EARLY LIFE EXPERIENCES INFLUENCE SEIZURE SUSCEPTIBILITY OF 14-DAY OLD RAT PUPS IN A DAM-DEPENDENT AND SEX-DEPENDENT MANNER

By

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Submitted in partial fulfilment of the requirements
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DALHOUSIE UNIVERSITY

DEPARTMENT OF ANATOMY & NEUROBIOLOGY

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今までずっと私を支えてくれた

お父さんとお母さんへ

ありがとう

の言葉とともに贈ります

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Abstract

Epilepsy is a devastating disorder characterized by recurrent seizures. The pathophysiology of the disorder is not well understood. In this study the effects of early life, including pre- and post-natal, experiences on the seizure susceptibility of offspring was determined. Sprague-Dawley rats were air transported prior to breeding (In-House), on gestation day 9 (G9), or G16. The maternal behaviour was scored from P2-P13. On P14, seizure susceptibility of pups was assessed by randomly assigning the pups into Naïve (control), Saline, lipopolysaccharides (LPS; 200 µg/kg), Kainic acid (KA; 1.75 mg/kg) or Febrile Convulsion (FC; LPS followed by KA) groups. No effect of prenatal transport was found on seizure susceptibility. Licking and grooming (LG) maternal behaviour was associated with higher FC seizure susceptibility of offspring. Male pups were more susceptible to FC seizure than female pups. These results emphasize the dam-dependent and sex-dependent effects of early life experiences on seizure susceptibility of offspring.

List of Abbreviations and Symbols Used

AB: Arched back nursing

AB1: Arched back nursing during first postnatal week

AB2: Arched back nursing during second postnatal week

AB10: Arched back nursing during first 10 postnatal days

BAL: Basolateral nucleus of amygdala

BL: Blanket

BL1: Blanket during first postnatal week

BL2: Blanket during second postnatal week

BL10: Blanket during first 10 postnatal days

BST: Bed nuclei of Stria Terminalis

CA1: Cornus ammonis 1 of hippocampus

CA2: Cornus ammonis 2 of hippocampus

CA3: Cornus ammonis 3 of hippocampus

DG: Dentage gyrus of hippocmapus

DO: Dam off

DO1: Dam off during first postnatal week

DO2: Dam off during second postnatal week

DO10: Dam off during first 10 postnatal days

FC: Febrile Convulsion

GR: Glucocorticoid receptor

HPA-axis: Hypothalamic-pituitary adrenal axis

KA: Kainic Acid

LG: Licking and grooming

LG1: Licking and grooming during first postnatal week

LG2: Licking and grooming during second postnatal week

LG10: Licking and grooming during first 10 postnatal days

LPS: Lipopolysaccharides

MeA: Medial nucleus of amygdala

MR: Mineralocorticoid receptor

PA: Passive

PA1: Passive during first postnatal week

PA2: Passive during second postnatal week

PA10: Passive during first 10 postnatal days

PVN: Paraventricular Nucleus of Hypothalamus

WDS: Wet Dog Shakes

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Please come visit me in Japan

最後まで私のわがままを聞いてくれた両親へ感謝の気持ちを伝えたいと思います。こうしてカナダの大学院を卒業できるのも、ずっと支えてくれた両親がいたからです。私がやりたいと言った事は何でも、何も言わずにサポートしてくれました。イライラして辛く当たった時も、笑顔で背中を押してくれました。今まで何度も励まされてきました。本当にありがとう、感謝しています。

Chapter 1: Introduction

Epilepsy

Epilepsy is one of the most common neurological disorders affecting more than 50 million people worldwide with an annual incidence of 47 per 100,000 people (Kotsopoulos et al., 2002; Ngugi et al., 2010). The disorder can be clinically classified into 3 categories by etiology; either genetic, structural/metabolic, or unknown. Having a gene that increases the likelihood of epilepsy development or epileptogenesis is the cause of genetic epilepsy (Johnson and Shorvon, 2011; Scheffer and Berkovic 2003). Structural/metabolic epilepsy results from disturbances in cellular physiology or in alterations of the structure or functions of the brain caused by traumatic brain injury, infection and stroke (Berg et al., 2010). The majority of the cases (50-60%) are classified as unknown because there is no obvious cause despite intensive investigation (Hauser et al., 1993).

The characteristic of epilepsy is recurrent seizures. Seizure is an abnormal excessive synchronous brain activity that causes transient disturbances in cognitive and physical functions. These disturbances sometimes lead to dangerous outcomes including head injuries, vehicle accidents, and drowning and can lead to death in severe cases. In fact, mortality among patients with epilepsy is 2-3 fold higher than that of the general population (Tomson et al., 2004). In addition, patients with epilepsy are faced with stigmatisms in the general population and a low quality of life, leading to a higher rate of bullying among children with epilepsy (Hamiwka et al., 2009; Johnson and Shorvon, 2011; Rantanen et al., 2012). Therefore, it is important to treat or control seizure activity among patients with epilepsy.

There are several treatments for epilepsy including pharmacotherapy, surgery, and a ketogenic diet that has been purported to be effective in controlling seizures. The mainstay of treatment is pharmacotherapy using anticonvulsants. Although anticonvulsants control seizures, they do not modify or cure the disease itself. In addition, the most widely used drugs such as carbamazepine and valporic acid have severe side effects including drowsiness, fatigue, headache, sedation, gastrointestinal disturbance and liver impairment such that some patients have better quality of life without using the anticonvulsants (Perruca and Tomson, 2011). Moreover, in 1/3 of the cases of epilepsy, pharmacotherapy fails to control the seizures despite the intense investigations. This is due to the complexity of seizure activity and that the most effective drugs act on multiple sites. Therefore, a better understanding of the pathophysiology of epilepsy is required for improved treatment or possibly prevention of the disorder. As epilepsy consists of recurrent seizures, the first step to understand the pathophysiology of epilepsy is to understand the mechanism of seizure.

Seizure

Seizure is an abnormal synchronous brain activity that is caused by an imbalance of excitation and inhibition. While the incidence of epilepsy is 46 per 100,000 people, the incidence of seizure in the population is about 56 per 100,000 people. The highest incidence of seizures occurs in the first couple years of life in humans (Hauser, 1993; Kotsopoulos et al., 2002). There are two broad types of seizures; partial onset and primarily generalized (Berg et al., 2010). Partial onset seizures originate in a focal group of neurons and they remain restricted to that area or are propagated to other regions of the brain. If the seizure activity propagates to other regions, the seizure type is classified as

partial onset with secondary generalization. In contrast, primarily generalized seizures involve both hemispheres of the brain simultaneously. The most common type of seizure is a partial complex seizure that typically originates in the limbic system including the hippocampus (Hauser, 1993).

The mechanism of seizure is complex, but all seizures are triggered by brain activity that is shifted toward excitation. Under normal conditions, brain activity is maintained by a delicate balance of three neurotransmitter systems; excitatory, inhibitory, and modulatory.

The major excitatory neurotransmitter system in the brain is glutamate. Glutamate neurotransmitters depolarize cells upon binding to alpha-amino-3-hydroxy-5-methyl-r-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors. All these receptors are ionotropic channels that are permeable to sodium and calcium. AMPA receptors open most rapidly upon binding to glutamate and are known as the rapid excitatory transmission system in a brain. Kainate receptors are much like AMPA receptors but have higher and selective affinity to its agonist, kainic acid (KA). NMDA receptors are unique in that activation and opening requires depolarization of membrane in addition to binding of glutamate. However, opening of the NMDA receptors also allows sodium and calcium conductance and depolarization of the cells. NMDA receptors are also unique with a role in synaptogenesis and memory formation. There are also metabotropic receptors (mGlu) for the glutamatergic system. These mGlu receptors are not ion channels and have modulatory effects on AMPA, kainate and NMDA receptors through the second messenger system.

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter system of the brain. Inhibitory neurotransmitter GABA binds to $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are ionotropic channels and permeable to chloride ions that flow into the cell upon binding of GABA. $GABA_B$ receptors are metabotropic channels and trigger potassium channel opening upon GABA binding. Activation of both receptors results in hyperpolarization of cells in adult brain. It is worth noting that GABA acts as an excitatory neurotransmitter system in immature brain and contributes to high seizure susceptibility in newborns (Rakhade and Jensen, 2009). In immature brain, potassium-chloride cotransporter 2 (KCC2) expression is lower while sodium-potassium-chloride cotransporter 2 (NKCC2) expression is higher than in adult brain. KCC2 export chloride while NKCC2 import chloride. Thus, a low ratio of KCC2/NKCC2 results in a higher intracellular chloride concentration in immature brain. Therefore, the opening of the chloride channel triggers an efflux of chloride ions and causes depolarization in immature brain.

Other neurotransmitter systems in the brain including acetylcholine, dopamine, serotonin, and norepinephrine are classified as modulatory neurotransmitter systems. These neurotransmitter systems have both excitatory and inhibitory effects on cells depending on what type of receptors they bind to and which area of the brain. In general, these neurotransmitters enhance or reduce excitatory or inhibitory inputs of glutamate and GABA. Other modulators including glucocorticoids and cytokines also impact cell activity, although these are not traditionally classified as neurotransmitters.

Brain activity is an outcome of the delicate balance of these three systems. Alterations in any of the systems that favor excitation can result in increased seizure

susceptibility. For example, KA, pentylenetetrazol, and pilocarpine are convulsants and induce seizures by acting on these 3 systems *in vivo* and *in vitro* (Raol and Brooks-Kayal, 2012). KA binds to kainate receptors and activates the glutamate system while pentylenetetrazol binds to $GABA_A$ receptors and inhibits their action. Pilocarpine is a muscarinic receptor antagonist. Muscarinic receptors are a type of acetylcholine receptor that enhances the excitatory glutamate neurotransmitter system. Although they all act on different systems, the outcome is the same as they all shift brain activity toward excitation and increase seizure susceptibility.

Based on animal models of seizures, previous studies have developed anticonvulsants that control and stop seizures. However, as discussed earlier, there are problems in the pharmacotherapy including severe side effects, failure in 1/3 of the cases, and most importantly, they do not modify the underlying pathophysiology of seizure and thus fail to prevent epileptogenesis. Therefore, there is an urgent need for the development of better models that address the question: what factors contribute to higher seizure susceptibility? Answering this question will not only lead to development of better treatments, but may also identify the etiology of the most common form of epilepsy that has no known identified cause.

High comorbidities of epilepsy with other neurological disorders in humans suggest that all of these disorders are symptoms of abnormal brain functions. As most neurological disorders including epilepsy do not have obvious structural abnormality, the alterations of molecular phenotype of a brain may lead to abnormal function. There are several studies that link altered molecular phenotype of a brain and psychiatric disorder such as depression, a neurodevelopmental disorder highly comorbid with epilepsy (see

Kupfer et al., 2012 for review). This molecular phenotype is programmed during the neurodevelopment that occurs in early life. Therefore, it is important to understand neurodevelopment and factors that impact development that may lead to the modification of the programming of the molecular phenotype of a brain leading to a higher seizure susceptibility.

Neurodevelopment

Development of the brain begins with neurulation, a formation of the neural tube that gives rise to a whole central nervous system. This process begins around gestational day 18 (GD18) in humans and GD8 in rats (Rice and Barone, 2000). Disturbances in this period may result in severe abnormal central nervous system development such as spina bifida. Neurulation is followed by four basic neurodevelopmental processes; proliferation, migration, differentiation, and synaptogenesis. Proliferation is a process by which brain cells divide and increase in number. Migration follows after proliferation and is a movement of brain cells to specific locations of the developing brain. Differentiation refers to a process in which cells become specialized cell types such as glutamatergic neurons. Synaptogenesis is the formation of cell-to-cell contacts and is important in the wiring/connectivity of the neurons. These four processes begin early in the gestational period. However, each of the processes has a time period in which they are occurring rapidly during which the cells are susceptible to disturbances (Rice and Barone, 2000). This time period for each process differs depending on the region of the brain and the type of cells. However, in general, the most rapid and intensive proliferation and migration of brain cells takes place during the prenatal and early postnatal period in rats. In contrast, intensive differentiation and synaptogenesis takes place during the first

couple of postnatal weeks in rats (Rice and Barone, 2000). Any disturbances of these systems during this time may lead to alteration in the brain development and function including number of cells, receptor phenotypes and plasticity without altering obvious structure of the brain.

Neurodevelopment is controlled by genetic and environmental factors. Environmental factors such as toxins can directly affect neurodevelopment. For example, alcohol consumption during pregnancy increases seizure susceptibility and risk of epilepsy in offspring (Bell et al., 2010). However, toxins usually lead to obvious structural abnormalities in brain such as smaller brain size. Other environmental factors such as stress can also impact brain development but in indirect ways and does not necessarily lead to an obvious structural alteration. Stress exposure during the gestational period has been linked with higher risk of neurodevelopmental disorders including depression and anxiety that have a high comorbidity with epilepsy (Charil et al., 2010; Lupien et al., 2009; Sandman et al., 2010). Therefore, the possibility exist that prenatal maternal stress alters neurodevelopment and may result in abnormal brain functions including higher seizure susceptibility and thus higher risk for epileptogenesis.

Prenatal Stress and Neurodevelopment

The key player that mediates the effects of prenatal stress on neurodevelopment of offspring is the stress hormone, glucocorticoid, released following activation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress stimulates cells in the paraventricular nucleus of the hypothalamus (PVN) to release corticotropin-releasing hormone (CRH) that in turn stimulates release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH then stimulates the adrenal cortex to release glucocorticoids.

Glucocorticoid negatively feedbacks on cells in the PVN and pituitary gland to stop CRH and ACTH release, respectively. The glucocorticoid also activates hippocampal neurons that inhibit PVN neurons from releasing CRH. Thus, the hippocampus has an important role in the regulation of glucocorticoid.

The regulation of glucocorticoid is altered in offspring from mothers that are prenatally stressed, changing behavioural phenotypes of the offspring toward heightened anxiety and an increased risk of depression (Charil et al., 2010; Harris and Seckl, 2011; Lupien et al., 2009; Oitzl et al., 2010; Sandman et al., 2010). The hippocampus is involved in the most common type of seizures as well as in the HPA-axis regulation. Thus it is possible that prenatal stress alters the HPA-axis and glucocorticoid regulation resulting in altered seizure susceptibility.

The mechanisms involved in the effects of prenatal stress on fetal brain development are complicated and involve at least 3 homeostasis systems of mothers; the HPA-axis, the sympathetic nervous system (SNS), and the immune system. These 3 systems interact and regulate each other, making it hard to separate the effect of each system on neurodevelopment as any treatment that activates one system also activates the other two. However, the HPA-axis is thought to be the major system involved in altered behaviour of offspring including anxiety and depression.

Prenatal stress induces HPA-axis activation and release of glucocorticoids in the dams. Excess glucocorticoids have deleterious effects on neurodevelopment of offspring directly or indirectly. Maternal glucocorticoid can cross the placenta and directly affect fetal neurodevelopment. About 10 – 20 % of maternal glucocorticoids are transferred into the fetus. The key mechanism controlling the amount of glucocorticoids transferred into

the fetus is placental 11-beta hydroxysteroid dehydrogenase 2 (11**β** -HSD2; Charil et al., 2010; Glover et al., 2010; Green et al., 2011; Harris and Seckl, 2011). 11**β** -HSD2 is an enzyme that breaks down glucocorticoids into the less active metabolite, cortisone. Prenatal maternal stress reduces placental 11**β** -HSD2 and thus increases fetal glucocorticoid exposure (Mulder et al., 2002).

Prenatal maternal stress also alters the transfer of nutrients into the fetus by altering levels of placental glucose transporters (GLUT; Gitau et al., 2001; Harris and Seckl, 2011; Mairesse et al., 2007). Prenatal restraint stress decreased placental GLUT1 by half and increased GLUT3 and GLUT4 (Mairesse et al., 2007). Consequently, weight and plasma glucose level were significantly decreased in prenatally stressed male fetuses compared to controls (Mairesse et al., 2007). These results suggest that prenatal stress reduces nutrients transferring from mother to fetus by altering GLUT and may impact the development of the offspring.

The alterations in maternal physiology are reflected in changes in fetal neurodevelopment. At the molecular level, prenatal stress alters the morphology of neurons and also the excitatory, inhibitory, and modulatory neurotransmitter systems (Barros et al., 2006; Jia et al., 2010; Laloux et al., 2012; Zuena et al., 2008). For example, prenatal restraint stress decreased the dorsal hippocampal NR1 subunit of NMDA receptors in female rat offspring and decreased the dorsal hippocampal and amygdala mGlu2/3 and mGlu5 receptors in both male and female rat offspring (Jia et al., 2010; Laloux et al., 2012; Zuena et al., 2008). The NR1 subunit contains a glycine-binding site of the NMDA that enhances the NMDA-induced ion current upon binding with glycine. The mGlu2/3 receptors are largely expressed in presynaptic neurons and decreases

glutamate release upon activation (Chaki et al., 2012). The mGlu5 receptor is largely

expressed on postsynaptic GABAergic interneurons and increase GABA activity thereby

decreasing glutamatergic neurotransmission (Chaki et al., 2012). In addition, prenatal

restraint stress decreased dorsal hippocampal benzodiazepine binding sites of GABA

receptor levels and amygdala gamma 2 subunit of $GABA_A$ in adult male offspring

(Barros et al., 2006; Laloux et al., 2012). Moreover, prenatal restraint stress increased

baseline plasma corticosterone and decreased glucocorticoid receptor (GR) and

mineralocorticoid receptor (MR) in hippocampus in adult rat offspring (Brunton and

Russel, 2010; Green et al., 2011). Prenatal stress also changes functional phenotypes of

brain cells in the offspring. Prenatal restraint stress and cat exposure decreased excitatory

postsynaptic potential (EPSP) of CA1 pyramidal neurons of young (P15) offspring

(Saboory et al., 2011). In addition, long-term potentiation (LTP) was impaired but long-

term depression (LTD) was enhanced in the CA1 region of hippocampus in prenatally

stressed adult rat offspring (Yang et al., 2006; Yeh et al., 2012).

These changes in molecular and functional phenotypes in the brain are reflected in

the behavioural alteration of offspring. Prenatal stress decreased the number of entries

into and the percentage of the time spent in open arms as well as increasing the latency to

enter open arm in the elevated plus maze test (Barros et al., 2006; Brunton and Russel,

2010; Laloux et al., 2012; Zuena et al., 2008). These results suggest that prenatal stress

increases anxiety-like behaviour in the rat offspring. In addition, prenatal stress increased

escape latency and decreased the number of times rats cross the quadrant that contain the

hidden platform suggesting impairment of spatial memory (Yang et al., 2006). Again,

both anxiety and memory impairment are highly comorbid with epilepsy pointing out that prenatal stress may also have effects on seizure susceptibility.

All of these studies suggest prenatal stress has a long-term or permanent effect on neurodevelopment of offspring. Prenatal stress impacts various areas of the brain; however, limbic regions appear to be more sensitive due to their role in HPA-axis. This is reflected in higher rates of depression and anxiety, in which the limbic system is known to be involved, in prenatally stressed humans and rats. As mentioned previously, the limbic system is an important area of the brain for the most common type of seizure. Therefore, it is possible that prenatal stress affects seizure susceptibility of offspring through neurodevelopmental programming of limbic system.

In fact, the results of previous experiments in our laboratory suggest that prenatal maternal stress in the form of air-transport may have an influence on seizure susceptibility of the pups. The pups from dams that were air-transported during middle gestational period (G9-G11) showed more severe seizure behaviour than the pups from the dams that were transported during early gestational period (G3-G5). During air-transport, the dams were exposed to different environment, loud noises, and changes in pressure. As the novel environment and loud noises are shown to induce a stress response in rats, it is possible that air-transport affected the seizure susceptibility of the offspring (Charil et al., 2010). The pressure change in the air can cause decreased uterine artery blood flow and could cause acute hypoxia, potentially affecting the brain development of offspring (Harris and Seckl 2010; Zamudio et al., 1995).

Maternal Behaviour and Postnatal Brain Development

I have discussed the importance of prenatal experience on neurodevelopment of offspring. Postnatal experiences are also important factors that can impact neurodevelopment of offspring. The brain is still under rapid and intense development after birth (Rice and Barone, 2000). Most prominent proliferation and migration occurs during the prenatal period; however, synaptogenesis is more active during first few postnatal weeks (Rice and Barone, 2000). Synaptogenesis is an important developmental process where a neuron is integrated into the neural network. Any perturbation of this process may have an impact on neural networking. This critical period of brain development lasts for about 3 weeks postnatally in rats (Rice and Barone, 2000). After this period, the brain is less plastic and these developmental processes are limited.

Of all the environmental experiences neonates receive, maternal care is an essential experience for survival. Maternal care received by individual offspring varies greatly and this variation is associated with different outcomes. For example, childhood adversity such as child abuse and neglect increases risk for mental disorders including anxiety and depression (see Afifi et al., 2011 for review). In rodents, levels of some maternal behaviours influence behaviours of the offspring such as anxiety-like behaviours, fear response, and fear memory (Champagne et al., 2008; Francis et al., 2002). These behaviours by offspring are associated with the alterations in their brain development including alterations in receptor phenotypes, morphology of neurons, and plasticity (Caldji et al., 1998; Caldji et al., 2003; Champagne et al., 2003; Champagne et al., 2008; Liu et al., 1997; Zhang et al., 2010). Therefore, both prenatal stress and maternal care are important factor during prenatal and early postnatal period resulting in

long-term or permanent modification of the molecular and functional phenotype of the

brain and behaviour of offspring (Charil et al., 2010; Harris and Seckl, 2011; Kaffman

and Meaney, 2007).

In rodent models, licking and grooming (LG) and arched back nursing (AB)

during the first postnatal week and first 10 postnatal days are positively correlated with

significantly higher hippocampal GR expression and less HPA-axis response (Caldji et

al., 1998; Francis et al 199; Liu et al 1997; Menard et al 2004). Interestingly, LG during

the second postnatal week was not associated with the hippocampal GR expression

suggesting that this effect is time dependent. In addition, the offspring of High LG-AB

dams have significantly less methylation of exon 17 GR promoters than offspring of Low

LG-AB dams, at postnatal day 1 (P1) and this difference persisted until P90 (Weaver et

al., 2004). This reduction in methylated exon 17 GR promoters is associated with a

change in hippocampal GR levels of offspring.

Maternal care not only affects the HPA-axis of offspring but also alters

neurotransmitter systems including GABA and NMDA in a brain of offspring (Caldji et

al., 1998; Caldji et al., 2000; Caldji et al., 2003; Kaffman and Meaney, 2007; Liu et al.,

2000; Zhang et al., 2010). For example, 15 minutes and 180 minutes of maternal

separation during the first two postnatal weeks (P1-P14) significantly reduced $GABA_A$

receptor expression levels in various brain areas including the frontal cortex, amygdala,

and locus coeruleus (Caldji et al., 2003). Another study found significantly lower

glutamic acid decarboxylase (GAD), the enzyme for GABA synthesis, in the

hippocampus of pups from Low-LG dams compared to pups from High-LG dams (Zhang

et al., 2010). In addition, a higher methylation of *GAD1* promoter is seen in pups from

Low-LG dams (Zhang et al., 2010). Moreover, nerve growth factor-inducible factor A (NGFI-A) expression was lower in pups from Low-LG dams. NGFI-A binds to the *GAD1* promoter and initiates transcription of GAD. Therefore, Low-LG may be associated with lower GABA activity. Interestingly, NMDA receptor mRNA was also lower in the hippocampus of the pups from Low-LG dams suggesting that excitatory glutamate activity is lowered (Liu et al., 2000).

These changes in molecular phenotype are reflected in the function of cells in the brain of offspring. Champagne et al., (2008) showed shorter dendritic branch length and lower spine density in Cornus ammonis 1 (CA1) cells in adult offspring of Low-LG dams compared to High-LG. Impaired long-term potentiation (LTP) in cultured hippocampal slices of Low-LG offspring was seen compared to High-LG offspring. Interestingly, after corticosterone treatment, LTP was enhanced in Low-LG offspring but not in High LG offspring. This LTP difference was reflected in the contextual fear memory performance (i.e., mimics corticosterone treatment) such that Low-LG offspring performed better compared to High-LG offspring. This indicates that maternal care is associated with changes in cellular response to a stimulus and thus the functional phenotype of the offspring.

Cross-fostering studies showed that these effects are due to maternal behaviours or "mother-infant interaction" rather than genetic factors. When the pups born to Low-LG dams were cross-fostered with High-LG dams, they were comparable to the pups born to and raised with High-LG dams in their GR mRNA expression and GR promoter methylation in the hippocampus, GABA$_A$ levels in the amygdala and spatial memory (Caldji et al., 2003; Francis et al., 1999; Liu et al., 2000; Weaver et al., 2004). This was

also true in the opposite direction in that the pups born to High-LG dams and cross-fostered with Low-LG dams were indistinguishable in these parameters with the pups born to and raised by Low-LG dams.

These studies suggest that maternal care contributes to programming of neurodevelopment and behaviours of offspring. To my knowledge, no study has looked at the contribution of maternal care on seizure susceptibility of offspring. However, maternal behaviour contributes to programming of HPA-axis and affect the limbic system development. Again, the limbic system is an important region of the brain that is involved in seizure activities. Therefore, maternal care may be associated with seizure susceptibility of offspring.

Importance of the Limbic System

I have discussed the importance of early experiences including prenatal maternal stress and maternal behaviour on brain development, behaviour, and possibly the programming for disorders such as epilepsy in offspring. The limbic system, especially the hippocampus is the area of the brain where these factors act. First, the hippocampus is the origin of seizure in the most common type of seizure. Second, the impact of prenatal stress is greatest in the hippocampus where expression of GR is reduced and consequently leading to enhanced stress response. Third, maternal care also acts on the hippocampus altering GR expression and thus the HPA-axis of the pups. In addition, the hippocampus is an important brain area involved in learning and memory and emotions. The alterations in these functions are observed in patients with epilepsy and also in comorbidities such as depression. Thus, the hippocampus is an important structure for this study.

In recent studies, the dorsal and ventral hippocampi have been shown to have different anatomical connections and functions (Fanselow and Dong, 2010). For example, dorsal CA1 have massive bidirectional connections with the dorsal subiculum and retrosplenial and anterior cingulated cortices (Fanseloq and Dong, 2010). These regions are involved in visuospatial information and memory processing and thus this network of connections with dorsal hippocampus is thought to control spatial memory. In contrast, ventral CA1 have massive bidirectional connections with the amygdala complex that is thought to have a role in fear memory and emotions (Fanselow and Dong, 2010; Pitkanen et al., 2000). In fact, ventral hippocampus projects to the anteromedial bed nuclei of stria terminalis (BST) that then directly projects to the PVH to innervate CRH neurons (Dong and Swanson, 2006). This suggests that the ventral hippocampus has an important role in controlling the HPA-axis. Therefore, it is important to consider the dorsal and ventral hippocampus separately.

Summary and Research Hypothesis

In summary, early life, including prenatal and postnatal experiences contribute to the developmental programming of the brain of offspring. The adverse early experiences such as prenatal stress and adverse childhood experiences can result in abnormal brain development that may be manifested as increased risk for disorders including epilepsy. In the present experiment, I hypothesized that prenatal maternal stress in the form of air-transport and maternal behaviour alters seizure susceptibility of rat offspring.

To test this hypothesis, I used rat pups from dams that were transported at different time points; 2 weeks before pregnancy (In-House), gestational day 9 (G9), and

gestational day 16 (G16). The maternal behaviour was scored from postnatal day 2 (P2) through P13 and the seizure susceptibility of the pups was assessed on P14.

Chapter 2: Methods

Experimental Design

This study was designed to examine the effect of transporting pregnant rats on seizure susceptibility of their pups. Thus the studies were designed to compare In-House bred litters to those from dams transported at two gestational time points: gestational day 9 (G9) and gestational day 16 (G16). The overall design of the experiment is shown in Figure 1. The two transport time dates were chosen based on the stage of brain development. Neurulation, formation of the neural tube, occurs around G8-G10. Thus if G9 transport affects brain development, the effects will be more broad and general to whole brain. On G16, rapid neurogenesis in the rat hippocampus takes place. Therefore, the effects of stress, if any, will be more specific in the hippocampus on G16.
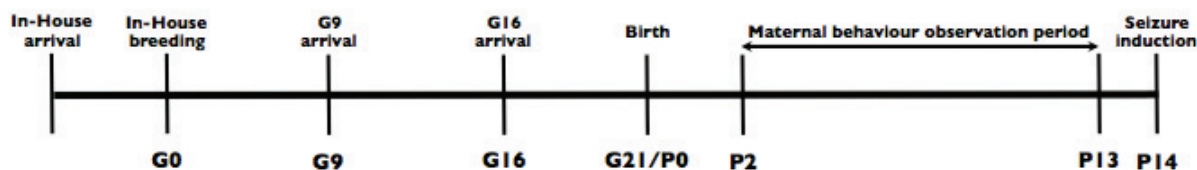
Figure 1. Experimental design. Dams were transported prior to breeding (In-House), on gestational day 9 (G9) or on gestational day 16 (G16). Females assigned to be In-House were given 2 weeks of acclimatization period prior to breeding. During the 2 weeks, the vaginal smears were collected to determine their estrous cycle. Once the estrous cycle was determined, we arranged mating so that In-House group and G9 and G16 groups gave birth on a same day or within 24 hours of each other. Starting from P2 through P13, maternal behaviour was observed and scored. On P14, seizure susceptibility of the pups was assessed.

Animals

All experiments were approved by University Committee on Laboratory Animals and done in accordance with Canadian Council on Animal Care guidelines. A total of 12 male and 12 female Sprague-Dawley rats were ordered from Charles River (Montreal, Canada) prior to being bred in-house. Of these 12 females, 10 successfully conceived and were used in this study. A total of 16 timed-pregnant dams were transported at G9 (8 dams) or G16 (8 dams) from Charles River. All the rats were housed in the same colony room with 12-hour light/dark cycle (7 am/7 pm) and were provided food and water *ad libitum*. A total of 150 pups were assessed for seizure susceptibility on postnatal day 14.

A total of 4 experimental runs were conducted in this study. For each experimental run, 2-3 In-House, 2 G9 and 2 G16 dams and their offspring were used. All the environmental conditions were kept the same throughout all experiments except for a room change with the Carlton Animal Care Facility of Dalhousie University for the fourth experimental run.

Estrous Cycle Determination

Estrous cycle was determined for the dams that were in-house bred to ensure they were on a similar cycle with the transported timed-pregnant dams. A vaginal smear was collected everyday starting 2 days after their arrival in the vivarium and ending 2 days before they were mated. This procedure was done between 10:30-11:00am.Vaginal smears were obtained by injecting 0.1 ml saline into their vagina with a syringe (no needle). The vaginal discharge was collected and smeared on to superfrost slides and left at room temperature to dry. The vaginal smears were then fixed and stained using Diff-Quick staining kit (VWR), which consists of 3 solutions: a fixative or methanol, solution

I to stain for cytoplasm, and solution II to stain for cellular nuclei. Each slide was dipped into these 3 solutions in sequential order as follows: fixative, solution I, then solution II. The smears were then dried again at room temperature.

Smears were viewed and images were captured using Leica microscope connected to PixeLINK camera using a PixeLINK OEM capturing program at 10x magnification. These images were compared to previously collected specimens to determine estrous cycle.

Maternal Behaviour

Maternal behaviour was scored daily from postnatal day 2 (P2; day of birth was P0) through postnatal day 13 (P13) for 1 hour at 8:00 am, 11:00 am, and 2:00 pm. During each one-hour session, behaviors were scored every 3 minutes as previously described (Champagne et al. 2008). The eight maternal behaviours scored during the observation period include; licking and grooming, arched back nursing, passive nursing, blanket, eating/nesting, dam off, pup retrieval, and pup removal (Table 1). All the behaviours were not mutually exclusive and when there were two or more behaviours observed simultaneously, they were considered to be ½ (2 behaviours), 1/3 (3 behaviours), or ¼ (4 behaviours).

To analyze maternal behaviour, percentage of the time (minutes) dams spent on each maternal behavior during first postnatal week (P2-P7), second postnatal week (P8-P13), and first 10 days (P2-P10) was calculated as previously described (Champagne et al 2008). The cumulative time during first and second postnatal week, and first 10 days was divided by total time of observation period to get the percentage. Then, for each behaviour, the group mean were used to divide the dams into High and Low groups for

first and second postnatal week, and first 10 days. In previous study (Champagne et al., 2003), dams were categorized into High (above the mean + 1 SD) or Low (below the mean – 1 SD) based on their time spent in each behaviour. However, in this study, this classification could not be used due to the limitation of smaller number of dams. Therefore, I categorized the dams as High if the relative frequency of the time they spent in the behaviour was above the population mean, and Low if the relative frequency of the time they spent in the behaviour was as below the population mean.

For the nomenclature of maternal behaviour, abbreviations were used as follows: Licking and grooming (LG), Arched Back nursing (AB), Passive (PA), Blanket (BL), and Dam Off (DO). Further, LG groups divided based on first (1) and second (2) postnatal week and first 10 days (10) were designated as LG1, LG2, and LG10, respectively. This categorization was applied for all maternal behavior analysis. Therefore, High-LG1 dams refer to a group of dams that spent a greater amount of time on licking and grooming behaviour compared to population average during the first postnatal week.

Ultrasonic Vocalization (USV) Recording

Ultrasonic vocalization (USV) was recorded in the housing room during the fourth experimental run using Zoom H4n Handy Mobile 4-Track Recorder (with 16-bit/96kHz sampling frequency). The recording started 1 week before the arrival of In-House group until a day before seizure induction day (P13). To analyze the USV, audacity 1.3 beta was used.

Table 1. Description of maternal behaviour

Behaviour	Description
Licking and Grooming	Dam is licking the pups body parts.
Arched Back Nursing	Dam actively positionher back up forming arched back. Most pups should be under the belly.
Passive	Dam is with the pups but on their back or side and can be sleeping.
Blanket	Dam is covering the pups but their butt is not in the air and the back is flat.
Eating and nesting	Dam is with the pups but caring for herself including eating, drinking and grooming.
Dam Off	Dam is not in any proximity of the pups.
Pup retreival	Dam moves the pups that are away back into the nest
Pup removal	Dam selectively remove a pup from the nest due to abnormal condition (i.e unhealthy).

Seizure Induction

A general time line for the seizure induction day is shown in Figure 2. On postnatal day 14, the pups were randomly assigned to one of the 5 treatment groups; Naïve, Saline, Lipopolysaccharides (LPS; 200 μg/kg; Sigma-Aldrich), Kainic Acid (KA; 1.75mg/kg; A.G. Scientific, Inc.), and Febrile Convulsion (FC; 200 μg/kg LPS followed by 1.75 mg/kg KA). All chemicals were dissolved in sterile saline and an equivalent volume was used in animals that served as saline controls. At 9 am, pups were removed from their cage and the Saline, LPS, and FC groups were intraperitoneally injected with Saline, 200 μg/kg LPS, and 200 μg/kg LPS respectively. These pups were then returned to their home-cage with their dams for 2.5 hours. After 2.5 hours, all the pups were separated from the dams and home-cages and placed into clean cages in a room where temperature was kept at 30 °C. Then the KA and the FC groups were intraperitoneally injected with 1.75 mg/kg of KA. The behaviour of all the pups was recorded for 3 hours for later scoring. However, the pups were observed during seizure provocation to monitor for any adverse events. After the 3-hour behavioural recording, pups were returned to their home-cage with their dams for 2 hours. After 2 hours in home-cage, pups were returned again to the cages in the greenhouse and their behaviour was recorded for a further1 h and then returned to their home-cage overnight.
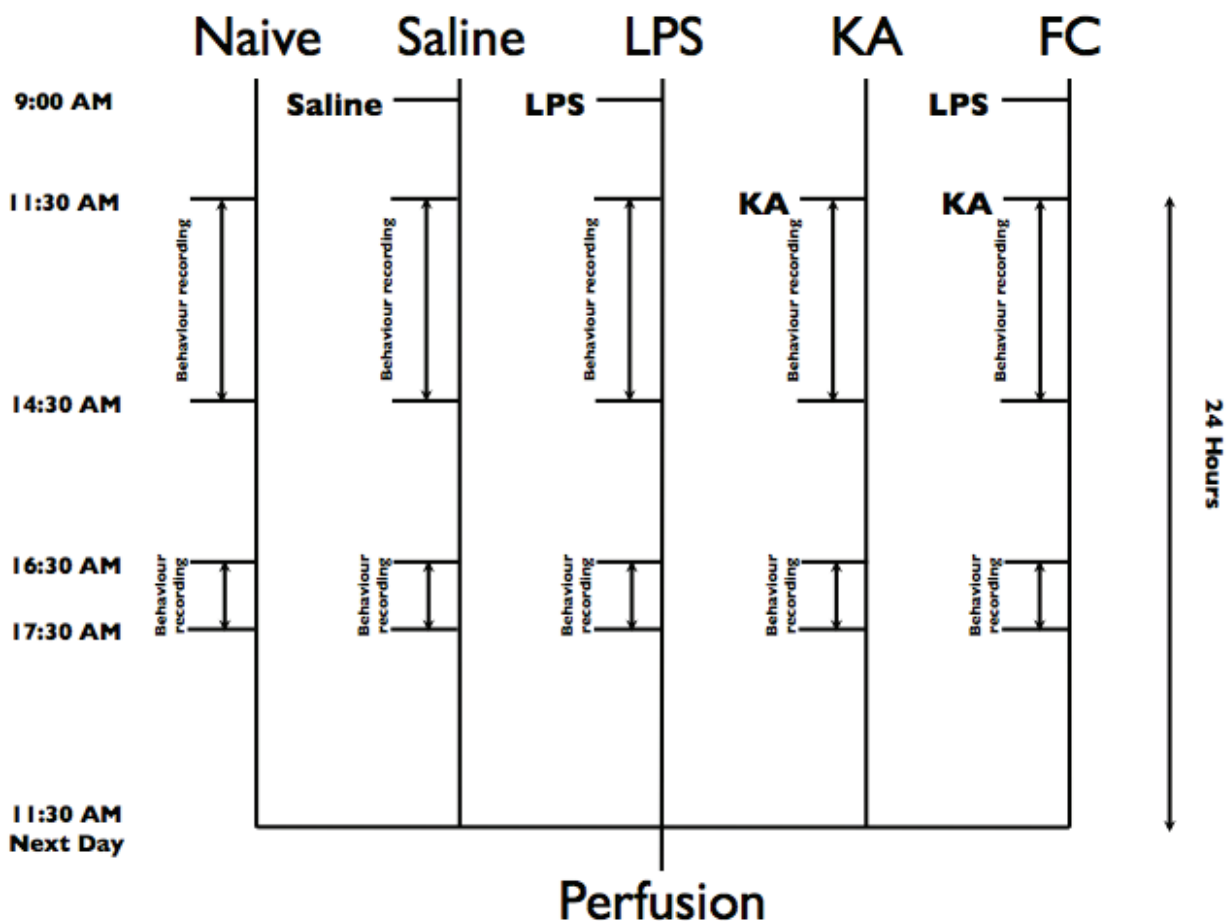
Figure 2. Timeline of the seizure induction day. Induced seizure susceptibility of pups was assessed on P14. At 9 am, all the pups were removed, weighed and coloured for identification. Then, pups assigned to Saline, LPS (200 µg/ kg), and FC (LPS; 200 µg/kg) received their respective intraperitoneal (IP) injection and were returned to home cages with their dams for 2.5 hours. Pups were then placed in a cage with their littermates (2-5 per cage) in a temperature-controlled room (30 °C). The KA and FC treatment group received KA (1.75 mg/kg) injection. Following KA injection, pups behaviour was recorded for 3 hours for later scoring. Following the recording, pups were returned to their homecage with their dams for 2 hours. After the 2 hours, the pups were placed in the cages again for 1 hour behavioural recording. Finally, the pups were returned to their dams for the night. The pups were perfused following day, 24 hours after seizure provocation, and their plasma and brains were collected.

Seizure Scoring

Pup behaviour was scored for 3 hours following KA treatment by two raters. Their behaviour was classified into two categories; normal and abnormal behaviours. Normal behaviours include sleeping, exploring, and grooming. Abnormal behaviours include myoclonus, wet-dog-shakes, continuous scratch, scratch-tonic, tonic-scratch, bicycling, tonic/clonic, ataxia, barrel rolling, non-reactive, consistent with the seizure scoring parameters previously described (Qulu et al., 2012). The score ranged from 0 to 5 where 0 being normal and 5 being most severe seizure behaviour (Table 2**).** The behaviour of each pup was scored every minute during behavioural recordings.

For seizure scoring analysis, the behavioural score was grouped into 5-minute bins (36 bins total) and the most severe score from the bin was used for analysis. The cumulative seizure score was determined by adding the score from all 36 bins (lowest of 0 to highest of 180). Onset of seizure was determined by the exact time (not 5 minute bin) of first seizure behaviour observation minus the exact time of KA injection (latency to the first seizure behaviour observation). The duration of seizure was determined as the length of time (minutes) that the pup continued to show seizure behaviour. The severity of seizure was calculated by taking the most severe score (0-5) over a 3-hour observation period from each pup.

Table 2. Description of seizure behaviour

Behaviour	Definition
Myoclonus	Twitching of the body
WDS	Wet dog shake. Rapid shaking of the body from head to butt
Cont Scr	Long (more than 5 minutes) of scratching the side of their face with their forelimbs
Scr-tonic	Intermittent scratching and tonic phases (increased tone of the body) with longer scratching phase than tonic
Tonic-Scr	Intermittent scratching and tonic phases (increased tone of the body) with longter tonic phase than scratching
Bicycling	Limbs are circling in the air (not touching faces) like riding a bicycle
Tonic/clonic	Intermittent increase in the tone and relaxing of the body (No scratching)
Ataxia	Lack of coordination of muscle. Eg. Staggering or stumbling
Barrel rolling	Turning their body on the floor
Non-reactive	No reaction to any stimulation such as poking or stumping by litter mates.

Perfusion and Brain Sectioning

Twenty-four hours after seizure induction (on P15), all the pups were overdosed with sodium pentobarbital. Blood from each pup was collected and the plasma was extracted (see below). The pups were then, exsanguinated with 0.9% saline and perfused using 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB; pH 7.4). The brains from the pups were harvested and stored in 0.4% PFA for at least 2 weeks before the sectioning. The brains were cryoprotected in 10% sucrose until they sank. The brains were sectioned at 10 μm and mounted on superfrost slides and stored in -20 °C freezer. Only 24 slides worth of sections (4 sections per each) were cut at 10μm and the rest were cut into 35μm thick and stored in 1x Millonigs at 4 °C.

Plasma Extraction

Immediately following blood collection, the samples were centrifuged for 10 minutes at 3000 rpm at 4 °C. Then supernatant (plasma) was collected and stored at -80 °C for later use in a corticosterone assay described below.

FosB Immunolabeling

Thirty-five μm thick brain sections were used for immunohistochemical localization of the protein product of the immediate early gene FosB. The brain sections were transferred into beakers and washed with phosphate buffer saline with 0.1% triton-X (PBST) 3 times for 10 min each. The sections were then incubated in 1% H_2O_2 diluted in PBS2T (0.2% Triton-X) for 45min at room temperature (2 ml per each beaker). The sections were washed with PBS2T (0.2% Triton-X) 3 times for 10 min each. Then, the

sections were incubated in primary antibody solution (2% goat serum [Vector] 1:1000 rabbit anti-FosB [Santa Cruz] in PBST (0.1% Triton-X)) over night at 4 °C.

Following incubation in primary antibody, the sections were washed 3 times in PBST (0.1% Triton-X) for 10 min each. Then, the sections were incubated in secondary antibody solution (biotinylated goat anti-rabbit IgG [Vector] in PBST (0.1% Triton-X)) for 60 min at room temperature. The sections were washed 3 times in PBST (0.1% Triton-X) for 10 min each. Then, they were incubated in ABC solution at a dilution factor of 1:500 in PBST (0.1% Triton-X)) for 90 minutes at room temperature. After the incubation, the brain sections were then washed 3 times in PBST (0.1% Triton-X) for 10 min each. Next, the brain sections were incubated in 0.05% DAB + 0.003% H_2O_2 in PBS for 14 minutes. Finally, the sections were washed with PBST (0.1% Triton-X) 2 times for 10 min each and stored in 1 x Millonigs at 4 °C until mounted.

The immunolabeled sections were mounted on superfrost slides and dried at room temperature for at least 24 hours. The sections were incubated in 50%, 70%, 95%, 100%, 100% ethanol and Xylene in order for 2 minute each. Then they were incubated in new Xylene for at least 5 minute before they were cover slipped with cover glass (VWR) using cytoseal (Thermo scientific).

Images for FosB-ir were captured with Leica microscope (LEICA DM2000) connected to PixeLINK camera using a PixeLINK capture OEM capturing program. Photographs of CA1, CA2, CA3 and DG for dorsal hippocampus and CA1, CA3 and DG for ventral hippocampus were captured from at least 3 sections for each animal (see Figure 3 and 4). In addition a photograph of medial nuclei of amygdala (MeA) and basolateral nuclei of amygdala (BAL) were captured for each animal (see Figure 4).

These photographs were analyzed with ImageJ software (ImageJ 1.45s). The optical density threshold was determined by visual inspection and kept constant for all animals within the same area of interest. For the analyses, density of FosB-ir cells (number of FosB-ir cells / mm^2) was used.

For analyses of FosB-ir, pups that died during behavioural recording were excluded. In addition, pups that did not develop seizure (i.e., 0 cumulative seizure score) were excluded except for correlational analyses.

FosB Double Immunofluorescent Labeling

To determine which cell type was activated following seizure induction, sections including dorsal and ventral hippocampi and amygdala were double immunolabeled with FosB and either NeuN, GFAP, and IBA1.

Brain sections (10 μm) on slides were thawed at room temperature and each section was circled with an Immunopen (Vector laboratories, Burlington, Ontario). The sections were incubated in 4% PFA for 10 min in 1 x PBS2T (0.2% Triton-X) containing 100 mM glycine for 15min and then washed three times in 1 x PBST for 15 min each. The sections were incubated in blocking solution [5% normal Donkey serum (Millipore), 1% bovine serum albumin (BSA; IgG and protease-free, Sigma), and 0.05 Tween 20 in 1 x PBS2T (0.2%Triton-X)] for 1.5 hours at room temperature. These slices were then incubated overnight at 4 °C in primary antibody solutions [5% normal goat serum, 1% BSA (IgG and protease-free) in 1 x PBS2T (0.2% Triton-X)] containing the primary antibodies (see below for details). The following day, the sections were incubated further in primary antibody solution for 30 min at 37 °C. Sections were washed in 1 x PBS2T (0.2% Triton-X) for 15 min, then incubated in secondary antibody solution [5% normal

donkey serum, in 1 x PBS2T (0.2% Triton-X), see below for antibodies used] for 1 h at 37 °C. After this incubation, sections were washed again in 1 x PBS2T (0.2% Triton-X) and then in double distilled H_2O for 15 min each. The sections were cover slipped using Vector Hardset mounting media with Hoechst for cellular nuclei staining.

The primary antibodies used in the labeling are: Rabbit anti-FosB [1:50; Santa Cruz], mouse monoclonal anti-NeuN [1:500; Millipore], mouse monoclonal anti-Glial Fibrillary Acidic Protein [1:1000; Sigma], and mouse monoclonal anti-Iba1 [1:500; abcam].

The secondary antibodies used in the labeling are: Alexa Fluor 488 Donkey anti-mouse IgG [1:500; Invitrogen] and Alexa Fluor 555 Donkey anti-rabbit IgG [1:500; Invitrogen]

Fluoro-Jade C Staining

Thirty-five μm brain sections were mounted on gelatin-coated slides. These sections were dried for at least for 24 hours. The sections were then immersed in basic alcohol [0.0025% NaOH in 100% alcohol] for 5 minutes. Next, the sections were immersed in 70% alcohol for 2 minutes. Then, the sections were rinsed in distilled water for 2 minutes. The sections were incubated in potassium manganite (KMnO4) for 10 minutes. Following incubation, the sections were rinsed in distilled water for 2 minutes. Next, the sections were incubated in 0.0001% Fluorojade-C + 0.1% acetic acid in distilled water for 10 minutes. Following incubation, the sections were rinsed 3 times in distilled water for one minute per rinse. The sections were then dried at least for 24 hours. The sections were immersed in Xylene for 15 minutes and cover slipped using Vector Hardset mounting media.

Images were captured using Zeiss axiovert 200M inverted microscope connected to a Hamamatsu ORCA-R2 digital camera.

Gamma-H2AX + NeuN, IBA1, or GFAP Double Immunoflourescent

Brain sections (10 μm) including dorsal and ventral hippocampi and amygdala were thawed at room temperature and each section was circled with an Immunopen (Vector laboratories, Burlingham, California). The sections were incubated in 4% PFA for 10 min in 1 x PBS2T (0.2% Triton-X) containing 100 mM glycine for 15 min and then washed three times in 1XPBST for 15 min each. The sections were incubated in blocking solution [5% normal Donkey serum (Millipore), 1% bovine serum albumin (BSA; IgG and protease-free, Sigma), and 0.05 Tween 20 in 1 x PBS2T (0.2%Triton-X)] for 1.5 hours at room temperature. These slices were then incubated overnight at 4 °C in primary antibody solutions [5% normal goat serum, 1% BSA (IgG and protease-free) in 1 x PBS2T (0.2% Triton-X)] containing the primary antibodies (see below for details). The following day, the sections were incubated further in primary antibody solution for 30 min at 37 °C. Sections were washed in 1 x PBS2T (0.2% Triton-X) for 15 min, then incubated in secondary antibody solution [5% normal donkey serum, Alexa Fluor 488 Donkey anti-mouse IgG (1:500; Invitrogen), Alexa Fluor 555 Donkey anti-rabbit IgG (1:300; Invitrogen) in 1 x PBS2T (0.2% Triton-X)] for 1 h at 37 °C. After this incubation, sections were washed again in 1 x PBS2T (0.2% Triton-X) and then in double distilled H_2O for 15 min each. The sections were cover slipped using Vector Hardset mounting media with Hoechst for cellular nuclei staining.

Immunofluorescent images of CA1, CA3 and DG (see Figure 4) for dorsal and ventral hippocampus were captured using a Zeiss axiovert 200m inverted microscope

connected to a Hamamatsu ORCA-R2 digital camera. γ-H2AX foci were analyzed using ImageJ software. The density threshold for foci detection was determined by visual inspection and kept constant for all animals within the same area.

Antibodies used for γ-H2AX + NeuN double labeling were monoclonal rabbit anti-phopho-histone H2Ax (1:300; Millipore) and mouse monoclonal anti-NeuN (1:1000; Millipore).

Corticosterone Enzyme Immunoassay

Corticosterone EIA kit (Enzo Life sciences, Catalog No: 900-097) was used to assess baseline corticosterone of 15-day old pups. The plasma collected from the pups was diluted to 1:5 with Assay buffer (tris buffered saline containing proteins and sodium azide as preservative) provided in the kit prior to the assay. Each sample was analyzed in duplicated and the average of duplicate was used for analysis. Only the samples that have lower than 15 % intra-assay coefficient were used. The procedure provided in the kit is as follows: In each 96 well plate, the 100 μl of diluted plasma samples were pipetted in duplicate. Then, alkaline phosphatases conjugated with corticosterone and sheep polyclonal antibody to corticosterone were added and the samples were incubated for 2 hours at room temperature on a plate shaker at 250 rpm. After the incubation, the samples were washed 3 times with tris buffered saline containing detergents provided in the kit. Then, 200 μl of p-nitrophynyl phosphate in buffer was added into each well and incubated for 1 hour at room temperature. Following the incubation, 50 μl of stop solution was added to each well and the plate was read at 405nm immediately using a microplate reader (Thermo Labsystems Multiskan Ascent).

Statistical Analyses

Statistical Package for the Social Sciences (SPSS) program was used to analyze the data. One-way ANOVA were used for analysis of the prenatal transport stress effects on litter size, maternal behaviours, seizure onset, duration, severity, and cumulative scores, and FosB-immunoreactivity. Following one-way ANOVA, Bonferroni's post-hoc tests were used to determine the differences between specific prenatal transport groups. Student's *t*-tests were used for the analysis of the effects of maternal care on litter size, seizure onset, duration, severity, and cumulative scores, and FosB-immunoreactivity. For the correlational analysis, Pearson's correlation was used.
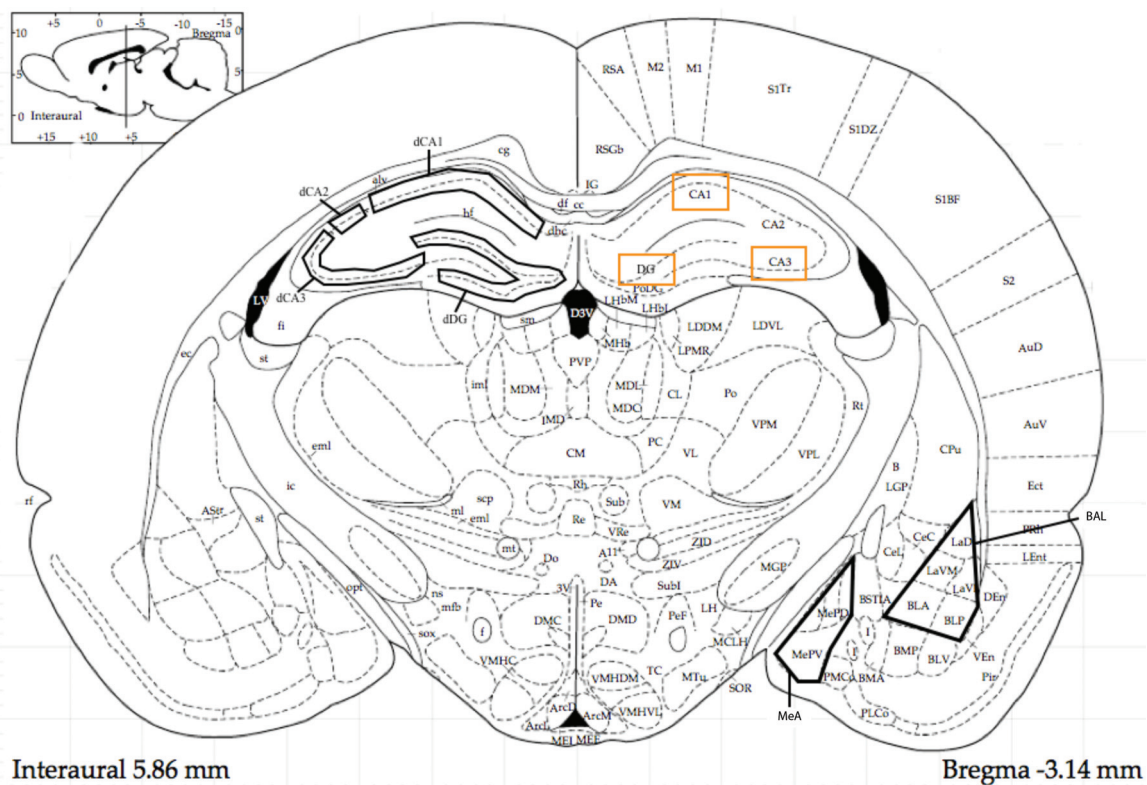
Figure 3. The regions of the dorsal hippocampus and amygdala used for immunohistochemical analysis. For the purpose of Fosb-ir analyses, areas demarcated by the black lines are used for dCA1, dCA2, dCA3 and dDG. BAL consistes of lateral and basal nuclei of the amygdala. MeA consists of medial nuclei of the amygdala. The similar sections are used across different pups. The square areas highlighted in orange lines were used for gamma- H2AX immunoflourescent labeling. Adopted from Figure 32 of The Rat Brain in stereotaxic coordinates, 4th ed by Paxinos and Watson (1998) and used with permission from Elsevier.
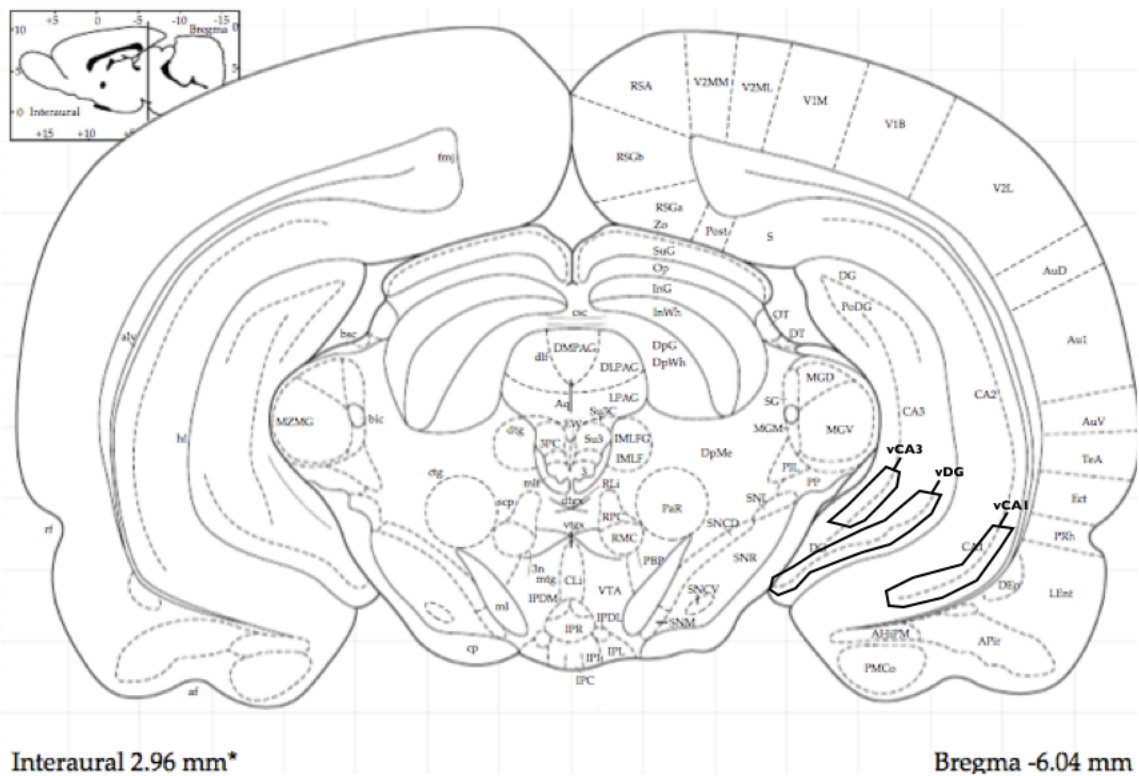
Figure 4. The regions of the ventral hippocampus used for immunohistochemical analysis. For the purpose of Fosb-ir analyses, areas demarcated by the black line were used. Adopted from Figure 44 of The Rat Brain in stereotaxic coordinates, 4th ed by Paxinos and Watson (1998) and used with permission from Elsevier.

Chapter 3: Results

Maternal Behaviour over Time

Figure 5 shows the change in maternal behaviour over time for the High and Low maternal behavioural groups based on the first postnatal week. The maternal behaviour pattern for LG and AB were similar to previous findings (Champagne et al., 2008; Liu et al., 2000). LG was significantly different between the High and Low dam groups during the first postnatal week but this differences decreased over time and diminished by P12. There was also a significant difference in AB behaivour during the first postnatal week but the difference diminished in the second postnatal week. PA and BL behaviour appeared similar to the LG and AB pattern; however, the variability was very high especially after first postnatal week. DO had the opposite characteristics and the significant difference between the High and Low groups was mostly found during the second postnatal week. Maternal behaviour over time between dams in the High and Low groups based on the means from the second postnatal week and the first 10 postnatal days were also analyzed. The pattern was similar to that shown in Figure 5 for the first postnatal week (data not shown).

Previous studies found that restraint stress during pregnancy decreases LG behaviour of dams (Champagne and Meaney, 2006). Therefore, I assessed the effect of transport on maternal behaviour (Figure 6). There was no significant difference between transport groups in LG, AB, PA, or BL behaviours during first and second postnatal weeks or first 10 postnatal days.

In summary, the natural variation in maternal behaviours and its time course found in this study was similar to that noted in previous studies (Champagne et al., 2008;

Liu et al., 2000). The frequency of LG, AB, PA, and BL behaviours was significantly different between High and Low dam groups in the first postnatal week while the difference diminished or was less apparent in the second postnatal week. In contrast, a clear and larger difference was observed between the High and Low dam groups in the second week for DO behaviour. Also, there was no apparent contribution of prenatal transport stress on the LG, AB, PA, BL, or DO pattern in this study.
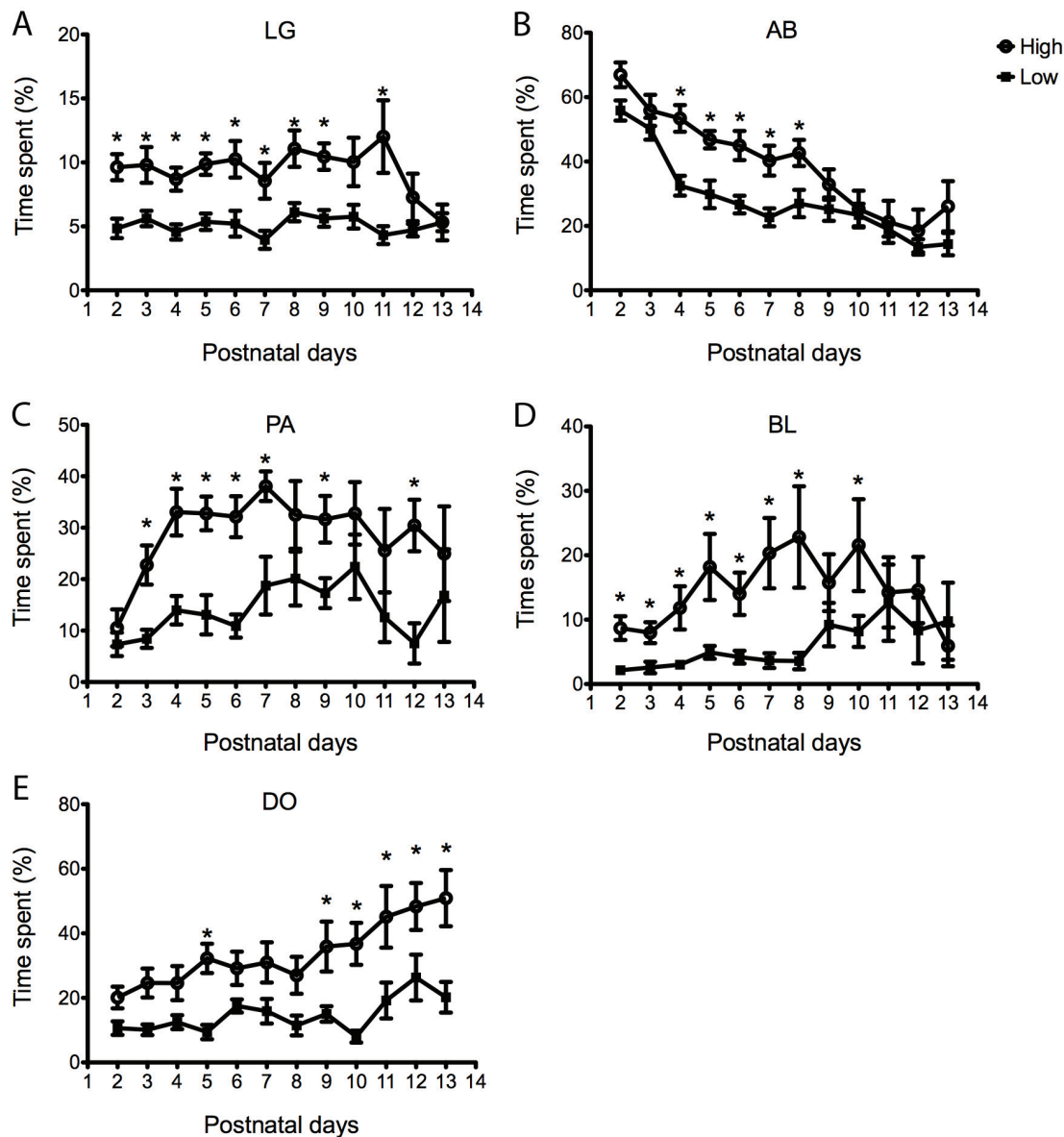
Figure 5. Maternal Behaviour over time. The percentage of time spent in each maternal behaviour for each dam was calculated from P2 through P13. Then the dams were grouped into High (above the mean) and Low (below the mean) groups based on population mean derived from first postnatal week. (**A – D)** High LG, AB, PA, and BL dams spent significantly longer time compared to Low groups during most of first week. During the second week, variability increased and the difference was only found on couple days. (**E**) In contrast, the difference between High- and Low-DO was more apparent during second week as compared to the first week.
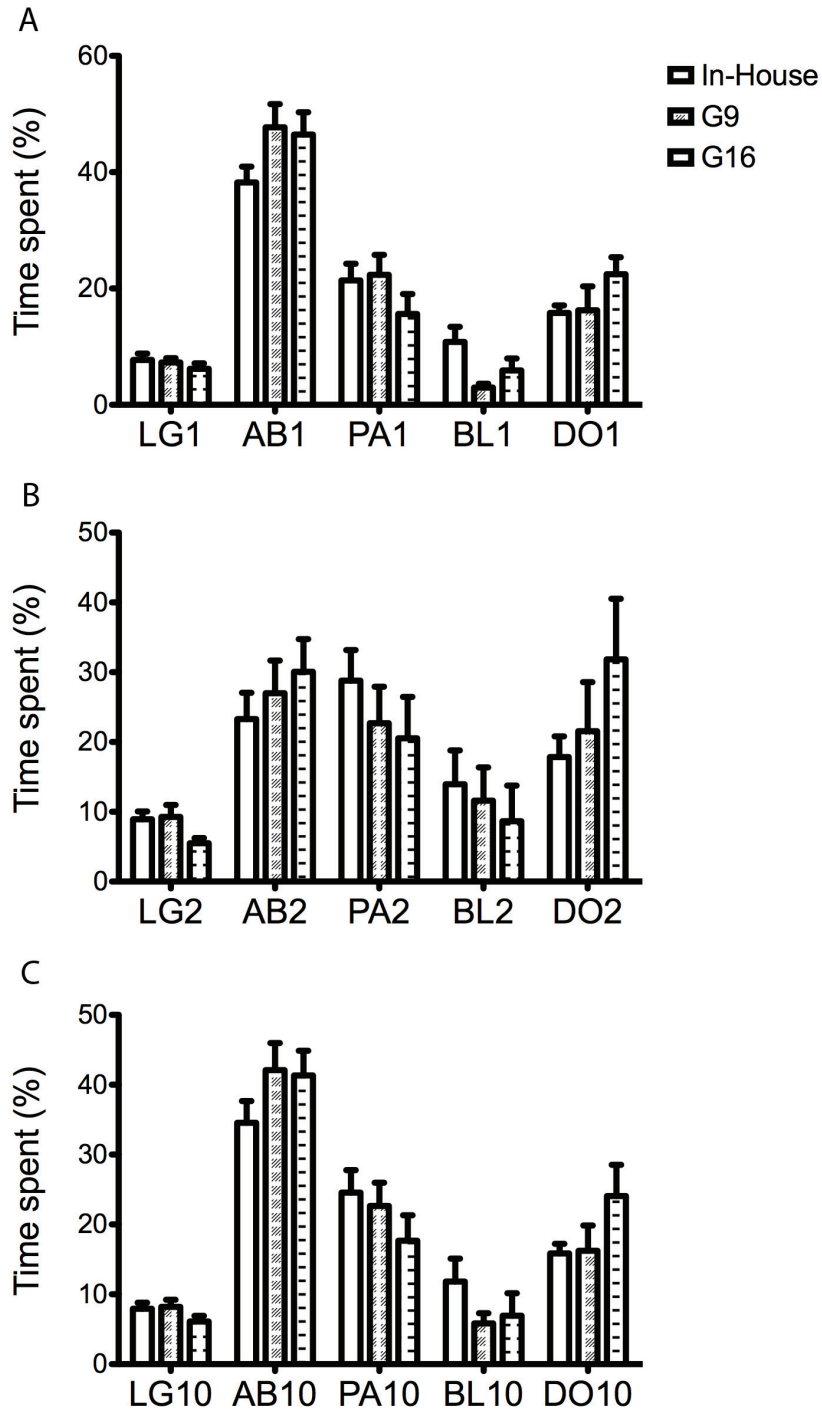
Figure 6. Maternal behaviour as a function of transport groups. Dams were grouped into In-House, G9 and G16 based on the original design. The percentage of time spent on each maternal behaviour was averaged for each transport group and were compared. There were no differences between transport groups for LG, AB, PA, or BL behaviours for (**A**) the first postnatal week, (**B**) the second postnatal week, or (**C**) the first 10 postnatal days.

Litter Size

Overall, the average litter size for all the dams (regardless of prenatal transport stress or maternal behaviour) was 6, which is smaller than the average expected litter size of 10-12 for Sprague-Dawley rats (Palmer and Uldrich, 1997). Eighteen out of 25 dams (72%) had a litter size smaller than 10. In addition to the small litter size, the most significant period of pup attrition for the dams that had a litter less than 10 at P14, started after the first postnatal week (Figure 7). This pup attrition is abnormal as anticipated pup loss usually occurs in first few postnatal days (Palmer and Uldrich, 1997). Figure 7 shows the litter size over time between dams with larger than 10 (Large) and smaller than 10 (Small) litter sizes at P14. Both groups started with a comparable litter size and this continued until P7. However, the litter size started decreasing for the Small group and continued to decrease until P14. A significant difference in litter size was observed at 3 time points, P9 ($t(24) = 2.41\ p < 0.05$), P12 ($t(24) = 4.44\ p < 0.001$), and P14 ($t(24) = 5.58\ p < 0.0001$).

Effect of Prenatal Transport: Litter size as a function of transport groups was also analyzed (Figure 8). There was no significant effect of prenatal transport stress on litter size at P14. However, a significant difference in litter size on P4 [$F(2,18) = 5.32, p < 0.05$] and P7 [$F(2, 16) = 5.29, p < 0.05$] between transport groups was found. The G16 group had a significantly smaller litter size at P4 compared to In-House ($t=2.86, p < 0.05$) and G9 ($t=2.67, p < 0.05$). The G16 group also had significantly lower litter size compared to G9 ($t=3.17, p < 0.05$) at P7. After P7, the litter size in the transport groups was comparable to one another.

Effect of Maternal Care: Litter size was also analyzed as a function of maternal behaviour during the first and second postnatal week and the first 10 postnatal days (Figure 9). High-LG behaviour was associated with smaller litter size as compared to Low-LG behaviour for all three time periods (LG1, LG2 and LG10) in the analysis. High-BL during the second week, but not the first week or first 10 days, was also associated with smaller litter size (Figure 9C). In contrast, Low-DO during the second week was associated with smaller litter size.

Ultrasonic Vocalization: Following the observation of abnormal pup attrition, we tried to determine whether the dams were under distress as stress exposure during pregnancy sometimes results in a smaller litter size (Cabrera et al., 1999; Gots et al., 2007). To determine this, we recorded ultrasonic vocalizations (USVs), to look for 22kHz (ranges between 18 – 32 kHz) USVs that rats produce when stressed (Kim et al., 2010; Yoav et al., 2007). Figure 9 shows the USV recordings in the room without any rats (Figure 9A), with pregnant females (Figure 9B) and with dams (i.e., after birth; Figure 9C). There was no USV recorded in the empty room (Figure 9A). However, the 22 kHz USV was observed in the room during pregnancy and after birth. Interestingly, the frequency of 22 kHz USVs decreased after birth (sample is from P4).

Summary of Litter Size: In the current study, abnormal pup attrition was observed: (1) 72% of the dams showed smaller litter size than average for the strain, (2) prenatal transport stress on G16 reduced the litter size at P4, and (3) the litter size was associated with natural variation in LG and DO behaviours where High-LG was associated with smaller litter size while Low-DO during the second postnatal week was associated with larger litter size.
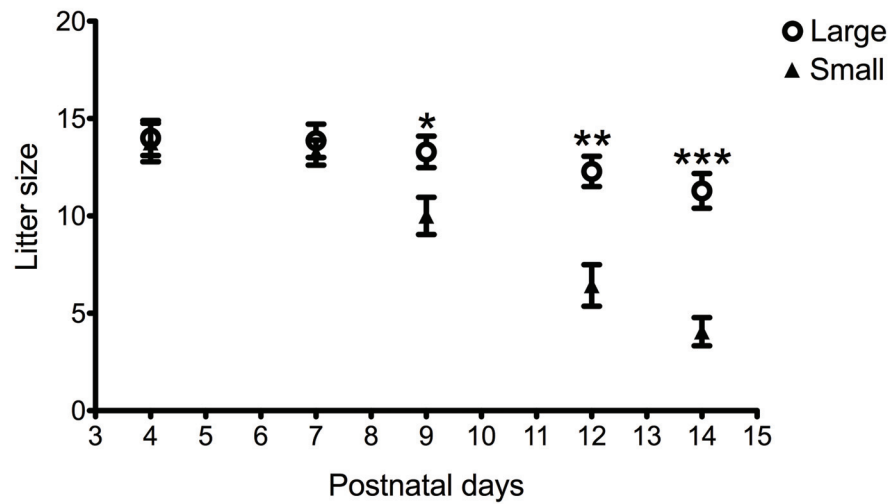
Figure 7. Change in litter size over time. Dams and litters were separated and categorized as Large (n=7, litter size ≥ 10) and Small (n=18, litter size < 10). On P4 and P7, the average litter size between Large and Small dam groups were not different. However, a significant difference between the groups was found at P9, P12, and P14 as compared between groups at the individual time point. [* $p < 0.05$,** $p < 0.001$, *** $P < 0.0001$]
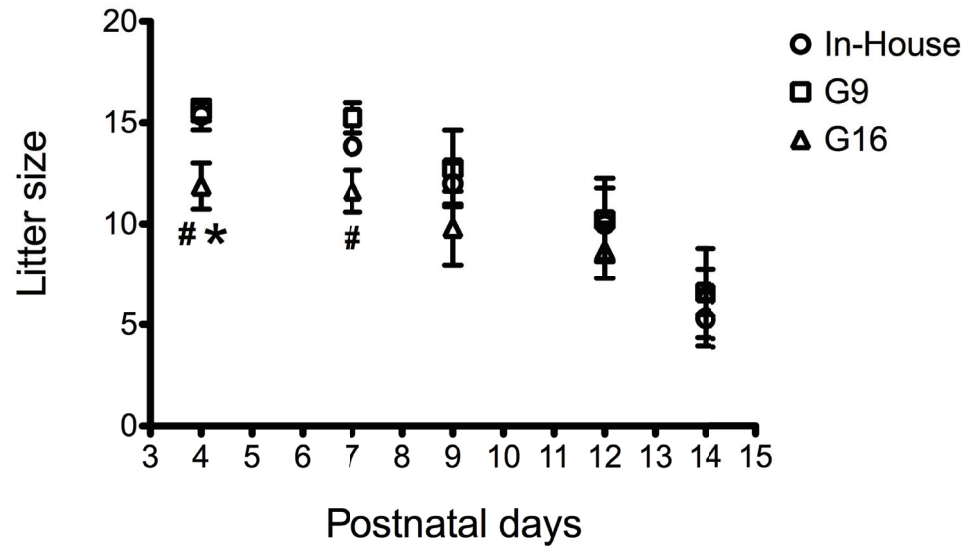
Figure 8. Litter size over time as a function of prenatal transport groups. Dams were grouped into In-House, G9 and G16 based on the original design. The average litter size of G16 group was smaller than In-House [t=2.86, $p < 0.05$] and G9 t=2.67, $p < 0.05$] groups on P4. The G16 group also had a significantly smaller litter size than G9 on P7 [t=3.17, $p < 0.05$].
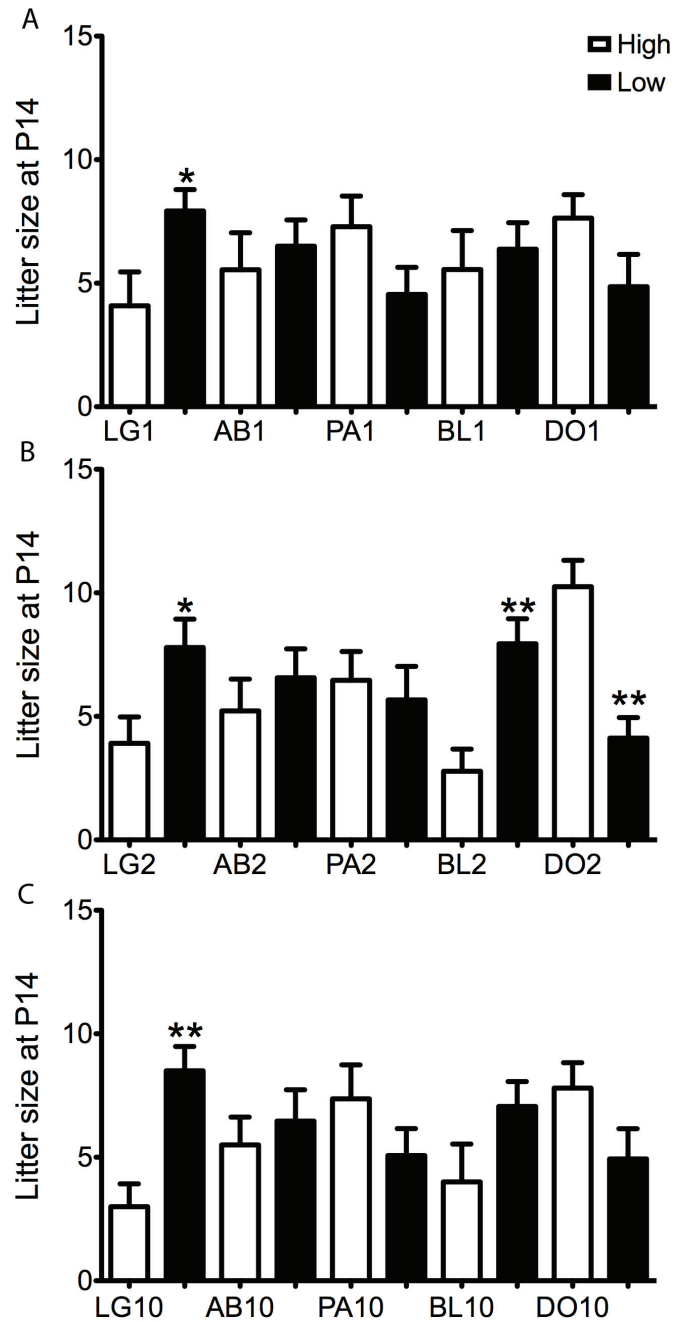
Figure 9. Litter size at P14 and relationship to maternal behaviour . (**A**) Low-LG1 dams had larger litter size than High-LG1 dams [$t(23) = 2.41$, $p < 0.05$]. (**B**) Low-LG2 and Low BL2 dams had larger litter size at P14 than High-LG2 dams [$t(23) = 2.41$, $p < 0.05$] and High-BL2 dams [$t(23) = 3.40$, $p < 0.01$]. In contrast, High-DO2 dams had larger litter size at P14 compared to Low-DO2 dams [$t(23) = 4.31$, $p < 0.001$]. (**C**) Low-LG10 dams had larger litter size at P14 compared to High-LG10 dams [$t(23) = 2.41$, $p < 0.001$].

Figure 10. Representative traces of twenty-two kHz ultrasonic vocalizations (USV) in the housing room. (**A**) Recording of housing room without rats demonstrating the absence of background 22 kHz noise. (**B**) The 22 kHz USV in housing room with In-House female and males before mating. (**C**) The 22 kHz USV present in the room only with In-House dams after mating, (**D**) with In-House and G9 dams, and (**E**) with In-House, G9 and G16 dams.

Sex Ratio

As part of the analysis of the effect of prenatal transport stress on the dams and the litters, the sex ratio of the litters was also examined. In total, there were 81 male pups and 70 female pups on P14. The male ratio of the pups was analyzed as a function of the prenatal transport groups and maternal care, but no significant differences were found.

P14 Pup Weight

The relationship between weight and prenatal transport stress or maternal care was analyzed. There was a significant effect of prenatal transport on weight of the pups [$F(2,147)=3.18$, $p < 0.05$] (Figure 11). However, post-hoc tests failed to find a difference between specific groups. Table 3 shows pup weight relative to the maternal behaviour during the first postnatal week, second postnatal week and first 10 postnatal days, respectively. Overall, pups in the High-LG and AB during the first postnatal week weighed less. Similarly, pups in the High-PA and Low-BL groups during the second postnatal week also weighed less than their comparative groups. In contrast, pups in the High-DO during the second postnatal week weighed more than those in the Low-DO group.

Pup weight was also examined in terms of its relationship to sex and prenatal transport group. However, there was no significant difference in pup weight between male and female pups nor was there a difference in pup weights between prenatal transport groups within a sex.

I have also analyzed the pup weight as a function of sex and maternal behaviours. No sex difference within High- and Low-maternal behaviour groups was found. There were however, differences between maternal behaviour groups within a sex. Low PA was

associated with heavier weight in both female and male pups. In contrast, High DO was associated with heavier pup weight only in female pups.

Finally, I analyzed the correlation between litter size and the weight of the pups. There was no significant correlation between litter size and the weight of the pups on P14. This suggests that the weight difference between High- and Low-LG is not due to difference in competition for milk.

Summary of Sex Ratio and Pup Weight: In summary, the sex ratio of the pups was not different between the prenatal transport groups or maternal behavior groups. The weight of the pups was associated with maternal behaviours where pups from Low maternal behaviour groups tended to have a heavier weight.
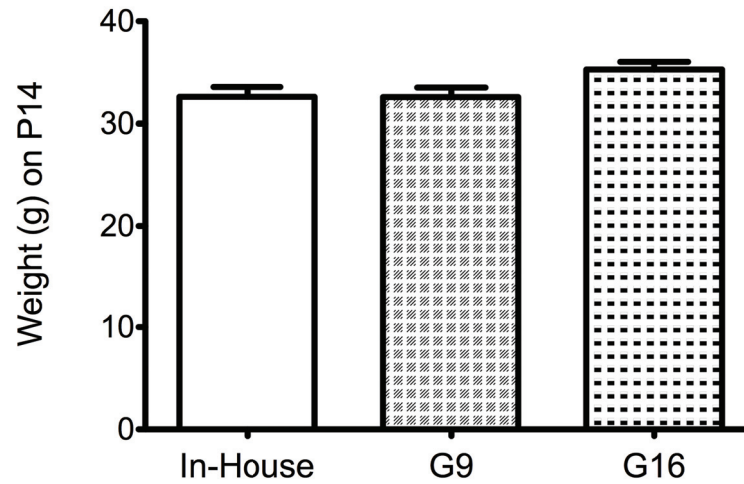
Figure 11. Pup weight as a function of prenatal transport groups. There was a significant effect of prenatal transport group on pup weight [$F(2,147)=3.18$, $p < 0.05$], but no specific difference was found. However, G16 tended to have greater pup weight than In-House and G9 groups.

Table 3. Pup weight as a function of maternal behaviours

	LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
All	L**	L**						L***	L***			L**	H**		H**
Male	L*							L***	L**						
Female								L***	L *			L*	H**	H*	H**

The group with heavier weight is indicated in each cell. $n = 22 - 56$ per group. H = High, L = Low. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ as compared to opposite maternal group

Mortality Rate of Pups in LPS Group

Effect of the Treatments: A high mortality rate was found in LPS treated pups (Figure 12A). Of the 23 pups that received LPS alone, 5 died (2 males and 3 females) during behavioural recording or overnight. In contrast, of the pups that received KA (n = 26) or FC (LPS + KA; n=56), only 1 and 2 pups died, respectively. This indicates that LPS itself has a strong adverse effect on the pups. Interestingly, the FC pup group that also received LPS, showed a lower mortality rate than the LPS alone.

Effect of Prenatal Transport Stress: The mortality of the pups in the LPS treatment group was analyzed for its relationship to prenatal transport stress (Figure 12B). Five pups out of 8 pups died within the In-House group while no pups died in either the G9 (n = 7), or G16 (n = 8) groups. Chi-square analysis revealed this to be a significant difference in mortality [χ^2 (2, N= 23), p = 0.01]. These 5 pups were from 4 different litters indicating this is not just an anomaly of one litter. In addition, 3 of the litters came from 3 different experimental runs (4 sets of experiments were conducted) suggesting that this is not the abnormal environmental condition in one experimental run.

Effect of Maternal Care: The mortality of LPS treated pups was analyzed for a relationship to maternal behaviour (Table 4). The mortality rate was significantly higher in the Low-AB1 [χ^2 (1, N= 23), p = 0.046] and Low-DO2 [χ^2 (1, N=23), p = 0.014] groups compared to High-AB1 and High-DO2, respectively.

Summary of Mortality Rate: I found that LPS treated pups showed higher mortality than the KA and FC treated pups and this high mortality was associated more with litters from the In-House group and from the Low-AB1 and Low-DO2 dam behaviour groups.
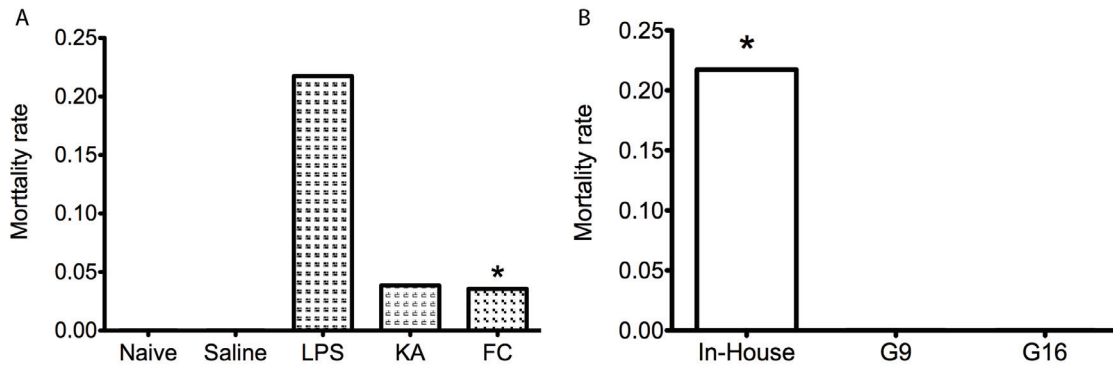
Figure 12. Mortality rate. (**A**) No pups died in Naïve or Saline treatment groups. Among LPS treatment groups, 5 out of 23 pups died. In KA and FC treatment groups, only 1 out of 26 and 2 out of 56 pups died, respectively. Mortality rate of LPS was higher than that of FC [χ^2 (1, N= 79), $p = 0.03$]. (**B**) Five out of 8 pups that were assigned to LPS treatment from In-House group died following the treatment. In contrast, all pups that were assigned to LPS treatment from G9 (n=7) and G16 (n=8) survived. The chi-square analyses found a significant difference in mortality rate of pups between the transport groups.

Table 4. Mortality of pups following LPS treatment as a function of maternal behaviour

	LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High	4	4	4	0	3	3	4	2	2	2	1	2	3	0	3
Low	1	1	1	5*	2	2	1	3	3	3	4	3	2	5*	2

The numbers in a cell are the number of pups that died following LPS treatment. Significant difference was found between the mortality rate when the pups were categorized based on AB1 and DO2 behaviours. * $p < 0.05$

Seizure Susceptibility of Pups

Effects of the Treatments: The pups were randomly assigned to Naïve, Saline, LPS, KA or FC (Figure 3). First, I analyzed the effects of the pup treatment on mortality and seizure susceptibility regardless of the prenatal transport stress or maternal behaviour. The number of pups assigned to each treatment is listed in Table 3.

The seizure susceptibility of the pups in each treatment group was analyzed. No seizure behaviour was observed in any of the control group; however, it was noted that some LPS treated pups had low mobility compared to others. There was no difference in seizure induction rate between the KA and FC treatment groups.

Effect of Prenatal Transport Stress: The number of pups that developed seizure following KA and FC treatment was also analyzed as a function of prenatal transport groups (Figure 13) but there was no difference between transport groups in the rate of seizure induction.

Next, I analyzed seizure onset, duration, cumulative score, and severity as other measures of seizure susceptibility of the pups (Figure 14). No significant effects of transport on seizure onset was found in KA or FC treated pups. There was no effect of transport on seizure duration in the KA treated pups. However, there was a significant effect of prenatal transport on the seizure duration in the FC treated pups [$F(2, 34) =$ 3.42, $p <0 .05$]. Post-hoc analysis revealed that pups born to In-House bred dams had a significantly lower seizure duration compared to those born to the G9 group [t=2.62$, p <$ 0.05]. There was no significant effects of transport on seizure severity in KA or FC treated pups. Similarly, no significant effect of transport was found on cumulative seizure score in the KA or FC treated pups. However, in the FC treated pups, the G9 group

tended to have higher cumulative seizure score compared to the In-House and G16 groups. This pattern is consistent with seizure duration in the FC treated pups.

Effect of Maternal Care: The parameters of seizure susceptibility were then analyzed in terms of the relationship to maternal behaviour groups (Table 5 and Table 6). A greater FC seizure rate was associated with the Low-DO10 group [χ^2 (1, N= 56) = 3.42, $p < 0.05$] but not with other maternal behaviour groups. Overall, the High-LG2, AB2, and PA2, as well as the Low-DO groups were associated with seizure susceptibility based on the seizure scores. This association was more prominent in the FC treatment pup group.

Sex Differences in Seizure Susceptibility: The pups were further divided into male and female within a treatment group and the seizure susceptibility was compared (Figure 15). Although there was a trend that the female pups appeared less susceptible to KA-induced seizure this difference was not statistically significant. In contrast, in FC treated pups, there was a significant effect of sex on seizure susceptibility [χ^2 (1, N= 56) = 6.33, $p = 0.01$] as 29/30 (97%) of male pups, as compared to 19 out of 26 (73%) female pups developed seizure. These results suggest that the male pups are more susceptible to FC-induced seizure than female pups at P14. However, there was no significant difference in the measures of seizure onset, duration, cumulative score, or severity between the two sexes in either the KA or FC groups (Figure 16).

I also analyzed sex difference in seizure susceptibility within a prenatal transport group. However, there was no significant difference within or between sexes in terms of the parameters of seizure onset, duration, cumulative scores or severity within the In-House, G9, or G16 groups.

In contrast to the lack of interaction with transport group, sex differences in seizure susceptibility was observed when the pups were categorized into maternal behaviour groups. Sex difference was observed mostly in seizure severity and within High-maternal behaviour groups (Table 6). In addition, there were differences between High- and Low-maternal behaviour groups only within the male pups (Table 7).

Inter-rater reliability: For seizure onset, duration, severity, and cumulative scores, I assessed inter-rater reliability between two raters. For all measurements, Cronbach's Alpha was greater than 0.85 suggesting that the scores were in agreement across raters.

Summary of Seizure Susceptibility: To summarize, I found that prenatal transport does not have an effect on seizure induction rate; however, there was a tendency where G9 showed higher seizure scores during FC-induced seizure. I also found that High-PA1, Low-DO2 and Low-DO10 were associated with higher FC seizure induction rate. Finally, the male pups had a higher FC seizure induction rate and also tended to have a more severe FC seizure.
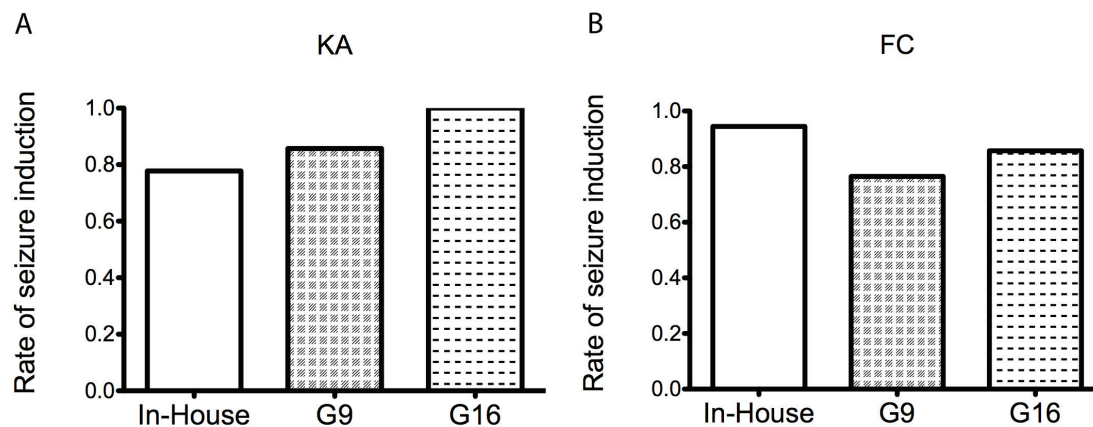
Figure 13. Rate of seizure induction following KA and FC treatment as a function of prenatal transport stress. Rate of seizure induction was determined by the number of pups that developed seizure divided by the total number of pups that received the treatment. (**A**) Following KA treatment, there was not significant difference in seizure induction rate between In-House (7 out of 9), G9 (6 out of 7), and G16 (10 out of 10). (**B**) Similarly, there was no significant difference in seizure induction rate between In-House (17 out of 18), G9 (13 out of 17), and G16 (18 out of 21).
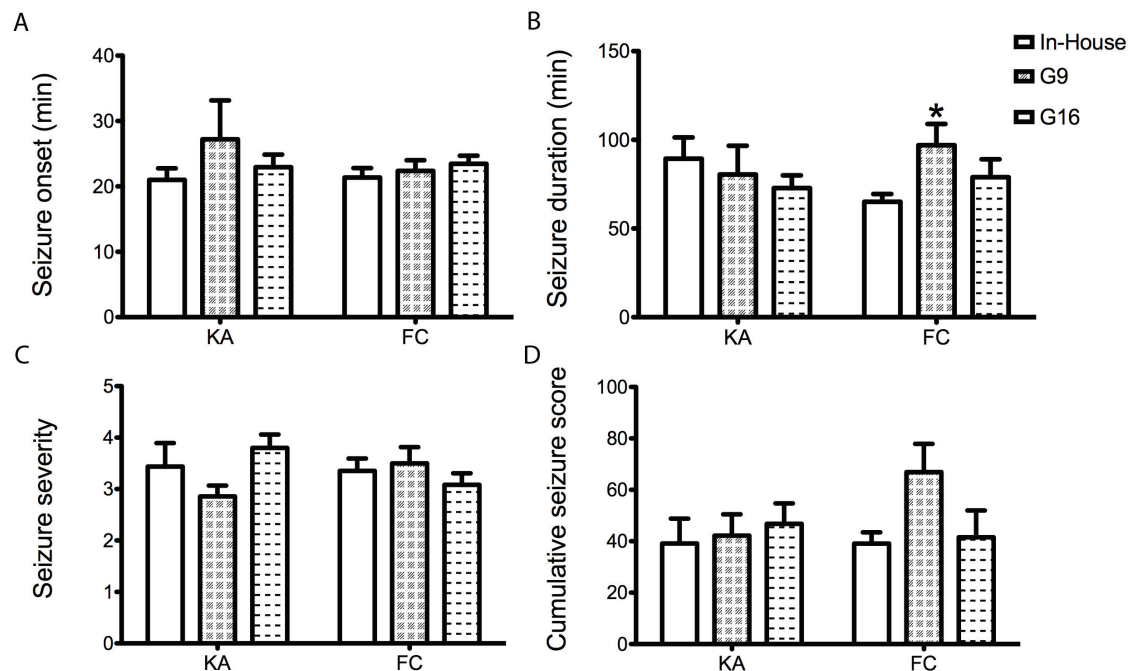
Figure 14. Seizure behaviours in KA and FC treatment groups as a function of prenatal transport groups. (**A**) Prenatal transport did not have effects on KA or FC seizure onset (latency to first seizure behaviour). (**B**) Significant effect of prenatal transport on seizure duration in FC treatment group was found [$F(2, 34) = 3.42, p < 0.05$]. G9 pups showed significantly longer FC seizure duration compared to In-House [$t=2.62, p < 0.05$]. However, no difference was found in KA seizure duration between transport groups. (**C**) Seizure severity (highest seizure score during a 3-hour period) was not affected by prenatal transport in KA or FC treated pups. (**D**) Similarly, prenatal transport did not have effect on cumulative seizure score over a 3-hour observation period.

Table 5. Seizure induction rate

FC	LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High	14/1	13/2	14/1	25/2	16/2	20/2	28/6	30/3	24/3	18/1	6/2	13/1	26/7	27/5	24/7
Low	34/7	35/6	34/7	23/6	32/6	28/6	20/2	18/5	24/5	30/7	42/6	35/7	22/1	21/3	24/1*

Number of pups that developed seizure/Number of pups that did not develop seizure.

Table 6. Seizure behaviour as a function of maternal behaviour

		LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
	Onset															
	Duration															
	Score								H*							L**
KA	Severity															
	Onset		L*													
	Duration				H*											
	Score	H*	H*		H*				H*					L*		L**
FC	Severity		H*						H**						L*	L*

A group with higher minutes and score is indicated in each cell. H = High, L = Low. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$
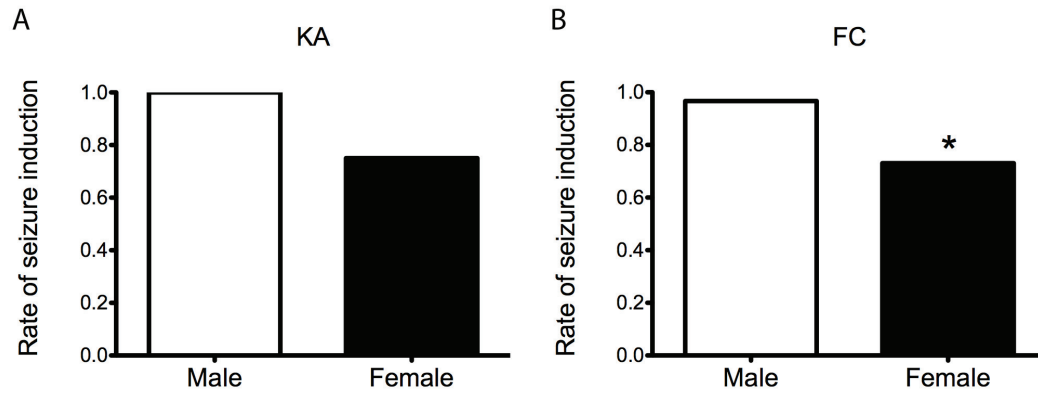
Figure 15. Rate of seizure induction in KA and FC treatment groups as a function of sex. (A) Following KA treatment, all 14 male pups developed seizure while 9 out of 12 did in female pups. This difference was not statistically significant. **(B)** Twenty-nine out of 30 male pups developed seizure following FC treatment while 19 out of 26 female pups did and this was statistically significant [$\chi2$ (1, N= 56) = 6.33, p = 0.01].
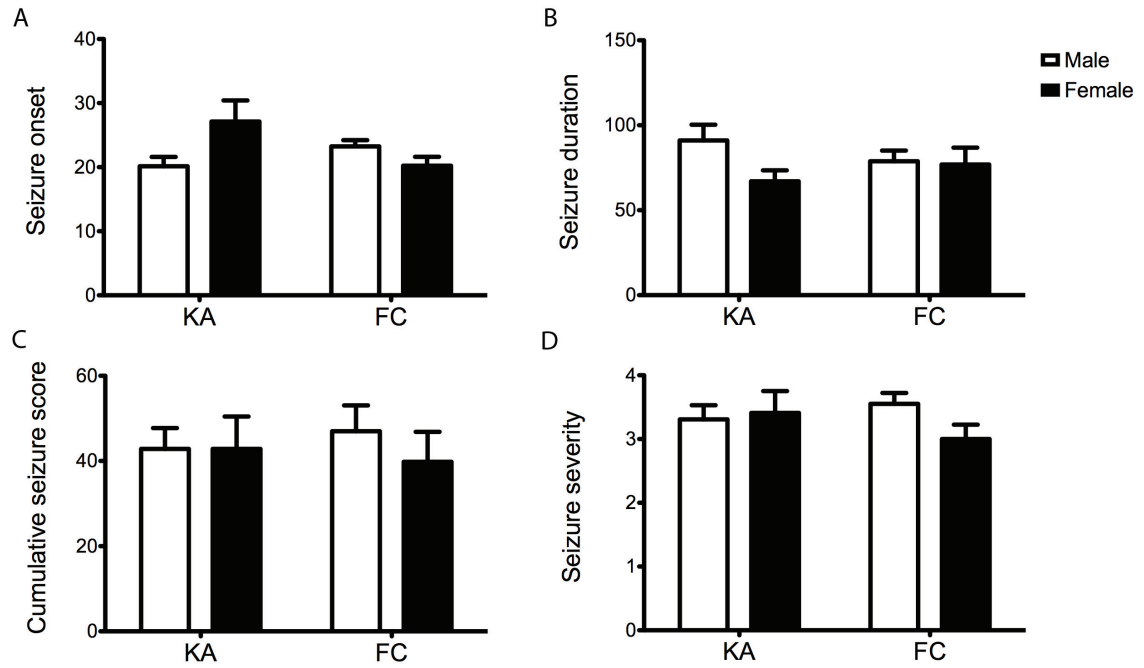
Figure 16. Seizure behaviour parameter as a function of sex. (A) – (D) There was no significant differences between sex or between KA and FC within a sex in seizure onset, duration, cumulative scores or severity.

Table 7. Seizure behaviours between sex as a function of maternal behaviours

			LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High	Onset	KA															
		FC															
	Duration	KA															
		FC															
	Cumulative scores	KA															
		FC												M*			
	Severity	KA															
		FC	M**		M*					M*		M*					
Low	Onset	KA												F*			
		FC															
	Duration	KA															
		FC							M*								
	Cumulative scores	KA															
		FC															
	Severity	KA															
		FC							M*								

A sex that showed significantly longer minutes (onset and duration) or higher scores (cumulative and severity) is shown in each cell. n = 3 – 20 for each sex. M = Male pups, F = Female pups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Table 8. Seizure behaviours between maternal behaviour groups as a function of sex

			LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
Male	Onset	KA															
		FC															
	Duration	KA	H*														
		FC				H**			L*								
	Cumulative scores	KA	H**														
		FC	H*			H*											L**
	Severity	KA		L*													
		FC	H*				H*			H**							L**
Female	Onset	KA															
		FC		L*													
	Duration	KA															
		FC															
	Cumulative scores	KA															
		FC															
	Severity	KA															
		FC															

A group that showed significantly longer minutes (onset and duration) or higher scores (cumulative and severity) is shown in each cell. n = 3 – 20 for each group. H = High, L = Low. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

FosB-immunoreactivity (ir) Analyses in Hippocampus and Amygdala Nuclei

Effects of the treatments: FosB-ir in the dorsal and ventral hippocampus of the pups was analyzed and compared between the Naïve (n=20), Saline (n=12), LPS (n=16), KA (n=19), and FC (n=36) treatment groups (Figure 17, 18 & 19). There was no difference in FosB-ir between the treatment groups (Naïve, Saline, LPS, KA, or FC) in any of the sub-regions of the dorsal hippocampus. However, while there was no difference in the CA1 and DG regions of the ventral hippocampus between the treatment groups, there was a significant effect of the treatment on FosB-ir in the vCA3 [$F(4,93) = 10.47, p < 0.0001$] where the FC group had the most FosB-ir labeling (Figure 18).

FosB-ir was also analyzed in the medial nuclei of amygdala (MeA) and the basolateral sub-nuclei of the amygdala (BAL; Figure 19), but a significant treatment effect was only seen in the MeA [$F(4,75) = 5.51, p < 0.05$]. I found that Saline treated pups had lower FosB-ir cell density compared to Naïve pups but that FC had higher FosB-ir than Saline treated pups. There was no significant difference in BAL between the treatment groups, although a similar trend where Saline treated pups had the lowest FosB-ir was also observed.

Effect of Prenatal Transport Stress: Figures 20 – 22 show FosB-ir in the dorsal and the ventral hippocampus, and medial and basolateral nuclei of amygdala. No significant effect of prenatal transport in any of the treatment groups was found in dCA1 or dCA2. In dCA3, however, a significant effect of prenatal transport was found in the Saline group [$F(2,9)=11.75, p < 0.01$] and LPS [$F(2,12)=17.91, p < 0.01$]. In the Saline treated group, pups from the In-House group had more FosB-ir cells compared to those in the G9 group. Similarly, pups in the In-House group also had higher FosB-ir compared to

those in the G9 and G16 in the LPS treatment group. In the vCA3, prenatal transport stress had an effect on FosB-ir only in the FC treatment group [$F(2,40)=4.07$, $p < 0.05$], but no specific difference between the prenatal transport groups was found. In the vDG, there was an effect of prenatal transport on baseline FosB-ir [$F(2,18)=12.41$, $p < 0.01$] where the In-House group had more FosB-ir compared to the G9 and G16 groups. Finally, there was no effect of the prenatal transport group on FosB-ir in MeA or BAL.

Effects of Maternal Care: Next, I analyzed FosB-ir as a function of maternal behaviours. Tables 9 - 13 summarizes the significant differences between the High and Low maternal behaviour groups and the correlation between maternal behaviour and FosB-ir. Overall, maternal behaviour was associated with FosB-ir in pups from the Naïve, LPS and FC treatment groups. There were some differences between the High and Low maternal behaviour groups in the Saline and KA treatment groups; however, no specific patterns were found in these groups.

In the Naïve treatment group, LG, AB and BL behaviours were associated with FosB-ir in the MeA. Specifically, LG2, LG10 and AB2 showed positive correlations with FosB-ir in MeA suggesting that the longer the dam spent on LG and AB behaviour, higher the baseline FosB-ir in pup MeA. In the LPS treatment group, LG and DO behaviours were correlated with FosB-ir in the vDG, and BAL. Specifically, LG and DO during the second week was highly correlated with the FosB-ir. Interestingly, the directions of their relationship with FosB-ir was opposite that LG was positively and DO was negatively correlated with FosB-ir in the vDG and BAL. In comparison, AB behaviour negatively correlated with the FosB-ir in the vCA3. Following FC treatment,

the differences between maternal behaviour groups were only found in the MeA and BAL. Specifically, LG was positively correlated with FosB-ir in both the MeA and BAL.

Because the previous literature found a within-litter variation in amount of LG individual pup receive is correlated with morphology and plasticity of neurons and behaviours of offspring, I have analyzed within-litter variation of FosB-ir in each sub-region following FC seizure (van Hasselt et al., 2011; van Hasselt et al., 2012b). There was large within-litter variability in FosB-ir. For example, FosB-ir in vCA3 following FC seizure is shown in Figure 23.

Sex Difference: I analyzed whether there was a difference in FosB-ir between the male and female pups. I analyzed sex differences within each treatment group regardless of the prenatal transport or maternal behaviour. A significant difference was found in the MeA following saline treatment [$t(7)=2.50$, $p < 0.05$] and in the dDG following LPS treatment [$t(13)=2.30$, $p < 0.05$] where male pups showed higher FosB-ir than female pups (Figure 24).

Then, I analyzed sex differences within each prenatal transport group. A significant difference was only found in the dCA3 of pups in the Naïve group within the G9 dams. Again, male pups showed more FosB-ir compared to female pups [$t(8) = 2.49$, $p < 0.05$].

I also analyzed sex differences in a relation to maternal behaviour groups. Due to a small n, I could not assess sex differences within all the maternal behaviour groups. The male pups tended to have more FosB-ir than the female pups (n > 3). This trend of more FosB-ir in the male pups was consistent with a higher seizure score and a higher seizure susceptibility in the male pups compared to female pups.

Finally, I correlated each maternal behaviour and FosB-ir within a sex. I found significant positive correlations between LG and FosB-ir in MeA and BAL only in female brains following FC seizure (Table 14).

Summary of FosB-ir: To summarize the main finding in this section, I found that (1) an increase in FosB-ir following FC induction was greatest in MeA, (2) the In-House group had higher FosB-ir compared to the G9, and G16 at baseline and following Saline and LPS treatment, (3) LG behaviour was positively correlated with FosB-ir in the MeA of Naïve and FC treated pups, (4) the male pups tended to show higher FosB-ir than the female pups, and (5) only female pups showed FosB-ir correlations with LG behaviour.
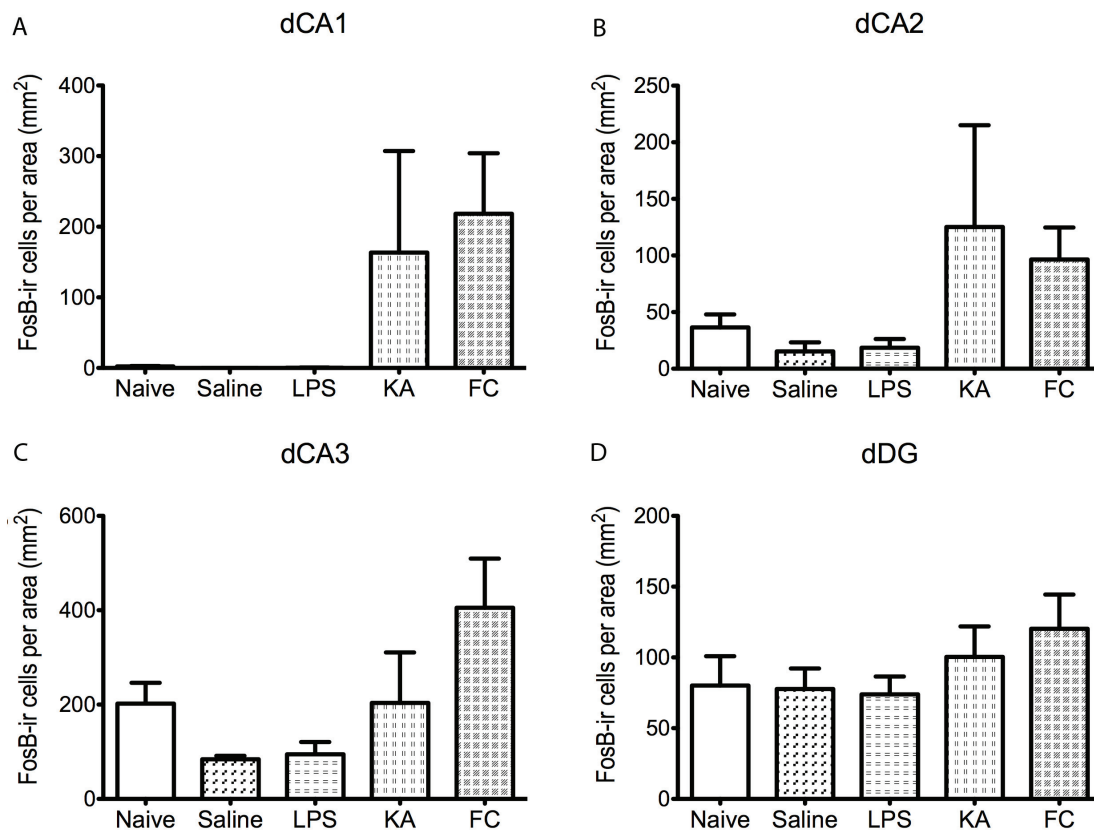
Figure 17. The effect of treatments on FosB-ir in the dorsal hippocampus.(A) Most of the pups showed no FosB-ir expression regardless of the treatments in the dCA1 and there was no difference between the treatment groups. (**B**) Similarly, no significant difference was found between the treatment groups in the dCA2. (**C**) In the dCA3, there was a trend where FC had higher FosB-ir than other treatment groups. (**D)** The dDG showed comparable FosB-ir expression across different treatments.
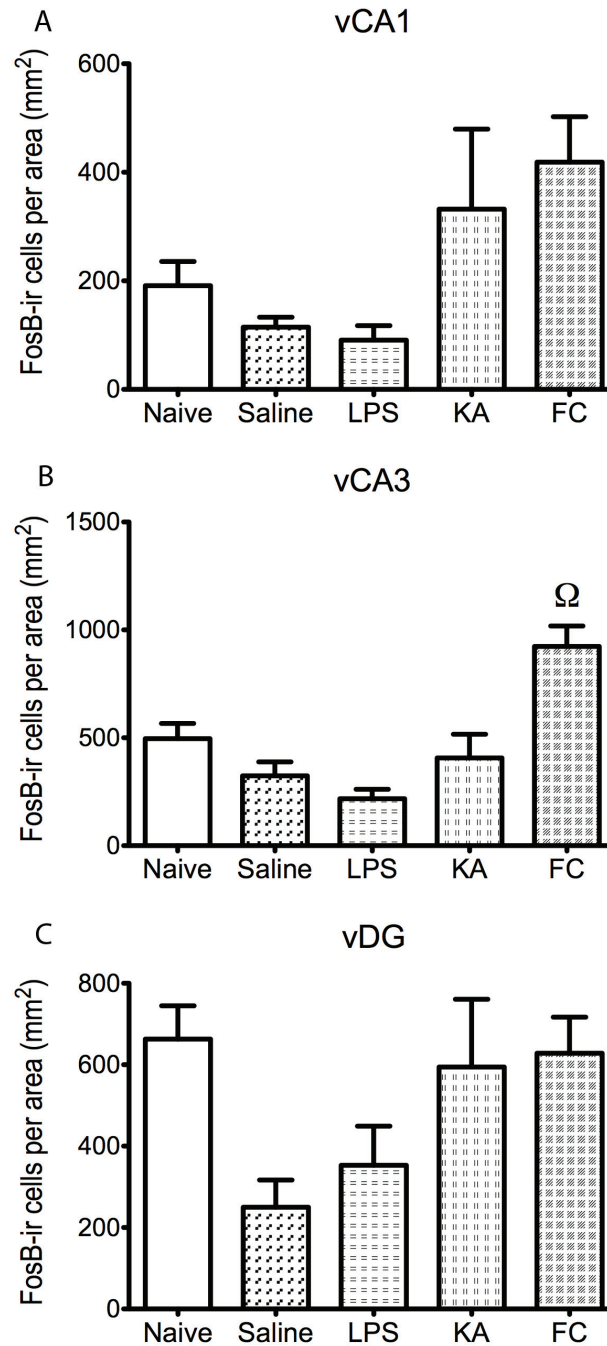
Figure 18. The effects of the treatments on FosB-ir in ventral hippocampus. (**A**) The vCA1 showed a trend where FC group showed higher FosB-ir. (**B**) There was a significant effects of the treatment on FosB-ir expression in the vCA3 [$F(4,94) = 5.26$, $p < 0.001$] with more FosB-ir in the FC group as compared to all other treatment groups. (**C**) Naive pups tended to have a high FosB-ir expression compared to other treatment groups in the vDG. In addition, Saline pups tended to have a low FosB-ir expression than other groups. Ω significant difference from Naïve, Saline, LPS, and KA groups at $p < 0.05$.
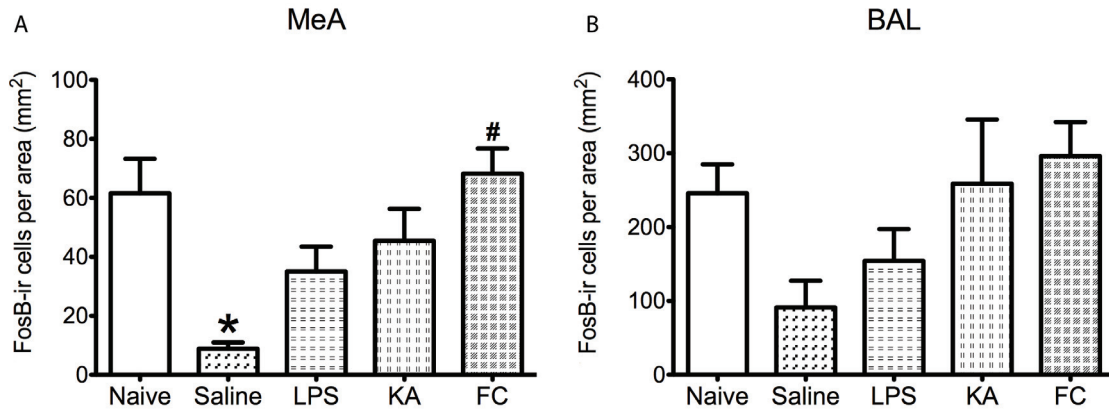
Figure 19. The effect of treatment on FosB-ir in medial amygdala (MeA) and basolateral amygdala (BAL). (**A**) FosB-ir levels in the MeA demonstrating significant effects of the treatment [$F(4,84) = 4.83$, $p < 0.01$] where Saline group had significantly lower FosB-ir as compared to Naive group, and FC group had significantly higher FosB-ir as compared to Saline group. (**B**) There were no significant effects of treatments on FosB-ir expression in BAL. However, a similar trend as in MeA was observed. *$p < 0.05$ as compared to Naive. # $p < 0.05$ as compared to Saline.
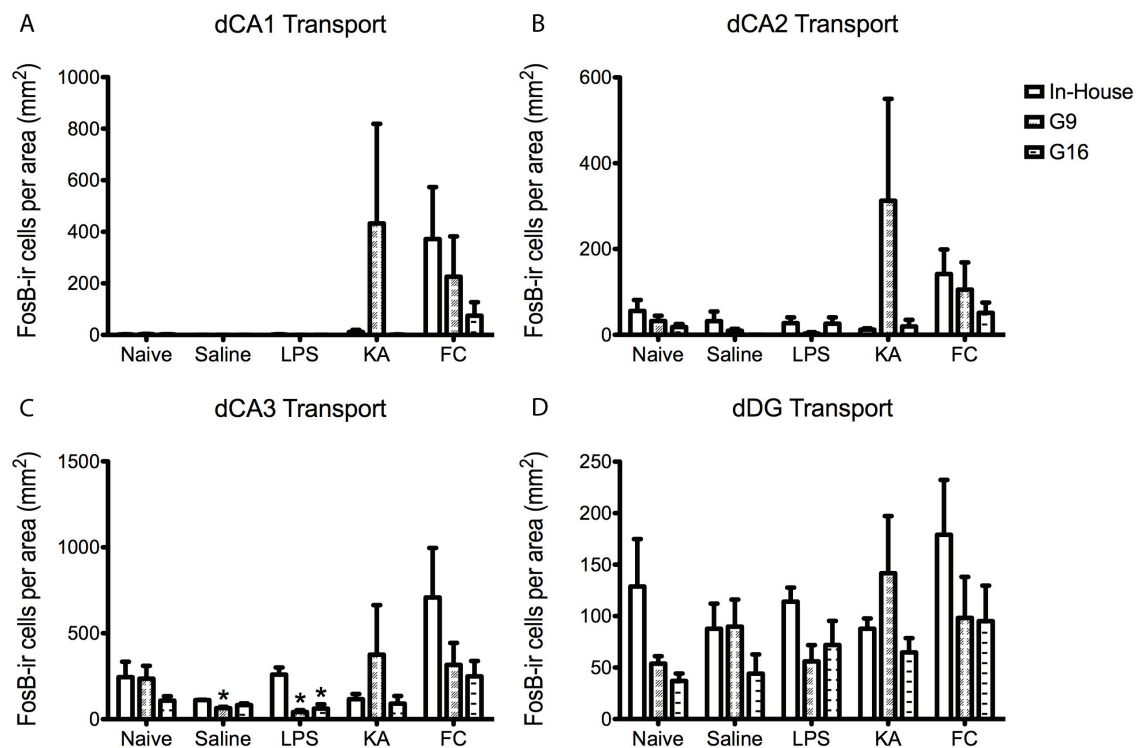
Figure 20. Dorsal hippocampal FosB-ir labeling as a function of prenatal transport and experimental groups. (**A**) There was no difference in the dCA1 FosB-ir levels between the prenatal transport groups within a treatment or between the treatment groups within a transport group. (**B**) Similarly, no difference was found in the dCA2 FosB-ir levels between the prenatal transport groups or between the treatments groups within a transport group. (**C**) There was a significant difference in the dCA3 FosB-ir levels between In-House and G9 following Saline treatment. In addition, there was a significant difference between In-House and G9 and G16 in the dCA3 FosB-ir levels following LPS treatments. There was no difference between the treatments within a transport group. (**D**) There was neither effect of prenatal transport within a treatment nor the effects of treatments within a transport group. *$p < 0.05$ as compared to In-House.
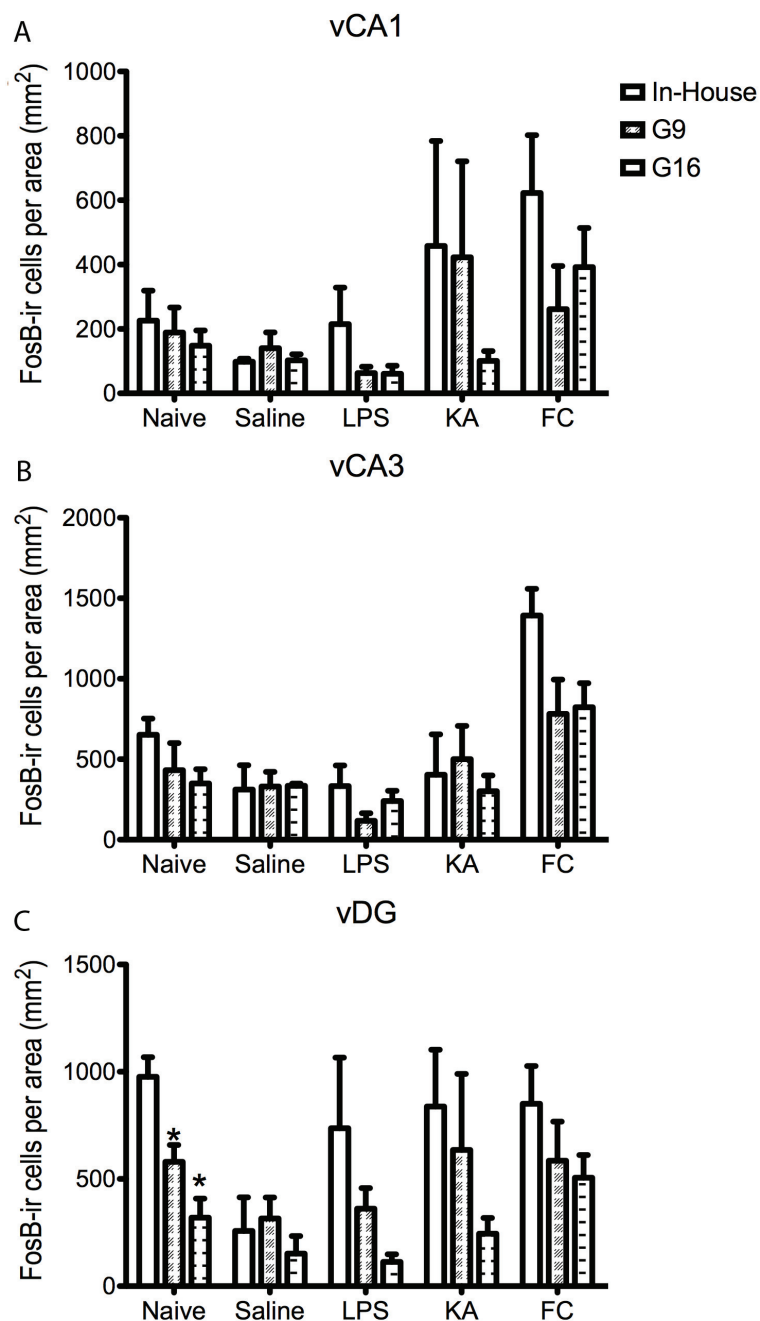
Figure 21. Ventral hippocampal FosB-ir levels as a function of prenatal transport stress groups. (**A**) There was no significant difference in vCA1 FosB-ir between the prenatal transport groups or between the treatment groups within a transport group. (**B**) There was no significant difference between the prenatal transport groups in FosB-ir levels within a treatment group. However, FC treated pups had higher FosB-ir level compared to KA treated pups [t(16) = 2.62, p < 0.05] within In-House group. (**C**) In-House showed higher baseline FosB-ir than G16. *p < 0.05 as compared to In-House. # p < 0.05 as compared to KA group within a prenatal transport group.
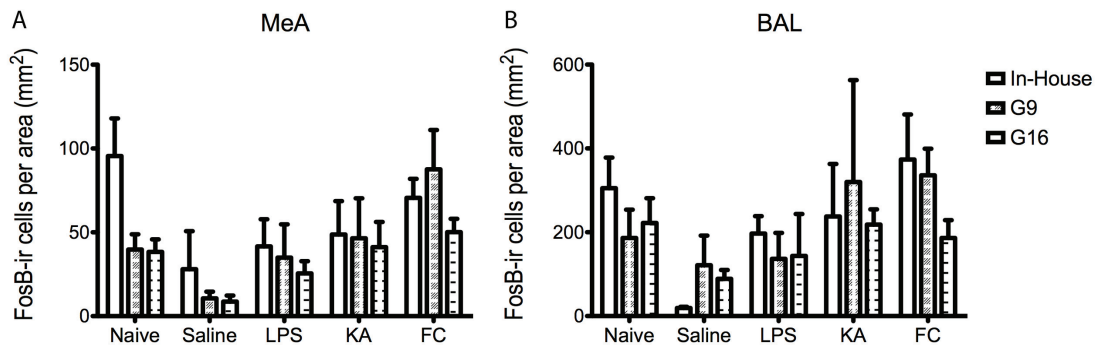
Figure 22. FosB-ir in medial nuclei of amygdala (MeA) and basolateral nuclei of Amygdala (BAL) as a function of prenatal transport stress groups. (A) There was no significant effect of prenatal transport on FosB-ir in MeA between the transport groups. (**B**) Similarly, there was no significant difference found in FosB-ir levels between the prenatal transport groups in BAL.
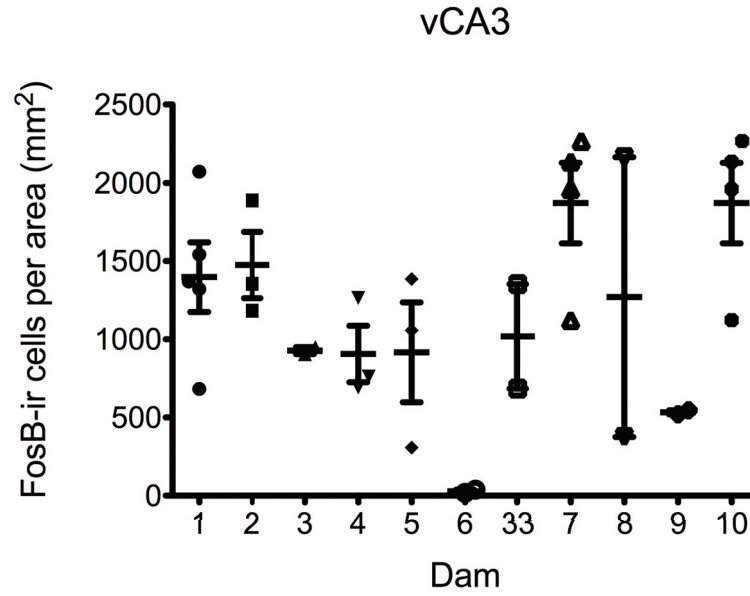
Figure 23. Within-litter variation in the vCA3 FosB-ir levels following FC seizure.
Each symbol in the scatterplot depicts individual animal FosB-ir density levels for all
pups within the litter (designated by dam number on x-axis). For each litter the mean and
SEM are shown demonstrating the large variability.
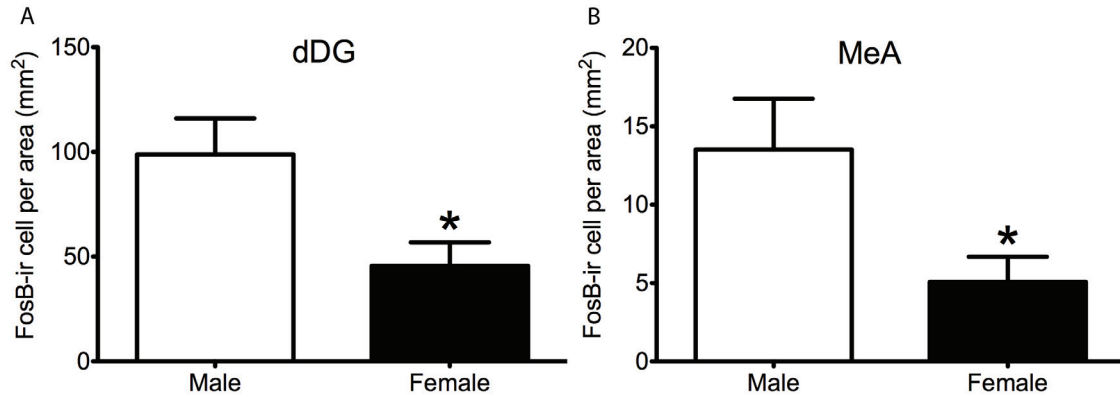
Figure 24. Sex differences in FosB-ir following Saline and LPS treatment. (**A**) Male pups had higher FosB-ir in dDG following LPS treatment as compared to female pups [$t(13)=2.30, p < 0.05$]. (**B**) Similarly, male pups also had higher FosB-ir in MeA following Saline treatment as compared to female pups [$t(7)=2.50, p < 0.05$].

Table 9. Summary of significance in FosB-ir as a function of maternal care groups: Naïve

Naïve		LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High v Low	dCA1															
	dCA2															
	dCA3															
	dDG															
	vCA1		*			**										
	vCA3					*										
	vDG														*	
	MeA	*	*	*	*	*						**	**			
	BAL					*	*				*	*	*			
Correlation	dCA1															
	dCA2															
	dCA3															
	dDG		0.563**	0.409*												
	vCA1															
	vCA3					-0.442*										
	vDG															
	MeA		0.643*	0.568*		0.547*										
	BAL					0.702**										

Top table shows results of student t-tests between High and Low maternal behaviour groups. Bottom table shows correlation between FosB-ir and maternal behaviour. Highlighted in red is where there was significant difference and correlations.

n = 16 - 24 for each High and Low group. * p < 0.05, ** p < 0.01

Table 10. Summary of significance in FosB-ir as a function of maternal care groups: Saline

Saline		LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High v Lo	dCA1															
	dCA2															
	dCA3				*										*	*
	dDG															
	vCA1															
	vCA3											*			*	
	vDG															
	MeA								*	*						
	BAL															
Correlation	dCA1															
	dCA2	0.666*	0.682*	0.642*												
	dCA3															
	dDG															
	vCA1													0.686*	0.604*	0.628*
	vCA3		-0.770**	-0.676*												
	vDG															
	MeA								-0.772*							
	BAL															

Top table shows results of student t-tests between High and Low maternal behaviour groups. Bottom table shows correlation between FosB-ir and maternal behaviour. Highlighted in red is where there was significant difference and correlations.

n = 11 - 21 for each High and Low group. * p < 0.05, ** p < 0.01

Table 11. Summary of significance in FosB-ir as a function of maternal care groups: LPS

LPS		LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High v Low	dCA1															
	dCA2															
	dCA3		*		*				*							
	dDG															
	vCA1						*									
	vCA3				*	*	**				*					
	vDG		**	**										*	*	*
	MeA		*													
	BAL		**												**	
Correlation	dCA1		0.563*													
	dCA2															
	dCA3		0.556*													
	dDG				-0.553*		-0.520*									
	vCA1															
	vCA3				-0.555*	-0.592*	-0.626*				0.554*					
	vDG	0.580*	0.811**	0.716**											-0.726**	
	MeA		0.567*			0.557*									-0.584*	
	BAL		0.707**												-0.598**	

Top table shows results of student t-tests between High and Low maternal behaviour groups. Bottom table shows correlation between FosB-ir and maternal behaviour. Highlighted in red is where there was significant difference and correlations.

n = 14 - 23 for each High and Low group. * p < 0.05, ** p < 0.01

Table 12. Summary of significance in FosB-ir as a function of maternal care groups: KA

KA		LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High v Low	dCA1															
	dCA2															
	dCA3															
	dDG															
	vCA1															
	vCA3															
	vDG															
	MeA					**	*				**					
	BAL															
Correlation	dCA1															
	dCA2															
	dCA3															
	dDG															
	vCA1															
	vCA3															
	vDG															
	MeA					0.849**	0.663**				-0.691**		-0.687**		-0.518*	
	BAL					0.515*										

Top table shows results of student t-tests between High and Low maternal behaviour groups. Bottom table shows correlation between FosB-ir and maternal behaviour. Highlighted in red is where there was significant difference and correlations.

n = 18 - 26 for each High and Low group. * p < 0.05, ** p < 0.01

Table 13. Summary of significance in FosB-ir as a function of maternal care groups: FC

FC		LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
High v Low	dCA1															
	dCA2															
	dCA3															
	dDG															
	vCA1															
	vCA3															
	vDG															
	MeA	*	*								**				*	
	BAL								*		*					*
Correlation	dCA1															
	dCA2															
	dCA3															
	dDG															
	vCA1															
	vCA3															
	vDG															
	MeA	0.408*	0.556**	0.484*		0.434*					-0.362*					
	BAL	0.532**		0.486**		0.396*			0.462*						0.398*	

Top table shows results of student t-tests between High and Low maternal behaviour groups. Bottom table shows correlation between FosB-ir and maternal behaviour. Highlighted in red is where there was significant difference and correlations.

n = 36 - 56 for each High and Low group. * p < 0.05, ** p < 0.01

Table 14. Maternal behavoiur and FosB-ir correlation in female pups following FC seizure

	FC	LG1	LG2	LG10	AB1	AB2	AB10	PA1	PA2	PA10	BL1	BL2	BL10	DO1	DO2	DO10
Female	dCA1															
	dCA2															
	dCA3															
	dDG															
	vCA1															
	vCA3															
	vDG				-0.662**											
	MeA	0.870**	0.871**	0.897***											-0.587*	
	BAL	0.667*		0.606*												

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Celllular Identification of FosB-ir Cells

We tried to determine which type of cell/s was activated following seizure induction using immunohistochemical double labeling of FosB-ir cells with NeuN for neurons and GFAP for astrocytes. Figure 25 shows representative images of ventral hippocampus from FC treated pup. Almost all the FosB (red) labeled cells were NeuN positive (Green) suggesting that they were neurons.

Fluoro-Jade C Staining

Next I analyzed the effects of prenatal transport stress and maternal behaviours on vulnerability of brain cells to seizure using Fluoro-Jade C as a marker of cell death. While there were high levels of Fluoro-Jade C imunoreactivity in positive control sections (stroke tissue), no Fluoro-Jade C immunoreactivity was found in the dorsal or ventral hippocampi, or in the amygdala in any of the pups, regardless the prenatal transport or maternal behaviours.

Gamma-H2AX and NeuN Double Immunofluorescent Labeling

As no cell death was observed in any of the pups using Fluoro-Jade C, I then sought to determine whether the cells were injured but may not have progressed along a cell death pathway. This was done using Gamma-H2AX (γ-H2AX) protein immunohistochemistry as a marker of DNA double strand breaks (DSB) that are often seen when cells are injured following exposure to a significant stressors such as prolonged seizures (Crowe et al., 2011).

γ-H2AX labeling in the dorsal and ventral hippocampus was compared between High- and Low-LG, and DO maternal behaviour groups since LG and DO behaviour

were most associated with FosB-ir. There were no differences in γ-H2AX

immunolabeling levels.
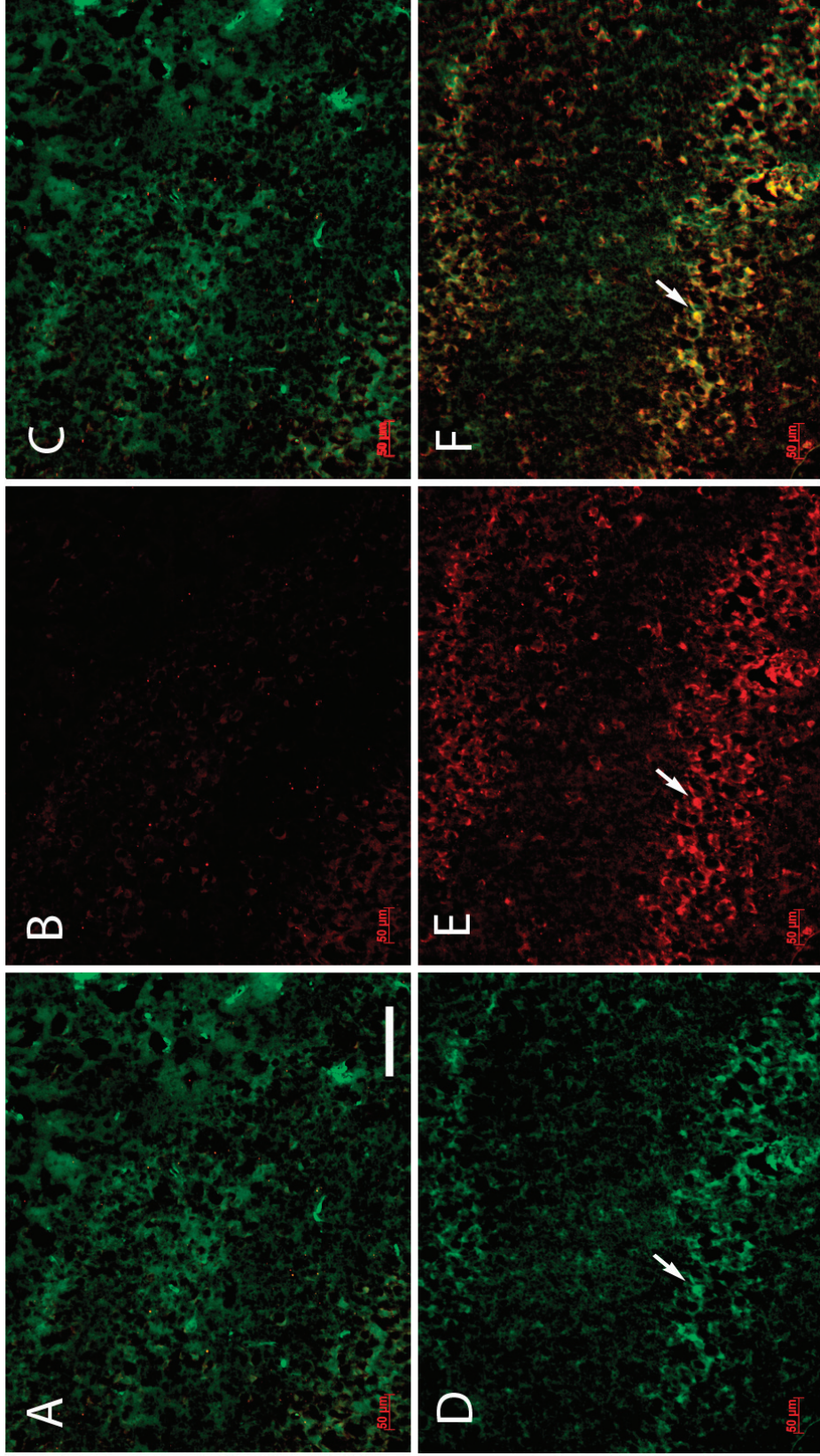
Figure 25. FosB and NeuN double immunofluorescence. Ventral hippocampi of pups in (**A** - **C**) Naïve and (**D** - **F**) FC groups were stained with NeuN (Green: **A & D**) and FosB (Red: **B & E**). No FosB-ir was observed in Naïve (**B**) pup while there was abundant FosB-ir in FC (**E**) pup. (**C**) & (**F**) Merged images of NeuN and FosB shows that FosB-ir is mostly in neurons. The arrows indicate co-labeled cell in (**D** - **F**). Scale bar in (**A**) indicates 100 μm in all panels.

Chapter 4: Discussion

The goal of the present study was to determine whether prenatal transport stress has an impact on seizure susceptibility of 14-day old rat pups. Because maternal behaviour has been shown to affect neurodevelopment, and can itself be affected by environmental stress, the modulatory effect of maternal care on seizure susceptibility of offspring was also examined. The main findings were that (1) there was abnormal late pup attrition regardless of maternal prenatal transport stress groups suggesting all the dams were stressed, (2) pups from In-House dams had a higher mortality rate following LPS treatment, (3) specific maternal behaviours were correlated with neuronal activation in the ventral hippocampus and amygdala following FC seizure, and (4) male pups were more susceptible to FC seizure than female pups. These findings suggest, as I will discuss below, that early experiences have an important role in shaping the brains of offspring and perhaps the seizure susceptibility of offspring.

Abnormal Pup Attrition Suggesting a Stressful Condition

In the present study, the average litter size was 6 (n = 25) at P14 where only 7 out of 25 litters had a litter size greater than 10. The litter size was independent of prenatal transport stress. On the other hand, maternal behaviour, specifically LG was negatively correlated with litter size while DO was positively correlated. The average litter size under normal conditions for Sprague-Dawley rats is about 10.5 at weaning (P21) (Palmer and Ulbrich, 1997). Small litter size generally implies adverse maternal or environmental conditions including stress (Serriere et al., 2011). Another finding in this study was that most of the attrition occurred after P7. Under normal conditions, it is natural for a few pups to die but this usually occurs before P4 (Palmer and Ulbrich, 1997).

After the observation of pup attrition that was consistent across 3 sets of experimental runs across all experimental groups, I decided to explore what might have caused this abnormal pup attrition. As there was no obvious cause for high pup attrition, I recorded ultrasonic vocalizations (USVs) within the housing room in the 4th experimental run. USVs at 22 kHz were observed before, during, and after the gestational period. The recording was from the room where all the dams were housed and the individual/s that emitted USV could not be determined. However, the presence of 22 kHz USV suggests that at least one dam was stressed. Rats emit USVs in a range of 18 – 32 kHz (i.e., 22 kHz) when the rats are under threatening, fearful, or stressful conditions (Kim et al., 2010; Litvin et al., 2007; Portfors, 2007). For example, rats emit the 22kHz USVs when exposed to a predator such as a cat or loud noises or when receiving an inescapable foot shock (Litvin et al., 2007; Portfors, 2007). In addition, the 22 kHz USV function as an alarm call that triggers anxiety-like behaviour and freezing response in other rats (Kim et al., 2010). Therefore, it is possible that 22 kHz USVs emitted by at least one dam induced a stress-response in other dams.

Interestingly, the rats in my experiments did not appear to habituate to stress, as suggested by the 22 kHz USV throughout the experiment over 7 weeks. Rats usually habituate to a stressor, decreasing their stress response over time. However, an unpredictable stressor (i.e., intermittent stressors) prevents habituation and even facilitates a stress response to a new stressor (Grissom and Bhatnagar 2009). Therefore, in this experiment, the rats may have been exposed to multiple unpredictable stressors.

There is a individual variability in stress response to stressors, as I will further discuss in later section. This may explain why maternal care is associated with litter size while prenatal transport stress is not.

It is worth noting that the USV was recorded only for set 4 dams. However, the attrition of the pups and general features of the initial 3 sets of dams were similar to that of set 4. It seems reasonable to suggest that some of the dams in the first 3 sets may also have emitted the 22 kHz USV during the gestational period and parturition that would have been detectable. Unfortunately, there was no identifiable stress-provoking factor. In addition, the room was switched within the animal care facility but did not change the litter attrition suggesting that it was not the particular housing room.

To summarize, there was no effects of prenatal transport stress on litter size. However, maternal behaviour, specifically, LG and DO behaviour were associated with the different mortality rates suggesting that litter health and pup survival was dam-dependent.

Mortality Rate Following LPS Treatment

An unexpected and somewhat surprising result was the high mortality rate in LPS treated pups as compared to not only the Naïve and Saline groups but also to the induced seizure groups. About 22 % of pups died following LPS treatment; a rate that was significantly higher than the 3-4% mortality rate in FC treated pups that also received LPS. The pups came from 4 dams that were from different experimental sets (i.e., they were ordered at different times) thus excluding the possibility that this was an isolated finding based on one set of experimental variables. In addition, as the weight of the pups

was not different between surviving and dead pups, gross malnutrition was not the factor either.

The pups that died following LPS alone (from 4 dams in different experimental sets) were all within the In-House group. This is interesting because prenatal stress suppresses the innate immunity of offspring (Bellinger et al., 2008). For example, prenatal stress decreases the number of peripheral blood lymphocytes including $CD4^+$ and $CD8^+$, and increases LPS-induced fever production and corticosterone levels in rats (Bellinger et al., 2008; Gotz and Stefanski 2007; Hashimoto et al., 2001). Since immunocompromised individuals are more prone to septic shock sometimes leading to death, one would expect prenatally stressed pups (G9 and G16 transport) to be more susceptible than the non-transported pups from the In-House bred rats. However, the 22 kHz USV was present before the arrival of the G9 and G16 dams as well as before the mating of the In-House dams. Therefore, the In-House dams were exposed to the 22 kHz USV for a longer time than the G9 and the G16 transported dams. This may explain the high mortality rate of the pups from the In-House bred dams.

The high mortality rate in LPS treated pups was also associated with time specific maternal behaviour. Specifically, Low-AB during the first postnatal week and Low-DO during the second postnatal week was associated with a high mortality rate. The previous literature that assessed effects of maternal care on immunity of offspring is limited and the evidence comes from maternal separation experiments rather than variation in maternal care. Previous studies have reported that 15 minutes and 6 hours of daily maternal separation during the first 2 postnatal weeks augmented inflammatory response and sickness behaviours induced by either bacterial and viral infection compared to a

handled control group in mice (Avitsur et al., 2006; Avitsur and Sheridan, 2009; Neveu et al., 1994). My finding of an association between Low-DO (i.e., dam spends more time with pups) during the second postnatal week with high mortality rate is not supported by these previous literature (Avitsur et al., 2006; Avitsur and Sheridan, 2009; Neveu et al., 1994). Experiments examining the effects of natural maternal behavioral differences on immunity of offspring appear not to have been done. However, these studies need to be done because the immune system can interact with HPA-axis as well as sympathetic nervous system and possibly affect excitability of the neurons.

Baseline Corticosterone Level of the Pup

In this study, the baseline corticosterone level of the pups was not different between sexes, prenatal transport groups, or maternal behaviour groups. In some previous literature, prenatally stressed pups and the pups born to Low-LG dams have a higher baseline corticosterone levels compared to non-prenatally stressed or the pups born to High-LG dams, respectively (Claessens et al., 2011; Green et al., 2011; Saboory et al., 2011; Weaver et al., 2004). However, other studies have not found this difference in baseline corticosterone levels (Champagne et al., 2008; Liu et al., 1997). More consistent results were found in differences in stress response following stress exposure that prenatally stressed pups and the pups from Low-LG dams show augmented corticosterone levels following stress exposure (Brunton and Russell, 2010; Charil et al., 2010; Classens et al., 2011; Glover et al., 2010; Harris and Seckl, 2011; Liu et al., 1997). These augmented responses are concurrent with a decrease in GR proteins and mRNA levels and increase in methylation on GR promoters in the dorsal hippocampus (Galeeva et al., 2010; Velisek, 2005; Weaver et al., 2004;). As activation of GR in the

hippocampus is part of the negative feedback to the HPA-axis, a decrease in GR levels in the hippocampus in the prenatally stressed and Low-LG explains the augmented stress response. Therefore, further investigation on GR expression are required to fully understand the effects of prenatal stress and maternal care on stress response of offspring.

Methodological differences may also have contributed to the inconsistencies between the results of the present study and those of the previous studies (Champagne et al., 2008; Green et al., 2011; Liu et al., 1997; Saboory et al., 2011; Weaver et al., 2004). The present study used transport stress that involves both air- and land-transportation that could last for about 24 hours. In contrast, some previous studies used restraint stress as a stressor at different developmental time-points (G14-birth, G11-birth, G15-G17; Green et al., 2011; Saboory et al., 2011; Zuena et al., 2008). The type, frequency, controllability, and predictability of stressor appeared to influence the outcome (Koolhaas et al., 2011). A chronic, uncontrollable and unpredictable stress appears to result in most severe outcome (Koolhaas et al., 2011). The stressor used in the present study was acute, uncontrollable and likely unpredictable (e.g. loud noise, changes in temperature and pressure, novel environment) while commonly used restraint stress is chronic, uncontrollable, and predictable stress. As these parameters of stress are important factors, it is difficult to compare the result of this study and those of previous literature.

Alternatively, prenatal transport stress might have affected the baseline corticosterone but in a dam dependent manner. Previous literature suggested that there is variability in coping style such that a stimulus could be perceived as a stressor in one rat while it is perceived as non-stressor in another rat (Koolhass et al., 2011). Therefore, individual differences in perception of stressor may lead to high variability within a

prenatal transport stress group. For this reason, non-significance in baseline corticosterone levels between the transport groups might be found.

Also, due to the small number of dams in this study, dams were categorized into High maternal behaviour group as above the population mean and Low maternal behaviour group as below the population mean. In contrast, previous studies categorized the dams into High maternal behaviour groups as 1 SD above the population mean and Low maternal behavior group as below the population mean. Previous studies have used a more stringent selection of dams while we included all dams. This categorization difference may have contributed to the non-significant difference in corticosterone found between the groups in this study due to high variability within a group.

Anatomical Differences in Neuronal Excitability Following Treatment

Clear anatomical differences were evident in neuronal activation (detected by FosB-ir) following KA and FC induced seizures. In our experimental paradigm, the vCA3 region and MeA appeared to have higher levels of neuronal activation following KA and FC seizures as compared to other regions. In addition, within the hippocampal formation, there was a greater neuronal activation in the ventral hippocampus compared to the dorsal hippocampus. This greater neuronal activation in the ventral hippocampus did not appear to be due to the vulnerability to excitotoxicity as there was no apparent cell death as indicated by Fluoro-Jade C staining. Similarly, the neuronal activation was not associated with any changes in cell injury such as double strand breaks (i.e., DNA damage) as indicated by H2AX labeling. Immature brains of rats are more tolerant to excitotoxicity than mature brains even with higher doses of KA (Haut et al., 2004). Thus the finding of no cell death in the present study is not surprising. However, other

molecular markers for cell death including caspases and TUNNEL may need to be assessed to verify the results of this study, as they may be more sensitive in this paradigm.

The sub-regional difference in FosB-ir may be due to the auto-associative connections of CA3 that contribute to the hyper-excitability of the CA3 compared to other sub-regions of the hippocampus (Anderson, 2007; Fisahn, 2005). The auto-associative connections are a loop of neuronal inputs between CA3-CA3 neurons that contribute to a self-positive feedback within CA3. Moreover, CA3 has the highest kainate receptor gene expression in the dorsal hippocampus (Bloss and Hunter, 2010). Kainic acid has the highest affinity to kainate receptors and acts mostly through kainate receptors (Bloss and Hunter, 2010). Therefore, the CA3 region of the hippocampus may be more responsive to kainic acid due to the combination of auto-associative connections and high kainate receptor levels.

The higher neuronal activation levels in the ventral hippocampus compared to the dorsal hippocampus in the present study may be due to functional and connectivity differences. For example, the ventral hippocampus has a major inhibitory role in the HPA-axis regulation and is heavily connected to the hypothalamus. An injection itself could be a stressor that triggers the HPA-axis response that leads to activation of ventral hippocampus to provide negative feedback. This may explain why the ventral hippocampus had a greater activation than the dorsal hippocampus.

In addition, the ventral hippocampus has a strong bidirectional connection to the amygdala. This bidirectional connectivity may promote recurrent neuronal activation within the hippocampal-amygdala connections. This hippocampal-amygdala connectivity

also explains why MeA as well as vCA3 had higher levels of FosB-ir following seizures. Why the MeA showed higher responsiveness than the BAL is not clear. The BAL has more anatomical connections to the ventral hippocampus than the MeA and is a well-known structure that is involved in fear memory and emotions (Pitkanen et al., 2000). Therefore, one would assume the BAL to be more responsive to seizures considering the greater number of connections to the ventral hippocampus. However, the BAL is a combination of basal and lateral sub-nuclei that have different connections (Pitkanen et al., 2000). The basal nucleus of the amygdala receives projections from the CA1 and projects back to both the CA3 and the CA1 regions of the hippocampus while the lateral nucleus receives projections from the subiculum and sends projections to the parasubiculum. These connections suggest that the basal nucleus of the amygdala has more direct connections to the Ammon's horn of the hippocampus than the lateral nucleus.

In summary, the results of this study suggest that there are hippocampal and amygdala sub-regional differences in neuronal activation following seizure induction. Many of the previous studies examining the effects of prenatal stress and maternal behaviour have focused on the dorsal hippocampus only and sometimes the hippocampus as a whole (i.e., no sub-regional separation). However, this study illustrates the importance of the ventral hippocampus as well as the point that experimental conclusions can be significantly different depending on the regions of the interest. Future studies should assess other structures that are involved in the HPA-axis such as PVN and the bed nucleus of stria terminalis (BST). The ventral hippocampus sends afferent outputs to and activates the BST neurons. These BST neurons then send projections to the PVN where

they inhibit the CRH neurons to have negative feedback on the stress response. Therefore, PVN and BST may also be activated during seizure activity and thus needs to be assessed in the future for FosB-ir.

Effects of Prenatal Transport Stress on Seizure Susceptibility

In the present study, prenatal transport stress had no effect on seizure induction rate following KA and FC treatment. However, the pups born to G9 transported dams showed a higher FC seizure duration compared to the pups born to In-House bred dams. This difference in seizure duration was not reflected by increased neuronal activation as indicated by FosB-ir in any of the hippocampal or amygdala regions. Because behavioural scoring is a subjective measure while FosB-ir is a more objective measure, based on behavioural measurement only a tentative conclusion can be made that prenatal stress increased seizure susceptibility. As I will discuss later, prenatal stress probably had no direct effects, but may have had indirect effects on seizure susceptibility.

Increased FC seizure severity was found in prenatally stressed rat offspring compared to non-stressed rat offspring (Qulu et al., 2012). Prenatal stress impacts the morphology of neurons, receptor phenotypes, plasticity of the brain and consequently the behaviour of offspring (Barros et al., 2006; Brunton and Russel, 2010; Glover et al., 2010; Green et al., 2011; Harris and Seckl, 2011; Jia et al., 2009; Laloux et al., 2012; Saboory et al., 2011; Yang et al., 2006; Yeh et al., 2012; Zuena et al., 2008). Such studies suggest that prenatal stress affects the functional phenotype of the brain of offspring.

The effects of prenatal stress vary across studies (see review Charil et al., 2010). Variability across studies may be due to different types of stressors, the intensity of the stress, the timing of the stress exposure, controllability and predictability of stressors, the

sex of the offspring, and the age of the offspring (Charil et al., 2010; Koolhaas et al., 2011). For example, there is a strain difference in the effects of prenatal stress on the stress response and patterns of gene expression in offspring (Neeley et al., 2011). Also, the intensity of stress determines the direction of the effects of the prenatal stress (Mychasiuk et al., 2011). For example, 10 minutes twice-daily prenatal stress decreased brain weight, body weight, and increased anxiety level, and global methylation in prefrontal cortex of both male and female offspring (Mychasiuk et al., 2011). In contrast, 30 minutes twice-daily of prenatal stress altered these parameters in the opposite way. In addition, the effects of 30 minutes stress was sex-dependent where male offspring showed a greater increase in brain weight, body weight, and decreased anxiety level and in global methylation in prefrontal cortex (Mychasiuk et al., 2011).

The prenatal transport stress may have had an indirect effect on seizure susceptibility of pups by affecting maternal behaviour. This idea is supported by a previous study showing that prenatal stress decreases LG behaviour of dams (Champagne and Meaney, 2006). As LG behaviour can influence the neurodevelopment and behaviour of offspring, modulation of maternal behaviour may affect seizure susceptibility. In fact, in this study, an association between maternal behaviours and seizure susceptibility fits with this idea. To summarize, results of the present study suggest that prenatal transport stress did not have a direct effect on the seizure susceptibility of 14-day old rat offspring, but further study is required to assess the effect of prenatal transport stress on maternal behaviour and indirect effects on seizure susceptibility of offspring.

Maternal Care is Associated With Seizure Susceptibility

The present study also assessed the contribution of maternal behaviour during first and second postnatal week, and first 10 postnatal days (e.g., LG1, LG2, and LG10). I demonstrated an association of maternal care with the FC seizure susceptibility of 14-day old rat offspring. More specifically, LG and DO appeared to contribute to the FC seizure susceptibility. The pups born to Low-DO10 dams showed a higher FC seizure induction rate than High-DO10. The High-LG1 and the High-LG10 groups also tended to have a higher FC seizure induction rate, although it was not significant. In addition, the High-LG, AB, and PA, and Low-DO was associated with higher cumulative seizure scores and severity. Moreover, FC induced FosB-ir in MeA and BAL were significantly correlated with High-LG1. Although some results are not consistent across the measurements, overall these results suggest that High-LG and Low-DO are associated with higher seizure susceptibility in 14-day rat offspring.

The association of High-LG with higher seizure susceptibility is somewhat counterintuitive as LG behaviour is thought to be a good maternal behaviour. For example, the pups born to High-LG dams show a lower stress response and enhanced spatial memory (Champagne et al., 2008; Francis et al., 2002; Liu et al., 2000). In addition, there is an increase in *GAD1* promoter methylation and decreased *GAD1* mRNA in the hippocampus of the pups born to the Low-LG dams (Zhang et al., 2010). GAD1 is an enzyme required to synthesize GABA neurotransmitters. Thus a decrease in GAD1 mRNA leads to a decrease in GABA neurotransmitter level and less inhibition of the brain. Also, the levels of α1, α5, β2, β3, γ1, and γ2 GABA receptor subunits in the amygdala are lower in offspring from Low-LG dams (Caldji et al., 2003). These studies

suggest lower inhibitory activities in brains of offspring born to Low-LG dams. Therefore, one might expect the offspring of Low-LG dams to have higher seizure susceptibility than the pups born to High-LG dams. However, previous studies suggest GABA activity is more complicated and sometimes can induce depolarization even in adult brains (see Avoli and Curtis 2011 for a review). The effects of maternal behaviour on seizure susceptibility are complex and probably affect many variables including receptor phenotypes.

Another variable that maternal behaviour can influence is the morphology of neurons. The spine density and dendritic length of CA1 pyramidal neurons are higher in pups of High-LG dams compared to pups of Low-LG dams. Since the majority of excitatory neurotransmission occurs at spines and dendrites and inhibitory neurotransmission is more common in soma or axons, increased spine density may be associated with an increase in excitatory synaptic transmission. In fact, the spine density was positively correlated with plasticity of the neurons and the excitatory postsynaptic potentiation (EPSP) was larger in pups of High-LG dams compared to pups of Low-LG dams following tetanic stimulation (Bagot et al., 2009; Champagne et al., 2008; van Hasselt et al., 2012a). This enhanced plasticity was reflected by better spatial memory in offspring of High-LG dams (Liu et al., 2000). This alteration in spine density could explain why the pups of High-LG dams are more susceptible to seizure. High-LG behaviour might enhance memory formation in offspring by increasing spine density and plasticity but also increase the seizure susceptibility as a result of enhanced excitatory transmission required for memory.

Alterations in MR and GR expression in the hippocampus may also contribute to differences in seizure susceptibility of the pups (Groeneweg et al., 2011). There are high levels of MR and GR in the hippocampus of offspring born to High-LG dams compared to pups born to Low-LG dams (Champagne et al., 2008; Francis et al., 2002; van Hasselt et al., 2012a). Stress exposure (forced swim and elevated platform) immediately before KA injection increased the severity of seizure in 21-day old male rats that was reversed by MR antagonists (Maggio and Segal, 2012). Also, stress exposure 1 hour prior to KA injection reduced seizure severity of the rats that was reversed with GR antagonist (Maggio and Segal, 2012). The MR and GR selective agonist application in hippocampal slices showed enhanced excitatory transmission immediately following MR agonist application but slow increase (30 minutes – 1 hour) in inhibitory postsynaptic potential following GR agonist. These data suggest that seizure susceptibility can be modified by corticosterone in a receptor dependent manner. Further analysis of GR and MR levels in the hippocampus and amygdala is required to understand the possible mechanism underlying higher seizure susceptibility in the pups born to High-LG dams in the present study.

The results of this study suggested that maternal care affects neurodevelopment as manifested as altered seizure susceptibility of offspring. Future studies should assess the MR and GR levels in the hippocampus and amygdala and their relationship to neuronal excitability, and the effects of cross-fostering and within-litter variation of maternal care on seizure susceptibility. Nevertheless, this study and others emphasized the importance of dam dependent effects on offspring development (van Hasselt et al., 2011; van Hasselt et al., 2012a).

Sex Differences in Seizure Susceptibility

In the present study, more male pups developed FC seizure compared to female pups regardless of prenatal transport and maternal behaviour. In addition, there were significant positive correlations between LG behaviour and FosB-ir in MeA and BAL in the female pups following FC seizure but not in male pups. These results suggest that sex is an important factor to consider when assessing seizure susceptibility. Interestingly, the incidence of epilepsy is higher in human males than females (Kotsopoulos et al., 2002). In addition, sex-dependent differences in neurodevelopment also support these results. GABA, normally an inhibitory system in adults, functions as an excitatory neurotransmitter system in newborns contributing to synaptogenesis in the developing brain that requires high excitatory stimulation. This excitatory GABA system decreases over time while inhibitory GABA increases. In Sprague-Dawley rats, female pups showed inhibitory responses through $GABA_A$ receptors by P4 while male pups showed excitatory responses through $GABA_A$ receptors until about P14 (Galanopoulou et al., 2008). Thus, the lower FC seizure susceptibility in female pups that I observed may be due to this early developing inhibitory GABA system.

Sex differences are also observed in the effects of prenatal stress on neurodevelopment. For example, prenatal stress increases spine density and dendritic length in CA1, CA3 and DG neurons in male pups but decreases in female pups (Weinstock, 2011). As the majority of excitatory synapses form on dendritic spines, an increase in spine density may reflect increases in excitatory synapses in male offspring. Dendritic length of CA3 pyramidal neurons, hippocampal glutamate concentration, and expression of NR1 subunit of NMDA receptors in CA1, CA3, and DG was lower in

prenatally stressed female offspring compared to control female offspring (Jia et al., 2009). As glutamate is a major excitatory system in the brain, decreases in glutamate concentration and NMDA receptor subunits reflect lower seizure susceptibility in prenatally stressed female offspring.

The contribution of maternal care to neurodevelopment and behaviour outcome of offspring also can be sex-dependent. In the present study, neuronal activation level in male pups was not different between the pups of High- and Low-LG dams. In contrast, female pups from High-LG dams tended to have a higher neuronal activation levels than the female pups from Low-LG dams. Considering male pups had higher seizure induction rate and tended to have a higher neuronal activation levels than female pups, male pups are more susceptible to seizure regardless of maternal care. In contrast, the seizure susceptibility of female pups is more affected by maternal care. Interestingly, LG is positively correlated with dendritic complexity of CA1 pyramidal neurons in male offspring but not in female offspring (van Hasselt et al., 2011a). Subsequently a negative correlation was found between dendritic complexity of DG granule cells and LG in female offspring but no correlation in male offspring. Also, a positive correlation was seen between post-tetanic EPSP amplitude and LG in both male and female offspring (van Hasselt et al., 2012a). Overall, these studies illustrate that the maternal care affects morphology of neurons and plasticity of neurons in sub-region of the hippocampus and sex-dependent manner. To my knowledge, there are no studies that have observed the sex-dependent effects of maternal care on the development and function of neurons in MeA and BAL. Therefore, future studies are needed to illustrate underlying mechanism of higher seizure susceptibility in male pups.

To summarize, the results of this study suggest that sex is an important factor that determines the direction of the effects of prenatal stress and maternal care on neurodevelopment of offspring. Some previous studies support this but there are many studies that have only used either male or female offspring, which does not tell a complete story. Further study is needed to increase sample size of each sex to confirm the results of this study. In addition, the underlying mechanism of higher seizure susceptibility in male offspring needs to be determined. An important point this experiment makes is that sex difference needs to be considered more carefully in the design of experiments.

Seizure Susceptibility is Dam and Sex Dependent?

Taking the results of the present and other studies, seizure susceptibility of rat offspring appears dependent upon dam as well as sex of the offspring. As discussed previously, prenatal stress did not have a direct effect on seizure susceptibility of the pups; however, prenatal stress may have changed maternal behaviour, which then influenced seizure susceptibility. In fact, prenatal stress has been shown to alter maternal behaviour (Champagne and Meaney, 2006). The response of the dams to prenatal stress is dependent on the innate stress response of the individual dam that is transmitted from their mothers through maternal care and is reflected in GR mRNA levels in the hippocampus. The variation in the innate stress response predicts the direction of how the maternal behaviour is altered after stress exposure in pregnant dams (Clinton et al., 2008). Female rats were classified as more anxious or less anxious by their behaviour using the elevated plus maze (Clinton et al., 2008). After the classification, they were mated and were randomly assigned to either control (no stress) or stress group where they

received chronic unpredictable stress from G2 to G19. Then the anxiety behaviour of all the dams was again assessed on G20 using the elevated plus maze. The previously classified less anxious dams exhibited increased anxiety-like behaviour after stress exposure during pregnancy. In contrast, previously classified more anxious dams exhibited decreased anxiety-like behaviour following stress exposure making them comparable to previously classified less anxious dams in control group (Clinton et al., 2008). This anxiety-like behaviour of dams is also associated with LG and AB behaviour where less anxious dams showed lower LG and AB than more anxious dams (Clinton et al., 2007). Therefore, prenatal stress may have opposite effects on maternal behaviour in more and less anxious dams. These results illustrate the challenges in interpretation of the effects of prenatal stress on seizure susceptibility without knowing the innate anxious behaviour of individual dams.

Cross-fostering studies also emphasize the dam dependent effects by illustrating the importance of dam-newborn interactions on neurodevelopment and behaviour of offspring (Francis et al., 1999). Maternal behaviour is shaped by the innate stress responsiveness (i.e., anxiety behaviour) of the dam and her experiences prior to parturition. The results of the present and other studies suggest dam-dependent effects on offspring neurodevelopment and behaviour including seizure susceptibility.

Another important factor is sex of offspring. Although most previous studies only used one sex, either male or female (mostly male offspring), the present study emphasizes the importance of sex-dependent effects on neurodevelopment and seizure susceptibility. As discussed previously, both prenatal stress and maternal behaviour influences neurodevelopment and the behaviour of offspring in a sex-dependent manner.

In addition, there are sex differences in the rate of neurological disorders including epilepsy (Kotsopoulos et al., 2002). Although there is sexual dimorphism, many animal models only use one sex. More specifically, most of the studies used male animals because of hormonal changes throughout the estrous cycle of female animals could complicate the results. In fact, the seizure susceptibility changes throughout the estrous cycle in humans and rodents where seizure susceptibility is lower during the estrus stage of the cycle (Reddy, 2012). Assessing both sexes could complicate the experiments; however, to understand the underlying mechanisms that can be translated into clinical setting, one needs to consider sex-dependent effects.

To summarize, there are many factors that contribute to the neurodevelopment of offspring and their behavioural outcome. The results of this study highlight the contribution of maternal care to the seizure susceptibility of 14-day rat offspring. In addition, based on the previous literature, maternal care is influenced by the innate responsiveness of the dam and their experiences before and during pregnancy. Moreover, the maternal care affects the neurodevelopment and outcome of the offspring in a sex-dependent manner. Therefore, the neurodevelopment and the seizure susceptibility of offspring is a result of complex effects of experience of dams and sex of the offspring.

This sex dimorphism as well as prenatal experience and maternal care may explain the difficulties in developing a drug that works for all individuals with epilepsy. As each person has different prenatal and postnatal experiences that interact with sex, the underlying physiology of seizures or epilepsy may be different. The complexity of the effects of early experiences and sex explains why a drug like carbamazepine that acts on

multiple sites including sodium channels and GABA have higher efficacy than other drugs that only act on one site.

Conclusion

In conclusion, the present study and previous literature suggest complex effects of prenatal and postnatal experiences including prenatal stress and maternal care on neurodevelopment and the seizure susceptibility of the pups. Importantly, the result of this study emphasizes the contribution of variation in maternal behaviour to alterations in seizure susceptibility of offspring. Maternal behaviour is shaped by the individual dams experiences before and during the pregnancy. More specifically, the innate responsiveness of the individual and stress exposure during pregnancy predicts the maternal behaviour of the dams. In addition, the effects of maternal behaviour are sex-dependent. Therefore, more complex experimental design analyzing the innate responsiveness, maternal care, and sex of the offspring may be required to understand the pathophysiology of neurological disorders such as epilepsy. Further assessment in this area may lead to the better treatment or possible prevention of epileptogenesis by screening of high-risk individuals for seizures and epilepsy.

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