

The Effects of Prenatal Predator Exposure and Postnatal Environmental Enrichment on
Febrile Convulsions, FosB- and CRH-immunoreactivity

by

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DALHOUSIE UNIVERSITY
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This work is dedicated to Randy and Kim Korgan. Without them, I would have never gotten here.

TABLE OF CONTENTS

LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	ix
LIST OF ABBREVIATIONS USED	x
ACKNOWLEDGEMENTS	xii
CHAPTER 1	
INTRODUCTION	1
1.1 Neurodevelopment of Epilepsy	1
1.2 HPA-axis	7
1.3 Prenatal Stress	9
1.4 Prenatal Stress and Epilepsy	14
1.5 Environmental Enrichment	15
CHAPTER 2	
METHODS	18
2.1 Animals and Breeding	18
2.2 Prenatal Stress Exposure and Postnatal Environmental Enrichment	19
2.3 Fecal Glucocorticoid Extraction and Assay	20
2.4 Febrile Convulsions - PD14	21
2.5 Sacrifice and Tissue Generation	23
2.6 CRH-ir Groups	23
2.7 FosB Immunoreactive Labeling (FosB-ir)	24
2.8 CRH Immunoreactive Labeling (CRH-ir)	25
2.9 FosB-ir and CRH-ir Analysis	25
2.10 Statistical Analyses	26
CHAPTER 3	
RESULTS	28
3.1 Effects of PS on Fecal GC Levels During Pregnancy	28
3.2 Effects of PS on Litter Characteristics on PD0 and PD14	28
3.3 Effects of PS and EE on PD14 Seizure Behaviour	29
3.4 Litter Effects on PD14 Seizure Behaviour	30

3.5	Effects of PS and EE on FosB-ir Density in the Limbic System.....	32
3.6	Effects of PS, EE, and Seizure on Plasma GC Levels After Seizure.....	35
3.7	Effects of PS and EE on CRH-ir Density in the PVN	35
CHAPTER 4		
	GENERAL DISCUSSION	36
	BIBLIOGRAPHY	52
APPENDIX A:		
	TABLES	66
APPENDIX B:		
	FIGURES	69

LIST OF TABLES

Table 2.1	Limbic brain regions in which FosB-immunoreactivity density was quantified	66
Table 3.1	FC non-responders per prenatal and postnatal group	67
Table 3.2	Regions in which there were significant main effects or interactions among Seizure, Prenatal Treatment, Postnatal Treatment, Sex, on FosB-immunoreactivity density	68

LIST OF FIGURES

Figure 2.1	Timeline of prenatal and postnatal treatment procedures	69
Figure 3.1	Total GCs measured in fecal samples collected from dams in NC or PS condition	70
Figure 3.2	Body weight of male and female pups at PD0 and PD14	71
Figure 3.3	Seizure behaviour in male and female pups	72
Figure 3.4	Cumulative seizure score of pups by individual dams.....	73
Figure 3.5	Relationship between pup seizure behaviour and maternal GC levels	74
Figure 3.6	FosB-ir density in various brain regions	75
Figure 3.7	Representative FosB-ir density images.....	77
Figure 3.8	FosB-ir density in various brain regions	78
Figure 3.9	Representative FosB-ir density images.....	79
Figure 3.10	Plasma GC levels in PD15 pups	80
Figure 3.11	CRH-ir density levels in NC and PS pups experiencing severe seizures.....	81
Figure 3.12	CRH-ir density levels in SC and EE pups; FC and seizure naïve.....	82
Figure 4.1	Connectivity flow-chart	83

ABSTRACT

Epilepsy, a relatively common and chronic neurological condition, affects 1-2% of the population. The underlying pathophysiology of epileptogenesis is not completely understood. To identify potential antecedents to seizure, the effects of maternal stress and environmental enrichment (EE) were investigated. Maternal stress was modeled by exposing pregnant rats to a prenatal stress (PS; an ethologically relevant predatory threat). At birth, PS and naïve control (NC) dams and litters were either maintained in standard cages or transferred to EE until postnatal day (PD) 14. A model of febrile convulsions (FC) was used to determine seizure susceptibility of all offspring. Pup brains were processed for detection of FosB (FosB-ir) from structures in the limbic system and corticotrophin-releasing hormone (CRH-ir) from the paraventricular nucleus of the hypothalamus (PVN). Our results suggest pre- and postnatal dam-dependent effects. PS increased glucocorticoid (GC) levels in dams and decreased pup birth-weights. Seizure scores on PD14 were highly individualized and litter dependent, suggesting a dam-dependent and variable effect of controlled pre- and postnatal factors. EE increased FosB-ir within the hippocampus but, in other regions, EE decreased FosB-ir. EE also significantly decreased CRH-ir in the PVN. Our results support the concept that both pre- and postnatal environmental influences affect fetal programming and neurodevelopment.

LIST OF ABBREVIATIONS USED

11b-HSD2	11b-hydroxysteroid dehydrogenase type 2
AcbC	Accumbens nucleus, core
ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
Arc	Arcuate nucleus
BLA	Basolateral amygdaloid nucleus, anterior
CRH	Corticotrophin-releasing hormone
dCA1	Dorsal CA1 hippocampus
dCA2	Dorsal CA2 hippocampus
dCA3	Dorsal CA3 hippocampus
dDG	Dorsal dentate gyrus (DG)
EE	Environmental enrichment
Ent	Entorhinal cortex
F	Female
FC	Febrile convulsions
GABA	Gamma-Aminobutyric acid
GC	Glucocorticoid
GD	Gestational day
GluR	Glutamate receptor
GR	Glucocorticoid receptor
HPA-axis	Hypothalamic-pituitary-adrenal axis
i.p.	Intraperitoneal
IEGs	Immediate early genes
KA	Kainic acid
LC	Locus coeruleus
LPS	Lipopolysaccharide
LS	Lateral septum
M	Male
Me	Medial amygdaloid nucleus
MN	Mammillary nucleus
MPA	Medial preoptic area
MR	Mineralocorticoid receptors
MRI	Magnetic resonance imaging
NAcc	Nucleus accumbens, core
NC	Naïve control
NMDA	N-methyl-d-aspartate
PBS	Phosphate buffered saline
PD	Postnatal day
Pir	Piriform cortex
PMCo	Posteromedial cortical nucleus of the amygdala
PoDG	Polymorphic layer of the dentate gyrus
PS	Prenatal stress
PVN	Paraventricular nucleus of the hypothalamus

PVT	Paraventricular nucleus of the thalamus
SN	Substantia nigra
SRSs	Spontaneous recurrent seizures
TLE	Temporal lobe epilepsy
V	Vehicle
vCA1	Ventral CA1 hippocampus
vCA3	Ventral CA3 hippocampus
vDG	Ventral dentate gyrus
WDS	Wet dog shakes

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CHAPTER 1 INTRODUCTION

Epilepsy is a relatively common neurological disorder, which affects people of all ages (Browne and Holmes, 2001), with the highest incidence of new onset seizures occurring within the first few years of life (Hauser, 1994). The etiology underlying the development of epilepsy during early life can be broadly grouped as symptomatic (hypoxic –ischemic encephalopathy, tumor, trauma), genetic (channelopathies, neurodevelopmental diseases), and cryptogenic (etiology unknown) (Manford et al., 1992, Bozzi et al., 2012). In cryptogenic epilepsies the lack of diagnostic yield despite considerable investigations suggests a problem at the molecular or ultra-structural levels consistent with clinical observations that approximately 50-60% of all people with epilepsy have no identified etiology despite extensive investigations (Manford et al., 1992). To gain a better understanding of the factors leading to the development of epilepsy, an appreciation of the pathophysiology underlying an increased susceptibility for having seizures is required. One potential approach to this question is to examine factors affecting neurodevelopment and brain organization, as these have significant influences on the adult brain and its susceptibility to disease. In the current study, we will examine the effects of prenatal stress (PS) and postnatal environmental enrichment (EE) on the development of febrile convulsions (FC).

1.1 Neurodevelopment of Epilepsy

Neurodevelopment proceeds along a course that is affected by both genetics and the environment, (reviewed in (Welberg and Seckl, 2001). Exposure to known teratogens

such as toxic exposure (Mendola et al., 2002) or injury during early life (Adams-Chapman, 2009) has well recognized effects, but the role of more subtle factors related to the prenatal environment, such as maternal stress, is less understood, (reviewed in (Bale et al., 2010). The prenatal environment is largely dictated by maternal health and experience, therefore factors that affect either parameter may influence the offspring's neurodevelopmental trajectory, neurophenotype, and risk of developing disorders of brain function. To this end, studies are continuing to demonstrate an association between an adverse maternal environment and a risk of developing disorders of brain function such as schizophrenia (van Os and Selten, 1998, Koenig et al., 2002, Khashan et al., 2008), anxiety (Watson et al., 1999, Weinstock, 2008, Laloux et al., 2012), and attention deficit disorders (Linnet et al., 2003, Rodriguez and Bohlin, 2005, Huizink et al., 2007).

Although less conspicuous than a teratogen, maternal stress is common and has diverse and long-lasting effects on neurodevelopmental outcome of offspring in a variety of species (Weinstock, 2008). Further, the high rate of co-morbid association between epilepsy and neuropsychiatric, as well as neurodevelopmental disorders (Gaitatzis et al., 2004, Tellez-Zenteno et al., 2007, Caplan, 2012), provides further support for a link between PS and seizure susceptibility and epilepsy.

Epileptogenesis, the process by which epilepsy develops, and the occurrence of febrile convulsions have been linked in both human (Rantala et al., 1995, Harkin et al., 2002, Audenaert et al., 2006, Dibbens et al., 2010) and animal studies (Holtzman et al., 1981, Morimoto et al., 1991, Germano et al., 1996, Toth et al., 1998, Jiang et al., 1999, Heida and Pittman, 2005, Schuchmann et al., 2006). Despite the evidence for febrile convulsions having a potential role in epileptogenesis, the etiology and mechanisms of

FCs remain unclear. In humans, correlational studies show that individuals experiencing prolonged (>30 min) FCs are more likely to develop epilepsy, specifically temporal lobe epilepsy (TLE), the most common type of epilepsy (Scantlebury and Heida, 2010), necessitating further research that investigates causal mechanisms.

The risk for prolonged FCs is higher in young children and rats, compared to adults, (Nelson and Ellenberg, 1976, Stafstrom et al., 1993), although this does take into account genetic factors or potential early life experiences that may increase susceptibility for seizure (Dubé et al., 2010), altering the initial set-point, or vulnerability, for FCs. With or without increased vulnerability, FCs are more common in young animals because the neo-natal brain is easily excitable (Ben-Ari and Holmes, 2006). During early life, gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the adult nervous system (Freund and Buzsaki, 1996), has excitability capabilities that persist, at least in the hippocampus, until approximately postnatal day 13 (PD13) in the rat (Khazipov et al., 2004). These atypical febrile convulsions are correlated with the development of spontaneous recurrent seizures (SRSs) following the initial seizure (Stafstrom et al., 1992). Animal models can be used to begin to unravel the complex relationship between genetic factors, pre- and postnatal experiences on FC development and even epileptogenesis.

Over and above genetic background (Gilby et al., 2009), age of onset has been shown to be an important factor in determining seizure severity and eventual epileptogenesis. In humans, fever-like symptoms occurring during the first five years of life followed by

acute seizure episodes (febrile convulsions) are common, occurring 1-14 percent of the time (Nelson and Ellenberg, 1976), and are often are often linked to epileptogenesis through this initiating event (Hauser, 1994). Normally, these episodes are mild and do not result in prolonged deficits. However atypical, prolonged febrile convulsions can have long-term effects, including increased risk of developing temporal lobe epilepsy (TLE), the most common epilepsy in humans (Sokol et al., 2003, Holmes, 2005, Dubé et al., 2007). The risk of developing epilepsy is reduced when seizures are medically controlled within two years of the initial convulsive episode (Hauser, 1994), suggesting that these early episodes contribute to the later development of epilepsy and that early treatment is most effective.

Recently, several methods of inducing febrile convulsions have shown promise in animal models of epileptogenesis and epilepsy, but there is a split among researchers regarding the best method to induce the febrile state. One method utilizes hyperthermia to increase core and brain temperatures to mimic a fever (Toth et al., 1998, Dubé et al., 2000) while the other uses a bacterial endotoxin, lipopolysaccharide (LPS), to induce fever. Issues persist with both techniques. First, fever from LPS injection cannot be reliably induced in suckling rodents (Heida et al., 2004) because some injected pups will not confer febrile convulsions. Second, vastly different mechanisms are involved in the increased temperature from fever and hyperthermia (Berg, 1993). A necessary factor in identifying valid models of epilepsy is the occurrence of SRSs. Several models of provoked epilepsy have shown evidence for SRSs, including chemically induced convulsions using pilocarpine or kainic acid (KA) (Stafstrom et al., 1992) or electrically induced

convulsions, such as those that occur during the phenomenon of kindling (reviewed in (Velíšková et al., 2009, Auvin et al., 2012)). Hyperthermia models have also shown that SRSs occur following FCs in rats (Dubé et al., 2000). When given along with the LPS, subthreshold levels of KA, compared to levels necessary for seizure in a non-febrile organism, induce seizures that most closely mimic febrile convulsions and their effects in humans, mainly characterized by a low incidence of mortality and a lack of cell death (Heida et al., 2004).

KA is a neuroexcitatory agent, derived from the seaweed *Digenea simplex* (Ben-Ari, 1985) which acts to increase glutamatergic neurotransmission and induce seizure activity by acting as an agonist at glutamate receptor 6 (GluR6) (Mulle et al., 1998) on pyramidal neurons in area CA3 of the hippocampus and, to a lesser extent, by binding to GluR5 on interneurons primarily in area CA1 (Vignes et al., 1998). This GluR binding leads to hyperexcitability, usually originating within the CA3 (Ben-Ari and Cossart, 2000) and is similar to that observed in seizures characteristic of TLE (Ben-Ari, 1985).

Hyperexcitability, which can spread throughout the hippocampus to the amygdala and several other cortical regions leads to excitotoxicity throughout these structures. With chronic over firing, excitotoxicity can lead to the development of new synapses, which increases GluR6 expression in CA3 providing the framework for future seizures (Represa et al., 1987). The excitotoxicity that follows KA induced seizure is also associated with cell death in the hippocampus (Pollard et al., 1994) and throughout the limbic system (Pereno et al., 2011).

Several methods have been used to measure the excitotoxic effects following chemically induced seizures. One common method is the measurement of immediate early genes (IEGs) responsible for recruiting transcription factors in response to cellular activity. C-jun, C-fos, and FosB are among the most well known IEGs (Herdegen and Leah, 1998). These proteins are part of the AP-1 transcription factor complex, are associated with long-term plasticity, and can remain elevated following initial status epilepticus (Morris et al., 2000). FosB activation remains elevated for up to one year following KA treatment (Morris et al., 2000), indicating an increase in baseline levels of transcription following epileptogenesis and supporting its validity as a marker for KA-induced seizure.

The activation of IEGs in specific brain regions enables tracking of affected areas and thus can provide insight regarding specific regions that might be more affected by the effects of excitotoxicity (Peng and Houser, 2005). The hippocampus, specifically the dentate gyrus, has been implicated as the 'gate', responsible for protecting the limbic system from excitotoxicity and synchronized firing (Heinemann et al., 1992). For this reason, our current understanding of the effects of increased IEG activity in the hippocampus is greater than many other areas. Chemically induced seizures result in neurodegeneration of neurons in the hilus of the dentate gyrus and pyramidal neurons in CA3. These regions, especially the granule cell layer of the dentate gyrus, also show the highest activation of IEGs (Silveira et al., 2002, Peng and Houser, 2005) although IEG (Fos) activation is also elevated in the amygdala, especially when seizure-like behavior is present (Silveira et al., 2002). Fos activation in the amygdala, as in the hippocampus, has been linked to neurodegeneration (Pereno et al., 2011).

1.2 HPA-axis

In mammals, arousal, for example from perceived threat (Figueiredo et al., 2003), pain (Palkovits et al., 2001), or fear conditioning (Van de Kar et al., 1991), is registered through excitatory and inhibitory inputs from sensory and threat processing areas to the paraventricular nucleus (PVN) of the hypothalamus. From hypophysiotropic neurons in the medial parvocellular division of the PVN, corticotropin-releasing hormone (CRH) is synthesized and transported via the hypophysial portal veins at the level of the median eminence to receptors in the anterior pituitary gland. This stimulates the release of adrenocorticotrophic hormone (ACTH) into circulation which, when bound to receptors in the adrenal glands, causes the release glucocorticoids (GCs) into the blood stream (Antoni, 1986, Whitnall, 1993, Herman et al., 2005).

Ultimately, GCs bind to receptors throughout the body to prepare an organism's 'flight or fight' response. Specifically, within the hypothalamic-pituitary-adrenal-axis (HPA) axis and limbic system activation of these receptors regulate negative feedback loops responsible for returning the organism to homeostasis. The two main receptors for GCs are the glucocorticoid receptor (GR) and mineralcorticoid receptors (MR). GRs are highly expressed in the brain and have a relatively low affinity to GCs (5-10 nM) and are typically bound only during times of high GC secretion (Reul and de Kloet, 1985). MRs have a much higher affinity (25-100 nM) and are quickly saturated by high stress levels of GC exposure (Reul and de Kloet, 1986). Based on these receptor attributes, research has provided ample evidence that GRs predominately mediate GC feedback following

stress while MRs are involved in regulating basal HPA-axis activity (de Kloet et al., 1998).

Three levels of negative feedback for the HPA-axis exist; fast, intermediate, and slow, (reviewed in (Keller-Wood and Dallman, 1984). Fast feedback involves receptors in the PVN responding to GCs on the cell membrane (Di et al., 2003), which decreases the secretion of CRH and ACTH from the PVN and anterior pituitary, respectively.

Intermediate feedback also acts to decrease the release of CRH and ACTH, as well as decreasing the synthesis of CRH. This is accomplished, again, by GCs binding to membrane receptors. Importantly, the ACTH ‘pool’ is not affected by intermediate feedback, suggesting that precursor synthesis is not affected. Finally, slow feedback decreases the ACTH ‘pool’ by restricting the transcription of pro-opiomelanocortin, an ACTH precursor. Slow feedback may also act in the regulation of circadian driven increases in GCs.

Clearly, the limbic system plays a key role in FCs and epileptogenesis. This system also plays a key role in regulating the HPA-axis, (reviewed in (Herman et al., 2005, Maguire and Salpekar, 2013). The most common view is that the hippocampus inhibits HPA-axis activity (Jacobson and Sapolsky, 1991), as a high number of GRs and MRs found in the hippocampus (Reul and de Kloet, 1985, 1986). MRs, responsible for basal HPA-axis functioning, are found throughout the brain. GRs, on the other hand, are found predominantly within the hippocampus, supporting the theory that the hippocampus is crucial for regulating the HPA-axis following stress exposure (Reul and de Kloet, 1985,

1986). Furthermore, the hippocampus plays a crucial role in neurogenesis (discussed below) and if this process is inhibited, the regulation of the HPA-axis will also be disrupted (Schloesser et al., 2009).

Aside from the hippocampus, the amygdala also plays a role in modulating activity of the HPA-axis. Depending on the specific sub-nucleus activated, electrical stimulation has varying effects on HPA-axis activation, (Dunn and Whitener, 1986), as measured by plasma GC levels. In fact, amygdala activation is more efficacious in stimulating HPA-axis activity than direct stimulation of hypothalamic nuclei around the CRH-producing PVN (Redgate and Fahringer, 1973). Like the hippocampus, the amygdala has a high number of GRs, further implicating its role in regulating negative feedback in the HPA-axis (Arriza et al., 1988).

1.3 Prenatal Stress

Epidemiological human research has uncovered several associations between disease and chronic maternal stress. Children born following a severe ice storm (Laplante et al., 2004), in New York post 9/11 (Yehuda et al., 2005), or to mothers with depression (Davis et al., 2007), or chronic or severe stress during pregnancy present with increased susceptibility to anxiety disorders and dysregulation of the HPA axis. Animal studies have shown that the prenatal period is critical for normal programming throughout the brain, including the HPA axis (Koehl et al., 1999) and the serotonergic system (Peters, 1989). Stressful life events (Entringer et al., 2009) or even perceived stress (Tollenaar et

al., 2011) in humans, or restraint, bright light and heat in rats (Williams et al., 1999), can result in abnormally high levels of stress hormones and result in negative birth outcomes, including HPA axis dysregulation in offspring, reduced birth weight, and propensity to disease (Champagne and Meaney, 2006). It is important to note, however, that the effects of PS on neurodevelopmental disorders is highly malleable, and can be influenced by a number of factors such as maternal genotype (Jones et al., 2011, Neeley et al., 2011), offspring sex (Mychasiuk et al., 2011a, Schulz et al., 2011), exposure timing (Edwards et al., 2002a) and the intensity of the stressor (Mychasiuk et al., 2011b). Recent research has focused on the importance of the type of stressor and significant differences have been documented in the neural and behavioural responses to physical and psychosocial stressors (Bowers et al., 2008). This is particularly notable in PS paradigms (Pohl et al., 2007). Most prior studies (Edwards et al., 2002a, Sadaghiani and Saboory, 2010, Qulu et al., 2012) have used physical stressors such as restraint but it can be argued that psychosocial stressors are more representative of 'natural' stress exposure, and perhaps also a better model of typical stressors experienced by pregnant women. In comparison, predator odour exposure has been shown to increase anxiety behaviour in tests such as social interaction, elevated plus maze, or hole board test, (reviewed in (Blanchard and Blanchard, 2003).

Under normal conditions prenatal HPA axis activation appears to be adaptive by increasing an organism's ability to cope with the environment in adulthood (Fujioka et al., 2006). Over-activation of GRs and MRs at the fetal stage, as occurs during exposure to PS, disrupts normal development and subjects the offspring to many serious health risks, ranging from anxiety (Salomon et al., 2011) and depression (Butkevich et al., 2009)

to epilepsy (Ahmadzadeh et al., 2011) and diabetes (Simmons et al., 2001). At the molecular level, prenatal overstimulation of GCs can severely alter the transcription of several genes, crucial for axonal growth, regulation of ion channels and transporters, trafficking synaptic vesicles and neurotransmitter release (Welberg and Seckl, 2001, Bogoch et al., 2007). PS impacts HPA-axis development (Henry et al., 1994) and can directly impact the development of fetal PVN neurons (Fujioka et al., 1999). Developmentally, PVN neurons contain GRs as early as gestational day 14 (Yi et al., 1994). This early development of the HPA-axis highlights the importance of fetal programming and early life experiences. For example, PS increases CRH levels and results in downstream adrenal deficits, reducing plasma GCs (Chung et al., 2005), suggesting another route for HPA-axis dysregulation. This includes increased apoptosis along with decreased length and branching of the dendritic processes in CRH-containing neurons of the PVN. CRH immunoreactivity is also decreased in the median eminence following chronic PS, providing further evidence that chronic stimulation of the HPA axis may be responsible for disrupting normal function (Fujioka et al., 1999). Fetal exposure to GCs has also been shown to decrease transcription of MR and GR receptors throughout the limbic system, severely altering negative feedback of the HPA-axis, resulting in elevations of baseline GCs (Henry et al., 1994, Chung et al., 2005). Several functional and morphological changes throughout the limbic system follow chronic up-regulation of GCs, including significant decreases in hippocampal volume in humans (Buss et al., 2007), decreased hippocampal pyramidal neurons (Martinez-Tellez et al., 2009) and decreased branching and length of these pyramidal neurons in animal models (Fujioka et al., 2006). These changes are associated with decreased cognitive function, measured by

performance, in both learning and memory tasks. One important factor involved in decreasing hippocampal volume is reduced neurogenesis, typified by inhibited mossy fiber sprouting within the sub granular zone of the dentate gyrus (Mandyam et al., 2008). PS inhibits neurogenic growth in primates (Coe et al., 2003) and rats bred for high anxiety, though this may be related to a deficiency in the enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (11-beta-HSD2) in high anxiety rats, thus increasing fetal exposure to GCs. Briefly, 11-beta-HSD2 is responsible for catalyzing the inactivation of maternal GCs, so failure to produce normal levels of 11b-HSD2 may leave the fetal brain vulnerable to the deleterious effects of maternal GCs (Lucassen et al., 2009), program receptor levels in the amygdala (Welberg et al., 2000), and impact birthweight (Harris and Seckl, 2011).

One hallmark biomarker for long-term effects of PS is alterations in birth weight. Several studies have linked low birth weight to increased susceptibility to disease, although the mechanisms linking birth weight with disease outcomes remain to be elucidated. In humans, reduced birth weight is associated with prenatal teratogen exposure, maternal stress (Rice et al., 2010), and FCs (Greenwood et al., 1998). Reported levels of maternal stress in an *in vitro* fertilization study showed that both related and unrelated mothers' stress levels were correlated with birth weight, gestational length, anxiety and antisocial behavior (Rice et al., 2010). In animals, more direct studies are possible that aim to uncover causal relations. Decreased birth weight as a result of PS has been demonstrated in several species (Wadhwa et al., 1993, Welberg et al., 2000, Ahmadzadeh et al., 2011). In primates (Mustoe et al., 2012) natural variations in circulating GCs have been

associated with decreased birth weight. Further, studies have used PS or synthetic GCs (dexamethasone) to increase fetal exposure to stress hormones, which leads to decreased birthweight in animal models (Nyirenda et al., 2001, Ahmadzadeh et al., 2011), and humans (Bloom et al., 2001, Murphy et al., 2012). Along with birth weight, significant alterations in brain morphology, especially within the HPA axis have been observed.

Cognitive deficits seem to be linked to morphological disruptions associated with PS. Epidemiological human studies have shown cognitive deficits in learning and memory (Gutteling et al., 2006), as well as delayed intellectual and language development following PS (Laplante et al., 2004). Magnetic resonance imaging (MRI) studies reveal reductions in hippocampal volume and implicate HPA axis over-activation as being a deleterious agent in cognitive tasks (Buss et al., 2007). Similar deficits in learning have been shown in rats, measured with the Morris water maze (Yang et al., 2007). Again, decreases in hippocampal volume were associated with these deficits in cognitive tasks (Yang et al., 2007). Further, behavioural responses to stress can be passed on to offspring, as an effect of epigenetics and maternal care (Francis et al., 1999). Indeed, PS could exert many of its effects via altered maternal care (Champagne and Meaney, 2006), which has a significant impact on hippocampal and cognitive development (Liu et al., 2000, Bredy et al., 2003a).

1.4 Prenatal Stress and Epilepsy

In addition to the above effects on the structure and function of the limbic system and its involvement in stress responding, PS also affects the severity, rate and overall development of epilepsy (Edwards et al., 2002a, Sadaghiani and Saboory, 2010, Qulu et al., 2012). This is not surprising since it is well established that the HPA-axis plays a crucial role in epileptogenesis and seizure outcome. In humans, life stress or feeling stressed is a commonly reported antecedent to seizure (Nakken et al., 2005). Molecular data also support this idea. Children with generalized epilepsy show increased levels of CRH, CRH-binding protein (CRH-BP) and the CRH receptor (CRH-R1) (Wang et al., 2001). In young animals, CRH-R1 is the first receptor bound during stressful experiences (Baram et al., 1997a). Intra-ventricular administration of CRH produces seizure behavior in adult (Marrosu et al., 1988) and juvenile rats (Baram and Schultz, 1991). The ensuing seizure significantly raises plasma GC levels (Baram and Schultz, 1991).

Prior work demonstrated that repeated restraint stress during the last week of gestation lowers the after-discharge threshold and increases the kindling rates in postnatal day (PD) 14 male and female pups, and in adult males (Edwards et al., 2002a). In addition, maternal PS increases the severity, and the risk of mortality, in pilocarpine-induced seizures, particularly in male pups (Sadaghiani and Saboory, 2010), and the severity of evoked seizures in a model of febrile convulsions (Qulu et al., 2012).

1.5 Environmental Enrichment

Clearly, the limbic system can be shaped by events in the early environment. In addition to negative effects observed after PS, positive effects of the early environment can also be observed in the developing nervous system. One of the more commonly recognized influences on neurodevelopment is environmental enrichment (EE) (Chapillon et al., 2002, Hellemans et al., 2004). EE can reverse the effects of poor maternal care (Bredy et al., 2003a) or maternal separation (Francis et al., 2002), buffer stress responding (Larsson et al., 2002), promote neurogenesis (Pham et al., 1999), and reduce seizure susceptibility (Korbey et al., 2008). In several models, EE results in increased basal levels of dentate granule cell layer neurogenesis (Kempermann et al., 1997), increased levels of surviving neurons stemming from the granule cell layer (Nilsson et al., 1999), as well as recovery of normal behaviour in both infant and adult animals (Francis et al., 2002, Bredy et al., 2003b, Morley-Fletcher et al., 2003, Fox et al., 2006). In adult animals, EE promotes neurogenesis following several different traumatic experiences, including psychological stress (Yang et al., 2007), malnutrition (Katz and Davies, 1983), ischemia (Komitova et al., 2002) and induced seizure (Auvergne et al., 2002).

In addition to determining risk factors that may increase seizure susceptibility, there is significant clinical and laboratory interest in identifying factors that may ameliorate these deleterious influences. In adult rats, EE has been shown to prevent or delay the development of seizures (Young et al., 1999, Auvergne et al., 2002, Korbey et al., 2008), as well as prevent the development of depressive symptoms after seizures early in life

(Koh et al., 2007). Most research to date in this area has focused on prevention in adults, or on recovery after initial status epilepticus, (reviewed in (Dhanushkodi and Shetty, 2008), while fewer studies have focused on an earlier prevention/intervention. Though, the possibility for EE to function as an early preventative is convincing, as it is known to have a number of effects on hippocampal neurogenesis, including recovering the capacity for long-term potentiation following PS (Yang and Lee, 2008) and recovering deficits induced by PS (Lemaire et al., 2006) in early life, especially as many of these factors are also involved in the development of seizures.

Having a heightened potential for neural plasticity, infant animals have increased levels of neurogenesis and behavioural recovery with EE. Many trauma models discussed above have also been effective in infant animals that were exposed to psychological stress (Morley-Fletcher et al., 2003, Lemaire et al., 2006), malnutrition (Katz and Davies, 1983), hypoxia (Pereira et al., 2007), and chemically induced seizure (Young et al., 1999). Seizures promote neurogenesis (Nakagawa et al., 2000, Kuruba et al., 2009). Increased activity in dentate progenitor cells and aberrant sprouting of mossy fibers, characteristic of atypical hippocampal reorganization (Parent et al., 1997), suggests that brain morphology may be altered following status epilepticus, although many of the mossy fiber sprouts in the hippocampus may be vulnerable to degeneration (Covolan et al., 2000). To disentangle the concurrent effects of EE and epileptogenesis on seizure susceptibility and neurogenesis, rats were raised in EE, in isolation, and in isolation until kindling procedures were started (PD 28) and found that EE raised rats were more resistant to kindling than those that were isolated or those that only experienced the

enriched environment during the kindling procedure (Auvergne et al., 2002). However, both EE procedures produced a significant increase in new dentate neurons, suggesting that preconditioning was key in reducing epileptogenic manifestation and that EE promotes healthier dispersion of dentate granule cells and interrupts epileptogenesis. Following status epilepticus on PD21, EE recovers cognitive performance without affecting seizure threshold in adulthood rats (Wang et al., 2007).

The effects of both prenatal and postnatal experiences, especially their effects on the HPA-axis and its regulation by the limbic system, lays a framework for understanding the regions and systems involved in post-seizure hyper-excitability. In this paper, we investigate the role of EE from birth in reducing/preventing vulnerability to initial febrile convulsions, both as a stand-alone treatment and as a potential ‘rescue’ measure to prevent effects potentially induced by PS. Specifically, we expect to see increases in fecal GC levels of predator exposed dams as well as decreased birth weight in the offspring of these dams. Next, we expect to see an interaction, indicating rescued weight at PD14 of EE raised pups. During seizure, we expect increased seizure behaviour in PS pups, though some buffering by EE. Following seizure, we expect neural activity, measured with FosB, to be increased, especially within the limbic system. Further, we anticipate an increase in stress hormones of the HPA-axis in PS conditions, especially following severe seizures.

CHAPTER 2 METHODS

2.1 Animals and Breeding

Twenty-four female Long-Evans hooded rats (Charles River Canada, Quebec) were ordered at 60 days of age and allowed to acclimate to the colony room for at least 2 weeks prior to breeding. Rats were housed in same-sex pairs upon arrival, but individually once breeding began. After acclimation, females' estrous cycles were monitored daily with vaginal smears and cytological analysis. Females in proestrus were paired overnight with a sexually experienced Long-Evans male from our colony. Successful mating was confirmed the next morning by the presence of sperm in vaginal smears, and was considered gestational day 0 (GD0). Mated females were then removed from males' cages and rehoused with one other recently inseminated female until GD12. At that point, females were singly housed and remained singly housed with their litters throughout the remainder of the experiment. Rats were housed in polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids, containing pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada) and a black piece of PVC tubing (12 cm length, 9 cm diameter). Rat chow (Purina Lab Chow) and tap water were available *ad libitum*. A 12h:12h reversed light cycle (lights off at 07:00 h) was maintained in the colony room, which was kept at a temperature of $21^{\circ}\pm 2^{\circ}\text{C}$. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals. An effort was made to use the

minimum number of animals required for statistical comparison to minimize pain and suffering in experimental subjects.

2.2 Prenatal Stress Exposure and Postnatal Environmental Enrichment

The experimental timeline for perinatal manipulations is shown in Figure 3.1. Starting on GD13, pregnant females were randomly assigned to one of four experimental conditions using prenatal naïve control (NC) or prenatal predator stress (PS), and postnatal standard cage (SC) or postnatal environmental enrichment (EE), with groups as follows; 1) PS/SC (n=6) 2) PS/EE (n=6) 3) NC/SC (n=6) 4) NC/EE (n=6). From GD13-GD20 dams in the PS groups were exposed 3 times a day (30 min/exposure; 08:30h, 11:30h, 14:30h) to a predatory threat. During each predator exposure, PS dams were first taken to a designated anteroom and placed in clean cages to avoid any contamination of the home cages and colony rooms with volatile predator odour cues. Dams were then transported to one of the three cat colony rooms in our department and their cages were placed on the floor, allowing the cats to investigate. All exposures took place under red light. During each exposure period, dams designated as NC were similarly placed in clean cages but were maintained in the colony room under red light for the duration of the 30 min. For both PS and NC dams, at the completion of the 14:30h exposure period (PM), fecal boli were collected from the cage and stored at -20°C for future assay. As circulating glucocorticoids (GCs) take approximately 6 hours to be excreted in the feces (Cavigelli et al., 2005), fecal boli deposited during the 30 min exposure period from 14:30 - 15:00h represent circulating levels at the time of the first exposure of the day from 08:30 –

09:00h. After each exposure period, PS and NC dams were returned to their home cages and the colony room.

All dams were checked daily for pups starting on GD20 near the start of the dark cycle. On the day of delivery, designated postnatal day 0 (PD0), litters were sexed, counted and weighed. This was performed quickly to minimize pup handling. Dams and pups designated to receive postnatal EE were transferred to enrichment cages at this time and remained in EE until the end of the experiment (PD15). EE cages are approximately 2.5x the volume of standard cages and EE cages are divided into an upper section (50.5 cm x 50.5 cm x 33.5), where food and water are available *ad libitum*, as well as a PVC tube, and a lower section (50.5 cm x 50.5 cm x 14 cm) simulating a burrow. Dams and pups designated as SC controls were transferred to a fresh standard cage (described in section 2.1) on PD0.

2.3 Fecal Glucocorticoid Extraction and Assay

Circulating levels of the GCs were estimated in both NC and PS females by examining fecal GC in samples taken on GD 13, 15, 18 and 20. In the rat, corticosterone is metabolized via two pathways, and in addition to corticosterone, the major metabolites tetrahydrocorticosterone and 11-dehydrocorticosterone are also present in fecal samples (Cavigelli et al., 2005). During extraction, samples were allowed to thaw to room temperature and the number of fecal boli and the wet mass (g) of each sample was recorded. The samples were frozen in liquid nitrogen, manually homogenized using a

mortar and pestle, after which 0.1g of sample was combined with 1ml of 80% methanol, vortexed for 30 min, and centrifuged at 2500xg for 15 min. The resulting supernatant was collected and stored at -80°C. Corticosterone metabolites were quantified from supernatant, diluted 1:40 in duplicate using Assay Design's Corticosterone correlate EIA™ Kit (Assay Designs, Michigan, USA) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation for this kit range between 6.6 - 8.0% and 7.8 - 13.1%, respectively. This assay kit had the following cross-reactivities with steroid compounds of importance to fecal sampling: corticosterone 100%; 11-deoxycorticosterone 28.6%; tetrahydrocorticosterone 0.18%.

2.4 Febrile Convulsions – PD14

On PD14, eight pups (4M/4F) from each dam were randomly selected for seizure testing and those pups were then randomly assigned to either febrile convulsion (FC; n=3M, 3F) or vehicle (n=1M, 1F) groups. Seizure induction took place at the start of the light cycle to allow for better visibility of convulsive behaviours. Pups were first injected intraperitoneally (i.p.) with 200 µg/kg of LPS (Sigma-Aldrich: MO, USA; dissolved in sterile saline; FC group) or a saline vehicle (V), and marked with permanent non-toxic ink marker for easier video identification and returned to their respective dams. After two hours, pups were separated from dams and placed into clean cages in groups of 3-4 littermates, and FC pups were injected i.p. with 1.75 mg/kg kainic acid (A.G. Scientific: CA, USA; dissolved in sterile saline) 2.5 hours after injection with LPS (Heida et al., 2004). All pups were monitored during seizure provocation and videotaped for 180 min

for later scoring of convulsive behaviours as described below. Cages were placed in an isolated chamber with the temperature maintained at approximately 30°C for the duration of the testing to approximate nest temperature (Spiers and Adair, 1986). Pups were then returned to their home cages, with their dams, for the 24 hours after the initial injection, and any mortality was recorded.

Videotapes of all pups were analyzed for convulsive behaviours according to a previously defined scale (Racine, 1972) and further developed by others (DAmbrosio and Miller, 2010). Non-seizure PD14 behaviours included sleeping (with normal reactivity to the external environment), grooming, exploration, and brief non-repetitive paroxysmal movements such as myoclonic jerks. Seizure behaviours consisted of repetitive brief paroxysms such as myoclonus or wet dog shakes (WDS), stereotypies such as continuous non-directed scratching movements, chewing automatisms, repetitive blinking, ataxia, and seizure like behaviours including tonic, clonic, and tonic-clonic limb or jaw movements. Animals that were immobile were assessed for reactivity and if found to be non-reactive were included in the severe category. For each 5-minute bin, a score was assigned based on the most severe behaviour present in that bin, using the following schema: 0 = normal behaviours; 1 = repetitive myoclonus, wet dog shakes (WDS), staring, mouth clonus, facial automatisms; 2 = continuous scratching-like hind limb movements with preserved reactivity; 3 = continuous scratching with intermittent periods of tonicity, non-reactivity or ataxia; 4 = periods of prolonged unresponsiveness with intermittent expression of stereotypies; 5 = persistent tonicity, tonic-clonic movements, bicycling behaviour, or non-reactivity; 6 = death. The scores for each 5-min bin were summed across 180 min to provide a total seizure score for each pup. The number of bins

in which pups had shown any seizure behaviour (score of 1 or higher) was defined as seizure duration. Finally, the severity of seizures were assessed by categorizing seizures as severe (scores of 4-5), moderate (scores of 2-3) or mild (score of 1) and summing the number of bins of each type of seizure for each animal. Two trained observers, who were blind to the pups' treatment group, completed all behavioural scoring and their scores were averaged to determine each pups' final seizure score. An inter-rater reliability analysis using the Kappa statistic was performed to determine consistency between raters, the inter-rater reliability for the raters was found to be $Kappa = 0.323$ ($p < 0.001$).

2.5 Sacrifice and Tissue Generation

Twenty-four hours after seizure initiation (PD15), pups were sacrificed with an overdose of euthanyl (i.p.) and perfused transcardially for 5 min with 0.9% NaCl (at 4°C) followed by 100 ml of 4°C paraformaldehyde PFA (4%) in 0.2 M phosphate buffered saline (PBS; pH 7.2). Blood was taken by a tail vein puncture at this time. Dissected brains were post-fixed overnight in 4% PFA at 4°C and then placed for at least 2h in a cryoprotectant solution of 0.01M PBS (pH 7.2) containing 20% sucrose, before being frozen in vapors of liquid nitrogen and preserved at -80°C. Free-floating serial coronal cryostat sections (35 μ m) were collected and preserved in 1x Millonigs phosphate buffer fixative.

2.6 CRH-ir Groups

Tissue used for CRH-ir experiments consisted of 166 offspring from 22 dams. Although most of the same dams that were used for FosB-ir are represented here, there are some

differences, and thus, basic physiological variables such as dam GC levels and birth weight are reported again here including only animals from whom tissue was used for CRH-ir processing.

2.7 FosB Immunoreactive Labeling (FosB-ir)

For protein immunohistochemical processing, sections were rinsed in PBS containing 0.1% Triton-X (PBS-T, 3 x 10 min), then incubated for 45 min with agitation at room temperature in 0.2% PBS-T containing 1% H₂O₂ to inactivate endogenous peroxidases. After rinsing (0.2% PBS-T, 3 x 10 min), sections were incubated for 72 hours at room temperature under agitation with the rabbit anti-FosB (Santa Cruz Biotechnologies, Santa Cruz, California) primary antibody diluted 1/1000 in 0.1% PBS-T and 2% Normal Goat Serum (Mandel). Next, sections were rinsed (0.1% PBS-T, 3 x 10 min) and incubated for 60 min at room temperature with a biotinylated goat-anti-rabbit secondary antibody (Vector Laboratories, Burlington, Ontario) diluted 1/500 in 0.1% PBS-T. Sections were rinsed (0.1% PBS-T, 3 x 10 min), then incubated 90 min at room temperature in the ABC complex (Vectastain, Vector Laboratories, Burlington, Ontario) diluted 1/1000 in 0.1% PBS-T. After rinsing (0.1% PBS-T, 3 x 10 min), visualization was achieved using 0.05% diaminobenzidine diluted in 0.1% PBS-T + 0.003% H₂O₂. Sections were then rinsed (0.1% PBS, 2 x 10 min) and put into 1X Millonigs and stored at 4°C until mounted.

Tissue was poured into a 25:25:50 mix of PBS-T, PBS, and distilled water to be mounted on gelatin coated slides. After mounting, tissue was allowed to dry for 48-96 hours. Once

dried, slides were dehydrated, immersed in xylene, air-dried at room temperature, and coverslipped using Cytoseal (Thomas Scientific, Swedesboro, NJ, USA).

2.8 CRH Immunoreactive Labeling (CRH-ir)

The same protocol described for FosB-ir was utilized for CRH protein immunohistochemical processing but, sections were incubated for 24 hours at 4°C under agitation with the with the rabbit anti-CRH (Gift from Paul E. Sawchenko, Salk Institute) primary antibody diluted 1/10,000 in 0.1% PBS-T and a biotinylated goat-anti-rabbit secondary antibody (Vector Laboratories, Burlington, Ontario) diluted 1/500 in 0.1% PBS-T.

2.9 FosB-ir and CRH-ir Analysis

Table 2.1 contains a list of the brain structures analyzed and the corresponding anatomical coordinates. Immunostained sections were examined with a light microscope (Nikon Eclipse E400) for each area of interest. Briefly, RGB images were captured and digitized by a high-resolution CCD video camera system (Nikon DXM1200) and then converted into 8-bit grayscale images. The grayscale images of a representative image for each area of interest were then thresholded to identify positive nuclei by referencing the raw brightfield sections. Once established, these threshold parameters remained unchanged throughout the analysis of each area, except for when image brightness varied between sections. Quality control for accuracy of the generalized thresholding parameters was done using random sections and no discrepancies were found. Therefore the parameters

were consistent for each brain area across all animals. Density analysis was performed using NIH imaging software (ImageJ, v10.2, NIH, USA) with the number of immunoreactive nuclei counted and the specific area of analysis noted for each section. Density measures were then calculated as a function of the number of immunopositive nuclei relative to the region's area. All counts were completed by an experimenter blinded to treatment group. Data were expressed as the mean number of positive nuclei per mm² of surface area.

2.10 Statistical Analyses

Differences between groups of dams in body weight gain and litter size were ascertained using separate Student's t tests. As fecal samples were collected during a defined 30-minute period, some dams (particularly in the NC condition) did not produce samples in the allotted time. As a result, repeated measures ANOVA was not feasible for these data and Student's t-tests were used to analyze differences between NC and PS dams on each day separately. Pup body weight on PD0 was analyzed using ANOVA, with prenatal treatment (NC or PS) and sex (M or F) as between-subject factors. Analyses of PD14 pup body weight, total seizure score, seizure duration, and seizure severity were performed using ANOVA with prenatal treatment, postnatal treatment (SC or EE) and sex as between-subject factors. Differences in FosB for each brain regions were assessed using 4-factor ANOVAs with prenatal treatment, postnatal treatment, sex, and seizure group (Mild, Moderate, High; as defined above) as between-subject factors. In cases of significant interactions, simple effects analyses were performed. Details of other analyses

are outlined in the appropriate section below. In all cases, an alpha level of 0.05 was considered an acceptable error level.

CHAPTER 3 RESULTS

3.1 Effects of PS on Fecal GC Levels During Pregnancy

PS dams had significantly higher evening GC levels compared to NC dams on Day 1 ($t(19) = 4.69, p < .0001$) and Day 3 ($t(14) = 2.17, p = .02$) (Fig. 3.1). However there were no significant differences between the maternal groups at Day 6 ($t(13) = 0.95, p = .18$) or Day 8 ($t(14) = 1.63, p = .06$). The two conditions show different slopes; a steady decrease in GC levels in the PS dams, consistent with our expectations of habituation, and relatively flat, stable GC levels in NC dams.

3.2 Effects of PS on Litter Characteristics on PD0 and PD14

All dams gained a similar amount of weight during pregnancy (NC = 149.3 ± 14.2 g vs. PS = 154.3 ± 8.8 g) and litter size was also similar between the two groups of dams (NC = 14.8 ± 0.9 pups vs. PS = 14.5 ± 1.1 pups) as well as sex distribution of pups; females (NC = $7.36 \pm .58$ F vs. PS = $6.27 \pm .62$) and males (NC = $7.55 \pm .62$ vs. PS = $8.73 \pm .59$). On PD0, male offspring weighed significantly more than females ($F(1, 40) = 9.17, p = .004$) (Fig. 3.2A) and pups, of both sexes, born to PS dams weighed significantly less than those born to NC dams ($F(1, 40) = 21.85, p < .0001$) (Fig. 3.2A).

Males still outweighed females significantly on PD14 ($F(1, 116) = 6.62, p = .01$) (Fig. 3.2B-C) and there was a significant interaction between PS and EE ($F(1, 116) = 23.36, p$

< .0001). Post hoc analyses revealed that male and female pups born to PS dams housed in SC weighed significantly less than male and female pups born to PS dams housed in EE ($p < .05$) (Fig. 3.2B-C). Additionally, female pups born to NC dams housed in EE weighed significantly less than female pups born to NC dams housed in SC ($p < .05$) (Fig. 3.2C).

3.3 Effects of PS and EE on PD14 Seizure Behaviour

EE significantly decreased the total seizure score (Postnatal Treatment main effect, $F(1,7) = 3.99, p = .048$) (data not shown). Analysis of the profile of each group revealed a higher percentage of females to be non-responders (defined as pups that did not achieve a seizure score > 1 at any time) (Table 3.1). Pearson's chi-square analysis of the proportion of non-responders revealed a trend towards more non-responders in the EE groups, ($\chi^2 = 2.89, p = .08$), and more of these pups were females, ($\chi^2 = 4.87, p = .068$).

When all non-responders were excluded from analyses there were no significant effects of Prenatal Treatment, Postnatal Treatment, or Sex on either total seizure score or seizure duration, but there was a significant difference in the amount of time spent in severe seizures ($F(1,7) = 5.17, p = .025$). As previous analyses of overall duration suggested there is an important role of sex, males and females were also analyzed separately. In males, there were trends towards a Prenatal Treatment X Postnatal Treatment interaction for moderate, ($F(1,7) = 3.43, p = .069$) (see Fig. 3.3A), and severe seizures, ($F(1,7) = 2.93, p = .09$) (Fig. 3.3B). In females, there was no effect of PS, but animals in the EE

group had a significantly lower time spent in severe seizures (Postnatal Treatment main effect; $F(1,7) = 5.56, p = .022$) (Fig. 3.3B).

3.4 Litter Effects on PD14 Seizure Behaviour

A high degree of variability was observed in the effects of prenatal and postnatal treatment on seizure behaviour that appeared to be dependent on litter (dam). Therefore, within each treatment group, a univariate ANOVA examining the influence of Dam on total seizure score, and duration of seizures, was performed.

Naïve Control/Standard Cage Dams: In NC/SC dams, there was a significant main effect of Dam on total seizure score, ($F(1,4) = 12.03, p < .0001$) (Fig. 3.4A) and for seizure duration, ($F(1,4) = 2.69, p = .047$). Pairwise comparisons revealed that the litter from Dam 9 had significantly lower total seizure scores than pups from all other NC/SC litters except Dam 24, (all p 's $< .001$). No significant pairwise comparisons for seizure duration were found.

Naïve Control/Environmental Enrichment Dams: In NC/EE dams, there was a significant main effect of Dam on total seizure score, ($F(1,4) = 5.46, p = .0012$) (Fig. 3.4B).

Pairwise comparisons revealed that the litter of Dam 21 had significantly lower seizure scores than pups from Dams 1, 10, and 17 (p 's $< .01$). Likewise, the time spent in seizures showed a litter-dependent effect ($F(1,4) = 3.17, p = .017$) (data not shown) and pairwise comparisons confirmed that pups from Dam 21 spent less time engaged in seizure behaviours relative to pups from Dams 10 and 17 (p 's $< .05$) (data not shown).

Prenatal Stress/Standard Cage Dams: In PS/SC dams, there was a significant main effect of Dam for total seizure score ($F(1,4) = 9.70, p < .0001$) (Fig. 3.4C), but no effect on the duration spent in seizures. Pairwise comparisons revealed that pups from Dam 4 had significantly higher total seizure scores than pups from all other PS/SC dams ($p < .05$ for all except $p < .001$ for Dam 23) while pups from Dam 5 had higher total seizure scores than Dam 23 ($p < .05$).

Prenatal Stress/Environmental Enrichment Dams: In PS/EE dams, there was a significant main effect of Dam on total seizure score, ($F(1,4) = 4.20, p = .006$) (Fig. 3.4D), but no effect for duration of time spent in seizures. Pairwise comparisons revealed that pups from Dam 8 had significantly higher total seizure score compared to the litters from Dam 12 and Dam 19 (p 's $< .05$).

Correlational analyses between dam GC levels and pup's behavioural seizure scores were also affected by prenatal condition. The relationship between seizure scores and maternal GC levels in offspring of PS dams was best fit by a curve predicted by a second-order polynomial equation ($df = 53, r^2 = 0.319$) (Fig. 3.5B) with low and high maternal GCs being associated with mild seizure behaviour and medium maternal GCs correlating with more severe seizure behaviours. Meanwhile, the relationship between seizure behaviour and maternal GC levels in offspring of NC dams was not strongly predicted by a linear or a second-order equation ($df = 53, r^2 = 0.023$) (Fig. 3.5A). A comparison test of the goodness of fit of the curves from the PS vs NC offspring revealed that they were significantly different ($p = 0.025$).

3.5 Effects of PS and EE on FosB-ir Density in the Limbic System

Table 3.2 summarizes the highest order effects in regions that showed significant effects, and a list of all brain regions analyzed is in Table 2.1.

Effects of Seizure: The only brain region in which the highest order effect was a main effect of Seizure was the PVN ($F = 4.230, p = .018$) (Table 3.2). Post hoc analyses revealed that FosB-ir density was significantly greater in the PVN of pups that had experienced moderate or severe seizures relative to mild ones.

There were a number of inter-related limbic regions in which seizure interacted with Prenatal and/or Postnatal Treatment, and Sex. Significant Seizure X Prenatal Treatment X Postnatal Treatment interactions were found for dCA1 ($F = 4.304, p = .017$) (Fig. 3.6A), PoDG ($F = 3.700, p = .029$) (Fig. 3.6B), BLA ($F = 6.115, p = .003$) (Fig. 3.6C), PMCo ($F = 3.826, p = .026$) (Fig. 3.6D), and vCA1 ($F = 3.774, p = .028$) (Fig. 3.6E). For each of these regions, simple effects analyses were run separately for moderate and severe seizure groups using Prenatal and Postnatal Treatments as factors, and collapsing across sex. Only those effects for which a confidence level greater than 95% will be discussed. Within both the dCA1 and the PoDG, simple effects analyses revealed that there was a significant interaction of pre- and postnatal treatment in pups that experienced moderate seizures, and post hoc tests showed that pups from NC-EE (Fig. 3.7B) dams had significantly greater levels of FosB-ir relative to those from NC-SC (Fig. 3.7A) dams and

PS-EE dams, but not from PS-SC. In pups that experienced severe seizures, there was a main effect of postnatal treatment for both dCA1 ($F = 17.519, p < .001$) and PoDG ($F = 24.796, p < .001$), resulting in greater levels of Fos-B-ir in EE pups relative to SC pups, regardless of prenatal treatment. Effects observed in the BLA were very different from those in the hippocampus. In pups that experienced moderate seizures, levels of FosB-ir were significantly elevated by dam exposure to PS relative to naïve controls.

Additionally, pups in the PS-EE (Fig. 3.7D) group had levels of FosB-ir that were significantly lower than those in either PS-SC (Fig. 3.7C) or NC-EE groups and were not significantly different from those observed in NC-SC pups. Interestingly, in pups that experienced severe seizures, there was an overall main effect of postnatal treatment ($F = 40.252, p < .001$), with FosB-ir density being significantly lower in EE animals relative to SC animals. In the PMCo, significant simple effects were only found in pups experiencing moderate seizures, in which the combined effects of PS and EE (Fig. 3.7F) yielded lower FosB-ir levels in pups relative to pups from NC-EE or PS-SC dams (Fig. 3.7E). Finally, in the vCA1, analyses of the 3-way interaction revealed that in pups that experienced moderate seizures, pups from NC-EE dams had significantly higher levels of FosB-ir relative to pups from NC-SC dams. In pups that experienced severe seizures, PS and postnatal enrichment together led to increased levels of FosB-ir such that levels were higher in pups from PS-EE dams relative to pups from PS-SC dams.

ANOVAs also revealed a significant Postnatal Treatment X Seizure X Sex interaction for PMCo ($F = 4.075, p = .047$) (Fig. 3.6F) and simple effects analyses revealed a statistical trend ($p = .05$), such that in male pups that experienced moderate seizures, EE (Fig. 3.7F) resulted in lower levels of FosB-ir relative to SC (Fig. 3.7E). There was no significant

effect in females although there was a trend toward more FosB-ir labeling in EE pups. No effects were seen in animals that experienced severe seizures.

Effects of Prenatal and Postnatal Treatments: The only brain region to demonstrate a main effect of prenatal treatment alone was Pir ($F = 5.081, p = .027$) (Table 3.2), with levels of FosB-ir being significantly lower in pups from dams exposed to PS relative to NC. For a number of brain regions, there were main effects or interactions involving Postnatal Treatment (Table 3.2). In some of these regions, FosB-ir levels were greater in EE pups relative to SC pups including: dCA2 ($F = 7.037, p = .010$), dCA3 ($F = 10.759, p = .002$), dDG ($F = 16.438, p < .001$), and Pir ($F = 10.133, p = .002$). In contrast, in AcbC ($F = 22.009, p < .001$), PVN ($F = 4.563, p = .036$), and PVT ($F = 24.311, p < .001$), levels of FosB-ir were significantly lower in EE pups relative to SC pups.

There were significant Prenatal Treatment X Postnatal Treatment X Sex interactions revealed for both the vCA1 ($F = 5.509, p = .022$) (Fig. 3.8A) and the vDG ($F = 13.433, p < .000$) (Fig. 3.8B). For each region, simple effects analyses for each sex were run using Pre- and Postnatal Treatment as factors, collapsed across seizure group. In vCA1, males from NC-EE dams (Fig. 3.9B) had significantly greater levels of FosB-ir relative to males from NC-SC dams (Fig. 3.9A), and there were no effects in females. In the vDG, there were also no effects in females, but for males, those that were born to NC-EE dams (Fig. 3.9B) had significantly greater FosB-ir levels relative to each of the other groups of males (Fig. 3.9A).

3.6 Effects of PS, EE, and Seizure on Plasma GC Levels After Seizure

There was a significant interaction between prenatal, postnatal and seizure groups for PD15 plasma GCs ($F(1,104) = 5.68, p = .019$) (Fig. 3.10). Post hoc analysis revealed a significant decrease in plasma CORT in PS-SC pups experiencing moderate seizures compared to NC-SC pups experiencing moderate seizures.

3.7 Effects of PS and EE on CRH-ir Density in the PVN

Effects of Seizure and Prenatal Treatment: There was a significant interaction between prenatal condition and seizure group on CRH-ir density ($F(2,117) = 3.515, p = .033$). Post hoc analysis revealed that CRH-ir was significantly higher ($p = .045$) (Fig. 3.11A) in PS pups experiencing severe seizures (Fig. 3.11C) than in NC pups experiencing severe seizures (Fig. 3.11B).

Effects of Postnatal Treatments: There were also significant differences in CRH-ir based on postnatal treatment. In pups experiencing seizures CRH-ir was significantly lower ($F(1,117) = 8.306, p = .005$) (Fig. 3.12A) in EE conditions compared to SC. This difference was also found in control pups that did not receive KA injections ($t(47) = -3.23, p = .002$) (Fig. 3.12B).

CHAPTER 4 GENERAL DISCUSSION

Our novel PS paradigm had significant effects on dams and offspring. Specifically, relative to NC dams, PS dams had significantly greater fecal GC levels and gave birth to significantly lighter offspring, an effect that was maintained at 2 weeks of age. These results suggest that repeated predator exposure is a valid and significant source of psychological stress during late pregnancy, providing an important new ethologically relevant model of assessing the impact of early life adversity. Despite these effects, there were few consistent effects on seizure behaviour or FosB-ir levels in any brain region. In fact, seizure behaviour in offspring was influenced more by the dam to which the pups were born than whether the dam was exposed to PS or not: a finding that is perhaps not surprising since it is the individual dams' response to the stressor that dictates the magnitude of the effect imparted on the pups. In contrast, our postnatal enrichment protocol ameliorated the PS-induced lower body weight at two weeks of age, resulted in a lower severity of seizures in female offspring. Environmental enrichment also affected levels of basal and seizure-induced FosB-ir with opposing effects on FosB-ir levels in hippocampal versus extra-hippocampal regions. CRH-ir in the PVN was also affected, suggesting this model exerts powerful effects on the developing limbic system.

Our results highlight the importance of individual dams with respect to moderating effects of prenatal manipulations. These intra-condition differences could be potentiated by within-litter variance in levels of maternal care (Cavigelli et al., 2010), which could lead to differences in GR and MR density and organization in offspring (Liu et al., 1997). The effects of our manipulations on dam-specific variables, including maternal behaviour, could be the topic of future study.

Exposure of pregnant dams to a predator resulted in a significant physiological stress response (higher fecal GC levels) on the first two days of exposure. Levels of GC gradually decreased over exposures to this psychological stressor as a result of habituation, which was similar to previous findings using physiological prenatal stressors, including electric shock (Takahashi et al., 1998), heated restraint (Williams et al., 1999) and a randomized stress protocol of restraint, crowding, and force-swim (Murmu et al., 2006). Although it has been demonstrated that there can be differing effects produced by physical versus psychological stressors (Bowers et al., 2008), others (Ahmadzadeh et al., 2011) have found that dams' GC levels were elevated following exposure to either a physical or psychological stressor and followed a habituation pattern similar to those found in other physical stressor models (Takahashi et al., 1998, Williams et al., 1999).

Both male and female pups of dams that had been exposed to prenatal predator stress weighed significantly less at birth, compared to those from non-stressed dams, providing converging evidence that predator stress had a significant physiological effect on the dams. This decrease in birth weight is consistent with other studies using PS (Welberg et al., 2000, Ahmadzadeh et al., 2011). Low birth weight in human infants is thought to be a marker for prenatal maternal stress and later disease susceptibility (Cardwell, 2013). Further, this apparent outcome of PS coincides with increased prenatal GC in the dam and postnatal GC in the pup (Takahashi et al., 1998, Ahmadzadeh et al., 2011). Despite a decrease in birth weight, litter size was not significantly affected by PS or exposure, which is consistent with a recent study (Ahmadzadeh et al., 2011). Also, PD15 GC levels showed an odd interaction, with NC-SC pups showing increased GC levels compared to PS-SC pups. This may have been a result of dysregulation of the pups' HPA-axis (Chung

et al., 2005) or an artefact of taking plasma from an animal during perfusion. Such prenatal effects on the developing fetus have been associated with alterations to HPA-axis regulation (Seckl and Meaney, 2004), as well as vulnerabilities for anxiety (Welberg and Seckl, 2001) and seizures (Edwards et al., 2002a, Ahmadzadeh et al., 2011) in adulthood.

At 15 days of age, offspring were subjected to a febrile convulsion protocol in order to examine seizure susceptibility. Previous studies have shown a decreased latency to immobility (associated with tonic-clonic seizures) and increased mortality following prenatal restraint stress (Sadaghiani and Saboory, 2010, Ahmadzadeh et al., 2011) as well as decreased seizure thresholds in a kindling model of epilepsy (Edwards et al., 2002a). In the present study, behavioural outcomes of induced febrile convulsions were not heavily influenced by prenatal treatment, as seen using other models (Edwards et al., 2002b, Sadaghiani and Saboory, 2010, Ahmadzadeh et al., 2011, Qulu et al., 2012). However, most of these models used a restraint stress protocol, which has been shown to have very different outcomes (Bowers et al., 2008, Briand and Blendy, 2013) compared to other stressors, such as our predator exposure. Further, methodological and strain differences (discussed below) beyond differences in the type of prenatal stressor exist. FCs, induced by LPS and subthreshold KA, (utilized in the current study and in (Qulu et al., 2012), do not generally produce neuronal damage or mortality (Heida et al., 2004). Other methods used in several studies (Edwards et al., 2002a, Sadaghiani and Saboory, 2010, Ahmadzadeh et al., 2011) include suprathreshold injections of proconvulsant drugs (KA) or kindling. High levels of glutamate agonists, such as KA, induce excitotoxic neuronal damage (Wasterlain et al., 2002), especially in the hippocampus (Schauwecker, 2010), and may induce effects that are more similar to TLE (Ben-Ari, 1985). This difference in

seizure induction technique could exacerbate any neuronal differences initiated by PS (Fujioka et al., 2006, Mandyam et al., 2008) and subsequent seizure-induced cell death.

As mentioned, seizure outcomes were more affected by the dam to which the offspring had been born. Apart from direct effects of fetal GCs on pup development, maternal behaviour is also impacted by PS and has a notable influence on the offspring. There was significant inter-litter variability with respect to offspring seizure behaviour and this was significantly correlated with the dams' fecal GC levels, represented by an inverted-U curve. The source of the litter variation is unclear without further empirical study. It is likely however that the interplay between maternal care, maternal experience, and genetic factors could play a role (Champagne and Meaney, 2006). Chronic stress or anxiety can impact the relationship between mother and offspring, which was not measured in the current study, and, in turn, reduce the quality and quantity of maternal care (Caldji et al., 2000a, Seckl, 2004). In rat strains bred for high and low levels of licking and grooming, a measure of maternal care, prenatally stressed-high licking and grooming dams display similar levels of maternal care behaviour to rats bred for low levels of licking in grooming, without a prenatal stressor (Champagne and Meaney, 2006). This suggests that maternal phenotype can significantly impact later development of offspring and be altered by previous experience.

Consistent with past work (Auvergne et al., 2002), demonstrating a delay in the time to reach seizure in animals reared in EE conditions, we showed an overall decrease in seizure severity, but only in females. The number of animals that were non-responsive to the induction of a febrile convulsion was also greater in the EE pups, suggesting the

potential of resistance. It is possible to speculate that this resistance to seizure induction could be due to an increase in the regulation of the HPA-axis (Morley-Fletcher et al., 2003) in animals from EE conditions, specifically involving decreased GC secretion, which, along with other stress-related hormones, has been shown to promote seizures, (reviewed in (Maguire and Salpekar, 2013) and as has been shown in several models of PS seizure potentiation (Edwards et al., 2002a, Sadaghiani and Saboory, 2010, Ahmazadeh et al., 2011, Qulu et al., 2012).

The postnatal environmental enrichment utilized in the current study is quite different from that traditionally used. Briefly, enrichment usually consists of increased potential for physical and social development. Methods for this vary, but usually include social housing, novel stimuli (toys) and exercise (e.g. running wheel, bigger cage) (van Praag et al., 2000, Chapillon et al., 2002). Rather than allowing the enriched groups access to running wheels and/or toys, we attempted to replicate a more natural environment for the dam and developing pups and potentially avoid the variability in the types and timing of classic enrichment procedures (Fox et al., 2006). Nonetheless, for rat moms, our enrichment could have had profound effects on her behaviour. The increased space and burrow gives the dam an opportunity to leave the pups for short periods of 20-30 minutes, as is often seen in naturalistic settings (Jans and Woodside, 1990, Rosenblatt, 1994). This leads to the possibility that our EE cages may have simulated a form of brief maternal separation or acute postnatal stress, which has been shown to increase the amount of licking and grooming behaviour observed in the mother after being reunited with the pups (Francis et al., 1999, Mashoodh et al., 2009). Postnatal ‘handling’, a model which includes removing the pups from the home cage daily and handling them (Meaney et al.,

1991) increases maternal behaviour (Levine, 1957, Mashoodh et al., 2009) and provides the neural framework for appropriate HPA-axis regulation (Meaney et al., 1991, Meaney et al., 1992, Fujioka et al., 1999), including reduced CRH and ACTH responses to acute stress, increased GR mRNA in the hippocampus, and decreased CRH mRNA in the PVN (Liu et al., 1997). Thus, quantity and/or quality of maternal care are mechanisms by which our environmental enrichment paradigm could exert effects on seizure induction. Previous research has shown that EE can ameliorate some of the deficits in cognitive function caused by low levels of maternal care (Bredy et al., 2003b), although the mechanisms of this interaction are not fully known. Recently, simple maternal care behaviours in humans, such as stroking, were shown to improve an infant's response to a mild stressor (Sharp et al., 2012).

The hypothesis that our EE altered maternal care also fits with the decreased CRH-ir in EE reared pups, regardless of seizure experience. Overall, the effects of EE and early life handling may act on similar neural processes (Fernandez-Teruel et al., 2002). Housing dams in our enriched environment from GD16 onwards marginally increases levels of arched-back nursing across the first week after parturition (unpublished observation). This may be an important effect as both EE (Pham et al., 1999) and maternal care (Liu et al., 2000) have the ability to promote cell survival, and increase axonal sprouting and synaptogenesis by increasing neurotropic factors, specifically BDNF. In turn, this may have led to increased neuronal plasticity, and hence, neuronal resilience following seizures in EE reared pups. The effects of our novel EE treatment still require further evaluation, especially regarding levels of maternal care and changes in the development of the HPA-axis. They do, however, indicate that an enriched environment does not need

to be complex or over-stimulating. Simple efforts to increase maternal care are sufficient to improve overall well being.

The only brain region to show a significant main effect of seizure was the PVN, where animals that had moderate or severe seizures had higher levels of FosB-ir than those that had mild seizures; a pattern that is consistent with previous literature (Silveira et al., 2002). The PVN secretes corticotrophin hormone/factor (CRH) and increased levels of CRH have strong pro-convulsive properties (Baram and Schultz, 1991), suggesting that PVN activation is important in epileptogenesis. Currently, we identified increased CRH-ir following severe seizures in pups born to PS dams, compared to those born to NC dams. However, further studies will be needed to determine whether animals subjected to PS and early life seizures do in fact go on to develop epilepsy, and whether there is a link between the epileptogenesis and the stress axis (particularly the PVN). Further, CRH-ir was increased following severe seizure in pups born to PS dams, compared to those born to NC dams.

EE reduced CRH-ir in the PVN of both FC and naïve pups. This was interesting, because it suggests that the EE condition has an effect on HPA-axis activation in pups following seizure, a finding that has not been reported before, though studies have suggested other neurogenic benefits of EE in seizure models (Young et al., 1999, Auvergne et al., 2002, Faverjon et al., 2002, Kazl et al., 2009). This decrease in HPA-axis activation, measured by CRH-ir is similar to other studies that lack a seizure or FC component (Francis et al., 2002, Morley-Fletcher et al., 2003). Thus, it is not surprising that seizure naïve pups also showed significant decreases in CRH-ir. These decreases further support evidence that EE

is beneficial in both seizure and PS models and that these effects are regulated by dam-dependent experiences.

Interactions between seizure severity and early life manipulations were much more common for a number of regions. In the dCA1 and PoDG, greater levels of FosB-ir, following severe seizure, were found in pups from the EE group. In terms of EE, more FosB-ir was detected within the hippocampus (dCA1, dCA2, dCA3, dDG, PoDG, vCA1, and vDG), as well as the Pir, compared that from SC pups. Specific increases in FosB-ir within the hippocampus could be an effect of increased neurogenesis and/or cell survival induced by the EE (Pham et al., 1999, Yutsudo et al., 2013). As mentioned before, EE conditions have been shown to increase the amount of synaptogenesis in dCA1 (Rampon et al., 2000), dCA3 (Altschuler, 1979), and the dDG (Juraska et al., 1985). Such increases could be associated with increases in connectivity between hippocampal regions, or with neurogenesis, resulting in higher FosB-ir following seizure. However, complicating the interpretation of the presence of FosB, especially after seizure, is the notion that neurogenesis may not be beneficial (Jessberger et al., 2005). Thus, at present, levels of FosB-ir cannot be causally linked to either beneficial or deleterious consequences, and thus more work is needed.

Other, extra-hippocampal regions, such as the BLA, PMCo, AcbC, PVN, and PVT were affected by the EE manipulation much differently. Specifically, these regions showed greater FosB-ir in animals raised under SC conditions relative to those housed in EE. This result is consistent with literature supporting a decrease in seizure susceptibility following EE conditions (Auvergne et al., 2002). These regions are especially critical to our

findings because, unlike the hippocampus, they do not show neurogenesis following EE or seizure and would not be subject to any extraneous effects that neurogenesis may have on FosB-ir measurements. Further, these regions are associated with stress response and HPA-axis activation. Lower levels of FosB-ir in these regions suggest that EE had a moderating effect on PS with respect to the regulation of HPA functioning. This is also consistent with our findings that, at least in females, EE decreased seizure susceptibility and severity. Less FosB-ir in these regions (especially PVN and PVT) could suggest a more regulated HPA-axis. As shown previously (Henry et al., 1994), prenatal stress can inhibit development of the HPA-axis by increasing glucocorticoid and mineralocorticoid receptors throughout the system. Further, Morley-Fletcher (Morley-Fletcher et al.) has shown a recovery of this system's functioning following early life EE exposure.

However, behaviorally, especially in females, and molecularly, when considering FosB-ir outside of the hippocampus, in the BLA, PVN, PVT, AcbC, and PMCO, EE decreased seizure severity. These regions are particularly interesting, because they all have key roles in seizure behaviour and/or stress responding, connectivity between regions is outlined in Fig. 4.1. The amygdala, important for processing fearful (ie. predator) stimuli (Choi and Kim, 2010), receives direct inputs from the hippocampus, specifically the ventral hippocampus (Pitkänen et al., 2000, Petrovich et al., 2001), explaining its pronounced increases in excitability following FC and is highly influenced by EE (Sztainberg et al., 2010), potentially due to decreases in CRH receptors following EE. Also receiving inputs from the ventral hippocampus is the PVN (Petrovich et al., 2001), which showed high FosB-ir in mild and severe seizures, though this was mediated by EE. The PVN is important because direct stimulation induces seizure behavior reminiscent of grooming

and chewing (Baram and Schultz, 1991) and stress (i.e. from FC) appears to increase CRH levels. FosB-ir was also increased in PVT following seizure. This structure, particularly the reuniens nucleus, serves as a junction, which connects efferent signals from the ventral hippocampus to the dorsal (Risold et al., 1997). The ventral hippocampus is also responsible for sending and receiving olfactory information through amygdalar nuclei, including the PMCo (Saunders et al., 1988). The dorsal hippocampus sends projections rostrally, to the AcbC, which is partially responsible for stimulating motor neurons in the cortex (Groenewegen et al., 1996). Together, FosB-ir in the PMCo and AcbC show that epileptiform activity can travel beyond the hippocampus and that EE can decrease seizure severity outside of the hippocampus, potentially through these mediating regions.

As stated, EE had an effect on both FosB-ir and CRH-ir. The increase in FosB-ir in the hippocampus may be due to increased neurogenesis in EE conditions. Increased seizure severity following environmental enrichment in adult rats has been reported, as well as increased neurogenesis following EE and kindling (Faverjon et al., 2002, Young et al., 2004). The assumption that increased neurogenesis, following EE, provides a framework for increased excitotoxicity in the limbic system is not unfounded, as increased seizure susceptibility following postnatal handling in adult rats has also been reported (Lee and Kalynchuk, 2002). Finally, significant age-dependent effects have been reported in studies utilizing FosB-ir as a measure for neural activation in the hippocampus (Silveira et al., 2002). Together, these studies suggest that an increase in neural plasticity may increase an organism's vulnerability for excitotoxicity, especially when induced with seizure.

In the current study, increased FosB-ir following seizures within the hippocampus and of EE raised pups could imply increased neurogenesis. This seems even more likely when considering the contradictory effects of EE on FosB-ir in regions outside of the hippocampus. It is well understood that EE increases neurogenesis within the hippocampus, resulting in more granule cells within the DG (Kempermann et al., 1997) and increasing synaptogenesis in the dCA1, dCA3, and DG (Altschuler, 1979, Juraska et al., 1985, Rampon et al., 2000) of adult rats and mice. The potential for neuronal survival following EE in infants is even greater and this increase in cell survival, neurogenesis and synaptogenesis could lead to increased FosB-ir, suggesting more severe seizures in EE raised rats.

Recently, studies have suggested a role for FosB in neurogenesis. This transcription factor is indicative of cellular activation and often used as a marker for excitotoxicity (Morris et al., 2000, Silveira et al., 2002, Peng and Houser, 2005, Pereno et al., 2011). However, FosB is also required for neurogenesis, which, in some cases, may play a role in mitigating seizure severity (Yutsudo et al., 2013). This may be due to its role in healthy neural stem cell proliferation. Further, reducing FosB expression leads to abnormal neuronal differentiation (Kurushima et al., 2005), which may also lead to an increased vulnerability for seizure (Yutsudo et al., 2013). This supports our idea that increased FosB-ir may correlate with neurogenesis, especially in EE conditions. Furthermore, as stated previously, individual dams reacted to the stress paradigm differently, possibly affecting the quantity or quality of maternal care in both EE and control conditions, further impacting HPA-axis development in their pups (Champagne and Meaney, 2006).

However, to confirm this, future studies will be necessary to determine if the effects of brief maternal separation, in our two-section enrichment condition, correlates with increased maternal behaviours.

Following the induction of febrile convulsions, FosB-ir was examined throughout the limbic system as a measure of brain activation. Prenatal stress exerted very few effects on its own, but interactions between prenatal treatment, postnatal treatment and seizure occurred for a number of brain regions. Other studies using a prenatal stressor to potentiate seizures utilized Wistar (Sadaghiani and Saboory, 2010, Ahmadzadeh et al., 2011) and Sprague-Dawley rats (Edwards et al., 2002a, Qulu et al., 2012), which have been shown to be more sensitive to KA-induced seizures than Long-Evans hooded rats (Xu et al., 2004), especially at low doses (Golden et al., 1995), as used in the current study. Inbred strains, such as the Wistar-Furth and Fisher 344 rats have greater sensitivity to KA induced seizure than outbred strains, such as Sprague Dawley and Long-Evans hooded rats (Golden et al., 1995). This may be due to underlying differences in the development of inhibitory GABA-ergic and excitatory glutamatergic systems.

In rats, the development of GABAergic neurons begins in the fetus (Seress and Ribak, 1988), but GABA's inhibitory effect does not occur here. Instead, GABA appears to have some capacity for excitability until approximately PD13 (Khazipov et al., 2004), probably due to a delay in the development of a chloride exporter (Ben-Ari, 2002). This excitability of the developing brain helps elucidate the increased vulnerability to seizure associated with this specific age (Ben-Ari et al., 1997, Ben-Ari, 2001, Ben-Ari, 2002), which aligns

with vulnerable ages in other studies utilizing febrile convulsion models (Baram et al., 1997b). Critically, the age-dependency of seizure sensitivity may depend on genetic (Golden et al., 1995) and epigenetic factors (Caldji et al., 2000b).

Glutamatergic systems are undergoing developmental changes during this stage as well. During the first two weeks of life, in rats, the KA-sensitive glutamate receptors (GluR5 and GluR6) are capable of binding KA and increasing synchronized (epileptiform) activity (Tremblay et al., 1984, Khalilov et al., 1999). Throughout maturity, the presence of GluR5 is lost in the hippocampus (Lauri et al., 2005, Lauri et al., 2006) and the effect of KA is reduced. It is suggested that this may be related to the loss of GluR5 receptors (Lauri et al., 2006) or the loss of downstream signaling mechanisms (Sallert et al., 2007), the specific mechanisms are not understood (Lauri and Taira, 2011). Regardless, this further suggests that early life, especially PD10-15, have a unique susceptibility to seizure.

Increased seizure susceptibility or severity does not necessarily indicate impaired behaviour in other contexts. Young et al. (2004) describe reduced fear behaviours in the open field test, along with increased seizure susceptibility following EE in adult rats. This suggests that the neural mechanisms that promote seizure may not lead to comorbidities, especially if an attempt (e.g. EE) is made to restore normal functioning. Differences in the strains (Konkle et al., 2010), the timing of EE or the type of EE exposure could also lead to differences in the dispersion of neural progenitor cells, potentially increasing an organisms vulnerability to seizure. Further research should explore the neural mechanisms of this EE induced seizure susceptibility.

The work of Young et al. (2004) also contradicts other research (Auvergne et al., 2002), which showed a neuroprotective effect of EE in adult animals subjected to kindling. However, the effect of EE on seizure susceptibility is unclear, as EE housed rats have improved Morris water maze performance following seizure and decreased fear behaviour, without reducing seizure severity (Faverjon et al., 2002, Young et al., 2004). These three studies offer an interesting perspective on potential strain differences in response to EE and kindling-seizure. Two studies (Young et al., 1999, Young et al., 2004) used Long-Evans hooded rats, as used in the current set of experiments, another (Auvergne et al., 2002) used Wistar rats, and another (Faverjon et al., 2002), used Sprague-Dawley rats which have both been shown to be more sensitive to chemically induced seizure (Golden et al., 1995, Xu et al., 2004) than the Long-Evans hooded rats. One of the few studies using chemically induced seizure in Long-Evans hooded rats (Kazi et al., 2009) found that EE had no significant effect on seizure severity or latency to seize, however, EE decrease the amount of neuronal damage, measured as DNA fragmentation and microglia activation. These examples provide more evidence that genetic differences in rat strains have a profound effect on seizure susceptibility.

Interestingly, full limbic motor behaviours, characterized as severe seizures in the current study, have not been recorded in pups before. However, the Long-Evans hooded rat has not been extensively studied in regards to febrile seizure. Ben-Ari et al. (1984) reported that male Wistar rat pups younger than PD 19 show clonic-tonic seizure behaviour with asynchronous movement of the limbs, often referred to as bicycling or swimming behaviours. This was further supported with similar asynchronous movement in PD 7 and

PD 13 Sprague-Dawley male rats (Silveira et al., 2002). At PD 20 more complete behavioural profiles of limbic seizures become apparent, including synchronized clonic movements of the limbs and facial clonus. Further, both studies found changes in the activation of the BLA around day 19, suggesting a role for this region in the behavioural changes noted around that age. Kainic acid-induced seizures on PD 7 and PD 13 did not result in increased Fos-ir in the BLA, but by PD 20 and PD 60, seizures were associated with greater Fos-ir (Silveira et al., 2002). Differences in both behavioural and FosB-ir patterns, observed to exist between the present study and these past studies, could be associated with strain differences.

Though FCs during early life may be linked to epileptogenesis, they are not directly causal. Changes in neuronal networks, specifically in the hippocampus and amygdala, are not necessarily indicative of changes that lead to epilepsy (Heida et al., 2005), though they increase an organism's susceptibility to induced seizure. KA induced seizure, without the FC component, has been shown to increase SRSs (Stafstrom et al., 1992), however, no research has attempted to identify SRSs in an LPS/KA-induced FC model. Future research is necessary to identify the usefulness of this model in the study of epileptogenesis, especially utilizing potential prenatal and postnatal antecedents.

In conclusion, the results of our study support current evidence that both prenatal and postnatal factors are capable of impacting normal developmental trajectories that could impact the development of neurological disease and the neural mechanisms underlying them. This suggests that early life experiences could have an impact on the susceptibility to prolonged febrile convulsions in human children. Human studies of cryptogenic

epilepsies, especially those originating from FCs should also consider the impact of prenatal GC exposure. First, we show that individual dam's responses to stressors has a significant impact on their offspring, and provide evidence that the impact of prenatal factors may be dam-dependent. Secondly, our data suggests sex-specific differences in seizure susceptibility, which may be mediated by early life experiences and have an impact on the severity of seizures. Finally, our novel postnatal environmental enrichment manipulation had very strong effects on FosB-ir throughout the brain and provides an opportunity for continued research. When attempting to diagnose and manage febrile convulsions, considerations should be made regarding prenatal and early life experiences. Further, to improve a child's responsiveness to stress, and potentially their vulnerability to seizure, they should be nurtured and attempts to enrich their environment should be pursued, though these do not require costly, extravagant tools.

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Appendix A: TABLES

Table 2.1. Limbic brain regions in which FosB-immunoreactivity density was quantified. Coordinates are from Paxinos & Watson (2nd ed.).

Region	Abbreviation	Bregma's Coordinates
accumbens nucleus, core	AcbC	1.7 to 1.6
lateral septum	LS	0.7 to 0.2
paraventricular nucleus of the hypothalamus	PVN	-1.3 to 1.8
paraventricular nucleus of the thalamus	PVT	-1.3 to 1.8
medial preoptic area	MPA	-0.26 to -0.3
arcuate nucleus	Arc	-2.3 to -2.8
basolateral amygdaloid nucleus, ant.	BLA	-2.8 to -3.3
dorsal CA1 hippocampus	dCA1	-2.8 to -3.6
dorsal CA2 hippocampus	dCA2	-2.8 to -3.6
dorsal CA3 hippocampus	dCA3	-2.8 to -3.6
dorsal dentate gyrus (DG)	dDG	-2.8 to -3.6
medial amygdaloid nucleus	Me	-2.8 to -3.6
piriform cortex	Pir	-2.8 to -3.6
polymorphic layer of the dorsal DG	PoDG	-2.8 to -3.6
posteromedial cortical nucleus of the amygdala	PMCo	-4.5 to -4.8
substantia nigra	SN	-4.52
ventral CA1 hippocampus	vCA1	-4.8 to -5.3
ventral CA3 hippocampus	vCA3	-4.8 to -5.3
ventral DG	vDG	-4.8 to -5.3
entorhinal cortex	Ent	-4.8 to -5.6
mammillary nucleus	MN	-5.2 to -5.3
locus coeruleus	LC	-10.3 to -9.68

Table 3.1. A number of PND15 offspring did not display a seizure score >1 at any time during the febrile convulsion protocol. The number of non-responders was calculated based on group designation, as depicted below, and statistical analyses of seizure behaviour were performed with and without the non-responders.

Prenatal Treatment	Postnatal Treatment	Sex	Number of Non-responders
NC	SC	F	1
NC	SC	M	1
NC	EE	F	3
NC	EE	M	1
PS	SC	F	1
PS	SC	M	0
PS	EE	F	4
PS	EE	M	1

Table 3.2. Regions in which there were significant main effects or interactions among Seizure, Prenatal Treatment, Postnatal Treatment, Sex, on FosB-immunoreactivity density. Only the highest order effects are indicated for each region. For main effects, the direction of effects is indicated and for interactions, the Figure displaying the significant simple effects is listed. A full list of the regions analyzed and abbreviations can be found in Table 1.

Region	Seizure	Seizure x Pre x Post	Seizure x Post x Sex	Prenatal	Postnatal	Pre x Post x Sex
dCA1		Fig. 3.6A				
dCA2					EE > SC	
dCA3					EE > SC	
dDG					EE > SC	
PoDG		Fig. 3.6B				
BLA		Fig. 3.6C				
AcbC					EE < SC	
Pir				PS < NC	EE > SC	
PVN	Mod > Mild; Severe > Mild; Mod = Severe				EE < SC	
PVT					EE < SC	
PMCo		Fig. 3.6D	Fig. 3.6F			
vCA1		Fig. 3.6E				Fig. 3.8A
vDG						Fig. 3.8B

Appendix B: FIGURES

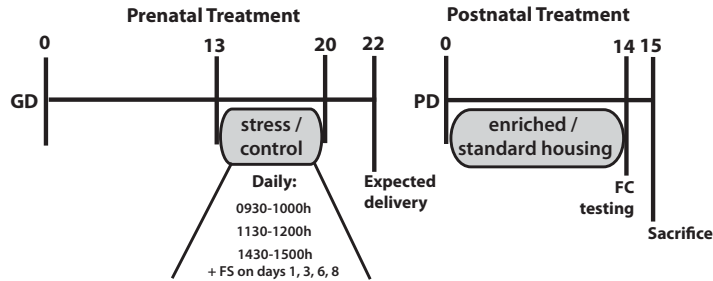


Figure 2.1. Timeline of prenatal and postnatal treatment procedures. Female rats were bred and beginning on gestational day (GD) 13, were exposed daily for 30 min to a predatory stress (live cat) or a control condition, at three times during the day. Stress and control exposures were performed following placement of the pregnant dam into a fresh home cage. On days 1, 3, 6, and 8 of the exposure period, fecal samples (FS) were collected from the fresh cages at the completion of the 1430-1500h exposure period in order to provide a sample to be used for glucocorticoid (GC) analysis. On the day of parturition (postnatal day (PD) 0), dams and pups were randomly assigned to standard or enriched housing. On PD14, male and female pups from dams belonging to each experimental group underwent febrile convulsion (FC) testing. Twenty-four hours later, all pups were sacrificed and brains removed for future immunohistochemical analysis.

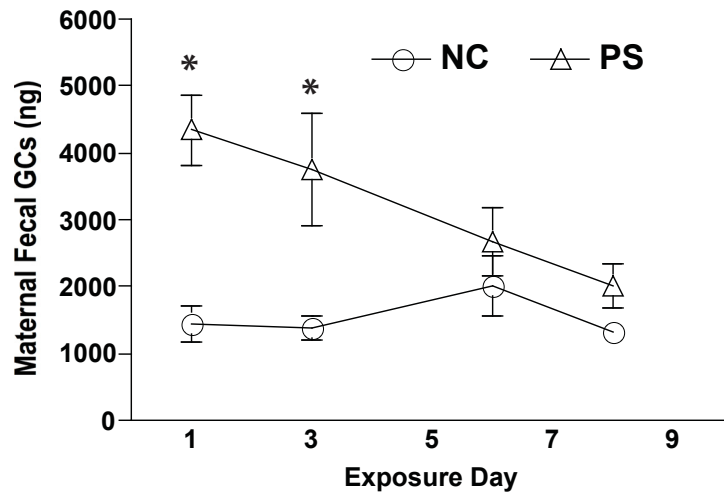


Figure 3.1. Total glucocorticoids (GCs) measured in fecal samples collected from dams randomly assigned to a prenatal naïve control (NC) or prenatal stress (PS) condition. Fecal samples were collected following the third and final daily cat exposure session on days 1, 3, 6, and 8 of the gestational stress period (spanning gestational days 13 – 20). *Significantly different from NC for that day, $p < 0.05$.

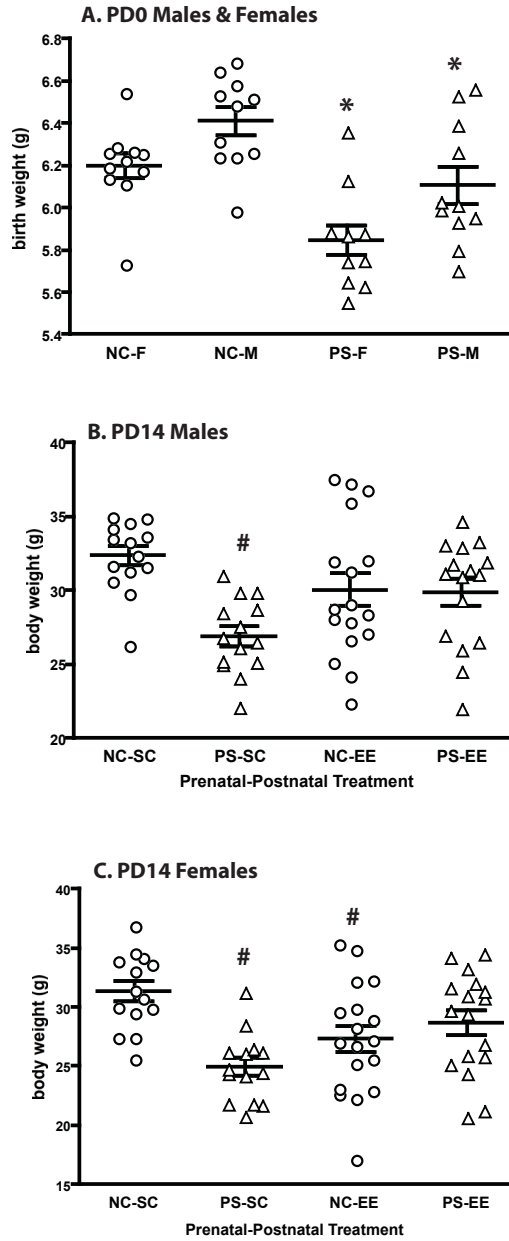


Figure 3.2. **A)** Range, mean, and standard error of the mean for birth weight (postnatal day (PD) 0) of female (F) and male (M) pups born to naïve control (NC) dams or dams that received prenatal predator stress (PS); Range, mean, and standard error of the mean for body weight measured at postnatal day (PD) 14 in **B)** males, and **C)** females, born to dams exposed to prenatal naïve control (NC) or prenatal predator stress (PS), followed by postnatal standard cage (SC) or postnatal environmental enrichment (EE). See Methods for details. *Significantly different from NC, collapsed across sex ($p < 0.05$), #Significantly different from NC-SC of same sex ($p < 0.05$).

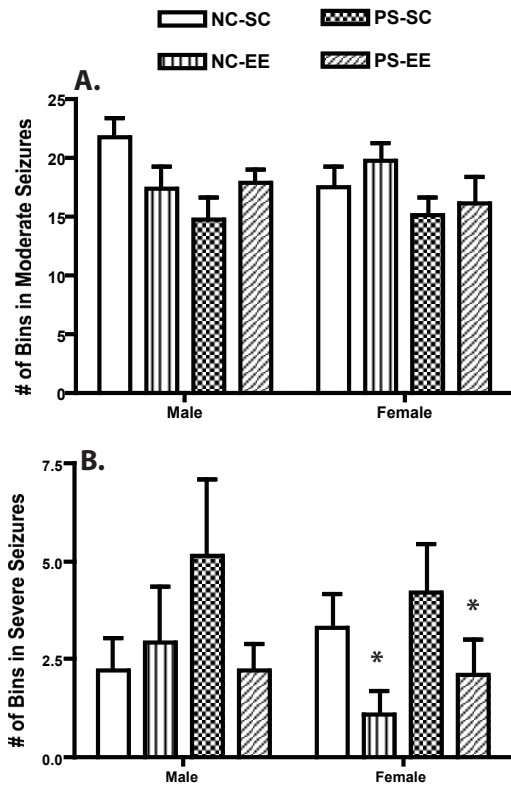


Figure 3.3. The number of 5-min bins in which male and female pups born to dams exposed to prenatal naïve control (NC) or prenatal predator stress (PS), followed by postnatal standard cage (SC) or postnatal environmental enrichment (EE), experienced **A**) moderate, and **B**) severe, seizures. *Significantly different SC animals of same sex ($p < 0.05$).

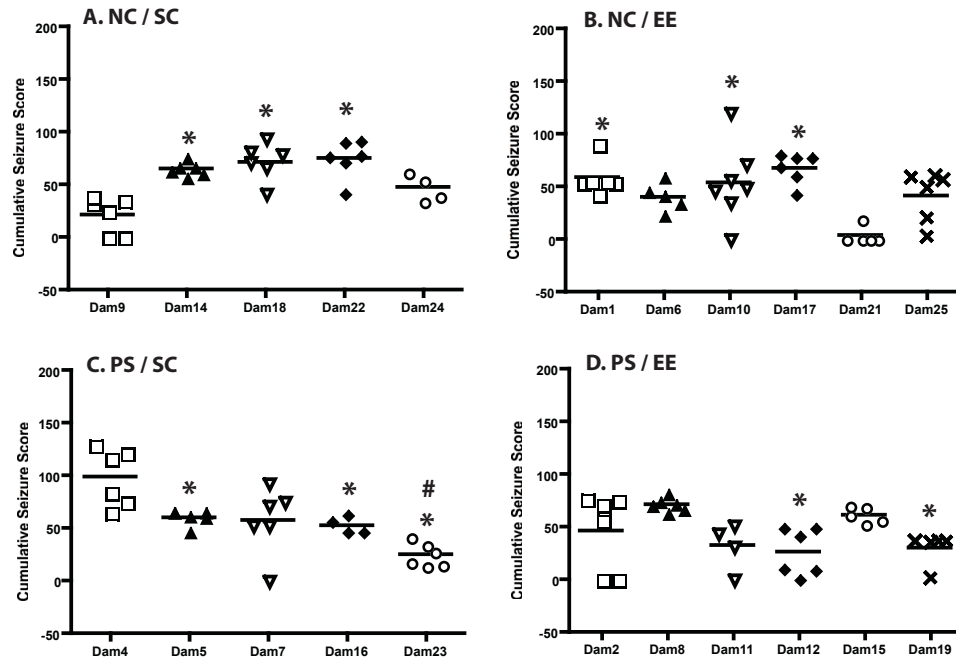


Figure 3.4. Cumulative seizure score of pups born to dams exposed to prenatal naïve control (NC) or prenatal predator stress (PS), followed by postnatal standard cage (SC) or postnatal environmental enrichment (EE), demonstrating the variability in this measure as a function of Dam. **A)** Range, mean, and standard error of the mean for pups of NC / SC dams *Significantly higher than Dam 9; **B)** Range, mean, and standard error of the mean for pups of NC / EE dams *Significantly higher than Dam 21; **C)** Range, mean, and standard error of the mean for pups of PS / SC dams *Significantly lower than Dam 4, #Significantly lower than Dam 5; **D)** Range, mean, and standard error of the mean for pups of PS / EE dams *Significantly lower than Dam 8.

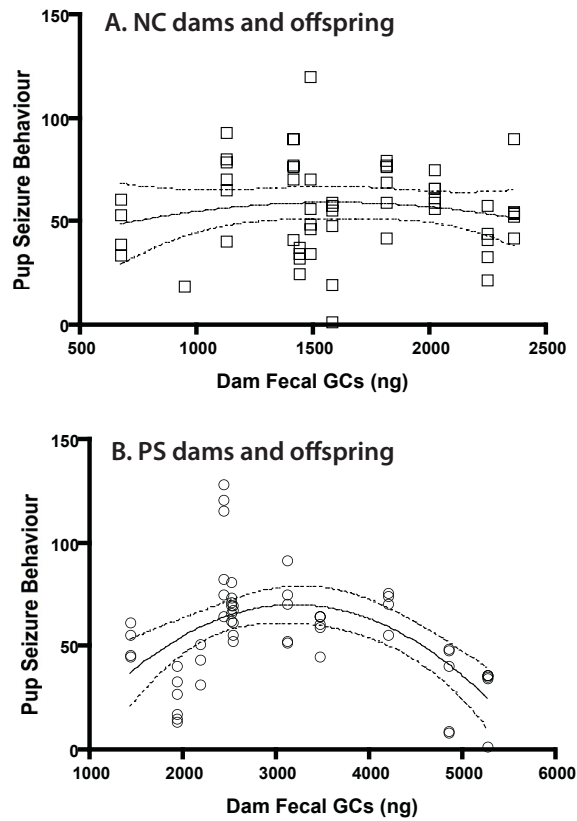


Figure 3.5. Relationship between pup seizure behaviour and maternal glucocorticoid (GC) levels in **A)** naïve control (NC) dams and offspring and **B)** dams and offspring exposed to prenatal stress (PS).

Although there was no significant linear relationship for either group, the relationship was best fit by a second-order polynomial equation for the PS dams and offspring. The relationship in the NC dams and offspring could not be fit by this equation and a comparison of fit of the curves in **A** and **B** revealed a significant difference. Note a higher level of maternal GC levels in PS dams (**B**) compared to NC dams (**A**).

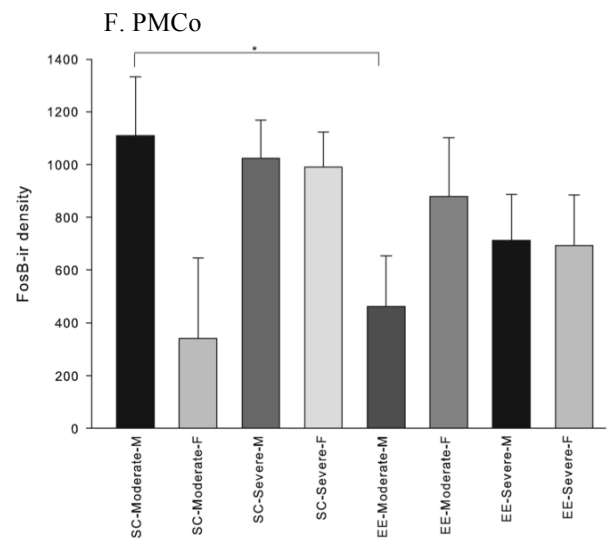
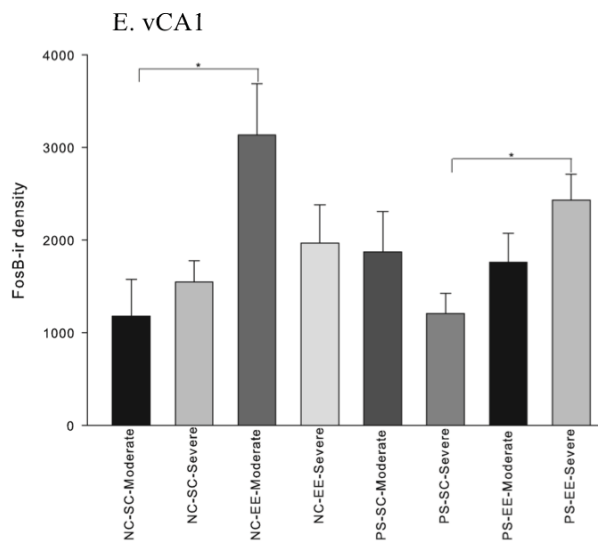
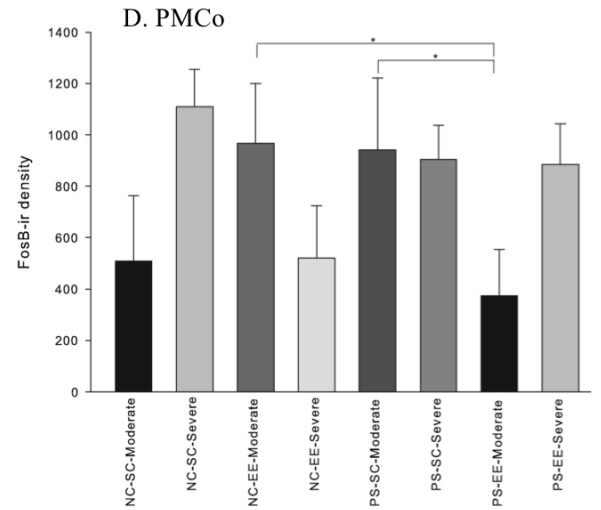
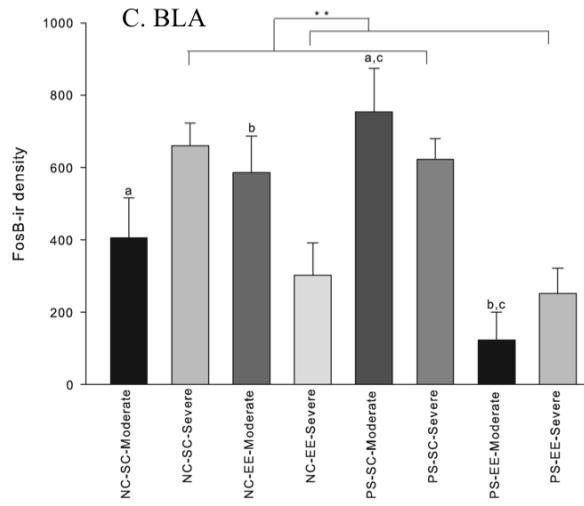
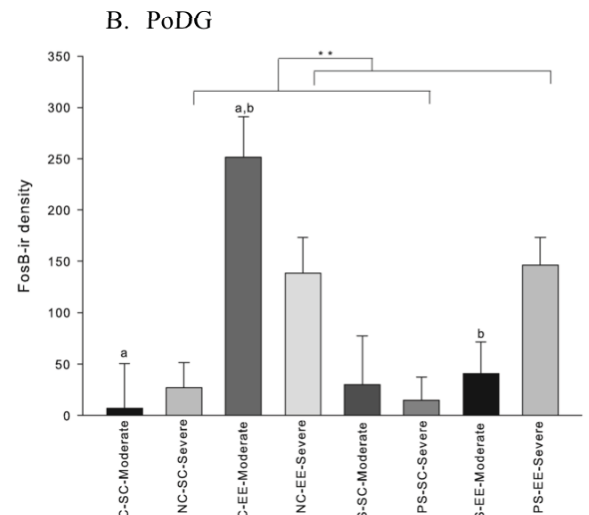
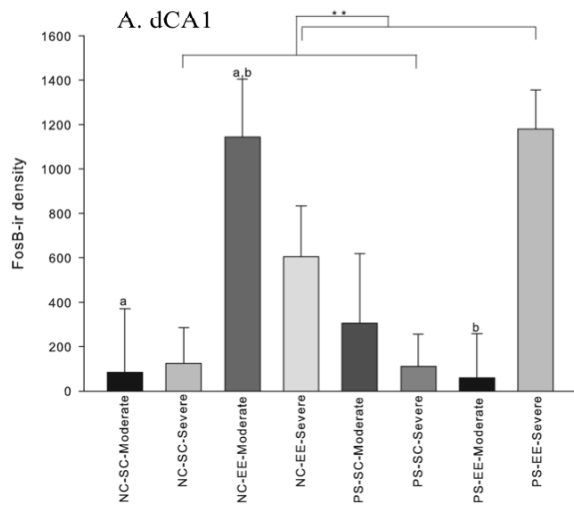


Figure 3.6. A – F) FosB-immunoreactive (-ir) density in various brain regions of male (M) and female (F) pups born to dams exposed to prenatal naïve control (NC) or prenatal predator stress (PS), followed by postnatal standard cage (SC) or postnatal environmental enrichment (EE), separated by severity of seizures (Moderate, Severe) experienced during febrile convulsion testing on postnatal day 14. Density analysis was performed using NIH imaging software (ImageJ, v10.2, NIH, USA) with the number of immunoreactive nuclei counted and the specific area of analysis noted for each section. Density measures were then calculated as a function of the number of immunopositive nuclei relative to the region's area. See the Methods section for more detail. * Significantly different ($p < 0.05$); ** Significantly different ($p < 0.001$); ^{a,b} Significantly different from groups with the same letter at $p < 0.05$; ^c Significantly different from groups with the same letter at $p < 0.000$; dCA1 - dorsal CA1 hippocampus, PoDG - polymorphic layer of the dorsal DG, BLA - basolateral amygdaloid nucleus, ant., PMCo - posteromedial cortical nucleus of the amygdala, vCA1 - ventral CA1 hippocampus

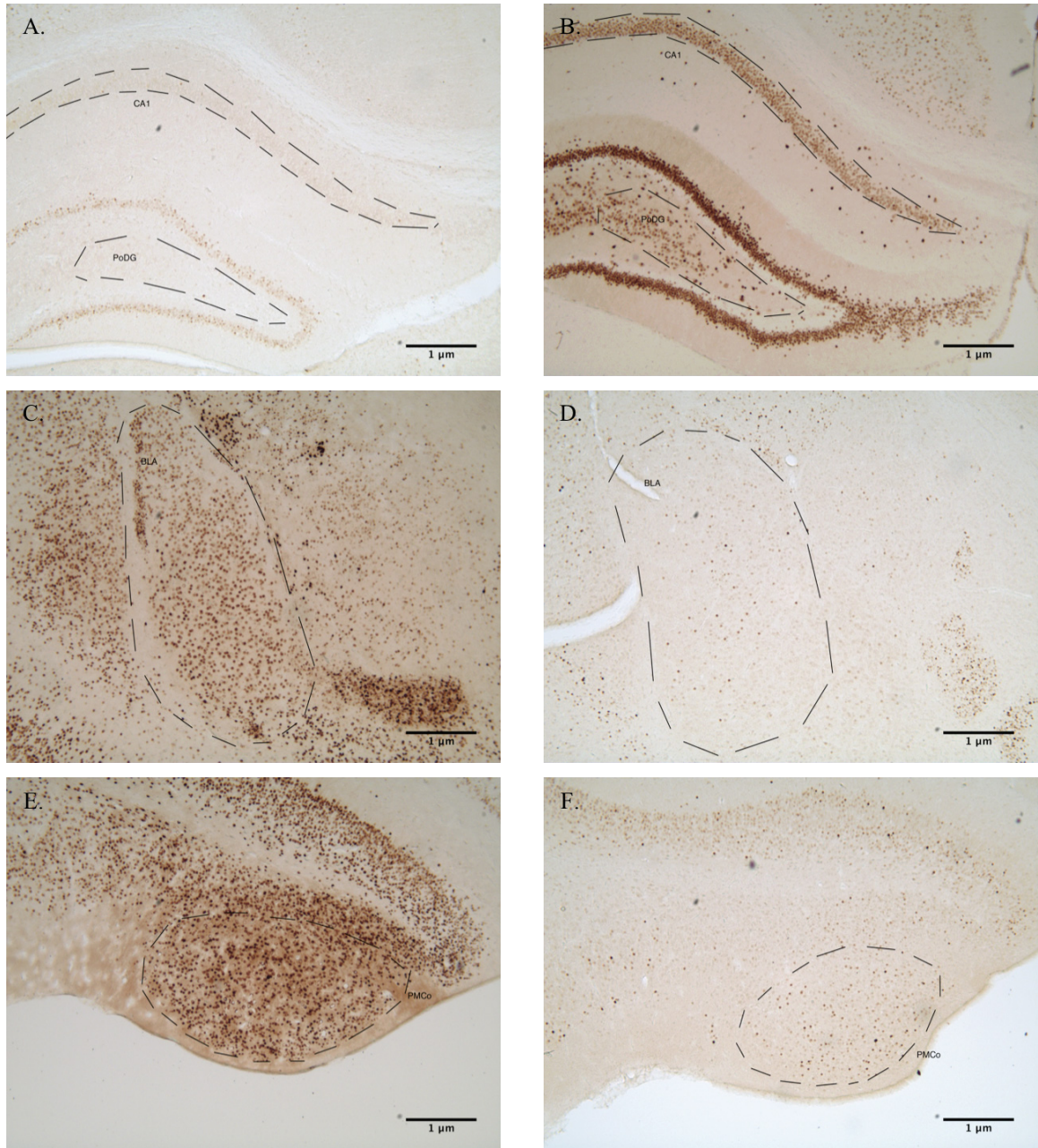


Figure 3.7. A – F) Representative FosB-immunoreactive (-ir) density images. Hippocampus with labeled CA1 and PoDG from **A)** SC and **B)** EE raised pups. Amygdala with labeled BLA from **C)** SC and **D)** EE raised pups. Posterior cortex with labeled PMCo from **E)** SC and **F)** EE raised pups.

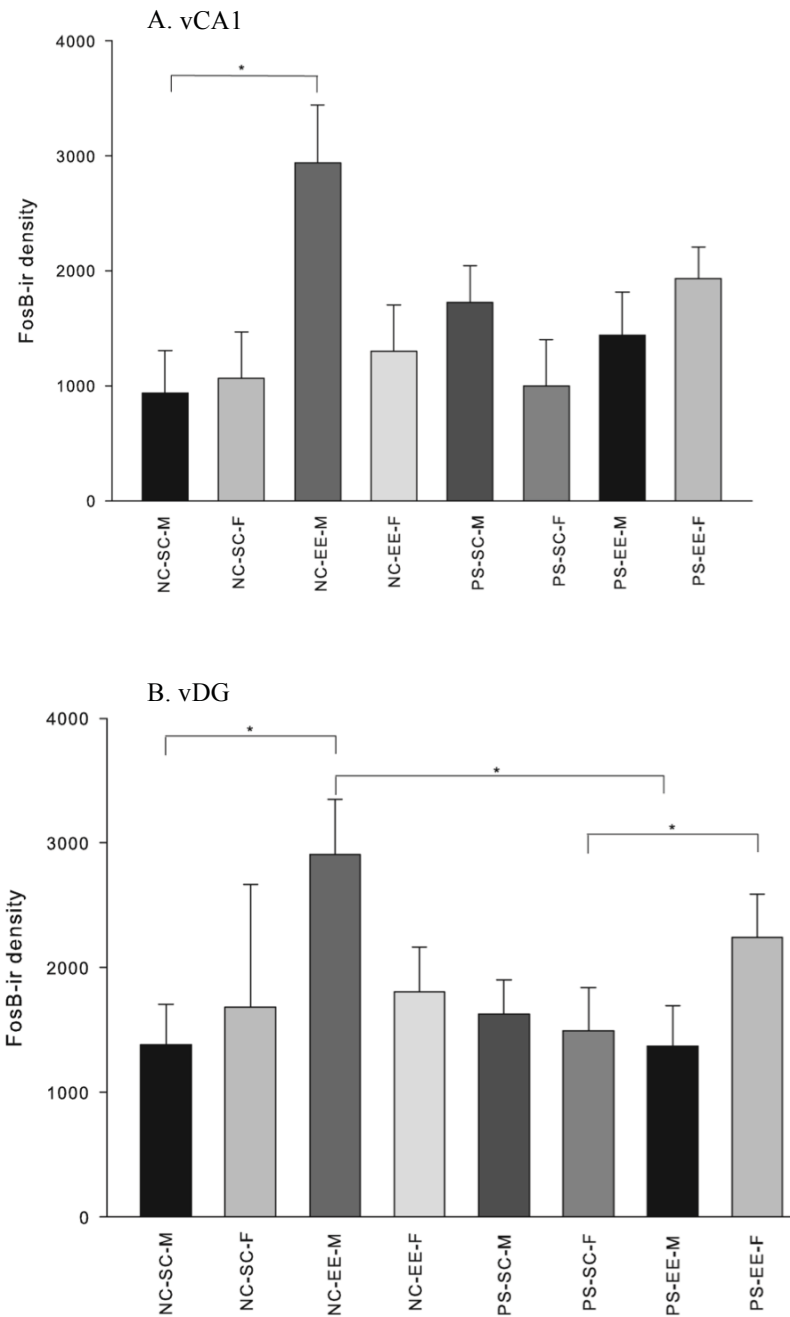


Figure 3.8. FosB-immunoreactive (-ir) density in **A)** ventral CA1 hippocampus (vCA1) and **B)** ventral dentate gyrus (vDG) of male (M) and female (F) pups born to dams exposed to prenatal naïve control (NC) or prenatal predator stress (PS), followed by postnatal standard cage (SC) or postnatal environmental enrichment (EE). * Significantly different ($p < 0.05$).

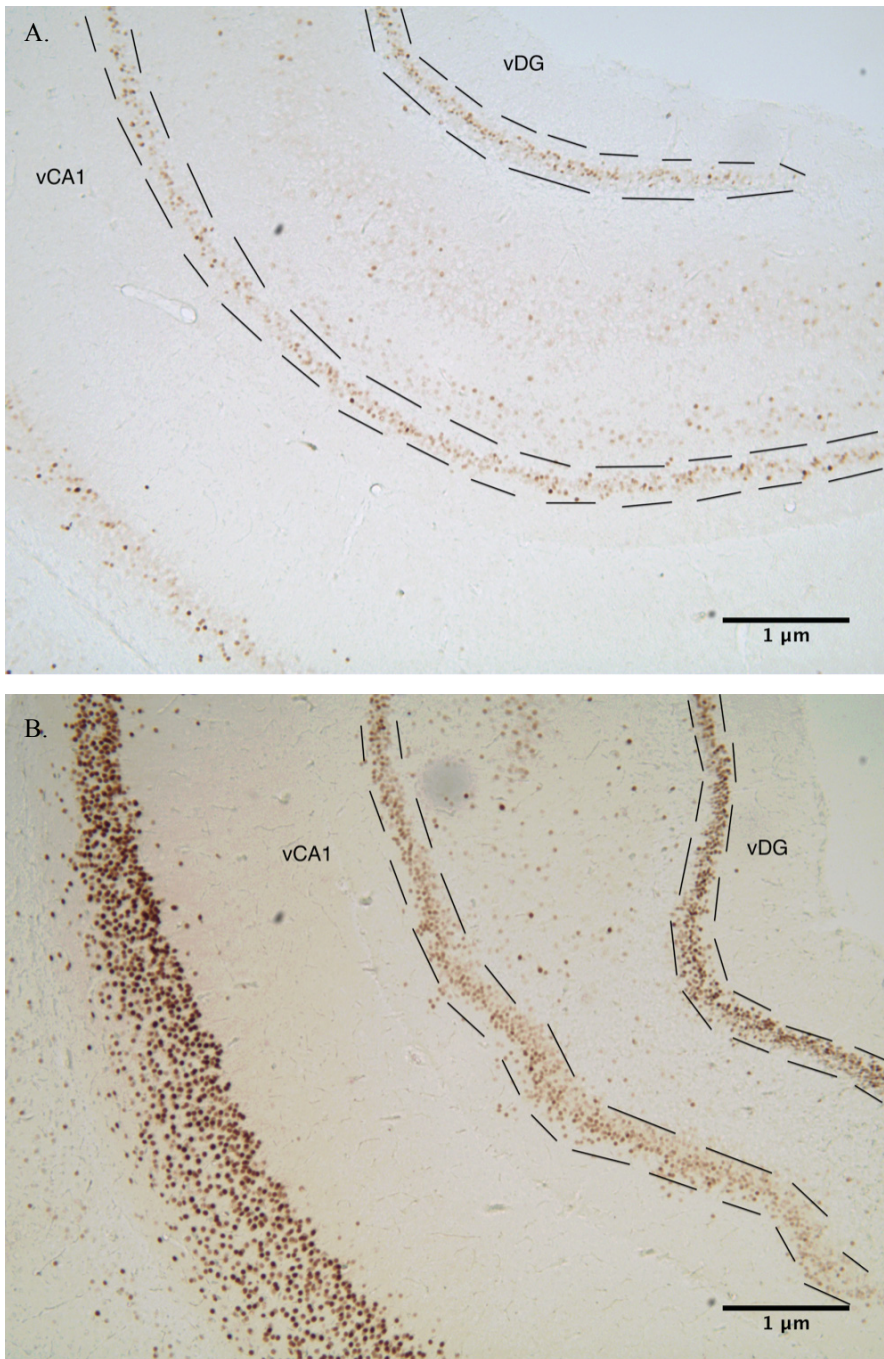


Figure 3.9. Representative FosB-immunoreactive (-ir) density images. Ventral hippocampus with labeled vCA1 and vDG from **A)** SC and **B)** EE raised pups.

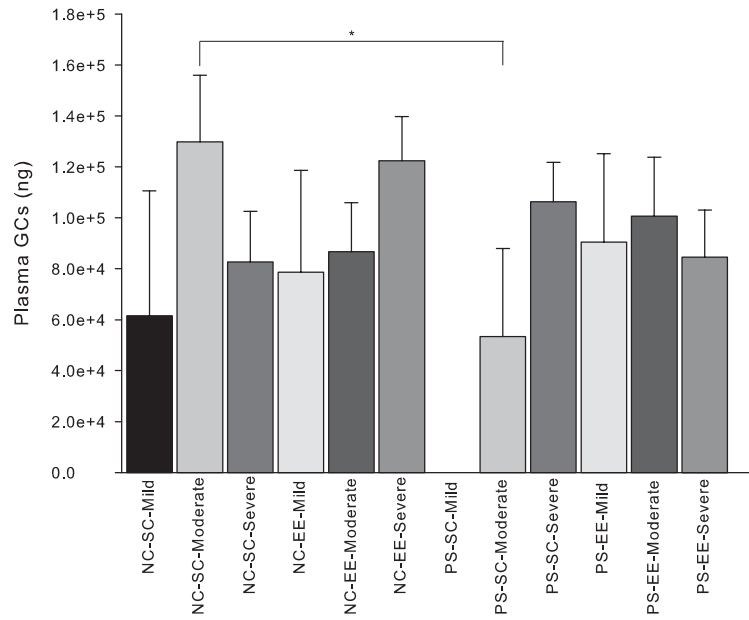


Figure 3.10. Mean and standard error plasma GC levels in PD15 pups. NC-SC had significantly higher GCs than PS-SC. * Significantly different ($p < 0.05$).

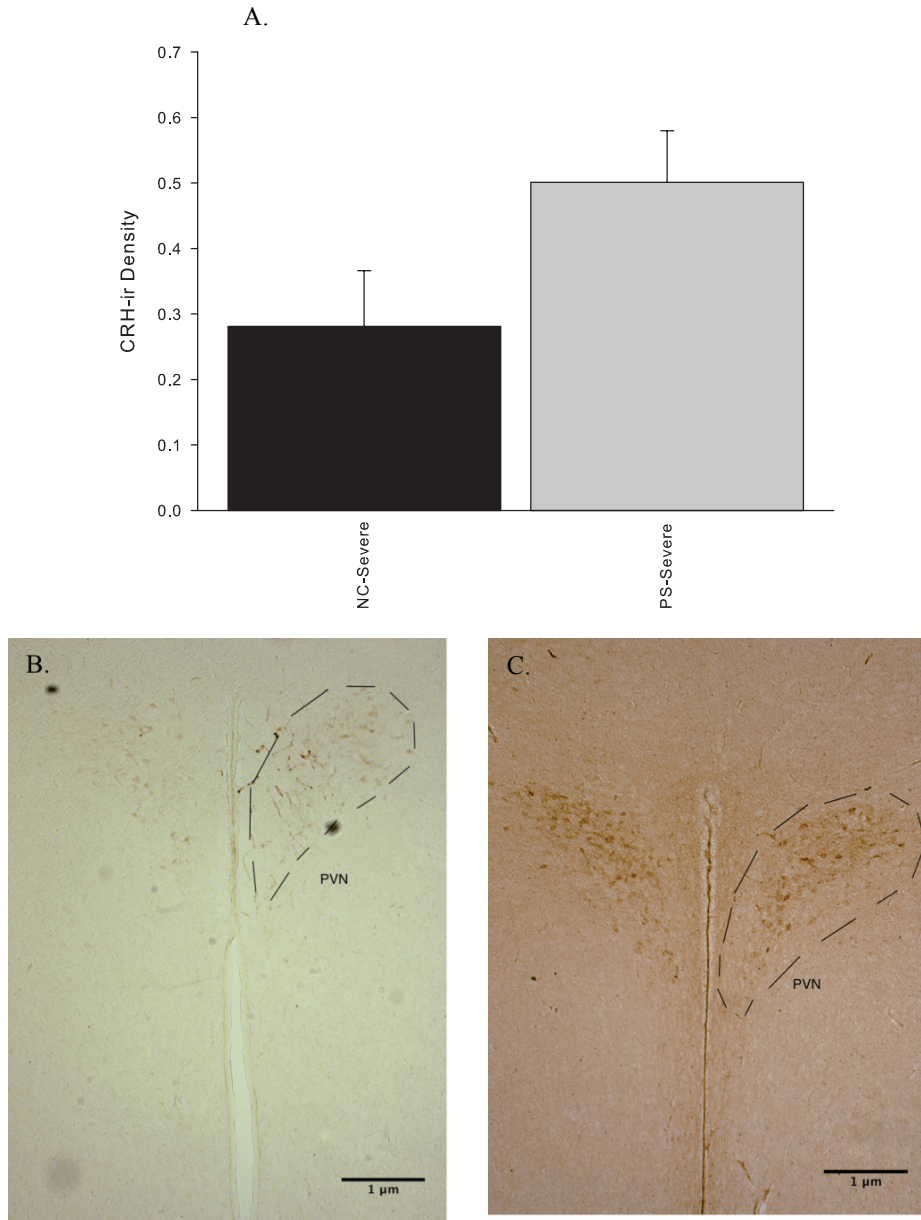


Figure 3.11. A) Mean and standard error CRH-ir density levels in PD15 pups. NC pups experiencing severe seizures had significantly lower CRH-ir than PS pups experiencing severe seizures, $p < .05$. Images of CRH-ir in the PVN of pups in **B)** NC-Severe and **C)** PS-Severe conditions.

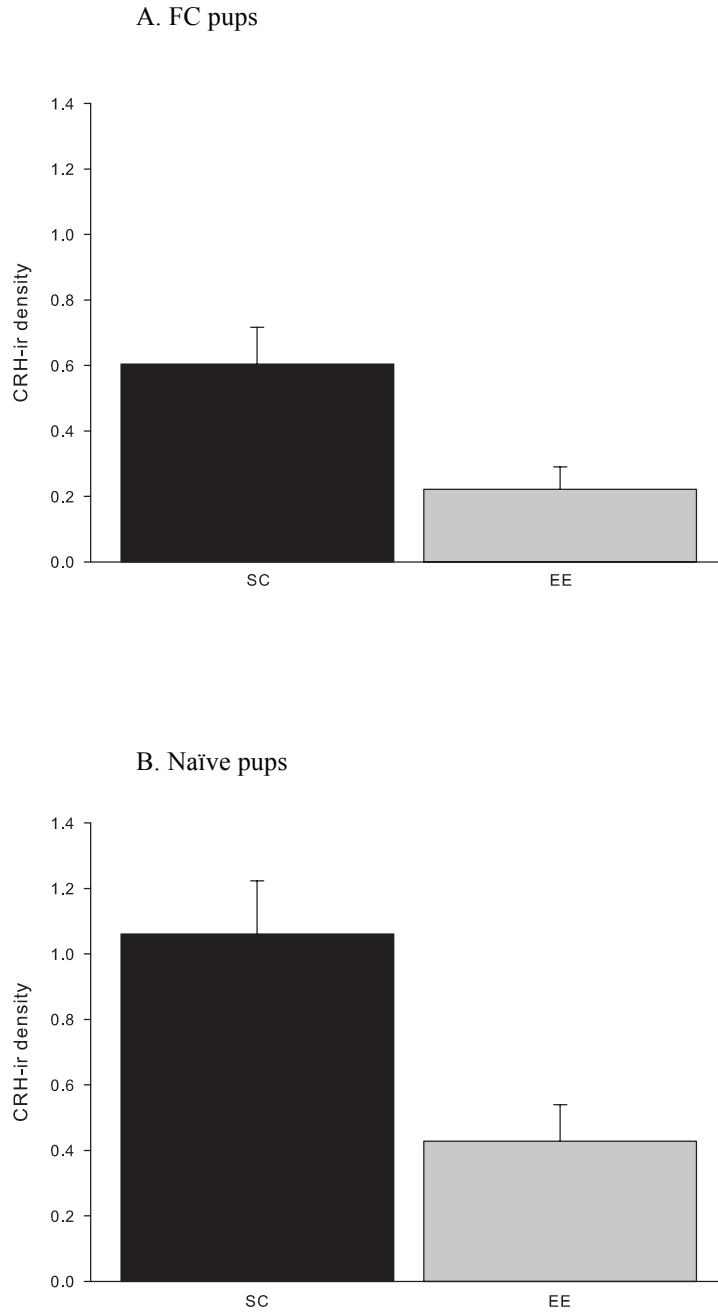


Figure 3.12. Mean and standard error CRH-ir density levels in PD15 pups. EE pups seizures had significantly lower CRH-ir than SC pups in both FC (**A**) and naïve conditions (**B**), $p < .05$.

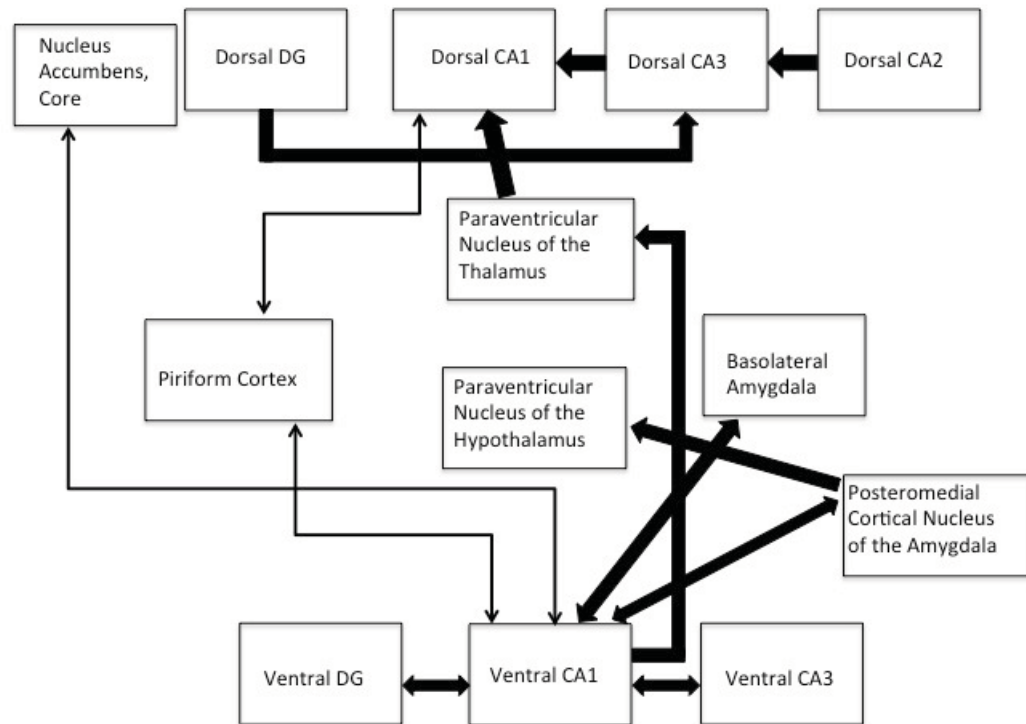


Figure 4.1. Connectivity flow-chart of regions that were affected by pre- or postnatal manipulation and/or FC.