Field, Smith, and Pellis, 1997).

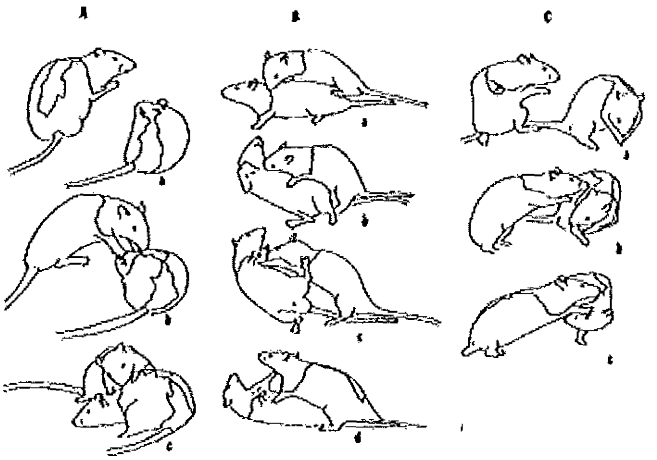


Figure 143. Illustration of three primary defense tactics used during play behaviour in rats (from Pellis et al, 1998, with permission). (A) Evasion is characterized by the opponent lateral swerve away from the attack. (B) Complete rotation involves the defending rat rotating to supine in an attempt to hide its nape from the attacker. (C) Partial rotation occurs when the defender of an attack swerves around its opponent with hind feet standing to gain the upper hand in a play bout.

Females generally defend a playful attack by evading (a) but also use the complete rotation tactic (b) during the juvenile stages. Both evade and complete rotation defense tactics are also common in adulthood in females. The number of defensive responses to playful attacks decline in maturity, especially when initiated by another female (Pellis, Field, Smith, and Pellis, 1997).

Previous experiments have manipulated sex-dependent hormones, or cortical-dependent behaviours to determine that interferences in either system significantly affect the social capabilities of the experimental animals. If rats were gonadectomized in adolescence, the development of dominance-subordinate relationships in rat play fighting behaviour was abolished along with some motivational mechanisms required for forming a hierarchical status among a colony. Age-related declines were still evident (Pellis, et al., 1997).

Rats with early cortical lesions to the orbital-frontal region of the prefrontal cortex were found to have interferences in the perception of their play partners as well as in their species-typical social signals that resulted in an incapability of modulating defense responses according to the dominant status of the play partner (Pellis, Hastings, Shimizu, Kamitakahara, Komorowska, Forgie, and Kolb, 2006). The number of attacks in the lesion animals was higher than in control animals and the proportion of complete rotations declined with age rather than increasing in subordinate males (Pellis, et al., 2006).

Recently, Bell, McCaffrey, Forgie, Kolb, and Pellis (2009) examined the effects of medial prefrontal cortex (mPFC) lesions with play behaviour. The lesions were administered on postnatal day three or in adulthood in the experimental animals. Bell and Pellis found that animals with neonatal mPFC injury were unable to correctly assemble the actions for supination into a complete rotation, but were able to respond appropriately according to the social status of their partners. Similar changes in other defense tactics were also observed in animals with neonatal mPFC injury. Adult mPFC lesions tended to produce play behaviour high in evade tactics and was thought to be a function of

diminished reward in play. Adult operates also showed less play initiation in attack frequency and counter-attack (Bell, et al., 2009). The adult injuries were helpful to delineate some of the mechanisms responsible for some of the play actions when compared to the behavioural actions of rats with injury in adjacent areas of the motor cortex or orbitofrontal cortex. It appears the mPFC is considered to be responsible for the sequencing of playful movements, but also with the rewarding aspects of play-fighting.

Agullar (2010) discusses the complete rotation defense tactic in play-fighting behaviour to be very rewarding in rodents and considers it a primary component of high quality rough-and-tumble play. Rats that have been handled in early postnatal life have the same behavioural phenotype as pups exposed to high licking and grooming during infancy (Agullar, 2010). These pups grew into rats that display high levels of complete rotations during play-fighting which was considered driven by a high motivation to play.

The purpose of the present experiment was to determine play behaviour with regard to: 1) the effects of a medial prefrontal cortical injury induced on postnatal day 10 (P10); 2) rats that were exposed to stress during gestation (G12-18); 3) the effects of a combination of prenatal stress with an early cortical injury (P10); and, 4) the effects of condominium housing in prenatally stressed rats with or without frontal lesions.

Rats were grouped into pairs of the same sex (1 control vs. 1 experimental), and filmed during the juvenile age of 40-45 days and at the young adult age of 60-65 days. The dominance-subordinate identity of the rats in their home environment was not determined prior to the play analysis as it is in Pellis' observations.

Experiments 1 and 2

A subset of animals from Experiments 1 and 2 were selected randomly to participate in social play. The experimental groups and sexes are displayed in Table 35.

Subjects

Table 35. Number of subjects of each sex in groups tested.

Groups	Female (N)	Male (N)
Control	6	6
Control Frontal	8	6
Prenatal Stress	10	9
Stress Frontal	10	5
Stress Condo	5	5
Stress Condo Frontal	5	5

Control Animals

Fourteen rats (7 males, 7 females) were used as controls for comparison with the experimental rats (frontal lesion, stress, and stress with frontal lesions) in the final analysis of play behaviour. They all had undergone a period of habituation followed by 24-h isolation prior to testing (filming) and none had been identified with respect to hierarchical relationships. All experimental groups were compared to the control pairs

that were selected randomly (ie. as a combination of dominant and submissive animals). During filming in adulthood (Play II) rats were paired with the same partners as in Play I.

Frontal Lesion Animals

Animals with frontal lesions that were not experimental with respect to stress or treatment were compared with normal control animals. All animals that had prenatal stress, condo or diet treatments, or the combination with frontal lesions were compared to control animals with frontal lesions. This was preferred because the statistics for all groups with frontal lesions resulted in a large lesion effect when compared to non-lesion control animals. By comparing the behaviours against the untreated control frontal animals, the stress and treatment effects can be revealed.

Table 36. Example of variable set in SPSS statistical analysis.

Rat #	Prenatal Stress	Lesion	Defense (eg.): Partial I
1	**Control**	Control	.272
2	**Prenatal stress**	Control	.586
3	**Control**	Frontal	1.23
4	**Prenatal Stress**	Frontal	1.53

Each experimental rat played with a control rat. Previous studies have shown that control rats will modify their play responses, if necessary, to accommodate a new partner in a play bout. Independent variables also include sex and treatment.

Statistics

The general linear model repeated measures analysis was carried out for comparisons of Play I (juvenile) and Play II (adult) (see Table 36). Each dependent variable of play behaviour is analyzed for between group comparisons of prenatal stress, lesion, sex, and treatment (condo housing or VS supplemented diet) compared to untreated control animal pairs. Within group comparisons were also reported to acknowledge the behaviours exhibited from the juvenile stages to the adult stages (Play I to Play II).

The total defense profile includes the 1) complete rotation, 2) partial rotation, 3) evade, 4) facing defense, and 5) counter attack. For the purpose of this thesis, four independent variables were of interest in the behavioural analysis: 1) attack frequency; 2) complete rotation; and, 3) partial rotation. Attack is expressed as frequency (raw number), whereas the two defense measures are converted into measures of a proportion of the total defenses performed. For example, when a control animal attacks its opponent to initiate a play bout, the probability that its partner (experimental animal) responds is [(***sum of defenses*** *made by the experimental animal, subtract the number of* ***no responses*** *by the experimental animal), then divide by the* ***number of attacks*** *from the control animal*]. For example, #Total defenses– #No Responses (experimental animal) / # Attacks by control partner. The defense measures are reported as the probability that the experimental animals will perform the defense of interest (ie *complete* rotation) divided by the *number of attacks* the control opponent initiated.

To simplify the statistical results, they are displayed in a table for each experimental group. The results of: 1) the probability of responding with a defense tactic;

2) the attack frequency; 3) the probability of using the complete rotation defense; and, 4) the probability of using a partial defense are demonstrated as arrows up, or down to indicate whether the experimental animals demonstrated a significantly higher, or lower play behaviour with respect to the play exhibited by the normal control pairs. If there were no significant differences with the comparison, it was displayed with an equal sign. Arrows were displayed for within group results even if there were no significant between group differences.

Normal (Control) Play Behaviour

Similar patterns of juvenile play and adult play were found in the present group of control rats as has been described in Pellis' rats used to determine the normal development of dominant-subordinate relationships between two rats. An exception is for attack frequency where females exhibited a similar frequency as males during the juvenile stages. In males, the frequency of attacks declined slightly in Play II, whereas measures in number of complete rotations declined from juvenile into adulthood and partial rotations increased slightly. In females, attack frequencies declined in Play II, whereas measures of complete rotation increased slightly and partial rotation declined from Play I to Play II.

Control frontal lesion

The frontal lesion animals showed an increased propensity to initiate play bouts with attack frequency and had a higher probability of defending an attack (see Table 37).

Animals with frontal lesions showed a decrease in the use of complete rotation with an increase in partial rotation defense tactics throughout both the juvenile and adult stages.

The low rate of complete rotations appear to be replaced in part, by partial rotations suggesting that the frontal lesion animals might not be able to perform the sequences of behavioural actions to arrive at a complete rotation. In addition to the inflexibility of movement, the frontal lesion rats were usually of lower body weight and might have increased their partial defense tactics to prevent becoming pinned into a complete rotation and to maintain a strategic counter-attack position. Thus, the lesion animals did not adhere to the age-related modulation of play or to the recognition of their partner regarding a dominate-subordinate relationship. The frontal lesion could interfere with the reward of play as suggested by the low level of complete rotation defense tactic, but might rather increase the animals' competitive nature with an increase in partial defense maneuvers.

Table 37. Summary of Control Frontal lesion animals in play behaviour.

Control Frontal Lesion						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	⇩	⇩	=	=	L: p=. 002	P.D.: p=. 002 PDxL: p<. 0001
Attack	⇩	⬆	⬆	=	N.S.	Attack: p< .0001 AxS: p .012 AxLxS: p .012

Complete	⇩	⇩	⇩	⇩	L: p= .003	N.S.
Partial	⬆	⬆	⬆	⬆	L: p= .002	PxL: p= .007 PxS: p= .006

L= Lesion, AxS= Attack x Sex interaction, AxLxS= Attack x Lesion x Sex interaction, PxL= Partial x Lesion interaction, PxS= Partial x Sex interaction, P.D.= Probability of Defense, PDxL= Probability of Defense x Lesion interaction, N.S. = Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Moderate prenatal stress

The primary findings for prenatally stressed animals were that they played considerably similar to control animal pairs (see Table 37). The attack frequency declines in adulthood (Play II) as it does in control animals, however, the partial rotation increased in both sexes in adulthood, suggesting they played as dominant partners.

Table 38. Summary of prenatally stressed animals with control non-stressed animals.

Moderate Prenatal Stress						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	=	=	=	=	N.S.	N.S.
Attack	⬆	⬆	⇩	⇩	N.S.	Attack: p< .0001

Complete	=	=	=	=	N.S.	N.S.
Partial	⇩	⇩	⬆	⬆	N.S.	PxS: p= .033

PxS= Partial x Sex, N.S. = Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Moderate prenatal stress with frontal lesions

The combination of moderate prenatal stress with frontal lesions revealed higher increases in attack frequency as well as in complete rotation defense tactics in both sexes during Play I (see Table 38). Males continued to use the complete rotation tactic with less partial defense into the adult stages suggesting that the stress might have alleviated the lesion animal's inability to complete the complex series of maneuvers to perform a complete rotation. These males might also find play more rewarding. Prenatal stress might also have subdued the lesion animal's propensity to persevere in play initiation.

Table 39. Summary of prenatally stressed animals with frontal lesions with compared to control non-stressed, non-lesion animals.

Moderate Prenatal Stress with Frontal Lesion						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	=	=	⇩	⇩	Str: p< .0001	P.D.: p< .0001 PDxStr: p< .0001
Attack	⬆	⬆	⇩	⇩	N.S.	Attack: p< .0001 AxStr: p< .0001 AxS:p= .001

Complete	⬆	⬆	⇩	⬆	S:p= .033 StrxS: p= .042	C: p= .005 CxStr: p= .001
Partial	=	⇩	⇩	⇩	Str: p< .0001	PxStr: p= .002 PxS: p= .011

Str= Stress, S= Sex, StrxS= Stress x Sex interaction, AxStr= Attack x Stress interaction, AxS Attack x Sex interaction, C= Complete, CxStr= Complete x Stress interaction, PxStr= Partial x Stress interaction, P.D.= Probability of Defense, PxS= Partial x Sex Interaction, PDxStr= Probability of Defense x Stress interaction, N.S. = Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Moderate prenatal stress housed in condominiums vs. moderate prenatal stress with frontal lesions housed in condominiums

The following sets of subjects were also statistically compared to untreated control animals as those analyzed above. There is an important exception with these groups however, that could have affected the way they played. One group of animals had frontal lesions, the other did not, and both are prenatally stressed. These two groups played against each other and are housed together, therefore, play I is not a first meeting for the pairs. Each group's activity is outlined into tables and their summaries combined. Because they played with each other this method can more adequately explain their play behaviour.

Animals with and without frontal lesions housed in condominiums greatly increased play behaviour in adulthood, as well as in their likelihood to respond to a playful attack (see Table 39). The complete rotation was a more predominant defense

tactic than the partial rotation through the juvenile and adult stages in both sexes. This suggests the animals have an ability to flexibly execute the behavioural sequences of a complete rotation, as well as receive the reward of play-fighting.

Animals with frontal lesions that were prenatally stressed and reared in condominiums played similar to control animals with the exception of a high attack frequency in adulthood as well as in the juvenile stages. The preference for using a complete rotation defense suggests a high reward for play in these animals.

Table 40. Summary of prenatally stressed animals reared in condominiums compared to untreated control animals.

Condo-housed with Prenatal Stress						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	⬆	⬆	⬆	⬆	T: p= .002	PDxT: p= .008
Attack	=	⬆	⬆	⬆	T: p<.0001	Attack: p< .0001
Complete	⬆	⬆	⬆	⬆	T: p= .004	N.S.
Partial	⇩	⇩	⇩	⬆	N.S.	P: p= .027 PxStr: p= .023 PxS: p= .028 PxStrxS: p= .050

Condo-housed with Prenatal Stress and Frontal Lesions

Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	⬆	⬆	⇩	⇩	Str: p<.0001 T: p=.001	PD: p<.0001 PDxStr: p<.0001 PDxS: p= .021
Attack	⬆	⬆	⬆	⬆	T: p=.001	Attack: p<.0001 AxStr: p<.0001 AxT: p<.0001 AxS: p<.0001
Complete	⬆	⬆	⬆	⬆	T: p<.0001 StrxS: p= .031 TxS: p= .006	CxStr: p= .001
Partial	⇩	⇩	⇩	⇩	Str: p<.0001 T: p= .048	PxStr: p<.0001 PxS: p= .009

T= Treatment, Str= Stress, TxS= Treatment x Sex interaction, StrxS= Stress x Sex interaction, AxStr= Attack x Stress interaction, AxT= Attack x Treatment interaction, AxS= Attack x Sex interaction, CxStr= Complete x Stress interaction, PDxT= Probability of Defense x Treatment interaction, PDxS= Probability of Defense x Sex interaction, PDxStr= Probability of Defense x Stress interaction, PDxSxStr= Probability of Defense x Sex x Stress interaction, N.S.= Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Experiments 3 and 4

Subsets of animals from Experiments 3 and 4 were selected randomly to participate in social play. The numbers for the experimental groups and sexes are displayed in Table 40. A discussion for social behaviour among groups follows the table illustrations. It is important to keep in mind that the treatment groups are compared statistically to control pairs. The results also do not indicate the partner's (control) responses.

The present experiment was employed to determine: 1) the effects of a medial prefrontal cortical injury induced on postnatal day 10 (P10); 2) the behavioural effects in rats that were exposed to MILD stress during gestation (G12-18); 3) the effects of a combination of mild prenatal stress and an early cortical injury (P10); 4) the possibility that a vitamin/mineral supplemented diet would make any changes in control animals without stress or lesions; and, 5) the possibility that a vitamin/mineral supplemented diet would make any changes in prenatally stressed rats with or without frontal lesions.

As in the previous set of experiments, rats were grouped into same sex pairs (1 control vs. 1 experimental) and filmed during the juvenile age of 40-45days as well as the adult age of 60-65 days. The dominant-subordinate identity of the rats' in their home environment was not ascertained prior to the task.

Subjects

Table 41. Number of subjects of each sex in groups tested.

Groups	Female (N)	Male (N)
Control	7	7
Control Frontal	4	4
Stress	8	6
Stress Frontal	4	4
Control VS	5	5
Control VS Frontal	4	4
Stress VS	4	4
Stress VS Frontal	4	4

Mild prenatal stress

Similar to the moderately stressed animals, animals exposed to mild prenatal stress played like control animals with the exception of the juvenile period (see Table 42). The stressed animals might have experienced uncertainty with the novelty in play partners. Both sexes showed a normal play of complete rotation, but also with a preference in the use of partial maneuvers into adulthood suggesting a reward with play-fighting.

Table 42. Relationship of prenatally stressed animals compared to untreated control animals.

	Mild Prenatal Stress					
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	⇩	⇩	=	=	Str: p= .003	PD: p= .006 PDxStr: p= .004
Attack	⇩⇩	⇩	=	=	N.S.	Attack: p= .001 AxStr: p= .004
Complete	=	=	=	=	N.S.	N.S.
Partial	=	=	=	⬆	N.S.	PxS: p= .053 PxStrxS:p= .024

Str. = Stress, AxStr= Attack x Stress interaction, PxStrxS= Partial x Stress x Sex interaction, PD= Probability of Defense, PDxStr= Probability of Defense x Stress interaction, N.S. = Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Control frontal lesion

The summary for control frontal animals is the same as in experiment 1, Table 36.

Mild prenatal stress with frontal lesions

The combination of mild stress with frontal lesions produced play behaviour similar to control animals and less like frontal lesion animals (see Table 43). Adult males show a decreased assertiveness with a decrease in attack frequency and partial defense tactics.

Table 43. Summary of prenatally stressed animals with frontal lesions compared to untreated control animals.

Mild Prenatal Stress with Frontal lesions						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	=	=	=	=	N.S.	N.S.
Attack	=	=	=	⇩	N.S.	Attack: p< .0001 AxS:p= <.0001
Complete	⬆	=	=	=	N.S.	CxS: p= .047 CxStrxS: = .046
Partial	⬆	⇩	=	⇩	S: p= .025 StrxS: p= .009	PxStr: p= .038

S= Sex, StrxS= Stress x Sex interaction, AxS= Attack x Sex interaction, CxS= Complete x Sex interaction, CxStrxS= Complete x Stress x Sex interaction, PxStr= Partial x Stress interaction, PDxS= Probability of Defense x Sex interaction, N.S.= Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Multivitamin supplemented diet

Animals reared on the supplemented diet showed a lower level of play initiation, and probability to defend their opponent's attack (see Table 44). There might have been a response to novelty during the juvenile stages of play. By adulthood, females preferred the use of a partial defense as opposed to the complete rotation, whereas males grew into control levels.

Mild prenatal stress and supplemented diet

The combination of the supplemented diet with mild prenatal stress also produced lower levels of play during the juvenile stages. The experimental males increased their probability to defend as adults however females exhibited lower levels of play with stress (see Table 44).

Overall, both groups supplemented on the diet increased play behaviour in adulthood giving the appearance of deficits in age-related modulation. This could be from novelty during Play I where the animals play as subordinates, but by Play II, the animals becomes more familiar with their opponents and more confident with play-fighting. These animals did not exhibit an anxiogenic response in the elevated plus maze however, suggesting that the novelty might not have been a stressful experience.

Table 44. Summary of control diet supplemented (VS) animals and the combination of VS diet with prenatal stress with respect to control non-treated animals.

	VS Diet					
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	⇩	⇩	=	=	T: p= .030	PD: p= .052 PDxT: p= .011
Attack	⇩	=	⬆	=	N.S.	Attack: p= .042 AxT: p= .006
Complete	⇩	⇩	⇩	=	T: p= .018	N.S.
Partial	=	=	⬆	=	N.S.	PxS: p= .002 PxTxS:p= .003

VS Diet and Prenatal Stress						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	⇩	⇩	=	⬆	Str: p= .010 T: p= .030 StrxT: p .018	PD: p= .052 PDxT: p= .011 PDxStrxT: p= .004
Attack	⇩	⇩	=	=	Str: p= .032	Attack: p= .042 AxT: p= .006 AxStr: p= .006
Complete	=	⇩	⇩	⇩	T: p= .018	N.S.
Partial	⇩	=	=	=	N.S.	PxS: p= .002 PxTxS:p= .003

Str = Stress, S = Sex, T= Treatment, StrxT= Stress x Treatment interaction, AxT = Attack x Treatment interaction, AxStr= Attack x Stress interaction, PxS= Partial x Sex interaction, PxStrxT= Partial x Stress x Treatment interaction, PD= Probability of Defense, PDxT= Probability of Defense x Treatment interaction, PDxStr= Probability of Defense x Stress interaction, PDxStrxT= Probability of Defense x Stress x Treatment interaction, N.S. = Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Supplemented diet with frontal lesions

Diet supplemented males with frontal lesions displayed a lower rate of play-fighting in all measures as juveniles, or were similar to control animals (see table 45). Both males and females increased their level of complete rotation defense tactics possibly

to the familiarity of their partners and the task, thus increasing the motivation and reward of play-fighting. On the other hand, it appears that the animals did not modulate their play behaviour according to age-related factors increasing activity rather than decreasing activity in Play II.

Supplemented diet with frontal lesions and Mild prenatal stress and supplemented diet with frontal lesion

The addition of prenatal stress decreased some measures in play behaviour, but overall restored many to control levels (see Table 45). The only increase in behaviour was seen in adult males that increased their use of complete rotation defense. It is possible that both the mild stress and diet treatments reversed some of the lesion effects seen in the control frontal animals. Although, there again appears to be an age-related modulation deficit in the frontal lesion animals, it is possible that they were sensitive to novelty in Play I.

Overall, the supplemented diet groups with frontal lesions appeared able to sequence the motor movements necessary to perform complete rotations during play-fighting.

Table 45. Summary of diet supplemented (VS) animals and the combination of stress with diet with frontal lesions with respect to control non-treated, non-lesion animals.

VS Diet with Frontal Lesions						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	=	=	⇩	⇩	T:p= .014 TxS:p= .004	PDxS: p= .005
Attack	⇩	⇩	⇩	⇩	N.S.	Attack: p= .001 AxS: p< .0001
Complete	=	=	⬆	⬆	S: p= .039	CxT: p= .024
Partial	=	⇩	⇩	⬆	TxS: p= .024	N.S.
VS Diet and Prenatal Stress with Frontal Lesions						
Behaviour	**Play I**		**Play II**		**Between** Subject P-Value	**Within** Subject P-Value
	Female	Male	Female	Male		
Probability of Defense	=	⬆	=	=	Str: p= .033 StrxS: p= .010 T: p= .014 TxS: p= .004 StrxT: p=.001 StrxTxS: p= .014	PDxS: p= .005 PDxStr:p= .026
Attack	⇩	=	=	=	StrxT: p= .016	Attack: p= .001 AxS: p< .0001
Complete	=	=	=	⬆	N.S.	C: p= .013 CxT: p= .003 CxS: p= .041

Partial	=	⇩	⇩	⇩	StrxS: p= .013 TxS: p= .024	PxStr: p= .016

Str= Stress, T= Treatment, StrxT = Stress x Treatment interaction, StrxS = Stress x Sex interaction, StrxTxS = Stress x Treatment x Sex interaction, TxS= Treatment x Sex interaction, AxS Attack x Sex interaction, C= Complete, CxT = Complete x Treatment interaction, CxS= Complete x Sex interaction, PxStr= Partial x Stress interaction, PDxS= Probability of Defense x Sex interaction, PDxStr= Probability of Defense x Stress interaction, N.S. = Not significant. ↑ = higher than control values, ↓= lower than control values, = denotes similar to control values.

Discussion

Play-fighting in normal animals is comprised of so many other factors than the isolated measurements of defense tactics, such as a complete rotation. Factors, such as the sequences of actions that determine the modulation of play responses with respect to learning hierarchical status rules during the late juvenile period, or factors that determine the modulation of play responses through partner identity with respect to an established hierarchical rule in adulthood. For example, juveniles are less bound by social status among a group and are free to play around the rules; however, juveniles and subordinate rats generally use the complete rotation defense tactic more often with an emphasis in adulthood (Pellis, and Pellis, 1990; Pellis et al, 2006).

In the present set of experiments, each member of the pair tested (control and experimental) would have developed a status within their home cages by adulthood. What each subject did not know was their new opponent's status because they are playing with an unknown and new partner in play I and become re-acquainted in Play II, with the exception of the condo-housed animals.

Normal play-fighting is quite fluid with each animal taking turns with various attack and defense tactics. One of the 'rules' learned with play-fighting between partners is to refrain from fully subduing each other in order to keep the play evolving, while at the same time maintaining a competitive edge (Pellis, et al, 2006; Pellis, et al, 1997). The animals with a high attack frequency such as the frontal lesion animals were considered *energetic* and the most persistent to initiate play bouts.

Medial frontal lesions

Damage to the frontal lobe is known to result in various aberrant social behaviours regardless of the time of injury, with the largest social changes apparent when damage is to the orbitofrontal region of the prefrontal cortex (Pellis, et al, 2006). Reasons for this behavioural dysfunction include an inability to adequately perceive species-typical social signals, whether observing humans, cats, or rats. Animals that failed to show the age-related modulation would likely exhibit an infrequent use of the complete rotation tactic during both juvenile and adult play that would be an independent factor from the deficits in normal age-related changes. Pellis' findings in postnatal day three orbitofrontal (OFC) injured animals were an increase in attack initiations and a failure to modulate responses with regard to the social status of their partner. This was defined by the use of the complete rotation tactic with the same frequency whether they were playing with a subordinate partner or a dominant partner. The Pellis paper concluded that the orbitofrontal region of the prefrontal cortex is critically important for the modulation of defense responses according to their partner's social status during play-fighting.

The relationship of the orbitofrontal region of the prefrontal cortex with the medial prefrontal cortex is intricate and reciprocal as both are necessary for all higher functions in mammalian species. The control frontal group (mPFC) in the current set of experiments also did not modulate their behaviour well according to age-related factors of a normal decline in attack frequency, in addition to the increase in use of complete rotations in adulthood. They also were very energetic displaying a high frequency of attack. When measured against control pairs their use of complete rotation defense tactics

were lower with a preference for partial defenses disregarding the social status, or size, of their partners. Finally, in pursuit of pinning down their opponent, the lesion animals would not recognize signals of 'no response' or 'evade' from their (usually dominant) partners, but rather tended to perseverate and continue attacking their opponent.

During play behaviour, the medial PFC (mPFC) is an area considered responsible for selecting appropriate behaviours based on the information coming from sensory and social signals with the inhibition of distracting stimuli by the OFC. Given the information that it receives, the mPFC elicits the proper sequencing (organizing, planning, and execution) of behaviour. Lesions to this area have been shown to interfere with the processes of sequencing behaviour and potentially with the production of a complete supination during play. The limbic or emotional brain areas are in the ventral portions of the brain including the orbitofrontal cortical region. Control males with frontal lesions demonstrated a higher amount of exploration on the open arms of the elevated plus maze suggesting they experienced less emotionality when exposed to a potentially dangerous situation. Their play behaviour also suggests inadequate orbitofrontal function with respect to partner identity.

Prenatal stress

During the juvenile stages, and in some cases adulthood, some of the prenatally stressed animals played very passively using a gentle nuzzle to their partner's nape to initiate a play bout. In other cases, animals were very energetic with frequent attacks. Takahashi, Haglin, and Kalin, (1992) defined the passive play response in juvenile rats as stressed-induced and considered prenatally stressed rats to have a heightened awareness

of a potentially stressful environment. Their hypothesis was supported by increased measures in plasma corticosterone and ACTH in young juvenile animals of 28 days. As the animals grew older, the significant differences diminished (Takahashi, Haglin, and Kalin, 1992). It does seem plausible that the prenatally stressed rats in the current experiments experienced a heightened awareness toward their novel partners displayed as an increase in attack rate. They also played more assertively as adults with the frequent use of partial defenses to keep an edge on their opponents. Whether this is from a heightened awareness into adulthood or stress from 24-hr. isolation is uncertain.

Rats exposed to mild prenatal stress played with a lower frequency during the juvenile stages, but grew into some normal play behaviour into adulthood. The increase in complete rotation during adulthood suggests they played as subordinates compared to normal control animals. They also might have been sensitive to the novelty of their play partner, although the rats exposed to mild stress did not exhibit an anxiogenic response in the elevated plus maze task. It is also possible that these rats spent some of their time exploring their environment, or they might not have experienced the 24-hr isolation as stressful and thus, requiring a release of energy.

Overall, both groups of prenatally stressed animals indicated an age-related deficit in their play-fighting behaviour. They could also be responding to a higher reward from their play in adulthood having already met their play partners. Prenatal Stress administered via dexamethasone (DEX) delivered during early pregnancy (days 42-48) in Marmoset monkeys revealed a reduction of social behaviour in infancy, but an increase in reward (Hauser, Dettling-Artho, Pilloud, Maier, Knapman, Feldon, and Pryce, 2008). Results from the Marmoset study also suggested aberrant motor development, which

might have produced the age-related deficits observed in play-fighting behaviour. The animals in the current study exposed to mild stress however did not have difficulty in the skilled reaching task and actually reversed the deficit shown by the frontal lesion animals.

Prenatal stress with frontal lesions

Some of the juvenile males and females were very assertive in their attack frequency and play maneuvers, regardless of having a lower body weight. By adulthood however, animals exposed to moderate prenatal stress showed declines in play behaviour, with the exception of the increased use of complete rotation in adult males. The lesion effects of an inability to perform the series of actions required for complete rotations appear to be reversed with exposure to prenatal stress. If rats perform complete rotations as a response to reward, the stressed rats would have also experienced reward in play-fighting.

Animals exposed to mild prenatal stress were closer to control values in play behaviour. Adult males exhibited lower attack rates and a lower use of partial defenses. Otherwise, the mild prenatal stress reversed the lesion effects in play-fighting.

Condominium housing

It is difficult to assess how this group of animals would have played if they were paired with novel control partners. A few non-stressed condominium housed animals were scored for play I, however, due to camera malfunction, the remainder of the tape did not record and for play II, the type of tape used for the camera was not compatible. What was realized with some control frontal females was a large inhibition of play. In one pair

the non-lesion female did not want to participate, in another pair, the non-lesion female was very passive and displayed bouts of freezing because her frontal partner was larger. And finally two pairs played well together with the frontal female acting quite aggressively. These groups of animals were all housed together and were not naive to each other when introduced into the play arena.

The prenatally stressed condo-housed rats were highly *energetic* with the frontal animals exhibiting the highest rate of attack and counter-attack. The more responsive frontal animals would take every opportunity possible to get into another play bout, which was well received by their non-lesion partners. These animals were all housed together and therefore were not experiencing anything novel socially.

The treatments of prenatal stress and frontal lesion, and the effects of condominium housing became apparent in adulthood. Both groups (lesion and non-lesion) demonstrated an inability to modulate play according to normal age-related declines in play behaviour. It also appears that if the frontal lesion animals recognized their partners' status they would be considered subordinate with the high rate of complete rotations. Males also merged into adulthood using the partial defense tactic as did the dominant males. It is possible that the reward to play might have exceeded other factors involved with social recognition.

Morley-Fletcher and colleagues (2003) found that prenatal stress decreased social play behaviour, but increased in stressed rats with environmental enrichment. Enrichment also increased the duration of rough-and-tumble play in prenatally stressed animals and slightly so in non-stressed animals. Plasma CORT was measured at time periods beginning after restraint stress. At baseline (0 min.) there were no differences among

groups, whereas at the peak CORT response (20 min.), the enriched environment decreased the level of CORT in prenatally stressed animals. A characteristic prolonged elevation of CORT was measured in prenatally stressed animals housed in standard shoe-box housing (Morley-Fletcher, Rea, Maccari, and Laviola, 2003).

The findings in the present study are similar in respect to higher activity and prolonged play behaviour during the 10-minute play period. Although their CORT levels are unknown, these animals showed a behaviourally anxiogenic response in the elevated plus maze. It is possible that the animals experienced more stress from the 24-hr. isolation prior to play.

Vitamin/mineral supplemented diet

The non-stressed supplemented animals played substantially less in play I, but played more in play II. It is possible that the first meeting of a novel play partner reduced assertiveness in juvenile play, or as with the moderately stressed animals, there might have been a delay in development with respect to play behaviour. Although the supplemented animals exhibited a higher rate of attack in adulthood, they also demonstrated proper partner modulation and sequencing of behaviour with respect to complete rotation frequency. An increase in complete rotation with a decrease of partial rotation defenses in adulthood suggests these rats (stressed and non-stressed) played as subordinate rats.

The effect of the supplemented diet on frontal lesions and prenatal stress appeared to neutralize the lesion effect of a perseveration of attacks with a high attack frequency. The frontal lesion animals were usually energetic exhibiting quick movements with the

relentless pursuit of a play bout. This was not the case with the diet supplemented animals. Partner identity is likely normal as the animals played similar to control animals with respect to their use of complete and partial rotations. They did not play as much as indicated by their low attack frequencies during both the juvenile and adult stages of play. Perhaps their level of reward, or stress was not as high as it was in the other groups.

Conclusions

The mediation of the many maneuvers necessary for successful social play requires the cooperation of both ventral and dorsal regions of the prefrontal cortex. The above results for the current study have suggested that prenatal stress, in addition to frontal lesions, affects the way animals learn to play-fight with new partners, and to what extent the animals could cope with the stress of a larger play partner. The treatments examined (condo housing and vitamin/mineral supplemented diet) as an intervention for animals with prenatal stress and/or medial frontal lesions suggest the treatments to be actually quite beneficial for coping with social stress, particularly with perseveration of attack and high attack frequencies.

So what is the significance of play? In Pellis' recent paper (2010), he discusses the development of the social brain and that when given opportunities to play through the juvenile stages of development, play serves to refine the motor, emotional and psychological development of the individual's maturation of the adult brain. Recall that rats are a highly social species and if they are reared in isolation, or with an adult, that deprives them of juvenile play they become socially incompetent in adulthood and have difficulty coordinating their movements with those of their partners in social situations

(Pellis, Pellis, and Bell, 2010). One of the proposed hypotheses that support the adaptability of play-fighting involves the juvenile training for the unexpected. Because animals in pairs, triads, or quadrads, experience various unexpected maneuvers that often lead to an unpredictable loss of control that ends with the pin into a complete rotation. Continuation of play-fighting into adulthood then provides the rats with the learned experience necessary to become adaptable in various social situations. This is especially important when subordinate males are required to modulate their social responses according to other male's behaviours.

Another very likely hypothesis for a functional purpose for play-fighting during the juvenile developmental stages is for the development of competent sexual behaviours into adulthood (Pellis and Pellis, 2009). A nuzzle at the nape of the neck is also part of the behavioural repertoire during sexual encounters between a male and female rat. Depending on the female's readiness for mounting, males will graduate from nuzzling the nape to mounting her from behind. The copulatory sequence of events is often not direct, but rather a form of play from ear wiggling to leading the males on a chase, similar to that found in play-fighting (Pellis and Pellis, 2009). Interestingly, male rats from mild prenatally stressed dams were observed to disregard the normal courtship practices and rather impulsively, attack the female directly. These tests were performed to determine the effects of prenatal stress in male rats' sexuality (Checknita, 2009).

Chapter Five: General Discussion

The primary goals of the current studies were: 1) to examine the effects of mild or moderate stress on normal brain and behavioural development; 2) to determine how mild or moderate stress could influence brain and behavioural recovery from perinatal prefrontal cortical injury; and, 3) to investigate whether potential therapeutic interventions (complex housing and vitamin/mineral supplements) might modulate the effects of prenatal stress and perinatal cortical injury.

Our primary general findings were that: 1) prenatal stress produced distinctly different behavioural and anatomical profiles in a dose-dependent manner; 2) there were sex differences in many behavioural and anatomical measures with prenatal stress; 3) the general outcome of prenatal stress with frontal injury is positive; and, 4) the treatments of condominium housing and a vitamin/mineral supplemented diet were effective in reducing the effects of the frontal injuries on most measures.

The effects of prenatal stress

Some of the earlier studies of stress during gestation arose from the children born to mothers that were pregnant during natural disasters, such as earthquakes, flooding, or the Second World War with results of giving birth to low birth weight infants that grew into disorders of schizophrenia or major depressive disorders, such as bipolar disorder (Weinstock, 2001). Although the purpose for some of the studies was not neurobehavioural in nature, it became apparent that the mothers in the study were exposed to many environmental insults; enough to make permanent neurobehavioural

effects on their children. One caveat of these studies is that they were "natural experiments" and thus confounding factors including drug use, smoking, alcohol consumption, and malnourishment were not controlled. In addition, it is difficult to include other factors such as genetic predispositions to behavioural disorders, the environment the infants are raised in, and the late onset of abberant behaviours (Weinstock, 2001).

Animal studies offer the advantage of more experimental control but are plagued by strain and stress protocol differences as well as differences in colony housing and experimental testing conditions. Typically, severe to moderate stress is produced by prolonged restraint (20-30 min) in a closed tube 2-3 times per day followed by forced swimming in tepid or cold water for 5 min (e.g., Morley-Fletcher, 2007; Maccari, 2004). A reduction in restraint time (e.g., 5-15 min) and swimming in warm water (e.g., 30°C) is thought to produce a milder stress reaction although we are unaware of any direct comparisons of corticosterone responses in the two types of paradigms. Recently, Mychasiuk (2010) compared the effects of twice daily prenatal exposure (10 min versus 30 min) on a small clear platform in the middle of an open field and found marked differences in the changes in global methylation, gene expression, and synaptic organization in the two paradigms. It appears that fairly subtle differences in stress protocols can produce large differences in perceived stress.

Most previous prenatal stress studies likely used a more intense stressor when compared to the present set of experiments. Experiments 1 and 2 used restraint stress for 20 minutes followed by forced swimming in tepid water. Relative to the literature, this is best considered to be a moderate stressor. Experiments 3 and 4 used restraint stress for

15 minutes followed by forced swimming in warm water. In addition, the stressed dams were given carbohydrate snacks after the stress to blunt the stress response. There was also one other important difference in the protocols. In the first set of experiments, the stressed dams were returned to cages and were paired with another stressed dam. Our previous experiences with prenatal stress experiments have shown that "bystander stress" occurs when a stressed dam resides with an unstressed control dam. The offspring of dams receiving bystander stress showed prenatal stress effects in DNA methylation and gene expression patterns (Mychasiuk, 2010). In contrast, the dams given milder stressors were returned to home cages with their male mates rather than another stressed partner. The following discussion will consider this stress paradigm as *mild stress.*

It is unfortunate that there are no direct measures of corticosterone or ACTH in the current study but there were marked differences in the behavior of the dams and offspring. For example, there was a reduction in food consumption in the dams with moderate stress in parallel studies done at the CCBN (G. Metz, personal communication). In addition, some litters of the moderately stressed dams were resorbed and the dams did not produce viable offspring. There were also differences in maternal care (see Appendix C) insofar as the stressed dams in Experiment 1 showed less licking and grooming relative to controls or the milder stressed dams. Lower levels of maternal licking and grooming has lead to different patterns of brain development than what was seen in more attentive dams (e.g., Ladd, 2000). With respect to the infants' behaviour the offspring of the moderately stressed moms had disturbed social behavior and impairments in skilled reaching that were not observed in the offspring of the mildly stressed moms. In

addition, the former animals showed an increase in behaviours of anxiety in the elevated plus maze that was not observed in the latter animals.

The current study also found differences in various anatomical measures, particularly changes in dendritic arbor and length. The largest difference between the two paradigms was in prefrontal regions of Cg3 and AID. Animals exposed to moderate stressors had decreased dendritic length in both regions, but increased dendritic arbor in Cg3. Mild stress produced an interaction with the sexes such that females had increased dendritic length and arbor in AID, whereas males showed a decrease. There were no differences from control animals in Cg3. Curiously, moderate stress in adult animals has been shown to produce a decrease in dendritic measures in Cg3 and an increase in AID (Liston, et al, 2006). It thus appears that not only does stress intensity matter but so does age at the time of stress.

Finally, although the adrenal weights in the current set of experiments are not direct measures of the stress experienced by the offspring pups, they have indicated differences between the stress paradigms. Heavier adrenal weights were measured in animals exposed to moderate stress whereas there were no differences in weights between control animals and animals exposed to mild stress.

In sum, the behavioural and morphological data suggest that the two stress paradigms have markedly different effects on the dams and the offspring. It is likely that the different intensities of stress altered the rate of cell proliferation and differentiation during neurogenesis. For example, Fujioka's (2001) found mild prenatal stress (30-minute restraint once daily) facilitated cell differentiation of fetal neurons of the

periventricular nucleus (PVN) and hippocampus, whereas more severe stress (120-minute restraint) induced neurotoxicity and apoptosis of neurons in the same brain areas.

Sex differences from prenatal stress

Some of the more recent research that included both females and males in their studies of prenatal stress has shown sex differences in most measures. In both non-stressed control and in prenatally stressed animals, males have been shown to spend less time entering an open field and when they do, they display less ambulatory activity. Similarly, males spend less time in the open arms of the elevated plus maze, and display more timid behaviours in novel environments (Weinstock, 2007). The general response of the HPA axis in animals that have been prenatally stressed and have been exposed to prolonged restraint stress as adults involves prolonged expression of the stress response and with more CORT released in response to environmental stress (Vallee, et al, 1999). This aberrant negative feedback has been found to be due to a reduction in glucocorticoid and mineralocorticoid receptors in the hippocampus (Maccari, 1995). In milder stress paradigms however, the sex differences become more apparent. Studies employing once daily maternal restraint, unpredictable noise, or saline injection as maternal stressors produced results in the offspring that affected the females only suggesting that females have a higher sensitivity to maternal stress hormones than males (McCormick, Smythe, Sharma, and Meaney, 1995).

In the current set of experiments moderate stress produced sex differences in behavioural parameters of lower locomotor distance and of greater behavioural anxiety in the elevated plus maze. Only the females showed altered circadian activity with increased

activity into the evening hours. The anatomical measures with normal control animals found males to have larger brain measures than females. The normal sex difference in brain weight (males>females) was not affected by prenatal stress. Male adrenal cortex thickness was thinner whereas the cortex in females was thicker following prenatal stress suggesting a greater adrenal response to stress in females.

In the mild stress paradigm, sex differences as a result of stress were observed in locomotor activity and the elevated plus maze with females showing higher levels of activity in behavioural tasks. Anatomically, dendritic branch and length in AID had differential patterns of growth with an increase in growth in females and a decrease in growth in males. Mild stress decreased the adrenal cortex thickness, but increased the size of the adrenal medulla, especially in males, suggesting an alternate energy source from the adrenal medulla, such as adrenalin and catecholamines instead of glucocorticoids. An interesting difference with mild stress was the increased brain weight in formaldehyde fixed brains in prenatally stressed males, but males also showed the thinnest cortex from prenatal stress. It is possible that some areas of the brain were enlarged by stress, such as was measured in the infralimbic and prelimbic regions of the prefrontal cortex, or that the brain cells might be denser in some regions.

The sex differences in measures from the current study reflect McCormick's (1998) findings in response to activation of the HPA axis with estrogen having excitatory effects and androgens inhibitory effects in HPA axis activation. Some of the aforementioned behavioural and anatomical measures from the current set of experiments have shown an increase in females and a decrease in males. There were sex differences in behavioural tasks of locomotor activity and in exploration of the elevated plus maze, with

large differences between stress paradigms. Using a more intense stress paradigm, Maccari and Fletcher (2007), have previously reported sex differences in metabotropic glutamate receptor-1 (inhibitory) with a decrease in receptor activity in males and an increase in activity in females. This could in part, explain the higher anxiogenic behaviour in males.

In conclusion, it appears that prenatal stress does affect the sexes differently, both behaviourally and anatomically.

Prenatal stress with frontal injury

Moderate stress also increased anxiogenic behaviour in animals with frontal lesions in the elevated plus maze in both sexes, which might correlate with the decreased arbor and length in AID. Locomotor activity and skilled reaching was not affected by stress.

Mild stress reversed the lesion effects in the Morris water maze task, the skilled reaching task, and reversed the moderate stress effect of anxiogenic behaviour in the elevated plus maze. Mild stress also decreased dendritic basilar arbor in Cg3.

The effects of the mild stress on the frontal symptoms could be related to FGF-2 expression. FGF-2 is normally expressed during neural development, differentiation, and synaptogenesis (Riva and Moccetti, 1991) and is upregulated after injury, stress, learning experience and physical activity thus contributing to synaptic plasticity and injury repair (Kolb, 1995; Gomez-Pinilla, et al, 1997). In animals that have been exposed to prenatal stress, postnatal exposure to either acute or chronic restraint stress has been shown to increase FGF-2 in the prefrontal cortex, whereas control non-stressed animals showed

decreased FGF-2 expression, potentially giving injured animals an advantage to injury repair if they were prenatally stressed (Fumagalli, et al, 2005). For example, postinjury injections of FGF-2 have been shown to enhance recovery from day 10 motor cortex injuries by stimulating neurogenesis (Monfils et al., 2005; 2006; 2007).

Condominium housing and supplemented diet treatments

Condominium housing, or environmental enrichment, has been employed to induce the plastic processes in the brain, particularly after injury (Comeau, et al, 2008; Chapillon, et al, 2002) and recently, to alleviate the adverse effects of intense prenatal stress (Laviola, et al, 2004; Morley-Fletcher, et al, 2003).

The current study did show the advantageous effect of an enriched environment in brain-injured rats in navigation in the Morris water maze, particularly in males with frontal lesions that were slightly impaired without condo treatment. Condo housing also reversed the skilled reaching impairment in non-stressed frontal animals, but it did not have any positive effects in behaviours of anxiety. The anxiogenic behaviour is not a commonly reported phenomenon in animals exposed to enriched environments from enriched housing. It is possible that the non-stressed animals experienced something during their perinatal life that affected them as a stressor. The dams used were older and gave birth to fewer pups with the appearance of smaller birth weights. There were however no differences among groups in adult body weight and they had the thinner adrenal cortex measures than group-housed control animals suggesting stress might not have been a factor in their behavioural outcome.

The prenatally stressed offspring that were condo-housed exhibited positive behavioural measures of a reduction in stress-induced locomotor activity, and facilitated navigation in the Morris water maze; however, skilled reaching and emotionality were not recovered from the effects of prenatal stress. Although there was a reduction in dendritic length from moderate stress in the condominium housed animals, there was an increase in branch arbor in AID with condo housing, which might be related to the increased flexibility in social play in both stressed and non-stressed animals with frontal lesions. Morley-Fletcher (2003) also found enriched animals to increase play behaviour that corresponded with a decrease in stressed-induced CORT in prenatally stressed animals and no effect in CORT levels in non-stressed animals. The current study showed that adrenal gland cortex thickness was greatest in the prenatally stressed condo-housed animals suggesting an overuse for glucocorticoid secretion, which could have contributed to the higher emotionality in these animals. There are substantial differences between the two studies however with respect to the size of standard housing and enriched housing and the stress paradigm.

The vitamin-supplemented diet treatment for moderate stress also reduced hyperactivity, improved spatial navigation, and reduced anxiogenic behavior in otherwise normal males in the elevated plus maze. The stressed and supplemented females also appeared to have reverted back to morning circadian activity as measured in the wheel running cages. The adrenal gland weights in the supplemented animals were also heavier owing to the increase in adrenal medulla area suggesting a greater use of medullary hormones adrenalin and catecholamine with low levels of glucocorticoids secreted from the adrenal cortex.

The supplemented-diet treatment in experiments using the mild stress paradigm lowered the higher activity levels in prenatally stressed females, was beneficial in spatial navigation in both intact control and frontal lesion animals and reversed the lesion effects in skilled reaching. The supplemented diet caused increased dendritic length in layer V of Cg3 in intact animals suggestive of increased cortico-striatal connectivity that was correlated with enhanced performances in various behavioural tasks. Prenatally stressed females with frontal lesions had decreased dendritic length, whereas males had an increase. On the other hand, intact stressed females had an increase in apical dendritic arbor. In area AID the supplemented diet reversed the effects of stress restoring basilar and apical growth patterns back to normal control levels in animals without lesions. In the frontal lesion group, basilar dendritic length was greater in the VS diet animals and apical branch was densest in males that had a combination of stress, and diet.

Adrenal cortex thickness was the smallest in the supplemented diet animals with larger medulla areal measures again suggesting a greater use of the medullary hormones in conjunction with lower levels of adrenal cortex hormones, as was found in animals treated with mild prenatal stress. Some of the improved behavioural effects could be from performing better with the stress reactivity that is required to facilitate learning, but with lower levels of stress hormones that could interfere with learning. Acute administration of glucocorticoids has been found to enhance learning whereas high circulating levels of glucocorticoids impairs memory retrieval and working memory performance (Nathan, Griffith, McReynolds, Hahn, and Roozendaal, 2004).

In addition to a having a well functioning hippocampus for coping with stressors, the integrity of the prefrontal cortex (Gratton, and Sullivan, 2005) and basolateral

amygdala (Nathan, et al, 2004) is critical for the regulation of physiological and behavioural responses to stressors. The ventromedial area of the medial prefrontal cortex, particularly the infralimbic region, works closely with orbitofrontal network and is considered the visceromotor system (Gratton, and Sullivan, 2005). This area receives a variety of neurological input from limbic regions such as the ventral hippocampus and the amygdala, which in turn provides output information through networks to the hypothalamus and brainstem neurons involved in emotion and stress regulation. In some aspects, both treatments of condominium housing and the supplemented diet could have improved the functioning of the prefrontal cortex which could be inferred from increased dendritic growth

Perinatal events have been found to alter some of the normal cerebral asymmetries that mediate stress responses, particularly prenatal stress. Prenatal stress of an intense nature has been found to increase anxiogenic behaviours in the plus maze, which was considered a reflection of the altered cerebral lateralization of dopaminergic (DA) systems (Fride and Weinstock, 1988). Fride (1988) also found that the right hemisphere DA neurotransmission increases in response to novel experiences and environments, such as during an anxiogenic response in the elevated plus maze and in escape from shock. Maternal separation and social isolation of pups are paradigms shown to produce anxiogenic behaviour in adults during the early postnatal period have been shown to result in abnormally high densities of DA and serotonergic (5-HT) terminals in the infralimbic cortex (Gratton, and Sullivan, 2005). Conversely, early postnatal handling stimulated the 'normal' development of cerebral lateralization, which showed a right-ward shift in benzodiazepine receptor binding in the infralimbic cortex and hippocampus

(Denenberg, 1981). Ladd (2000) has shown that the handling procedure in new rat pups produced an increase in growth hormones and in ornithine decarboxylase, an enzyme required to propagate the rate of the cell cycle between G1 and S phases.

Overall, these treatments have proved some beneficial results for functional recovery in animals with prenatal stress and/or postnatal frontal injury. Both treatments have a global effect on the brain and individual with a naturalistic approach to encouraging brain plasticity in favor of functional recovery from injury or stress.

Concluding remarks

The current experiments have demonstrated that prenatal stress can program the brain to function differently in response to novel tasks and novel play partners through many changes in the physiology of the stress response. Prenatal stress also appears to affect the brains response to injury, depending on the intensity of the stress during gestation. The intensity of the stressors employed during gestation is also a critical factor that can produce distinctly different behavioural outcomes. A number of researchers have proposed that the developing brain becomes shaped by its environment within the genetic profile that it has inherited. When the environment of an animal is under duress with a higher competition for food or in close proximity of predators, this adversity might shape the developing brain to mount a faster and prolonged stress response to promote survival of the animal. In animals, including and perhaps under some circumstances in humans, this hyperactive stress response would be advantageous. In humans living in a modern society however, this could be very disadvantageous and give rise to various metabolic disorders that are known to contribute to diabetes and heart disease in addition to the

development of neuropsychiatric disorders. The experimental findings suggest some positive interventions for children born into a stressful environment and/or a mother who has experienced stress during pregnancy.

The various biological mechanisms of the micronutrient formula have not yet been demonstrated in the present clinical or animal studies aside from the standard biomarkers in blood that measure nutrient status to ensure they remain within normal limits. On the other hand, Groff, Groper, and Hunt, (1995) periodically publish updated educational literature about vitamins and minerals, their peripheral mechanisms with respect to synthesis, deficiency, excesses and food sources amongst other factors. Every vitamin and mineral contributes to one or more pertinent biochemical reactions, either as cofactors or prosthetic groups for enzymes, and/or as substrates for necessary biochemical and cellular conversions. Through consistent replenishment, these micronutrients would restore some of the normal cellular and physiological processes in the body and brain and maintain cellular function. In the current study, animals reared on the supplemented diet had a number of significantly different results, both behaviourally and anatomically. One must keep in mind that even the standard rat chow diet is likely to be of higher nutritional quality than what some people are able to maintain through diet alone, particularly those suffering from disorders, such as bipolar disorder. It therefore makes sense to prescribe a micronutrient formula for various ailments.

Maintaining necessary cellular functions is critical for the proper physiological balance in the body and brain. Given that the micronutrient formula tested in the current study was administered beginning the first day of postnatal life, it would have an opportunity to correct some of the adverse prenatal events because the developing rats

were still within a critical developmental period of cell proliferation and migration. Similar to studies of postnatal handling (Meaney, et al, 2000), cross fostering to high licking/grooming dams (Lui, et al, 2000), nutritional supplementation (Niculescu, et al., 2004), and peripubertal environmental enrichment (Bredy, Zhang, Grant, Diorio, and Meaney, 2004), the postnatal intervention would be able to correct some of the pre-existing genetically expressed effects, or effects from perinatal adversity by changing the epigenetic expression of the genome. Much of the endocrine system develops postnatally in rats, mice and rabbits (altricial species) (Wadhwa, and Federenko, 2006) enabling treatments such as micronutrient supplementation beginning on day one to influence development. In humans, this time period would approximately correspond with Embryological Stage 17, or 18 when the embryo is about seven weeks developing and during the migration of neural cells to form the cortical layers.

A follow-up examination of a large population of individuals that were exposed to the Dutch Hunger Winter famine (1944-45) periconceptionally was performed to determine the presence of any persistent epigenetic marks six decades later (Heijmans, et al, 2008). The average caloric intakes were 667 calories derived from a very low animal protein source of 4% (total ~ 12%), a low-to-moderate fat content of 19%, and a high carbohydrate content of 69%. The periconceptionally exposed individuals had hypomethylated CpG sites of the insulin-like growth factor 2 (IGF2) that is a critical factor in human growth and development and is maternally imprinted. These data were also compared with data from same sex siblings that were not exposed to famine, as well as with individuals exposed during late gestation. There were no differences in methylation between individuals exposed to famine during late gestation to those not

exposed to famine. Furthermore, exposure to famine during late gestation resulted in infants having lower birth weights, but was not influenced by epigenetic changes. Conversely, those exposed periconceptionally had a reduction in DNA methylation, but did not have lower birth weights and were still affected developmentally. The authors concluded this study with the suggestion that birth weight might be a poor indicator for a compromised prenatal development and nutritional status, but also that the maternal diet was likely very low in methyl donors, such as methionine (Heijmans, et al, 2008).

It is also likely that the women who were pregnant during the Dutch Hunger Famine were exposed to additional stressors of cold and emotional stress (Heijmans, 2008), but the overall results from studies of undernutrition and environmental adversity continue to report changes in the HPA axis. Wadhwa, and Federenko (2006), have suggested that adverse birth outcomes are a result of multifactorial and heterogeneous entities that arise from a combination of environmental and genetic factors that reflect several pathophysiological processes. Chronic activation of the stress response could thereby lead to either over-activation in some physiological systems and/or under-activation in other systems simultaneously, producing a number of abberant metabolic effects that change cellular responses and epigenetic expression. Long-term effects could produce a number of disorders and neuropathologies, such that if a pregnant woman has inherited, or acquired metabolic disorders, some of the effects could be transferred to her fetus potentially altering homeostatic set points and promoting difficulties in fetal and infant development.

The degree of stress experienced by the mother would vary and it is uncertain to how mild stress in rats would correspond to mild stress in humans. Stressors could be

positive and consist of moving or marriage, but these events still activate the HPA axis and HPA axis activity is dependent on the mother's reactivity. In modern society, a working mother-to-be has enough stress to increase her blood pressure. The type of work and the length of time working while pregnant would determine the outcome of stress the fetus receives. In worst case scenarios of domestic abuse and substance abuse, it would be especially advantageous for women, or caretakers to understand the potential consequences for their newborn. Having the knowledge of interventions such as nutritional supplementation and enriching environments is necessary for the future well-being of many children and could provide caretakers exposed to perinatal stress and/or brain-injured infants better tools to provide a more positive outcome for the growing child.

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Appendixes

Appendix A: The Autonomic Nervous System

Appendix B: EMPower+ Supplement

Appendix C: Maternal Care

Appendix A: The autonomic nervous system

Sympathetic nervous system:

The cell bodies of the preganglionic (presynaptic) sympathetic neurons are in the lateral horns of the grey matter in the spinal cord extending from the first thoracic segment (T_1) to the second lumbar segment (L_2) (Spence, and Mason, 1992). These visceral motor neurons project their axons with somatic motor axons through the ventral roots into the ventral rami where all sympathetic nerves combine with an interconnected chain of sympathetic ganglia. Within the sympathetic chain, or trunk axons may extend upward, or downward prior to innervating postganglionic (postsynaptic) neurons. Postganglionic neurons innervate cells in, smooth muscle cells of blood vessels in skin, sweat glands, and arrector pili muscles of the hairs. Some postganglionic axons of the cervical and thoracic ganglia directly innervate structures in the head rather than entering the spinal nerves. Preganglionic nerves that do not enter the sympathetic chain ganglia form pathways called the splanchnic nerves that pass through the diaphragm to become part of a neural network called the abdominal aortic plexus. The aortic plexus is located on the anterior surface of the abdominal aorta, the large blood vessel that carries blood from the heart to be distributed throughout the body. Splanchnic nerves also innervate the adrenal medulla directly (Spence, and Mason, 1992) (see fig. 144).

Parasympathetic nervous system:

The cell bodies of preganglionic neurons in the parasympathetic nervous system are located either within the brainstem, or in the lateral grey matter horn of the spinal cord of the second, third, and fourth sacral segments (Spence, and Mason, 1992). Cell bodies located in brainstem nuclei travel to the head, thorax, and abdomen within the cranial nerves; occulomotor, facial, glossopharyngeal, and vagus nerves. Parasympathetic neurons do not travel within the rami of the spinal cord and therefore, do not affect sweat glands, skin cells, or hair cells. Preganglionic neurons located in the sacral region of the spinal cord travel through the ventral roots to form the pelvic splanchnic nerves, which interconnect in the hypogastric plexus and innervate the pelvic region (Spence, and Mason, 1992).

Preganglionic and postganglionic cells of the parasympathetic system are cholinergic, as are preganglionic neurons of the sympathetic nervous system (Spence, and Mason, 1992). The postganglionic cells of the sympathetic system are noradrenergic, with the exception of sweat glands that secrete acetylcholine.

The Autonomic Nervous System

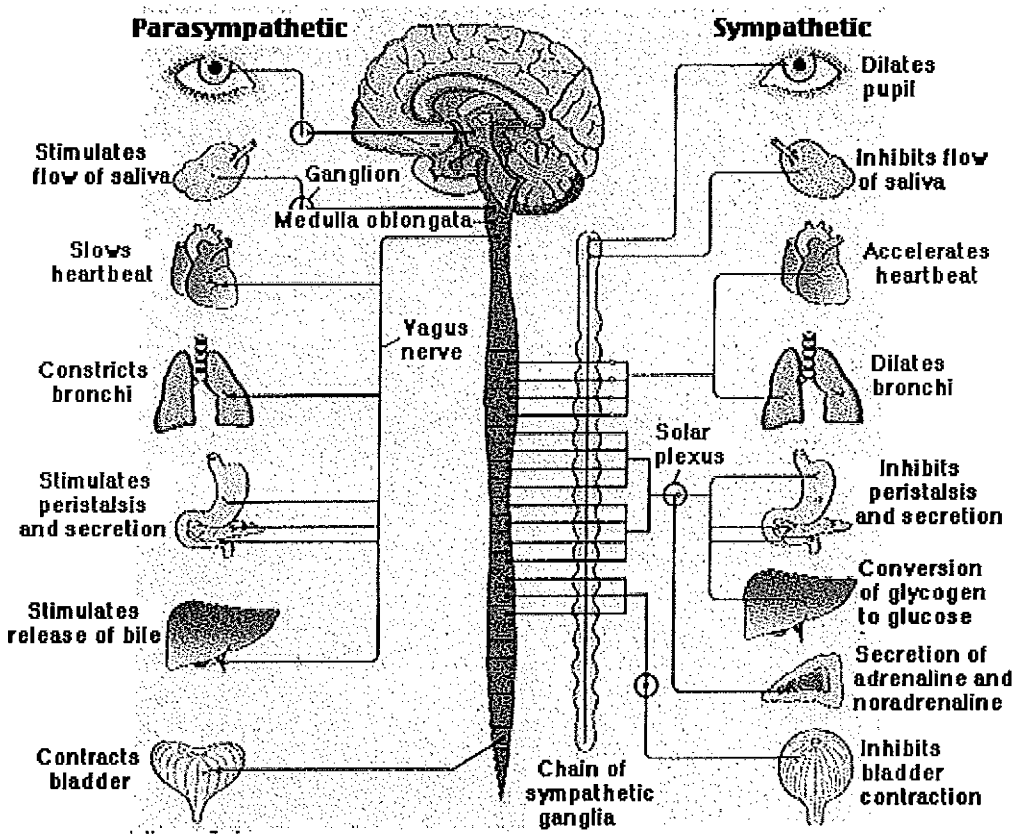

Figure 144. An illustration of the autonomic nervous system and function (From scienceblog.com/clock/2006/06/bio101_lecture).

Appendix B
EMPower+ Supplement

Table 46. Micronutrient formula: Serving Size is 4 Capsules. (Label Claim)
Loading dose is 18 capsules/ day to be gradually reduced.

Dietary Constituent	Amount Per Serving	% Daily Value
Vitamin A (retinyl palmitate)	1536 I.U.	30 %
Vitamin C (ascorbic acid)	160 mg.	270 %
Vitamin D (cholecalciferol)	384 I.U.	100 %
Vitamin E (d- α tochopherol succinate)	96 I.U.	320 %
Vitamin B1 (thiamine mononitrate)	4.8 mg.	320 %
Vitamin B2 (riboflavin)	3.6 mg.	210 %
Vitamin B3 (niacinamide)	24 mg.	120 %
Vitamin B6 (pyridoxine hydrochloride)	9.6 mg.	480 %
Vitamin B9 (folic acid)	384 mcg.	100 %
Vitamin B12 (cyanocobalamin)	240 mcg.	4000 %
Vitamin H (biotin)	288 mcg.	100 %
Vitamin B5 (d-calcium pantothenate)	5.8 mg.	60 %
Calcium (amino acid chelate)	352 mg.	35 %
Iron (amino acid chelate)	3.7 mg.	20 %
Phosphorous (phosphorous complex)	224 mg.	20 %
Iodine (from kelp)	75 mcg.	50 %
Magnesium (amino acid chelate, magnesium complex)	160 mg.	40 %
Zinc (amino acid chelate, zinc complex)	12.8 mg.	90 %
Selenium (amino acid chelate, selenium complex)	54.4 mcg.	80 %
Copper (amino acid chelate, copper complex)	1.9 mg.	100 %
Manganese (amino acid chelate, manganese complex)	2.6 mg.	130 %
Chromium	166.4 mcg.	140 %
Molybdenum (amino acid chelate, molybdenum complex)	38.4 mcg.	50 %
Potassium (potassium complex)	64 mg.	2 %

The central nervous system proprietary blend as part of this supplement consists of dl-phenylalanine, glutamate, citrus bioflavinoids, grape seed, choline, inositol, ginkgo biloba, methionine, organic germanium, boron, vanadium, and nickel.

Appendix C: Maternal Care

Licking and grooming

The results found the stressed dams to spend a significant decrease in time licking and grooming their pups than control dams (see Fig 145). Univariate analysis for licking and grooming characteristics of maternal attention the pups receive indicated a marginally significant treatment effect ($F(1,4)= 17.577$, $p= 0.025$).

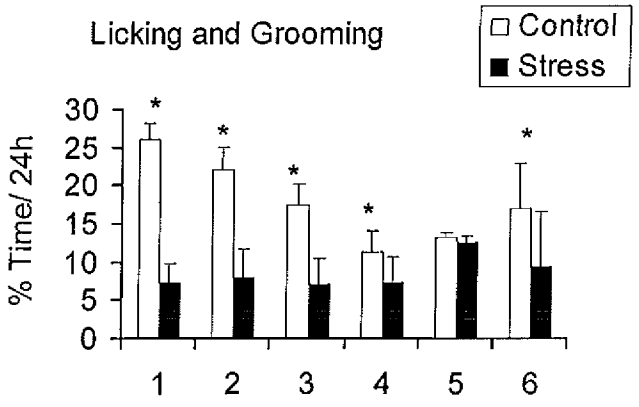

Figure 145. Mean percent frequency of licking and grooming of pups for their first 6 days in dams that were stressed during gestation. * Denotes statistically significant from non-stressed control dams ($p<.05$). Bars indicate ± mean SEM.

Passive nursing

The stressed dams spent much of their time passive nursing, whether it was with a blanket posture, or nursing on their side, it occurred often; likely from fatigue as a result of stress with the addition of caring for new pups. Occasionally, dams would lick and groom their pups while lying on their sides (see Fig. 146).

Univariate analysis did not find significant effects of stress ($F(1,4)= 0.429$, $p= 0.559$), among the first six days of monitoring, although the first three days could prove a different profile.

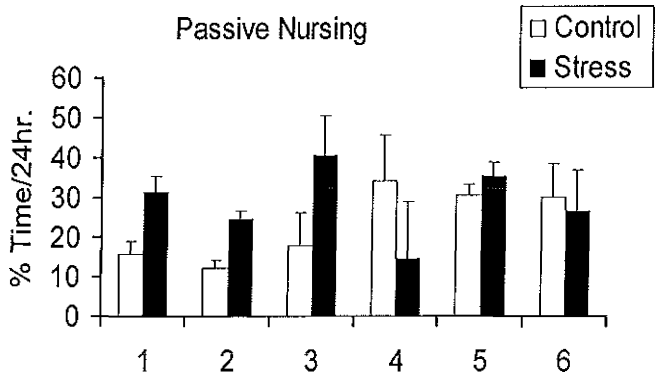

Figure 146. Mean sums of passive nursing behaviour during the pups' first 6 days in dams that were stressed during gestation. Bars indicate ± mean SEM.