DOES ONE PLUS ONE MAKE TWO?: INVESTIGATION OF PHARMACOLOGICAL EFFECTS AND CORTICAL INJURY ON THE DEVELOPING BRAIN

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Dedication

For Thérèse Hermans - van de Vijver

Ever since I was a little girl, you have had a very special place in my heart.
I am so grateful to have you as my grandmother.

Sinds ik een klein meisje was heb je altijd al een speciale plaats in mijn hart.
Ik ben erg dankbaar dat jij mijn oma bent.

Thesis Abstract

This thesis examined how pharmacological treatment and cortical injury during development affects brain plasticity. Rats were given either a low dose of perinatal fluoxetine or a mild postnatal day 7 Hypoxic-Ischemic (HI) injury, both, or neither. The functional outcome was assessed using a series of behavioral tasks and anatomical measures. To assess how HI affects the development of motor maps, forelimb motor maps were evoked at P19.

The findings indicate that fluoxetine treatment or HI injury mostly negatively affected functional outcome. The combined treatment with fluoxetine and HI injury only interacted on a limited number of measures. There was no delay in the emergence of evoked motor movements, or change in map location in the HI animals. These results suggest that the pharmacological treatment and cortical injury described in this thesis may have different mechanisms whereby plastic changes are induced and the interaction between these two mechanisms is limited.

Acknowledgements

I first would like to thank my supervisor, Dr. Bryan Kolb, for his willingness to take me on as a student. Thank you for all you have done for me. Dr. Robbin Gibb, your Brain and Behaviour Class sparked my interest in Neuroscience. Thank you for all your help and support you have given me since we met. To all the members of the Kolb and Gibb labs, thank you for your assistance and input. You made my time here so enjoyable. Special thanks to Preston Williams, Wendy Comeau, and Heather Bell, who were very generous with their time and invaluable advice starting from the first day I set foot in this lab and continuing every step along the way, including reading this document. Dr. Gerlinde Metz, Dr. Martin LaLumière, and Dr. Jerome Yager, thank you for serving on my thesis committee. To the agencies that provided me with financial assistance, your support is much appreciated. Katrina Perehudoff, thank you so much for reading and correcting pages and pages of text for me, for always being there for me and so much more. Adam Vossepoel, who kept my brain plastic through hours of thought experiments and mental excursions, thank you for reminding me there are no stupid questions, just stupid people asking them. Mom & Dad, who are always encouraging me to be to me, I want to thank you for providing me with such a strong foundation that I can build on. The older I get, the more I realize and appreciate all you have done for me and continue to do.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
AM	*Ante Meridiem*
ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
C	degree Celsius
CFA	Caudal forelimb area
cm	centimeter
COMT	Catechol-O-methyl transferase
E	Embryonic day
e.g.	for example
EPM	Elevated plus maze
Fig	Figure
g	gram
HA	Horizontal Activity
HI	Hypoxia-Ischemia
hr	hour
i.e.	that is
ICMS	Intracortial Microstimulation
Ip	Intraperitoneal
kg	kilogram
LSD	Fisher's least significant difference
mg	milligram
ml	milliliter
mm	millimeter
P	Postnatal day
PPC	Posterior parietal cortex
RFA	Rostral forelimb area
SPSS	Statistical Package for the Social Sciences
SSRI	Selective serotonin reuptake inhibitors
TOTDIST	Total Distance
VA	Vertical Activity
Val	Valine
WT	Water Task

CHAPTER ONE: GENERAL INTRODUCTION

Introduction

The brain is a remarkable structure that changes throughout life, particularly during development is the brain easily altered. Abnormal development of the brain is associated with a wide range of altered cognitive and motor capacities. Although there are a few well-studied conditions with obvious causes for the observed developmental disabilities (e.g., Down's sydrome, spina bifida, Tourette's syndrome), in the majority of cases the etiology is uncertain (e.g. cerebral palsy, mental retardation, attention deficit hyperactivity disorder (ADHD)). The first indication of perinatal compromise in the latter examples is usually the emergence of behavioral abnormalities in the absence of any other obvious neurological symptoms. The reason for the emergence of behavioral symptoms in many individuals could be the result of two or more sub-clinical events, the combination of which produces the unexpected symptoms.

The brain is most vulnerable to insult from a variety of factors such as injury, disease, and toxins (including psychoactive drugs) during early development (Kolb, Gibb, & Gorny, 2000). The effect of such early-life events can be disordered behavior, including a range of neurological and psychiatric conditions. A combination of these perinatal factors may put the individual at risk for the exaggerated effects of later factors. For example, prenatal exposure to stimulants or antidepressants may predispose an infant to respond differently to perinatal insults (e.g, subclinical hypoxic-ischemic (HI) insult).

As a result a large number of children with developmental disabilities likely experience a complex 'double-hit' that leads to neurological compromise

and later behavioral problems. There is, however, no good experimental model for the double-hit idea, nor any clear idea how brain development may be altered by the double-hit.

The goal of this thesis was to investigate the effects of (1) perinatal fluoxetine exposure, (2) a postnatal day 7 Hypoxic-Ischemic insult, and (3) both these experiences, on brain development and behavioral outcome.

Thesis Objectives and Rationale

The purpose of the current studies was to investigate how two sub-clinical factors affect brain development. More specifically, I investigated the effect of perinatal exposure of fluoxetine and/or a hypoxic-ischemic injury on postnatal day 7 (P7) on normal brain and behavioral development in rats.

A lot of literature on antidepressants has focused on the teratogenic effects. The research that does assess the behavioral and anatomical effects of selective serotonin reuptake inhibitor (SSRI) typically uses a high dose of fluoxetine that is higher than would normally be prescribed. In contrast, this thesis investigated the effects of a very low dosage of fluoxetine during the perinatal period on brain and behaviour.

Most reports in the literature on postnatal day seven Hypoxic-Ischemic injury include animals that suffered infarctation (Palmer, Vannucci, & Towfighi, 1990). In this thesis, only animals with non-cavity HI damage were included because our interest was primarily on the effects of subclinical perinatal events. Post-mortem, the animals that suffered infarctation were identified and excluded.

Thesis Format

Chapter One provides an introduction to the thesis, including a description of the general behavioral and anatomical measures. *Chapter Two* explores the effect of fluoxetine and/or P7 HI on body and brain weight, activity levels, anxiety levels, cognitive, and motor abilities. In addition, anatomical assessment of the size of hemispheres and brain structures, cortical thickness, and quantitative analysis of neuronal constituents is performed. In *Chapter Three*, the consequence of unilateral stroke on the emergence of forelimb motor maps in the frontal cortex is assessed. *Chapter Four* summarizes and provides a discussion of the results of this thesis.

Cortical Plasticity

Plasticity - What is it?

The capacity to change is a fundamental characteristic of the nervous system. The structural change in the brain as a result of experiences is referred to as *brain plasticity*.

The principle unit of plasticity is the synapse. In 1928, Ramón y Cajal was the first to propose the idea that learning results in morphological changes at the synapse. Later Donald O. Hebb (1947) expanded on that idea, by proposing that learning is mediated by increased efficiency of the synapse. A change in a single neuron is not sufficient to bring about functional plasticity. Plasticity is the result of a summation of changes in a group of synapses in a network (Kolb, 1995). In addition, axonal outgrowth, dendritic morphology, cell death, and neurogenesis are all mechanisms for plasticity resulting in varied cell

size and density, distribution of neurotransmitters and receptors, cortical thickness, and brain size.

Some parts of the brain possess more potential for plasticity. For example, areas that perform basic functions, such as respiration, are less susceptible to change. On the other hand, cognitive and motor abilities are extremely flexible behaviors, which makes the cerebral cortex the most likely candidate for plasticity (Kolb, 1995). The intrinsic cortical circuit is made up of local connections and it is thought that these connections are altered as a result of experience.

One of the underlying principles of neuroscience is the assumption that a change in behaviour reflects changes in the organization of the neural network that controls it, and vice versa. Behavioral changes due to plasticity may include learning, memory, addiction, aging, and recovery.

Influential Factors in Cortical Plasticity

The general term *experience* refers to a variety of factors that can influence plasticity. The synaptic organization of the brain thus can be influenced by a variety of factors during the stages of brain development, including but not limited to stress, neurotrophic factors, gonadal hormones, psychoactive stimulants, and injury (Kolb, Gibb, & Gorny, 2000). The last three factors will be analyzed in this thesis, but first the concept of plasticity will be further explored.

Cortical Development and Plasticity

Cortical Development and Plasticity in the Normal Brain

A key feature of cortical plasticity is the fact that the brain changes over the course of a lifetime, starting with when the brains develops. All mammalian brains go through the same genetically programmed stages of neural development: neurogenesis (cell birth), cell migration/cell proliferation, cell differentiation, cell maturation/dendritic and axonal growth, synaptogenesis (formation of synapses), cell and synaptic death, and myelogenesis (myelination of nerve fibers).

As soon as the first cells are produced, migration along radial glial cells starts. Cells in the cortex form six distinct layers. When the cells arrive in their designated locations, they differentiate into neurons. Neuron maturation is the growth of dendrites and axons and this process is guided by environmental cues and signals. Moreover, neurons make connections with other neurons for communication via synapses during synaptogenesis. The process of synaptogenesis goes on for a lifetime. Neurons and synapses are created in excess, thus programmed cell death takes place shortly after the onset of cell maturation. In the human brain up to 40%, and in the rat brain up to 10% of the synapses are eliminated in this process (Huttenlocher, 1994). Only the functional and meaningful connections are preserved. This is proposed to be a gateway/mechanism for environmental factors to influence brain development. Myelogenesis is the birth of oligodendrocytes and myelination is the last stage during which myelin is wrapped around the axons and serves as insulation to

increase the speed of nerve impulses, similar to the plastic surrounding an electrical wire. Myelination continues until adulthood.

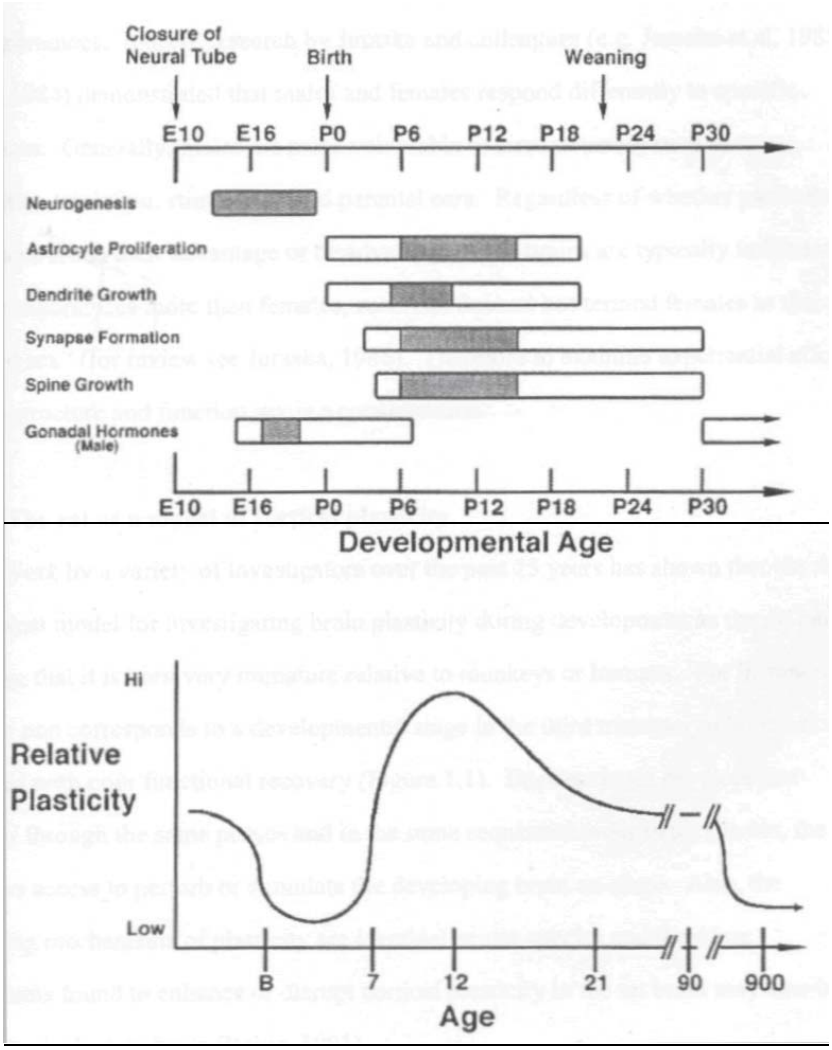

Figure 1.1. Developmental plasticity in the rat brain. Top: Cellular events associated with cortical plasticity. Bars represent the period of each cellular event, shaded area represents peak of the event. Bottom: Time-line with variations of cortical plasticity during the lifespan of the rat. Abbreviations: E = embryonic day; P = postnatal day) (Kolb, 1995).

Age Related Changes in Cortical Plasticity

The degree of brain plasticity varies with the stages of brain development. Cortical plasticity is relatively low during neuronal migration, which begins prenatally in rats and continues until about postnatal day 7 (P7). In contrast, the cortex is most plastic during the periods of dendritic and synaptic growth. In rats this translates into around embryonic P12. Thus, the first week after birth is a time of low cortical plasticity potential, followed by a period of high plasticity from P7 to P12. During adulthood cortical plasticity gradually declines, especially in senescence.

The variable stages of developmental plasticity illustrate the importance of the time of injury and other forms of experience in brain development (Fig. 1.1). The time point of injury dictates the mechanisms available for brain plasticity and the consequential behavioral outcomes.

It is important to keep in mind that birth date is irrelevant as an indicator of brain development. Different species are born at various stages of brain development (Fig. 1.2). For example, rats are born in a more immature state than humans. Rat pups have their eyes and ears closed and have hardly any fur at birth. The time of birth of rats is the developmental equivalent to a five month old human fetus. Furthermore, a newborn human baby is as developed as a seven day old rat.

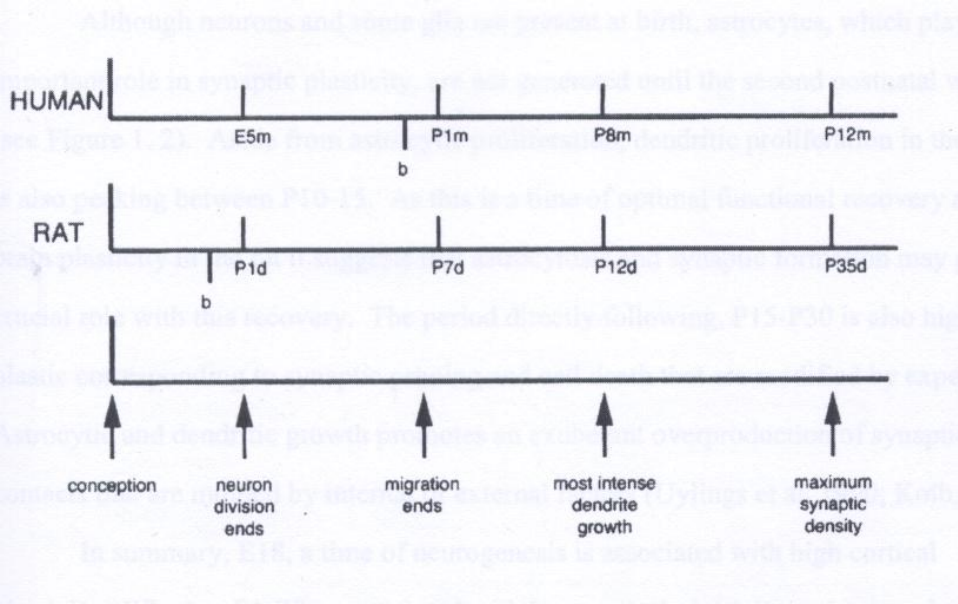

Fig 1.2. Schematic illustration of comparable developmental ages of the rat and human brain. Abbreviates: E = embryonic day; P= postnatal day; b = birth. (Kolb, 1995).

Factors Influencing Brain Plasticity

The brain not only changes during development, it also responds to other factors such as hormones, injury, toxins (psychoactive drugs) and others. These experiences have the potential to alter the brain's structure and function.

Sex Differences in Brain Plasticity

One of the earliest prenatal experiences to influence the brain is exposure to gonadal hormones, which either masculinize or feminize the brain. Gonadal hormones alter the structure of neurons, the number of cells and connections between different areas of the brain (Kolb & Stewart, 1991). In addition, these hormones also dictate how the brain will respond to future experience-dependent factors (Juraska, 1986). Regardless of positive or negative effects, male brains tend to be more affected by a range of factors than female brains (Yager, Wright, Armstrong, Jahraus, & Saucier, 2005). As such, it is important to consider sex when analyzing experimental effects on brain and behavior.

Brain Injury and Cortical Plasticity

Brain injury results in massive changes in the brain. The degree of recovery or compensation after brain injury is dictated by the level of brain plasticity. This makes injury a suitable candidate to investigate the mechanisms that drive and affect brain plasticity. For example, the level of brain plasticity changes dramatically over time.

A topic to be explored was how age at which injury occurred affects functional outcome. In the late 1930's, Margaret Kennard showed that in monkeys impairments as a result of unilateral motor cortex lesions are less severe if the lesion occurred in infancy rather than in adulthood (Kennard, 1942). The general conclusion from her work was that a brain injury early in life results in better functional outcome than if the injury is experienced later in life, which is commonly referred to as the Kennard Principle.

However, there are contrary observations in which cases of brain injuries result in worse outcome if they occur earlier in life. It has been noted that children with frontal lobe injuries have more deficiencies compared to adults with the same injury type (Hebb, 1947). Hebb (1949) concluded that some types of early injuries compromise or prevent the development of certain capacities imperative to normal cognitive development. Since then, numerous studies have supported the idea that developmental age predicts the functional outcome of early cortical injuries.

For example, Kolb and Gibb (1990) have shown that frontal cortical injury in rats of about seven days of age have good behavioral and anatomical recovery. The same lesion in three days old rats did not yield spontaneous behavioral nor anatomical recovery as it did in the day seven rat. Animals that received the lesion at embryonic day 18 showed behavioral recovery but abnormal cortical structure (Kolb, Cioe, & Muirhead, 1998). These findings reconfirm the claim that behavioral and anatomical outcomes are tightly linked to the precise developmental stage. Injury acquired outside the critical

maturation period, a time of high plasticity, results in poor behavioral and anatomical outcome.

Similar studies on the developmental stages in different focal cortical regions, such as posterior parietal cortex (PPC) and the temporal cortex have shown the same general conclusion. Lesions between days seven and ten have better outcomes than same lesion on days one to five and worse than adult. However, global lesions such as hemi-decortication show the opposite result. If hemi-decortication occurs between postnatal day one to five the functional outcome is better than the postnatal day seven to ten period (Kolb & Tomie, 1988) This finding indicates that there is more to the story than solely developmental age. There are at least three factors that are related to functional outcome following brain injury, age, location, and nature of the injury.

Nature of Injury – Hypoxia-Ischemia

Stroke refers to the interruption of flow of blood to the brain. Blood carries oxygen and glucose, which are required by neurons to produce energy. During a stroke the lack of oxygen and glucose results in the initiation of neuronal death (Huleihel & Golan, 2006). There are two types of stroke: hemorrhagic and ischemic. Hemorrhagic stroke is a bleeding in the brain. Ischemic stroke is the lack of blood flow in a brain area.

A common misconception is that stroke is a disease of the elderly. Data from the Canadian Pediatric Stroke Registry indicates that between 2.5 and 5% of children under the age of 15 years have suffered a stroke (deVeber, 2002). Five percent is most likely an underestimate given the fact that cerebro-vascular

accidents are common (25% pediatric population) in term and pre-term infants but often not included in stroke statistics (deVeber, 2002). More surprisingly, Looney (2007) showed that intracranial hemorrhage in asymptomatic neonates occurs in about 26% of all vaginal births (Looney et al., 2007).

Cerebral Hypoxia-Ischemia is one form of ischemic stroke that is a major cause of brain injury in newborns. One to six of every 1000 live term births suffer from HI (Ferriero, 2004). Hypoxia refers to the decrease in oxygen availability that leads to a decrease in blood pressure. Ischemia is a decrease in blood supply to the brain as result of a constricted or an obstructed blood vessel. These two conditions can lead to brain damage via the activation of various cytotoxic agents and other pathways that ultimately result in neuronal injury or death (Martin et al., 1998).

Several causes can result in HI injury such as premature placental detachment or other complications of pregnancy and delivery. The mechanism of the pathology is multi-factorial, complex and not very well understood. As a result there is currently no effective treatment available (Lorenz, Wooliever, Jetton, & Paneth, 1998). Because of its clinical relevance this injury model was chosen in this thesis.

HI is a major risk factor of a variety for neurological disorders such as motor and learning disabilities, cerebral palsy, epilepsy, seizures and even death (Huleihel & Golan, 2006). The severity of brain damage is dependent on the duration of the hypoxic-ischemic process and the gestational age of the infant. Premature infants that suffer form HI have mostly white matter damage, whereas gray matter damage is prevalent in term infants. Damage is seen in

selected areas of the cerebral cortex, hippocampus and striatum (Huleihel & Golan, 2006).

Depression, Psychoactive Drugs, and Cortical Plasticity

Unipolar depression consists of recurring episodes of dysphoria and negative thinking. It affects about 20% of the North-American population and is more common in females than in males (Nestler et al., 2002), especially during the childbearing years (Gotlib, Whiffen, Mount, Milne, & Cordy, 1989).

The brain has several nonspecific fiber systems that regulate neuronal activity by releasing neurotransmitters such as serotonin, noradrenalin, and acetylcholine. Altering the levels of these neurotransmitters during development will likely affect the cerebral circuits that they act on.

Serotonin (5-HT) is especially interesting in brain development because 5-HT projections precede the development of most other projections and thus can fundamentally influence synaptic organization. Serotonin belongs to the monoamine class and is synthesized from the amino acid tryptophan in a two-step biochemical pathway. After synthesis it is stored in synaptic vesicles before it is released. Serotonergic autoreceptors control 5-HT release. After release, 5-HT is removed from the synaptic cleft by 5-HT transporters. Most of the serotonergic fibers arise from the dorsal and median raphe nucleus, located in caudal midbrain and rostral pons. From these nuclei, fibers are sent to most forebrain areas including neocortex, striatum, nucleus accumbens, thalamus, hypothalamus, and the limbic system. Serotonin influences a wide array of

behaviours and physiological systems such as wakefulness, mood, and learning (Wenk et al., 1987).

Patients suffering from depression have abnormalities within the serotonergic system. One of the treatments for depression is the use of antidepressants. There are three classes that are clinically used; monoamine oxidase inhibitors, tricyclic compounds, and the newest class of antidepressants are selective serotonin reuptake inhibitors.

Fluoxetine is a selective serotonin reuptake inhibitor, which prevents the reuptake of presynaptic 5-HT, thus allowing an increase in serotonin in the synaptic cleft (Wong, Horng, Bymaster, Hauser, & Molloy, 1974). By changing the sensitivity and number of receptors or adjusting the pattern of transmitter movement across the synaptic membranes, the chemical imbalance of the serotonin system is readjusted after administration of fluoxetine (Blows, 2000).

As a result of fluoxetine's selectivity, efficacy and low risk of side effects, it is clinically accepted for use in pregnant women. Fluoxetine, and its metabolite norefluxotine, can both cross the placenta and are excreted in breast milk (Heikkinen, Ekblad, Palo, & Laine, 2003). As such, the fetus is exposed to these compounds during the prenatal and postnatal period.

Serotonin is important in the developing brain. It serves as a neurotransmitter throughout the central nervous system and acts as a signaling molecule involved in the regulation of cell migration, axon growth, and synapse formation (Whitaker-Azmitia, 2001). Alteration of the levels of serotonin in the developing brain could change the developmental organization of the cortex (Cases et al., 1996). For example, Xu, Sari, and Zhou (2004) have shown that

neonatal exposure to SSRIs during thalamocortical synaptic formation disrupts the organization of the barrel field cortex as a result of lack of refinement of thalamocortical afferents.

Double Hit Hypothesis

The double hit hypothesis originates from cancer research. It was proposed that if one of the two copies of a gene is faulty at birth, the second one is more likely to mutate and create abnormal growth of cells, also referred to as neoplasia (Knudson, 1971). The double hit notion is also used within the field of neuroscience. (Caspi et al., 2005) have shown that Catechol-O-methyl transferae (COMT) Valine/ Valine (Val/Val) genotypes combined with adolescent cannabis use have a three-fold increase in the likelihood of developing adult psychosis. Cannabis or COMT Val/Val genotype alone does not result in the increased susceptibility to developing psychosis.

This principle can be applied to the effects of perinatal experiences on brain development. For example, if perinatal exposure to fluoxetine is considered one hit, does the brain react differently to a second hit such as injury than if it had not experienced the first hit?

Behavioral and Anatomical Assessments

Behavioral Tasks

A range of behavioral tasks were used to examine both motor and cognitive consequences of the perinatal treatments.

Activity Box (Multifactorial open-field analysis)

Activity boxes are used to measure the general activity level by assessing motor and exploratory activities (Joutsiniemi, Leinonen, & Laakso, 1991). Measurement of spontaneous motor activity provides information on how various experimental manipulations affect changes in locomotor activity. A multivariate assessment of spontaneous locomotor activity, including horizontal activity, vertical activity, and total distance traveled, is obtained using a VersaMax automated animal activity monitoring system (Fig. 1.3).

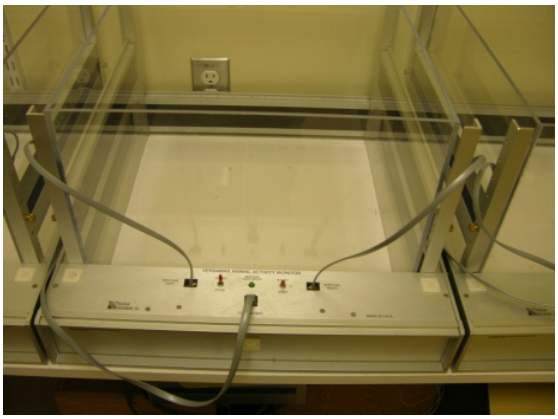

Figure 1.3. The activity box.

Footprints

Locomotion is a frequent behavior of a rat. As such, it is a crucial part of the behavioral analysis. In order to get an objective measure of the walking pattern, the animal's hind paws are inked and footprints are made as the animal crosses a one meter long sheet of paper. Angle of rotation, stride length, and stride width are measured (Fig 1.4) (Hruska, Kennedy, & Silbergeld, 1979).

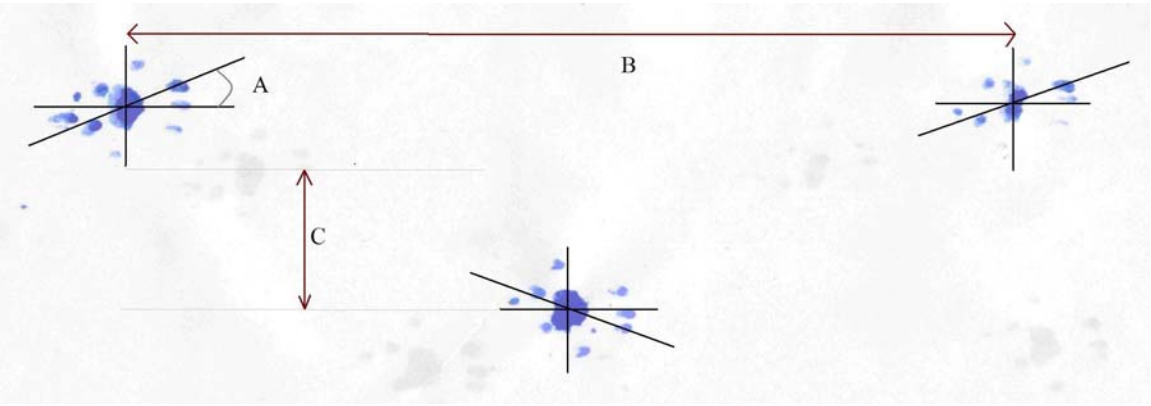

Figure 1.4. Footprints. The parameters angle of rotation (A), stride length (B) and stride width are shown.

Spatial Navigation Water Task

The spatial navigation water task (WT) is a spatial navigation learning task created by Richard Morris (Morris, Garrud, Rawlins, & O'Keefe, 1982). The WT requires animals to use spatial navigational skills to find a platform hidden under the water surface over multiple trials. The apparatus consists of a large circular pool that is filled with water in a room with visible distal cues (Fig. 1.5). A Plexiglas platform is submerged just underneath the water level that acts as an escape out of the water for the rat. Rats are naturally good swimmers, but prefer to be out of the water. They are therefore motivated to find the platform. Skimmed milk powder is added to the water in order to make the water opaque, thereby ensuring the invisibility of the platform.

There are multiple versions of the WT, but the central objective is the following: a rat is placed at one of the four starting locations and is allowed 90 seconds to find the platform. Rats learn this task quickly. Performance can be assessed by measuring various variables such as latency to find the platform or path length. Several cortical lesions have shown to cause a deficit in task performance. It is important to rule out that deficits are not due, in part, to motor problems but are rather a sole result of difficulty with navigation and learning the platform location. Analyzing the path length, instead of latency, is solely a measure of task acquisition, and thereby excluding the motor component.

Figure 1.5. The spatial navigation water task (Kolb & Whishaw, 2003, with permission).

Elevated Plus Maze

The elevated plus maze is a task that assesses anxiety levels (Pellow, Chopin, File, & Briley, 1985). The apparatus is made up of two open arms (50 cm x 10cm) and two enclosed arms of the same size that have additional 50 cm high walls (Fig. 1.6). These arms are referred to as the open and closed arms, respectively. This apparatus creates an environment of unfamiliarity, openness, and elevation which results in anxiousness to enter the open arm (File, 2001). The amount of time the animal spends in the open versus the closed arm is an indication of its anxiety level.

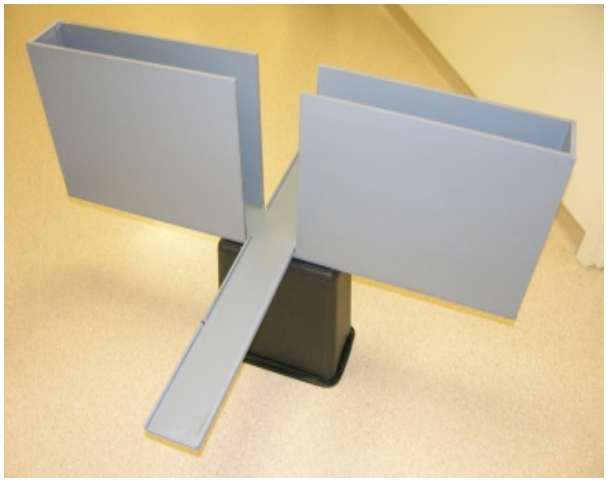

Figure 1.6. The elevated plus maze.

Tray Reaching Task

The tray reaching task, developed by Whishaw, O'Connor and Dunnett (1986), is a motor task to asses forelimb function. The animal is placed in a reaching box that consists of three solid Plexiglas walls on the left, right and top and thin vertical metal bars on the front and bottom (Fig. 1.7). On the outside of the box a tray filled with chicken food is on floor level. The animal is trained to reach through the bars, grasp the food with their forepaw and successfully bring it to their mouth to eat it. Performance is assessed by the total number of attempts and success rate in reaching for, and consuming the food.

Figure 1.7. The tray reaching task.

Forepaw Inhibition

When a rat swims in a straight line, it propels itself forward with its hindlimbs (Fig. 1.8), while the forepaws are usually inhibited and tucked under the chin (Schapiro, Salas, & Vukovich, 1970).

Kolb and Whishaw (1983) described that a unilateral cortical injury can disrupt motor control of forepaw inhibition. To evaluate forepaw inhibition, animals are placed in a rectangular aquarium and are required to swim to a visible platform located above the water level on the opposite end of the aquarium. The number of forelimb strokes that each limb makes is counted.

Figure 1.8. Forepaw inhibition during swimming.

Single Pellet Reaching Task

The single pellet reaching task is a motor task used to asses independent skilled forelimb functional use and was developed by Whishaw, Gorny, Pellis and Pellis (1991). This task is similar to the tray-reaching task and requires the rat to use its forepaw to retrieve food. However, the single pellet reaching task requires more fine motor control and bodily support adjustment than the tray reaching task because the target is a single food pellet. Animals are placed in a reaching box that has vertical slot in the front wall. Animals are trained to accurately reach through the slot for a single pellet, withdraw the paw through the slot, and release the pellet to the mouth to consume it (Fig. 1.9). Gharbawie, Gonzalez, and Whishaw (2005) have shown that although there is a quantitative improvement (i.e., reaching success) in the single pellet reaching task after middle cerebral artery stroke, there is still a distinctive qualitative impairment in those animals. Therefore, in addition to reaching success, reaching movements are analyzed through frame-by-frame video analysis, providing a detailed description of the separate components of the forelimb reaching movements.

Figure 1.9. The single pellet reaching task (Courtesy of O. A. Gharbawie).

Physiological and Anatomical Assessments

An examination of anatomical observations and measures were used to aide the determination of anatomical correlates of behavioral outcome.

Body and Brain Weight

A disruption in brain function can also cause a disruption in other behaviors such as eating and metabolic rates. It is therefore informative to monitor body weight after injury.

There is a possibility for a reduction in brain weight after a lesion that can be a result of missing tissue, but can also reflect atrophy and other developmental abnormalities of the brain tissue. Factors accounting for atrophy include (but are not limited to) fewer neurons, fewer connections, decreased dendritic branching, and decrease in myelination levels.

Gold Chloride Stain

The periventricular white matter is highly susceptible to HI insult and is a major contributor to chronic neurological dysfunction (Skoff et al., 2001). The level of myelination of the major axon pathways is therefore analysed.

In the past decade there have been several histochemical techniques developed to stain for myelin. In 1990, Laurence C. Schmued devised a gold chloride technique for myelinated axons that was comparable with the previous techniques but with improved speed, and metachromatic staining (the ability of the dye to stain different substances in different colors) and greater compatibility with frozen cut sections (Fig. 1.10).

The size of major axon pathways can be examined by tracing the surface area of structures of interest in a cross section. The standardized locations are chosen according to the atlas of Paxinos and Watson (1997). The plane at which the structure is the largest size is chosen. For example, if the structure comprises plate 16-36, the middle plate 19, is chosen.

Brains stained with Gold Chloride can be used for various anatomical measures. The dorsal surface area of each hemisphere without the cerebellum is measured to assess cortical surface area. Total hemisphere surface area as well as fimbria fornix and combined area of the corpus callosum, external capsule are measured on the coronal brain sections.

Cortical thickness can also be measured on the Gold chloride stained brains. Substantial loss or increase of neuron or glia number, dendritic branching, or axonal outgrowth, as a result of lesion or treatment will affect the thickness of the cortex.

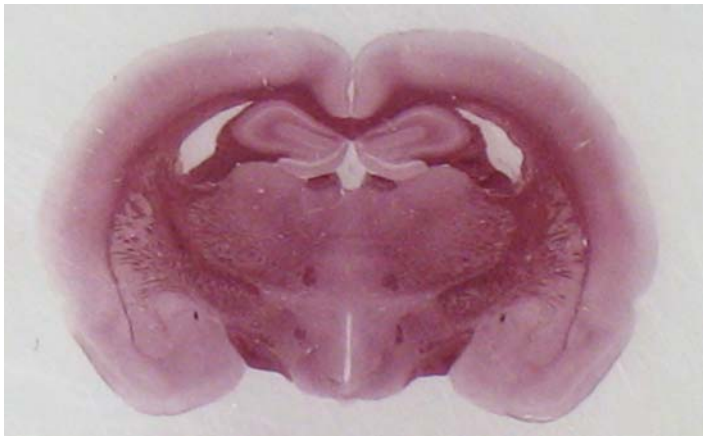

Figure 1.10. Gold chloride staining of whole brain section.

Golgi-Cox Stain

In 1873, Camillo Golgi developed a histological staining technique that is now known as the Golgi Stain (Fig. 1.11). With the Golgi stain, only one to five percent of the neurons are randomly and completely stained (Pasternak & Woolsey, 1975) and this ensures an unbiased representative selection of neurons. These characteristics allow for a quantitative analysis of the neuronal constituents such as the amount of dendritic space (DeFelipe & Jones, 1988), which provide information about the cortical organization (Douglas, Marin, & Whitteridege, 1989). In order to analyze the dendritic structure, a two-dimensional representation of the dendritic material is created using the *camera lucida* technique. This is an optical superimposition technique. A light microscope is equipped with a drawing tube that allows the neuron to be superimposed, so that the image and the drawing surface can be seen simultaneously. This allows for accurate duplication of the neuron on paper. Based on this image on paper, total dendritic length, and branch order of individual neurons is measured.

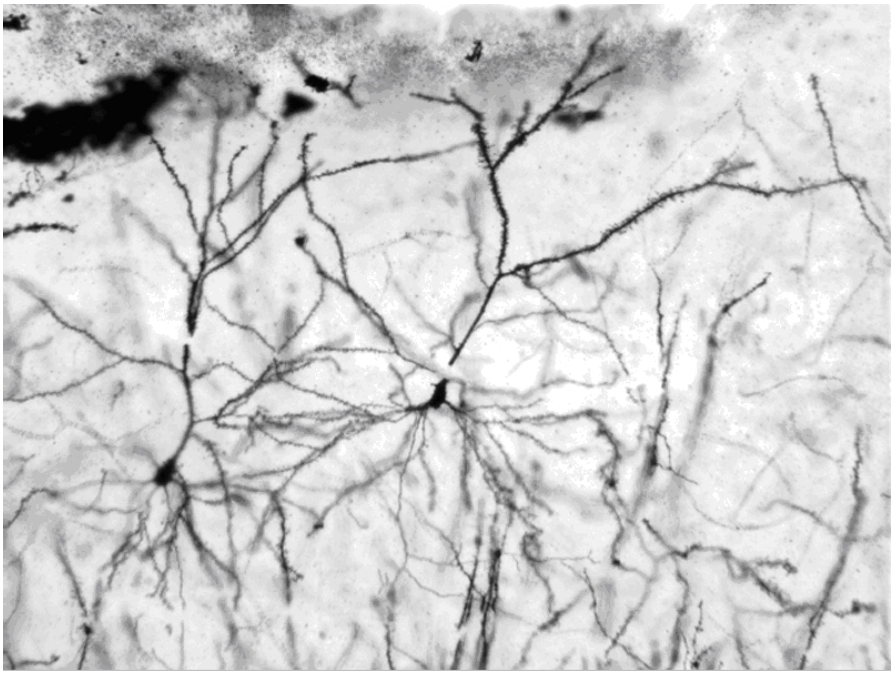

Figure 1.11. Image of pyramidal neurons as revealed by Golgi-Cox stain.

Intracortical Microstimulation Technique

Intracortical microstimulation (ICMS) is a technique that is used to examine the function of the motor cortex because it identifies the origin of the corticospinal pathways controlling movement. In 1870 Gustav Fritisch and Eduard Hitzig were the first to elicit movements by electrically stimulating the cortex of an anesthetized dog (von Bonin, 1960). Later, beginning in the 1940s, Wilder Penfield mapped the human cortex with electrical stimulation. These and other studies have shown that the neocortex is made up of regions that have localized functions. The regions that receive sensory inputs and motor outputs can each be represented by a motor and somatosensory projection map. The topographical representation of the body surface can be created on the cortex. There is a disproportionate representation in the maps of the human body. For example, the hands and face represent more space than the trunk.

In ICMS, movements are represented as cortical loci from which a specific muscle group can be activated (Fig. 1.12). In order to evoke a movement, an electrode is lowered into the corticospinal cells in layer V of the motor cortex. When a current is passed through the electrode, a small group of cortiospinal cells are activated, and produce movement of the body region that they connect to (Jankowska, Padel, & Tanaka, 1975). For each site of stimulation, the lowest current level to evoke the movement is recorded as well as the type of movement on a picture of the surface of the brain. After completion of an ICMS session, the results are presented as a mosaic map of the different movement categories on the motor cortex.

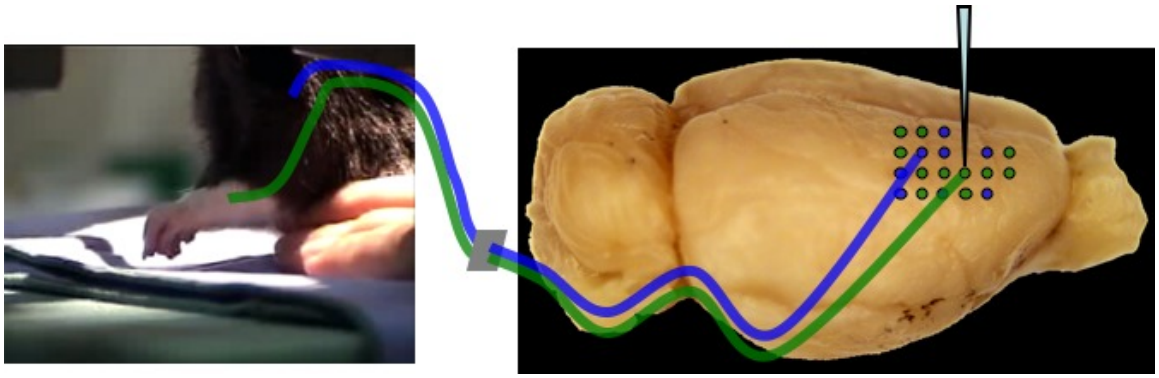

Figure 1.12. Intracortical microstimulation technique (Courtesy of P.T.J. A. Williams).

CHAPTER 2. EXPERIMENT 1: PERINATAL FLUOXETINE EXPOSURE AND POSTNATAL HYPOXIC-ISCHEMIC INJURY

Abstract

The effects of perinatal fluoxetine and mild postnatal day 7 Hypoxic-Ischemic lesion on behavioral, anatomical, and morphological measurements were investigated.

Background and Purpose: Brain injury and psychoactive drugs affect brain development and can have long-lasting behavioral implications. However, the impact of sub-clinical factors such as mild injury or low dose drug exposure is unclear. Furthermore, the effect of exposure to two of these factors is largely unknown. This experiment therefore examined the effect of a perinatal low dose of fluoxetine and moderate postnatal day seven Hypoxic-Ischemic insult.

Methods: Female Long-Evans rats received a low oral dose of fluoxetine (1 mg/kg), or vehicle during pregnancy and throughout the pre-weaning period until postnatal day 25. On postnatal day 7 offspring received a unilateral hypoxic-ischemic lesion (unilateral carotid artery ligation followed by 1.5 hours of exposure to 8% oxygen), or a sham surgery. The animals underwent a battery of behavioral tests to assess general activity, anxiety levels, cognitive and motor skills during adolescence and adulthood before being perfused for anatomical and morphological analysis. The females were randomly assigned for Golgi-Cox staining to assess dendritic arborization in the ipsi-leison and contra-lesion parietal layer III pyramidal cells and motor cortex layer V. The males were used for Gold Chloride staining to analyze cortical thickness, hemisphere surface area, and the degree of myelination of the corpus callosum and fimbria fornix.

Results: Fluoxetine-treated animals were more anxious, had poorer spatial learning, were less active, and had a reduction in body weight. The HI injury resulted in increased activity levels, release of forepaw inhibition during swimming and altered motor performances. Prenatal fluoxetine treatment improved the performance of the HI animals on certain measures of the activity box, footprints and tray reaching. In addition to behavioral consequences, anatomical measures were also influenced by the lesion and treatment. Animals exposed to fluoxetine had an increase in cortical thickness, whereas the HI group showed a decrease in cortical thickness. Furthermore, size of the ipsi-lesion hemisphere was reduced in the HI group and pyramidal cells in the parietal cortex layer III were more complex but shorter than sham-operated animals.

Conclusion: These results suggest that the lesion and treatment independently altered brain and behavioral development and that the combination failed to interact.

Introduction

The causes of developmental disabilities in children are uncertain in a majority of the cases and in the absence of any obvious neurological symptoms. The reason for the emergence of the behavioral symptoms is hypothesized to be the result of two or more sub-clinical events, the combination of which produces the unexpected symptoms. The developing brain is especially sensitive to agents

that affect the brain such as injury and drugs (Gressens, Mesples, Sahir, Marret, & Sola, 2001).

One class of these environmental factors is psychoactive drugs such as cocaine, nicotine, caffeine and prescription drugs such as antidepressants (e.g., fluoxetine). Most psychoactive drugs reduce plasticity potential (Marin, Perez, Duero, & Ramirez, 1999). For example, past treatment with nicotine interfered with the learning of a new skilled reaching task that was acquired in only a few days by saline-treated animals (Gonzalez, Gharbawie, Whishaw, & Kolb, 2005). Furthermore, exposure to psychoactive drugs can affect the ability of the brain to respond to later experiences, such as brain damage. For example, Day, Gibb and Kolb (2003) have shown that low maternal dose of fluoxetine exacerbate medial frontal cortex lesions.

The current experiment investigated the effect of prior drug experience on structural plasticity associated with recovery from brain injury in a clinically relevant model. A commonly used psychoactive drug used to alleviate depression symptoms which is also used by pregnant mothers, fluoxetine (Prozac), was chosen for peri-natal drug treatment. Hypoxia-Ischemia insult is linked to 25% of developmental disabilities in human cases (Shevell, Majnemer, Rosenbaum, & Abrahamowicz, 2001) and because there is a good rat pup model (Rice, Vannucci, & Brierley, 1981), HI was chosen as our injury model.

This study aimed to asses the effects of two sub-clinical hits. As such, a very low dose (1mg/kg) of fluoxetine was given, this dose is in the clinically-effective range in humans (D. Frost, personal communication, February 24, 2007).

Subjects, Housing, and Treatment Protocols

Subjects and Housing

Long-Evans rats raised at the University of Lethbridge vivarium were used in this study. The animals were housed in pairs under a 12 hr light/day cycle with lights on at 7:30 AM. All procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care at the University of Lethbridge.

All pups stayed with the dam in 46 x 23.5 x 20 cm clear plastic shoe-box cages until weaning. After weaning all litters were moved to a large 60 x 40 x 20 cm clear plastic housing tube, separated by sex around postnatal day 45.

Treatment

Eight Long-Evans dams (90 days old and weighing 400-600 g) were divided into two groups: *vehicle* and *fluoxetine*. Each dam received a plain Arrowroot biscuit or saturated with fluoxetine daily starting one week before pairing the female with a male and continuing throughout gestation and lactation. The *vehicle* group received plain biscuits. The fluoxetine group received biscuits saturated with a 1 mg/kg dose of fluoxetine. (Apo-Fluoxetine : Fluoxetine Hydrochloride Oral solution USP, Apotex Inc. Toronto, Canada). This dose is considered low and is commonly prescribed to human patients. Using a cross-litter design, the offspring of all dams were semi-randomly divided into two groups: *sham* and *Hypoxia-Ischemia* . The Hypoxic-Ischemia groups received surgery to induce insult at Post-natal day 7 (P7). *Post mortem* lesion size of the individual animals was determined. In this study the effect of minor

hits on anatomical and behavioral analyses was of interest, therefore animals with cavities as a result of HI surgery were excluded from all analysis. Only three animals from the HI group had cavities. The groups are summarized in Table 2.1. Due to collaborations, time constrains and technical problems, groups do not have equal numbers and not all animals participated in all behavioral and anatomical analyses (see appendix A1 and A2 for information).

All subjects had access to food and water *ad libitum*, except during the tray reaching task and single pellet task, in which the animals were placed on food deprivation while maintaining at least 85 % of their body weight.

treatment	lesion	sex	N
Flx	HI	M	8
		F	8
		Total	16
	Sham	M	10
		F	12
		Total	22
	Total	M	18
		F	20
		Total	38
Veh	HI	M	4
		F	5
		Total	9
	Sham	M	9
		F	9
		Total	18
	Total	M	13
		F	14
		Total	27
Total	HI	M	12
		F	13
		Total	25
	Sham	M	19
		F	21
		Total	40
	Total	M	31
		F	34
		Total	65

Table 2.1. Composition of experimental groups by *treatment*, *lesion* and *sex*.

Surgical Procedures

The surgical procedure was modified from (Rice, Vannucci, & Brierley, 1981). On P7, pups were anesthetized with isoflurane (5% induction, 2% maintenance). Under an operational microscope, an incision in the midline of the neck was made to expose the right common carotid artery (CCA). Although not always successful, attempts were made to separate the right CCA from the vagus nerve as we did not want to inflict direct nerve damage. The CCA was permanently ligated at two points with 5-0 silk surgery suture (25 yds silk non-absorbable surgery suture U.S.D. Dekuatel, inc. Fall River, MA, 02720 USA). The artery tissue between the ligation points was coagulated by passing electrical current using a bipolar coagulator. On completion, the incision was covered by tissue adhesive (3M Vetbond). For best results, the aim was to complete the surgery within five minutes so that the animal is not exposed longer than necessary to anesthesia.

Following the surgery, animals were placed in an incubator at 37˚C for 60 minutes to recuperate. The animals were then placed in containers that were maintained at 8% oxygen and 92% nitrogen at a pressure of 110mm/Hg for 90 minutes. The containers were 500 mL glass jars partially submerged in a 36.5˚C water bath to maintain a constant thermal environment. After completion of the hypoxic-ischemic insult and 15 minutes of recovery, pups were returned to their mothers in the home cages. Sham-operated animals consisted of littermates that underwent a similar surgical procedure, without ligating the CCA, followed by 90 minutes in the hypoxia environment. This HI surgery procedure has been

shown to result in permanent brain damage limited to the cerebral hemisphere ipsi-lateral to the CCA occlusion.

Methods and Timeline

Video Recording

All behavior, during task performance, was recorded by using a Canon ZR500 MD camcorder set at a shutter speed of 1/1000 s, 30 frames per second. During filming, additional illumination was supplied by a two arm Nikon Inc. MII cold light source. Frame-by-frame analysis was performed using a Sony digital videocassette recorder DSR-II GV-D1000 NTSC miniDV player.

Behavioral Tasks

Activity Box

Locomotor activity was measured using a VersaMax activity monitor (Accuscan Instruments Inc., Columbus, OH). The apparatus consists of a clear Plexiglas box (40cm x 40 cm x 30.5cm) covered with a sound-attenuating Plexiglas top with air holes. The box was surrounded by four separate infrared beams for a total of 16 beams on each side to measure horizontal activity, these beams were located 18 cm above the floor. In addition, there were two sets of beams to measure vertical activity which were located 10 cm, 13 cm and 18 cm above the floor. Animals were tested on P30, P45 and P60, respectively.

During the testing period, the experimenter was absent from the room, the room lights remained on and there was no water or food available in the

testing box. Animals were habituated to the activity boxes for 20 minutes each day for three days before the initial testing session.

The activity monitor measures the number of infrared beam breaks. This served as a measure of activity. The beam breaks were automatically recorded by a Digiscan Analyzer (Accuscan Instruments Inc., Columbus, OH) for selected measures. Data collection during a 30-minute interval included the following variables: horizontal activity (HA); number of beam breaks of lower photocells, vertical activity (VA); total number of beam interruptions in the vertical sensor and total distance (TOTDIST); the total distance traveled by the animal in cm.

Footprints

Footprint analysis was modified from de Medinaceli, Freed, and Wyatt (1982). A one meter long walkway was covered with a 7.6 cm wide strip of cash register paper. The walkway was enclosed by two pieces of Plexiglas that served as walls (one meter long, 15 cm wide) to ensure the animal followed a straight walking path. A dark box was positioned at the end of the walkway, which served as the motivator for the animal to walk across the paper. To begin the task, the animal's hindpaws were dipped in non-toxic ink (Parker Quink bottled in, blue/black, 57 ml, Parker Pen Company, Newhaven, England) followed by directly placing the animal on the paper at the start of the walkway. Animals were tested on three consecutive trials.

A series of at least three clearly defined, sequential steps for every trial was used to calculate a mean value for each of the following parameters: angle of rotation (angle between line parallel to the walking direction and the third digit

rotation was determined by the angle of the intersection of the line through the print of the third digit and the print of the metatarsophalangeal joint (MTP) parallel to the walking direction), stride length, and stride width.

Spatial Navigation Water Task

A circular pool (1.5m diameter, 45 cm height) was filled with water at a temperature between 25˚C - 27˚C. A water temperature comfortable to the rats was chosen to prevent increased stress levels. A Plexiglas platform (12 cm x 12 cm) was submerged 2 cm underneath the water level at least 20 cm from the pool wall in one of the quadrants of the pool. The platform serves as an escape out of the water for the rat. Water was mixed with 500 mL skim milk powder to make the water opaque, preventing the animal from seeing the platform and forcing it to use another method of navigation. The pool was located in a room with visible distal cues.

The WT was performed at P60. The standard place task version was based on the original task described by Morris (1982). The animals were tested for five consecutive days, four trials per day. The hidden platform remained at a fixed location in a particular quadrant throughout all the testing days. Every day the animals' starting positions were different, rotating each day between the quadrants (east, west, south and north) in pseudorandom order. To begin a trial an animal was placed at the edge, in the water facing the pool wall, released and allowed 90 seconds to find the platform to escape from the water. If the animal was unsuccessful in finding the platform after the allotted time, the

experimenter placed the rat on the platform. All animals were allowed 10 seconds on the platform before being returned to the holding cage.

The swim path (distance traveled) in meters(m), latency to find the platform measured in seconds (s), and swimming speed in meters per second (m/s) during all trials were recorded by a Poly Track video tracking system (San Diego Instruments). On the sixth testing day, the platform was removed and animals were released in the pool for an one 20 second trial. Dwell time in each quadrant was measured to assess if the animals preferred the quadrant, in which the platform was located in previous days. Quadrant preference for the previous platform location on the sixth testing day indicates that the animal learned to use spatial information to locate the platform from the previous five testing days.

Elevated Plus Maze

The elevated plus maze is a task that assesses anxiety levels (Pellow, Chopin, File, & Briley, 1985). The apparatus is made up of two open arms (50 cm x 10 cm) and two enclosed arms of the same size with 50 cm high walls, referred to as the open and closed arms, respectively. These conditions create an environment of unfamiliarity, openness and elevation (File, 2001). The open and closed arms are positioned opposite from each other creating a plus shape that is elevated 4 feet off the floor, so that the animal finds itself in an unfamiliar elevated environment (File, 2001).

The central square, where the arms cross, is referred to as the centre. It is at the centre that the animals are initially placed when the test starts. The

animals are free to walk in any direction on the apparatus for 300 seconds. Entry in a different arm was defined when the rat positioned its forepaws and 2/3 of the body into a different arm. The animals' behavior was videotaped and the percentage of time in open arms, closed arms and center was analyzed. An animal that spends more time in the closed arms, relative to the open arms, is considered more anxious than if it would spend more time in the open arm.

Tray Reaching Task

The tray-reaching box (30.5 cm high, 20.5 cm wide by 18.0 cm in length) consists of three solid Plexiglas walls in the front and a bottom, and 2 mm diameter bars metal bars, 9 mm apart edge to edge. A 4 cm wide and 5 cm deep tray was mounted outside the full length of the front wall and filled with chicken food pellets. There was a 5 cm distance between the bars and the food tray to prevent the animals from simply dragging the pellets in the cage. To obtain the chicken food, the rat had to reach through the bars, grab the food with its forepaw and successfully bring the pellet to the mouth in order to eat it. Any dropped food is inaccessible to the animal after it had fallen through the mesh bottom.

Animals were put on moderate food deprivation to motivate them to reach for the chicken food. Animals were allowed to use either forelimb to evaluate limb preference. Each rat was trained for a two-week period on consecutive days. During the first three days, animals were put in pairs in a tray reaching box and allowed to consume chicken food for 30 minutes. Animals were individually caged for the remaining 12 training session, each of 30 minute

duration. To see if HI injury would shift paw preference, animals were allowed to use their preferred paw. After 15 days all animals had learned the task. On day 16 the animals were video recorded for the first five minutes in the reaching box and the recordings were scored later. A reach was defined when the animal reached through the bars and grabbed the food. A successful reach resulted in successful retrieval and food consumption. Percentage of successes over attempts was calculated.

Forepaw Inhibition

Animals were trained for two days to swim to the visible platform at the end of a rectangular aquarium (120 x 43 x 50 cm). The water was maintained around 25˚C and at a depth preventing that the animals touched the bottom of the tank with their limbs or tail. Animals were released on one end of the tank and trained to swim directly, in a straight line, to the platform (26 cm high) without touching the walls on ten consecutive trials. On the third day, ten swim sessions for each animal were video recorded. The first three sessions where the animal swam in a straight line to the platform were analyzed. The numbers of forepaw strokes were counted for the left and right forelimb separately.

Single Pellet Reaching

The single pellet reaching task assesses skilled limbuse based on reaching success and movement performance. The reaching boxes were made of clear Plexiglas (40 cm x 45 cm x 13 cm). The front wall of the box had a 1.3 cm wide vertical slot, allowing the rats to reach for the food pellet located on a shelf

attached to the outside of the box. The shelf was located 4 cm above the floor on the front of the box. On the top of the shelf were two indentations (5 mm in diameter, and 1.5 mm deep), each aligned with one side of the slot. These indentations stabilized the pellet and were located 1.5 cm away from the front wall (Metz & Whishaw, 2000).

Prior to training, rats were food deprived to 85% of their free feeding body weight. In a training session, a rat was placed in the reaching box and a food pellet (45 mg each, Bio Serv, Frenchtown, New Jersey, USA) was placed contra-laterally to the rat's reaching paw. In order to test the hemisphere that suffered the insult in the HI animals, all rats were forced to reach with their left paw. The rats were trained to walk to the rear end of the box before reaching for a new pellet, which forced them to reposition themselves and prepare for the next reach. The rats were given 20 pellets per training and testing session. A successful reach was defined as obtaining the pellet on the first attempt, withdrawing the paw through the slot and releasing the pellet to the mouth where it was eaten. Success was calculated using the following formula:

$$\text{Success rate} = \frac{\textit{number of successful first reaches}}{20} * 100\ \%$$

The percentage of total pellets eaten was measured by counting the number of pellets eaten, regardless of whether the pellet was grasped and eaten on the first attempt. If the rat dropped the pellet, it did not count as a pellet eaten. The percentage of total success was measured using the following formula:

Percentage of total success= *number of pellets eaten* * 100%

20

During the first 10 days of training, animals were trained for 15 minutes with unlimited access to food pellets (45 mg). Animals were presented with 20 pellets each day for the remaining training sessions of the experiment. All animals were trained for a total of 15 days and their performance was video recorded on day 16.

Qualitative analysis of skilled reaching.

For qualitative analysis of single pellet reaching, the reaching movement was broken down into ten components (Whishaw, Pellis, Gorny, Kolb, & Tetzlaff, 1993), described as:

(1) *Digits to midline:* The limb is lifted from the floor with the upper arm and the digits adducted to the midline of the body. (2) *Digits close*: The forelimb continues to lift, the paw supinates so that the palm faces the midline of the body and the digits are semiflexed. (3) *Aim*: The forelimb continues to rise and the palm is in the midline of the rat's body and underneath the snout. As the forelimb is lifted the elbow moves in. (4) *Advance*: The forelimb moves forward through the slot and moves toward the pellet. As the forelimb advances the elbow continues to move in. (5) *Digits open*: As the limb is advanced the digits open and partially pronate above the pellet. (6) *Pronation*: The elbow is abducted, the palm is fully pronated over the pellet and the palm moves down in an arpeggio movement. (7) *Grasp*: The digits close around the pellet. Food is grasped by closure of the digits. (8) *Supination 1*: The elbow moves in as the

palm is withdrawn. As the palm is being withdrawn the palm turns 90°. (9) *Supination 2*: The palm is withdrawn from the slot and the palm is again supinated so that the palm faces the rat's mouth. (10) *Release*: The rat sits back and places the food in its mouth. The rat also raises its other paw to assist the reaching paw with eating.

Three successful reaches were analyzed through frame-by-frame analysis of the video recording on day 16. Each of the subcomponents was scored on a 3-point scale. A score of 0 was given if the movement was present, 0.5 if the movement was present but abnormal, or 1 if the movement was absent. The mean score from each component was averaged over three reaches. An average total score was calculated from the ten components.

Anatomical Methods

After the behavioural tasks were concluded, subsets of the animals were perfused for anatomical and morphological analysis. There were not enough animals available to analysis main effect of sex for the anatomical measure and therefore the brains of each sex were used for the same stain. The males of each group were used for Gold Chloride staining to stain for myelin. This measure was used to analyze cortical thickness at five different planes and degree of myelination of the corpus callosum, internal capsule and fimbria fornix. The females were assigned for Golgi-Cox staining to assess dendritic arborization of pyramidal cells in the ipsi-lesion and contra-lesion parietal layer III (Par) and forelimb area V (FL).

Body and Brain Weight

Animals were weighed on P60. After completion of the experiment, the animals' adult body weight was measured just before perfusion. After the completion of the perfusion, the brain was removed from the skull, trimmed in a consistent manner, and weighed.

Gold Chloride Stain

An overdose of sodium pentothal (Euthansol) intraperitoneal (ip) (0.9 mL for male adults, 0.5 mL for female adults) was administered to the animals. After bodyweight was measured the animals were prepared for perfusion. A needle was inserted into the bottom left ventricle of the heart and the animal was perfused with a solution of 0.9% saline in 0.1 M phosphate buffer (pH 7.2) followed by about 200 ml of fixative made of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and weighed. Brains were post-fixed, 24 hr later, in the same fixative with 30% sucrose.

Brains were freeze-sectioned on a cryostat at 40 μm thickness. Every tenth section was collected on 1% gelatin + 0.2% chromatin coated glass slides and dried overnight. Sections were incubated in the Gold Chloride Solution (0.2% gold chloride in phosphate buffer (1.8 g crystalline gold chloride, 0.33 g sodium phosphate monobasic monohydrate, 3.6 g sodium phosphate dibasic ahydrous, 9.0 g sodium chloride, 1000 mL distilled water) between 1-2 hours at 40˚C until myelinated bundles appeared in shades of purple/brown. This was followed by 5 minutes in distilled water, 5 minutes 2.5% Sodium Thiosulfate to

fix and a 30 minute rinse of slow running tap water. Sections were air dried and cover slipped with permount mounting media (Fisher Scientific).

White Matter Volume of Myelinated Structures

First, an image was taken using Zeiss Axiovision 4.3 (Zeiss, Germany) at 1x magnification. Using ImageJ (Image J, Betheseda, MD) software, the image was calibrated to transform pixel units into mm units. Subsequently, the structure of interest was traced in order to calculate the area size in mm^2.

In order to assess size of myelinated structures, the surface area of a structure of interest was measured in a cross section at a standardized location according to the atlas of Paxinos and Watson (1997). The plane at which the structure was largest was chosen. For example, if the structure comprised plate 16-36 the middle plate, 19, was chosen.

Size was examined by tracing the following structures; the dorsal surface area of each hemisphere without the cerebellum, total hemisphere surface area, the total surface area of the respective hemispheres (hem), combined area of: corpus callosum, external capsule and cingulum (cc) on the anterior plate and the fimbria fornix (fi) on the posterior plate. The following standardized locations were chosen according to the atlas of (Paxinos & Watson, 1997): anterior; Plate 19 (-0.30 mm from Bregma and Interaural 8.70 mm) and posterior; Plate 27 (-1.88 mm from Bregma and Interaural 7.12 mm) (Figure 2.1).

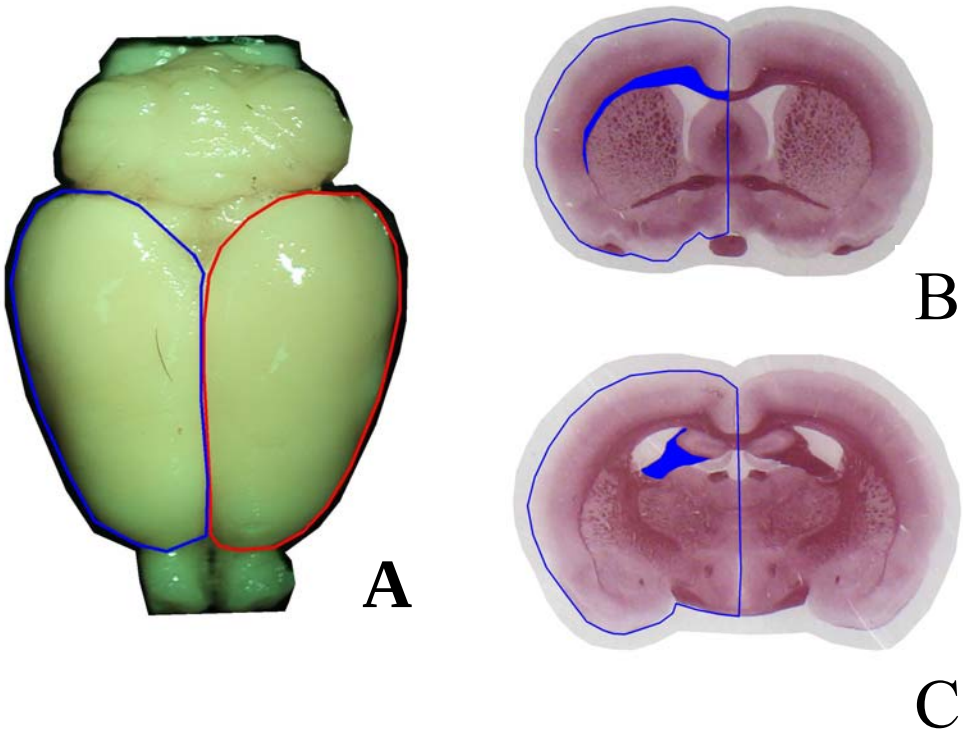

Figure 2.1. Photographs of a Vehicle Sham animal brain with areas outlined that were used in anatomical analysis. A) Dorsal hemisphere area as identified by the outlined segments. Red outline: left hemisphere, blue outline: right hemisphere. B) Plate 19, hemisphere surface and combined area of corpus callosum, external capsule and cingulum are identified with blue outlined segment. C) Plate 27, hemisphere surface and the fimbria fornix are identified with blue outlined segment.

Cortical Thickness

Cortical Thickness was measured from the Gold Chloride stained coronal sections. Only male brains were used for this analysis. Sections were projected on a Zeiss 2 POL petrographic projector at a magnification of 20X. A metric ruler was used to measure at four different points: lateral (L), medial (M), central (C) and at rhinal fissure (RF), bilaterally on each of the five planes corresponding to Zilles' levels +2.2, -0.3,-2.3,-4.8 and -6.3 relative to bregma (Figure 2.2). These planes correspond to the anterior tip of caudate-putamen, middle of anterior commissure, anterior tip of dorsal hippocampus (ammon's horn), posterior commissure and the posterior tip of ventral hippocampus, respectively (Stewart & Kolb, 1988).

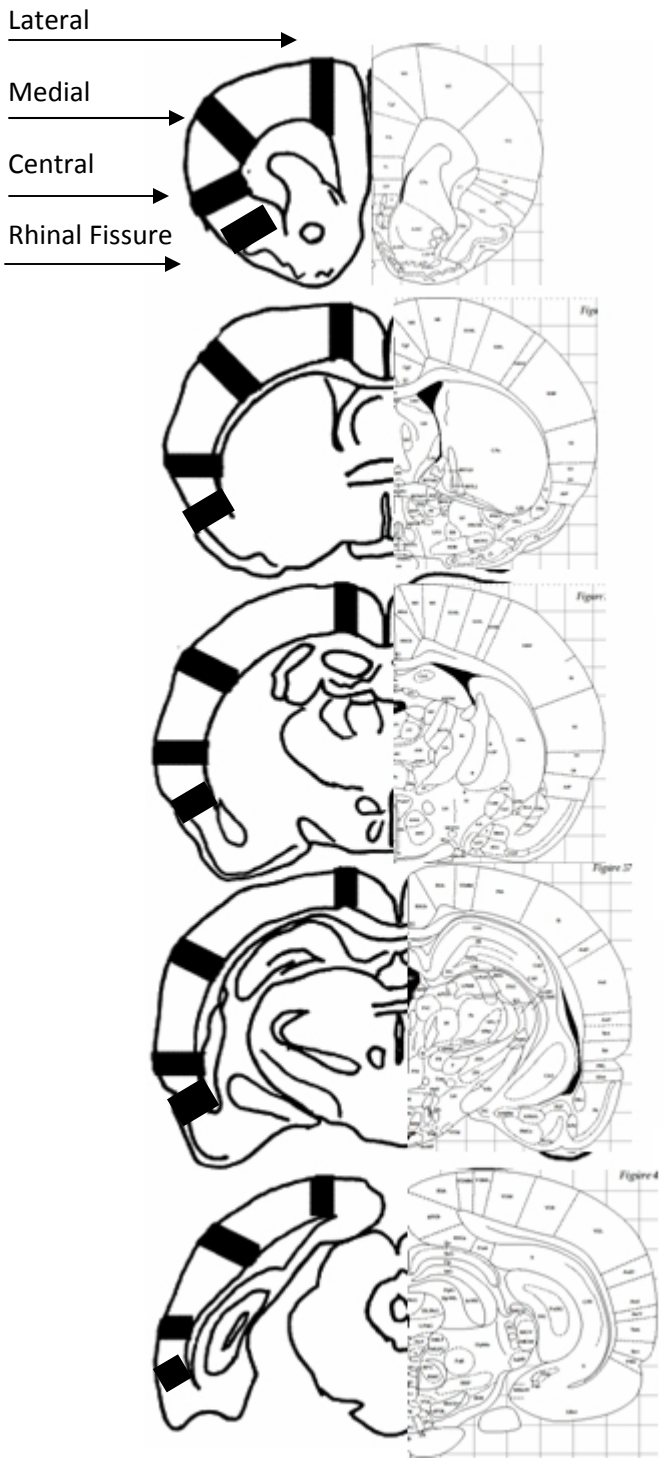

Figure 2.2. Coronal sections used for cortical thickness. Black bars on each plane are the four measures of cortical thickness that were made.

Golgi-Cox Stain

An overdose of sodium pentothal (Euthensol) ip (0.5 ml for female adults) was administered to the animals. After bodyweight was measured animals were prepared for perfusion.

A pump needle was inserted into the bottom left ventricle of the heart and the animal was perfused with about 200 ml of 0.9% saline solution. Brains were removed, weighed and submerged in Gogi-Cox solution (Glaser & Van der Loos, 1981). After being stored in the dark for 14 days, brains were transferred to a 30% sucrose solution for at least two days to post-fix. Brains were sectioned using a vibratome. The brain was mounted on the sectioning stage with cyanoacrylic glue and the vibratome reservoir was filled with 8% sucrose solution that covered the brain and sectioning blade. Sections were cut 200 μm thick and mounted on 2% gelatinized glass slides. Each full slide was then covered and moist bibulous paper was used to manually press the sections onto the slide. Slides were kept in a humidity chamber between 24 hr and 3 days before being processed using a procedure described by Gibb and Kolb (1998)

Processing of Golgi stain was done as follows: 1 minute distilled H_20, 40 minutes in dark chamber, ammonium hydroxide, 1 minute distilled H_20, 40 minutes in 1/2Kodak Fix for film / ½ distilled H_20 in a 1:1 ratio in a dark chamber, 1 minute distilled H_20, 1 minute distilled H_20, 1 minute 50% alchohol, 1 minute 70% alcohol, 1 minute 95% alcohol, 5 minutes 100% alcohol with VWR molecular sieve, 5 minutes 100% alcohol with VWR molecular sieve, 5 minutes 100% alcohol with VWR molecular sieve, 10 minutes 1/3 100% alcohol / 1/3

chloroform / 1/3 HemoDe in a 1:1:1 ratio, 15 minutes HemoDe, 15 minutes HemoDe.

Pyramidal cells in the parietal layer III and forelimb layer V (Zilles, 1985) were traced using camera lucida at 250X magnification. Five representative cells from each hemisphere were chosen. In order to be selected, the pyramidal cells had to fulfill the following criteria: the dendritic tree had to be intact, visible in the plane of the section and not obstructed by blood vessels or other structures. On the drawings the branch order was measured as described by Coleman and Riesen (1968). The branch order analysis for basilar dendrites used the branch originating from the cell body that was first order, and after one bifurcation, second order, and so on. Analysis for apical dendrites was done by selecting the first order branches of the apical dendrites which are those with branches that bifurcate from the primary apical dendrite.

The dendritic length was measured by the Sholl analysis (Sholl, 1956). The drawing of a neuron is covered with a transparent overlay that had concentric circles 20 μm apart, and placed such that the innermost ring covered the middle of the cell body. For all rings the number of dendrite-ring intersections were counted, which allowed for the calculation of the total dendritic length (number of intersections X 20).

Statistical Analysis

A three-way Analysis of Variance (ANOVA) or repeated-measures ANOVA (SPSS 11.0; SPSS Inc., Chicago, IL, USA) and standard errors were employed with Treatment (vehicle or fluoxetine), Lesion (sham or HI), Sex (male or female) and

Time for the repeated-measures as independent variables. In analyses with only one sex a two-way Analysis of Variance (ANOVA) was employed with Treatment (vehicle or fluoxetine), Lesion (sham or HI) as independent variables. For post hoc tests, a Fisher's LSD was used. Levene's test of homogeneity of variance was utilized. Only significant results are reported. In Repeated Measures, only meaningful (eg. learning) main effects of *Time* are reported.

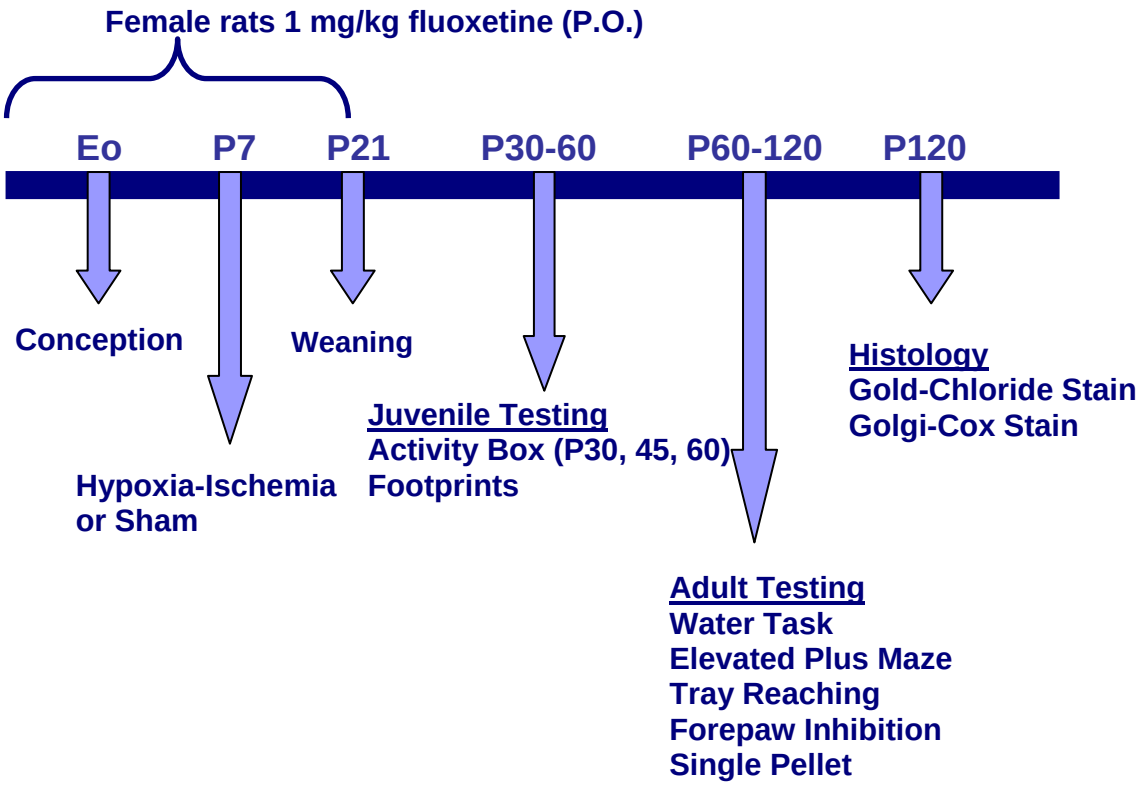

Figure 2.3. A timeline of methods performed in this experiment. Abbreviations E = embryonic day; P= postnatal day.

Results

General Observations

Seven pups died from the CCA occlusion, sham surgery or hypoxia exposure. In only three animals did the HI insult resulted in actual infarction of the brains and these animal were excluded from analysis. The HI group appeared normal in appearance except for characteristic asymmetry of the face. The ipsi-lesion side of the face appeared narrower with a ptotic eye (drooping upper eyelid).

Post-mortem inspection of the whole brain showed that only three HI rats (1.5%) had cavities. These animals were all male, one with peri-natal fluoxetine exposure, the other two without. The infarcts atrophied the posterior parietal, temporal and occipital cortex but did not encroach on the midline cortex or frontal cortex. In two of these three cases, the striatum was dramatically reduced in size. The cavity animals were excluded from all analyses in an effort to keep the HI group uniform, as this study intended to assess the effect of a sub-clinical insult.

Behavioral Results

Activity Box

Hypoxia-Ischemia did affect the subjects' general activity levels in adolescence, as there was an increase in activity level compared to the sham operated subjects; however, this effect was absent in adulthood. Fluoxetine treatment decreased the average total distance traveled and horizontal activity,

but increased the average vertical activity level. Both treatment and lesion groups were most active at P60. Overall, females traveled further than males.

Horizontal activity

For horizontal activity, a repeated-measures ANOVA (*Treatment x Lesion x Sex x Test Age*) revealed an *Age x Lesion* $F(2, 114) = 3.65$, $p = .029$ and *Age x Treatment* $F(2, 114) = 6.43$, $p = .002$ interaction (Fig. 2.4). Post-hoc analysis revealed that P30 HI animals were more active than sham ($p = .022$). The HI group was most active at P60 compared to P30 ($p = .004$) and P45 ($p = .045$). The sham group had similar findings, with P60 also being the most active time compared to P30 ($p < .001$) and P45 ($p < .001$). In addition, the sham animals were more active at P45 compared to P30 ($p = .003$).

At P60 females were more active than males ($p < .001$). The males were more active at P60 compared to P45 ($p = .032$) and P30 ($p = .009$). In addition they were more active at P30 compared to P45 ($p = .032$). Same results were found within the female group. At P60 the females were more active compared to P45 ($p < .001$) and P60 ($p < .001$). In addition they were more active at P30 compared to P45 ($p < .001$).

Collapsing across time, there was a main effect of Sex $F(1, 57) = 8.63$, $p = .005$. Females were significantly more active than males ($p = .005$). A main effect of Treatment $F(1, 57) = 11.795$, $p = .001$ showed that vehicle treated subjects were more active compared to fluoxetine treated animals.

Vertical activity

For vertical activity**,** a repeated-measures ANOVA (*Treatment x Lesion x Sex x Test Age*) on number of beam breaks showed a *Age x Treatment* interaction $F(2, 114) = 6.68$, $p = .002$ (Fig. 2.5). On P45 the fluoxetine treated subjects were more active ($p = .023$) compared to the vehicles. Fluoxetine animals as a group were less active at P30 compared to P45 ($p < .001$) and P60 ($p < .001$).

Collapsed across time, there was a *Lesion x Treatment* interaction $F(1, 57) = 6.22$, $p = .016$ (Fig. 2.6). In the vehicle-treated group, HI subjects were significantly more active compared to shams ($p = .019$) and within the sham group, the fluoxetine-treated subjects were significantly more active compared to the vehicle treated group ($p = .007$).

Total distance

For total distance traveled, a repeated-measures ANOVA (*Treatment x Lesion x Sex*) of the number of beam breaks showed an *Age x Sex* interaction $F(2, 114) = 6.514$, $p = .002$ and an Age x Treatment interaction $F(2, 114) = 7.094$, $p = .001$ (Fig. 2.7). Post-hoc analysis showed that at P60 the fluoxetine subjects were less active than the vehicle group ($p = .001$). Within the fluoxetine group the animals traveled more distance at P60 compared to P30 ($p = .017$) and P45 ($p < .001$). In the vehicle group the same results were found as the animals traveled more distance at P60 compared to P30 ($p = .017$) and P45 ($p = .000$). The level of activity between the sexes was comparable in adolescence ($p > .05$) but the females were more active than males at P45 ($p = .002$) and P60 ($p < .001$). Within the male group, there was more distance traveled at P60 compared

to P30 (p = .030) and P60 (p = .001). The same result was found in the females, who also traveled more distance at P60 compared to P30 (p < .001) and P45 (p < .001).

Collapsed across time, there was a main effect of *Sex*, with the females being significantly more active than males $F(1, 57) = 19.00$, $p < .001$. There were no other main effects or interactions.

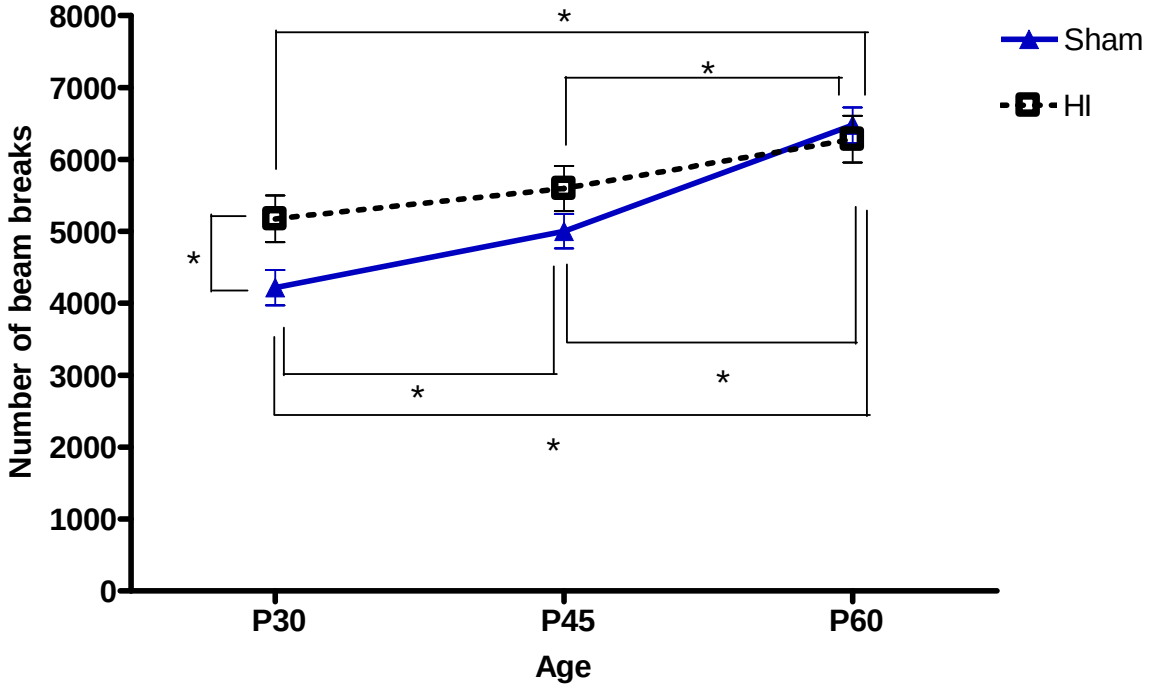

Figure 2.4. Activity box - Number of beam break for horizontal activity at P30, P45 and P60. At P30 the HI group was more active compared to the sham group. HI animals are more active at P60 compared to P30 and P45. The sham group is also more active at P60 compared to P30 and P45. In addition, sham animals had more beam breaks at P45 compared to P30. * indicates difference is significant at $p \leq .05$.

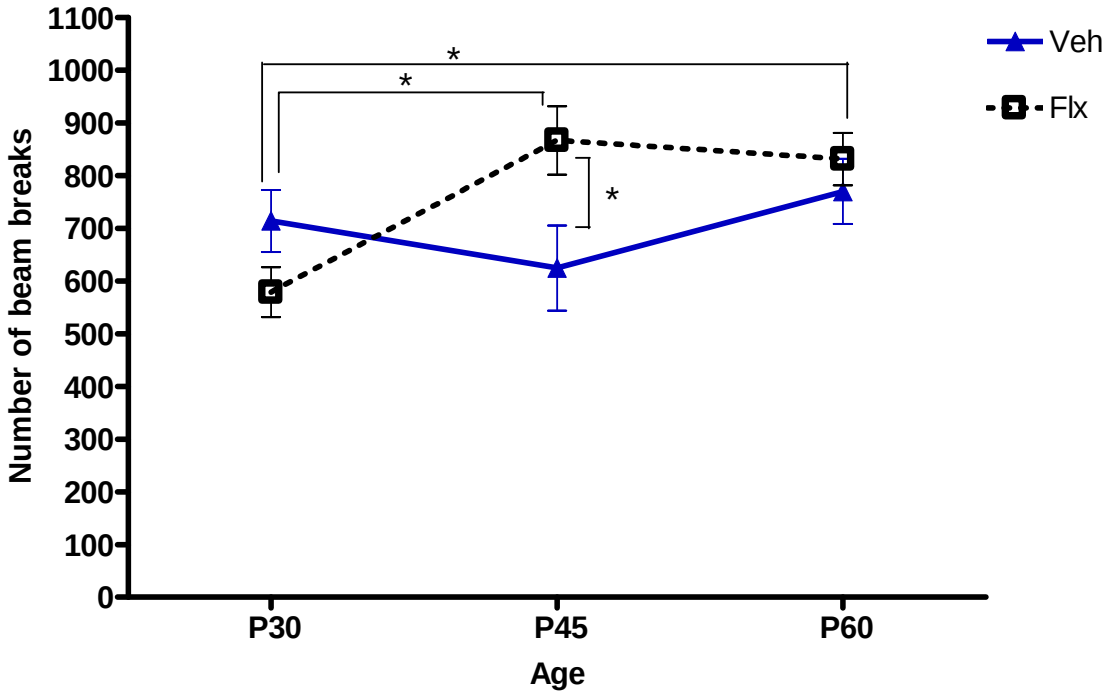

Figure. 2.5. Activity box - Number of beam breaks for vertical activity at P30, P45 and P60. At P45 the fluoxetine subjects were more active than the vehicles. The fluoxetine group was less active at P30 compared to P45 and P60. * indicates difference is significant at $p \leq .05$.

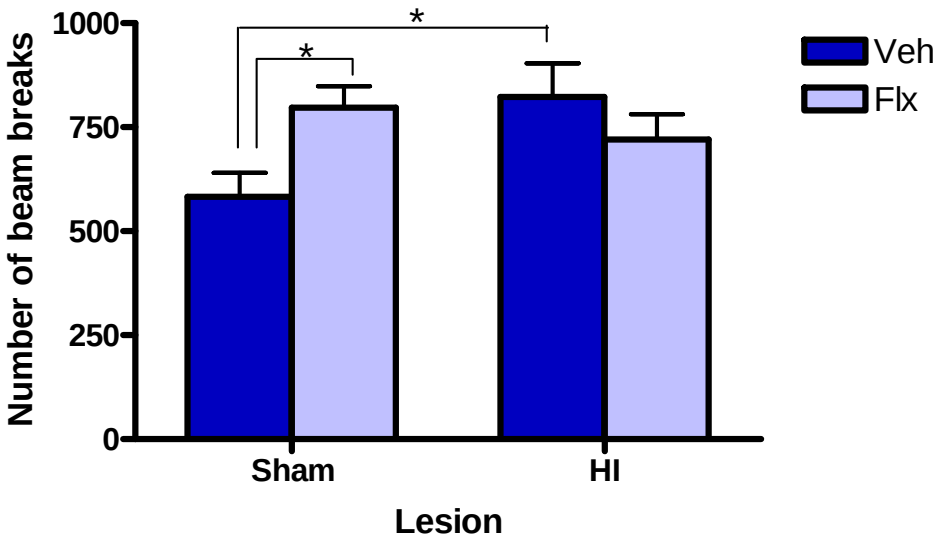

Fig. 2.6. Activity box - Number of beam breaks for vertical activity collapsed across *Time*. In the vehicle group, HI subjects were more active than the sham animals. Within the sham group, fluoxetine animals were more active than the vehicle group. * indicates difference is significant at $p \leq .05$.

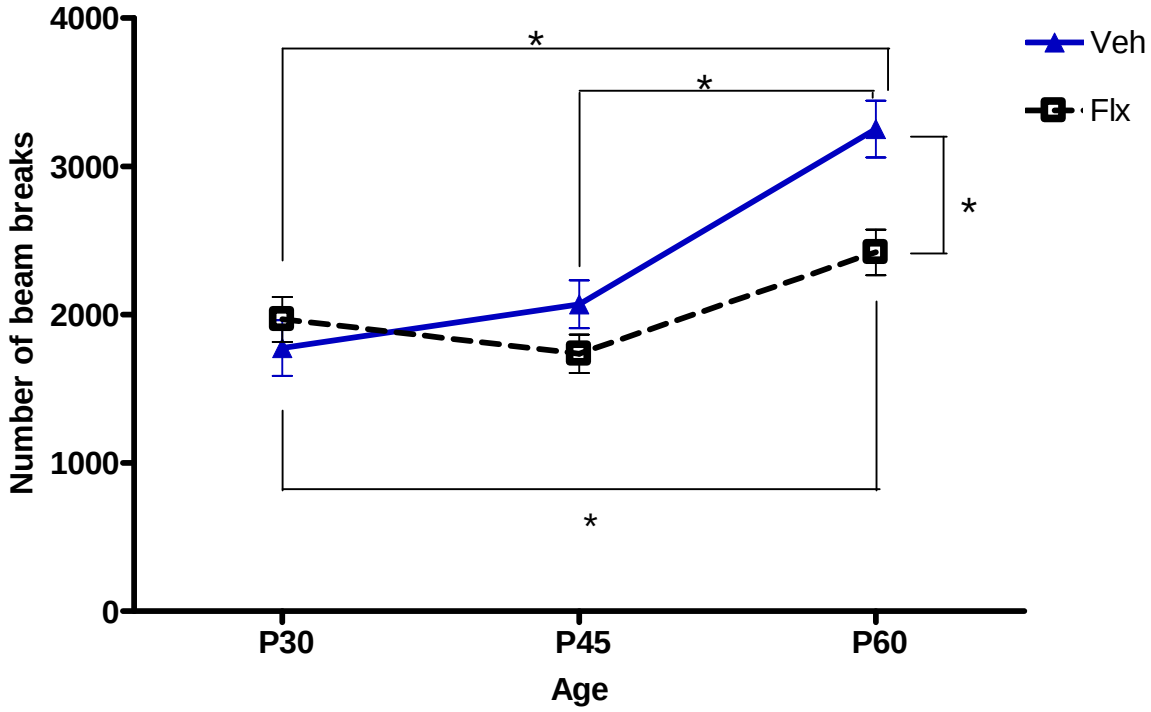

Figure 2.7. Activity Box - Number of beam breaks for total distance traveled. At P60 fluoxetine-treated animals were less active compared to vehicle-treated animals. In the vehicle group, animals were more active at P60 compared to P45 and P30. In the Fluoxetine group, animals were more active at P60 compared to P30. * indicates difference is significant at $p \leq .05$.

Footprints

Hypoxic-Ischemia resulted in larger angle of rotation in both the ipsi-and contra-lesion hindlimb. Fluoxetine subjects had, compared to vehicle treated subjects, a smaller angle of displacement and stride length in both the ipsi- and contra-lesion side.

A two-way ANOVA (*Treatment x Lesion*) on stride length revealed a main effect of Treatment in the ipsi-lesional $F(1, 21) = 11.28$, $p = .003$ (Fig. 2.8) and a marginal, but not significant, effect in the contra-lesional stride length $F(1, 21) = 4.02$, $p = .058$. In both cases, the vehicle subjects had a significantly longer stride length compared to fluoxetine treated subjects.

A two-way ANOVA (*Treatment x Lesion*) on angle showed a main effect of Treatment $F(1, 21) = 17.99$, $p < .001$ in the ipsi-lesion hindpaw, with a larger angle of rotation in the vehicle subjects compared to the fluoxetine animals. A main effect of Lesion $F(1, 21) = 5.50$, $p = .029$ in the ipsi-lesional hindpaw showed with HI subjects had a larger angle of rotation compared to the sham subjects.

There were no main effects ($p > .05$) but a significant *Treatment x Lesion* interaction $F(1, 21) = 9.34$, $p = .006$ (Fig. 2.9) was found in the contra-lesional angle of rotation. Post-hoc analyses showed that within the sham-operated subjects, vehicle-treated subjects had a higher angle of rotation compared to fluoxetine-treated subjects ($p = .002$). Within the fluoxetine-treated group, the HI subjects had a higher rotational angle ($p = .018$).

There were no main effects nor significant interactions found in the distance of stride width ($p > .05$).

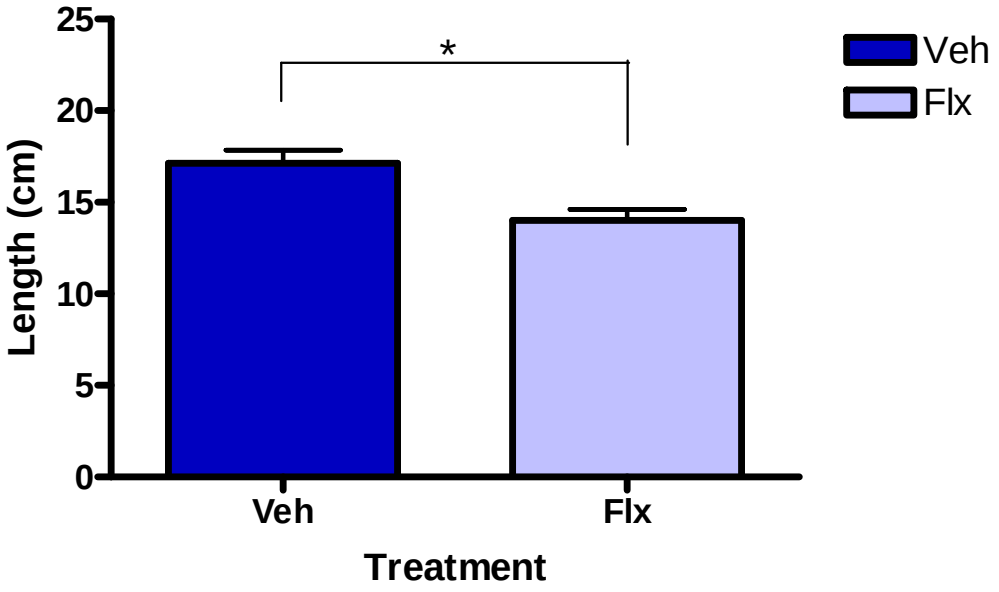

Figure 2.8. Footprints – Ipsi-lesion stride length. The fluoxetine group had a smaller stride length than the vehicle group. * indicates difference is significant at $p \leq .05$.

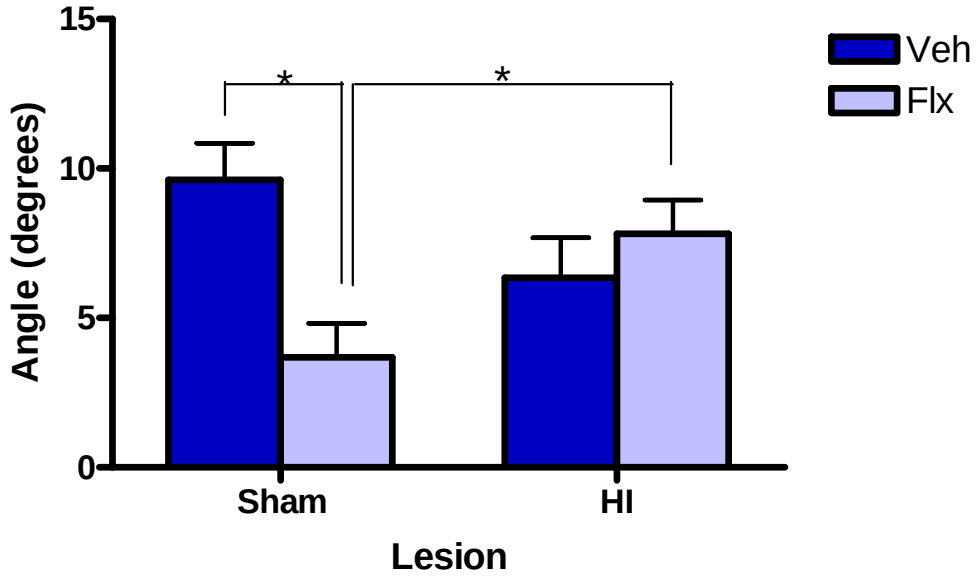

Figure 2.9. Footprints – Contra lesion angle of rotation. In the Sham group, the vehicle subjects had a higher angle of rotation. Within the fluoxetine-treated group, the HI subjects had a higher angle of rotation. * indicates difference is significant at $p \leq .05$

Spatial NaivagionWater Task

Fluoxetine treated animals swam significantly faster than the vehicle treated animals. As such, path length was analyzed instead of latency because this measure is independent of swim speed. Vehicle treated sham females had abnormally long path lengths and were therefore excluded from analyses and only males were used.

All animals were able to learn and locate the hidden platform. However, the perinatal fluoxetine treatment had a significantly longer swim path length to reach the platform from the starting location compared to the vehicle treated animals.

A Repeated Measures ANOVA (*Day x Treatment x Lesion*) on average swim speed on Day 1, 2, 3, 4 and 5 did not show a main effects or interactions (*F*'s < 1.38, *p*'s > .261). However, collapsing across Day revealed a main effect of *Treatment* $F(1, 24) = 10.800$, $p = .003$ with fluoxetine-treated subjects swimming significantly faster than the vehicle treated subjects.

A Repeated Measures ANOVA (*Day x Treatment x Lesion*) on average path length on Day 1, 2, 3 and 4 did not show a main effect of Day (*F*'s < .91, *p*'s > .404). Collapsing across Days revealed a main effect of *Treatment* $F(1, 24) = 5.77$, $p = .024$ with fluoxetine-treated subjects having a longer average path length (Fig.2.10). There were no other main effects nor significant interactions found on average path length for Day one to five.

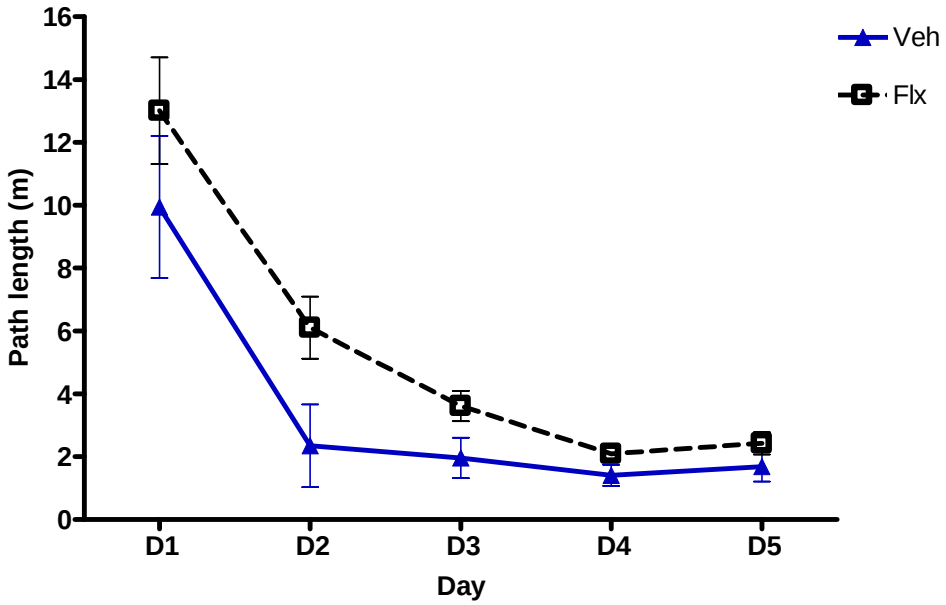

Figure 2.10. Water task – Average path length for day one to five. Fluoxetine animals have a longer path length compared to the vehicle treated subjects.

Elevated Plus Maze

Fluoxetine treated subjects were more anxious compared to the vehicle treated subjects.

A three-way ANOVA (*Treatment x Lesion x Sex*) found that fluoxetine treated subjects spent more time in the closed arm $F(1, 56) = 10.73$, $p = .002$ (Fig. 2.11) and less time in the centre $F(1, 56) = 6.27$, $p = .015$. There was a main effect of *Sex* in the centre time $F(1, 56) = 6.91$, $p = .011$ with males spending significantly more time than the females. There were no other main effects or interactions ($p > .05$).

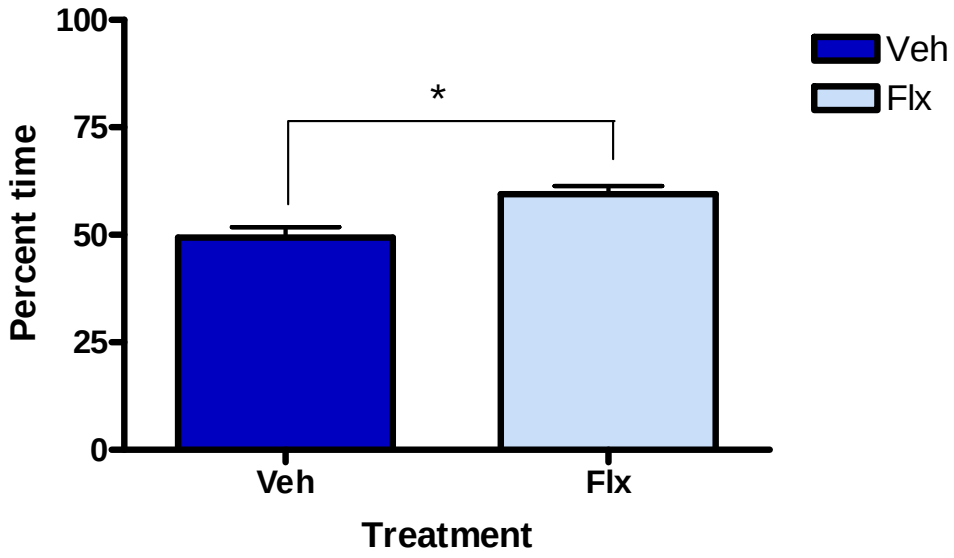

Figure 2.11. Elevated Plus Maze - Percent time in closed arm. Fluoxetine treated animals spent significantly more time in the closed arm than the vehicle treated animals. * indicates difference is significant at $p \leq .05$.

Tray Reaching

HI subjects did not have a significant preference for using their ipsi-lesion forelimb but rather all groups were equally likely to use the ipsi- or contralateral forelimbs. Vehicle-treated subjects with HI injury had significantly fewer total reaching attempts than sham operated subjects, regardless of treatment. The HI fluoxetine-treated animals on the other hand, had a significantly higher rate of attempts compared to the sham fluoxetine-treated animals.

Females did retrieve more pellets than the males with the contra-lesion forelimbs. Curiously, with the ipsi-lesion forelimb in the fluoxetine group, HI subjects had higher reaching success than the sham operated animals. A three-way ANOVA (*Treatment x Lesion x Sex*) on handedness did not show any main effect, including lesion $F(1, 55) = .93$, $p = .338$ or interactions.

A three-way ANOVA (*Treatment x Lesion x Sex*) on total attempts revealed a main effect of *Treatment* $F(1, 55) = 9.04$, $p = .004$, with fluoxetine animals having more attempts than the vehicle group. In addition, there was an interaction for *Treatment x Lesion* $F(1, 55) = 18.24$, $p = .000$ (Fig. 2.12), *Treatment x Sex* $F(1, 55) = 5.07$, $p = .028$ and *Lesion x Sex* $F(1, 55) = 6.80$, $p = .012$. Post hoc analysis showed that in the HI group, fluoxetine animals had more attempts than the vehicle animals ($p < .001$). Within the vehicle group the sham subjects made more attempts ($p = .001$), whereas within the fluoxetine group the HI animals had more attempts ($p = .011$). Fluoxetine-treated males made more attempts than the vehicle males ($p = .001$) and within the vehicle group the females attempted more than the males ($p = .038$). Sham-operated

females made more often attempts than HI females (p = .005) and within the sham group the females made more attempts than the males (p = .003).

A three-way ANOVA (*Treatment x Lesion x Sex*) on the number of successful reaches did not reveal a main effect but there was a *Treatment x Lesion* interaction with the ipsi-lesion forelimb $F(1, 55) = 4.45$, p = .039 (Fig.2.13). Post-hoc analysis revealed that within the fluoxetine animals, HI subject had more successful reaches compared to the Sham-operated subjects (p = .034). A main effect of *Sex* $F(1, 55) = 5.95$, p = .018 was found in the contra-lesion forelimb, indicating that females made more successful reaches than males with this forelimb. There were no other main effects or significant interactions found (p > .05).

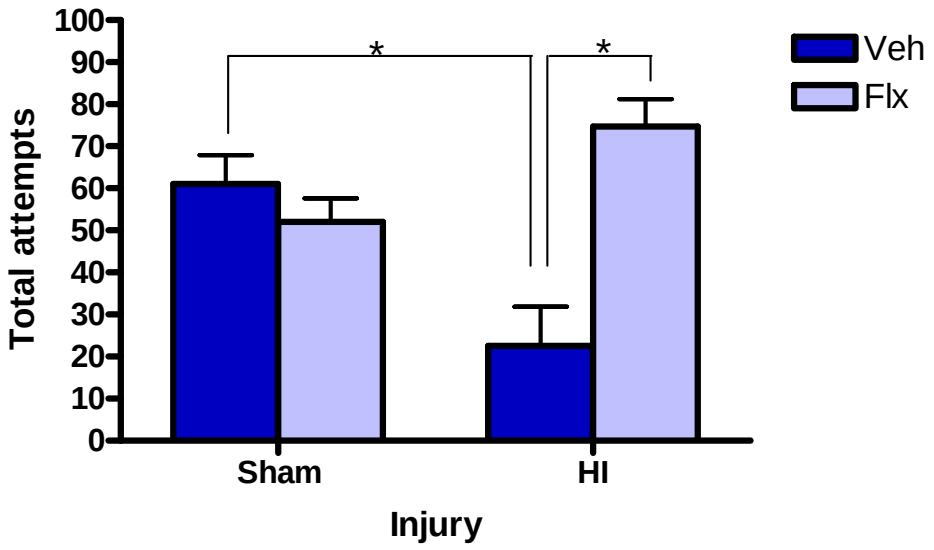

Figure 2.12. Tray Reaching – Total Attempts. In the vehicle group, sham animals reached more than HI subjects whereas in the Fluoxetine group, the sham subjects reached less than the HI subjects * indicates difference is significant at $p \leq .05$.

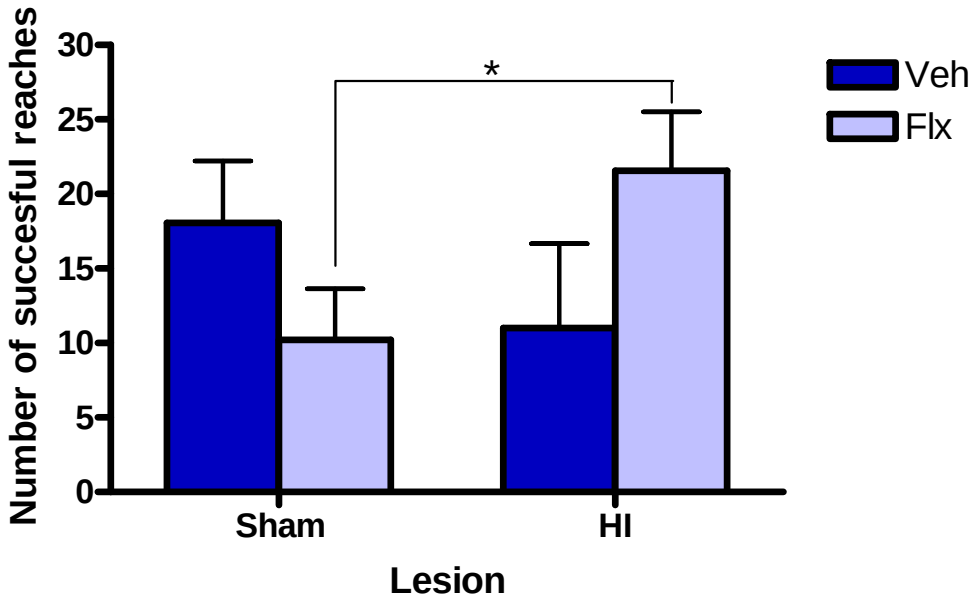

Figure 2.13. Tray Reaching – Number of successful reaches with ipsi-lesion forelimb. Within the fluoxetine animals, the HI subjects had more successful reaches compared to sham-operated subjects * indicates difference is significant at $p \leq .05$.

Forepaw Inhibition

Sham-treated animals mostly inhibited their contra-lesion forepaw during swimming, whereas the HI subjects did this significantly less with their contra-lesion paw. On the ipsi-lesion side, the fluoxetine subjects made more forepaw strokes than the sham subjects. In the contra-lesion side males made more fore-paw strokes than females.

A three-way ANOVA (*Treatment x Lesion x Sex*) on ipsilesional forepaw strokes revealed a main effect of *Treatment* $F(1, 57) = 4.20$, $p = .045$]indicating that fluoxetine subjects made significantly more strokes than the vehicle treated animals.

In the contra-lesion side there was a main effect of *Sex* $F(1, 57) = 12.67$, $p = .001$, males made more forepaw strokes compared to females. A main effect of *Lesion* $F(1, 57) = 4.68$, $p = .035$ indicated that HI subjects made more forepaw strokes. In addition there was a *Sex x Lesion* interaction $F(1, 57) = 5.77$, $p = .020$ (Fig. 2.14). Post-hoc analyses showed that in the HI-group, males made significantly more strokes than females. Within the males ($p = .001$), HI subjects made significantly more strokes than the sham operated subjects ($p = .005$). There were no main effects nor a significant interaction found ($p > .05$).

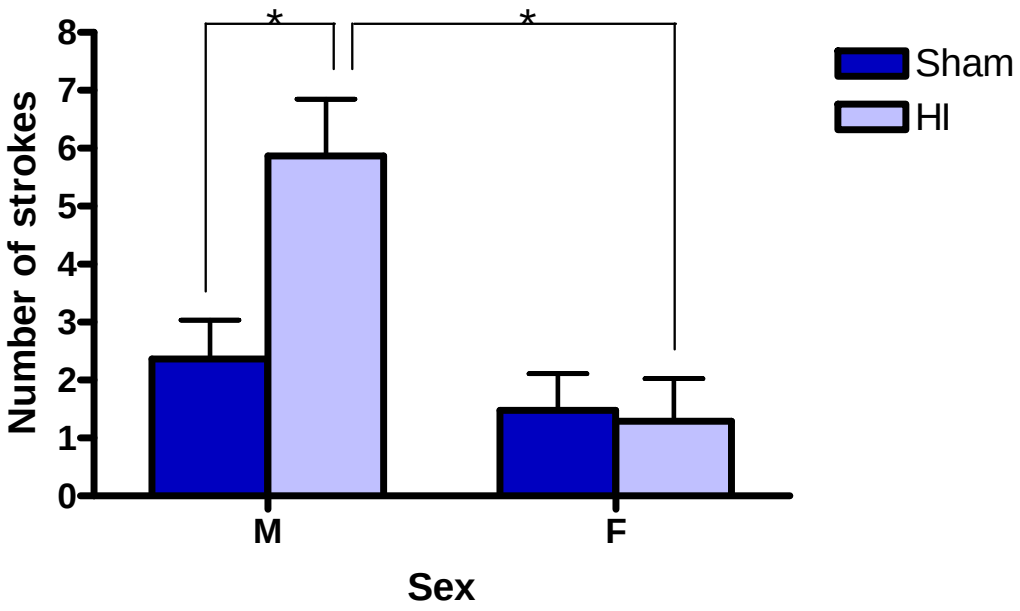

Figure 2.14. Forepaw Inhibition – Contra-lesion forelimb strokes. In the HI-group the males made significantly more strokes than the females. Within the males, HI subjects made more strokes than the sham-operated subjects * indicates difference is significant at $p \leq .05$.

Single Pellet Reaching

There was no effect of Injury or Treatment on percent score. In the kinematics qualitative analysis, HI animals had a better release technique compared to the sham operated animals.

A Repeated Measures ANOVA (*Day x Treatement x Lesion*) on percent score did show a main effect of *Day* $F(5, 80) = 3.617$, $p = .005$, indicating that all the animals improved over time. No other main effect or interactions were found on any of the other measures ($p > .05$).

A two-way ANOVA (*Treatment x Lesion*) on the qualitative reaching components, only revealed a main effect of *Lesion* $F(1, 16) = 6.614$, $p = .025$ in Release. HI animals performed better than the Sham-operated group.

Anatomical Results

Body and Brain Weight

Body weight at P60 showed the normal pattern of sexual dimorphism, with the males being heavier than females. In addition, fluoxetine had a negative effect on female body weight as they were lighter than the vehicle treated females. In the sham group the vehicle treated animals were heavier, whereas in the HI group the fluoxetine animals were heavier. Body weight at perfusion as well as brain weight only showed an effect of sexual dimorphism, with the females being lighter in both measures.

A three-way ANOVA (*Treatment x Lesion x Sex*) on adult body weight did show a main effect of *Sex* $F(1, 58) = 286.66$, $p < .001$, indicating that females were lighter compared to males. In addition a *Treatment x Lesion* interaction

$F(1, 58) = 40.05$, $p < .001$ (Fig. 2.15) was found, this effect was not driven by sex as analysis of each sex individually gave the same results. In addition a *Treatment x Sex* interaction $F(1, 58) = 6.02$, $p = .017$ was revealed. Post hoc analysis showed that all comparisons were significant, except in the males, where there was no difference between the fluoxetine and vehicle treated group. In the fluoxetine group, HI animals were heavier ($p < .001$), in the *Vehicle* group, the sham animals were heavier ($p < .001$). In the HI group the fluoxetine-treated animals weighed more ($p = .005$), whereas in the *Sham* group the vehicle subjects were heavier ($p < .001$). In both the fluoxetine and vehicle group the females weighed less (both $p < .001$). Within the females, the fluoxetine-treated group were lighter ($p = .003$).

A three-way ANOVA (*Treatment x Lesion x Sex*) on body weight at perfusion did show a main effect of *Sex* $F(1, 64) = 524.55$, $p = .000$. Males were heavier than females.

A three-way ANOVA (*Treatment x Lesion x Sex*) on brain weight indicated a main effect of *Sex* $F(1, 64) = 12.83$, $p < .001$. The brains of females weighed less compared to males' brains. There were no other main effects or significant interactions found ($p > .05$).

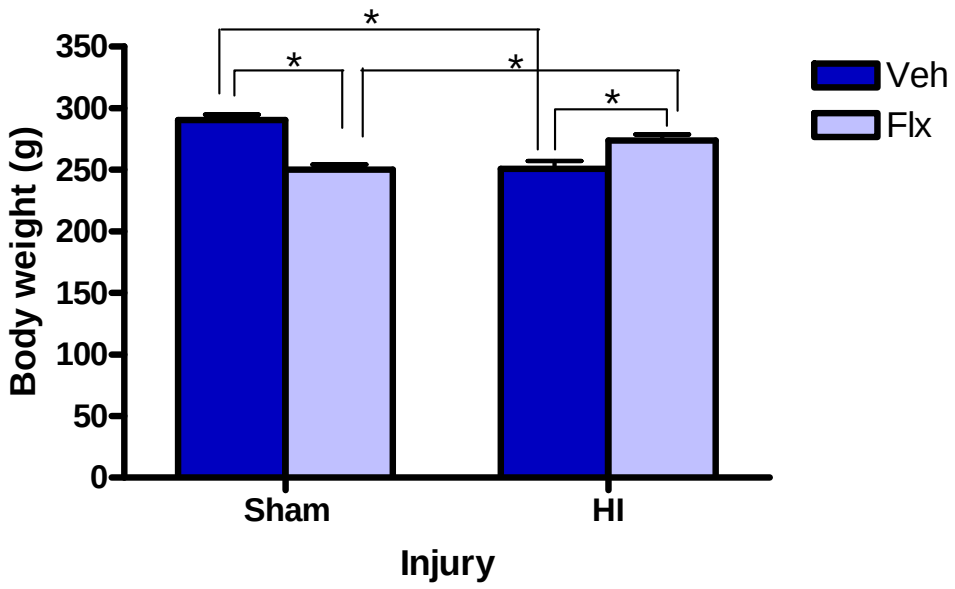

Figure 2.15. Postnatal day 60 body weight. In the fluoxetine group, HI animals were heavier than sham-operated animals. In the HI group, the fluoxetine-treated animals weighed more than vehicle-treated subjects. * indicates difference is significant at $p \leq .05$.

Gold Chloride Stain Histochemistry

General observations.

In some of the HI animals, the coronal sections of the ipsi-lesion hemisphere showed enlargement of the lateral ventricle, smaller caudate putamen and a smaller hippocampus (Fig. 2.16). The thalamus and hypothalamus appeared normal, as did the anterior commissure and cingulum. There was no obvious patchy cell death within the cortex.

Vehicle-Sham Vehicle-HI Fluoxetine-Sham Fluoxetine-HI

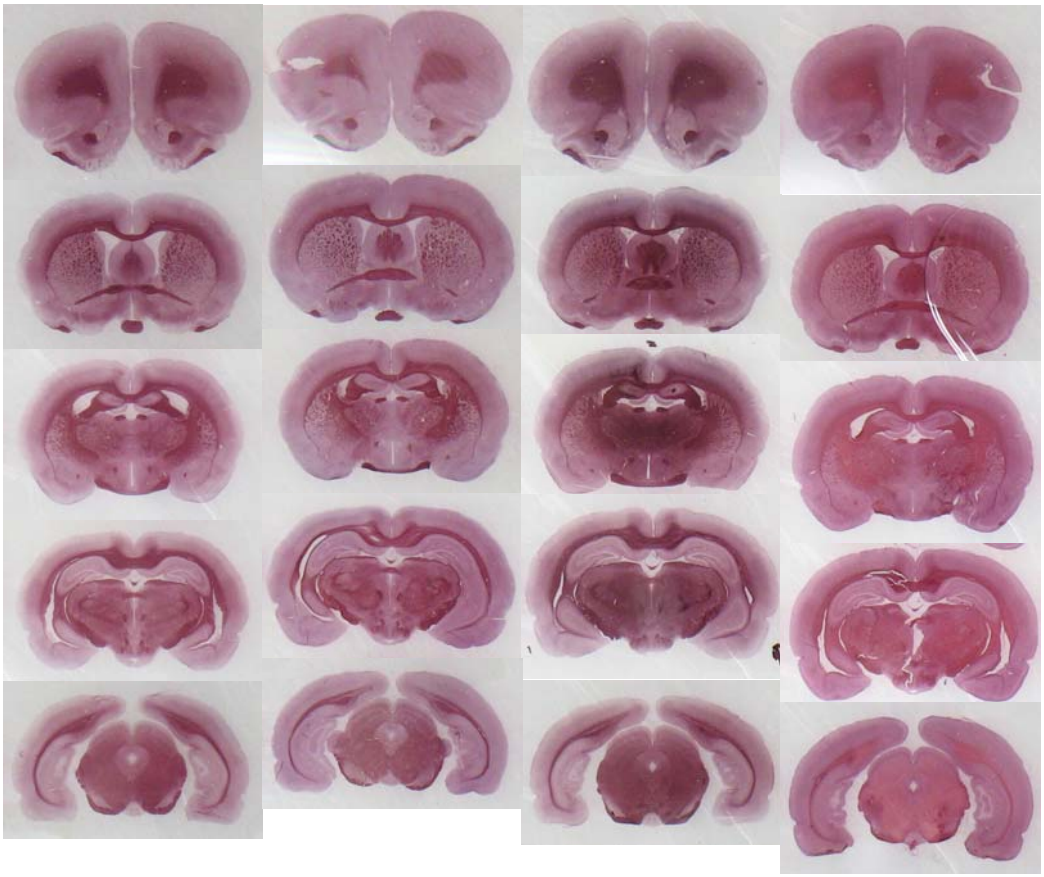

Figure 2.16. Coronal Sections stained with Gold Chloride. Plate 10, 18, 25, 37, 45, from top to bottom for Vehicle Sham (column 1), Vehicle HI (column 2), Fluoxetine Sham (column 3), and Fluoxetine HI (column 4).

Dorsal whole brain picture analysis.

HI affected the relative size of the ipsi- versus contra-lesion hemisphere. When the contra-lesion area was subtracted from the ipsi-lesion area, HI animals had a significantly higher value, indicating that their ipsi-lesion hemisphere was smaller than their contra-lesion hemisphere.

A two-way ANOVA (*Lesion x Treatment*) on total area on ipsi-lesion and contra-lesion dorsal surface area of the whole brain did not reveal main effects or interactions (F's ≤ 2.72 and p's ≥ .116). When the ipsi-lesion hemisphere size was divided over the total area of both the ipsi-lesion and contra-lesion hemisphere of plate 19 (other studies have found that there was no significant difference between the contralateral hemisphere of hypoxic animals and that of normal controls (Towfighi, Housman, Vannucci, & Heitjan, 1994)) a main effect of *Lesion* $F(1, 13) = 5.45$, $p = .036$ (Fig. 2.17) was found. This finding indicates that in the HI animals the percentage of ipsi-lesion hemisphere was smaller compared to the sham animals. There were no other main effects or significant interactions found ($p > .05$).

Coronal cortical area.

HI injury resulted in a significantly smaller cortical area of the ipsi-lesion hemisphere of both the anterior plane (plate 19) and posterior plane (plate 27).

A two-way ANOVA *(Lesion x Treatment*) on coronal cortical surface area of the ipsi-lesion and contra-lesion hemisphere in the anterior plane (plate 19) and posterior plane (plate 27) did reveal a main effect of *Lesion* in the ipsi-lesion anterior hemisphere $F(1, 13) = 5.02$, $p = .043$, with sham animals having a larger

ipsi-lesion surface area. No other main effects or interactions were found. There were no other main effects or significant interactions found (p >.05).

Corpus callosum, cingulum, and external capsule.

Neither *lesion* nor *treatment* affected the combined surface area of the corpus callosum, cingulum and external capsule. A two-way ANOVA (*Lesion x Treatment*) on the combined surface area of the corpus callosum, cingulum and external capsule showed no main effect or interactions (p > .05). There were no other main effects or significant interactions found (p > .05).

Fimbria of the hippocampus area.

Neither *lesion* nor *treatment* affected the surface area of the fimbria of the hippocampus. A two-way ANOVA (*Lesion x Treatment*) on the surface area of the fimbria of the hippocampus showed no main effect or interactions (p > .05). There were no other main effects or significant interactions found (p > .05).

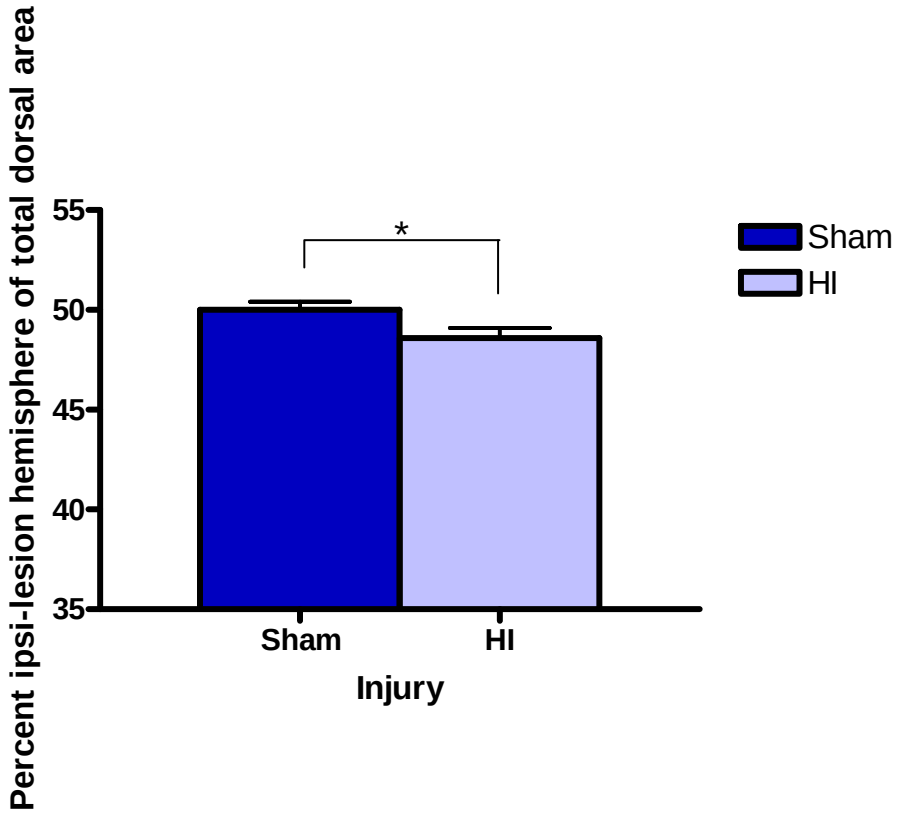

Figure 2.17. Dorsal whole brain picture analysis. In HI animals, the lesion hemisphere provides a smaller relative contribution to the total dorsal area compared to sham controls. * indicates difference is significant at $p \leq .05$.

Cortical Thickness

The HI lesion resulted in a decrease in cortical thickness in the central part of the contra-lesion hemisphere in plate 5. Fluoxetine treatment resulted in increased cortical thickness in the lateral part of the contra-lesion hemisphere in plate 1.

A Repeated Measures ANOVA (*Treatment x Lesion*) on the ipsi-lesion and contra-lesion hemisphere at the medial, central, lateral and rhinal fissure region for plate 1, 2, 3, 4, and 5 revealed a *Plate x Lesion* interaction for the contra-lesion hemisphere in the central area $F(4, 52) = 2.94$, $p = .029$, on plate 5 the HI animals have a smaller cortical thickness ($p > .038$).

Collapsed across Plate, a main effect of *Lesion* was found in the ipsi-lesion hemisphere at the level of the rhinal fissure $F(1, 13) = 5.51$, $p = .035$, indicating that cortical thickness across plates was larger in the sham animals. No other main effects or interactions were found.

A two-way ANOVA (*Treatment x Lesion*) on the sum of the entire plate on the ipsi-lesion and contra-lesion hemispheres showed a main effect of *Treatment* at plate one at the contra-lesion hemisphere $F(1, 13) = 7.59$, $p = .016$, showing that the fluoxetine treatment resulted in a larger cortical thickness. A main effect of *Lesion* at plate five of the contra-lesion hemisphere $F(1, 13) = 5.75$, $p = .032$ (Figure. 2.18) indicated that HI resulted in a decrease of cortical thickness at this plate. No other main effects or interactions were found.

Further analysis for plate one and plate five revealed significance, in order to reveal in which specific area there was a significant difference in cortical thickness. A two-way ANOVA (*Lesion x Treatment*) found a main effect

of treatment in the medial area of the ipsi-lesion hemisphere in plate1 $F(1, 17) = 5.62$, $p = .034$ and a main effect of Lesion on the central level of the ipsi-lesion hemisphere in plate 5 $F(1, 17) = 5.31$, $p = .038$ was found. No other main effects or interactions were found in any other areas on these plates in the ipsi-lesion hemisphere.

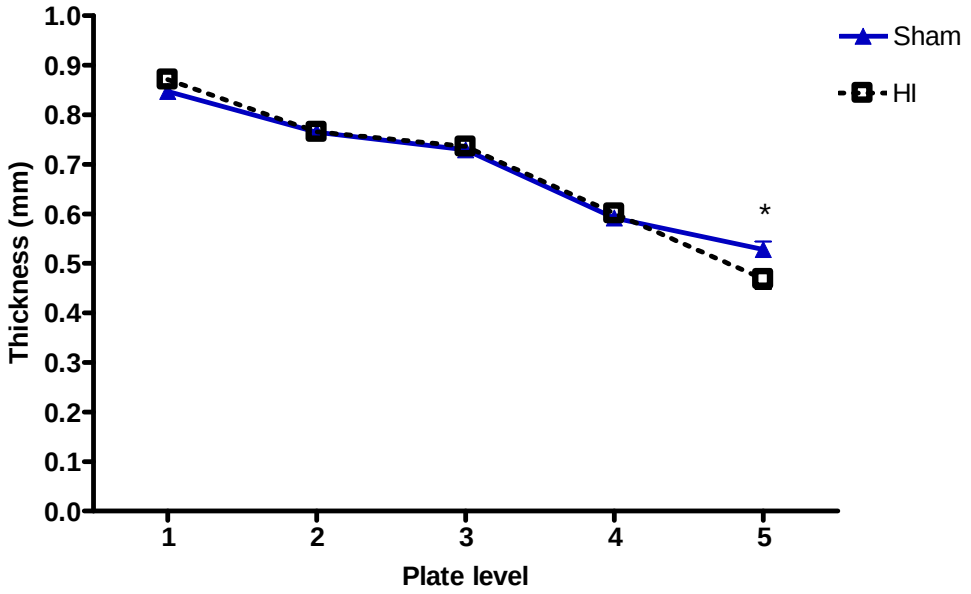

Figure 2.18. Cortical thickness – Central cortical thickness of contra-lesion hemisphere across five plates. On plate five the HI animals have a significantly thinner cortex than the sham subjects. * indicates difference is significant at $p \leq .05$.

Golgi-Cox Analysis

Parietal cortex- layer III.

HI injury resulted in shorter, but more complex pyramidal cells in both the ipsi- and contra-lesion hemisphere compared to sham operated animals. Fluoxetine treatment resulted in the contra-lesion hemisphere in smaller basilar fields.

Branch order.

Apical dendrites.

In order to assess the amount of dendrites that branch off the soma, the numbers of branches at Level One were determined. A two-way ANOVA (*Treatment x Lesion*) on branch order one showed no main effect or interaction ($p > .05$). The apical field did not exhibit any significant difference on any other measures ($p > .05$).

Basilar dendrites.

A Repeated-Measures ANOVA (*Branch x Treatment x Lesion*) on the basilar branch order in the ipsi-lesion hemisphere showed a *Branch x Lesion* interaction (Mauchly's test indicated that the assumption of sphericity had been violated, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity) $F(2.739, 52.043) = 3.14$, $p = .037$ (Fig 2.19). HI animals had more arborization at level 4 ($p = .012$),5 ($p = .001$) and 6 ($p = .016$). Collapsed across *Branch* there was a main effect of *Lesion* $F(1, 19) = 7.93$, $p = .011$, again indicating that HI animals had more arborization.

A two-way ANOVA (*Treatment x Lesion*) on the basilar total branch order of the ipsi-lesion hemisphere showed a main effect of *Lesion* $F(1,19) = 8.31$, $p = .010$, HI subject had higher dendritic complexity. The basilar field did not exhibit any significant difference on any other measures ($p > .05$).

Dendritic Length.

Dendritic length is inferred from the number of dendrites crossing each of the 16 concentric rings placed over the drawings. The rings thus represent increasing distances from the soma.

Apical dendrites.

A Repeated-Measures ANOVA (*Ring x Treatment x Lesion*) on the apical field in the contra-lesion hemisphere revealed a *Ring x Lesion* interaction (Mauchly's test indicated that the assumption of sphericity had been violated, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity) $F(1.652, 31.383) = 3.72$, $p = .043$, revealing that sham animals had a larger branch order at ring crossing 16 ($p = .038$).

A Repeated Measures ANOVA (*Ring xTreatment x Lesion*) on the apical field in the ipsi-lesion hemisphere showed a *Ring x Lesion* interaction (Mauchly's test indicated that the assumption of sphericity had been violated, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity) $F(1.697, 32.243) = 4.71$, $p = .021$, indicating that sham animals had longer dendrites.

A two-way ANOVA (*Treatment x Lesion*) on the total of the distal apical dendrites revealed a main effect of *Lesion* on the contra-lesion hemisphere [$F(1, 23) = 5.06$, $p = .037$] and ipsi-lesion hemisphere $F(1, 19) = 4.64$, $p = .044$. In both cases the sham animals had larger dendrites than the HI group. There were no other significant differences on any other measures ($p > .05$).

Basilar dendrites.

A Repeated Measures ANOVA (*Ring xTreatment x Lesion*) on basilar length in the ipsi-lesion hemisphere showed a *Ring* x *Lesion* interaction (Mauchly's test indicated that the assumption of sphericity had been violated, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity) $F(2.456, 46.669) = 3.42$, $p = .032$, reflecting that HI animals had more dendritic material at Rings 3 and 4.

A two-way ANOVA (*Treatment x Lesion*) on the proximate portion of the basilar tree in the ipsi-lesion hemisphere revealed a main effect of *Lesion* $F(1,19) = 4.84$, $p = .040$, showing that HI animals had a larger proximal basilar tree.

A two-way ANOVA (*Treatment x Lesion*) on the distal portion of the basilar tree in the contra-lesion hemisphere found a main effect of *Treatment* $F(1,19) = 5.17$, $p = .035$ (Fig.20) , with a larger distal basilar tree in the vehicle animals.

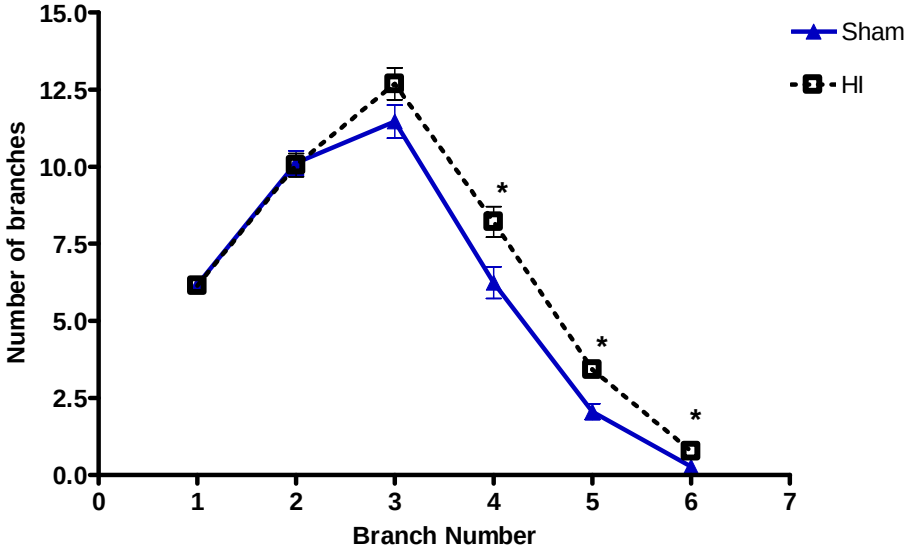

Figure 2.19. Golgi-Cox – Basilar Branch order of ipsi-lesion hemisphere. HI animals have more branches at the level 4, 5 and 6. * indicates difference is significant at $p \leq .05$.

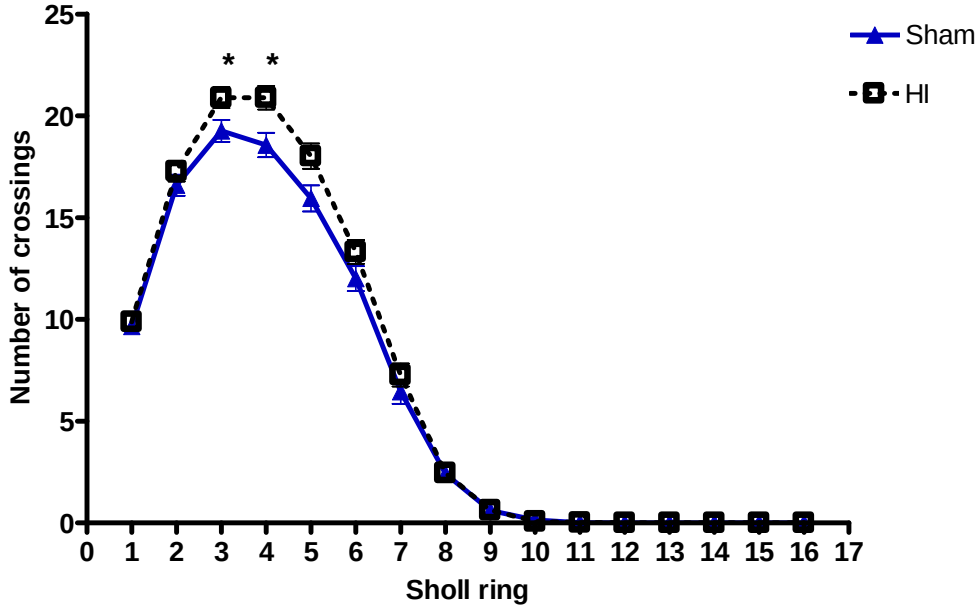

Figure 2.20. Golgi-Cox – Basilar Dendritic Length. HI subjects have higher branch orders at level 3 and 4, compared to sham subjects. * indicates difference is significant at $p \leq .05$.

Forelimb area - layer V.

HI lesion and/or fluoxetine treated did not alter the morphology of pyramidal cells in forelimb area layer V, in either the ipsi-lesion or contra-lesion hemisphere. A two way ANOVA (*Treatment x Lesion*) did not show any main effect or interactions on any of the variables (F's ≤ 3.79 and p's ≥ .067).

Discussion

In this experiment the effect of peri-natal fluoxetine exposure on a P7 HI injury was assessed. There were two main findings. First, fluoxetine treatment or HI injury alone had mostly negative affects on behavioral and anatomical measures. Second, although the combined treatment of fluoxetine and HI insult did interact on specific measures, the combination did not lead to exacerbated effects (see table 2.2 for summary of results). These findings will be discussed in turn.

Table 2.2
Summary of the Results of Experiment One

	Fluoxetine	HI	Fluoxetine + HI	Sex
Activity Box	↓	↑	–	♀↑
EPM	↓	–	–	–
Footprints	↓	Δ	–	–
Forepaw Inhibition	–	↓	–	–
WM	↓	–	–	–
Tray Reaching - nr attempts	–	↓	↑	–
Tray Reaching - success	–	–	↑(ipsi-lesion)	♀↑ (contra-lesion)
Single Pellet	–	–	–	–
Adult Body Weight	↓	–	–	♀↓
Brain Weight	–	–	–	♀↓
Cortical Surface Area	–	↓ (ipsi-lesion)	–	–
Cortical Thickness	↑(contra-lesion, Plate 1)	↓ (contra-lesion, Plate 5)	–	–
Golgi-Cox Par III	↓ (contra-lesion, basilar)	↑(complex) ↓ (shorter) (both ipsi-and contra-lesion)	–	–
Golgi-Cox FL V	–	–	–	–

↑ = Increase compared to controls
↓ = Decrease compared to controls
– = Similar to controls
♀ = Females only
♂ = Males only
Δ = Different from controls, both increase and decreases seen

Fluoxetine

Perinatal fluoxetine treatment resulted in a reduction in body weight, decrease in general activity, and increased anxiety. Furthermore, fluoxetine treatment affected cognitive skills as these animals were less accurate in the water maze, as seen by their increased swim distance. Motor behavior was also affected as these animals had smaller stride lengths and angles of displacement of the hindlimbs during walking. In the anatomical analysis, fluoxetine treatment resulted in a smaller basilar tree in the contra-lesion side in pyramidal cells in parietal cortex and an increase in cortical thickness in the contra-lesion side in plate one.

The reduction in body weight was not a result of malnutrition nor other abnormal physical characteristics. However, the reduction in uterine blood flow, due to increased plasma serotonin, has been proposed as a mechanism responsible for reduced growth (Morrison, Chien, Riggs, Gruber, & Rurak, 2002). The absence of effect on brain weight was also reported by Vorhees et al. (1994).

The results of our study agree with previous findings of Bairy, Madhyastha, Ashok, Bairy and Malini (2007), who reported a decrease in general activity as a result of exposure to 7 mg/kg fluoxetine prenatally. There are several reports that link the altered serotonin levels to the altered motor abilities seen in the fluoxetine-treated group. The ascending serotonergic pathways have an activating effect on the neocortex and hippocampal formation, which is coupled to motor activities (Vanderwolf, 1988). Futhermore, Wilkinson, Auerbach and Jacobs (1991) have shown a correlation between serotonergic function and motor activity. They showed that extracellular 5-HT levels are

elevated during active behavioral states. Bairy, Madhyastha, Ashok, Bairy, and Malini (2006) have suggested that the increased serotonin levels in the perinatal period have an inhibitory effect on motor neuronal development.

File, Hyde, and MacLeod (1979) showed that the increase in anxiety levels in the fluoxetine group was not surprising as there was a link between the serotonin system and anxiety. For example, a lesion in the dorsal and medial raphe nuclei produced anxiolytic effects. On the other hand, increasing 5-HT levels by fluoxetine treatment resulted in anxiogenic effects (To, Anheuer, & Bagdy, 1999). The increased anxiety and decrease in locomotor activity after fluoxetine treatment have been proposed to be mediated by the activation of 5-HT2C and 5-HT1A receptors, respectively, as selective receptor antagonists were able to reverse the SSRI-induced effects (Bagdy, Graf, Anheuer, Modos, & Kantor, 2001).

Xu, Sari, and Zhou (2004) have shown that neonatal SSRIs disrupt the organization of the barrel field cortex as a result of lack of refinement of thalamocortical afferents. An increase in 5-HT levels in cortex can affect the cytoarchitectonics of cortical neurons and this study confirmed this by reporting a change in the morphology of pyramidal cells in layer III in the fluoxetine treated animals. Futhermore, it is not surprising that the morphological changes were found in the basilar field, as they receive more connections from across columns and from other brain areas than the apical field (Douglas, Markham, & Martin, 2004).

The other anatomical result, namely the increase in cortical thickness, could be a consequence of reduced programmed cell death in the cortical

neurons. Persico et al. (2003) showed that in serotonin transporter knockout mice, the elevated 5-HT levels reduced the number of apoptosis in the striatum, hypothalamus, cerebral cortex and hippocampus. A link between serotonin and cortical thickness is also reported in the clinical population. Autistic children are known to have elevated levels of serotonin (Anderson, Horne, Chatterjee, & Cohen, 1990), and this is associated with a 16% increase of frontal lobe gray matter volumes.

Serotonin plays a crucial role in various aspects of brain development (Whitaker-Azmitia, 1991). Serotonin$_{1A}$ receptors develop early in development, in humans around 16-22 weeks of gestation (Bar-Peled et al., 1991), and this peaks around prenatal day 15 in rats (Daval et al., 1987). This receptor is involved in the stabilization of the cytoskeleton and therefore plays a role in cell proliferation and cell differentiation. Clinically, this receptor acts as a anxiolytic (Azmitia, 2001). The 5-HT$_2$ receptor becomes more prominent during the neonatal and adult period. It is sensitive to developmental experiences, such as postnatal stress, and therefore called a programmable receptor (Meaney et al., 1994). This receptor destabilizes the internal skeleton, which allows for cell proliferation, synaptogenesis, and apoptosis.

The current experiment has shown that altering the levels of 5-HT in the perinatal period changes the brain and behavior in adulthood. Changes in serotonin levels during development are thought to have two main effects. First, because serotonin autoregulates itself, alterations in serotonin levels will lead to altered serotonin neuron morphology. The serotonin system regulates the

maturation of cells it projects to, which would be affected by altered serotonin levels (Whitaker-Azmitia, 1991).

It is clear that disruption of serotonergic development can leave permanent alterations in brain function and behavior. However, it is important to note that the behavioral and anatomical alterations shown in this experiment are not solely due to alterations in the development of the serotonergic system but, although to a lesser extent, also in systems of other catecholamines. Analysis of basic pharmacology of fluoxetine indicates that it is the least selective SSRI. Fluoxetine also results in an increase of catecholamine levels such as noradrenaline and dopamine (Stanford, 1996). For example, there is a convergence of serotonin and dopamine fibers onto both pyramidal cells and GABAergic interneurons in the medial prefrontal cortex in the rat. When the nucleus raphe dorsalis, a major input centre of 5-HT, is removed during the neonatal period there is a significant increase in dopamine fibers (Benes, Taylor, & Cunningham, 2000). Futhermore, Favaro, Costa and Moreira (2008) showed that maternal exposure to fluoxetine in mice resulted in a decrease in dopaminergic-mediated behaviours such as drug-induced stereotypical behavior induced by apmorphine. Activation of the dopaminergic system is thought to result in the stereotypical-behavior (Bedingfield, Calder, Thai, & Karler, 1997). These results suggest that as a result of prenatal fluoxetine exposure, the pups had an altered dopaminergic-system. The decrease in general activity could thus be a result of decreased dopaminergic activity as a result of the fluoxetine exposure in the perinatal period.

Hypoxia-Ischemia

The HI group mainly showed deficits on motor tasks, as is reported in other animal (Tomimatsu et al., 2002) and clinical papers (Trauner & Mannino, 1986). Footprints of HI subjects had a larger angle of rotation, indicating that the compensated paw placement to gain more stability during walking. This gait instability is also reported in children and adolenscents with cerebral palsy (Johnson, Damiano, & Abel, 1997). There are also forelimb deficits as the vehicle HI animals made less attempts in the tray-reaching task than sham animals.

Surprisingly, there was no *Lesion* effect in success rate in the single pellet reaching task. It could be that the intensive training and testing in the tray reaching task facilitated the development of compensatory strategies that were successfully used in the single pellet reaching task. For example, Gharbawie, Gonzalez, Williams, and Kleim (2005) showed that training in tray reaching prior to testing significantly improves the performance on the single pellet test after a middle cerebral artery occlusion. The qualitative change seen in the movement component of HI subjects during the kinematics analysis could be a sign of altered strategy used to perform the task successfully.

Forepaw inhibition was only lost in the contra-lesion forepaw of the males. In the rat most of the corticospinal tracts are crossed so this means that the ipsi-lesion hemisphere creates the impairment. Other studies with similar injuries like middle cerebral artery stroke, have also shown asymmetry in contra-lesion limb inhibition (Gonzalez, Gibb, & Kolb, 2002).

Both hyper- and hypoactivity have been described following neonatal hypoxia–ischemia. Antier et al. (1998) chronically implanted a radiotelemetry

device and found that animals were hypoactive during a 24 hour recording period. When a similar method was employed as in our study however, a single session of 30 min in automated motor activity boxes, HI animals were also hyperactive at P21 which disappeared at P90 (Balduini, De Angelis, Mazzoni, & Cimino, 2000).

Numerous reports have found that as a result of HI injury the ipsi-lesion hemisphere was smaller relative to the contra-lesion hemisphere (Chou et al., 2001; Ikeda et al., 2001; Jansen & Low, 1996; Rice, Vannucci, & Brierley, 1981). This study was the first to shown a smaller ipsi-lesion hemisphere in brains that underwent HI insult but that did not result in infarction.

The general lack of changes in cortical thickness was not surprising as it was also reported in P8 HI animals by Towfighi, Housman, Vannuci, and Heitjan (1994). The decrease in the contra-lesion hemisphere in plate 5 was surprising, and we are not able to account for this.

The increase in arborisation of the pyramidal neurons in layer III of the HI animals probably reflects the reorganization of the sensorimotor networks. The lack of changes in forelimb layer V were surprising as changes in this area have been reported after middle cerebral artery occlusion in adults (Gonzalez & Kolb, 2003) and unilateral damage to the forelimb representation of the sensorimotor cortex .

HI affects the middle cerebral artery-perfused regions such as parietal cortex, hippocampus, and striatum (Rice, Vannucci, & Brierley, 1981). For example, HI decreased the regional bloodflow of ipsilateral subcortical white matter, neocortex, striatum and thalamus to 15, 17, 34, 41% of controls,

respectively (Vannucci, Lyons, & Vasta, 1988). As such, striatal damage likely contributed to motor deficits seen in this experiment, which is an important predictor of early motor deterioration. Kim et al. (2008) suggest that the posterolateral striatum is vulnerable to ischemic damage as there are no collateral vessels in that area. Because the corticospinal tract is anatomically closely related to the posterolateral striatum, damage in the lateral structure likely results in motor damage (Kim et al., 2008).

Fluoxetine and Hypoxia-Ischemia

There were a few measurements that showed interactions between perinatal fluoxetine exposure and HI insult. The level of vertical activity in the activity box, contra-lesion angle rotation in footprints, total attempts and ipsi-lesion success in tray reaching and P60 body weight showed an interaction between *Treatment* and *Lesion*. In only the tray reaching and body weight measures did perinatal fluoxetine treatment alter the behavioural outcome after HI injury, in the other cases fluoxetine treatment significantly altered the outcome of the sham group. Perinatal fluoxetine positively affected the outcome of the HI animals: higher reaching attempt and higher success rate in the HI subjects and an increased bodyweight compared to the vehicle treated HI animals.

Our hypothesis was that fluoxetine would negatively impact the HI subjects with the combined treatments acting as a double hit. However, in adult rats chronic treatment with SSRI's has been shown to stimulate neurogenesis and increase maturation, differentiation and survival in newborn neurons

(Fujioka, Fujioka, & Duman, 2004; Malberg, Eisch, Nestler, & Duman, 2000). The neuroplastic benefits have been shown to facilitate recovery in stroke patients (Dam et al., 1996). It, therefore, could be that perinatal fluoxetine exposure via the same mechanism has similar beneficial effects on P7 HI insult.

This does not explain, however, why there were no interactions found in the anatomical measures. Furthermore, although several interactions were found, most behavioral measures did not reveal a *Treatment* x *Lesion* effect. Neuroplastic benefits of perinatal fluoxetine on a P7 HI injury can only account for part of the results. The lack of beneficial result of prenatal fluoxetine for HI animals on several motor measures suggest that prenatal fluoxetine is not capable of completely decreasing the susceptibility of neurons and glia to HI injury.

An alternate explanation could be that for two perinatal factors to have an additive effect, they need to act on the same brain system or have similar mechanism by which they alter brain plasticity. It is reasonable to see how this was not the case in this experiment. Fluoxtine alters the levels of serotonin in the brain. Given the early appearance of serotonergic neurons with their wide distribution of terminals, it plays a key role in brain development. Hypoxia-Ischemia on the other hand results in mostly apoptotic cell death in the hippocampus, cortex, and thalamus. The fact that each factor affected mostly different tasks and behavioral measures provides evidence for the idea that perinatal fluoxetine and P7 HI affect different aspects in the brain and/or mechanisms of plasticity.

CHAPTER THREE. EXPERIMENT 2: DEVELOPMENT OF FORELIMB CORTICAL MOTOR MAP FUNCTION: EARLY ASSESSMENT FOLLOWING NEONATAL STROKE IN RATS

Modified from a paper in preparation by: Preston T.J.A. Williams, Gharbawie, O.A., Ph.D., M.Sc., Linda T.A. van Waes, B.Sc., Bryan Kolb, Ph.D.

Abstract

Background and Purpose: In experiment one the HI animals had mostly motor deficits, similar results are seen in survivors of pediatric cerebral stroke who often experience long-term motor disability. We have previously demonstrated abnormally small motor maps in the ipsi-lesion hemisphere when assessed in adulthood. One possibility is that the developmental trajectory of cortical functions, i.e. maps, are compromised rendering abnormal, and potentially disruptive, neural representations of movement in one or both hemispheres. In an attempt to account for the motor deficits in the HI animals, following unilateral neonatal stroke, forelimb motor maps were evoked in both hemispheres at timepoints during map emergence.

Methods: Hypoxia-ischemia stroke was achieved by ligating and permanently occluding one common carotid artery and then exposing the pups for 90 minutes in hypoxia (8% oxygen) on postnatal day 7 (HI). Forelimb motor maps were evoked in both hemispheres with intracortical microstimulation between postnatal ages 19-20. Histological sections were gathered to assess the size of each hemisphere and their major axon fiber pathways were stained with gold chloride.

Results: The size of the ipsi-lesion hemisphere was reduced, but there were no changes in the corpus callosum or fimbria. There was no delay in the emergence of evoked motor movements in the neonatal stroke group in either hemisphere, or change in map location, nor stimulation threshold. The size of the forelimb

motor maps in HI animals were comparable to shams, but with less elbow and shoulder representation in the ipsi-lesion hemisphere.

Conclusion: There is no delay for motor maps to emerge and the map size is spared in both ipsi- and contra-lesion hemispheres, indicating that the postnatal stroke has little acute effect after two weeks. Abnormalities in map organization occur later in maturation when behavioural skills with the forelimbs are integrated.

Introduction

One important high-risk group for stroke is infants, and the majority of diagnosed cases report ischemia within blood flow territories of the middle cerebral artery. Hypoxia-ischemia to one hemisphere is a type of stroke that does have prevalence in newborns. The prognosis typically includes chronic motor impairments such as limb spasticity, hemiparesis, poor dexterity and coordination, and cerebral palsy in severe cases (Hill, Martin, Daneman, & Fitz, 1983). Given the brain is undergoing tremendous maturation perinatally, it is imperative to assess the developmental trajectory of neurophysiological function. Thus, there is a gap in understanding how neurological abnormalities from neonatal stroke progresses, if at all.

An important milestone in the development of motor behaviour is the emergence of motor maps in frontal cortex (Lawrence & Hopkins, 1976; Martin, 2005). The motor deficits from unilateral neonatal stroke are curious because the primary motor cortex is typically spared, raising the possibility that cortical

motor maps are dysfunctional. Indeed, previous studies in our lab indicate that the ipsi-lesion motor map is abnormally small in adulthood following P7HI (Williams, Davidov, Steed, Arif & Kolb, 2007). It is unknown if the ipsi-lesion motor maps are abnormal from the outset, or if the changes occur later in maturation when motor skills increase in complexity. An asymmetry in map function may be disruptive on corticospinal tract development. Generally, magnetic resonance imaging of pediatric stroke infarct volume shows poor correlation with motor deficits, although decreased corticospinal tract volume does predict motor outcome (Kirton, Shroff, Visvanathan, & deVeber, 2007). A direct measure with higher resolution of corticospinal network function, albeit not permissive in most human cases, is intracortical microstimulation (ICMS). Used to characterize learning- or stroke- induced reorganization of motor maps in adult monkeys and rats (Kleim, Barbay, & Nudo, 1998; Kleim, Jones, & Schallert, 2003), ICMS can also be used to characterize the developmental emergence of motor map representations (Chakrabarty & Martin, 2000) and the influences of early experience (Martin, Friel, Salimi, & Chakrabarty, 2007).

A widely used experimental model of unilateral human stroke at birth is the rat postnatal day 7 hypoxia-ischemia (HI) procedure (Rice, Vannucci, & Brierley, 1981). We took advantage that this model does not require craniotomy or cause cerebral damage mechanically, and is permissive to reperfusion, in order to study the consequence of neonatal stroke on the emergence of forelimb motor maps in frontal cortex for both hemispheres. Post-mortem histological analysis with gold chloride staining for myelin targeted the corpus callosum fiber pathway and the fimbria of the hippocampus. This study provides the first

insights into map plasticity following neonatal stroke.

Subjects and Housing

Long-Evans male rat pups (N=15) from two dams bred at the Canadian Centre for Behavioural Neuroscience breeding colony were used in this study. Group assignment was counterbalanced within and across litters. Rats were mapped on postnatal day 19 (Sham=5; P7HI=5). Rats were housed in standard laboratory cages with food and water available *ad libitum*. Weaning occurred on postnatal day 23 and consisted of separating the litter by sex. This study was approved by University of Lethbridge Animal Care Committee review and procedures followed institutional and the Canadian Council for Animal Care guidelines.

Surgical Procedures

On postnatal day 7, the litter was removed from the dam and placed in a standard cage on a heating pad to maintain body temperature. Pups were initially anesthetized in an induction chamber with isoflurane before transfer to an operating table where they were maintained under anesthesia through a mini nose cone. The ventral neck was cleaned and an incision along midline of the neck was made. The exposed muscles were then separated allowing access to the common carotid artery (CCA). The right CCA was then ligated and the vagus nerve separated. The CCA was tied with 2 sutures (5-0 silk) 2-3 mm apart caudal to internal and external carotid artery branches. The exposed arterial tissue was permanently occluded with bipolar electrocoagulation. The muscles

were repositioned and the incision was closed using Vetbond tissue adhesive. The pups were then placed in an incubator, set at 37 °C, for one hour to recuperate. Then, exposure to hypoxia for 90 min was achieved by placing counterbalanced small groups of pups in a glass jar with the temperature set at 36.5°C humidified in a water bath and a tube inserted delivering 8 % oxygen (balance nitrogen) at 110 mm Hg. The pups were then returned to the incubator and, after 10 minutes and mobile, were returned to their dam. Sham surgery did not include right CCA occlusion (or vagus nerve separation). There was no mortality from CCA occlusion or hypoxia exposure.

Physiological Assessment

Intracortical Microstimulation (ICMS)

Motor mapping was used to probe the emergence of forelimb motor maps using intracortical microstimulation (ICMS). In all rats, the ipsi-lesion and contra-lesion hemispheres were mapped. The ipsi-lesion hemisphere was of primary interest and was mapped first, but some sessions did include transferring the electrode to the opposite hemisphere between stimulation sites. Rats were anesthetized with ketamine hydrochloride (30 mg/kg, ip), which maintains muscle tone, and supplements were given when necessary (10 mg/kg, ip). The dorsal head was shaved and wiped with betadine, and the eyes were protected with ointment. An incision along the midline scalp was made and the skull exposed. The skull was trephinated to access the dorsal frontal cortex and anterior parts of the parietal cortex. A pilot hole in the skull was achieved with a dental drill bit and a window at least 3 mm X 3 mm over frontal cortex was made

with rongeurs. The cisterna magna was punctured with a 30 gauge needle to drain cerebrospinal fluid and manage swelling. The dura was retracted and the exposed cortex was covered with inert silicon fluid (37◦ C).

The head was secured into stereotaxic ear-bars with the body in a prone position. The surface of the cortex was digitally photographed with a CCD camera mounted on a surgical microscope and a grid (500 μm^2) was superimposed onto the digital image using Canvas (ACD Systems, http://www.deneba.com) to guide and record electrode penetration sites. Inter-penetration distance was 350 μm^2 while avoiding vasculature branches on the surface of the brain.

The electrode consisted of a platinum filament, inserted in a borosilicate glass micropipette (2-4 m tip diameter, 15 bevel) filled with concentrated saline (3.5 M). The electrode was fastened to a tower affixed to the stereotaxic instrument and was manually lowered so the tip of the electrode penetrated to 1000 μm beneath the surface of the cortex which evoked movements at the lowest stimulation threshold. Stimulation trains of thirteen, 200 μs, 350 Hz cathodal pulses, were delivered from a stimulation isolation unit. At each site, current intensity was gradually increased from 0 A up to 60 A, or until a movement was evoked. In the event of a forelimb movement, the current intensity was decreased until the movement no longer persisted and was deemed the threshold.

During stimulation trains, an experimenter supported the rat's forelimb from underneath the elbow and visually classified evoked forelimb movements as Proximal (elbow/shoulder) or Distal (wrist/digit). In the case of two

simultaneous movements, the movement obtained at the lowest threshold was recorded. Movements of hindlimb, trunk, vibrissae, jaw, and neck were also recorded. At least one experimenter was blind to the rat's group membership, but it was known that stimulation in the right hemisphere was possibly a lesion hemisphere.

At the completion of motor mapping under standard parameters mentioned above, forelimb representations were abnormally small or absent in some rats. In such cases, the non-responsive sites surrounding the forelimb representations were reinvestigated, first with current intensities between 0 μA and 60 μA and if still unresponsive, then current intensities between 60 μA and 150 μA were used. Also, the tip of the electrode was lowered to varying depths between 400 μm and 1600 μm in steps of 200 μm increments beneath the surface of the cortex to accommodate changes in cortical thickness that may have shifted the relative depth of layer V pyramidal cell bodies. In most cases, it was possible to stimulate the ipsi-lesion hemisphere in homotopic sites and depths that evoked forelimb movements in the contra-lesion hemisphere.

Areal measurements of forelimb movement representations were made to scale using the software Canvas. The midpoint distance between outskirt forelimb movements and neighbouring non-forelimb movements was outlined and the enclosed area was calculated. Amongst forelimb movements, areas of Proximal (elbow and shoulder) and Distal (wrist and digit) representations were outlined and measured.

Brain and Body Weight

Before the intracortical microstimulation session, body weight was measured. After the mapping session and perfusion, the brain was harvested, trimmed caudal to the cerebellum, and weighed.

Histology

After completion of each intracortical microstimulation session the rats were given an overdose of Euthensol (0.1 mL ip). Rats were cardiac perfused with saline wash and fixed with 4% formalin. Brains were removed, trimmed flush with the cerebellum, weighed, and post-fixed for 24 hours and then transferred to 4% formalin and 30% sucrose solution for cryoprotection. Digital whole brain pictures were captured with a CCD camera. For sectioning, brains were trimmed at the pole of the frontal lobe, and freeze-sectioned on a cryostat at 40 μm thickness. Every tenth section was collected on 1% gelatin + 0.2% chromatin coated glass slides and dried overnight.

Gold Chloride Myelin Histochemistry

Two major axon pathways were assessed quantitatively, the corpus callosum and the fimbria of the hippocampus, by measuring the area from digital pictures. The measurements were guided according to the atlas of Paxinos and Watson (1991), Plate 19 (-0.30 mm Bregma) and Plate 27 (-1.88 mm Bregma) to represent anterior and posterior planes. Other pathways, such as the internal capsule, were not amenable to quantify because of their diffuse course through the brain, however, qualitative assessments were made.

Sections were incubated in Gold Chloride solution (0.2% gold chloride in phosphate buffer (1.8 g crystalline gold chloride, 0.33 g sodium phosphate monobasic monohydrate, 3.6 g sodium phosphate dibasic anhydrous, 9.0 g sodium chloride, 1000 mL distilled water) at 40˚C for 1-2 hours until myelinated bundles appeared in shades of purple/brown. This was followed by 5 minutes in distilled water, 5 minutes in 2.5% Sodium Thiosulfate to fix and a 30 minute rinse of slow running tap water. Sections were air dried and cover slips fastened with Permount mounting media (Fisher Scientific).

Images were gathered with a Zeiss Axiovision 4.3 (Zeiss, Germany) at 1x magnification outfitted with a CCD camera. Using ImageJ (Image J, Betheseda, MD) freeware, each image was internally scaled to units in mm. Subsequently, the structure of interest was outlined and area calculated. In addition, the area of each entire hemisphere was taken.

Statistics

For Body Weight, Brain Weight, Histology, and Intracortical Microstimulation data, Analysis of Variance (ANOVA) for (SPSS 11.0 Inc., Chicago, IL, USA) was used with Group (Sham and P7HI) as the between-subject factor unless otherwise stated.

Results

Body and Brain Weight

The P7HI group appeared normal except for a characteristic asymmetry of the face: the ipsi-lesion side of the face appeared narrower with a ptotic eye.

There were no differences in body weight between Sham and HI rats $F(1, 8) = 0.40$, $p < .05$. Brain weights of the P7HI operates were not different from Sham operates $F(1, 8) = 0.48$, $p > 0.05$.

Gold Chloride Myelin Histochemistry

General Observations

There was variability in brain morphology depending on the severity of the hypoxia-ischemia lesion. Post-mortem inspection of whole brains determined that HI cases did not show evidence of an infarct core, but the ipsi-lesion hemisphere appeared smaller. There was no effect of P7HI on myelination in the corpus callosum or fimbria.

In nearly all P7HI cases, coronal sections of the ipsi-lesion hemisphere showed enlargement of the lateral ventricle, smaller caudate putamen, smaller hippocampus, and reduced myelin staining in the internal capsule. The thalamus (ventromedial nuclear group) and hypothalamus appeared normal, as did the anterior commisure and cingulum. There was no evidence of patchy cell death within residual cortex and the laminar organization appeared spared, although distorted with a less prominent layer IV in the ipsi-lesion hemisphere.

Anterior Plane (Plate 19)

There was no differences in percent ipsi-lesion hemipshere size of the total hemisphere size $F(1, 8) = 1.80$, $p > .229$. There were no effects of *Group* for corpus callosum size in the ipsi-lesion hemisphere $F(1, 8) = 0.66$, $p > .05$. Further, there were no *Group* differences found for corpus callosum size in the

contra-lesion hemisphere $F(1, 8) = 0.70$, $p > .05$, or in corpus callosum total size $F(1, 8) = .072$, $p > .05$.

Posterior Plane (Plate 27)

There was a differences in percent ipsi-lesion hemipshere size of the total hemisphere size $F(1, 8) = 7.2$, $p = .028$, HI animals had a lower value (see Fig. 3.1).

There were no size effects of *Group* for the corpus callosum in the Contra-lesion hemisphere $F(1, 8) = 1.57$, $p > .05$, or in the total size of the corpus callosum $F(1, 8) = 0.53$, $p > .05$.

The area of the fimbria fornix was the same for *Group* in the Ipsi-lesion hemisphere $F(1, 8) = 0.01$, $p > .05$ and in the contra-lesion hemisphere $F(1, 8) = 0.01$, $p > .05$.

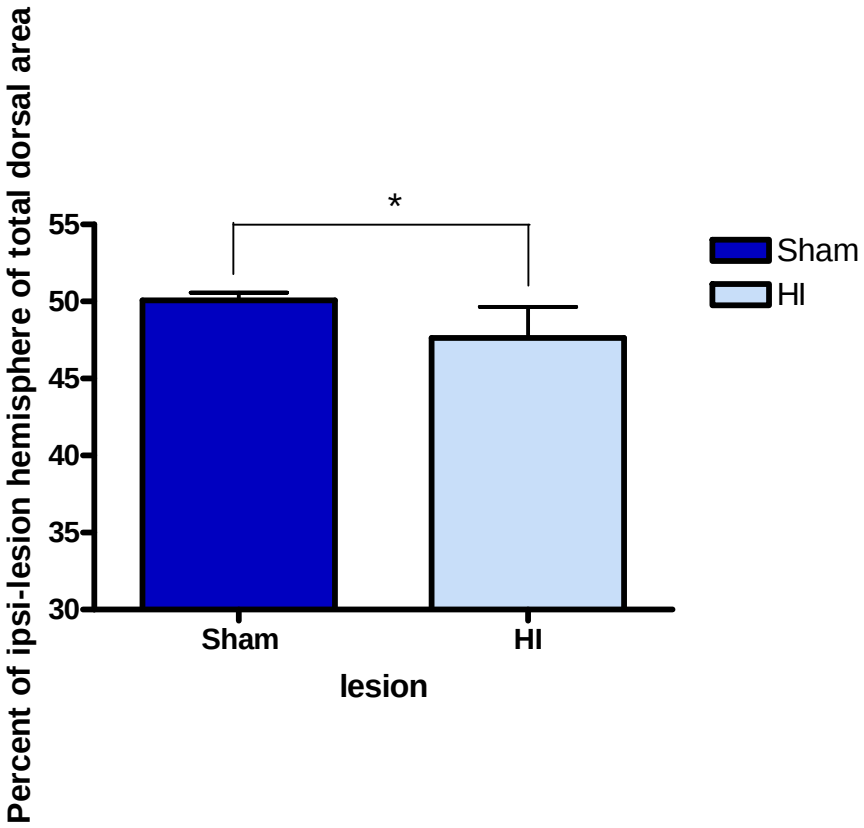

Figure 3.1. Hemisphere size. Percent of Ipsi-lesion hemisphere. In HI animals, the lesion hemisphere takes up a smaller relative contribution to the total dorsal area compared to sham controls. * indicates difference is significant at $p \leq .05$.

Intracortical Microstimulation (ICMS)

Forelimb motor maps were assessed in both hemispheres at developmental time points when forelimb representations can be first evoked in the developing rat brain. There was no difference in the number of stimulation sites probed between sham and HI subjects Ipsi-lesion: $F(1, 8) = 0.89$; Contra-lesion: $F(1, 8) = 0.03$, p's > 0.05). There was no difference in average stimulation threshold to evoke forelimb movements ipsi-lesion: $F(1, 7) = 0.47$; contra-lesion: $F(1 ,6) = 0.31$, p's > .05. An overlay of the maps from each group demonstrates that similar areas of cortex were investigated, and that forelimb movements were consistently found within the same regions, although the individual organization is different (Fig. 3.2). There were no cases that ICMS, in either hemisphere, evoked ipsilateral or bilateral movements.

HI rats had the same Total area of forelimb representations in the ipsi-lesion motor cortex as sham $F(1, 8) = 2.62$, $p < .05$. Further analysis indicated that the HI group had less proximal representation of shoulder and elbow movements (Fig. 3.3), but no difference in distal representation of wrist and digit movements compared to sham Proximal: $F(1, 8) = 6.34$, $p < .05$; Distal: $F(1, 8) = .03$, $p > .05$.

There were no differences found in analyses of areal representations in the contra-lesion hemisphere between *Group* Total: $F(1, 8) = 0.12$; proximal: $F(1, 8) = 0.91$; distal: $F(1, 8) = 0.01$, $p > .05$, p's > .05.

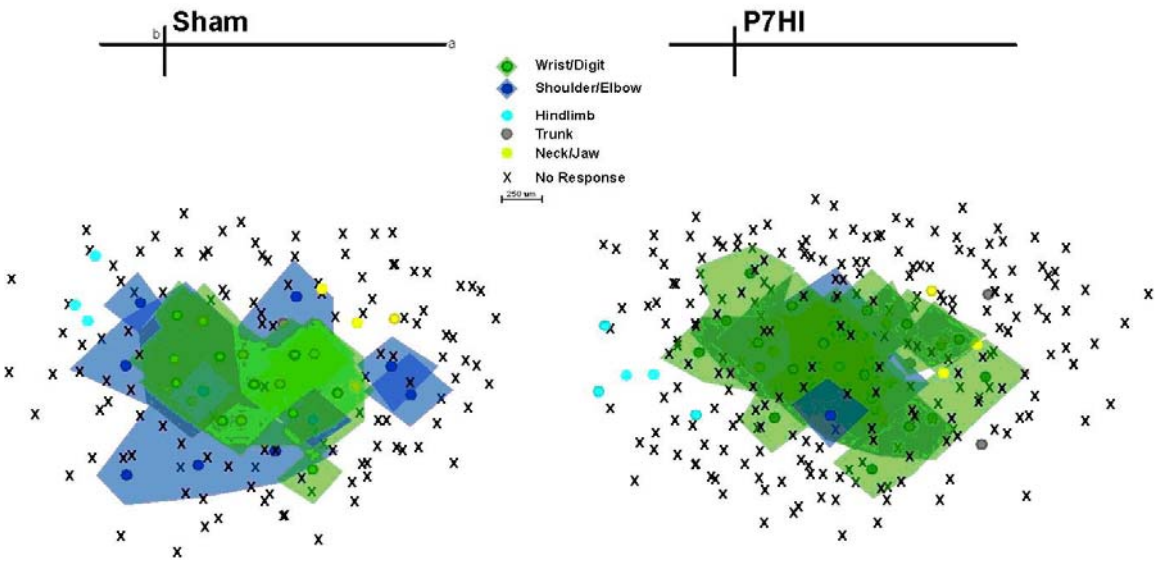

Figure 3.2. Overlay of ipsi-lesion forelimb motor maps for every sham (left) and postnatal day 7 hypoxia-ischemia (HI) (right) case. The cortical region investigated with intracortical microstimulation is comparable between the two groups. There was some individual variation in the location and organization of the motor representations, but forelimb maps closely overlap (darkest shaded areas show the most overlap) as well as areas of non-forelimb movements (hindlimb, neck/jaw), in both groups suggesting there was no shift in the topography of the motor maps due to the neonatal stroke. Each mark represents a stimulation site and coded green:wrist/digit; blue:shoulder/elbow; turquoise:hindlimb; grey:trunk; yellow:neck/jaw; x:no response. *b* is bregma and *a* is anterior.

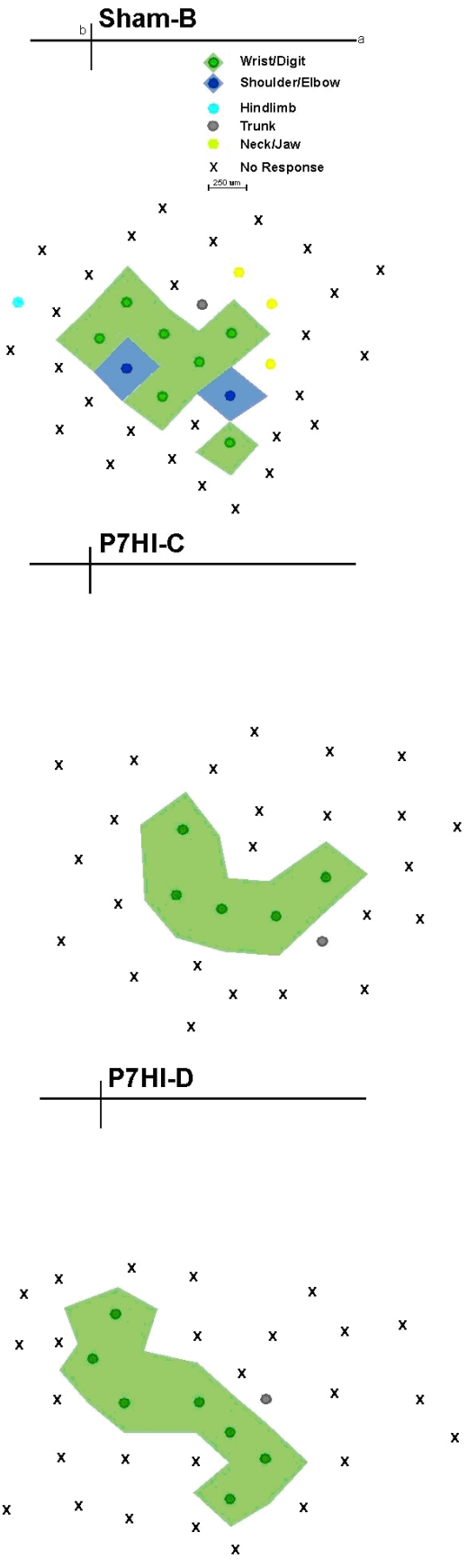

Figure 3.3. Intra-areal organization of forelimb movement representations for Sham (P7HI-B) and postnatal day 7 hypoxia-ischemia (P7HI-C, P7HI-D). P7HI rats had a smaller ipsi-lesion shoulder/elbow representation than Sham (* $p < .05$) whereas there was no difference for wrist/digit representation.

Discussion

The most commonly presented impairment from stroke at any age is a motor deficit. We took a systems approach to test the influence of postnatal day 7 hypoxia-ischemia (HI) stroke on motor map emergence. The major findings are that HI (1) did not impose a delay in motor map emergence, (2) reduced the size of proximal representation of shoulder and elbow movements in the ipsi-lesion hemisphere hemisphere motor map, and (3) had no effect on the contra-lesion hemisphere motor map.

HI effects on brain morphology

There was no mortality during or after the induction of hypoxia-ischemia. Body and brain weights were measured nearly two weeks after the stroke and there were no reductions in the HI group. Following the same lesion procedures, we have noted reduced body weight in HI operates when assessed in adulthood. Additionally, other studies have shown reduced body weight following neonatal stroke, suggesting that the weight differences occur post-weaning. Thus, the acute maintenance of normal body weight indicate that following hypoxia-ischemia there were no immediate changes in body set-point or food intake (i.e. suckling), which can be a factor leading to mortality in other stroke models.

Post-mortem inspection of the whole brains showed that none of the HI cases had overt infarcts. Ipsi-lesion hemisphere size was reduced compared to the contra-lesion hemisphere in HI operates at posterior, but not anterior, planes. Infarcts reported from HI occur in posterior planes of the brain, whereas

the frontal cortex is less vulnerable because of anastomoses between anterior cerebral and middle cerebral arteries via the azygomatic communicating artery, even in severe cases. Coronal sections of the ipsi-lesion hemisphere showed enlargement of the lateral ventricle, smaller caudate putamen, smaller hippocampus. The thalamus appeared normal, particularly in the ventromedial nuclear group that sends afferents to motor cortex. There was no evidence of patchy cell death within remaining neocortex and the integrity of cortical lamina appeared spared except for a less prominent layer IV in the ipsi-lesion hemisphere. In all cases, motor cortex was intact. There was a marked reduction in the intensity and frequency of staining in the internal capsule in the ipsi-lesion hemisphere in almost all HI cases.

Interestingly, HI subjects had an abnormally appearing ipsi-lesion side to the face. The misshapen face is likely due to occlusion of the common carotid artery caudal to the internal and external carotid branches, thus disrupting blood supply to the face with limited avenues of receiving collateral blood flow.

In analysis of the corpus callosum we did not find a decrease in size compared to shams. Other volumetric studies examining the effects of neonatal hypoxia-ischemia on the ipsi-lesion hemisphere have shown reductions in hippocampus, basal ganglia, internal capsule, and corpus callosum. Our comparison is limited because we were unable to dissociate myelinated and non-myelinated axons or assess the number of fibers, or evaluate the degree of myelination. Another possibility is that our measurements were taken at a time point when axons are still maturing and adding myelin, and therefore may not reflect adulthood outcomes and/or ongoing anterograde degeneration. It has

been shown that oligodendrocytes are vulnerable to hypoxia ischemia rendering CNS axons compromised (Skoff et al., 2001). Studies on children afflicted with neonatal stroke have found considerable anterograde Wallerian degeneration within major fiber pathways such as the corticospinal tract (Khong et al., 2004; Kirton, Shroff, Visvanathan, & deVeber, 2007).

Motor map emergence following HI

There is a protracted time course in postnatal development during which intracortical microstimulation of frontal cortex can evoke movements in naïve animals (Martin, 2005). A key component of motor system development is the completion of lateral and ventral corticospinal tract refinement of cortical layer V pyramidal neuron axon mono- and poly-synaptic contacts with spinal motoneurons and interneurons respectively. Less well understood are the sculpting processes of intracortical circuitry and the embedding of horizontal connections within and between hemispheres.

ICMS evoked movements demonstrate a sufficient capacity within the corticospinal motor network to elicit movement via direct layer V pyramidal cell depolarization at the electrode tip (Stoney, Thompson, & Asanuma, 1968), and indirect pyramidal cell depolarization by trans-synaptic recruitment along horizontal connections and *en passant* fibers (Fetz & Cheney, 1980). It is known in rats that lateral corticospinal projections have reached their targets and begin assembling synaptic contacts by postnatal day 14 (Terashima, 1995) (ventral corticospinal tract unknown). A previous study from our group has characterized the emergence of motor maps in naïve rats with the same

intracortical microstimulation methods and found that forelimb movements, including shoulder, elbow, wrist, and digit, could be first evoked on postnatal day 17, but was not reliable in every case until postnatal day 19 (Williams, van Waes & Kolb, 2008). It is important to note that Chakrabarty and Martin (2000) showed the time course of map emergence is similar in anesthetized and awake preparations.

A major contribution of this study is that there was no delay in the emergence of motor maps in the HI group. Following HI, movements could be evoked from the ipsi-lesion hemisphere at electrode depths and stimulation thresholds on postnatal day 19, the same day maps emerge in shams. It is remarkable that there was no developmental delay imposed on the ipsi-lesion hemisphere considering the stroke occurred on postnatal day 7 coinciding with maximal synaptogenesis in the spinal cord, particularly within the cervical enlargement (Gribnau, de Kort, Dederen, & Nieuwenhuys, 1986). One interpretation could be that the onset of motor map representations is more dependent on refinement in cortical organization. The total size of the forelimb representation was spared in the ipsi-lesion hemisphere of HI rats. A separate series of experiments in our lab has investigated the organization of forelimb movements in adulthood following the same HI parameters (Williams, Davidov, Steed, Arif & Kolb, 2007) and found that moderate and severe infarcts dramatically reduce the amount of forelimb representation, even though motor cortex is spared. Together these results suggest that ipsi-lesion motor cortex map ontogeny is not a good indicator of motor map organization in adulthood. The importance of these results is that the map size reduction observed in

adulthood, but not early on, indicates either that anterograde degeneration continues into adulthood affecting motor pathways, or the capacity for experience-dependent map maturation (Martin, Friel, Salimi, & Chakrabarty, 2007) is compromised, or both. Nevertheless, rehabilitation after map emergence may be an important period for intervention following neonatal stroke.

There was a difference in the organization of forelimb maps in the HI rats compared to shams, marked by a decrease in proximal movements (shoulder and elbow). This is the first report to show lesion-induced reorganization of motor maps from neonatal stroke. The reduced proximal forelimb representation is curious because the emergence of motor representations in cats shows only proximal movements initially and later elaboration of the map to include distal and multi-joint movements (Chakrabarty & Martin, 2000). In rats, all types of forelimb movement types could be evoked when the motor map emerges. A diminished shoulder/elbow representation following HI may represent a compensatory strategy to spare control of wrist/digit movements that arguably underlie the unique contributions of motor cortex to the motor system (Piecharka, Kleim, & Whishaw, 2005). Furthermore, wrist/digit representations show tremendous plasticity and demonstrate re-organization from skilled reach training in adult monkeys and rats (Kleim, Barbay, & Nudo, 1998).

The profile of evoked movements from the contra-lesion hemisphere was the same in HI as in Shams. The implication is that following HI the intact hemisphere does not show evidence of compensatory motor map

reorganization. This is the first report on the effects of unilateral neonatal stroke on the contra-lesion hemipshere. That there was no difference between ipsi- and contra-lesion map size suggests the function of the motor pathways from each hemiphere is similar. There were no ipsilateral or bilateral movements evoked in any of the cases, perhaps because the ventral corticospinal tract develops differently. Mirror movements have not been reported following pediatric stroke, but mirror movements are present following adult stroke (Kim et al., 2003). It has been observed in children with subcortical stroke that there is increased inter-cortical inhibition exerted from the contra-lesion hemisphere. Applying repetitive transcranial magnetic stimulation showed that artificial activation of the contra-lesion hemisphere was effective in reducing the motor deficits (Kirton et al., 2008).

The protracted maturation of motor maps continues after weaning. In adult rat motor maps, there are two regions in motor cortex that evoke forelimb movements, a large caudal forelimb area (CFA) and small rostral forelimb area (RFA), separated by a band of whisker, neck and jaw responses (Neafsey et al., 1986). It is likely that the movements evoked in the current study represent the caudal forelimb area because of their close location relative to bregma, and neck responses were found rostral to the forelimb maps. Future studies will be required to determine the emergence of the RFA, and the longitudinal instatement of both forelimb representations. We have found that the RFA is spared in HI cases mapped in adulthood and may be an important compensatory mechanism (Williams et al., in preparation). Indeed, in adult rats with middle cerebral artery occlusion, RFA is also spared (Gharbawie, Williams, Kolb, &

Whishaw, 2008). However, rats with neonatal prefrontal cortex lesions have a dysfunctional RFA in adulthood that can be partially rescued with rehabilitation (Williams, Gharbawie, Kolb, & Kleim, 2006). Further studies will be needed to characterize the emergence of RFA and its influence on motor map function following stroke. A plausible strategy might be to further enhance the plasticity of CFA, or RFA, depending on where the lesion is and what the behavioral symptoms are (Gharbawie, Karl, & Whishaw, 2007).

CHAPTER FOUR. GENERAL DISCUSSION

Introduction

The goals of this thesis were to examine: (1) the effects of perinatal fluoxetine and post-natal day 7 Hypoxic-Ischemic injury on the developing brain; and, (2) how these two factors in combination affect the developing brain. A low dose of fluoxetine and mild HI insults were selected for this thesis to address the effects of minor brain perturbations, alone and in combination, on brain and behavioral development.

Principal findings

There were several important findings. First, fluoxetine exposure or HI injury alone mostly had negative effects on behavioral and anatomical measures. Second, the combined treatment with fluoxetine and HI injury only interacted on a limited number of measures. In the measures where the two factors did show an interaction, fluoxetine positively affected the outcome of the HI injury animals. Third, HI injury, which was largely outside the motor cortex, did not delay the emergence of, nor the size of, the motor map in infancy.

Effects of Perinatal Fluoxetine exposure on behavioral and anatomical measures

Perinatal fluoxetine-treated animals weighed less and were less active but more anxious as adults. Moreover, the fluoxetine group performed poorly in the cognitive task and had altered motor skills such as smaller stride length and angle of displacement of the hindfoot. The anatomical measures revealed that the cells in certain areas of the parietal cortex were simpler although the cortex was thicker in the anterior plane.

The cortical plasticity and altered behavior seen after perinatal fluoxetine exposure is likely a result of the disruption of development of a normal serotonergic system and its target tissue. For example, lesion studies have shown that the serotonergic system plays a key role in motor behaviours and anxiety (File, Hyde, & MacLeod, 1979; Vanderwolf, 1988), and changes in the serotonergic system are likely to affect these behaviors. Moreover, there is evidence that neonatal SSRIs disrupt normal cortical development (Xu, Sari and Zhou, 2004) and reduce apoptosis (Persico et al., 2003), which likely accounts for the anatomical findings in this thesis. Given the impact of alterations of serotonin levels on the autoregulation of serotonergic neurons and the development of the target tissue, including other monoamine systems, it is understandable that alteration of serotonin levels during brain development leads to permanent alterations in brain and behavioral functions.

It is interesting to note that other psychoactive drugs and other factors that alter brain development also alter serotonin levels. Drugs such as alcohol (Sari, Powrozek, & Zhou, 2001) and nicotine (Muneoka et al., 1997), as well as malnutrition (Blatt, Chen, Rosene, Volicer, & Galler, 1994) and social isolation can affect serotonin (Whitaker-Azmitia, Zhou, Hobin, & Borella, 2000). It is conceivable that the serotonergic alterations play a key role in the behavioural abnormalities that medicate the developmental effects of the described factors.

Abnormal behaviors in the clinical population are also linked to serotonergic dysfunction, for example autism. Autistic children have an increased level of serotonin in their blood (Anderson, Horne, Chatterjee, & Cohen, 1990; Naffah-Mazzacoratti et al., 1993). Serotonin reuptake blockers have beneficial effects for these children, indicating

that altered serotonin levels have a causal role in this disorder (Buitelaar & Willemsen-Swinkels, 2000; McDougle, Kresch, & Posey, 2000).

Effects of Hypoxic-Ischemic Injury on behavioral and anatomical measures

The HI group was mostly impaired on the motor tasks. The HI animals had a lower success rate in tray reaching, larger angle of rotation of hindfoot, and were hyperactive in the activity box. However, compared to other reports in the literature (Balduini, De Angelis, Mazzoni, & Cimino, 2000; Jansen & Low, 1996; Tomimatsu et al., 2002) the motor deficits reported in this thesis are rather mild. For example, there was no effect found in the qualitative analysis of the single pellet task, a test known to be very sensitive to motor impairments (Metz & Whishaw, 2000). The mild behavioral results are accompanied by lack of damage in structures typically reported in the literature after HI injury, such as damage to the subcortical and periventricular white matter, striatum, and hippocampus (Rice, Vannucci, & Brierley, 1981; Towfighi, Yager, Housman, & Vannucci, 1991; Towfighi, Zec, Yager, Housman, & Vannucci, 1995).

An explanation for the discrepancies in the findings in this thesis and the literature is related to differences in methodology - this thesis included solely non-cavity HI subjects. Cavity animals could only be excluded *post mortem* and were therefore run on the behavior tasks such as single pellet. Deficits were detected in the qualitative analysis of single pellet reaching in the cavity animals (data not included). These observations provide support for the idea that there is a significance difference in functional outcome between cavity and non-cavity subjects.

There was no delay in motor map emergence nor an effect on map size after early assessment at P19 in HI rats. This was surprising as it has previously been shown

in the Kolb lab (Williams, Davidov, Steed, Muhammed & Kolb, 2007) that HI subjects have smaller ipsi-lesion motor maps in adulthood. There is a known correlation between behavioral capacity and the size of motor maps (Kleim et al., 2002). The motor map results suggest that mild HI does not affect the time of onset of the motor map, but that it affects the mechanism involved in developing a normal adult motor map.

The Kolb lab has extensively studied the effects of frontal cortex lesions in the perinatal period and the associated behavioral and anatomical outcome. The mild behavioral abnormalities as a result of the non-cavity P7 HI insult suggests that this injury fits the plasticity paradox somewhere in the middle of high and low plasticity level (see Figure 1.1), as functional outcome is neither severely compromised nor excellent.

Grafe (1994) showed that rats between P3 and P6 were more susceptible to HI injury than P7, providing evidence that the HI model follows the same pattern of functional outcome as focal frontal lesions. However, an increased amount of damage was observed after HI insult between P7 and P30 (Towfighi, Mauger, Vannucci, & Vannucci, 1997), which is not in accordance with the finding of the focal frontal lesions. It seems that the degree of vulnerability of HI injury starts off similar to focal frontal lesions, but continues to increase as the developmental age of insult increases. There is currently no single theory that accounts for the changes in vulnerability, but Towfighi, Mauger, Vannucci and Vannucci (1997) suggest that it is the result of the complex regional anatomic and functional changes as the brain matures and we can hypothesize that they are different for the focal injuries. For example, whereas the HI injuries usually produce subcortical effects, this is not typical of the focal injuries.

Combined treatment of Fluoxetine & HI

Fluoxetine treatment altered the performance in the activity box, both in total attempts and success rate in tray reaching, and the angle of foot rotation during walking. The reason for the limited interaction of fluoxetine treatment on HI insult outcome could be that in order for two perinatal factors to have an additive effect, they need to act on the same brain system or have similar mechanism by which they alter brain plasticity. It is reasonable to see how this was not the case in this thesis. Because fluoxetine is a serotonin reuptake inhibitor, it alters the levels of serotonin in the brain. Given the early onset of serotonergic neurons with their wide distribution of terminals, serotonergic neurons play a key role in brain development. Hypoxia-Ischemia on the other hand results in mostly cell death in the hippocampus, cortex, and thalamus (Rice, Vannucci, & Brierley, 1981). The fact that each factor affected mostly different behavioral measures supports the idea that perinatal fluoxetine and P7 HI affect different aspects in the brain and/or mechanisms of plasticity.

In the few instances were fluoxetine and HI interacted, it was positive; the HI animals mostly performed better than if they had not received the perinatal fluoxetine. This is interesting, given that Day, Gibb and Kolb (2003) reported that prenatal exposure to fluoxetine impaired the normal development and recovery following P3 and P10 frontal cortex lesions. It could be that the difference in result is due to the nature of the lesion (focal and anterior vs global and posterior), or age (P3 and P10 vs P7), or a combination of the two.

Limitations and Caveats

There are several limitations and caveats in this thesis that should be addressed. First, as a result of the experimental design it is not possible to have a true counterbalanced, randomized group assignment for all subjects. For example, it not possible to have one pup in the vehicle group and another pup from the same litter in the fluoxetine group. As such, there is the risk of running into pseudoreplication, which refers to the problem of potential litter effects that could confound the results for the fluoxetine main effect. One solution to this problem is to have a very large number of treated litters but this leads to animal welfare issues related to using so many animals. A second solution is to specifically look for litter effects in the fluoxetine-treated litters. There were no litter effects in the current studies. Second, as mentioned earlier in the thesis, owing to collaborations, time constrains, and technical problems, groups do not have equal numbers and not all animals participated in all behavioral and anatomical analyses.

Another concern is the extensive behavioral testing. It is well documented that one of the most beneficial treatments for optimizing functional brain recovery is being exposed to a complex, stimulating environment. For example, Whishaw, Zaborowski and Kolb (1984) found that placing animals in an enriched environment after hemidecortication at birth, allowed the animals to perform significantly better on a spatial navigation task than standard lab-raised animals. It could be the case that the extensive behavioural testing had the similar benefits as an enriched environment and was therefore rehabilitative for tasks given towards the end of the training protocol. It would be interesting to run the same experiment but only test the animals on one behavioral task in adulthood.

On the same topic, the subjects were raised after weaning in a large tub environment, that has larger in volume than standard lab caging and rats have a larger social group. This housing condition could potentially be beneficial to functional outcome of the drug and injury treatment. It is possible that the housing condition might have served as a mild enriched environment, through increased locomotor activity and social interaction, with the associative benefits of better functional outcome. As a result, comparison with other studies is somewhat difficult.

Future Directions

Reports in the literature on the behavioral and anatomical effects of perinatal fluoxetine or HI are sometimes inconclusive or contradictory. These inconclusive results may be related to methodology or time of assessment. For the fluoxetine literature, different doses, and exposure duration and method of administration are variables that can, and do, vary. Age of insult, duration of hypoxia exposure, percent of oxygen used are all factors that all affect the behavioral and functional outcome. In addition, there are strain differences in susceptibility to HI injury in mice (Sheldon, Sedik, & Ferriero, 1998) and Walberer et al. (2006) showed that ischemic lesion volume is different between Wistar and Sprague-Dawley rats. Future studies should be directed at explaining the discrepancies between different studies and attempt to account for them by revealing the mechanism that drives the behavioral and anatomical results.

More specifically related to the double hit hypothesis, it would be interesting to see how different doses and timing of administration of fluoxetine would affect the outcome of a mild HI insult. Brain development is comprised of many cellular events that are associated with specific sensitive periods. In this thesis, fluoxetine was given

during the whole perinatal period. However, it could be the case that fluoxetine has a negative impact during the prenatal period, but if it was given only around the time of the HI injury it has beneficial effects. If that was the case this thesis would not be able to detect differences between the two. For example, it is known that brain-derived neurotrophic factor (BDNF) has highly age-dependent effects. When given at P0 it exacerbates injury, at P5 it protects against excitotoxicity, whereas at P10 it has no effect on lesion outcome (Husson et al., 2004). Administration of fluoxetine solely at the peak of certain cellular events could potentially result in better, or worse, behavioral and anatomical outcome after the HI insult.

In this thesis there was limited interaction between the fluoxetine and HI insult. One hypothesis for this result is that for two factors to have additive or synergistic effects they act on similar mechanisms by which they alter brain plasticity. The behavior category that was mainly affected in the HI subjects was motor abilities, so in order to test this hypothesis, a drug that affects the dopaminergic system should be tested because it is known that dopamine plays a key role in coordination of movement. Alternatively, a different injury model could be explored. As noted earlier fluoxetine exacerbates P3 and P10 frontal cortex lesions.

An interesting question did arise from experiment number two. There was no difference in time of onset or map difference in the HI subjects, but it has previously been shown in our lab that there is a difference in the map size in adulthood (Williams, Davidov, Steed, Arif, & Kolb, 2007). Future studies should address this discrepancy, by looking at what the differences and similarities are between the postnatal and adult map. One study that would be particularly interesting is to longitudinally map HI animals in order to detect at which age the map of HI begins to differ from the control

animals. When the time point of map size difference in the HI group is identified, follow-up studies could start to investigate the mechanism that causes this effect.

Chemical neurotransmitters play a central role in the transfer of information between neurons, however it is in the neural networks that information is stored (Hua & Smith, 2004). Furthermore, histological and physiological analyses document brain changes after certain experiences, but they do not address the mechanism (i.e., address the "how" question) by which this occurs. Kolb (2003) therefore suggests that the final analysis should include investigation of the change in proteins that drives the effects and ultimately alters gene expression. Having insight into the mechanism of how genes are changed by experience will be beneficial in understanding how to enhance beneficial plasticity after experiences that negatively affect brain function. Exploring the mechanism that drives the described results in this thesis will be crucial in the future.

Conclusion

Brain development is dynamic and adaptive. The capacity for plasticity in the developing brain is considerable, but there are limits. Not all neuronal systems exhibit the same level of plasticity and the windows of plasticity are temporally constrained. Furthermore, there is not always an interaction between plasticity mechanisms of several experiences such as drugs and injury. For example, Kolb, Gorny, Li, Samaha, and Robinson (2003) showed that after drug treatment, exposure to enriched environment was not associated with the usual beneficial effects. The conclusion was that drug treatment blocked dendritic changes typically found after a period of enriched environment.

This thesis provided an example of the constraints of plasticity. Specifically, psychoactive drugs and cortical injury during the perinatal period resulted in widespread changes in behavioral and anatomical measures, but there was very limited interaction between the two particular factors. We can summarize by saying that brain plasticity allows for adaptation, but it has limits and must fit within the developmental process (Stiles, 2008).

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APPENDIX ONE

Summary of Rat Subjects Utilized and Their Respective Behavioral Assessments

Litter 2006-34										
Rat#	**Sex**	**Treatment**	**Lesion**							
				1	**2**	**3**	**4**	**5**	**6**	**7**
47	F	Vehicle	Sham		√		√	√		√
48	F	Vehicle	Sham		√		√	√		√
53	F	Vehicle	Sham		√		√	√		√
58	F	Vehicle	Sham		√		√	√		√
49	M	Vehicle	Sham		√		√	√		√
43	M	Vehicle	Sham		√		√	√		√
50	M	Vehicle	Sham		√		√	√		√
Litter 2006-28										
Rat#	**Sex**	**Treatment**	**Lesion**							
				1	**2**	**3**	**4**	**5**	**6**	**7**
62	F	Vehicle	Sham		√		√	√		√
64	F	Vehicle	Sham		√		√	√		√
66	F	Vehicle	Sham		√		√	√		√
67	F	Vehicle	Sham		√		√	√		√
71	M	Vehicle	Sham		√		√	√		√
73	M	Vehicle	Sham		√		√	√		√
Litter 2006-38										
Rat #	**Sex**	**Treatment**	**Lesion**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
32	F	Fluoxetine	HI	√	√		√	√		√
34	F	Fluoxetine	HI	√	√		√	√		√
35	F	Fluoxetine	HI	√	√		√	√		√
37	F	Fluoxetine	HI	√	√		√	√		√
38	F	Fluoxetine	HI	√	√		√	√		√
30	M	Fluoxetine	HI	√	√		√	√	√	√
31	M	Fluoxetine	Sham	√	√	√	√	√	√	√
33	M	Fluoxetine	HI	X	X	X	X	X	X	X
36	M	Fluoxetine	HI	√	√	√	√	√	√	√
39	M	Fluoxetine	HI	√	√	√	√	√		√
40	M	Fluoxetine	HI	√	√	√	√	√		√

Litter 2006-37

Rat #	Sex	Treatment	Lesion	1	2	3	4	5	6	7
16	F	Fluoxetine	Sham	√	√		√	√		√
17	F	Fluoxetine	Sham	√	√		√	√		√
18	F	Fluoxetine	Sham	√	√		√	√		√
19	F	Fluoxetine	Sham	√	√		√	√		√
20	F	Fluoxetine	Sham	√	√		√	√		√
21	F	Fluoxetine	Sham	√	√		√	√		√
22	F	Fluoxetine	Sham	√	√		√	√		√
24	F	Fluoxetine	Sham	√	√		√	√		√
26	F	Fluoxetine	Sham	√	√		√	√		√
27	F	Fluoxetine	Sham	√	√		√	√		√
14	M	Fluoxetine	Sham	√	√	√	√	√		√
15	M	Fluoxetine	Sham	√	√	√	√	√	√	√
23	M	Fluoxetine	Sham	√	√		√	√	√	√
25	M	Fluoxetine	Sham	√	√	√	√	√		√
28	M	Fluoxetine	Sham	√	√		√	√		√
29	M	Fluoxetine	Sham	√	√	√	√	√	√	√

Litter 2006-36

Rat #	Sex	Treatment	Lesion	1	2	3	4	5	6	7
1	F	Fluoxetine	Sham	√	√		√	√		√
2	F	Fluoxetine	HI	√	√		√	√		√
4	F	Fluoxetine	Sham	√	√		√	√		√
5	F	Fluoxetine	HI	√	√		√	√		√
8	F	Fluoxetine	HI	√	√		√	√		√
10	F	Fluoxetine	HI	√	√		√	√		√
11	F	Fluoxetine	HI	√	√		√	√		√
3	M	Fluoxetine	HI	√	√		√	√	√	√
6	M	Fluoxetine	HI	√	√	√	√	√		√
7	M	Fluoxetine	Sham	√	√	√	√	√	√	√
9	M	Fluoxetine	HI	√	√	√	√	√	√	√
12	M	Fluoxetine	Sham	√	√	√	√	√	√	√
13	M	Fluoxetine	Sham	√	√	√	√	√	√	√

Litter 2007-3

Rat #	Sex	Treatment	Lesion	1	2	3	4	5	6	7
125	M	Vehicle	HI	x	x	x	x	x	x	x
126	F	Vehicle	Sham	√	√		√	√		√
127	M	Vehicle	Sham	√	√	√	√	√	√	√
128	M	Vehicle	HI	x	x	x	x	x	x	x
129	F	Vehicle	HI	√	√		√	√		√
130	F	Vehicle	HI	√	√		√	√		√

Litter 2007-4

Rat #	Sex	Treatment	Lesion	1	2	3	4	5	6	7
131	M	Vehicle	HI	√	√	√	√	√	√	√
132	M	Vehicle	HI	√	Died	√	Died	√	Died	Died
133	M	Vehicle	HI	√	√	√	√	√	√	√
134	M	Vehicle	Sham	√	√	√	√	√	√	√
135	M	Vehicle	HI	√	√	√	√	√	√	√
136	F	Vehicle	HI	√			√	√		√
137	F	Vehicle	HI	√			√	√		√
138	M	Vehicle	Sham	√		√	√	√	√	√
139	F	Vehicle	HI	√			√	√		√

Litter 2008-9

Rat #	Sex	Treatment	Lesion	1	2	3	4	5	6	7
140	F	Vehicle	Sham	√						
141	F	Vehicle	Sham	√						
142	F	Vehicle	Sham	√						
143	F	Vehicle	Sham	√						
144	F	Vehicle	Sham	√						
145	F	Vehicle	Sham	√						
146	F	Vehicle	Sham	√						
147	F	Vehicle	Sham	√						
148	M	Vehicle	Sham	√	√					
149	M	Vehicle	Sham	√						
150	M	Vehicle	Sham	√	√					
151	M	Vehicle	Sham	√						
152	M	Vehicle	Sham	√						
153	M	Vehicle	Sham	√	√					

√ = included
blank = did not participate
Died = died during the experiment

1= Activity Box
2= Elevated Plus Maze
3= Footprints
4= Forepaw Inhibition
5= Water Maze
6= Single Pellet
7= Tray Reaching

APPENDIX 2

Summary of Rat Subjects Utilized and Their Anatomical Assessments.

Litter 2006-34										
Rat#	**Sex**	**Treatment**	**Lesion**							
				1	**2**	**3**	**4**	**5**	**6**
47	F	Vehicle	Sham		√			√	
48	F	Vehicle	Sham		√				
53	F	Vehicle	Sham		√				
58	F	Vehicle	Sham		√				
49	M	Vehicle	Sham		√				
43	M	Vehicle	Sham		√	√	√	√	
50	M	Vehicle	Sham		√	√	√	√	
Litter 2006-28										
Rat#	**Sex**	**Treatment**	**Lesion**							
				1	**2**	**3**	**4**	**5**	**6**
62	F	Vehicle	Sham		√				√
64	F	Vehicle	Sham		√				√
66	F	Vehicle	Sham		√				
67	F	Vehicle	Sham		√				√
71	M	Vehicle	Sham		√				
73	M	Vehicle	Sham		√				
Litter 2006-38										
#	**Sex**	**Treatment**	**Lesion**							
				1	**2**	**3**	**4**	**5**	**6**
32	F	Fluoxetine	HI	√	√				√
34	F	Fluoxetine	HI	√	√				√
35	F	Fluoxetine	HI	√	√				√
37	F	Fluoxetine	HI	√	√				
38	F	Fluoxetine	HI	√	√				
30	M	Fluoxetine	HI	√	√			√	
31	M	Fluoxetine	Sham	√	√				
33	M	Fluoxetine	HI	x	x	x	x	x	
36	M	Fluoxetine	HI	√	√	√	√	√	
39	M	Fluoxetine	HI	√	√	√	√	√	
40	M	Fluoxetine	HI	√	√			√	

Litter 2006-37

#	Sex	Treatment	Lesion	1	2	3	4	5	6
16	F	Fluoxetine	Sham	√	√				
17	F	Fluoxetine	Sham	√	√				
18	F	Fluoxetine	Sham	√	√				
19	F	Fluoxetine	Sham	√	√				
20	F	Fluoxetine	Sham	√	√				√
21	F	Fluoxetine	Sham	√	√				√
22	F	Fluoxetine	Sham	√	√				√
24	F	Fluoxetine	Sham	√	√				√
26	F	Fluoxetine	Sham	√	√				√
27	F	Fluoxetine	Sham	√	√				√
14	M	Fluoxetine	Sham	√	√			√	
15	M	Fluoxetine	Sham	√	√			√	
23	M	Fluoxetine	Sham	√	√			√	
25	M	Fluoxetine	Sham	√	√	√		√	
28	M	Fluoxetine	Sham	√	√	√	√	√	
29	M	Fluoxetine	Sham	√	√	√	√	√	

Litter 2006-36

#	Sex	Treatment	Lesion	1	2	3	4	5	6
1	F	Fluoxetine	Sham	√	√				
2	F	Fluoxetine	HI	√	√				√
4	F	Fluoxetine	Sham	√	√				
5	F	Fluoxetine	HI	√	√				√
8	F	Fluoxetine	HI	√	√				
10	F	Fluoxetine	HI	√	√				
11	F	Fluoxetine	HI	√	√				
3	M	Fluoxetine	HI	√	√	√	√		
6	M	Fluoxetine	HI	√	√	√	√	√	
7	M	Fluoxetine	Sham	√	√	√	√	√	
9	M	Fluoxetine	HI	√	√				
12	M	Fluoxetine	Sham	√	√				
13	M	Fluoxetine	Sham	√	√	√	√	√	

Litter 2007-3

#	Sex	Treatment	Lesion	1	2	3	4	5	6
125	M	Vehicle	HI	x	x	x	x		
126	F	Vehicle	Sham	√	√				√
127	M	Vehicle	Sham	√	√	√	√	√	
128	M	Vehicle	HI	x	x	x	x		
129	F	Vehicle	HI	√	√				√
130	F	Vehicle	HI	√	√				√

Litter 2007-4

#	Sex	Treatment	Lesion	1	2	3	4	5	6
131	M	Vehicle	HI	√	√	√	√	√	
132	M	Vehicle	HI	√	√				
133	M	Vehicle	HI	√	√	√	√	√	
134	M	Vehicle	Sham	√	√	√	√	√	
135	M	Vehicle	HI	√	√	√	√	√	
136	F	Vehicle	HI	√	√				
137	F	Vehicle	HI	√	√				√
138	M	Vehicle	Sham	√	√	√	√	√	
139	F	Vehicle	HI	√	√				√

Litter 2008-9

#	Sex	Treatment	Lesion	1	2	3	4	5	6
140	F	Vehicle	Sham	√					
141	F	Vehicle	Sham	√					
142	F	Vehicle	Sham	√					
143	F	Vehicle	Sham	√					
144	F	Vehicle	Sham	√					
145	F	Vehicle	Sham	√					
146	F	Vehicle	Sham	√					
147	F	Vehicle	Sham	√					
148	M	Vehicle	Sham	√					
149	M	Vehicle	Sham						
150	M	Vehicle	Sham						
151	M	Vehicle	Sham						
152	M	Vehicle	Sham						
153	M	Vehicle	Sham						

Litter # Yillin	Sex	Treatment	Lesion	1	2	3	4	5	6
11	F	Vehicle	HI	√					√
14	F	Vehicle	HI	√					√
13	F	Vehicle	HI	√					

√ = included
blank = did not participate

1= Body Weight
2= Brain Weight
3= Cortical Thickness
4= White coronal
5= White whole
6= Golgi

(Antier et al., 1998; Bairy, Madhyastha, Ashok, Bairy, & Malini, 2007; Cases et al., 1996; Chou et al., 2001; Douglas, Markham, & Martin, 2004)

(Favaro, Costa, & Moreira, 2008; Gharbawie, Gonzalez, & Whishaw, 2005; Golgi, 1873; Grafe, 1994; Hebb, 1949)

(Ikeda et al., 2001; Kim et al., 2003; Kolb & Gibb, 1990) (Kolb, 2003; Kolb, Gorny, Li, Samaha, & Robinson, 2003; Kolb & Tomie, 1988; Kolb & Whishaw, 1983, , 2003)

(Persico et al., 2003; Schmued, 1990; von Bonin, 1960; Vorhees et al., 1994; Walberer et al., 2006; Whishaw, Zaborowski, & Kolb, 1984; Wilkinson, Auerbach, & Jacobs, 1991) (Xu, Sari, & Zhou, 2004)