

**NEONATAL STROKE IN RATS IMPAIRS BEHAVIOUR, ANATOMY,
AND NEUROPHYSIOLOGY IN ADULTHOOD**

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Dedication

To my Mom and Dad

Abstract

In Canada, every ten minutes someone will have a stroke injury to the brain. According to the Heart and Stroke Foundation of Canada 15% of the patients will die and only 10% of patients will recover normal behaviour completely. The remaining 75% of stroke patients will experience one or many cognitive and motor neurological deficits. This thesis examines a rat model of human perinatal stroke populations most at risk to study the long-term behavioural, neurophysiological, and anatomical outcomes in maturity. Evidence is provided showing that the nature of motor deficits is dependent on the age-of-stroke and earlier ages do not lead to better outcomes. These data are important because they show that motor learning requires an optimal organization of the motor cortex to support motor behaviours. Early experiences, such as a stroke, can impair motor skills and the organization and function of the motor system in adulthood.

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Table of Contents

Title Page	
Approval/Signature Page	
Dedication	iii
Abstract	iv
Acknowledgments	v
Table of Contents	vi
List of Figures	viii
 1. General Introduction	 1
1.1. Overview	2
1.2. Behaviour-Brain Development And The Motor System	3
1.2.1. Behaviour-Brain Development	3
1.2.2. Organization Of The Motor System	4
1.3. Stroke Affects Behaviour-Brain and The Motor System	6
1.3.1. Types Of Stroke	6
1.3.2. Neonatal Stroke	6
1.3.3. Stroke Affects Behaviour-Brain	7
1.3.4. Stroke Affects The Motor System	7
1.4. Treatment Strategies For Brain Injury	8
1.5. Rationale For The Methods	9
1.5.1. Subjects And Ages	9
1.5.2. Hypoxia Ischemia	9
1.5.3. Drug Treatment	13
1.5.4. Behavioural Assessment	15
1.5.5. Motor Mapping	17
1.5.6. Anatomy	21
1.6. Objectives And Hypotheses	22
1.7. Research Questions	23
 2. Hypoxia ischemia on postnatal day 7 does not impinge forelimb motor map development in rat pups	 27
2.1. Abstract	28
2.2. Introduction	29
2.3. Methods	31
2.4. Results	37
2.5. Discussion	48
2.6. Conclusion	57
 3. Abnormal ipsi-lesion motor maps in adulthood following postnatal day 7 hypoxia ischemia in rats	 64
3.1. Abstract	65

3.2. Introduction	66
3.3. Methods	67
3.4. Results	73
3.5. Discussion	83
3.6. Conclusion	87
4. Nicotine or fibroblast growth factor treatment partially ameliorates the deficits in forelimb skills and diminished ipsi-lesion motor maps following postnatal day 7 hypoxia ischemia in rats	91
4.1. Abstract	92
4.2. Introduction	94
4.3. Methods	96
4.4. Results	105
4.5. Discussion	121
4.6. Conclusion	125
5. Motor skill deficits are associated with abnormally large motor maps in adulthood following postnatal day 3 stroke in rat pups	129
5.1. Abstract	130
5.2. Introduction	131
5.3. Methods	132
5.4. Results	138
5.5. Discussion	147
5.6. Conclusion	151
6. Nicotine treatment does not improve reaching skill impairments or motor map deficits following postnatal day 14 hypoxia ischemia in rats	155
6.1. Abstract	156
6.2. Introduction	158
6.3. Methods	159
6.4. Results	167
6.5. Discussion	180
6.6. Conclusion	182
7. General Discussion	185
7.1. Overview	186
7.2. Novel Findings	186
7.3. Proposed Mechanisms	189
7.4. Caveats	199
7.5. Conclusions	203
7.6. Future Directions	204

List Of Figures

1.1	Schematic organization of the motor system	5
1.2.	Hypoxia ischemia stroke method <i>in vivo</i>	12
1.3.	Behavioural methods	18
1.4.	Motor mapping methods <i>in vivo</i>	21
2.1.	Reconstructed motor maps using intracortical microstimulation across postnatal days	39
2.2.	Comparison of hemisphere size for postnatal day 7 (P7HI) cases	42
2.3.	Histology and corresponding motor maps for Sham and postnatal day 7 hypoxia ischemia (P7HI) cases	44
2.4.	Overlay of forelimb motor maps for Sham and postnatal day 7 hypoxia ischemia (P7HI) cases	46
2.5.	Developmental timecourse of forelimb motor maps determined by intracortical microstimulation (ICMS)	47
3.1.	Timeline of experimental procedures	68
3.2.	Infarct variability in adulthood following postnatal day 7 hypoxia ischemia (P7HI)	74
3.3.	Anatomy in adulthood following postnatal day 7 hypoxia ischemia (P7HI)	76
3.4.	Behavioural performance in juveniles following postnatal day 7 hypoxia ischemia	80
3.5.	Motor map organization in adulthood following postnatal day 7 hypoxia ischemia (P7HI)	81
3.6.	Reconstructed motor maps in adulthood following postnatal day 7 hypoxia ischemia (P7HI)	82
4.1.	Timeline of experimental procedures	97
4.2	Infarct variability in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	106

4.3.	Anatomy in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	108
4.4.	Food maneuvering performance in juveniles following postnatal day 7 hypoxia ischemia (P7HI) and treatment	110
4.5.	Reaching performance in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	113
4.6.	Reaching savings in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	115
4.7.	Reaching forgetting in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	116
4.8.	Motor map organization in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	119
4.9.	Reconstructed motor maps in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment	120
5.1.	Timeline of experimental procedures	133
5.2.	Photographs of whole brains from dorsal, lateral, and ventral views	140
5.3.	Reaching success in adulthood following postnatal day 3 hypoxia ischemia (P3HI)	142
5.4.	Motor maps in adulthood following postnatal day 3 hypoxia ischemia (P3HI)	144
5.5.	Reconstructed ipsi-lesion motor maps in adulthood following postnatal day 3 hypoxia ischemia (P3HI)	145
6.1.	Timeline of experimental procedures	160
6.2.	Anatomy in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment	169
6.3.	Histology in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment	170
6.4.	Juvenile motor performance following postnatal day 14 hypoxia ischemia (P14HI) and treatment	173

6.5.	Reaching performance in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment	175
6.6.	Motor map organization in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment	177
6.7.	Reconstructed motor maps in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment	178
7.1.	Model of stroke injury from neonatal hypoxia ischemia	190
7.2.	Facial abnormalities following neonatal hypoxia ischemia	202

Chapter 1

General Introduction

1.1. Overview

Humans are at high-risk for a cerebral stroke to occur during development with a rate of 1/4000 birth's (Lynch et al., 2002). Over 50% of childhood stroke survivors will develop cognitive, sensory, and/or motor neurological disorders that typically persist throughout life. Pediatric stroke patients often show enduring impairments in the ability to perform motor skills necessary for feeding and manipulating objects and will never gain independence because treatment options are limited. A fundamental question is whether the nature of the motor deficits from stroke is similar across developmental age groups. If so, it may be possible that new treatments for stroke would be beneficial equally across age groups. The goal of this thesis was to use behavioural, neurophysiological, and anatomical analyses to determine if there are similarities in the nature of motor deficits following neonatal stroke at different ages. A further goal was to determine if there are drug treatments, such as nicotine or fibroblast growth factor, that can at least partly reverse the motor deficits from neonatal stroke. Delving to obtain these goals will foster a more complete understanding of stroke and contribute to provide a brighter outlook for stroke patients. The following introduction is structured to review behaviour-brain development related to the motor system, provide background about stroke and how it affects behaviour-brain relationships and the motor system, propose treatment strategies for brain injury, and give rationale for the methods used in the experiments. The last sections present the research objectives and hypotheses and finally, the research questions.

1.2. Behaviour-Brain Development And The Motor System

1.2.1. Behaviour-Brain Development

Behaviour is overtly expressed with movements of the body. Movements are classified as reflexive, rhythmic, or voluntary and seem to be performed effortlessly with a mature nervous system. Behaviour typically develops from simple movements after birth, such as reflexive suckling during nursing, to complex movements later in maturation, such as voluntarily using a limb for feeding. Humans are an altricial species with a limited behavioural repertoire at birth and require extensive parental care for many years. Humans develop personal independence as they successfully expanded the behavioural repertoire from simple to more complex movements. Thus, movements are adaptive throughout life and are mediated by altering brain structure and function (Kolb, 1995).

Changes to behaviour-brain relationships are referred to as plasticity. William James was the first to apply the term plasticity to the brain as the interplay between sensory inputs and motor acts. James said: "The currents, once in, must find a way out. In getting out they leave their traces in the paths in which they take. The only thing they *can* do, in short, is to deepen old paths or make new ones" (James, 1890). The notion of plasticity was modernized by the Canadian Donald Hebb who postulated that repeated activity of presynaptic and postsynaptic neural elements leads to changes in 'phase-sequences' (i.e, neural networks) that adapt behaviours from experiences, such as perceptual learning or brain injury (Hebb, 1949). Exactly how the underlying neural circuitry is plastic to support adaptive behaviours is an exciting field of neuroscience.

A number of brain areas and their connections are involved in controlling movements with the limbs. Indeed, it can appear daunting to understand the neural

underpinning of motor control. Fortunately, neural development of the central nervous system (CNS) is similar across many species and is characterized by four stages of growth: neurogenesis, migration, differentiation, and maturation (Kolb, 1995). One important consideration is that the rate of CNS development differs across species. For instance, the absolute gestation period of humans is nine months whereas the gestation period of rats is three weeks. However, rats at birth are relatively comparable to the cerebral maturation of third-trimester humans. It is not fully understood how the CNS is able to establish, refine, and sustain appropriate neural networks of activity to adapt behaviour.

1.2.2. Organization Of The Motor System

The neocortex is an important structure for producing and adapting motor behaviours, especially for voluntary complex movements (Porter and Lemon, 1995). Figure 1.1 shows a simplified schematic of the motor system. A feature of primary motor cortex (M1) organization is that movement fields, conceptually similar to visual fields in visual cortex, are coded in the activity of neural networks that represent maps for movements of the body. For example, M1 has maps of movement fields of the proximal (upper) and distal (lower) forelimb. M1 can mediate muscle activity directly via corticospinal tract (CST) projections to motor units in the spinal cord. The formation of the CST during development is led by pioneer fibers that navigate from the cortical mantle to the spinal grey. Pioneer fibers are steered by attractant and deterrent molecular cues detected by growing axons along which subsequent CST fibers follow (Joosten et al., 1987; Tessier-Lavigne and Goodman, 1996). The CST descends from the cortex via the internal capsule

and is parceled into two components; one forming the lateral corticospinal tract and crosses midline at the pyramids (~90% of fibers); and, one forming the ventral corticospinal tract that does not cross midline. Interestingly, the developing CST originates from many cortical regions and becomes restricted to limited cortical regions a few weeks after birth (Joosten et al., 1987). The mature CST only descends from

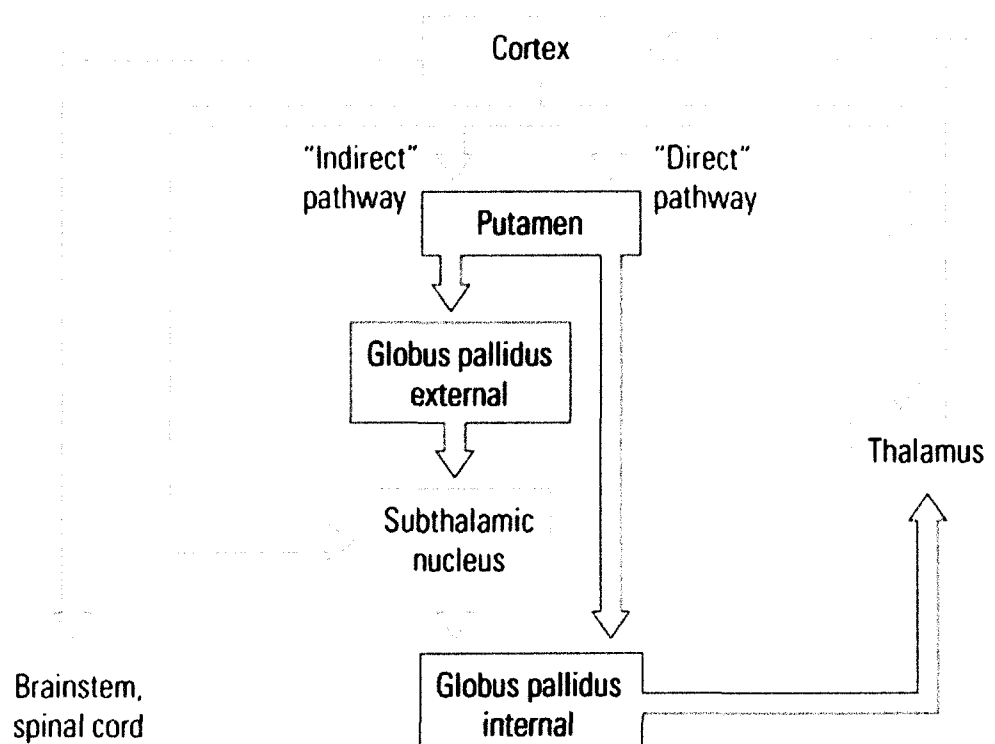


Figure 1.1. Schematic organization of the motor system. The motor system sends motor commands for the control of movement via connections to the brain stem (corticorubral) and spinal cord (corticospinal). An efferent copy from the cortex is conveyed to the basal ganglia. The basal ganglia distribute the efferent copy from the putamen to either the external globus pallidus forming the “indirect” pathway, or the external globus pallidus forming the “direct” pathway. The indirect pathway has an excitatory effect (*blue*) and activates the thalamus and cortex to amplify movements. The indirect pathway has an inhibitory effect (*red*) and shuts- down the thalamus and cortex and curtails movements. Thus, the cortico-basal ganglia-thalamus loops modulate the ‘final common pathway’ to the spinal cord for the production of movement. Adapted from (Kolb and Whishaw, 2003), reprinted with permission.

pyramidal cell axons in layer V in M1, prefrontal cortex, and in somatosensory cortex (Bates and Killackey, 1984). The late development of complex behaviour and the CST are likely more than just coincidental.

1.3 Stroke Affects Behaviour-Brain And The Motor System

1.3.1. Types Of Stroke

Stroke is defined by the National Institute of Neurological Disorders and Stroke (NINDS) as a cerebrovascular accident that disrupts blood flow enough to interrupt brain function. There are two fundamental types of stroke that can occur to arterial or venous vessels of the vasculature. One type of stroke is hemorrhagic (Greek *haima* is blood + *rrhagia* is excessive) and is the least common type to affect humans (~20%). Hemorrhagic stroke is diagnosed when blood flow is disrupted due to a break in a vessel wall, often resulting from an aneurysm rupturing. The other type of stroke is called ischemia (Greek *ischein* is restriction + *haima*) and is the most common type to affect humans (~80%). Ischemic stroke is diagnosed when blood flow is disrupted due to restriction in a vessel, often resulting from a clot inside the lumen that forms onsite (thrombolism) or is displaced from somewhere else in the vasculature (embolism) and restricts blood flow.

1.3.2. Neonatal Stroke

For every 100,000 children in Canada under 19 years old, 6.7 will have a stroke according to the Heart and Stroke Foundation of Canada. Stroke is the leading cause of death in children and is most prevalent in the first year of life. The perinatal period is the highest risk period for stroke to occur and rates among the leading causes of infant death

or enduring cognitive and motor impairments. The NINDS defines perinatal stroke as cerebrovascular accidents occurring between gestation (28 weeks) and the early postnatal period (28 days) in humans. The NINDS defines childhood stroke as cerebrovascular accidents occurring between 0-18 years in humans. One third of pediatric stroke cases have no identifiable cause. Arterial or venous occlusion is caused by similar events across young ages, such as vascular disease, arteriopathies, infection, hypercoagulation, and hypoxia ischemia (Lynch et al., 2002). Hypoxia ischemia stroke is prevalent during the perinatal period and the majority of cases report ischemia within blood flow territories of the middle cerebral artery.

1.3.3. Stroke Affects Behaviour-Brain

There are approximately 300,000 Canadians currently living with the aversive effects of stroke according to the Heart and Stroke Foundation of Canada. The five acute signs that a stroke may have occurred in an adult are: sudden loss of muscle strength or numbness in one side of the body; an inability to articulate or comprehend speech; loss of vision; severe headache; and, dizziness/loss of balance. These five signs may be present individually or in any combination and require immediate medical evaluation.

A fundamental difference between adult and neonatal stroke is that an infant has a limited behavioural repertoire so the signs of a stroke can be more difficult to evaluate. Additionally, because the infant brain is organized differently at the time of injury compared to an adult brain, the neural mechanisms available to respond to the stroke may support alternative outcomes. Another difference is that adult stroke patients will have learned how to perform many motor skills once already, whereas neonatal stroke patients

will not have had this opportunity. Thus, the behavioural effects of stroke are often more severe in pediatric cases because the ability to properly learn new motor skills may be compromised.

1.3.4. Stroke Affects The Motor System

Stroke can affect reflexive, rhythmic, or voluntary movements although impairments in performing voluntary movements can be the most cumbersome to overcome (Twitchell, 1951). Several studies using adult primate or rat models have shown enduring abnormal organization of motor maps following stroke. For example, direct ischemic lesions cause motor map dysfunction of the “core” tissue at the site of the infarct, as well as dysfunction within regions adjacent to the infarct in “penumbra” tissue (Nudo and Milliken, 1996; Nudo et al., 1996). Furthermore, the motor skill deficits from adult stroke are associated with dysfunctional motor maps (Gharbawie et al., 2005; Kleim et al., 2003a; Nudo and Friel, 1999).

There have been no neonatal stroke studies in rats investigating changes in motor map organization. This is surprising given that development is a particularly vulnerable period to interfere with neural wiring of the motor system (Hicks and D'Amato, 1975).

1.4. Treatment Strategies For Brain Injury

The goal of rehabilitation therapy following brain injury is to restore normal behavioural performance. It is generally accepted that the behavioural compensation associated with recovery from stroke is supported by concomitant neural compensation (Kleim et al., 2003b; Nudo, 2003). Two general possibilities exist for neural compensation after injury:

(1) the brain can reorganize existing circuits; or, (2) the brain can develop new circuits mediated by new connections and/or new neurons (Kolb, 1995). The extent of lesion-induced compensatory ‘re-wiring’ depends on many conditions, but likely involves a combination of anatomical and neurophysiological compensation. An intriguing idea is that the immature brain may be able to harness at least some ongoing developmental plasticity to augment the mechanisms of recovery from early brain injury and enhance behavioural outcomes. Drugs may be useful to further augment developmental plasticity to attenuate behavioural deficits from neonatal brain injury.

1.5. Rationale For The Methods

1.5.1. Subjects And Ages

The behaviour, vasculature, CNS organization, and neurophysiology of rats are homologous with humans (Kolb and Tees, 1990). Given the biology between humans and rats is similar, rats are useful experimental models to precisely investigate stroke. The ages to induce a stroke in the thesis experiments were chosen to model high-risk populations of pediatric stroke patients. Postnatal day 3, 7, or 14 rat pups show analogous cerebral maturation of human infants at premature birth (<38 weeks gestation), full-term birth (38 weeks gestation – 6 months), and infants (0.5-1 year) respectively.

1.5.2. Hypoxia Ischemia

There is no experimental model that can be used in animals to exactly replicate a stroke in humans. However, two general approaches have been developed to model ischemia in animals and each model has unique advantages and disadvantages for understanding

aspects of stroke. One general approach to model ischemia is by directly constricting blood flow to a focal region of brain tissue. Direct stroke models typically occlude cerebral vasculature or inject thrombus (i.e., vasoconstrictive) agents into a region of brain tissue to induce ischemia. The major advantage of direct stroke models is they typically produce a focal infarct that can be reproduced with less variability between cases. The major disadvantage of direct stroke models is they are mechanically invasive to the integrity of the skull, dura, pia, and blood brain barrier, which may have secondary effects unlike human stroke.

A second general approach to model ischemia is by indirectly restricting blood flow to global regions of brain tissue. Indirect stroke models typically occlude vessels peripheral to the cerebrum, such as the common carotid or vertebral arteries (one or more), to restrict blood flow to downstream tributaries irrigating the forebrain or hindbrain respectively. The major advantage of indirect stroke models is they do not mechanically disrupt the skull, dura, or pia, and may permit reperfusion of blood flow after an ischemic episode. The major disadvantage of indirect stroke models is they produce a global infarct that is less reproducible across cases clouding the ability to attribute behavioural deficits to precise brain areas across cases.

The thesis experiments adopted an indirect approach of ischemia known as hypoxia ischemia. Most importantly, hypoxia ischemia was the choice of model because it is recognized as a predominant type of stroke in human neonates (Yager and Ashwal, 2009). Additionally, hypoxia ischemia is well characterized for neonatal rats (Vannucci and Vannucci, 2005) and it was useful to maintain the integrity of the skull, dura, and pia to avoid confounding the neurophysiological experiments performed in adulthood.

Hypoxia ischemia is achieved by surgically accessing a common carotid artery via an incision in the neck near the thyroid gland. The artery is separated from the adjacent vagus and sympathetic nerves and is occluded caudal to the internal and external carotid artery, which alone does not produce ischemia. The subject is subsequently exposed to an hypoxic environment and, in combination with prior common carotid artery occlusion, together produces unilateral ischemia in the hemisphere ipsilateral to the occluded artery (Fig 1.1). Ischemia is suspended when the subject is returned to a normoxic environment, even if the carotid artery is permanently occluded.

The objective of the thesis was to model moderate stroke injuries because they are the most prevalent in pediatric stroke populations. There is a positive linear relationship between severities of infarct pathology with the duration of hypoxia. Previous studies have determined that hypoxia for 90 minutes in an 8% oxygen environment typically produces moderate brain pathology signified by global reductions in size of the lesion hemisphere, although severe lesions that produce widespread atrophy may show signs of porencephaly. To allow for more direct comparisons between the experimental age groups (P3, P7, or P14) the hemisphere targeted for inducing ischemia (right hemisphere) and the duration of hypoxia (90 minutes) was kept consistent.

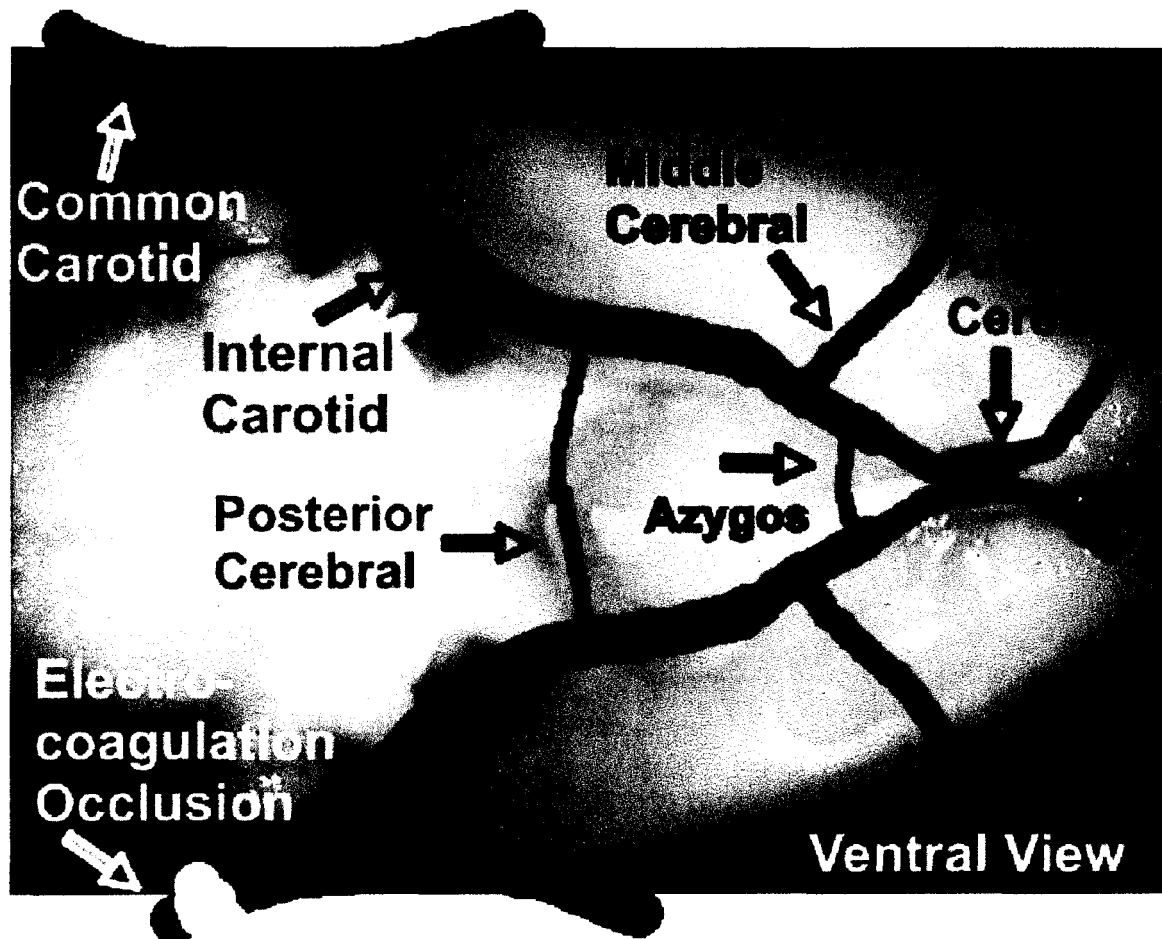


Figure 1.2. Hypoxia ischemia stroke method in vivo. Two main sources of blood to the forebrain are via the common carotid arteries; one for each hemisphere. The common carotid bifurcates forming the internal (and external) carotid artery that irrigates the cerebrum. Occluding one common carotid with electro-coagulation (*bottom left*) may potentially reduce blood flow to the ipsi-occlusion hemisphere affecting the internal (and external) carotid and its branches, the middle cerebral and anterior cerebral arteries. Common carotid artery occlusion alone does not produce ischemia because of collateral anastomoses with the opposite hemisphere; the middle cerebral via the arterial circle formed by junctions of the internal carotid arteries and also the posterior cerebral artery; and, the anterior cerebral artery via the azygos communicating artery linking the hemispheres. An ischemic stroke is produced by further reducing blood pressure to the whole brain during subsequent exposure to low oxygen for an extended period of time (8% O₂ for 90 minutes).

1.5.3. Drug Treatment

There is an urgent need to develop new treatments for stroke patients to promote better outcomes. Only a few treatments are available for stroke related disorders, but none completely restore normal behaviour. A barrier to evaluating new treatments for stroke is that very little is known about when and how much treatment to give to patients. Drugs that do provide some benefit to a small population of adult stroke (ischemia) patients, such as restoring blood flow with the clot buster tissue plasminogen activator (tPA), are effective if administered within a few hours after the stroke occurs. Administration of tPA is not approved for pediatric stroke and may be ineffective in most cases because the average time after a stroke a child presents at hospital and receives diagnosis is 24 hours (Lynch et al., 2002).

The thesis studies began administrations of drug treatment 24 hours after inducing hypoxia ischemia stroke. The treatment continued for one week to ensure the drug would be available to the brain during stages of intense neuronal growth and refinement (see above).

Nicotine: Otto Loewi was the first to show compelling evidence that neural activity is modulated by transmitting chemicals between synapses in his breakthrough experiments in 1922. One of the chemicals Loewi was studying was isolated and identified as acetylcholine. It is now recognized that the acetylcholine neurotransmitter system is implicated in a variety of brain functions including neuronal growth, synaptic efficacy, learning and memory, as well as reward (Jones et al., 1999). Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels with multiple subunits found on

presynaptic and postsynaptic neurons throughout the cholinergic system. The concentration of nAChRs in hippocampus and cortex is most dense during development.

Nicotine is a drug with high affinity to nAChRs and readily crosses the blood-brain barrier when administered peripherally. There is evidence that the function of nAChRs declines in human ageing and is adversely affected in cognitive disorders, such as Alzheimer's disease. Recent studies show that low dose administration of nicotine can promote beneficial outcomes for cognitive (Brown et al., 2000) and motor (Gonzalez et al., 2006) impairments in adult rats, presumably by altering synaptic connections in existing networks to support behavioural compensation (Brown et al., 2000; Robinson and Kolb, 2004). The therapeutic potential of nicotine administration following neonatal stroke has not been explored.

bFGF: A remarkable experiment by Rita Levi-Montalcini and Viktor Hamburger in 1951 showed that when a mouse sarcoma was implanted in a chick embryo, the implanted tumor released a peptide (nerve growth factor) that amplified growth of sympathetic nerve buds. Their findings stimulated many researchers to look for other peptides that regulate cellular growth and several 'growth factors' have been identified. An important class of growth factors for brain maturation is the fibroblast growth factor family. Fibroblast growth factors play a role in the embryo to signal proliferation of mesodermally and ectodermally derived cells. Two families of fibroblast growth factors have been characterized if the compound is acidic (FGF-1) or basic (FGF-2, also known as bFGF).

bFGF is expressed in neurons and glia throughout the lifespan and readily crosses

the blood-brain barrier when administered peripherally. Extracellular bFGF exerts its effects by binding to cellular surface receptors. Interestingly, bFGF is especially important for regulating cortical size (Vaccarino et al., 1999) and can nourish existing circuits or promote implementing new circuits to support behavioural compensation (Monfils *et al.*, 2006). The therapeutic potential of bFGF administration following neonatal stroke has not been explored.

1.5.4. Behavioural Assessment

Cylinder Task

The cylinder task is a commonly used task to investigate asymmetries (i.e., preferences) in forelimb use during exploration (Schallert et al., 2000). The cylinder task is an ecologically-relevant behaviour that does not require training and can be used in juvenile or adult rats. In this task, the subject was placed in the middle of a vertical cylinder and was permitted to freely explore. In a bout of exploration a rat will spontaneously rear by raising the body up and touch a forepaw on the wall of the cylinder for support. The rat then scans the cylinder with successive stepping movements with the forelimbs on the cylinder wall (Fig 1.2. *Top*). A bout of exploration is completed when the rat rears down and touches the floor with the forelimbs (Gharbawie et al., 2004).

The cylinder task was typically administered in the thesis experiments on postnatal day 30 to obtain an early indication of limb preferences in the juvenile period. Numerous variables were scored across several bouts of exploration including: the forelimb used to make initial contact with the cylinder wall upon rearing; the number of total touches with each forelimb across rears; and, the first forelimb to make contact with

the floor when descending from a rear.

Food Maneuvering Task

The food maneuvering task assesses a rats' ability to manipulating food items with their forelimbs and digits during feeding (Whishaw et al., 1997). The food maneuvering task is an ecologically-relevant behaviour that does not require training and can be used in juvenile or adult rats. In this task, a rat familiar to the food items and testing environment will spontaneously begin to eat a food item placed on the floor immediately. The forelimbs and digits are used to grasp and maneuver the item into the mouth (Fig 1.2. *Middle*).

The food maneuvering task was administered in the thesis experiments between postnatal days 42-45 to obtain a measure of limb preferences in the juvenile period. The behaviour of the rat is scored on their preference to use ipsi-lesion forelimb and digits to facilitate feeding. The performance of juvenile rats in this task has not been previously described following neonatal stroke.

Skilled Reaching Task

The skilled reaching task assesses a rats' ability to use a forelimb to reach for a single small food item and put it in the mouth (Whishaw et al., 1991). The skilled reaching task is an ecologically-relevant behaviour that is goal-directed requiring training over several daily sessions and can be used in adult rats. In this task, a rat is trained to use a forelimb to reach through an opening in the testing apparatus box, grasp a food item, and transport the food item to the mouth (Fig 1.2. *Bottom*).

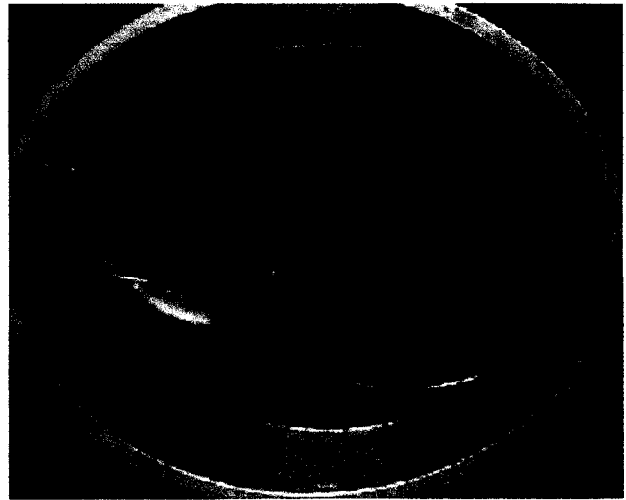
The skilled reaching task was administered in the thesis experiments during adulthood. Reaching success and the number of attempts across 14 training session was measured. Only the contra-lesion forelimb was trained to facilitate relating motor learning with changes in neurophysiology. The performance of adult rats in this task has not been previously described following neonatal stroke.

1.5.5. Motor Mapping

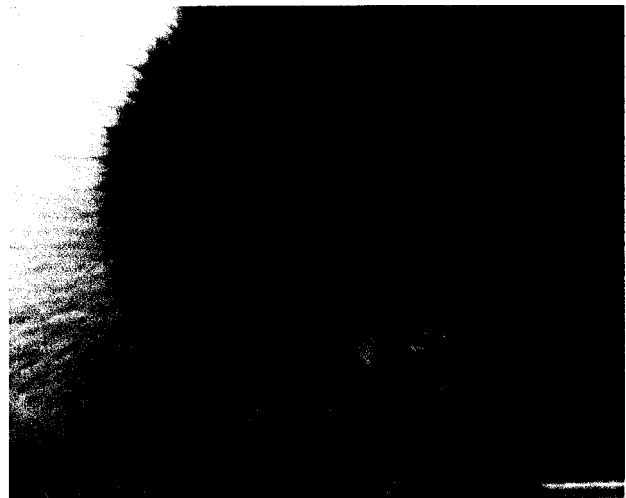
The discovery that electrical stimulation to regions of the cortex could evoke movements generated a great deal of interest in “mapping” the organization of the motor system. Superficial stimulation, intracortical microstimulation (ICMS), and more recently, transcranial magnetic stimulation, techniques have been developed to investigate cortical motor maps in numerous species, including humans (Penfield, 1958; Woolsey and Erickson, 1950), non-human primates (Kaas, 1991; Sherrington, 1947; Woolsey, 1963), and rodents (Asanuma and Sakata, 1965; Donoghue and Wise, 1982; Neafsey et al., 1986). Motor mapping techniques have the common effect of activating the CST to evoke movements, primarily on the contra-stimulation side of the body. The details of corticospinal tract activation differ between motor mapping techniques and will be discussed briefly.

Superficial stimulation and transcranial magnetic stimulation are considered indirect motor mapping techniques because current is applied to the scalp or subdurally. The major advantage of indirect techniques is that they are minimally invasive, relatively easily administered, and can be performed more readily in longitudinal studies in humans. A major disadvantage of non-invasive techniques is that they are regarded as

Cylinder Task



Food Maneuvering Task



Skilled Reaching Task

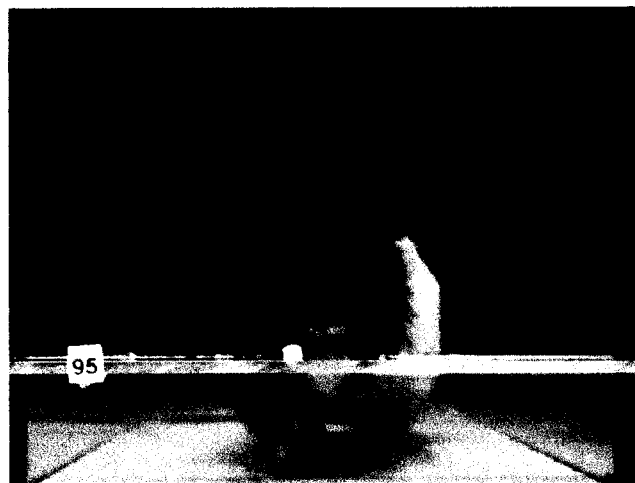


Figure 1.3. Behavioural methods. Still frames of rats performing movements with their forelimbs during behaviour. See text for task descriptions.
low-resolution mapping because a relatively large current pulse is required to activate the

corticospinal tract descending from pyramidal neurons in the deep layers of cortex. Furthermore, because a widespread amount of neuropil is electrically stimulated across cortical layers, it is less clear if the evoked-movements are attributable to either a discrete area of corticospinal tract activation, a broader region of corticospinal tract fibers that could be recruited by neural elements across cortical layers, or both.

ICMS is recognized as a direct motor mapping technique because electrical current is applied through a fine-tip electrode into layer V of motor cortex. A major advantage of ICMS techniques is they are considered high-resolution mapping because a small current pulse is delivered from an electrode lowered into the cortical proximity of the corticospinal tract pyramidal cell bodies (i.e., close to the axon hillock). Furthermore, because a small amount of neuropil is activated, the evoked-movements are attributable to fewer corticospinal neurons and subtle changes in map organization are more likely to be detected. A major disadvantage of ICMS techniques is that they are not easily administered and are less amenable for longitudinal studies, at least in rats.

The thesis experiments used ICMS to investigate the organization of motor maps. The forelimb motor map was investigated to relate potential differences in forelimb abilities observed in the behavioural tasks with the corresponding neural representations. Forelimb representations are topographically organized and are subdivided into two subregions in the cortex of rats; the caudal forelimb area (CFA) and the rostral forelimb area (RFA) motor maps (Neafsey and Sievert, 1982). Each region consists of a mosaic of wrist and digit (distal) as well as elbow and shoulder (proximal) representations. The organization of forelimb motor maps was investigated, in both hemispheres in several of the thesis experiments, to determine the source of: a) stroke-induced changes; b) post-

stroke treatment-induced changes; and, c) post-stroke motor skill learning-induced changes. Maps in the ipsi-lesion hemisphere were of priority and were assessed first. Pilot experiments determined that there were no obvious differences which hemisphere was mapped first, or if mapping one hemisphere altered the forelimb map borders in the opposite hemisphere (data not shown).

The details of the ICMS mapping parameters were selected because they are known to detect changes in map organization from experiences, such as early brain injury, and reorganization from experiences, such as motor learning in adulthood (Williams et al., 2006). The chosen ICMS protocol is considered ‘threshold’ mapping because the least amount of current intensity, below a maximum of 60 μ A, was used to allocate evoked-movements to the motor map. To further ensure that the least amount of current was used for mapping, the fewest number of stimulation sites necessary to complete the forelimb map at high-resolution was used. That is, only forelimb maps and their immediate borders were investigated. Motor mapping started by locating a site that evokes forelimb movement and moving the electrode in steps to neighbouring sites to make an outline the forelimb map, and then to fill in the map completely (Fig 1.3). The location and size of the CFA is more predictable and larger than the RFA, so the CFA was mapped first, followed by the RFA. After mapping the CFA and RFA, multiple border sites of the forelimb map in medial, lateral, posterior and anterior orientations were re-tested for confirmation.

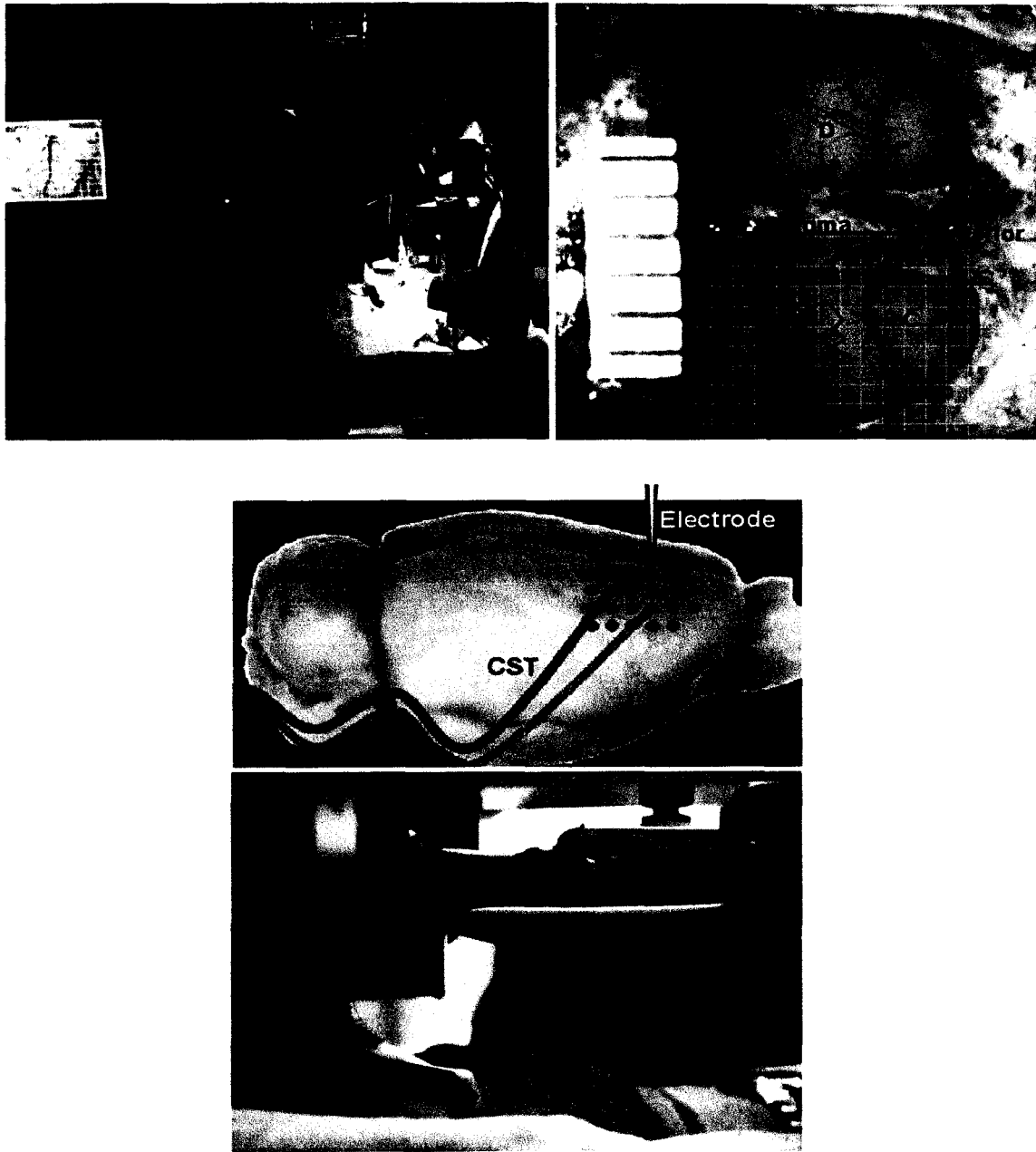


Figure 1.4. Motor mapping methods in vivo. *Top Panel:* Surgery (*left*). A rat was anesthetized and the skull over frontal cortex surgically removed. Order of motor mapping (*right*). A photo of the rat's cortical surface was taken and used to plot intracortical microstimulation sites (grid = 0.5 mm²). A forelimb movement site was located in the caudal forelimb area (A₁) and outlined in a clockwise sequence of steps (A₂) and then filled-in (B). Then the rostral forelimb area (C) was completed and then the contra-lesion hemisphere (D). *Middle Panel:* Microstimulation. Electrical current is applied through an electrode in layer V of motor cortex (M1) to evoke the corticospinal tract (CST). *Bottom Panel:* Evoked movement. An experimenter monitors the rat's elbow and shoulder (blue), and wrist and digit (green), as well as the rest of the body, for movements corresponding with microstimulation.

1.5.6. Anatomy

Body and brain weights were measured to compare the general growth of the rats. The most common approach to describe lesion pathology in models of stroke is to measure the size of an infarct core. Hypoxia ischemia does infarct brain tissue, however, it can be difficult to determine the extent of an infarct core because the ischemia is global. There is less known about the organization of the residual brain tissue following neonatal stroke and is surprising considering that residual tissue presumably mediates the motor outcomes in adulthood.

The histological approach of the thesis experiments describes the pathology from neonatal hypoxia ischemia stroke, if any, in residual brain structures important for the control of motor behaviour. Cortical thickness measured in cresyl violet stained sections provided a gross assessment of cortical integrity in regions with corticospinal projections (cingulate, motor, and somatosensory areas). Myelin histochemistry (Schmued, 1990) provided a measure of axonal integrity in pathways connecting each hemisphere (corpus callosum), or pathways connecting to the spinal cord (internal capsule).

1.6. Objectives And Hypotheses

The main objectives of the present thesis were to characterize the nature of motor deficits in a model of neonatal stroke. A secondary objective was to determine if motor deficits could be ameliorated with treatments. Based on previous work in the Kolb lab indicating that age, and extent, of brain injury are the most influential factors to predict outcomes, a reasonable hypothesis is that there are differences in the nature of the deficits following hypoxia ischemia occurring on different neonatal ages. However, the direction of the

effects (for better or worse) is not easily predicted for age and extent of injury following hypoxia ischemia because the stroke model shows focal (ischemia core), and diffuse (whole hemisphere) pathological features. That is, if brain injury from hypoxia ischemia is mainly focal, then the P7 group may show the best outcomes (Kolb and Whishaw, 1985; Kolb and Cioe, 2000), whereas if the brain injury is mainly global, then the P3 group may show the best outcomes (Kolb and Tomie, 1988; Kolb et al., 1992). The treatments are likely to show the most benefit in neonatal stroke age groups with the worst outcomes.

1.7. Research Questions

The foremost question of the thesis studies asks whether varying the neonatal age-at-stroke produces similar outcomes in motor behaviour, anatomy, or motor maps in adulthood. A second question asks to what effect post-stroke drug treatments promote recovery of the deficits, if any. Chapter 2 investigates the short-term (12 days post-stroke) effects of postnatal day 7 hypoxia ischemia (P7HI) on anatomy and development of the motor map. Chapter 3 investigates the long-term effects of P7HI on juvenile behaviour, adult anatomy, and adult motor maps. Chapter 4 investigates the effects of drug treatment, either nicotine or bFGF, following P7HI on juvenile and adult motor behaviour, adult anatomy, and adult motor maps. Chapter 5 investigates the effects of postnatal day 3 hypoxia ischemia (P3HI) on adult motor behaviour, adult anatomy, and adult motor maps. Chapter 6 investigates the effects of postnatal day 14 hypoxia ischemia (P14HI) on juvenile and adult behaviour, adult anatomy, and adult motor maps. New evidence is provided about the mechanisms of behaviour-brain plasticity.

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Chapter 2

Hypoxia ischemia on postnatal day 7 does not impinge forelimb motor map development in rat pups.

2.1. Abstract

Survivors of pediatric stroke often experience long-term neurological motor disabilities. It is possible that neonatal stroke alters the development of cortical functions, such as motor maps, leading to abnormal control of movement. The objective of this study was to determine the timecourse of motor map emergence in rats, and to assess the effects of neonatal stroke on map emergence. Intracortical microstimulation (ICMS) did not evoke motor representations reliably until postnatal day 19 so this age was used to compare the effects of neonatal stroke. Hypoxia ischemia stroke on postnatal day 7 (P7HI) was achieved by occluding one common carotid artery followed by 90 minutes of hypoxia (8% oxygen). After 12 days recovery, motor maps were assessed in both hemispheres for Sham and P7HI pups on postnatal day 19. Motor mapping indicated P7HI did not delay the age of onset of evoked movements, or change map location, nor stimulation threshold, in either hemisphere compared to Sham. P7HI ipsi-lesion maps had less shoulder and elbow representation, however. P7HI pathology showed a smaller ipsi-lesion hemisphere but no change in cortical or callosal thickness. Thus, early assessment of maps after P7HI showed a similar profile to Shams despite diffuse pathology in the ipsi-lesion hemisphere from P7HI. Post-map emergence may be a desirable period for treatments following neonatal stroke in effort to avert dysfunction in adulthood.

2.2. Introduction

Pediatric stroke is a leading cause of long-term neurological motor disabilities. The impairments from pediatric stroke are marked by limb spasticity, hemiparesis, poor dexterity and coordination, and cerebral palsy in severe cases. The prevalence of pediatric stroke is 1/4000 births, and is becoming increasingly recognized as a major contributor to childhood disorders (Lynch et al., 2002). The majority of perinatal ischemic strokes, as is true in adult strokes, affect brain areas irrigated by the middle cerebral artery and may directly or indirectly damage motor areas and their pathways. Indeed, cross-sectional imaging studies following pediatric stroke have shown that the development of the corticospinal tract (CST) is impaired (Berweck et al., 2008; Kirton et al., 2007). Integrity of the CST has been well established across many species as an important pathway for voluntary motor control (Iwaniuk and Whishaw, 2000).

A milestone of CST maturation is the emergence of neural networks capable to influence spinal motor circuitry for the control of movements (Martin, 2005). Motor cortex and the corticospinal tract (CST) may be vulnerable to neonatal stroke because both structures mature substantially during the postnatal period. Developmental studies using various types of neonatal lesions to directly damage motor cortex areas have shown tremendous plasticity of behaviour, the developing CST, and motor maps, when assessed in adulthood (Brus-Ramer et al., 2007; Castro, 1975; Eyre, 2007; Gibson et al., 2000; Kennard, 1936; Kennard, 1938; Kolb et al., 2000a; Kolb et al., 2000b; Monfils et al., 2005; Rouiller et al., 1991). It is less clear what the effects of diffuse and variable injuries sparing motor cortex, such as neonatal stroke, are on motor map development and this

was the focus of the current study. It is likely that the motor deficits from neonatal stroke are related to abnormal motor cortex and accompanying pathways, and the observed motor deficits are correlated with abnormal motor maps. Insights from adult studies suggest that stroke can lead to ipsi-lesion map dysfunction abruptly by altering cortical connections (Brown et al., 2007; Brown et al., 2008) and can last for several weeks (Gharbawie et al., 2005; Gharbawie et al., 2008; Nudo and Milliken, 1996).

Hypoxia ischemia induced on postnatal day 7 (P7HI) is the most commonly used rat model to study pediatric arterial ischemic stroke (Vannucci and Vannucci, 2005). Previous studies have shown that P7HI produces deficits in forelimb skills in adulthood (Grow et al., 2003; Tomimatsu et al., 2002) although the neural underpinnings of the deficits are poorly understood. To investigate the nature of potential changes to motor maps following neonatal stroke, P7HI was induced by common carotid artery occlusion followed by exposure to hypoxia (8% oxygen for 90 minutes). The timecourse of motor map emergence is unknown in the rat and warrants investigation considering that rats are the most widely used laboratory animals in stroke studies. The neurophysiological timecourse of motor map development can be studied in vivo with intracortical microstimulation (ICMS) (Chakrabarty and Martin, 2000). The earliest age that forelimb movements could be evoked was determined in normal rats using ICMS under light ketamine preparations on several postnatal ages. In a second experiment, a group was given P7HI and motor map emergence was investigated. The mapping results are discussed in relation to histological measures on hemisphere size, cortical thickness, and myelinated fiber pathways.

2.3. Methods

Subjects and Housing

Long-Evans male rat pups (N=30) from four dams bred at the Canadian Centre for Behavioural Neuroscience breeding colony were used in this study. There are two experiments in this report. Experiment 1 was a cross-sectional timecourse study in naïve pups to determine when movements can be first evoked by intracortical microstimulation (ICMS). ICMS was performed with separate pups on postnatal day (P) 10, 12, and 14 (N's=2); 15, 17 and 19 (N's=3). In Experiment 2, rat pups received Sham or Postnatal day 7 Hypoxia Ischemia (P7HI). ICMS mapping commenced on P19 (Sham N=5; P7HI N=5) or P25 (Sham N=2; P7HI N=3). Group assignment was counterbalanced within and across two litters used for Sham (N=7) and P7HI (N=8). One additional Sham was mapped prior to full adulthood on P52. Rats were housed in standard laboratory cages with food and water available *ad libitum*. Weaning occurred on P23 and consisted of separating the litter by sex. This study was approved by University of Lethbridge Animal Welfare Committee and procedures followed institutional and the Canadian Council for Animal Care guidelines. Use of animals was minimized and effort was taken to reduce discomfort.

Experiment 1

Motor Mapping

Intracortical microstimulation (ICMS) was used to map the emergence of forelimb representations during development. Both hemispheres were mapped in at least one rat at

each age tested. Rats were anesthetized with ketamine hydrochloride (30 mg/kg, i.p.), which maintains muscle tone, and supplements were given when necessary (10 mg/kg, i.p.). The dorsal head was shaved and wiped with betadine, and the eyes were bathed 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) to guide and record electrode penetration sites. The inter-penetration distance between microstimulation sites was 350 μm while avoiding vasculature branches visible on the surface of the brain.

The electrode consisted of a platinum filament inserted in a borosilicate glass micropipette (20-40 μm tip diameter, 15° bevel) filled with saline (3.5 M). The electrode was fastened to a tower micromanipulator on the stereotaxic apparatus. The electrode was manually lowered so the tip penetrated to 200-1600 μm beneath the cortical surface where microstimulation evoked movements with the lowest 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 μ A up to 60 μ 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 the completion of motor mapping under standard ICMS parameters outlined above, the cortex was re-investigated using thresholds to a maximum of 150 μ A. In such cases, non-responsive and new microstimulation sites were assessed, first with current intensities between 0 μ A and 60 μ A and if still unresponsive, then current intensities between 0 μ A and 150 μ A were used. Areal measurements of forelimb representations were conducted to scale using Canvas software. 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 separately outlined and measured.

Experiment 2

Postnatal Day 7 Hypoxia Ischemia (P7HI)

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 (4%). Pups were placed in a supine position on an operating table where they were maintained under Isoflurane (2%) delivered through a modified nose cone. The ventral neck was cleaned and an incision along midline was made. Exposed muscles were separated permitting access to the common carotid artery (CCA). The right CCA was ligated and 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. Exposed arterial tissue was permanently occluded with bipolar electrocoagulation. Muscles were repositioned and the incision was closed using Vetbond tissue adhesive. Pups were placed in an incubator (36.5°C) for 30 minutes to recuperate. Small groups of pups were placed in a glass jar maintained at 36.5°C with surrounding water bath. Hypoxic conditions were created by delivering 8 % oxygen (balance nitrogen) at 110 mm Hg via a tube inserted into the jar. Pups were returned to the incubator for 10 minutes of recovery. Once mobile, the pups were returned to their dam. Sham surgery included all procedures except right CCA occlusion (and vagus nerve separation). There was no mortality from CCA occlusion or hypoxia exposure.

Motor Mapping

The ipsi-lesion hemisphere in the P7HI group was of primary interest so the right hemisphere was mapped first (including Sham). Both hemispheres were mapped except for the contra-lesion hemisphere equivalent in one Sham. The electrode was transferred between hemispheres after every five microstimulation sites tested as a control procedure.

The experimenters were blind to group membership. See methods in Experiment 1.

Gross Anatomy

Brain and Body Weight

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

Histology

After each intracortical microstimulation session, an overdose of Euthensol (80 mg/Kg i.p.) was given. Rats were cardiac perfused with saline wash and 4% formalin fix. 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. Brains were freeze-sectioned on a cryostat at 40 μ m thicknesses. Every tenth section was collected on 1% gelatin + 0.2% chromatin coated glass slides and dried overnight. Digital images of the sections were gathered with a Zeiss Axiovision 4.3 (Zeiss, Germany) at 1x magnification outfitted with a CCD camera. ImageJ freeware (Image J, Bethesda, MD) was used for measurements. Coordinates for measurements correspond to Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998).

Myelin Histochemistry

Sections were incubated in 0.2% gold chloride in phosphate buffer at 40°C for 1-2 hours

until myelinated bundles appeared in shades of purple/brown (Gold chloride solution: 1.8 g crystalline gold chloride; 0.33 g sodium phosphate monobasic monohydrate; 3.6 g sodium phosphate dibasic anhydrous; and, 9.0 g sodium chloride in 1000 mL distilled water). The reaction was stopped by washing for 5 minutes in distilled water, fixing for 5 minutes in 2.5% sodium thiosulfate, and rinsing for 30 minute under slow running tap water. Sections were air-dried before cover slips were secured with Permount mounting media.

Hemisphere Size

The whole hemisphere was outlined and calculated for Anterior and Posterior planes (Plate 19, -0.30 mm Bregma; Plate 27, -1.88 mm Bregma, respectively).

Cortical Thickness

Cortical thickness was measured from areas with corticospinal projections in motor cortex (M1) and somatosensory (S1) cortex from planes Anterior, Central, and Posterior to Bregma. In addition, cingulate cortex (Cg1) in Anterior and Central planes, or retrosplenial cortex (RSGb) in the Posterior plane was measured (generally referred to as cingulate unless otherwise stated). The Planes corresponded to Plate 9, Plate 18, and Plate 29 (Bregma 2.70, -0.03, and -2.30) respectively.

Myelination

The area of axonal pathways was assessed for the corpus callosum and the fimbria of the

hippocampus from Anterior and Posterior planes (Bregma 2.70 and -2.30 respectively).

The structure of interest was outlined and area calculated. The internal capsule was not amenable to quantify because of the diffuse course through the brain; however, qualitative assessments were made.

Statistics

Analysis of Variance (ANOVA) was used in Experiment 2 to compare: body weight; brain weight; mean cortical thickness of M1, S1, and Cg1; myelin area of corpus callosum and fimbria fornix; and, motor maps. Group (Sham and P7HI) was the between-subject factor unless otherwise stated. Hemisphere size in P7HI operates was analyzed with paired t-test comparisons with Bonferroni-Holm's adjustments.

2.4. Results

Experiment 1

Motor Mapping

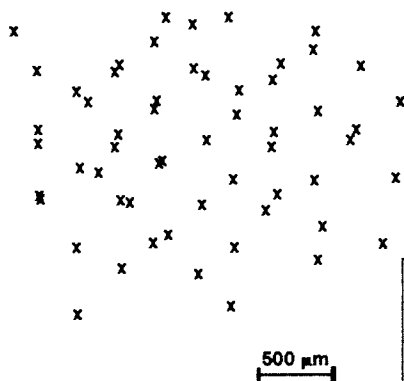
Timecourse mapping in developing rats indicated that movements were not observed from stimulation on postnatal days 10, 12, 14, and 15 (P10, P12, P14, P15; Fig 2.1). In two P15 cases, the hemisphere was extensively probed throughout the parietal and frontal cortex to ensure the localization of cortically evoked movements would be captured if present, but to no avail.

The age that forelimb movements could be first evoked was on P17 in one of three cases (see P17a and P17b; Fig 2.1). The types of forelimb movements observed in case

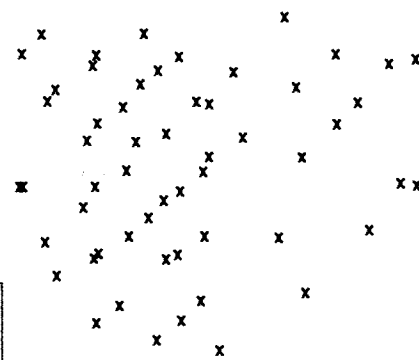
P17b consisted of both proximal movements (elbow and shoulder) and distal movements (wrist and digit). The lowest thresholds were obtained at penetration depths at 1100 μm below the cortical surface. These forelimb representations may be considered part of the caudal forelimb area based on the localization of these forelimb sites in relation to Bregma. There were no effective sites in the presumptive rostral rostral forelimb area. There were two sites that evoked vibrissae movements within the medial vibrissae representation that normally borders the forelimb representations.

Forelimb movements were evoked in every case on P19 and thereafter. Proximal (shoulder and elbow) and distal (wrist and digit) forelimb movements were evoked from the caudal forelimb area. The lowest thresholds were obtained at penetration depths of 1300 μm below the cortical surface. ICMS did not evoke ipsilateral or bilateral movements at any age. The pattern of motor map results was consistent for both hemispheres.

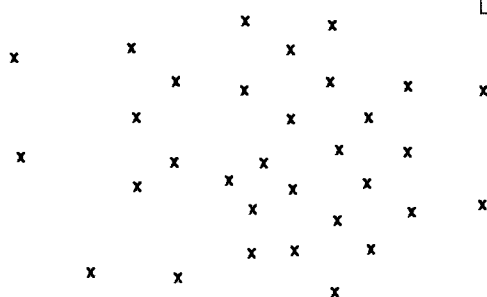
P12



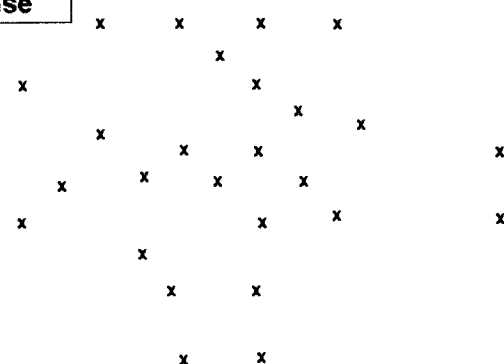
P14



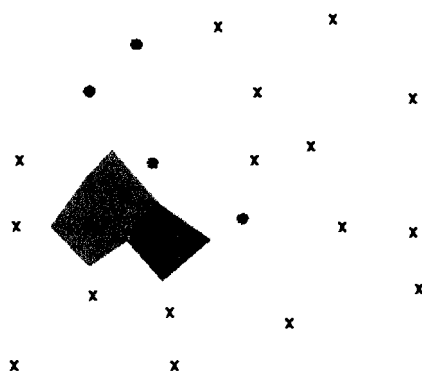
P15



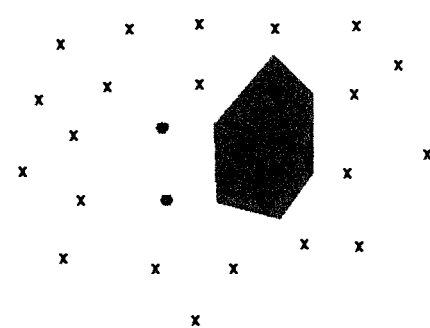
P17a



P17b



P19



	Anterior
	Bregma
●	Wrist
◆	Shoulder/Elbow
◆	Digit
●	Hindlimb
●	Trunk
●	Neck/Jaw
●	Vibrissae
X	No Response

Figure 2.1. Reconstructed motor maps using intracortical microstimulation across postnatal days (P). Movements could not be evoked prior to P17 and were found in only one of three cases at P17 (P17b). Movements could be evoked in every case on P19. Elbow, wrist, or digit movements were evoked at adjacent microstimulation sites. Non-forelimb movements that normally border the forelimb area could also be evoked. Data shown for P12 and P14 are overlays of two cases at each age. See inset for legend.

Experiment 2

Gross Anatomy

Body and Brain Weight

The P7HI group appeared physically normal except the ipsi-lesion side of the face appeared narrower with a ptotic eye. There were no differences in body weight between Sham and P7HI rats [$F(1,8)=0.40$, $P<0.05$]. Brain weights of the P7HI operates were not different than Sham operates [$F(1,8)=0.48$, $P>0.05$].

Histology

There was individual variability in brain morphology amongst the P7HI group. Histological inspection showed 75% of P7HI cases had moderate lesions with a smaller ipsi-lesion hemisphere (Fig 2.2 and 2.3). There were two severe cases of P7HI with ipsi-lesion porencephalic cavities in cortex posterior to Bregma. In addition, the severe P7HI cases showed extensive subcortical damage to striatum and internal capsule. The two severe P7HI cases were excluded from histological statistical analysis.

The ipsi-lesion hemisphere in P7HI cases showed enlargement of the lateral ventricle, smaller caudate putamen, smaller hippocampus, and reduced myelin staining in

the internal capsule (P7HI-A, P7HI-B; Fig 2.3). The anterior commissure and cingulum bundle appeared normal. There were no changes in cortical thickness or area of the corpus callosum or fimbria.

Hemisphere Size

There were no differences in overall size between the Ipsi-lesion and the Contra-lesion hemisphere amongst P7HI cases in the Anterior Plane [$t(4)=2.31$; $P > 0.05$]. The P7HI group has smaller Ipsi-lesion hemisphere in the Posterior Plane [$t(4)=2.65$, $P < 0.05$; Fig 2.2].

Cortical Thickness

The Ipsi-lesion thickness of M1, S1, or Cg1 showed no differences between the groups [M1 $F(1,8)=1.28$, S1 $F(1,8)=0.29$; Cg1 $F(1,8)=1.53$, $P's > 0.05$]. The Contra-lesion thicknesses also did not show differences between the groups [M1 $F(1,8)=0.41$, S1 $F(1,8)=0.01$; Cg1 $F(1,8)=0.01$, $P's > 0.05$].

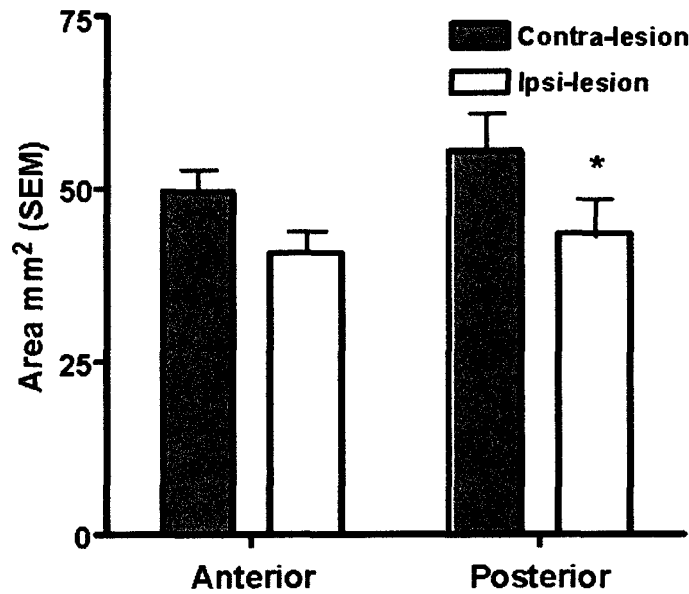


Figure 2.2. Comparison of hemisphere size for postnatal day 7 hypoxia ischemia (P7HI) cases. Hemisphere size assessed on postnatal day 19 was the same at the anterior plane (Plate 19), but smaller in the ipsi-lesion hemisphere at a posterior plane (Plate 27), compared to the contra-lesion hemisphere (* $P < 0.05$).

Myelination

Anterior Plane: There were no effects of Group for corpus callosum size in the Ipsi-lesion hemisphere [$F(1,8)=0.66$, $P > 0.05$]. Furthermore, there were no Group differences in corpus callosum size in the Contra-lesion hemisphere [$F(1,8)=0.70$, $P > 0.05$], or in corpus callosum Total size [$F(1,8)=.072$, $P > 0.05$].

Posterior Plane: There were no Group differences in corpus callosum size in the Ipsi-lesion hemisphere [$F(1,8)=0.03$, $P > 0.05$]. There were no Group effects for corpus callosum size in the Contra-lesion hemisphere [$F(1,8)=1.57$, $P > 0.05$], or in the Total size of the corpus callosum [$F(1,8)=0.53$, $P > 0.05$].

There were no Group differences were detected for the area of fimbria fornix in the

Ipsi-lesion hemisphere [$F(1,8)=0.01$, $P>0.05$] or in the Contra-lesion hemisphere [$F(1,8)=0.01$, $P>0.05$].

Motor Mapping

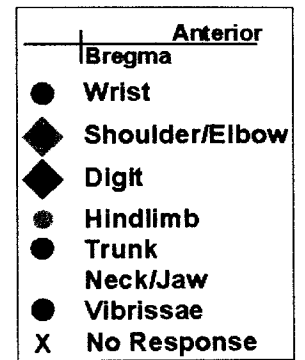
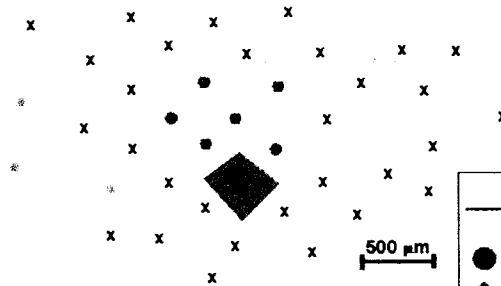
Forelimb motor maps were assessed in both hemispheres at developmental timepoints that ICMS evoked movements identified in Experiment 1. There was no delay of map emergence or reduction in size on P19 in the P7HI group with moderate lesions (Fig 2.3 and 2.5).

There was no difference in the number of microstimulation Sites probed between Sham and P7HI subjects examined on P19 [Ipsi-lesion: $F(1,8)=0.89$; Contra-lesion: $F(1,8)=0.03$, $P's>0.05$]. Additionally, there was no difference in average stimulation Threshold to evoke forelimb movements [Ipsi-lesion: $F(1,7)=0.47$ (one case missing); Contra-lesion: $F(1,6)=0.31$, $P's>0.05$ (two cases missing)]. The electrode tip depth that movements could be evoked with the lowest threshold was consistent between Sham and P7HI subjects (approximately 1300 μm).

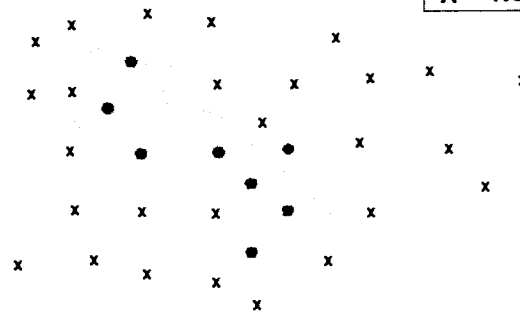
The Total area of forelimb representations in the Ipsi-lesion motor cortex was similar between Sham and P7HI [$F(1,8)=2.62$, $P>0.05$, Fig 2.5]. Further analysis indicated that the P7HI group had less area of shoulder and elbow movements, but there was no difference in area of wrist or digit movements compared to Sham [Proximal: $F(1,8)=6.34$, $P<0.05$; Distal: $F(1,8)=0.03$, $P's>0.05$].



Sham



P7HI-A



P7HI-B

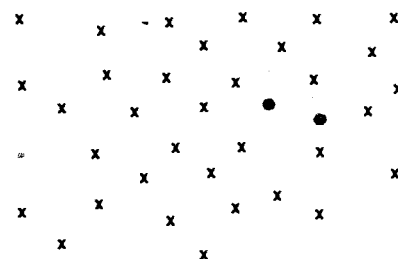


Figure 2.3. Histology and corresponding motor maps for Sham and postnatal day 7 hypoxia ischemia (P7HI) cases. *Left Panel:* A representative P7HI case (*middle*) shows a moderate lesion with an enlarged ventricle and smaller striatum and hippocampus in the ipsi-lesion hemisphere (right side). Porencephalic cysts were present in two severe P7HI cases although motor cortex was spared (*bottom*). Sections stained for myelin (Schmued) revealed that the internal capsule was diminished in the ipsi-lesion hemisphere yet the corpus callosum thickness was comparable to Sham and the pyramidal tract appeared symmetrical. White lines show areas of cortical thickness measurements. Numbers show distance to Bregma. Boxed area shows approximate region of the motor map. *Right Panel:* Intracortical microstimulation on postnatal day 19 in the ipsi-lesion hemisphere showed that moderate P7HI did not affect the size of the forelimb map (P7HI-A), whereas a severe P7HI case had an abnormally small map (P7HI-B, mapped on P25). Abbreviations: C is cingulate cortex area 1; cc, corpus callosum; Cp, caudate putamen; fi, fimbria of hippocampus; H, hippocampus; M, motor cortex; S, somatosensory cortex; St, striatum; R, retrosplenial granular cortex b; py, pyramidal tract; V, lateral ventricle.

There were no differences 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: $F(1,8)=0.01$, $P's > 0.05$].

There were no differences in motor map organization between P19 and P25 in Sham operates. Movements were evoked at thresholds below 150 μA and electrode tip depths of 1300 μm . Two P7HI operates that were mapped on P25 had abnormally small or absent forelimb representations in the ipsi-lesion hemisphere (P7HI-B, Fig 2.3). These two P7HI cases had severe infarcts, yet the ipsi-lesion motor cortex was spared. An additional P7HI case confirmed as a moderate lesion was found to have a motor map the same size as Sham, but the forelimb representation consisted of mostly Distal movements (86%), paralleling the observation in P7HI cases mapped on P19 (statistics not performed). The size of the forelimb representations in the contra-lesion hemisphere were not different than Sham.

An overlay of ipsi-lesion maps from Sham and P7HI cases demonstrates that similar areas of cortex were investigated, and that forelimb movements were consistently found within the same regions presumed to be the caudal forelimb area, although the individual organization was different (Fig 2.4). ICMS did not evoke ipsilateral or bilateral movements.

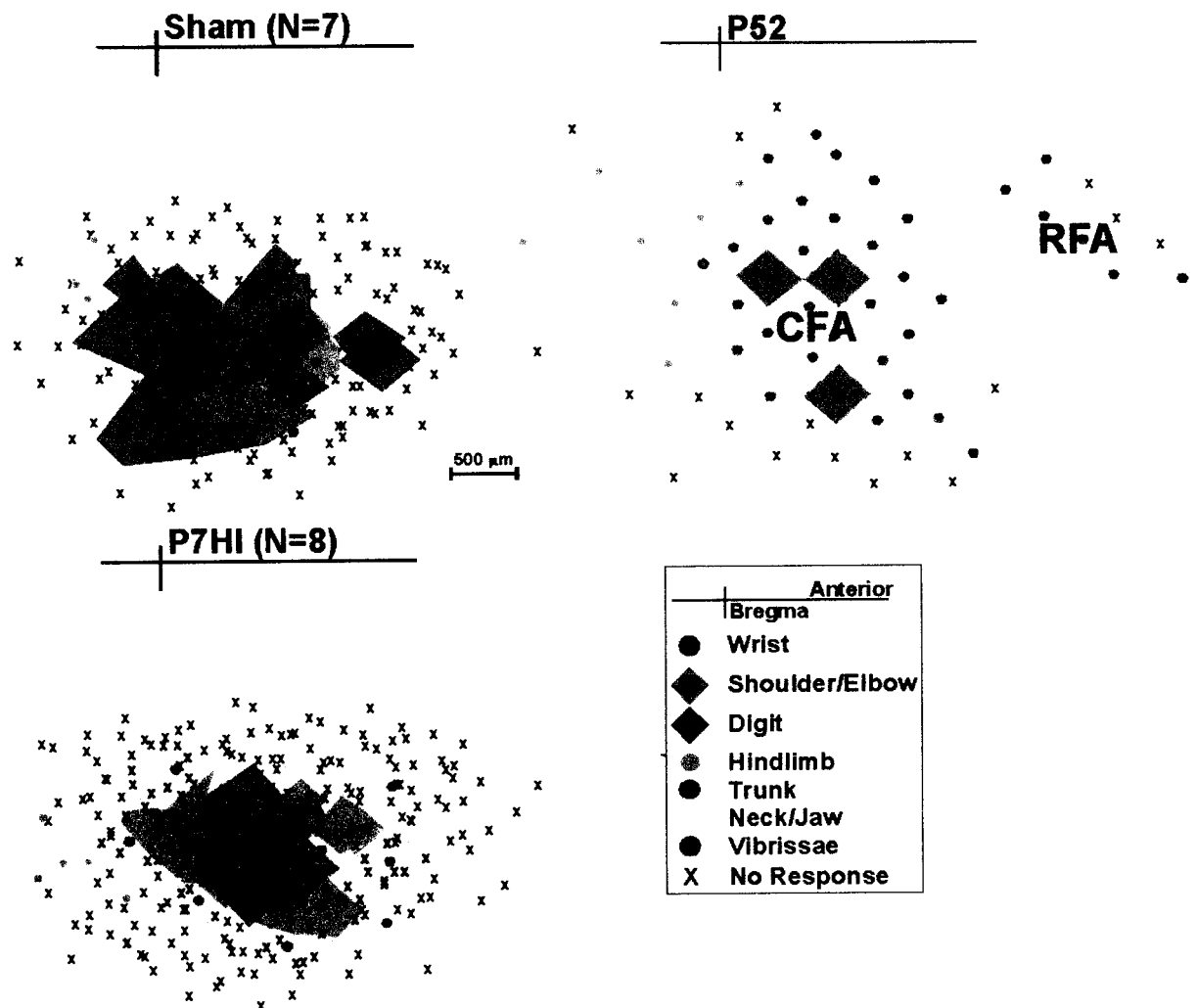


Figure 2.4. Overlay of forelimb motor maps for Sham (*top left*) and postnatal day 7 hypoxia ischemia (P7HI) cases (*bottom left*). For comparison, a Sham motor map on postnatal day 52 shows the adult-like organization of the caudal and rostral forelimb area (CFA and RFA respectively, *top right*). The overlay shows that the cortical region investigated with intracortical microstimulation is comparable between Sham (N=7) and P7HI (N=8). There was some individual variation in the location and organization of the motor representations, but the forelimb maps closely overlap (darkest shaded areas show the most overlap) within regions corresponding to the caudal forelimb area described in adults. Areas of non-forelimb movements (hindlimb, trunk, neck, jaw, vibrissae) also overlap with the adult pattern suggesting there was no shift in the topography of the motor maps following P7HI. Note there is less shoulder and elbow representation (blue) in the P7HI group. See inset for legend.

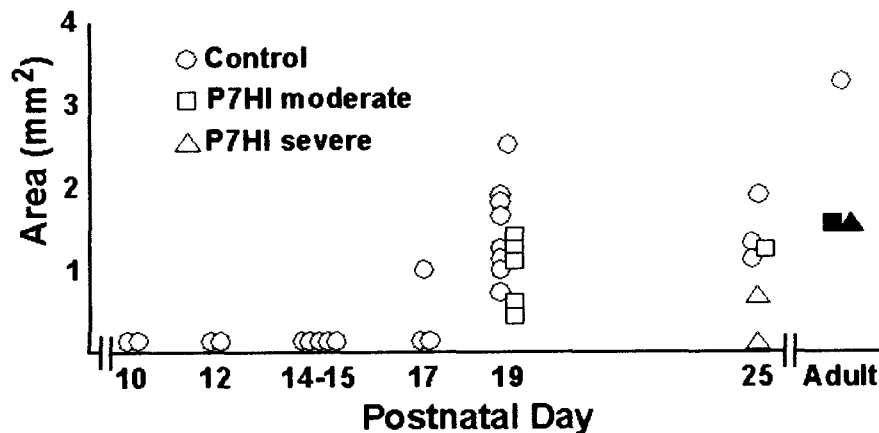


Figure 2.5. Developmental timecourse of forelimb motor maps determined by intracortical microstimulation (ICMS). Ages tested are postnatal day (P) 10, 12, 14, 15, 17, 19, 25, 52. Forelimb movements were reliably evoked on P19 and there was no further change in map size on P25. A control young adult map on P52 is nearly triple the size of maps from P19, but moderate or severe P7HI diminishes age-related map expansion (indicated by dark filled square and triangle; data from Williams 2007).

2.5. Discussion

The most common impairments from pediatric stroke presented at clinics are motor deficits, yet the effects of neonatal strokes on motor neurophysiology remains unclear. The normal timecourse of motor map emergence was studied with intracortical microstimulation (ICMS) and used to assess if motor map emergence is altered by postnatal day 7 hypoxia-ischemia (P7HI) stroke. The major findings are that (1) ICMS evoked movements reliably on P19, and (2) moderate P7HI showed variable lesion pathology but did not delay the motor map.

Motor map emergence

Soon after Fritsch and Hitzig's discovery that electrical stimulation to the surface of motor cortex produced contralateral movements of the body (Fritsch and Hitzig, 1870), Otto Soltmann demonstrated that similar stimulation in neonatal dogs could not evoke movements until the second postnatal week (see Finger et al., 2000). Soltmann used his observations to propose that a level of postnatal maturation in cortex and descending pathways, such as axonal myelination, was necessary before movements could be evoked with stimulation (Finger et al., 2000). A major advancement in motor mapping was the introduction of intracortical microstimulation (ICMS) techniques (Asanuma and Sakata, 1967). ICMS provides a more direct approach to stimulate layer V pyramidal cells compared to surface or transcranial electrical stimulation, and can be done at high resolution (columns). ICMS in layer V excites pyramidal neurons comprising the corticospinal tract (CST) that descend via the internal capsule making mono- and poly-

synaptic connections onto ventral horn motoneurons and interneurons in spinal cord leading to coordinated movements (Donoghue and Wise, 1982). Cortical layer V pyramidal neurons comprising the CST descend via the internal capsule and make mono- and poly-synaptic connections onto ventral horn motoneurons and interneurons in spinal cord. Thus, ICMS-evoked movements demonstrate a capacity within the CST network sufficient to evoke movements via direct layer V pyramidal cell depolarization at the electrode tip (Stoney et al., 1968), and indirect pyramidal cell depolarization by trans-synaptic recruitment along horizontal connections and *en passant* fibers (Cheney et al., 2000; Jankowska et al., 1975).

ICMS was used to characterize the developmental emergence of motor maps during motor system maturation. The ICMS parameters were similar to previous reports in developing and adult motor map organization in primates, cats, and rats (Chakrabarty and Martin, 2000; Kleim et al., 1998; Nudo et al., 1996). The current results show that forelimb movements, or bordering sites such as hindlimb, trunk, neck, jaw or vibrissae, could not be evoked with ICMS before P17 using microstimulation currents up to 150 μ A at various intracortical depths. Considering CST projections are initially splayed throughout the cortical mantle (O'Leary and Wilkinson, 1999) and begin to show mature connectional specificity on P14 in rat spinal cord (Clowry et al., 1997; Terashima, 1995), a rat on P15 was extensively probed to determine if areas outside the normal range of forelimb areas could evoke movements, but to no avail. The findings suggest that exuberant corticospinal fibers initially present are not capable of evoking movement. Thus, the role of exuberant corticospinal projections remains unclear, but likely does not

support the motor map. It is important to note that a previous study has compared the timecourse of motor map emergence in awake or ketamine-anesthetized cats and found the results to be similar between preparations (Chakrabarty and Martin, 2000).

The complement of proximal and distal forelimb movements was present from the earliest age that ICMS could evoke movements. Microstimulation evoked elbow, wrist, and digit movements in one P17 case (two other P17 cases did not show a motor map), and forelimb movements were found in every case assessed on P19. The intra-areal organization, but not the size, of proximal and distal representations varied between subjects. The region evoking forelimb movements is consistent with the caudal forelimb area described in adults because of the location relative to Bregma and also neck responses were found rostral to the forelimb maps (Neafsey and Sievert, 1982). Microstimulation sites evoking forelimb movements were adjoined suggesting that proximal and distal representations in cortex develop together. ICMS evoked an individual digit movement, which is rare in this experimental set-up even in adult rats with a mature CST. It is interesting that other representations emerged at the same timepoint as the forelimb, including vibrissa that is mediated by the corticobulbar tract. However, the presence of bordering representations was variable between cases and hindlimb movements were not evoked until P25. The relatively late emergence of hindlimb representations is reasonable considering that lumbar segments of spinal cord are the last to be innervated by the CST (Terashima, 1995; Vinay et al., 2000). The unitary organization of the emergent motor map suggests that representations of the body develop synchronously (Bruce and Tatton, 1980), presumably incorporating complimentary body segments used during motor skills.

A previous study in cats found that proximal forelimb movements preceded the emergence of distal forelimb movements and forelimb sites were not necessarily adjoining. The authors proposed a proximal-to-distal developmental timecourse of emerging map representations (Chakrabarty and Martin, 2000). The discrepancy in the type of movements comprising the early map between rats and cats could reflect species differences in rate of behavioural development.

The timing of motor map emergence is consistent with the notion that motor maps support complex skill learning. Rats are altricial mammals that are not weaned from the dam until more three weeks after birth. The second postnatal week corresponds to mature forelimb placing reactions (Donatelle, 1977) thought to be mediated by refined CST projections from sensory and motor areas that show features of adult-like organization by P14 (Bates and Killackey, 1984; Terashima, 1995). ICMS did not consistently evoke movements until P19 and there were no additional changes in map size on P25. The expansion of the motor map corresponds well to the expression of mature characteristics in fine motor control (Altman and Sudarshan, 1975). The relatively late emergence of motor maps provides a mechanism to accommodate the transition of motor movements from nursing to skilled movements acquired around the time of weaning in preparation for adulthood (Meng et al., 2004). Future studies will need to characterize the development of the RFA. Nevertheless, it is possible that the relatively late emergence of motor maps is tied to the maturation of other cortical areas, particularly in sensory areas that provide input to motor cortex. Other cortical maps such as visual (Crair et al., 1998), somatosensory (Zhang et al., 2001), and auditory (Eggermont, 1992) areas show

characteristic field tunings at ages younger than when motor maps emerge. Thus, the preceding maturation of sensory maps may provide important information for motor maps to develop the capacity to skillfully adapt movements. The maturation of the motor maps may also reflect learning and practicing motor behaviors, which at first are much less precise than they become.

Neonatal stroke effects on anatomy

There was no mortality during or after the induction of hypoxia ischemia stroke or obvious evidence of seizure activity. Body and brain weights measured nearly two weeks after stroke indicated no differences compared to Sham. The acute maintenance of normal body weight indicates a normal period of physical growth following hypoxia ischemia with no immediate changes in body set-point or food intake (i.e. suckling) within the litter, nor maltreatment from the dam, which can affect health status. P7HI subjects had abnormally appearing ipsi-lesion facial features (ptotic eye, smaller cranium) but were not predictive of lesion size. Occlusion of the common carotid artery, caudal to the internal and external carotid bifurcation, disrupts blood supply to the facial artery and may account for the abnormal facial features. Nevertheless, the brain pathology is similar following P7HI or selective MCA occlusion, although P7HI tended to produce larger infarcts (Ashwal et al., 2007).

P7HI produces variability in lesion size. Overall, the ipsi-lesion hemisphere size was reduced compared to the contra-lesion hemisphere in P7HI operates in posterior, but not anterior, regions. The ipsi-lesion lateral ventricle was enlarged, whereas the caudate

putamen and hippocampus were smaller in P7HI cases and are commonly found using the same parameters (Grow et al., 2003). There was no evidence of patchy cell death within residual neocortex and the integrity of cortical lamina appeared intact, except for a less prominent layer IV in the ipsi-lesion hemisphere in the P7HI group. Two P7HI operates had porencephalic cysts in parietal cortex and were classified as severe cases. Cortical thickness including presumptive cingulate cortex, motor cortex, and somatosensory cortex was spared following P7HI, even in severe cases. The source of the variability in lesion pathology is unknown, but is likely related to individual differences in architecture of the developing arterial network. Sparing of medial frontal areas is consistent with MCA occlusion in adults and is likely supported by collateral blood flow from the contralateral hemisphere (Gharbawie et al., 2005; Gharbawie et al., 2008).

There was variability in the extent of white matter injury from the neonatal stroke. There were no changes detected in the size of the corpus callosum or fimbria, but there was a marked reduction in the frequency and intensity of myelin staining in caudate putamen and internal capsule of the ipsi-lesion hemisphere in almost all P7HI cases. It is noted that the method used was unable to clearly dissociate myelinated and non-myelinated axons or count the number of fibers in a pathway, however. Another possibility is that the measurements were taken at an early developmental timepoint when oligodendrocytes are still maturing and producing myelin, and therefore our assessment may not capture evidence of ongoing anterograde degeneration or reflect adulthood outcomes. It has been shown that oligodendrocytes are vulnerable to hypoxia ischemia, albeit acutely (Liu et al., 2002; Skoff et al., 2001). Studies examining the effects

of neonatal hypoxia ischemia on white matter volume have shown transient reductions in corpus callosum, hippocampus, basal ganglia, and internal capsule in the ipsi-lesion hemisphere (Northington et al., 2001; Qiao et al., 2004; Tuor et al., 1998). Other indirect measures of white matter injury following P7HI have found delayed degeneration in hippocampal pathways using diffusion tensor imaging in mice (Stone et al., 2008). Studies on children afflicted with neonatal stroke have found considerable anterograde Wallerian degeneration within the CST using magnetic resonance imaging (Kirton et al., 2007).

Neonatal stroke effects on motor map emergence

A major contribution of this study is the early assessment of motor map function following neonatal stroke. The findings indicate that there was no delay in the emergence of motor maps in the P7HI group. Following P7HI movements could be evoked from the ipsi-lesion hemisphere at electrode depths and stimulation thresholds similar to Shams on P19. Furthermore, the total size of the forelimb representation was spared in the ipsi-lesion hemisphere of P7HI rats. It is remarkable that there was no functional delay in the ipsi-lesion maps considering the reduction in hemisphere size and evidence of an abnormal internal capsule. In addition, P7HI surgery coincides with the period of maximal CST synaptogenesis in spinal cord. Spinal areas are irrigated by the vertebral arteries and would likely not be directly affected by hypoxia ischemia, but this does not rule out the possibility that P7HI is disruptive to spinal cord networks and contributes to motor disorders in adulthood. Termination patterns of CST projections in spinal cord are disrupted by permanent and temporary neonatal motor cortex lesion, particularly if the

lesion is unilateral (Clowry et al., 2004; Friel and Martin, 2007), and is accompanied by a diminished motor map (Chakrabarty et al., 2008). The intra-areal organization of forelimb maps in the P7HI group showed a decrease in the area of proximal movements (shoulder and elbow). The reduced proximal representation in the caudal forelimb area is curious because the complement of forelimb movements appears to expand together under normal circumstances. It is possible that compensatory behaviours adapted by the P7HI group involve less than normal use of the shoulder and elbow. Additionally, a diminished area evoking shoulder and elbow movements in P7HI cases may represent a compensatory strategy to spare cortical control of wrist and digit movements that arguably underlie the unique contributions of motor cortex to the motor system (Lawrence and Kuypers, 1965; Lemon, 2008). For example, the percentage of wrist and digit representations in the CFA increases from skilled reach training (Kleim et al., 1998; Kleim et al., 2002; Nudo et al., 1996; Plautz et al., 2000). The emergence of an adult-like motor map could thus be dependent upon motor experience during the juvenile to adolescent period.

There were two severe P7HI cases found to have abnormally small forelimb representations. In one severe P7HI case, ICMS evoked forelimb movements only, and in a second severe P7HI case, ICMS did not evoke forelimb movements of any type, but there was a microstimulation site in the hindlimb area that did evoke hindlimb movements. A separate experiment using the same P7HI procedures to investigate the adult organization motor maps was performed. That study found that both moderate and severe P7HI had abnormally small caudal forelimb area in the ipsi-lesion map, even though motor cortex is spared in adulthood (Williams, 2007). Thus, mapping at a later

timepoint in these severe cases would likely not have shown a recovered motor map. In contrast to the diminished CFA in adulthood following P7HI, the above study found that organization of the RFA is spared in P7HI cases mapped in adulthood and may be an important compensatory mechanism (Williams, 2007). A parallel result has been found in adult rats with MCA occlusion stroke whereby the CFA is diminished but the RFA is spared (Gharbawie et al., 2008). The abnormal organization of motor maps following P7HI or adult MCA occlusion is similar and it is tempting to speculate that the CFA is a common target for neurophysiological disruption from stroke. Further studies are needed to characterize the emergence of RFA and its influence on motor map function and behaviour following neonatal stroke. A plausible strategy for stroke rehabilitation might be to further enhance the plasticity of CFA, or RFA, depending on where the stroke is and what behavioural symptoms ensue (Gharbawie et al., 2007).

There is increasing evidence that the effects of pediatric stroke may progress slowly in cognitive and motor systems (Eyre, 2007; Westmacott et al., 2009). Compelling results have been found in pediatric cases of middle cerebral artery stroke resulting in hemiplegic cerebral palsy (Eyre et al., 2007). In the Eyre study, motor areas were mapped with transcranial magnetic stimulation at three-month intervals during the first two years after birth. Their results at early timepoints did not show differences in CST function compared to controls whereas their within-subject results at later timepoints showed impairments in CST function. Some cases with normal function after birth showed complete dysfunction at later timepoints (Eyre et al., 2007). Together these results imply that early assessment of CST function from the ipsi-lesion motor map is not necessarily a

reliable predictor of map organization in adulthood, except perhaps in severe cases. The importance of these results is that abnormally small maps observed in adulthood, but not earlier in development, either indicates that anterograde degeneration of motor pathways continues into adulthood (Kirton et al., 2007), or the capacity for factors to promote map maturation, such as limb use (Martin et al., 2005), is compromised, or both (Martin et al., 2007). An important period to intervene with treatments after neonatal stroke may be following map emergence in order to avert motor map dysfunction in adulthood.

2.6. Conclusion

Motor impairments documented following pediatric stroke suggest that the neural control of motor behaviour is compromised, even when motor cortex is spared. Early assessment of motor map function indicated that unilateral P7HI does not impose a delay in the emergence of the forelimb motor map, does not alter the size or location of the cortical area ICMS evokes forelimb movements, but does reduce the area for shoulder and elbow movements. Given the emergent size of the motor map is similar between P7HI and Sham cases, but not the mature size of the motor map (Williams, 2007), suggests that neonatal stroke prevents the maps from flourishing during maturation. Thus, it appears that abnormalities in motor map function following neonatal stroke are present when behavioural skills with the forelimbs become more elaborate. Similarly, parallel cognitive results have been shown using a variety of neuropsychological measures in both children (Banich et al., 1990) and monkeys (Goldman, 1974) with early cortical injury. Future research can use these results and others to design treatment interventions for neonatal

stroke aimed at averting adult motor map dysfunction, and motor deficits.

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Chapter 3

**Abnormal ipsi-lesion motor maps in adulthood following postnatal day 7 hypoxia
ischemia in rats.**

3.1. Abstract

Pediatric stroke induces a range of behavioural deficits that may be severe and persist into adulthood. The neural correlates of the deficits remain poorly understood. Pediatric cases with motor impairments are particularly elusive because motor cortex is seemingly intact. The present study used the most common animal model of neonatal stroke to determine if the motor impairments are related to aberrant organization of cortical motor maps in adulthood. Postnatal day 7 hypoxia-ischemia (P7HI) was achieved by occluding a common carotid artery and exposure to hypoxia (8% oxygen for 90 min). Preferences in forelimb use in the cylinder task and during food manipulation were documented in juveniles. Forelimb motor maps were investigated with intracortical microstimulation in adulthood. Cortical thickness was obtained from sections stained with cresyl violet. Pathological variability from P7HI was classified as Moderate or Severe. The Moderate P7HI group did not show changes in motor, somatosensory, or cingulate cortex thickness, whereas the Severe P7HI group had thinner posterior somatosensory cortex that typically showed cortical cysts. The P7HI groups demonstrated a mild preference to use the ipsi-lesion forelimb for contact with the cylinder wall and when manipulating food items. Motor maps from the ipsi-lesion hemisphere indicated that the caudal forelimb area was abnormally small, regardless of lesion size, yet the rostral forelimb area was not affected in the P7HI groups. This is the first study to directly demonstrate that motor maps are mutable to neonatal stroke and may contribute to motor impairments in adulthood. That Moderate or Severe hypoxia ischemia diminished the maps similarly in adulthood suggests there may be a common trigger, not related to lesion size, to render motor map development abnormal.

3.2. Introduction

Cerebral ischemic stroke occurs in humans less than 30 days old at a rate of 1 in every 4,000 (Lynch et al., 2002). Pediatric stroke produces deficits in motor function in 30-40% of cases and the impairments may be diagnosed immediately or after months to years after the stroke (deVeber et al., 2003). An hypoxic ischemic episode at birth is most common, second only to premature births, and can lead to spasticity, poor fine motor control and skill learning, hemi-paresis or -plegia, and cerebral palsy in severe cases (deVeber, 2002; Eyre, 2007; Perlman, 2006). The ensuing neural correlates of the motor impairments following pediatric stroke are poorly understood.

Animal models of pediatric stroke can foster advancements in stroke diagnosis and treatment. An experimental model of human stroke at full-term birth has been developed for rats by inducing hypoxia ischemia on postnatal day 7 (P7HI) (Vannucci and Vannucci, 2005). Investigations of the long-term outcomes following P7HI demonstrate various cognitive and motor deficits (Grow et al., 2003), including symptoms of motor cortex damage such as deficits in reaching for food task (Kohzuki et al., 2006; Tomimatsu et al., 2002), although motor cortex was seemingly spared in those reports. Based on findings from adult stroke studies in primates and rodents, it is possible that the neurophysiology of motor cortex may be compromised despite its apparent anatomical intactness and this in turn could contribute to the motor deficits (Nudo, 2003).

The objective of this study was to investigate the long-term outcomes of P7HI on forelimb-use preferences and dexterity, and to determine if there were alterations in motor map organization in adulthood. Changes in motor map organization following stroke can be studied in detail with intracortical microstimulation (ICMS) (Frost et al.,

2003; Nudo and Milliken, 1996). Rat motor maps comprise two cortical regions that evoke forelimb movements from ICMS, a caudal and a rostral forelimb area (CFA and RFA respectively), and are known to reorganize from experiences such as adult learning and stroke injury (Gharbawie et al., 2005; Gharbawie et al., 2008; Kleim et al., 1998; Kleim et al., 2003; Teskey et al., 2002).

3.3. Methods

Subjects and Housing

Long-Evans rat pups (N=17) from two dams bred at the Canadian Centre for Behavioural Neuroscience breeding colony were used in this study. The study used a cross-litter design with roughly equal numbers of male and female rats in each condition. The experimental design comprised two Lesion conditions (4 Sham and 10 P7HI). In addition, one Sham and two P7HI cases were added for ICMS. Figure 3.1 outlines the experimental procedures.

Rats were weaned on postnatal day 23 and housed in same-sex groups of two or three in standard laboratory cages with food and water available *ad libitum*. Food was reduced to 85% daily serving during behavioural testing from postnatal day 42-45. Food was reduced to 25% daily serving 16-20 hours before motor mapping surgery to minimize variability in response to anesthetics. This study was approved by University of Lethbridge Animal Care Committee review and procedures followed institutional and the Canadian Council for Animal Care guidelines. Effort was taken to use the fewest possible animals and to minimize discomfort.

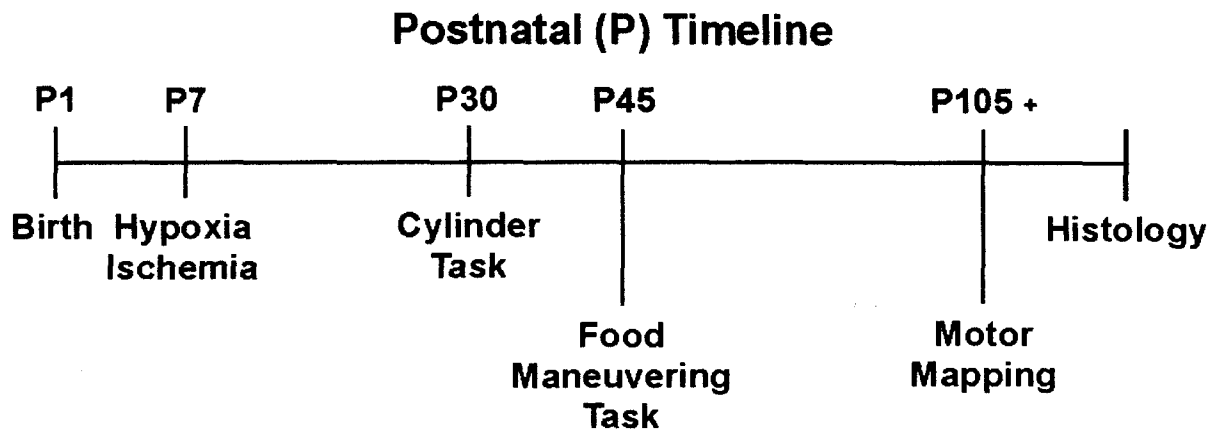


Figure 3.1. Timeline of experimental procedures.

Postnatal Day 7 Hypoxia Ischemia

The litter was removed from the dam on postnatal day 7 and placed in a standard cage on a heating pad. Pups were anesthetized with Isoflurane and maintained under anesthesia through a modified nose cone. The ventral neck was cleaned and a midline incision was made. The right common carotid artery (CCA) was ligated (not including vagus and sympathetic nerves) and tied with two sutures (5-0 silk) 2-3 mm apart caudal to the branches of the internal and external carotid artery. The CCA was occluded with bipolar coagulation. The muscles were repositioned and the incision was closed with Vetbond tissue adhesive. The duration of the surgery was typically 5 minutes. Pups were placed in an incubator (37 °C) for 30 minutes to recover. Pups were transferred in groups of three or four to a glass jar with the temperature set at 36.5°C and humidified in a water bath. Exposure to hypoxia for 90 min was achieved by delivering 8 % O₂ at 110 mm Hg through a tube into the jar. Pups were placed in the incubator until mobile (15-20 minutes) and returned to their dam. Sham surgery did not include right CCA occlusion. Group assignment was random across litters and there was no mortality.

Behavioural Assessment

Cylinder Task

Rats were individually placed into the cylinder for 5 minutes on postnatal day 30. Rats will spontaneously rear and use their forelimbs for support on the wall when placed in a narrow Plexiglas cylinder (Schallert et al., 2000). Videorecords were scored for the number of rears on the wall or in the centre, the forelimb used first for support during a wall rear, the total touches for each forelimb on the wall, and the first forelimb to touch the floor on descent from a rear. Forelimb preference scores were calculated for First forelimb to contact the wall upon rearing, Total forelimb touches on the wall, and first forelimb to contact the Floor after a rear, using the following formula: $[(\# \text{ ipsi-lesion} / \# \text{ ipsi-lesion} + \# \text{ contra-lesion}) * 100]$.

The cylinder was 20 cm in diameter and 30 cm in height placed on clear Plexiglas tabletop. A mirror was positioned on an angle below the tabletop to capture videorecords from a ventral perspective of the rat.

Food Maneuvering Task

Rats are adept in manipulating food items with their forelimbs and digits to present food items to the mouth (Whishaw et al., 1997). Rats were familiarized to the food items by placing small pieces of angel hair pasta (<1 cm dried) in the home cage for three days before testing. Testing began on postnatal day 42 and the rats were filmed on postnatal day 45 for scoring. Rats were placed in cylinder for two minutes for habituation purposes and then briefly removed to clean feces or urine in order to ensure unobstructed videorecords. Rats were returned to the cylinder and three Short pieces of 4 cm long

angel hair pasta (dried) were presented on the floor. After consumption of the short pasta pieces, three Long pasta pieces 8 cm in length were given followed by three large round Sugar Pellets (300 mg, banana flavoured). Three trials of each food item were scored on: 1) first forelimb to Contact and pick up each item; 2) type of Grasp used to pick up each item (between digits 2-3, 3-4; 4-5; or prehension grip); 3) number of forelimb Maneuvers (repositions) during item consumption; percent number of Contra-lesion forelimb Maneuvers during item consumption; and 4) Time to eat one item.

The testing apparatus was a Plexiglas cylinder 20 cm in diameter and 30 cm in height set vertically on a clear Plexiglas tabletop with a mirror below angled to allow viewing from underneath the rat.

Videorecording

Each rat was digitally videotaped during testing using a Cannon ZR 30 MC digital video camcorder with shutter speed at 1000th of a second. Additional lighting was provided by a cool florescent studio light source.

Motor Mapping

ICMS motor mapping was conducted to map the organization of the caudal forelimb area (CFA) and the rostral forelimb area (RFA) (Kleim et al., 1998). In all rats, the lesion hemisphere (right side) was assessed. Rats were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and supplemented when necessary. The skull over motor cortex was trephinated and dura retracted. The surface of the cortex was digitally photographed and a grid (500 μm^2) was superimposed onto the

image using Canvas (ACD Systems) to guide and record electrode penetration sites (Remple *et al.*, 2001). The electrode was made of a platinum filament inserted in a borosilicate glass micropipette (20-40 μm tip diameter, 15° bevel) filled with concentrated saline (3.5 M). The electrode tip was lowered to 1550-1600 μm beneath the cortical surface corresponding to layer V pyramidal cell bodies giving rise to corticospinal tract fibers (Donoghue and Wise, 1982). Microstimulation trains of thirteen, 200 μs , 350 Hz cathodal pulses, were delivered from a stimulation isolation unit. At each site (350 μm interpenetration distance) the current intensity was gradually increased from 0 μA up to 60 μA , or until a movement was evoked. During microstimulation trains, an experimenter supported the rat's forelimb from underneath the elbow and visually classified evoked forelimb movements as shoulder or elbow (proximal) or wrist or digit (distal). The movement obtained at the lowest threshold was recorded along with the threshold intensity.

After completion of motor mapping under these parameters, forelimb representations were abnormally small in some rats. In such cases, non-responsive sites were reinvestigated, first re-testing sites with the same parameters as above, and if still unresponsive, current intensities up to 150 μA were used across multiple depths between 1000 μm and 1800 μm (200 μm increments).

The midpoint distance between outskirt forelimb movements and neighbouring non-forelimb movements was outlined and the enclosed area calculated using Canvas software. Areas of shoulder and elbow or wrist and digit representations were outlined and separately measured for the CFA and RFA.

Anatomy

Body and Brain Weight

Body weight was measured before ICMS. Brain weight was measured after the mapping session and perfusion.

Histology

Rats were given an overdose of Euthansol and perfused with saline and 4 % formalin fixative. The brain was post-fixed in 4 % formalin and cryoprotected in 30 % sucrose in 4 % formalin solution in refrigeration. Coronal sections were cut at 40 µm with a freezing microtome. Every tenth section from the anterior to posterior pole of the brain was transferred to a glass slide prepared with a 1 % gelatin and 0.2 % chrome-alum coating. Sections were stained for Cresyl Violet and digital images were taken. ImageJ (NIH freeware) was used to measure hemisphere size and cortical thickness. The atlas plates correspond to Paxinos and Watson (Paxinos and Watson, 1998).

Hemisphere Size

Hemisphere size was estimated by measuring width from the rhinal fissure to the midline in a posterior coronal plane (Bregma -2.30, respectively). The percentage of ipsi-lesion hemisphere size was calculated $[\text{Ipsi-size} / (\text{Ipsi-size} + \text{Contra-size}) * 100$.

Cortical Thickness

Cortical regions with corticospinal projections including the cingulate (Cg1), motor (M1), somatosensory (S1), and retrosplenial (RSGb) were measured from anterior, central, and

posterior coronal planes. The sections correspond to Plate 9, Plate 18, and Plate 29 (Bregma 2.70, -0.03, and -2.30, respectively). Percentage of Ipsi-lesion cortical thickness was calculated using the formula: $[\text{Ipsi-thickness} / (\text{Ipsi-thickness} + \text{Contra-thickness}) * 100]$.

Statistical Analysis

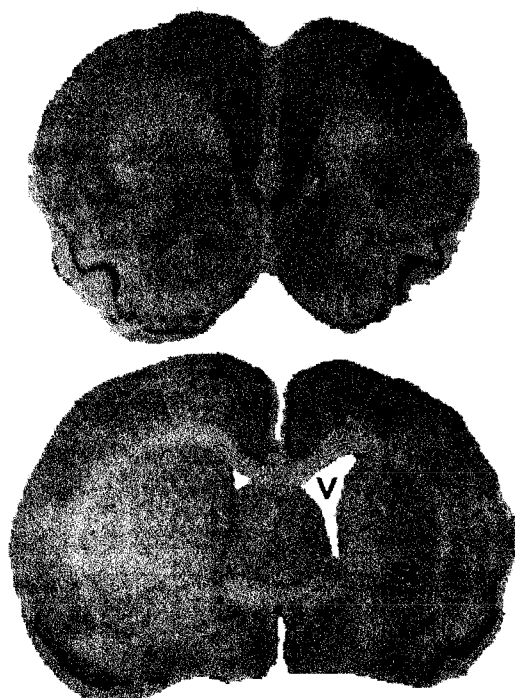
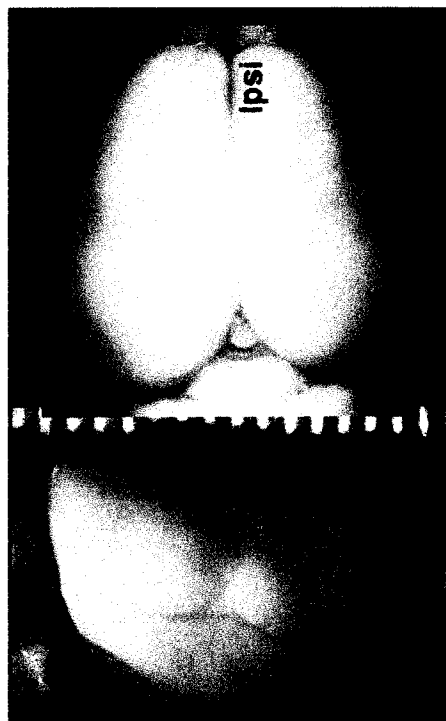
Continuous variables were tested with one-way Analysis of Variance (ANOVA). Discrete variables were analyzed by non-parametric Mann-Whitney U tests. Tukey's post-hoc tests were performed where appropriate. Preliminary analysis did not reveal sex differences on any of the measures so sex was collapsed unless otherwise stated. Statistics for significant results only are reported. The results are similar if P7HI is not separated by lesion size.

3.4. Results

Anatomy

There was variability in the lesion profile amongst P7HI cases (Fig 3.2). The lesion was characterized as Moderate if there was no obvious evidence of cysts. Moderate P7HI cases (N=5) typically showed an enlarged ventricle, smaller striatum, and smaller hippocampus in the ipsi-lesion hemisphere compared to the contra-lesion hemisphere. Severe P7HI cases (N=7) typically showed multiple cysts in regions of parietal, temporal, and/or occipital cortex and subcortex in the ipsi-lesion hemisphere in addition to the pathology observed in moderate cases (N=7).

Moderate P7HI



Severe P7HI

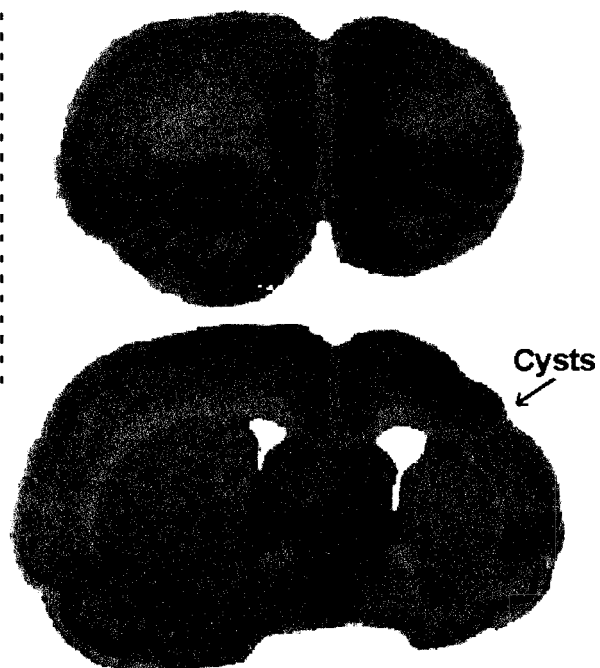
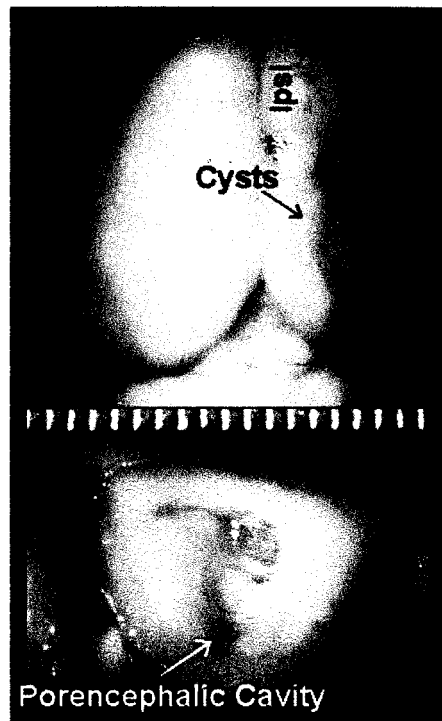


Figure 3.2. Infarct variability in adulthood following postnatal day 7 hypoxia ischemia (P7HI). The lesion was characterized as Moderate if there was no obvious formation of cysts. Moderate P7HI cases (*left*) typically showed an enlarged ventricle (V), smaller striatum (St), and smaller hippocampus in the ipsi-lesion (Ipsi) hemisphere compared to the contra-lesion hemisphere (N=5). Severe P7HI cases (*right*) typically showed pathological cyst(s) in the ipsi-lesion hemisphere, in addition to the pathology observed in moderate cases (N=7). The regions most affected by Severe P7HI were parietal, temporal, and/or occipital cortical and subcortical areas displaying porencephalic cysts and/or a porencephalic cavity (lateral view of Severe P7HI is from a different case).

Body and Brain Weight

There were Sex differences in body weight revealing that females had lower body weights than males. Subsequent analysis used Sex as a covariate and indicated that there were no Lesion differences in body weight.

There were Sex differences in brain weight revealing that females had lower brain weights than males. Subsequent analysis used Sex as a covariate and indicated the Severe P7HI group had smaller brains than the Sham and Moderate P7HI group, whereas Sham and Moderate P7HI groups did not differ [$F(2,13)=10.91$, $P<0.05$, Fig 3.2 A].

Histology

Moderate P7HI cases did not show changes in cortical thickness, whereas Severe P7HI cases had thinner somatosensory cortex in the ipsi-lesion hemisphere, particularly at posterior planes corresponding to the barrel fields in somatosensory cortex. Exploratory analysis used Sex as a covariate and the pattern of results was similar so the following results are reported collapsing Sex.

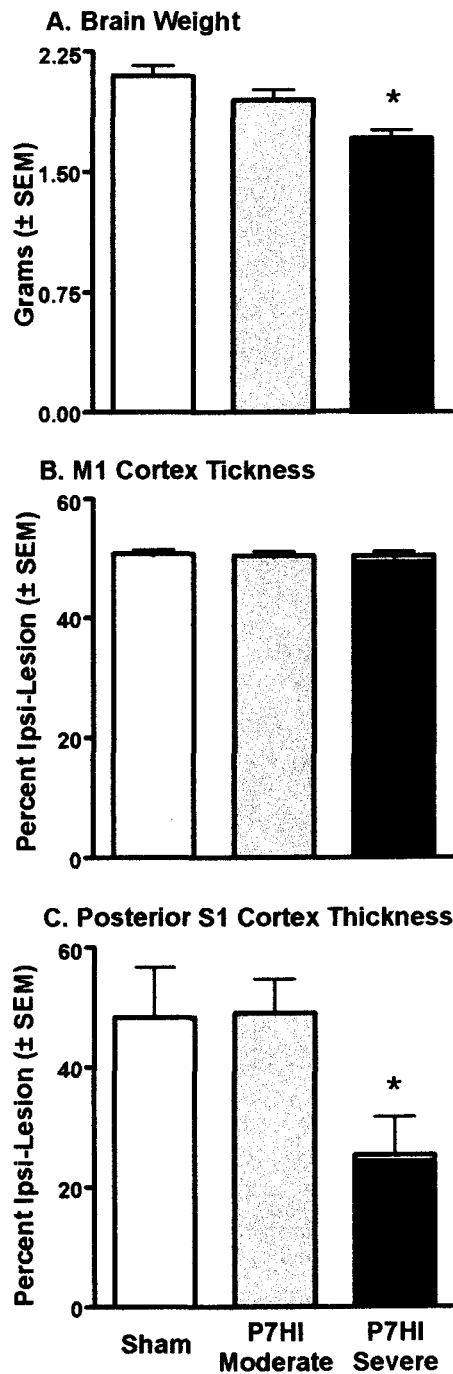


Figure 3.3. Anatomy in adulthood following postnatal day 7 hypoxia ischemia (P7HI). *A.* Brain weight was reduced in the Severe P7HI group (*differs from Sham). *B.* Motor cortex (M1) thickness was not affected by P7HI. Somatosensory cortex (S1) thickness was reduced in the Severe P7HI group and was most evident in the posterior plane corresponding to the barrel fields (* differs from Sham and Moderate P7HI).

Hemisphere Size

There were no differences between the groups in the size of the contra-lesion hemisphere although the Severe P7HI group showed a reduction in the ipsi-lesion hemisphere [Contra-lesion $F(2,13)=0.35$; Ipsi-lesion $F(2,13)=4.72^*$; $P<0.05$]. There were differences between the groups in percentage of posterior ipsi-lesion hemisphere size indicating a 25% reduction in the Severe P7HI whereas the Moderate P7HI group showed a 2% reduction [$F(2,13)=5.34$, $P<0.05$].

Cortical Thickness

There was no difference between the groups on percentage of ipsi-lesion cortical thickness in motor (Fig 3.3 B), cingulate, or retrosplenial cortex. However, Severe P7HI cases had thinner somatosensory cortex in the ipsi-lesion hemisphere compared to Moderate P7HI cases [$F(2,11)=3.83$, $P<0.05$]. Comparisons of each anterior, central, and posterior plane showed a regional change in posterior somatosensory cortex only. The Severe P7HI group showed a marked reduction in the posterior plane in [$F(2,11)=4.51$, $P<0.05$, Fig 3.3 C]. There were no differences between the groups in the contra-lesion hemisphere.

Behavioural Assessment

Cylinder Task

The general activity level was not different between the groups. Sham and Moderate P7HI groups showed similar behavioural profiles, whereas the Severe P7HI group used their contra-lesion forelimb less when first contacting the cylinder wall upon

a rear. There were differences between the groups for percentage of total touches with the ipsi-lesion forelimb across rears [$F(2,11)=5.93$, $P<0.05$, Fig 3.4 A]. There were no differences between groups for percentage of first touches with the ipsi-lesion forelimb across rears [$F(2,11)=3.68$, $P=0.06$]. For both results, the pattern was the same indicating that the Severe P7HI group used their contra-lesion forelimb less than the Sham group but were comparable to the Moderate P7HI group, whereas Moderate P7HI and Sham groups did not differ. There was no difference in total number of rears, in total number of touches, or percentage of landings with the contra-lesion forelimb across rears. There was a difference for number of landings using both forelimbs simultaneously [$F(2,11)=4.37$, $P<0.05$]. The Severe P7HI group used both forelimbs for landing more often than Sham and Moderate P7HI, but Moderate P7HI and Sham groups did not differ.

Food Maneuvering Task

General observations across food items showed that rats picked up the item with their mouth, grabbed the item with their digits, and stabilized their posture on their haunches. The forelimbs were used to make small rapid maneuvers of the item to facilitate presentation in the mouth. There did not appear to be deficits in use of the digits across groups.

Pasta Maneuvering: For the Short Pasta item the Severe P7HI group took longer to get the entire food item into the mouth compared to Sham, whereas the Moderate P7HI group did not differ from Sham [$F(2,11)=4.42$, $P<0.05$, Fig 3.4 B]. There were no differences between the groups on total number of Maneuvers, or the Percentage of maneuvers with

the contra-lesion forelimb. Mann-Whitney U tests found no group differences on first forelimb to Contact and pick up each item, or type of Grasp used to pick up each item. There were no differences between the groups for the Long pasta item.

Sugar Pellet Maneuvering: There was no difference in the amount of Time to feed the item into the mouth between the groups. The Moderate P7HI group used more total Maneuvers compared to Sham, whereas the Severe P7HI group did not differ from Sham [$F(2,11)=5.81, P<0.05$, Fig 3.4 C]. There were no differences on the Percentage of maneuvers with the contra-lesion forelimb between groups.

Motor Mapping

ICMS evoked movements in the ipsi-lesion hemisphere in every case but forelimb movements were not elicited in two Severe P7HI cases and one Moderate P7HI case. The cortical localization of the CFA and RFA motor maps was consistent between the groups in the ipsi-lesion hemisphere. There was no difference in the number of stimulation sites, stimulation threshold, or effective electrode depth (1550 μm) among the groups. The area of total forelimb representation was abnormally small in Moderate and Severe P7HI groups compared to Sham, whereas Moderate and Severe P7HI groups did not differ [$F(2,13)=20.41, P<0.05$, Fig 3.5 A]. The reduction in forelimb representations for both the Moderate and Severe P7HI group was evident in the CFA [$F(2,13)=25.17, P<0.05$, Fig 3.5 A and 3.6]. Further breakdown within the CFA showed no differences between the groups for shoulder/elbow representation, whereas the area of wrist/digit

representation was smaller in Moderate and Severe P7HI groups compared to Sham [$F(2,12)=19.07$, $P<0.05$, Fig 3.5 B].

There were no group differences in total RFA representations, RFA shoulder/elbow representations, or RFA wrist/digit representations, although there was one Moderate P7HI case that did not show evidence of the RFA (see P7HI-B, Fig 3.6).

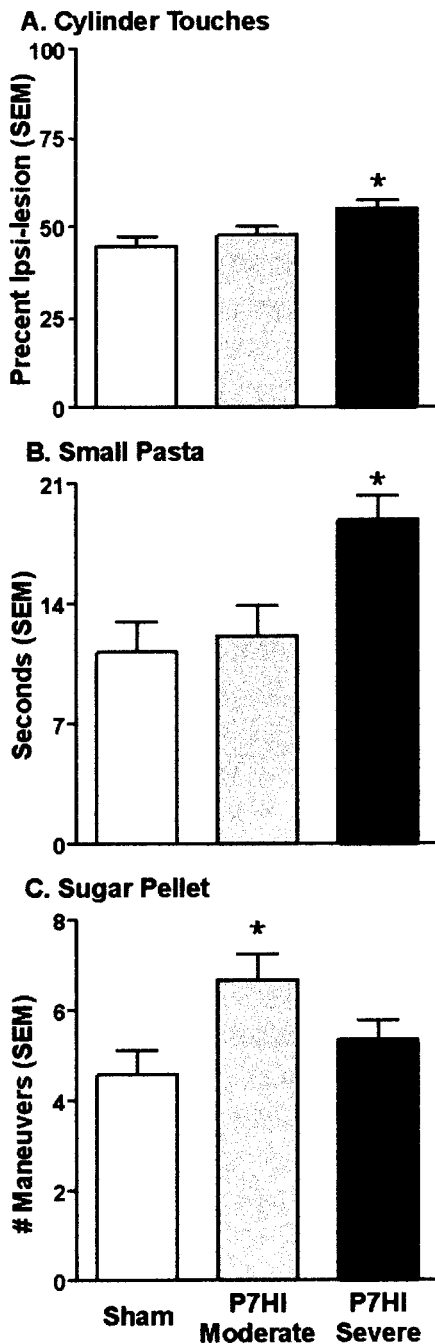


Figure 3.4. Behavioural performance in juveniles following postnatal day 7 hypoxia ischemia (P7HI). *A.* The Severe P7HI group used their ipsi-lesion forelimb more often during cylinder exploration (*differs from Sham). *B.* The Severe P7HI group took more time to get the entire small pasta item into the mouth (*differs from Sham and Moderate P7HI). *C.* The Moderate P7HI group made more maneuvers with both forelimbs to consume the sugar pellet (* differs from Sham and Severe P7HI).

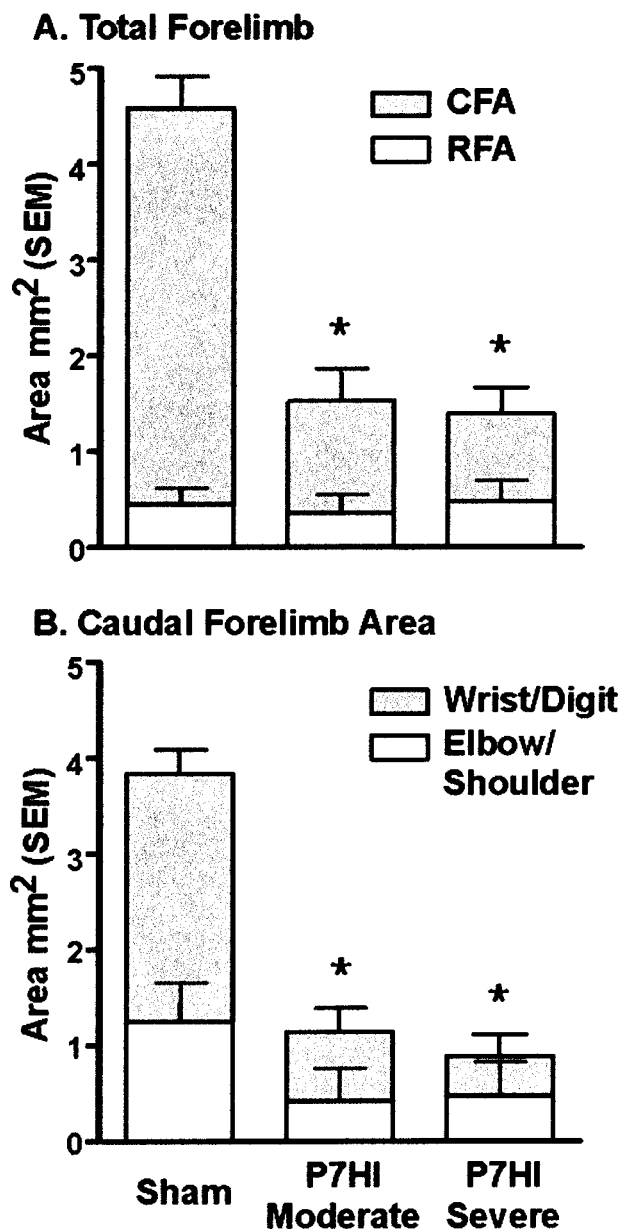


Figure 3.5. Motor map organization in adulthood following postnatal day 7 hypoxia ischemia (P7HI). *A.* The total area of forelimb movements was reduced in both P7HI groups attributable to a decrease in size of the caudal forelimb area (CFA, differs from Sham), but not the rostral forelimb area (RFA). *B.* The smaller CFA in P7HI groups corresponded to less wrist and digit representation (*differs from Sham).

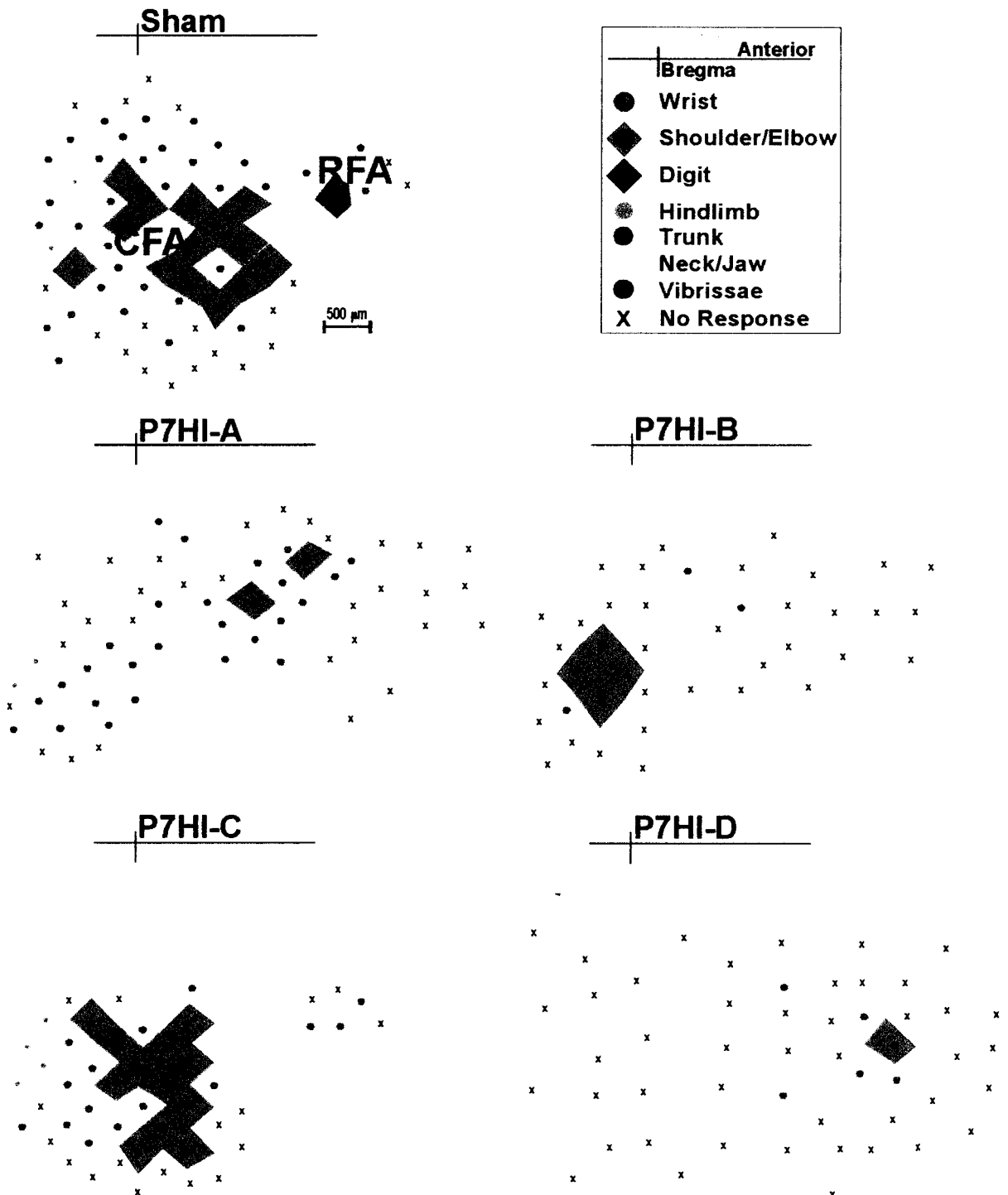


Figure 3.6. Reconstructed motor maps in adulthood following postnatal day 7 hypoxia ischemia (P7HI). Shown are a typical caudal forelimb area (CFA) and rostral forelimb area (RFA) (Sham, *top left*), and representative large and small maps following Moderate P7HI (P7HI-A and P7HI-B, *middle row*) and following Severe P7HI (P7HI-C and P7HI-D, *bottom row*). There was a Moderate P7HI case lacking a RFA (P7HI-B) and a Severe P7HI case lacking a CFA (P7HI-D). Note the location of CFA and RFA is relatively consistent between the groups.

3.5. Discussion

This study combined anatomical, behavioural, and neurophysiological measures to elucidate the underpinnings of motor impairments following neonatal P7HI stroke. The main findings are that P7HI: (1) produced a diffuse injury that reduced the size of the ipsi-lesion hemisphere somatosensory cortex in Severe P7HI cases; (2) mildly induced a preference to use the ipsi-lesion forelimb during cylinder exploration and food maneuvering; and, (3) diminished the size of the ipsi-lesion hemisphere motor map.

Anatomy in adulthood following P7HI

In the rat model of neonatal hypoxia ischemia there is a linear relationship between the infarct size and the duration of hypoxia (Welsh et al., 1982). The hypoxia ischemia parameters used in the present study were chosen because there is low mortality and typically produces a mild injury. Nevertheless, even under controlled conditions there is individual variability in lesion pathology that is poorly understood. To better represent the variability from P7HI, the presence of cortical cysts was used to classify the lesion as Severe (with cysts, often porencephalic) or Moderate (without cysts). There were no differences in body weight between the groups although the Severe P7HI group had lighter brains compared to the other groups. The anatomical measures focused on quantifying cortical thickness and found no reductions in any of the cortical regions

assessed in the Moderate P7HI group. However, somatosensory cortex was thinner in the Severe P7HI group, and was most apparent in posterior areas near the barrel fields. Somatosensory cortex thinning was proximal to the presence of cysts and together suggests that the lesion compromises middle cerebral artery (MCA) territories. However, lesions from neonatal hypoxia ischemia are typically broader than intra-luminal suture MCA occlusion alone (Ashwal et al., 2007). It was surprising that there were no changes in cortical thickness in motor or cingulate cortex, even in Severe P7HI cases, because these cortical areas also receive MCA irrigation. Furthermore, the somatosensory cortex is a major neural projection to the motor cortex. The anterior cerebral artery (ACA) receives collateral blood flow from the opposite hemisphere and may provide blood flow compensation to spare tissue near midline. The factors that contributed to individual lesion variability may include differences in vascular development and requires further study.

Motor behaviour in juveniles following P7HI

This study examined rats on two spontaneous motor behaviours to determine forelimb preferences during exploration or manipulation. The cylinder task revealed limb preferences in the Severe P7HI group only. The Severe P7HI group showed a preference to use the ipsi-lesion forelimb during rearing and this effect was greater for the first touch on a rear. The Severe P7HI group also used both forelimbs simultaneously during landing from a rear more often. Other reports using the cylinder task also show that P7HI can lead to ipsi-lesion forelimb preferences (Grow et al., 2003) and can be more pervasive if longer durations of hypoxia are used during surgery (Jones et al., 2008). It is interesting

that the Moderate P7HI group did not show preferences, and points to the importance of characterizing lesion size in this model.

A second behavioural task tested forelimb dexterity skills for manipulating different sized food objects, which has not been used in this stroke model, or at juvenile ages. The rats routinely picked up the item from the floor with their mouths and used both forelimbs to stabilize the item between the digits. The Severe P7HI group took more time to consume the short pasta item, whereas there were no differences between the groups for the long pasta item. There was a mild forelimb preference in the Moderate P7HI group requiring more maneuvers with the ipsi-lesion forelimb to consume the sugar pellet item. The short pasta and sugar pellet items were more sensitive than the longer pasta item, possibly because the long pasta item could be stabilized against the floor initially. Rats with adult stroke or motor cortex injury demonstrate more difficulty using the ipsi-lesion forelimb to maneuver food (Allred et al., 2008; Gharbawie et al., 2008). The mild impairment in forelimb maneuvering abilities following P7HI might have been due to the juvenile testing age. The rats were tested on postnatal day 45 when the lesion pathology could still be evolving and the characteristics of skilled manipulative behaviours were immature (Altman and Sudarshan, 1975). Previous studies have assessed reaching ability in adulthood following P7HI (Tomimatsu et al., 2002). The authors used the staircase reaching task in adulthood and showed that the contra-lesion forelimb was less successful than the ipsi-lesion forelimb following P7HI. The nature of adult motor impairments from P7HI is still poorly understood, and it is curious that P7HI produces motor impairments with the contra-lesion forelimb because neuroimaging (Meng et al.,

2006) and histological assessments (Tomimatsu et al., 2002; Towfighi et al., 1997) indicate that motor cortex is not directly damaged.

Motor map organization in adulthood following P7HI

Recent neuroimaging studies in humans suffering a stroke at birth have shown that the corticospinal system can undergo tremendous reorganization. TMS studies in perinatal stroke patients show abnormal output from motor cortex. For example, the ipsi-lesion hemisphere shows a reduction in CST conduction velocity and the contra-lesion hemisphere often produces bilateral movements (Berweck et al., 2008; Eyre et al., 2007). Intracortical microstimulation is widely used to assess the detailed organization of cortical motor maps and provides a more direct measure of CST function compared to TMS. The current results demonstrated that P7HI did not displace the motor map, effect microstimulation thresholds, or evoke bilateral movements in the lesion hemisphere. A major contribution of the current study demonstrated that P7HI diminished the size of ipsi-lesion caudal forelimb area map in adulthood. Surprisingly, the extent of map reductions was similar between Moderate and Severe P7HI. Figure 3.6 shows the largest and smallest motor maps from Moderate and Severe P7HI groups. The map reconstructions show two interesting cases with one Moderate P7HI not showing ICMS-evoked responses in the RFA and one Severe P7HI case not showing responses in the CFA. Several reports show that the CFA is more responsive to motor learning (Conner et al., 2003; Kleim et al., 1998; Kleim et al., 2002; Williams et al., 2006) and is more likely to

be diminished from adult stroke compared to the RFA (Gharbawie et al., 2008). Indeed, the P7HI groups showed sparing of the RFA (except one case) in adulthood.

In a separate study it has been shown that the developmental emergence of motor maps occurs in the second postnatal week and is not affected by P7HI in rats (Williams et al., 2009). A similar result has been reported in infants with hemiplegic cerebral palsy from perinatal stroke (Eyre et al., 2007). It is unknown why the CST system does not mature and support a normal motor map. The connectional specificity of CST terminals in the spinal cord are known to be disrupted by CST impairment during development and could account for the map dysfunctions in adulthood (Martin and Lee, 1999).

3.6. Conclusion

P7HI can lead to variable lesions that produce motor preferences to favour the ipsi-lesion forelimb. The novel findings are that P7HI dramatically diminished the forelimb representations in the lesion hemisphere even though motor cortex is not directly damaged. Interestingly, the reductions in motor representations were similar in both Moderate and Severe P7HI cases and provide new insight into the neural correlates underlying motor impairments from neonatal stroke. It remains to be determined if early treatments can avert map dysfunction, or if rehabilitation can restore the motor maps from P7HI.

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Chapter 4

Nicotine or fibroblast growth factor treatment partially ameliorates the deficits in forelimb skills and diminished ipsi-lesion motor maps following postnatal day 7 hypoxia ischemia in rats.

4.1. Abstract

Hypoxia ischemia stroke is a leading cause of neurological motor deficits arising from infant brain injury in humans. Psychomotor stimulants and growth factors are potential pharmacological treatments for neonatal stroke patients because they have been shown to alleviate motor impairments and to induce cortical changes in neural structure following several other types of lesion etiologies, including adult stroke and neonatal cortical lesions. To determine if psychomotor stimulants or growth factors promote beneficial outcomes in a neonatal stroke model, motor behaviour and skill learning using the forelimbs as well as neurophysiological motor map organization were assessed in adulthood. Rat pups on postnatal day 7 were given an hypoxic ischemic stroke (P7HI) by permanent occlusion of one common carotid artery and subsequent exposure to hypoxia (8% O₂ for 1.5 hr). Drug treatments commenced one day following P7HI using either nicotine (0.1 mg/kg, s.c.) or basic fibroblast growth factor (bFGF, 10ng/g, s.c.) with daily subcutaneous injections for seven consecutive days. Forelimb motor behaviours were assessed in juveniles during a food maneuvering task and in adulthood during a skilled reaching task using the contra-lesion forelimb. P7HI produced an enduring deficit in food maneuvering and reaching abilities, however the motor deficit was attenuated by nicotine or bFGF treatments. The motor skill learning from reach training was robust in Sham and P7HI with treatment groups when re-evaluated over 8 months later. Analysis of the ipsi-lesion motor maps with intracortical microstimulation indicated that P7HI diminished the size of the caudal forelimb area map whereas the rostral forelimb area map was abnormally large compared to Shams. P7HI rats treated with nicotine or bFGF showed maps that were more normal than non-treated P7HI counterparts, particularly with more

wrist representation in the caudal map. Maps in the contra-lesion hemisphere indicated no differences in organization between Sham, P7HI, and P7HI with treatment groups. Histological analysis indicated that P7HI did not alter cortical thickness in motor or somatosensory areas, but did reduce cortical thickness in cingulate areas compared to Shams. P7HI rats treated with nicotine or bFGF did not show the same reduction in cingulate thickness as the no treatment group. Together, these findings suggest that motor deficits from P7HI are attributable, at least in part, to abnormal ipsi-lesion motor maps and that nicotine or bFGF treatment attenuated the affected forelimb and motor map deficits in adulthood. This study provides evidence that two different treatment strategies may be beneficial following neonatal stroke.

4.2. Introduction

Hypoxia ischemia is a leading cause of perinatal brain injury in humans and induces persisting neurological motor deficits. There are no available treatments that can completely spare/recover the deficits following neonatal stroke. There is some evidence that hypothermia treatment following perinatal stroke can reduce mortality and promote beneficial outcomes in behaviour and cortical structure within the first year of life (Gluckman et al., 2005; Gunn et al., 2008) although the ensuing long-term neurodevelopment consequences in childhood and beyond are unknown. A drawback of hypothermia however, is that the treatment needs to be administered within the first few hours after the ischemic attack in order to provide the most beneficial outcomes (Gunn, 2000) and may not be beneficial for motor deficits (Bona et al., 1998). Furthermore, the administration of hypothermia requires a team of specialized practitioners to deliver and monitor the infant in hospital (Barks, 2008a; Barks, 2008b). Treatments that can be easily administered, such as drugs, to promote behavioural and neural compensation following brain injury, even if they are not administered until long intervals after the injury, are urgently needed (Kolb, 1995).

It is logical to look for compounds that alter behaviour and neural connectivity of the normal brain to determine if they can provide compensation or recovery from aberrant changes in behaviour and neural connectivity associated with brain injury. There is evidence in normal humans and rats that administering psychomotor stimulants alters existing neural circuitry. For example, peripheral administration nicotine, a nicotinic agonist, increases dendritic connectivity in cortex (Brown and Kolb, 2001; Gonzalez et al., 2005). Other compounds, such as growth factors, have been shown to play important

roles in cellular proliferation and survival, especially during development. For example, basic fibroblast growth factor (bFGF) potently modulates neurogenesis (Vaccarino, 1999) and cortical development (Raballo, 2000). Given stimulants or growth factors alter neural organization of normal brain, albeit in different ways, it is conceivable stimulants or growth factors may work to improve behavioural and anatomical outcomes following brain injury. Indeed, nicotine or bFGF treatments following neonatal and adult cortical injuries have been separately shown to alleviate motor (Gonzalez et al., 2006; Monfils et al., 2006) and cognitive (Brown et al., 2000; Brown et al., 2001; Comeau et al., 2008) impairments. The use of nicotine or bFGF following neonatal stroke however, is unknown.

The novelty of the current study is that nicotine or bFGF drug treatment following neonatal stroke was assessed on motor skills and neurophysiological outcomes in maturity. Rat pups were given an hypoxia ischemia stroke on postnatal day 7 (P7HI) by occlusion of one common carotid artery and subsequent exposure to hypoxia (8% O₂ for 1.5 hr). To explore the effects of nicotine or bFGF treatments following neonatal hypoxia ischemia, either nicotine (0.1 mg/kg, s.c.) or bFGF (10ng/g, s.c.) was administered with daily subcutaneous injections for seven consecutive days beginning 24 hours after ischemia. Forelimb motor behaviour was assessed in juveniles during a food maneuvering task and in adults during a reaching-for food task using the contra-lesion forelimb. The neurophysiological organization of forelimb motor maps was determined with intracortical microstimulation (ICMS) following reach testing.

It is pertinent to determine if treatment-induced improvements to motor skills show savings over long intervals. There is little known if motor skills initially learned

and practiced persist after a long interval without concurrent practice (Whishaw et al., 2008). If treatments after neonatal stroke do alter the savings of motor skills, it is more likely that alternative neural mechanisms were engaged during initial learning only transiently promoting compensation, and the impairments may re-appear. To assess if treatments following neonatal stroke alter savings of the learned motor skills, a subgroup of rats was re-tested on the reaching task 8 months later.

4.3. Methods

Subjects and Housing

Long-Evans rat pups (N=59) from five dams bred at the Canadian Centre for Behavioural Neuroscience breeding colony were used in this study. There were roughly equal numbers of male and female rats in each condition. The cross-litter design contained four groups of rats (Sham=18, P7HI=5, P7HI+Nicotine=18, and P7HI+bFGF=18). Figure 4.1 provides an outline of the experimental timeline.

Pups were weaned on postnatal day 23 and housed in same-sex groups of two or three in standard laboratory cages with food and water available *ad libitum*. Food was reduced to 85% daily serving during behavioural testing as a motivator for food reward (postnatal day 32-35 for the food maneuvering task; adulthood for three consecutive days before the single pellet reaching task). Food was reduced to 25% daily serving 16-20 hours before motor mapping surgery to minimize variability in response to anesthetics. The University of Lethbridge Animal Welfare Committee approved this study and procedures followed institutional and Canadian Council for Animal Care guidelines. Effort was taken to use the fewest possible animals and to minimize discomfort.

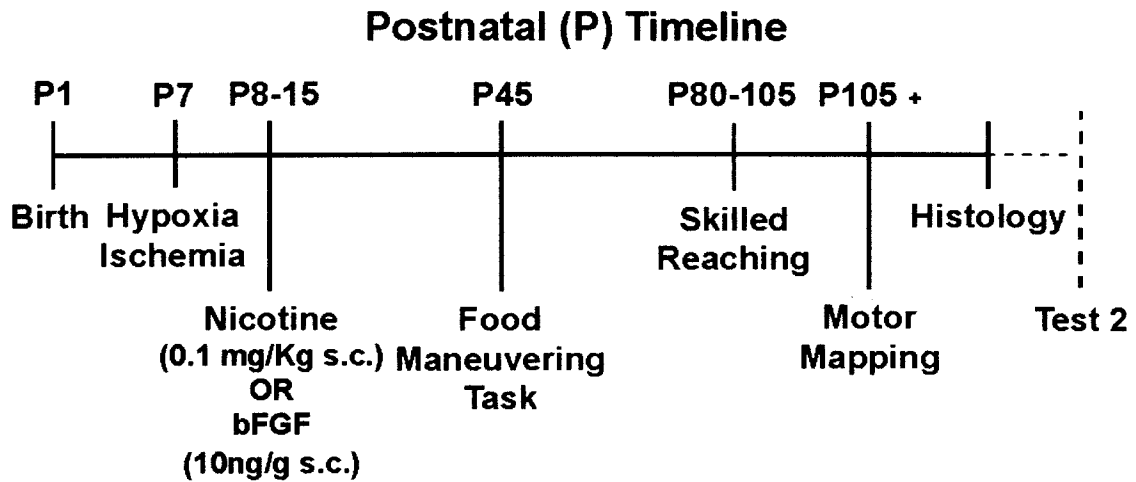


Figure 4.1. Timeline of experimental procedures. A subgroup of rats was re-tested in the skilled reaching task 8 months later and then motor maps were assessed (dotted line).

Postnatal Day 7 Hypoxia Ischemia (P7HI)

The litter was removed from the dam on postnatal day 7 and placed in a standard cage on a heating pad. Pups were anesthetized with Isoflurane and maintained under anesthesia through a modified nose cone. The ventral neck was cleaned and a midline incision was made. The right common carotid artery (CCA) was ligated (not including vagus and sympathetic nerves) and tied with two sutures (5-0 silk) 2-3 mm apart caudal to the branches of the internal and external carotid artery. The CCA was occluded with bipolar coagulation. The muscles were repositioned and the incision was closed with Vetbond tissue adhesive. The duration of the surgery was typically 5 minutes. The pup was placed in an incubator (37 °C) for 30 minutes to recover. Pups were transferred in groups of three or four to a glass jar (at least one Sham/jar) with the temperature at 36.5°C and humidified in a water bath. Exposure to hypoxia for 90 min was achieved by delivering 8 % O₂ at 110 mm Hg through a tube into the jar. Pups were placed in the incubator until

mobile (15-20 minutes) and returned to their dam. Sham surgery did not include right CCA occlusion Group assignment was random across litters and there was no mortality.

Drug Administration

Treatment with vehicle, nicotine, or bFGF began 24 hours after hypoxia ischemia on postnatal day 8. A drug was administered subcutaneously once a day for seven consecutive days at approximately 11:00 a.m. On each injection day the pups were removed from the dam and transferred to another room in a shoebox cage and placed on a heating pad. The pups were monitored for 20 minutes after drug injection and returned to the dam. The volume of subcutaneous injections was based on body weight for all treatments. The P7HI no treatment group did not receive vehicle administration.

Vehicle

The Sham group received subcutaneous injections of sterile saline (0.1ml/10g). Saline aliquots were stored at 4 °C and warmed to room temperature before daily administration.

Nicotine

Nicotine tartrate powder (N5260; Sigma-Aldrich, St. Louis) was mixed with sterile saline vehicle. Nicotine tartrate contains 25% pure nicotine and was prepared to yield a pure nicotine concentration of 0.1mg/Kg in a volume of 0.1ml/10g delivered subcutaneously. Nicotine aliquots were stored at 4 °C and warmed to room temperature before daily administration.

Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF-2) was converted from powder (R & D systems, Minneapolis, MN, USA) to liquid in bovine serum albumin (BSA 1g/ml) under sterile conditions to yield a bFGF concentration of 10ng/g in a volume of 0.1ml/10g delivered subcutaneously. bFGF aliquots were stored at -80 °C and warmed to room temperature before daily administration.

Behavioural Assessment

Behavioural Rotation

The pups were monitored for changes in activity for 20 minutes following administration of the drug treatments. If there was an obvious change in a rats' activity after drug administration the rat was positioned in the middle of an empty cylinder (20 cm in diameter and 30 cm in height) mounted on clear Plexiglas tabletop for further observation of locomotion and rotation movements. A mirror was positioned on angle below the tabletop to capture videorecords from a ventral perspective for a subgroup of rats for general observation.

Food Maneuvering Task

Forelimb abilities to maneuver food were tested between postnatal days 32-35 and videorecords on the final day of testing were used for scoring. Previous studies in adult rats have shown rats adept in manipulating food items with their forelimbs and digits to present food items to the mouth for feeding (Whishaw et al., 1997). In the present experiment, the rats were familiarized to the pasta by placing small pieces of angel hair

pasta (<1 cm dried) in the home cage for three consecutive days before testing. Three trials of each food item were scored on: 1) first forelimb to Contact and pick up each item; 2) type of Grasp used (between digits 2-3; 3-4; 4-5; or prehension grip); 3) number of forelimb Maneuvers (repositions) during item consumption; percent number of Contra-lesion forelimb Maneuvers during item consumption; and 4) Time to eat one item.

The testing apparatus was a Plexiglas cylinder 20 cm in diameter and 30 cm in height on a clear Plexiglas tabletop with a mirror below angled to allow viewing from underneath the rat. Rats were placed in cylinder for two minutes for habituation purposes and then briefly removed to remove feces or urine in order to ensure unobstructed videorecords. Rats were returned to the cylinder and three Short pieces of 4 cm long angel hair pasta (dried) were presented on the floor. Three trials (one piece of pasta for each trial) of food maneuvering were scored for Time to consume a pasta strand and for the number of forelimb Maneuvers (repositions) during item consumption.

Skilled Reaching Task

The reaching task was used to assess the rats ability to reach through an opening with one forelimb to grasp and obtain a food reward (Whishaw and Pellis, 1990). The reaching apparatus consisted of a Plexiglas box (50 cm length, 12 cm width, and 30 cm height) with an opening 1 cm wide on the front panel and a shelf mounted on the outside 2.5 cm above the floor. The contra-lesion forelimb (left forelimb in all rats) was trained by placing the pellet off-center on the contralateral side of the shelf (i.e. in the natural trajectory of the forelimb to cross midline of the body). Each rat was trained to approach the slot at the front panel, to determine if a pellet is present (using olfaction), and to

reach, grasp and transfer the pellet into the mouth in order to be scored a successful reach. The rat was required to re-set after each pellet by returning to the back of the box before a new trial. The use of a bracelet on the ipsi-lesion forelimb was required during initial training. The duration of each training session was 20 minutes for the initial sessions. Once the rat was reaching consistently, each training session consisted of 5 warm-up pellets followed by 20 trials.

Test 1: Rats were trained to reach with their contra-lesion forelimb (left forelimb in Shams). Rats were familiarized to the food target (45mg banana flavoured sugar pellets, Bioserv, Inc, Frenchtown) in their home-cage for the 3 days before training began. Training consisted of 20 trials/day for 14 sessions and then they were filmed. The number of attempts and success (# hits/20) were calculated for analysis.

Test 2: A subgroup was tested 8 months after Test 1 using the same methods with no additional training between Test 1 and Test 2. The rats were re-tested for seven days and the number of attempts and success rate on session 7 were compared with scores from session 14 in Test 1. Forgetting was determined by analyzing the first five trials on session 1 in Test 2 for attempts, success, and accuracy $[(\text{hits} / \text{attempts for hits}) * 100]$.

Videorecording

Rats were digitally videotaped during testing using a Cannon ZR 30 MC digital video camcorder with shutter speed at 1000th of a second. Additional lighting was provided by a cool florescent studio light source.

μ A were used across multiple depths between 1000 μ m and 1800 μ m (200 μ m increments).

The midpoint distance between outskirt forelimb movements and neighbouring non-forelimb movements was outlined and the enclosed area calculated using Canvas software. Areas of shoulder and elbow or wrist and digit representations were outlined and separately measured for the CFA and RFA.

Gross Anatomy

Body and Brain Weight

Body weight was measured before motor mapping in adulthood. Brain weight was measured after the mapping session and perfusion.

Histology

Rats were given an overdose of Euthansol and perfused with saline and 4 % formalin fixative. The brain was post-fixed in 4 % formalin and cryoprotected in 30 % sucrose in 4 % formalin solution in refrigeration. Coronal sections were cut at 40 μ m with a freezing microtome. Every ninth and tenth section to make two sets from the anterior to posterior pole of the brain were transferred to a glass slide prepared with a 1 % gelatin and 0.2 % chrome-alum coating. Sections were stained for cresyl violet or myelin and digital images were taken with ImageJ (NIH freeware).

Cortical Thickness

The regions of interest were motor cortex (M1) and somatosensory (S1) cortex from planes anterior, central, and posterior to Bregma. In addition, cingulate cortex (Cg1) in anterior and central planes, or retrosplenial cortex (RSGb) in the posterior plane was measured (generally referred to as cingulate unless otherwise stated). The planes corresponded to Paxinos and Watson (ref) Plate 9, Plate 18, and Plate 29 (Bregma 2.70, -0.03, and -2.30) respectively.

Myelin Histochemistry

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. The reaction was stopped by washing sections for 5 minutes in distilled water, 5 minutes in 2.5% Sodium Thiosulfate to fix, and a 30 minute rinse under slow running tap water. Sections were air-dried before cover slips were secured with Permount mounting media.

Myelination

General observations of lesion pathology were made in coronal brain sections stained for myelin. The internal capsule was not amenable to quantify because of the diffuse course through the brain although qualitative assessments were made.

Statistical Analysis

Behavioural, motor mapping, and histological data were tested with Analysis of Variance (ANOVA). Tukey's post-hoc tests were performed where appropriate. Preliminary analysis on behavioural and motor mapping data did not reveal sex differences on any of the measures so sex was collapsed. Statistics for significant results only are reported.

4.4. Results

Gross Anatomy

Body and Brain Weight

There were Sex differences for body weight with the expected effect that females weighed less than males [$F(3,22)=25.53$, $P<0.05$]. ANCOVA using Sex as the covariate did not find additional differences between the Treatment groups [$F(3,25)=1.47$, $P>0.05$]. There were Sex differences for brain weight revealing that females had less weight than males. ANOCOVA using sex as the covariate indicated that there were no differences in brain weight between the Treatment groups [$F(3,19)=1.74$, $P>0.05$].

Histology

There was individual variability in the lesion pathology in adulthood following P7HI although there were no obvious differences between non-treatment and treatment groups. A typical case showed a larger ventricle and smaller hippocampus and striatum in the ipsi-lesion hemisphere. There was no evidence of porencephalic cysts in any of the cases indicating that neonatal hypoxia ischemia produced a moderate stroke lesion (Fig 4.2).

Cortical Thickness

There were several brains that were not amenable to quantify cortical thickness either due to poor perfusion, poor staining, or misplacing the brain (remaining group sizes were Sham=9, P7HI=5, P7HI+Nicotine=10, P7HI+bFGF=6). Exploratory analysis used Sex as a covariate and the pattern of results was similar without the covariate so the following results are reported collapsing Sex.

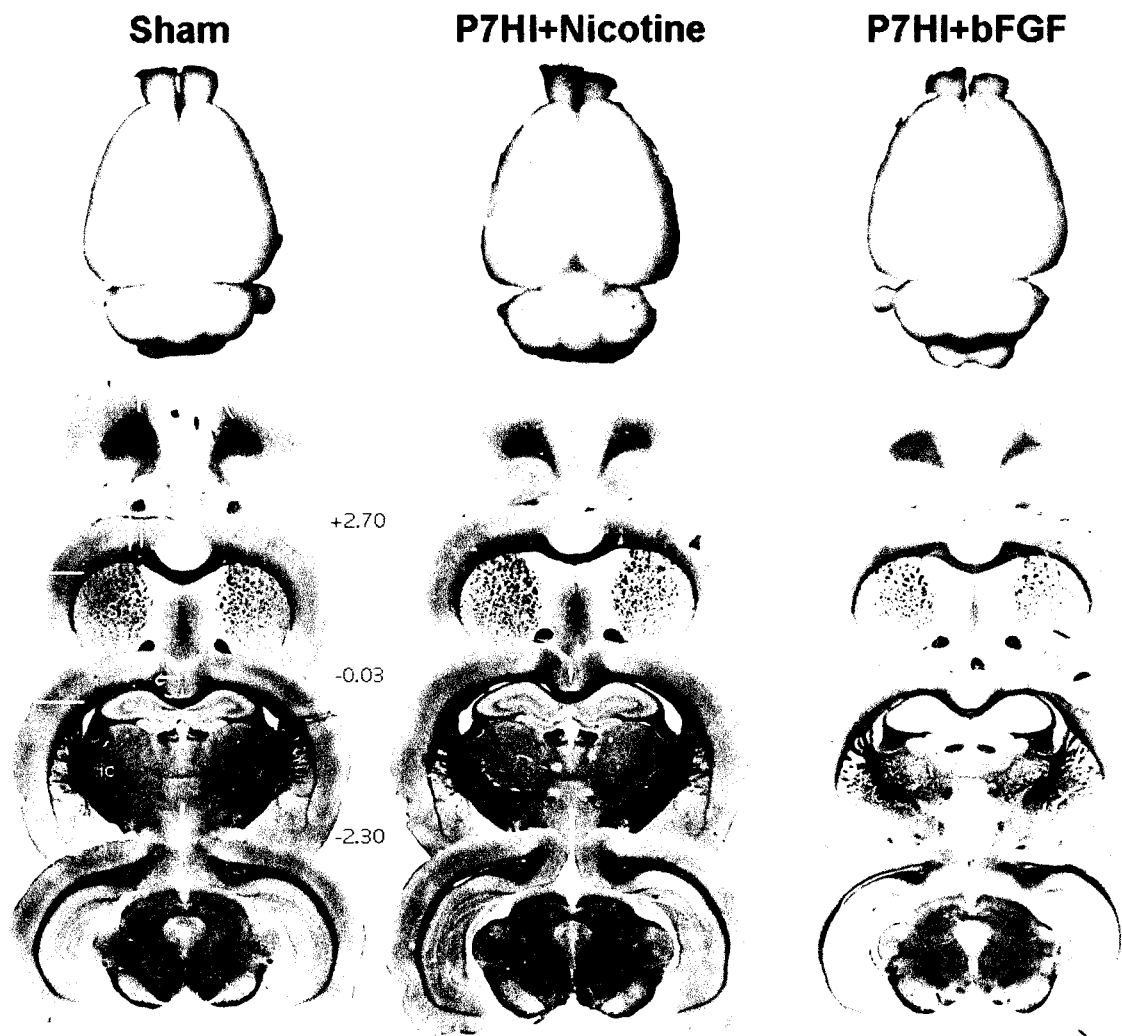


Figure 4.2. Infarct variability in adulthood following postnatal day 7 hypoxia ischemia (P7HI) with treatments. P7HI with treatment cases typically showed an enlarged ventricle (V), smaller striatum (St), and smaller hippocampus in the ipsi-lesion (Ipsi) hemisphere compared to the contra-lesion hemisphere. There was no evidence of porencephalic cysts indicating that lesion size was moderate.

There were regional changes in cortical thickness found in the ipsi-lesion hemisphere following P7HI. There were no differences between the Treatment groups in the Mean thickness found in the Ipsi-lesion motor cortex [$F(3,26)=1.32$, $P>0.05$] or the somatosensory cortex [$F(3,26)=0.44$, $P>0.05$]. In contrast, the P7HI group had a thinner cingulate cortex in the Ipsi-lesion hemisphere compared to the other groups. Follow-up ANOVA showed that the P7HI group had thinner cingulate cortex for each Plane measured [Anterior $F(3,26)=3.99$; Central $F(3,26)=8.75$; RSGB Posterior $F(3,26)=10.24$, $P's<0.05$]. The mean Percentage of Ipsi-lesion cortical thickness in relation to Contra-lesion cortical thickness was also analyzed and there were no differences for motor cortex or somatosensory cortex [M1 $F(3,26)=0.88$; S1 $F(3,26)=0.66$, $P's<0.05$, Fig 4.3 A and B]. There were differences in the Percentage of Ipsi-lesion cingulate cortex indicating that the P7HI+bFGF group had a lower percentage compared to the P7HI group, whereas the P7HI, P7HI+Nicotine, and Sham groups did not differ [$F(3,26)=11.74$, $P<0.05$, Fig 4.3 C].

There were regional changes in cortical thickness found in the Contra-lesion hemisphere following P7HI. There were no differences in thickness for motor or somatosensory cortex [M1 $F(3,26)=0.32$; S1 $F(3,26)=1.21$, $P's<0.05$]. However, the P7HI group showed a thinner cingulate cortex in the Contra-lesion hemisphere compared to the other groups [$F(3,26)=10.89$, $P<0.05$]. The data for each hemisphere was combined as an overall indication of cortical thickness and there were no differences for motor or somatosensory cortex [M1 $F(3,26)=0.88$; S1 $F(3,26)=0.66$, $P's<0.05$]. There was an overall difference for Cingulate cortex revealing that the P7HI group had thinner

Figure 4.3. Anatomy in adulthood following postnatal day 7 hypoxia (P7HI) and treatment. *A.* Motor cortex (M1) was not affected by P7HI or the treatments. *B.* Somatosensory cortex (S1) was not affected by P7HI or the treatments. *C.* Cingulate cortex showed an increased percentage of ipsi-lesion thickness in the P7HI group compared to the P7HI+bFGF group.

Myelination

The ipsi-lesion internal capsule was stained with less intensity indicating fewer myelinated fibers compared to the contra-lesion hemisphere in the P7HI groups (Fig 4.2).

There were no obvious treatment effects on lesion pathology.

Behavioural Assessment

Behavioural Rotation

The pups were monitored following injection of vehicle, nicotine or bFGF on postnatal days 9-15. There were no overt behavioural changes following bFGF treatment. However, the P7HI pups given nicotine treatment showed an increase in rotational locomotive activity that was displayed 2-3 minutes after injection and subsided in 10-15 minutes. Locomotion is not well developed at postnatal day 30; however, it appeared as though the P7HI+Nicotine pups stepped normally with their forelimbs but locomoted in a circular direction 'towards the lesion'. The P7HI+Nicotine pups were not able to pivot the hindlimbs quickly enough to orientate their posture while rotating. Typically, the ipsi-lesion hindlimb was outstretched with the plantar surface fixated on a point, and the hindlimb became over-extended as the pups rotated, sometimes causing the pup to do a complete roll with their body.

Food Maneuvering Task

A subgroup of subjects was tested although several cases in the P7HI+Nicotine (4 cases) and P7HI+bFGF (6 cases) group did not complete the task and were excluded. Amongst the remaining cases (Sham=5, P7HI=5, P7HI+Nicotine=6, P7HI+bFGF=4) there was a main effect of Treatment on Time to consume pasta strands showing that the P7HI group took more time to complete the task compared to Sham or P7HI+bFGF but did not differ from the P7HI+Nicotine group [$F(3,16)=4.68$, $P<0.05$, Fig 4.4 A].

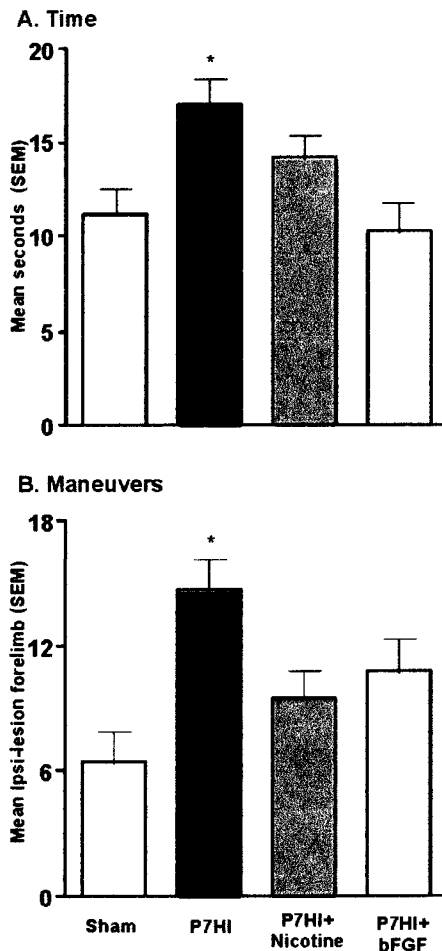


Figure 4.4. Food maneuvering performance in juveniles following postnatal day 7 hypoxia ischemia (P7HI) and treatment. *A.* The P7HI group took more time to feed the pasta item completely into the mouth (*differs from Sham and P7HI+bFGF). *B.* The P7HI group made more maneuvers with the ipsi-lesion forelimb to consume a pasta item (* differs from Sham).

Additionally, there was a difference between the Treatment groups for Total number of maneuvers revealing that the P7HI group made more maneuvers than Sham, whereas the P7HI+Nicotine or P7HI+bFGF groups did not differ from Sham or P7HI [$F(3,16)=3.29$, $P<0.05$]. Finally, there was a main effect of Treatment on the number of maneuvers indicating that the P7HI group made more maneuvers with the Ipsi-lesion forelimb compared to Sham, whereas the P7HI+Nicotine or P7HI+bFGF groups did not differ from Sham or P7HI [$F(3,16)=5.78$, $P<0.05$, Fig 4.4 B]. There were no differences between the Treatment groups for the number of maneuvers with the Contra-lesion forelimb [$F(3,16)=0.30$, $P>0.05$].

Skilled Reaching Task

All rats learned to reach by the end of training however, not all cases were reaching successfully for pellets within the first four sessions. The number of attempts decreased and the level of success increased across testing sessions. The use of a bracelet to restrict the limb not being trained was initially required in some rats (roughly equal percent of cases across groups, including Shams) but was not necessary by the end testing.

Test 1: Repeated Measures ANOVA was run on the dependent variable Attempts for Sessions 4,6,8,10,12, and 14 as the within-subjects factor and Treatment as the between-subjects factor. There was a main effect of Session [$F(5,255)=17.61$, $P<0.05$]. The Session X Treatment interaction was also significant revealing differences on Session 4 with the P7HI group making more reaching attempts than the other groups, whereas the P7HI+Nicotine or P7HI+bFGF groups made fewer attempts than P7HI or Sham and [$F(15,275)=6.98$, $P<0.05$, Fig 4.5 A]. There was an overall difference for Attempts

between Treatment groups indicating that the P7HI group made more attempts than the other groups however, the P7HI+Nicotine or P7HI+bFGF were not different than Sham [$F(3,55)=4.00$, $P<0.05$, Fig 4.5 A].

Repeated measures ANOVA run for Success (# hits/20trials) showed an effect of Session indicating that there was improvement in reaching success during testing [$F(5,255)=17.72$, $P<0.05$]. The Session X Treatment interaction was also significant and attributable to the P7HI group not showing improvement in success across testing sessions [$F(15,275)=2.63$, $P<0.05$, Fig 4.5 B]. Additionally, there was a difference for Success between Treatment groups indicating that the P7HI group was impaired at reaching success compared to Sham, but were not different from the P7HI+Nicotine or P7HI+bFGF groups, whereas the P7HI+Nicotine or P7HI+bFGF groups were not different than Sham. [$F(3,55)=4.00$, $P<0.05$, Fig 4.5 B].

Test 2: A subgroup of rats was re-tested eight months after Test 1 (Sham 7, P7HI+Nicotine 12, P7HI+bFGF 9). Not all of the rats were completing a session of 20 trials until Session 4. Repeated measures ANOVA run on Attempts for Session 4-7 indicated that there were no differences within Session, or Session X Treatment, nor between Treatments [$F(3,75)=1.24$; $F(6,75)=1.34$; $F(2,25)=0.41$, P 's > 0.05 respectively]. Success was analyzed for Session 4-7 and did not reveal any differences within Session, or Session X Treatment, nor between Treatments [$F(3,75)=3.75$; $F(6,75)=0.79$; $F(2,25)=0.59$, P 's >0.05 respectively].

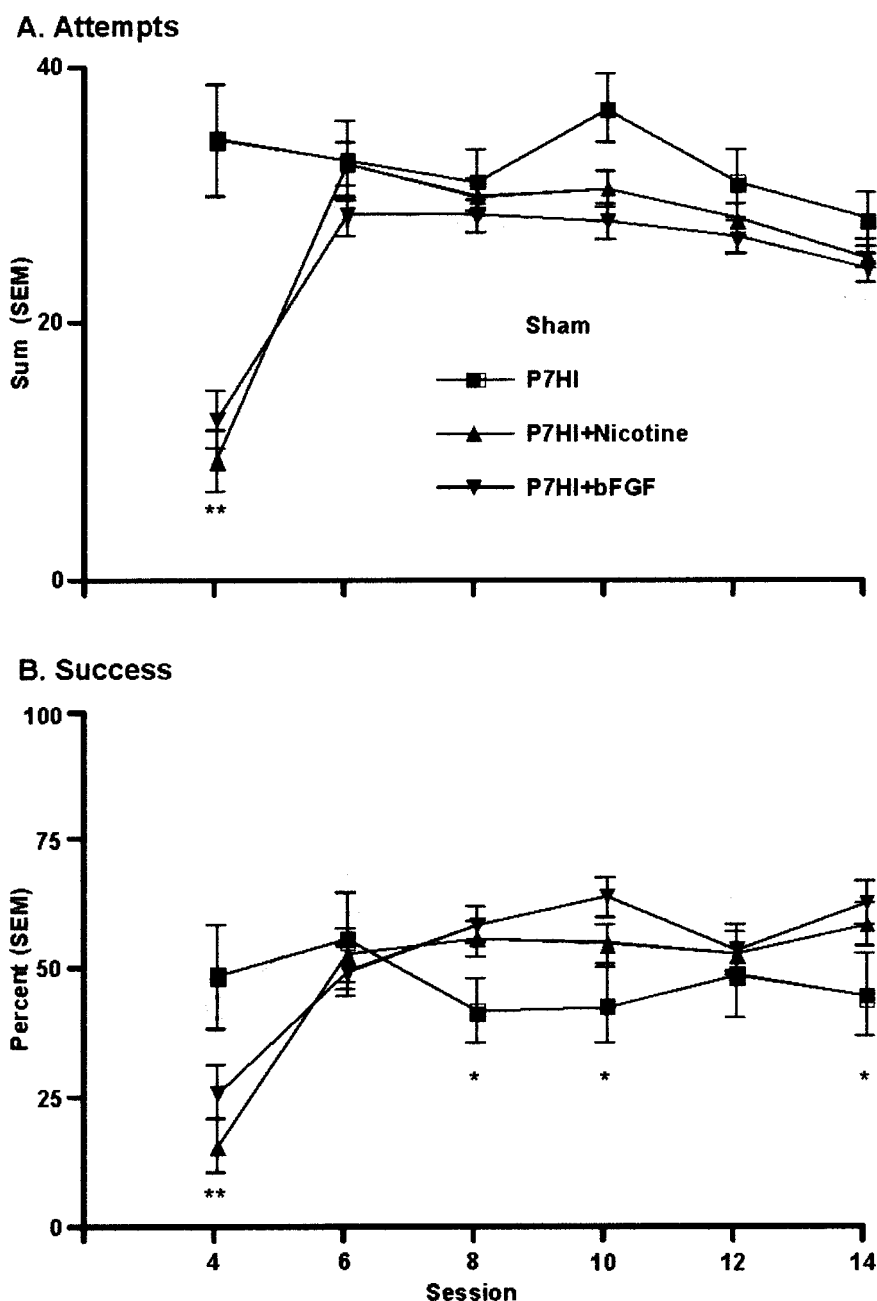


Figure 4.5. Reaching performance in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment. *A.* The P7HI+Nicotine and P7HI+bFGF groups made fewer attempts during Session 4, but there were no differences between the groups during Sessions 6-14 (** differs from Sham and P7HI). *B.* The P7HI+Nicotine and P7HI+bFGF groups were less successful initially at reaching during Session 4 (** differs from Sham and P7HI). The P7HI group was less successful at reaching during Sessions 8-14, except during Session 12 (*differs from Sham).

To determine savings of learned reaching skills, separate repeated measures ANOVA compared performance on the final session of Test 1 (Session 14) with the final session of Test 2 (Session 7). The analysis indicated that there were no group differences between Test 1 and Test 2 for Attempts [Session $F(1,25)=0.10$; Session X Treatment $F(2,25)=0.23$; Treatment $F(2,25)=0.68$, P 's >0.05 respectively, Fig 4.6 A]. Additionally, there were no group differences between Test 1 and Test 2 for Success [Session $F(1,25)=3.32$; Session X Treatment $F(2,25)=0.55$; Treatment $F(2,25)=1.50$, P 's >0.05 respectively, Fig 4.6 B].

To determine forgetting of learned reaching skills the first five trials on session 1 were analyzed and found no group differences for Attempts, Success, or Accuracy [(Attempts $F(2,28)=1.32$; Success $F(2,28)=0.69$; Accuracy $F(2,28)=0.70$, P 's >0.05 , Fig 4.7].

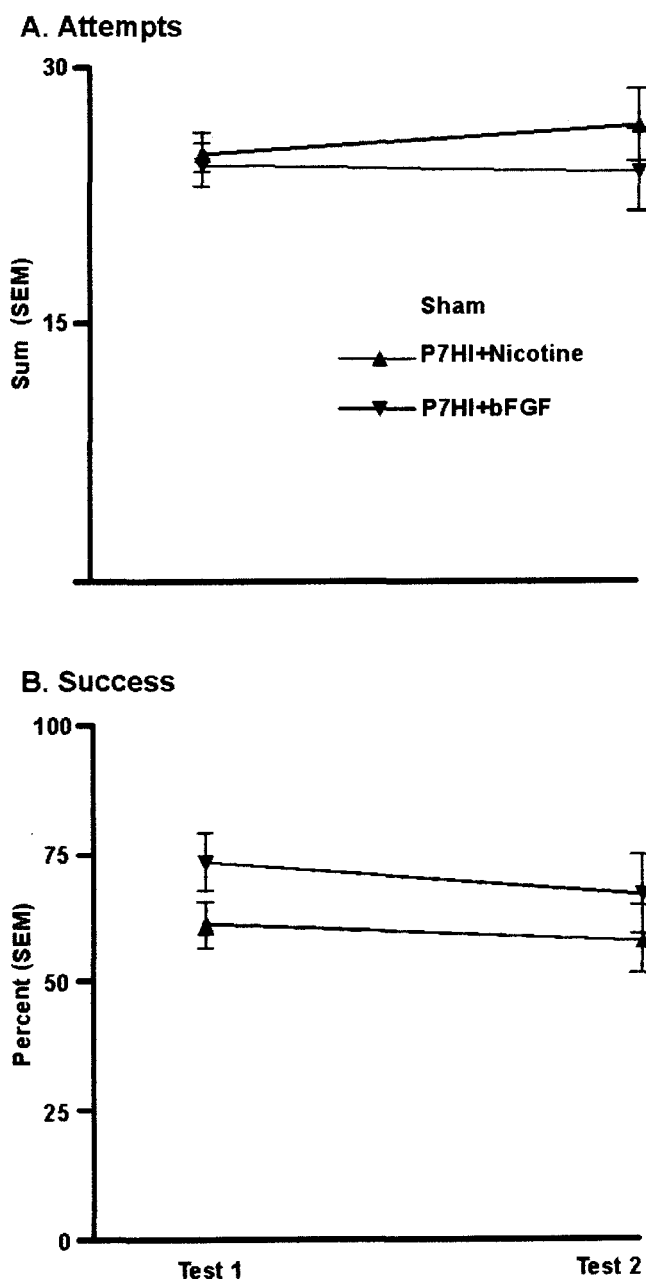


Figure 4.6. Reaching savings in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment. Test 1 is data from a subgroup of rats during Session 14 and Test 2 is data from Session 7 of the same cases re-tested 8 months later. *A.* There were no differences in reaching attempts between the groups in either test. *B.* There were no differences in reaching success between the groups in either test.

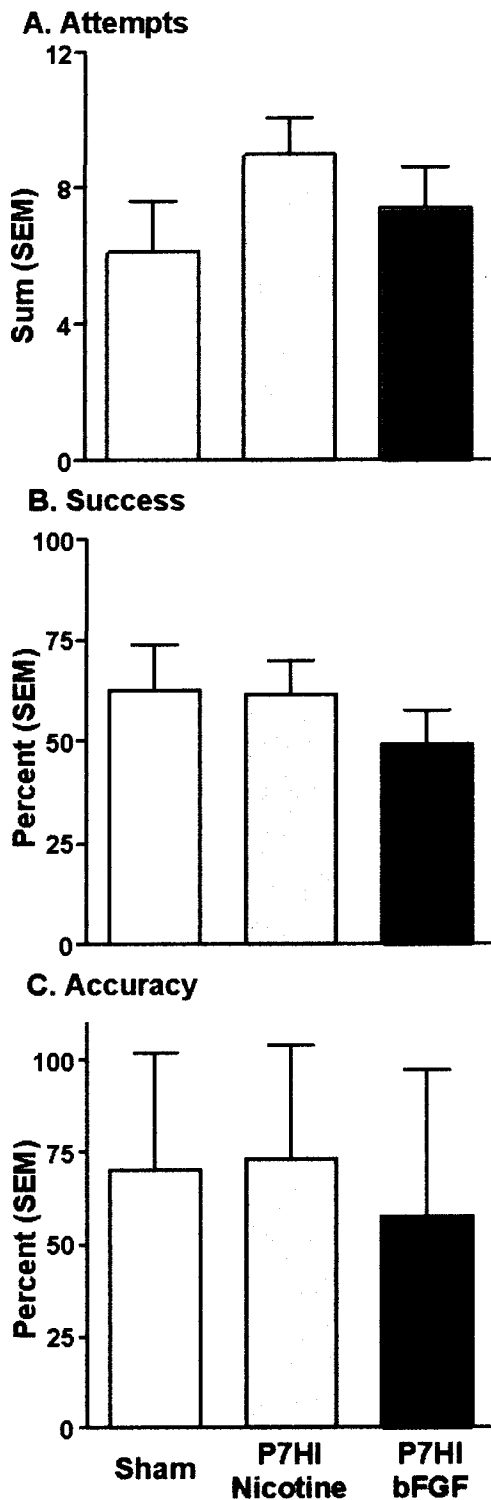


Figure 4.7. Reaching forgetting in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment. Shown are data from the first five trials on session 1 in Test 2. *A.* There were no group differences in the number of reaching attempts. *B.* There were no group differences in reaching success. *C.* There were no group differences in reaching accuracy [(hits/attempts for hits)*100].

Motor Mapping

ICMS evoked forelimb movements in the ipsi-lesion hemisphere motor cortex in every case. The cortical localization of the CFA and RFA motor maps in the ipsi-lesion hemisphere was consistent between the groups relative to Bregma. There was no difference in the number of stimulation sites, stimulation threshold, or effective electrode depth (1550 μm) amongst the groups.

The mapping surgeries occurred either after reaching Test 1 or reaching Test 2. To determine if there were Treatment differences in map size in groups mapped after Test 1 or Test 2 (see Skilled Reaching), an exploratory two-way ANOVA was conducted. The results show no group differences across tests in the ipsi-lesion maps for Total, CFA, and RFA size [Total: Treatment $F(2,27)=1.20$, Test $F(1,27)=0.34$, Interaction $F(2,27)=0.39$; CFA: Treatment $F(2,27)=1.49$, Test $F(1,27)=0.21$, Interaction $F(2,27)=0.21$; RFA: Treatment $F(2,27)=2.19$, Test $F(1,27)=0.48$, Interaction $F(2,27)=2.28$, P 's >0.05 respectively]. The mapping data for Test 1 and Test 2 were collapsed for subsequent analyses.

The area of Total forelimb representations in the Ipsi-lesion hemisphere was abnormally small in the P7HI group compared to Sham, whereas the P7HI+Nicotine or P7HI+bFGF groups did not differ from Sham or P7HI [$F(3,34)=4.57$, $P<0.05$, Fig 4.8 A]. The reduction in forelimb representations in the P7HI group was evident in a smaller ipsi-lesion CFA compared to Sham, whereas the P7HI+Nicotine or P7HI+bFGF groups did not differ from Sham or P7HI [CFA: $F(3,34)=6.20$, $P<0.05$, Fig 4.8 A]. Further analysis within the ipsi-lesion CFA showed the P7HI group had a smaller area of wrist and digit representation compared to the other groups [$F(3,34)=2.83$, $P<0.05$, Fig 4.8 B]. In

addition, the P7HI group showed a smaller area of ipsi-lesion elbow and shoulder representations compared to the other groups [$F(3,34)=4.60$, $P<0.05$, Fig 4.8 B]. Follow-up analysis within the ipsi-lesion RFA revealed that the P7HI group had a larger RFA compared to the other groups [$F(3,34)=2.86$, $P<0.05$, Fig 4.8 A]. There were no differences in RFA between the Treatment groups for the size of ipsi-lesion shoulder and elbow representations [$F(3,34)=0.70$, $P>0.05$] however, the larger RFA in P7HI rats was attributable to more wrist and digit representation compared to the other groups [$F(3,34)=3.19$, $P<0.05$]. Figure 4.9 shows reconstructed motor maps for comparison amongst the groups.

Motor map organization was also investigated in the contra-lesion hemisphere in a subgroup of rats (Sham 5, P7HI 5, P7HI+Nicotine 11, P7HI+bFGF 8). There were no differences between the Treatment groups in size of the Total area of forelimb representation [$F(3,25)=0.65$, $P>0.05$], or the CFA [$F(3,25)=0.50$, $P>0.05$], nor the RFA [$F(3,25)=0.79$, $P>0.05$].

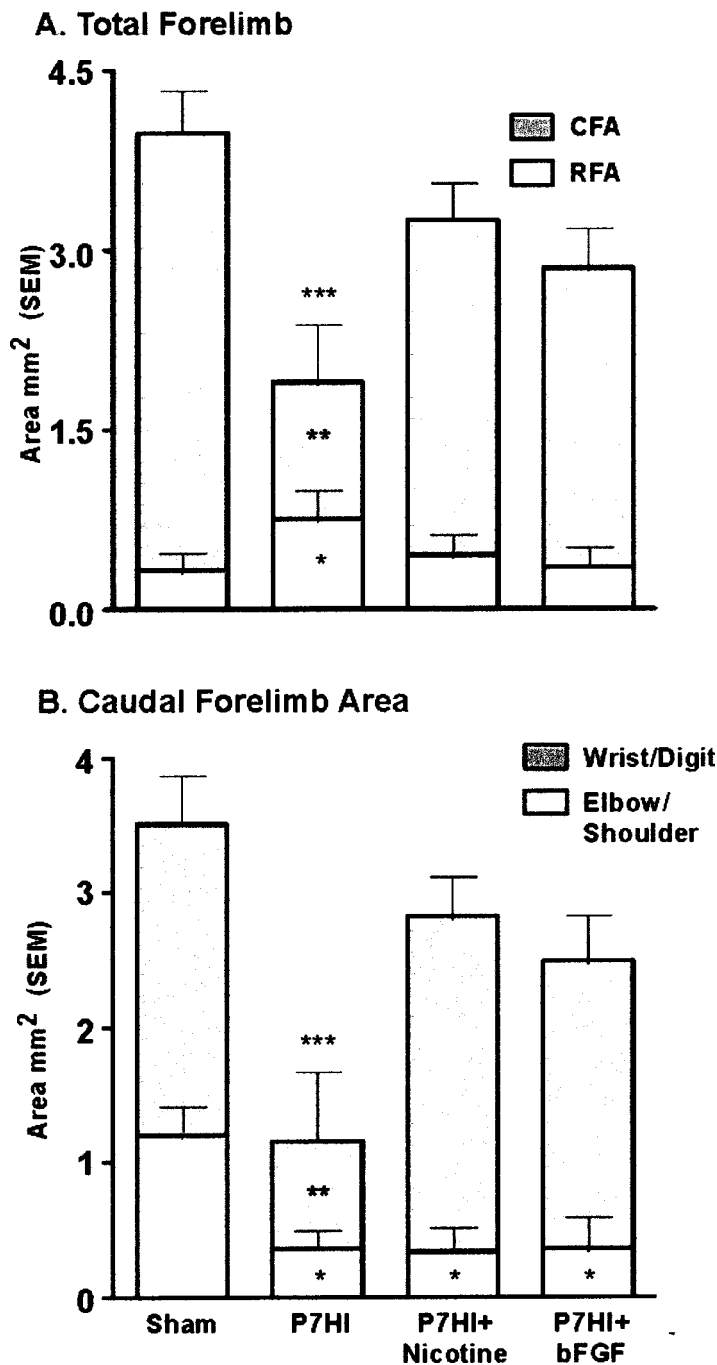


Figure 4.8. Motor map organization in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment. *A.* The total area of forelimb movements was reduced in the P7HI group that was attributable to a decrease in size of the caudal forelimb area (CFA) and rostral forelimb area, (***; **; *differs from Sham). *B.* The smaller CFA in the P7HI group corresponded to less wrist and digit representation (**differs from Sham). There was also less elbow and shoulder representation in the CFA of P7HI, P7HI+Nicotine, and P7HI+bFGF operates (*differs from Sham).

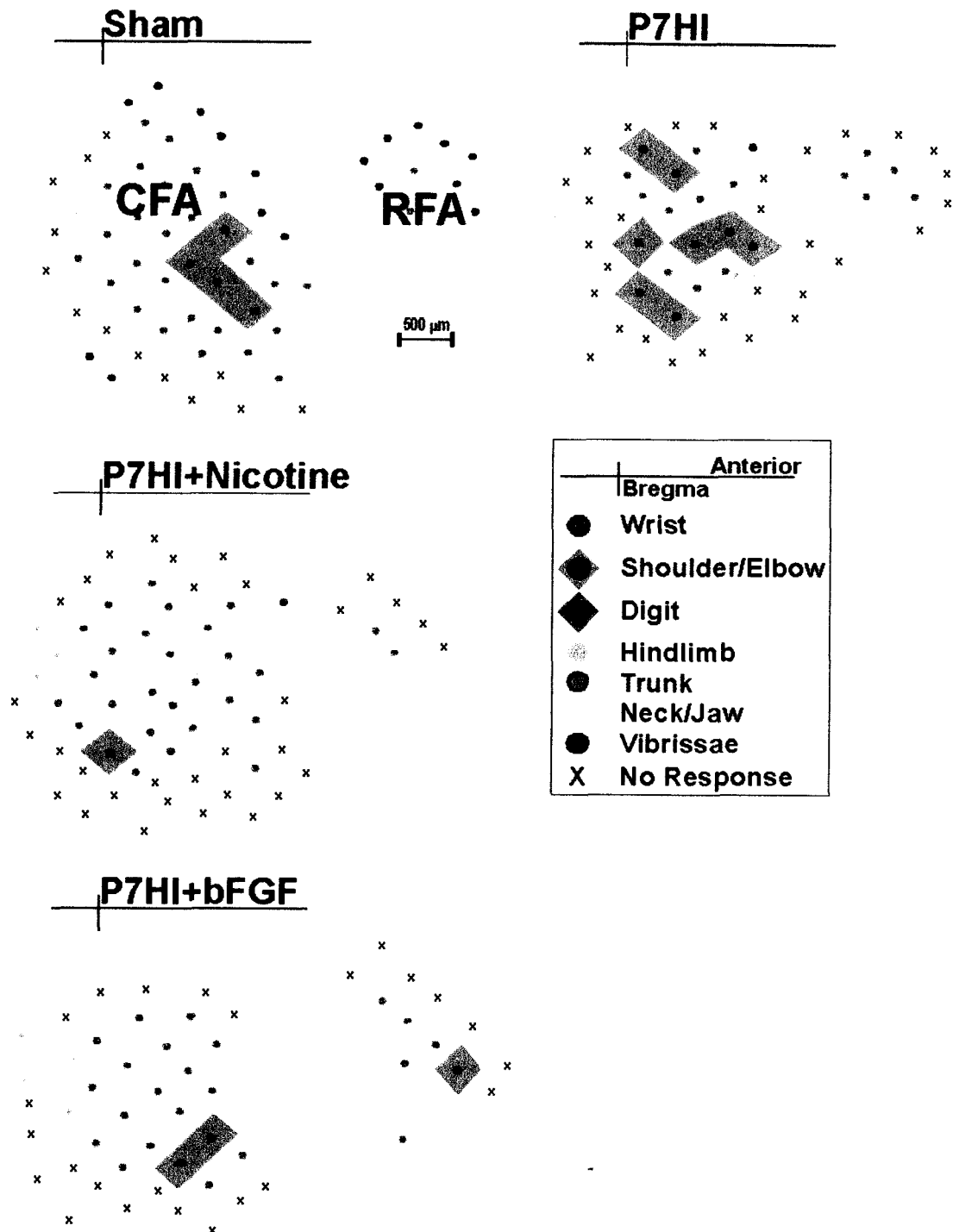


Figure 4.9. Reconstructed motor maps in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment. Shown are a typical caudal forelimb area (CFA) and rostral forelimb area (RFA) from Sham (*top left*), and representative maps from P7HI (*top right*), P7HI+Nicotine (*middle*), and P7HI+bFGF (*bottom*) groups. The P7HI group had an abnormally small CFA and large RFA maps compared to the other groups. Note the location of CFA and RFA is relatively consistent between the groups.

4.5. Discussion

This study combines anatomical, behavioural, and neurophysiological measures to determine the effectiveness of nicotine or bFGF treatment following neonatal hypoxia ischemia stroke. The main findings were that P7HI: (1) produced a moderate lesion although there was an increased the percentage of ipsi-lesion cingulate cortical thickness and this was partially ameliorated by the treatments; (2) impaired the contra-lesion forelimb in tests of maneuvering and reaching for food and this was partially ameliorated by the treatments; and, (3) diminished the size of the ipsi-lesion hemisphere motor map and again this was partially ameliorated by the treatments.

Anatomy in adulthood following P7HI and treatments

The results of the current experiment did not find that P7HI altered body or brain weight in adulthood. There were no changes in cortical thickness in motor or somatosensory cortex although the P7HI group showed a decrease in cingulate cortex thickness in both hemispheres compared to Sham and P7HI with treatment groups. The P7HI+Nicotine or P7HI+bFGF groups did not show reduced cortical thickness in either hemisphere. Unexpectedly, the percentage of ipsi-lesion cortical thickness relative to the contra-lesion hemisphere was increased in the P7HI group compared to the P7HI+bFGF group although neither group differed from Sham or P7HI+Nicotine. The cingulate regions are located distal from the infarct core and may indicate a compensatory response from damage in posterior parts of the cortex following neonatal hypoxia ischemia in the absence of treatment. Endogenous bFGF has been show to be upregulated in the lesion hemisphere following adult motor cortex lesions (Rowntree and Kolb, 1997).

Motor behaviour following P7HI and treatments

Casual observations noticed that nicotine administration in P7HI operates increased activity and induced rotation during locomotion that was not observed in the other groups. The P7HI+Nicotine group showed a turning bias towards the ipsi-lesion hemisphere indicating a behavioural asymmetry within 24 hours after hypoxia ischemia and was observed after each day of nicotine administration. The P7HI+Nicotine turning behaviour towards the lesion side is similar to that observed after amphetamine administration following adult unilateral basal ganglia damage (Hudson et al., 1993). Interestingly, the rotational behaviour in the current study was opposite compared to nicotine administration following adult basal forebrain cholinergic system damage (Abdulla et al., 1994). Thus, the ipsi-lesion cholinergic inputs to striatum are deficient and lead to rotational locomotion. Future studies should determine if psychomotor stimulant administration later in maturity also produces ipsilateral behavioural rotation following neonatal unilateral hypoxia ischemia. In addition, this study was not able to determine if the P7HI+bFGF groups would also show rotational behaviour if administered a psychomotor stimulant.

The P7HI group showed impairments in motor abilities that were not present in P7HI groups that received treatments. As juveniles the P7HI group required more time and favoured the ipsi-lesion forelimb to consume and maneuver pasta strands. As adults the P7HI group was able to learn the reaching task and were as active as the other groups although they showed a deficit in reaching success with the ipsi-lesion forelimb. Nicotine or bFGF treatments ameliorated the motor deficits ensuing from hypoxia ischemia to Sham performance levels. The findings that Nicotine or bFGF treatments were beneficial

to motor abilities have been noted in following other types of cortical injury (Gonzalez et al., 2006; Monfils et al., 2005) and blocking bFGF after cortical injury can have a detrimental effect on outcomes (Rowntree and Kolb, 1997). The beneficial endpoint behavioural measures should be taken with some caution because it is unclear why some cases in the treatment groups did not complete the food maneuvering task. Cases that were scored likely did not completely spare/recover motor skills and instead were compensating with postural and kinematics adjustments (Whishaw and Pellis, 1990). Nevertheless, the more normal endpoint measures in the P7HI with treatment groups suggest that the neural organization supporting adaptive postural and kinematics adjustments ensuing from neonatal stroke changed in favourable ways.

An important feature of motor skill learning in humans is that learned motor skills can be performed with proficiency even after a long interval without practicing. Human stroke patients show ‘forgetting’ of motor skills if there is a break in physical rehabilitation (Winstein et al., 1999). One intriguing study on the stability of learned motor skills revealed that motor cortex lesion in adult rats, but not controls, showed ‘forgetting’ in retention of a recently acquired motor skill after a two-week interval without practice, whereas savings of the memory was spared (Whishaw et al., 2008). The present study retested Sham and P7HI with treatment groups 8 months later on the skilled reaching task and found that the motor memory was not forgotten and the prior learning was saved. It is worth noting that although the motor aspect was unchanged over the long interval, the groups required a few days of training to regain the cognitive component of re-setting between trials. These findings suggest that the treatment effects following P7HI do not ‘wear-off’, even without practice over a long interval.

contributes to improvements in motor skills in adulthood (Monfils et al., 2005; Monfils et al., 2008). Moreover, children treated with intramuscular administration of bFGF following hypoxia showed improved cognitive outcomes (Aguilar et al., 1993). The findings from the current study suggest that nicotine or bFGF treatment-induced improvements to the motor maps, and motor behaviour, are long lasting.

4.6. Conclusion

The improved behavioural performance and more normal appearing ipsi-lesion motor maps following nicotine or bFGF are encouraging for promoting behavioural and neurophysiological plasticity following neonatal stroke. These results provide a brighter outlook for pediatric stroke patients, and their families.

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Chapter 5

Motor skill deficits are associated with abnormally large motor maps in adulthood following postnatal day 3 stroke in rat pups.

5.1. Abstract

Pre-term babies are one of the highest risk groups to experience an ischemic stroke. The effects of pre-term stroke on motor skill learning later in life are poorly understood. This study models stroke at an age in rat pups corresponding to the cerebral maturation of a pre-term baby to investigate motor learning and the accompanying changes to cortical motor maps. Hypoxia ischemia was administered on postnatal day 3 (P3HI) by unilateral common carotid artery occlusion followed with hypoxia (8% O₂ for 1.5 hours), or Sham operation. Motor learning was assessed in adulthood by training and testing rats to use their contra-lesion forelimb in a reaching for food task. The P3HI stroke did not achieve the same reaching success rate showing nearly a 20% disadvantage compared to Sham operates. Intracortical Microstimulation (ICMS) mapping in the ipsi-lesion hemisphere indicated that the caudal forelimb area was larger (25%), as well as the rostral forelimb area (35%), compared to Sham counterparts. Sham and P3HI rats that received reach training had a higher percentage of wrist and digit representation in the lesion (i.e. trained hemisphere) compared to those that were not trained. The caudal and rostral forelimb areas in the contra-lesion hemisphere did not differ between the groups. ICMS did not evoke bilateral movements in either hemisphere in any case. There were no differences in body or brain weight between the groups. The results indicate that P3HI induced the ipsi-lesion motor cortex to reorganize in a paradoxical manner by *increasing* forelimb map size. This is the first demonstration that stroke, at any age, shows motor skill deficits that are associated with abnormally large motor maps. Furthermore, skilled reach training partly ameliorated the motor deficits in the P3HI group that was accompanied by the same pattern of motor map reorganization from motor learning as Sham operates.

5.2. Introduction

Pre-term births account for 74% of all infant mortalities, although the majority of pre-term births do survive. Alarming, the rate of premature births is increasing; compare 9.5 % in 1981 to 12.7 % in 2005, (Goldenberg et al., 2008) and is a social and economic concern because pre-term babies are at increased risk for abnormal brain development producing long-term neurological impairments (deVries et al., 1997). Furthermore, the incidence of perinatal stroke is greater in pre-term compared to full-term infants (Benders et al., 2008). Umbilical cord or uteroplacental obstructions are serious complications to the fetus that can lead to intrauterine growth restriction (Rees et al., 2008; Yager and Ashwal, 2009) and/or episodes of hypoxia-ischemia brain damage (Lynch et al., 2002).

Pre-term infants are more susceptible to white matter injury and peri-ventricular leukomalacia (PVL) from hypoxic-ischemic episodes compared to later ages (Rees and Inder, 2005; van Haastert et al., 2008). PVL often occurs where the CST descends from the cortex at the transition of the corona radiata into the internal capsule. Damage to motor cortex or the CST early in development can produce impairments in fine motor skills, hemiplegia, spasticity, and the combination of stroke with pre-term delivery is recognized as a severe threat to trigger cerebral palsy (deVries et al., 1997; van Haastert et al., 2008). The preponderance of clinical studies has focused on stroke occurring in the postnatal period, however antenatal stroke is poorly understood. The rat is a useful model to study pre-term stroke because a third trimester human fetus is equivalent to the cerebral maturation of a postnatal day 3 (P3) rat (Yager and Ashwal, 2009). There are less than a handful of rat studies examining the effects of stroke in P3 pups (Grafe, 1994; Stadlin et al., 2003; Towfighi et al., 1997). The outcomes following postnatal day 3

hypoxia ischemia (P3HI) on motor skills or the neurophysiological function of motor cortex in adulthood are unknown.

The objective of the current study was to investigate the nature of motor skills and the neurophysiological organization of motor cortex in adulthood following P3HI. Rats were tested in adulthood to determine forelimb-use preferences in the cylinder task and motor skill learning with the contra-lesion forelimb in a reaching for food task. The functional integrity of motor cortex was assessed with intracortical microstimulation (ICMS) in both hemispheres. ICMS is the most direct assessment tool to measure the organization of motor maps (Donoghue and Wise, 1982; Martin et al., 2007) and provides insights into learning- and stroke-induced re-organization of the motor system (Nudo, 2003).

5.3. Methods

Subjects, Housing and Feeding

Long-Evans rat pups (N=36) from three litters bred at the Canadian Centre for Behavioural Neuroscience breeding colony were used in this study. The study used a cross-litter design with roughly equal numbers of male and female rats in each condition. The study design was composed of two lesion conditions (Sham and Lesion) and two training conditions (No training and Training) yielding four groups (No training: 7 Sham and 9 Lesion; Training: 7 Sham and 9 Lesion). A summary of the experimental timeline is provided in Figure 5.1.

Weaning occurred on postnatal day 23 and separated the litter by sex with food and water available *ad libitum*. Subjects were housed in pairs of two or three in standard

laboratory cages after the juvenile period. Rats were served 90% of daily chow ration for the immediate 5 days prior and during reach training in adulthood. Rats were served 25% daily chow serving 16-20 hours prior to motor mapping surgery to minimize variability in response to anesthetics. This study was approved by University of Lethbridge Animal Care Committee review and procedures followed institutional and the Canadian Council for Animal Care guidelines. Effort was taken to use the fewest possible animals and to minimize discomfort.

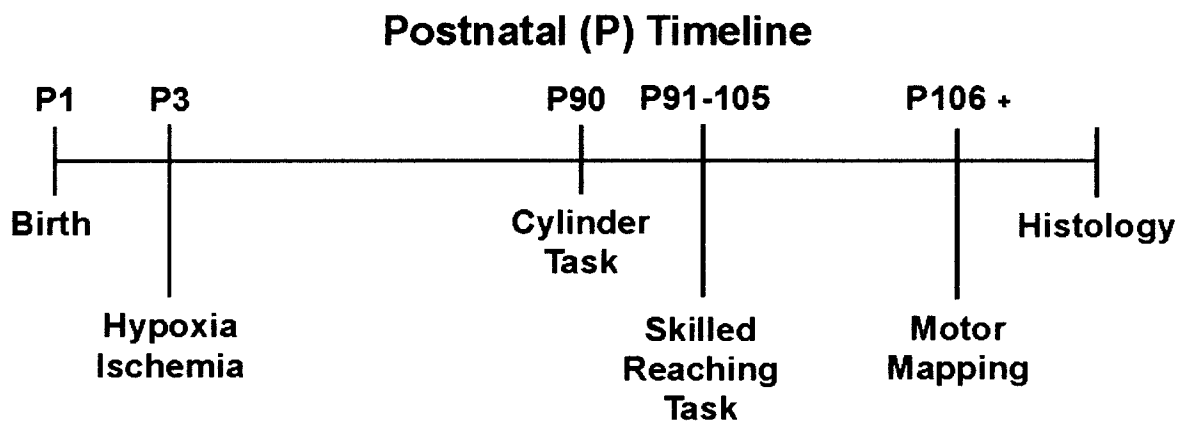


Figure 5.1. Timeline of experimental procedures.

Postnatal Day 3 Hypoxia Ischemia (P3HI)

A litter of pups was removed from the dam on postnatal day 3 and placed in a standard cage on a heating pad. Pups were anesthetized with Isoflurane and maintained under anesthesia through a mini nose cone on a heated aseptic operating table. The ventral neck was cleaned with saline and a midline scalpel incision was made. The exposed muscles were separated to expose the right common carotid artery (CCA) in all cases. The CCA was ligated and the vagus and sympathetic nerves separated. The CCA was tied with 2 sutures (5-0 silk) 2-3 mm apart caudal to the branch of the internal and external carotid

artery. The CCA was occluded between the 2 sutures with bipolar electrocoagulation. The muscles were repositioned and incision closed using Vetbond tissue adhesive. Surgery duration was kept under 5 minutes. Pups were placed in an incubator (37 °C) for 30 minutes to recuperate and transferred in groups of three or four to a glass jar with the temperature set at 36.5°C and humidified in a water bath. Exposure to hypoxia for 90 min was achieved by delivering 8 % at 110 mm Hg through a tube into the jar. Pups were returned to the incubator and once mobile they were returned to their dam. Sham surgery did not include right CCA occlusion (or vagus nerve separation). Group assignment was random across litters and there was no mortality.

Behavioural Assessment

Cylinder Task

Preferences in limb-use were assessed in adulthood during spontaneous exploration in the cylinder task (Schallert et al., 2000). The cylinder apparatus was 20 cm in diameter and 30 cm in height and was placed on a Plexiglas tabletop with an angled mirror underneath to permit filming videorecords from a ventral view of the rat. The first three minutes of exploration were scored for the number of rears on the wall or in the centre, the forelimb used first for support during a wall rear, the total touches for each forelimb on the wall, and the first forelimb to touch the floor on descent from a rear. Forelimb preference scores were calculated for first forelimb to contact wall, total forelimb touches on the wall, and first forelimb to contact the floor after a rear, using the following formula: $[(\# \text{ ipsi-lesion} / (\# \text{ ipsi-lesion} + \# \text{ contra-lesion})) * 100]$.

Skilled Reaching Task

The reaching task was used to assess the rats ability to reach through an opening with one forelimb to grasp and obtain a food reward (Whishaw and Pellis, 1990). Rats were trained to reach with their contra-lesion forelimb (left forelimb in Shams). Rats were habituated to the food target (45mg banana flavoured sugar pellets, Bioserv, Inc, Frenchtown) in their home-cage for 3 days before training began. The reaching test apparatus consisted of a Plexiglas box (50 cm length, 12 cm width, and 30 cm height) with an opening 1 cm wide on the front panel and a shelf mounted on the outside 2.5 cm above the floor. The contra-lesion forelimb (left forelimb in all rats) was trained by placing the pellet off-center on the contralateral side of the shelf (i.e. in the natural trajectory of the forelimb to cross midline of the body during a reach). Each rat was trained to approach the slot at the front panel, to determine if a pellet is present (using olfaction), and to make reaching attempts through the aperture with a forelimb to obtain the target. Multiple attempts were permitted until the target was displaced. The rat was required to re-set after each pellet by returning to the back of the box before a new trial. A successful trial was scored if the target was transferred into the mouth. The duration of each training session was 20 minutes for the initial sessions. Once the rat was reaching consistently, each training session consisted of 5 warm-up pellets followed by 20 trials. Training consisted of 20 trials/day for 16 sessions. The number of attempts, success (# hits/20), and reaching average (#hits per attempts) were recorded for analysis.

Videorecording

Each rat was digitally videotaped during testing using a Cannon ZR 30 MC digital video camcorder with shutter speed at 500th of a second. Additional lighting was provided by a cool florescent (Lowel Caselight 5, Brooklyn, NY, USA) studio light source mounted on a stand behind the camera.

Motor Mapping

Motor mapping was performed to investigate the organization of the caudal forelimb area (CFA) and the rostral forelimb (RFA) using intracortical microstimulation (ICMS) (Kleim et al., 1998). In all rats, the lesion hemisphere was assessed (i.e., trained hemisphere). In a subset of rats the contra-lesion hemisphere was also mapped (No training: Sham 3, P3HI 7; Training: Sham 5, P3HI 4). Rats were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and supplemented when necessary. The skull was trephinated to access most of the dorsal frontal cortex and anterior parts of the parietal cortex, and the cisterna magna was punctured to manage edema. The dura was retracted and the exposed cortex was covered with inert silicon fluid (37 °C). The cortical surface was photographed and a 500 mm² grid was superimposed onto the digital image with Canvas software (ACD Systems). The image was used to guide and record electrode penetration sites (Rempel et al., 2001) with an average interpenetration distance 350 µm while avoiding vasculature branches on the pial surface.

The electrode consisted of a platinum filament inserted in a borosilicate glass micropipette (20-40 µm tip diameter, 15° bevel) filled with concentrated saline (3.5 M).

The tip of the electrode was manually manipulated between points and lowered to a depth of 1550-1600 μm below the cortical surface targeting layer V pyramidal cell bodies giving rise to corticospinal tract fibers to evoke movements at the lowest threshold (Donoghue and Wise, 1982). Stimulation trains of thirteen, 200 ms, 350 Hz cathodal pulses, were delivered from a stimulation isolation unit. At each site, current intensity was gradually increased from 0 μA up to 60 μA , or until a movement was evoked. During microstimulation trains, an experimenter blind to group membership supported the rat's forelimb from underneath the elbow and visually classified forelimb movements (matching 1-second intervals of microstimulation) as proximal (shoulder or elbow) or distal (wrist or digit). In the case of two simultaneous movements, the movement obtained at the lowest threshold was considered representative of that cortical site. The minimal current threshold required to evoke a movement was recorded. Movements of hindlimb, trunk, neck, jaw and vibrissae were also marked, as well as sites that did not evoke a movement. Motor maps were reconstructed with Canvas software for analysis. The midpoint distance between outskirt forelimb movements and neighbouring non-forelimb movements was outlined and the enclosed area calculated. Outlines of forelimb movements were further separated into areas of shoulder and elbow (proximal) and wrist and digit (distal) representations for the CFA and RFA.

Anatomy

Body weight was measured before the mapping surgery. Brain weight was measured after the mapping surgeries and perfusion. After the motor mapping surgeries the rats were overdosed with Euthansol and perfused with 0.9 % saline and 4 % formalin.

Lesion Assessment

Digital photographs of the whole brains were taken of dorsal, lateral, and ventral orientations and inspected for porencephalic cavities.

Statistics

Cylinder task data were analyzed with ANOVA and Reaching Task data were analyzed with repeated-measures ANOVA on a subset of Training sessions (Sessions 6, 8, 10, 12, 14, 16). Post-hoc test were performed for within-subject effects with ANOVA or t-tests (corrected with Bonferroni-Holm's procedure). Motor mapping data was analyzed with ANOVA and comparisons between hemispheres were made with paired t-tests (corrected with Bonferroni-Holm's procedure). Analyses were conducted using SPSS software with an alpha level below 0.05 for significant results. Missing data were random. The factor Sex only affected the analyses for body weight. The results are similar if Sex is used as a covariate.

5.4. Results

Anatomy

The general appearance of the P3HI group was normal except for a ptotic eye on the lesion side. Ptosis can be caused by ischemia to the facial arteries and indicates that the hypoxia ischemia parameters used did produce a deficiency in CCA blood flow downstream of the occlusion. The ipsi-lesion hemisphere appeared reduced in the P3HI group (Fig 5.2).

Body and Brain Weight

The variable Sex had the expected effect on Body weight showing that females (mean 335g) weighed less than males (mean 487 g) [three-way ANOVA: Sex $F(1,23)=14.89$, $P<0.05$; interactions n.s.]. The groups were roughly equal between the lesion conditions so Sex was collapsed. Follow-up tests with two-way ANOVA showed there were no differences in Body weight between the Lesion [$F(1,32)=0.83$, $P>0.05$], or Training groups [$F(1,32)=3.38$, $P>0.05$], nor was there an interaction [$F(1,32)=0.38$, $P>0.05$].

Surprisingly, Sex did not have the same effect on the Brain showing that females (2.08g) had weights similar to males (2.05g) [three-way ANOVA: Sex $F(1,22)=0.03$, $P<0.05$; interactions n.s.]. Two-way ANOVA showed there were no differences in Brain weight between the Lesion [$F(1,28)=3.57$, $P>0.05$], or Training groups [$F(1,28)=0.17$, $P>0.05$], nor was there an interaction [$F(1,28)=2.86$, $P>0.05$].

Lesion Assessment

There was no obvious evidence of porencephalic cyst(s) suggesting the lesions were moderate (Fig 5.2). There were no obvious differences between training groups. The ipsi-lesion hemisphere appeared smaller than the contra-lesion hemisphere and the superior colliculi in the tectum were slightly more visible in the lesion hemisphere.

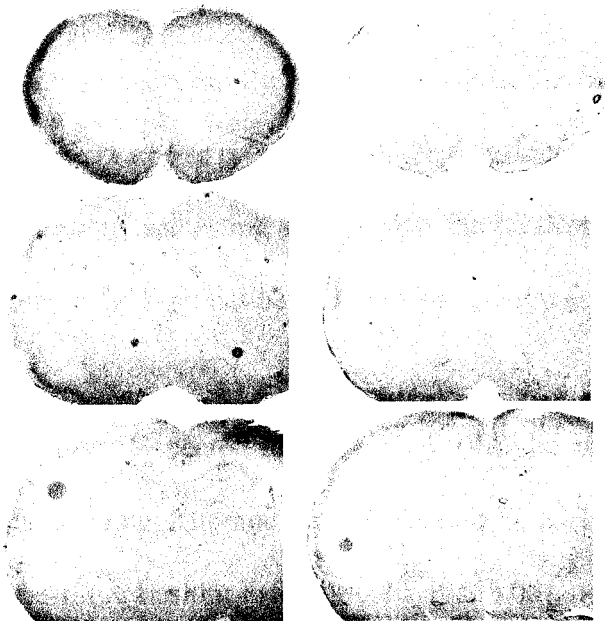
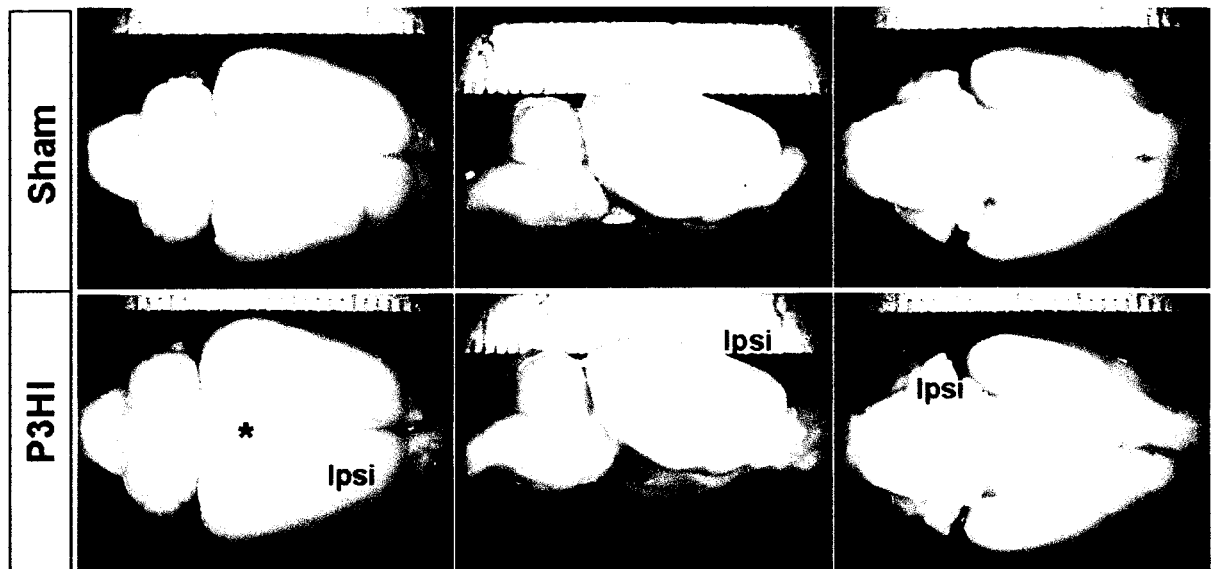


Figure 5.2. Photographs of whole brains from dorsal, lateral, and ventral views. *Top Panel:* A case is shown for the Sham group (*top row*) and the P3HI group (*bottom row*). Note the appearance of a smaller hemisphere on the ipsi-lesion side (ipsi) and more exposure of the tectum (*) in the P3HI group. *Bottom Panel:* Shown are fresh tissue sections from two P3HI cases (ipsi-lesion hemisphere on left).

Post hoc independent t-test comparisons for the Session X Lesion interaction revealed that the P3HI group was less successful at the end of the training regime (session 14 and 16) compared to Sham (Fig 5.3). Follow-up ANOVA confirmed a main effect of Lesion on reaching Success at the end of training (session 14 and 16) indicating that the P3HI group was less successful than Sham [Session 16: $F(1,14)=7.13$, $P<0.05$].

Success

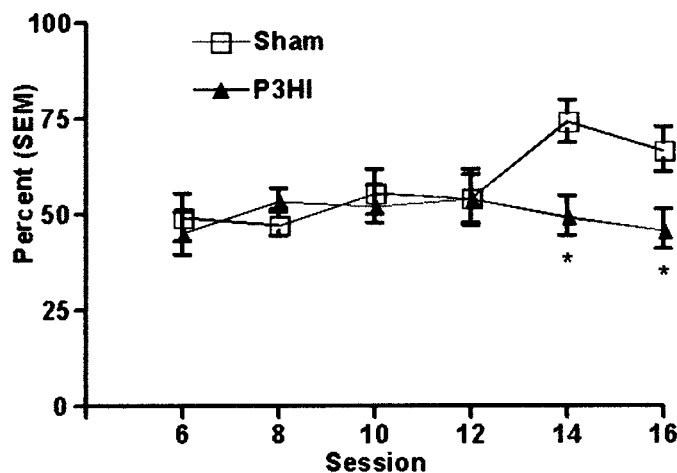


Figure 5.3. Reaching success in adulthood following postnatal day 3 hypoxia ischemia (P3HI). The P3HI group learned the task but was less successful at reaching and obtaining the food target at the end of the training period (* differs from Sham).

Accuracy. Repeated measures ANOVA did not indicate within-subjects differences on Hits per Attempts across Session 6, 8, 10, 12, 14, and 16 [Session $F(5,60)=1.61$; Session X Lesion $F(5,60)=1.88$, $P's>0.05$], nor or a between-subjects effect of Lesion [$F(1,12)=0.24$, $P>0.05$].

Motor Mapping

Informal observations revealed the P3HI group showed a locomotor turning bias moving 'towards' the lesion hemisphere after the initial injection of ketamine. The total duration of motor mapping surgeries and amount of anesthetic used was similar between the

groups. The region of cortex and optimal electrode depth for evoking forelimb movements were similar between the groups.

Ipsi-lesion Hemisphere: There were more microstimulation sites needed to complete the forelimb motor map in the P3HI group (21.5%) compared to Sham [$F(1,24)=14.304$, $P<0.05$], but there was no difference between the Training groups [$F(1,24)=0.36$, $P>0.05$] or an interaction [$F(1,24)=0.36$, $P>0.05$]. The Total size of the forelimb motor map (combined CFA and RFA) was larger in the P3HI group compared to Sham [$F(1,24)=17.34$, $P<0.05$, see 5.4. A and 5.5]. There was no further difference between Training groups [$F(1,24)=3.93$, $P>0.05$] nor an interaction [$F(1,24)=1.08$, $P>0.05$]. Analysis of the CFA showed that the P3HI group had larger CFA (24.5%) compared to Sham [$F(1,24)=16.88$, $P<0.05$]. There was no difference in CFA size between Training groups [$F(1,24)=3.64$, $P>0.05$] nor an interaction [$F(1,24)=0.83$, $P>0.05$]. Further analysis of the CFA comparing the Percentage of wrist and digit representation (distal) revealed that there was no difference between Lesion groups [$F(1,24)=0.75$, $P>0.05$]. However, rats that received reach training had a higher Percentage of wrist and digit representation compared to those that did not get training [$F(1,24)=13.25$, $P<0.05$, see 5.4 B and 5.5]. The interaction was not significant [$F(1,24)=1.31$, $P>0.05$].

Analysis of RFA showed that the P3HI group had a larger RFA (35.0%) compared to Sham operates [$F(1,24)=7.70$, $P<0.05$]. There was no difference in RFA size between Training groups [$F(1,24)=2.33$, $P>0.05$] nor an interaction [$F(1,24)=1.37$, $P>0.05$]. Further analysis of the RFA comparing the Percentage of wrist and digit representation (distal) revealed that there was no difference between Lesion groups

[F(1,24)=0.75, $P>0.05$], Training groups [F(1,24)=0.164, $P>0.05$] nor an interaction [F(1,24)=3.31, $P>0.05$].

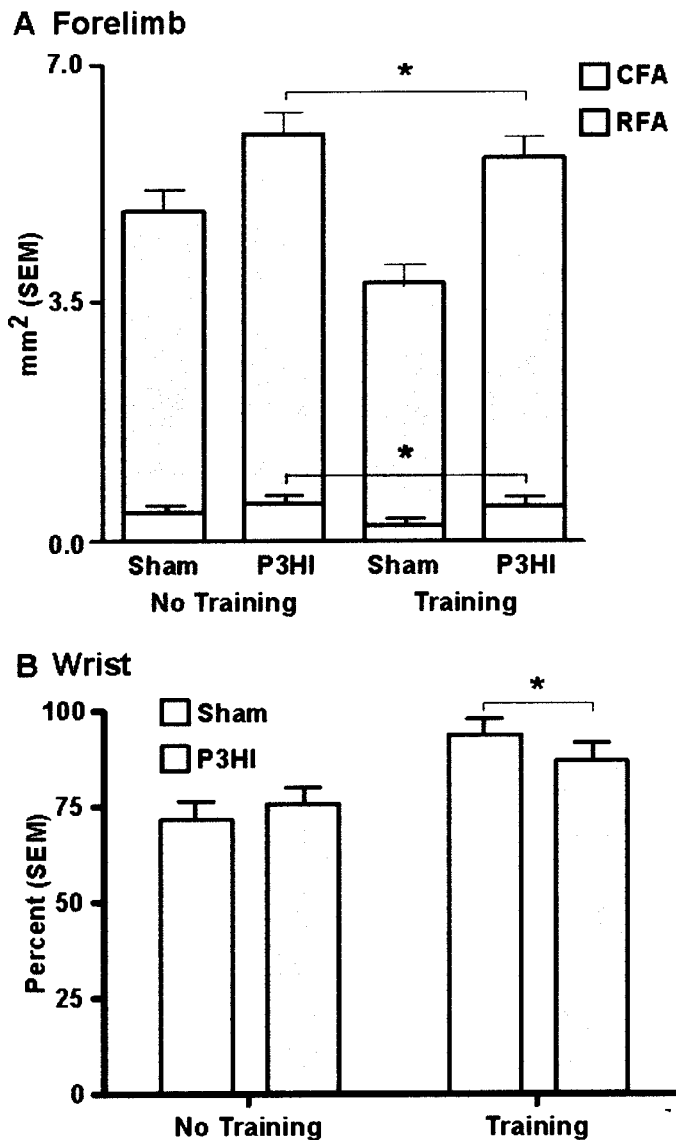


Figure 5.4. Motor maps in adulthood following postnatal day 3 hypoxia ischemia (P3HI). *A.* The total ipsi-lesion forelimb map was larger in the P3HI groups compared to Shams (*), but there was no effect of Training. The ipsi-lesion maps in P3HI groups comprised of larger caudal forelimb area, and rostral forelimb area (** CFA, *RFA, respectively), but there were no effects of Training. *B.* The caudal forelimb area map reorganized from Training by increasing the Percentage of wrist and digit representations (*differs from no-training groups) although there were no Lesion effects.

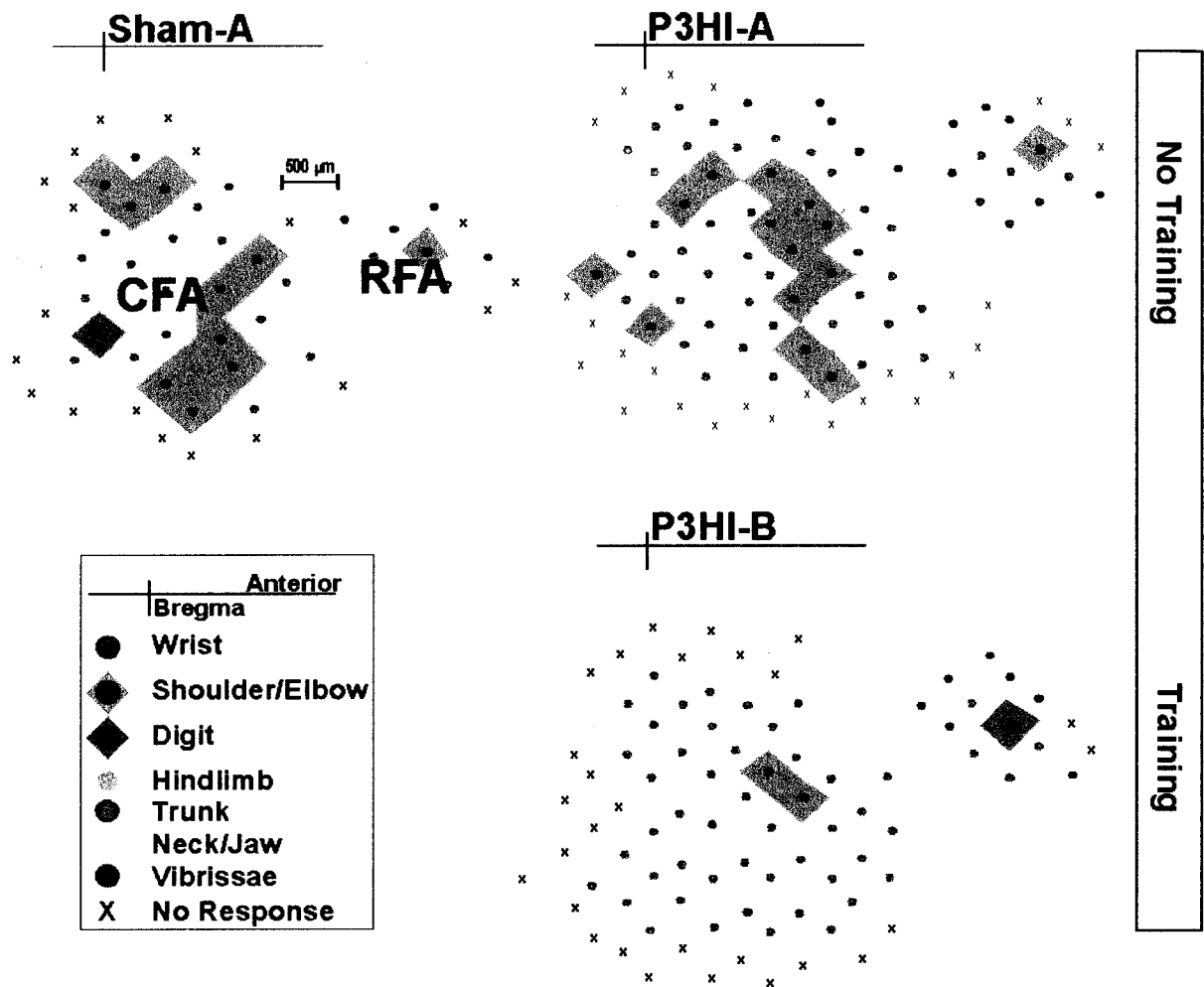


Figure 5.5. Reconstructed ipsi-lesion motor maps in adulthood following postnatal day 3 hypoxia ischemia (P3HI). Shown are a typical caudal forelimb area (CFA) and rostral forelimb area (RFA) in a Sham No-Training group (Sham-A, *top left*), and representative maps from P3HI No-Training (P3HI-A, *top right*) and P3HI Training (P3HI-B, *bottom right*) groups. Note the larger representation of wrist and digit in the trained CFA map. The location of CFA and RFA is relatively consistent between the groups.

Contra-lesion Hemisphere: In a subset of rats both hemispheres were mapped. There were no differences in the number of microstimulation sites tested for Lesion [$F(1,15)=0.82, P>0.05$], Training [$F(1,15)=1.14, P>0.05$] nor an interaction of Lesion X Training [$F(1,15)=0.82, P>0.05$]. There were no differences in Total size of the forelimb motor map (CFA and RFA) between the Lesion [$F(1,14)=2.11, P>0.05$], and Training groups [$F(1,14)=2.90, P>0.05$] nor was there an interaction [$F(1,14)=1.11, P>0.05$]. There were no differences in CFA size between the Lesion [$F(1,14)=1.73, P>0.05$], and Training groups [$F(1,14)=3.50, P>0.05$], nor an interaction [$F(1,14)=0.37, P>0.05$]. Further analysis of the CFA comparing the percentage of wrist and digit representation (distal) revealed that there was no difference between Lesion [$F(1,14)=1.89, P>0.05$], or Training groups [$F(1,14)=0.32, P>0.05$], nor an interaction [$F(1,14)=0.53, P>0.05$].

Analysis of RFA revealed that there were no differences in size between Lesion groups [$F(1,14)=0.81, P>0.05$]. There was no difference in RFA between Training groups [$F(1,14)=0.02, P>0.05$] nor an interaction [$F(1,14)=2.72, P>0.05$]. Further analysis of the RFA comparing the percentage of wrist and digit representation (distal) detected no differences between Lesion [$F(1,14)=2.39, P>0.05$], or Training groups [$F(1,14)=2.39, P>0.05$], nor was there an interaction [$F(1,14)=2.39, P>0.05$].

In order to compare maps between hemispheres, paired t-tests were performed for Sham and P3HI groups collapsed across Training on each of the above measures. The only difference found between hemispheres was in the Sham group who exhibited a smaller ipsi-lesion (i.e. trained) CFA map [$t(7)=2.89, P<0.05$]. There were no differences between hemispheres amongst the P3HI group.

5.5. Discussion

Motor deficits from stroke pose a problem across all ages, but the least is known about the effect of stroke in the perinatal period. This study used a rat pup model of hypoxia-ischemia stroke to investigate the long-term sequela on motor learning and motor map plasticity. The principal findings in adulthood were: 1) there was no evidence of limb preference during exploration although the P3HI group was less successful at reaching with the contra-lesion forelimb; 2) motor maps in the lesion hemisphere of P3HI operates were abnormally large; and, 3) reach training reorganized the ipsi-lesion map by increasing the percentage of distal representations.

Motor behaviour in adulthood following P3HI

There is evidence that the hypoxia ischemia parameters did produce a unilateral lesion. Although there were no changes in body or brain weight, the P3HI group did show common features of a unilateral stroke with ptosis and a smaller hemisphere on the ipsi-lesion side. There was no evidence of a porencephalic cyst suggesting the injury was mild. Previous detailed studies of histopathological changes have noted that P3 rats are less susceptible to severe lesions compared to P7 rats from similar hypoxia ischemia parameters (Grafe, 1994). Curiously, brain weight was not affected by sex, lesion, or training.

It is typical of adult stroke patients to use the affected limb less during motor activities, even if the task is simple (Twitchell, 1951). The P3HI stroke did not show limb preference during exploration bouts in the cylinder task nor did they need bracelets on the ipsi-lesion forelimb during reach training. The P3HI group did show a bias in locomotor

rotation behaviour after ketamine administration before the mapping surgeries (prior to profound anesthesia). Ketamine is a dissociative anesthetic that non-competitively blocks NMDA receptors. Interestingly, adult rats depleted of striatal dopamine with the neurotoxin 6-OHDA injected in the substantia nigra also show an ipsilateral turning bias if given ketamine (Johnson and Snell, 1985). The cortex is the major excitatory glutamatergic input to cholinergic cells in the striatum. It has been well documented following unilateral brain injuries that cholinergic antagonists like atropine and scopolamine produce ipsilateral turning towards the lesion hemisphere. It is possible that ketamine may indirectly affect cholinergic signalling in the striatum and could account for the turning (Johnson and Snell, 1985).

In a test of motor skill learning the P3HI group was less successful at reaching with the contra-lesion forelimb compared to Shams. The reaching success deficit in the P3HI group was not attributable to decreased attempts during training, and all of the cases learned the task. Future studies should also assess the ipsi-lesion forelimb because the deficits from stroke are often bilateral (Gonzalez et al., 2004).

Motor map organization in adulthood following postnatal day 3 hypoxia ischemia

An unexpected finding of the current study was that the ipsi-lesion maps were *larger* in the P3HI cases compared to Shams. This is the first report of a stroke at any age producing an increase in motor map representation in adulthood. In contrast, stroke at older neonatal ages or in adult rats typically diminishes the size of the ipsi-lesion maps, even if the infarct is relatively small (Gharbawie et al. 2008, Ch 3, see also Ch 6). In addition, other types of brain injury, for example, cortical injuries in infancy, which do

not directly injure the motor cortex, also produce smaller maps and in some cases to result in an absent RFA (e.g., Williams et al. 2006). Seizures have been shown to abnormally increase the size of the motor maps (Teskey et al., 2002; Young et al., 2009) and could account for these results, however, there were no obvious signs of seizures following P3HI.

It is increasingly appreciated that perinatal stroke can impair the development of cortex and/or the corticospinal tract and may contribute to motor deficits later in life. The corticospinal tract is the major descending motor pathway from the cortex and is rapidly maturing during the perinatal period. In humans, monosynaptic connections from motor cortex along the corticospinal tract (CST) to spinal motoneurons and interneurons are established in the third trimester. Transcranial magnetic stimulation (TMS) can produce EMG responses in proximal and distal muscles of the arm as early as 26 weeks postconception (Eyre et al., 2000). However, TMS in humans does not produce overt movements of the limbs until age two (Nezu et al., 1997).

The CST is capable of a remarkable rewiring following early brain injury (Hicks and D'Amato, 1975). An interesting feature of the CST believed to contribute to the capacity of the CST to rewire during development is the transient presence of CST fibers throughout the entire cortex (Joosten et al., 1987). In rats, CST connections are established in the first few postnatal days and show refinement in terminal connections lasting into adolescence (Clowry et al., 2004). Cortical injury during the first postnatal week in rats, and typically not at later ages, appears to disrupt the normal pruning of transient CST fibers. It has been shown, for example, that suction lesions of the cerebral cortex in the few days of life result in the failure of aberrant connections to be pruned

(Kolb et al., 1994). One possibility is that transient CST fibers normally pruned in the first postnatal week were retained following P3HI, and were capable of evoking movements from stimulation. There is evidence that transient CST connections do not evoke movements during normal development and likely do not support the maturation of the forelimb areas (Ch 2). The normal scenario may change following early brain injury, for example, bilateral frontal cortex lesion on postnatal day 4 in rats may also retain and incorporate otherwise transient CST connections into the mature motor map (Kleim et al., 2009). Thus, it is likely that P3HI retained transient CST fibers *and* incorporated them into mature networks to support the maps. Interestingly, the abnormal map expansion was limited to the ipsi-lesion hemisphere in CFA and RFA, and microstimulation did not evoke bilateral movements from either hemisphere. It is unclear if the mechanisms underlying the aberrant maps and associated circuitry breached regenerative or degenerative processes that mediate normal brain development, but they were specific to the ipsi-lesion hemisphere.

Motor map reorganization from learning in adulthood following P3HI

An adult control rat trained to reach for a single food target and practice the new reaching skills shows reorganization in the CFA maps opposite the trained limb whereby there is an increased percentage of wrist and digit in the CFA map (Kleim et al., 1998; Nudo et al., 1996; Williams et al., 2006). This study replicates those findings. It is interesting the ‘trained map’ can support motor learning without necessarily increasing (or decreasing) the size of the map. Rather, the intra-areal maps can reorganize existing circuitry, presumably by altering the number of local synaptic connections (Kleim et al., 2002).

Another mechanism of map reorganization is to adjust the physiological properties of the networks, for example, by altering LTP and LTD (Monfils et al., 2004; Rioult-Pedotti et al., 2000; Teskey et al., 2007).

An important question rarely considered in the neonatal stroke literature is whether the neural mechanisms that support motor learning are spared in maturity. The already grim prognosis of an aberrant map would be worse if the map was not adaptive to motor learning. This study suggests that the mechanisms supporting reorganization of the maps from learning and practicing a new motor skill in adulthood are spared following P3HI. Furthermore, this study was able to determine that reorganization from motor learning occurred in the ipsi-lesion (i.e., trained) map, whereas the contra-lesion (i.e., not trained) map did not reorganize from the neonatal lesion, or the training in adulthood.

5.6. Conclusion

There is an optimal size of the forelimb motor map that is often altered by brain injury such as stroke. The unique findings that postnatal day 3 stroke abnormally increased the size of the motor map and was accompanied by motor skill impairments in adulthood suggests that a bigger map is not necessarily better.

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Chapter 6

Nicotine treatment does not improve reaching skill impairments or motor map deficits following postnatal day 14 hypoxia ischemia in rats.

6.1. Abstract

Children who experience cerebral vasculature accidents are susceptible to show enduring neurological motor deficits. There are limited treatments available. The psychomotor stimulant nicotine is a promising treatment because it has been shown to alleviate motor impairments and induce cortical reorganization following brain injury. The objective of this study was to assess the effects of nicotine drug treatment on behavioural and neurophysiological outcomes following neonatal hypoxia ischemia in a rat model. Rat pups on postnatal day 14 were given an hypoxic ischemic stroke (P14HI) by occlusion of one common carotid artery and subsequent exposure to hypoxia (8% O₂ for 1.5 hr). Drug treatments commenced one day following P14HI with nicotine (0.1 mg/kg, s.c.) daily injections for seven consecutive days. Forelimb motor behaviour was assessed in juveniles in the cylinder task and during food maneuvering. The contra-lesion forelimb was assessed in adulthood during a skilled reaching for food task. The neurophysiological organization of forelimb motor maps was determined with intracortical microstimulation (ICMS) following reach testing. The results indicate that neonatal stroke produced a preference to use the ipsi-lesion forelimb more often during the cylinder task and was not attenuated by nicotine treatment. There were no group differences during the food maneuvering task. The P14HI group showed deficits in success at reaching with the contra-lesion forelimb. Motor map analysis in the ipsi-lesion hemisphere for P14HI rats without treatment indicated that the caudal forelimb area (CFA) was abnormally small although the rostral forelimb area (RFA) did not change compared to Shams. Rats that received nicotine treatment did not show attenuation of the behavioural deficits or abnormal CFA. Maps in the contra-lesion hemisphere indicated no differences in

organization between Sham and P14HI, and there were no effects of nicotine. Histological analysis suggested that the ipsi-lesion hemisphere was smaller than normal although there were no large cysts in any of the cases. Assessment of myelin indicated that the P14HI groups showed reduced staining in the internal capsule in the ipsi-lesion hemisphere. Together, these findings suggest that motor deficits from neonatal stroke are attributable, at least in part, to abnormal ipsi-lesion motor maps in adulthood and that nicotine treatment was not able to reverse the deficits. It thus appears that more heroic efforts are needed for treating childhood stroke.

6.2. Introduction

Stroke is a leading cause of death and enduring neurological morbidity following brain injury in children 0-18 years old. Childhood stroke occurring in ages less than 1 year accounts for 40% of cases reported (Lynch et al., 2002). Pediatric stroke cases are not typically diagnosed within the first 24, usually because they are not taken to hospital for examination immediately after the ischemic attack (deVeber, 2002). The behavioural outcomes in children who experience a stroke typically include cognitive and motor impairments (Deveber et al., 2008; Wu et al., 2004). There have been no treatments to stimulate complete recovery following childhood stroke (Whelan et al., 2008). Prior studies in the thesis modeled rat analogs of full-term or pre-term human stroke and found motor deficits in adulthood that were attributable, at least partially, to abnormal motor maps in the lesion hemisphere. Thus, it appears that the age-at-stroke influences the details in the nature of motor impairments and the accompanying neurophysiological motor maps.

There have been no experimental models studying the nature of motor deficits, if any, in an early childhood model of stroke. There is histological evidence in rats demonstrating that hypoxia ischemia on postnatal day 13 is more detrimental to cortical and hippocampal pyramidal cell health compared to a similar stroke occurring at younger ages (Towfighi et al., 1997). It is possible that abnormal cortical pyramidal cells may affect the neurophysiological function of motor maps and together, may contribute to motor deficits. It is important to understand the nature of the deficits in a childhood model of stroke to test the therapeutic value of treatments. Psychomotor stimulants have been shown to offer some improvements following brain injury and can be easily

administered (Kolb, 1995). Considering there were benefits of nicotine treatment following postnatal day 7 hypoxia ischemia (Ch 4), a similar treatment regime was employed in the current study.

The first objective of this study was to investigate motor skills following hypoxia ischemia in rats at an age analogous of early childhood stroke in humans (approximately 0.5-1 years old). A second objective was to determine if nicotine treatment promoted motor improvements. Rat pups on postnatal day 14 were given a hypoxic ischemic stroke (P14HI) by occlusion of one common carotid artery and subsequent exposure to hypoxia (8% O₂ for 1.5 hr) in a temperature controlled humidified chamber. Drug treatment commenced one day following P14HI using daily nicotine (0.1 mg/kg, s.c.) injections for seven consecutive days. The rats were assessed as juveniles for forelimb preferences and manipulation skills and as adults in a skilled reaching task. Motor maps in both hemispheres were assessed with intracortical microstimulation in adulthood. A subgroup of cases was also mapped in the contra-lesion hemisphere. Histology was performed to assess gross changes in hemisphere structure and myelination of major axonal pathways.

6.3. Methods

Subjects, Feeding and Housing

Long-Evans rat pups (N=39) from five dams bred at the Canadian Centre for Behavioural Neuroscience breeding colony were used in this study. The study used a cross-litter design with roughly equal numbers of male and female rats in each condition. The experimental design contained four groups (Sham=8, Sham+Nicotine=8, P14HI=13, and P7HI+Nicotine=10). A summary of the timeline is provided in Figure 6.1.

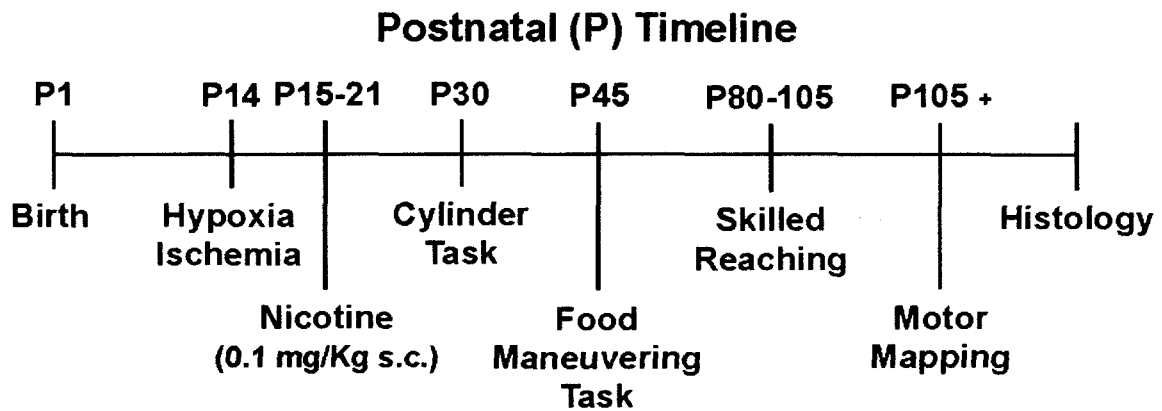


Figure 6.1. Timeline of experimental procedures.

Pups were weaned on postnatal day 23 and housed in same-sex groups of two or three in standard laboratory cages with food and water available *ad libitum*. Food was reduced to 85% daily serving during behavioural testing as a motivator for food reward (postnatal day 42-45 for the food maneuvering task; adulthood for three consecutive days before the single pellet reaching task). Food was reduced to 25% daily serving 16-20 hours before motor mapping surgery to minimize variability in response to anesthetics. The University of Lethbridge Animal Welfare Committee approved this study and procedures followed institutional and Canadian Council for Animal Care guidelines. Effort was taken to use the fewest possible animals and to minimize discomfort.

Postnatal Day 14 Hypoxia Ischemia (P14HI)

The litter was removed from the dam on postnatal day 14 and placed in a shoebox cage on a heating pad. A pup was anesthetized with Isoflurane and maintained under anesthesia through a modified nose cone. The ventral neck was cleaned and a midline incision was made. The right common carotid artery (CCA) was ligated (not including

vagus and sympathetic nerves) and tied with two sutures (5-0 silk) 2-3 mm apart caudal to the branches of the internal and external carotid artery. The CCA was occluded with bipolar coagulation. The muscles were repositioned and the incision was closed with Vetbond tissue adhesive. The duration of the surgery was typically 5 minutes. The pup was placed in an incubator (37 °C) for 30 minutes to recover. Pups were transferred in groups of three or four to a glass jar with the temperature set at 36.5°C and humidified in a water bath. Exposure to hypoxia for 90 min was achieved by delivering 8 % O₂ at 110 mm Hg through a tube into the jar. Pups were placed in the incubator until mobile (15-20 minutes) and returned to their dam. Sham surgery did not include right CCA occlusion. Group assignment was random across litters and there was no mortality.

Drug Administration

Drug treatment with nicotine began 24 hours after hypoxia ischemia on postnatal day 15. Nicotine or vehicle (equal volume of saline) was administered once a day once for seven consecutive days at approximately 11:00 a.m. On each day the pups were removed from the dam and transferred to another room in a shoebox cage placed on a heating pad. The pups were monitored for 20 minutes after drug administration for changes in activity and returned to the dam.

Vehicle

The Sham group received subcutaneous injections of sterile saline (0.1ml/10g). Saline aliquots were stored at 4 °C and warmed to room temperature before daily administration.

Nicotine

Nicotine tartrate powder (N5260; Sigma-Aldrich, St. Louis) was mixed with sterile saline vehicle. Nicotine tartrate contains 25% pure nicotine and was prepared to yield a pure nicotine concentration of 0.1mg/Kg in a volume of 0.1ml/10g delivered subcutaneously. Nicotine aliquots were stored at 4 °C and warmed to room temperature before daily administration.

Behavioural Assessment

Cylinder Task

Rats will spontaneously rear and use their forelimbs for support on the wall when placed in a narrow Plexiglas cylinder (Schallert et al., 2000). Rats were individually placed into the cylinder for 5 minutes on postnatal day 30. The cylinder was 20 cm in diameter and 30 cm in height placed on clear Plexiglas tabletop. A mirror was positioned on angle below the tabletop to capture videorecords from a ventral perspective of the rat. Videorecords were scored for the number of rears on the wall or in the centre, the forelimb used first for support during a wall rear, the total touches for each forelimb on the wall, and the first forelimb to touch the floor on descent from a rear. Forelimb preference scores were calculated for first forelimb to contact wall, total forelimb touches on the wall, and first forelimb to contact the floor after a rear, using the following formula: $[(\# \text{ ipsi-lesion} / \# \text{ ipsi-lesion} + \# \text{ contra-lesion}) * 100]$.

Food Maneuvering Task

Rats are adept in manipulating food items with their forelimbs and digits to present food items to the mouth (Whishaw et al., 1997). Testing began on postnatal day 42 and the rats were filmed on postnatal day 45 for scoring. Rats were habituated to the food items by placing small pieces of angel hair pasta (<1 cm dried) in the home cage for three days before testing.

The testing apparatus was a Plexiglas cylinder 20 cm in diameter and 30 cm in height set vertically on a clear Plexiglas tabletop with a mirror below angled to allow viewing from underneath the rat. Rats were placed in cylinder for two minutes for habituation purposes and then briefly removed to clean feces or urine in order to ensure unobstructed videorecords. Rats were returned to the cylinder and three Short pieces of 4 cm long angel hair pasta (dried) were presented on the floor. Three trials were scored on: 1) first forelimb to Contact and pick up each item; 2) type of Grasp used to pick up each item (between digits 2-3, 3-4; 4-5; or prehension grip); 3) number of forelimb Maneuvers (repositions) during item consumption; percent number of Contra-lesion forelimb Maneuvers during item consumption; and 4) Time to eat one item.

Skilled Reaching Task

The reaching task was used to assess the rats ability to reach through an opening with one forelimb to grasp and obtain a food reward (Whishaw and Pellis, 1990). Rats were trained to reach with their contra-lesion forelimb (left forelimb in Shams) in adulthood. Rats were familiarized to the food target (45mg banana flavoured sugar pellets, Bioserv, Inc, Frenchtown) in their home-cage for the 3 days before training began.

The reaching apparatus consisted of a Plexiglas box (50 cm length, 12 cm width, and 30 cm height) with an opening 1 cm wide on the front panel and a shelf mounted on the outside 2.5 cm above the floor. The contra-lesion forelimb (left forelimb in all rats) was trained by placing the pellet off-center on the contralateral side of the shelf (i.e. in the natural trajectory of the forelimb to cross midline of the body). Each rat was trained to approach the slot at the front panel, to determine if a pellet is present (using olfaction), and to reach, grasp and transfer the pellet into the mouth in order to be scored a successful reach. The rat was required to re-set after each pellet by returning to the back of the box before a new trial. The use of bracelets on the ipsi-lesion forelimb was required during initial training. The duration of each training session was 20 minutes for the initial sessions. Once the rat was reaching consistently, each training session consisted of 5 warm-up pellets followed by 20 trials. Training consisted of 20 trials/day for 14 sessions and then they were filmed. The number of attempts and success (# hits/20) were calculated for analysis.

Motor Mapping

ICMS motor mapping was conducted to map the organization of the caudal forelimb area (CFA) and the rostral forelimb area (RFA) (Kleim et al., 1998). The ipsi-lesion hemisphere (right side) was assessed after reach training in adulthood. Rats were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and supplemented when necessary. The skull over motor cortex was trephinated and dura retracted. The cortical surface was digitally photographed and a grid (500 mm²) was superimposed onto the image using Canvas (ACD Systems) to guide and notate electrode

penetration sites (Remple et al., 2001). The electrode was made of a platinum filament inserted in a borosilicate glass micropipette (20-40 μm tip diameter, 15° bevel) filled with concentrated saline (3.5 M). The electrode tip was lowered to 1550-1600 μm beneath the cortical surface corresponding to layer V pyramidal cell bodies giving rise to corticospinal tract fibers (Donoghue and Wise, 1982). Microstimulation trains of thirteen, 200 ms, 350 Hz cathodal pulses, were delivered from a stimulation isolation unit. At each site the current intensity was gradually increased from 0 μA up to 60 μA , or until a movement was evoked. An experimenter supported the rat's forelimb from underneath the elbow and visually classified evoked forelimb movements as shoulder or elbow (proximal) or wrist or digit (distal) during microstimulation trains (350 μm interpenetration distance). The movement obtained at the lowest threshold was recorded along with the threshold intensity.

After completion of motor mapping under these parameters, forelimb representations were abnormally small in some rats. In such cases, non-responsive sites were reinvestigated, first re-testing sites with the same parameters as above, and if still unresponsive, current intensities up to 150 μA were used across multiple depths between 1000 μm and 1800 μm (200 μm increments).

The midpoint distance between outskirt forelimb movements and neighbouring non-forelimb movements was outlined and the enclosed area calculated using Canvas software. Areas of shoulder and elbow or wrist and digit representations were outlined and separately measured for the CFA and RFA.

Gross Anatomy

Brain and Body Weight

Body weight was measured before motor mapping. Brain weight was measured after the mapping session and perfusion.

Histology

Rats were given an overdose of Euthansol and perfused with saline and 4 % formalin fixative. The brain was post-fixed in 4 % formalin and cryoprotected in 30 % sucrose in 4 % formalin solution in refrigeration. Coronal sections were cut at 40 μ m with a freezing microtome. Every tenth section from the anterior to posterior pole of the brain was transferred to a glass slide prepared with a 1 % gelatin and 0.2 % chrome-alum coating. Sections were stained for myelin and digital images were taken. Images were gathered with a Zeiss Axiovision 4.3 (Zeiss, Germany) at 1x magnification outfitted with a CCD camera.

Myelin Histochemistry

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 before cover slips were secured with PermOUNT mounting media.

Myelination

General observations of lesion pathology were made in coronal brain sections stained for myelin. The internal capsule was not amenable to quantify because of the diffuse course through the brain although qualitative assessments were made.

Videorecording

Each rat was digitally videotaped during testing using a Cannon ZR 30 MC digital video camcorder with shutter speed at 1000th of a second. Additional lighting was provided by a cool florescent studio light source.

Statistical Analysis

Behavioural and motor mapping data were tested with two-way Analysis of Variance (ANOVA) for Lesion and Treatment. Tukey's post-hoc tests were performed where appropriate. Preliminary analysis did not reveal sex differences on any of the measures so sex was collapsed unless otherwise stated. Differences across hemispheres for the mapping data was compared with multiple paired t-test for each group and corrected with Bonferroni-Holm's post hoc. Statistics for significant results only are reported.

6.4. Results

Gross Anatomy

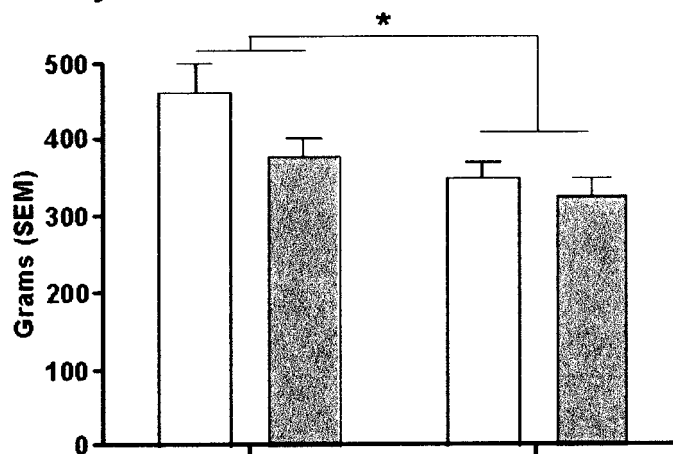
Body and Brain Weight

There was no mortality in any of the groups and there were no obvious seizures in any of the cases. There were Sex differences in body weight showing that males (446.5g) were

heavier than females (341.2g) although Sex did not interact with Lesion or Treatment [Sex $F(1,24)=12.56^*$; Sex X Lesion $F(1,24)=0.51$; Sex X Treatment $F(1,24)=1.46$, Sex X Lesion X Treatment $F(1,24)=0.11$, $*P<0.05$]. Sex was used as a covariate to determine the effect of Lesion and Treatment on Body weight and revealed that the P14HI groups had lower body weight compared to the Sham groups although there was no interaction [Lesion $F(1,27)=9.53^*$; Treatment $F(1,27)=3.66$; Interaction $F(1,27)=1.42$, $*P<0.05$, Fig 6.2 A].

There were Sex differences in brain weight with males (1.96g) showing heavier weights than females (1.81g) although Sex did not interact with Lesion or Treatment [Sex $F(1,13)=4.42^*$; Sex X Lesion $F(1,13)=0.01$; Sex X Treatment $F(1,13)=0.11$, Sex X Lesion X Treatment $F(1,13)=0.50$, $*P<0.05$]. Sex was used as a covariate to determine the effects of Lesion and Treatment on Brain weight and there were no differences between the groups [Lesion $F(1,15)=0.27$; Treatment $F(1,15)=0.84$; Interaction $F(1,15)=0.75$, $P's>0.05$, Fig 6.2 B].

A. Body



B. Brain

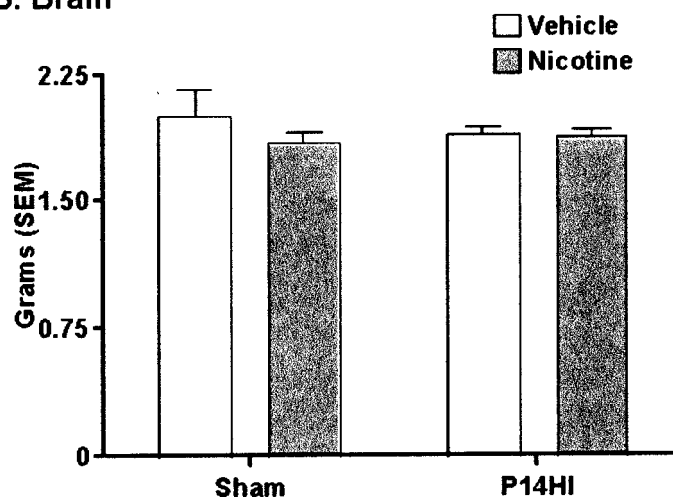


Figure 6.2. Anatomy in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment. *A.* The P14HI groups had lower body weights than the Sham groups (*P14HI groups differ from Sham groups). *B.* There were no group differences in brain weight.

Histology

There were no porencephalic cysts in any of the cases suggesting the lesion was moderate in the P14HI groups. The ipsi-lesion hemisphere appeared smaller with a notably diminished striatum and hippocampus in the P14HI groups (Fig 6.3).

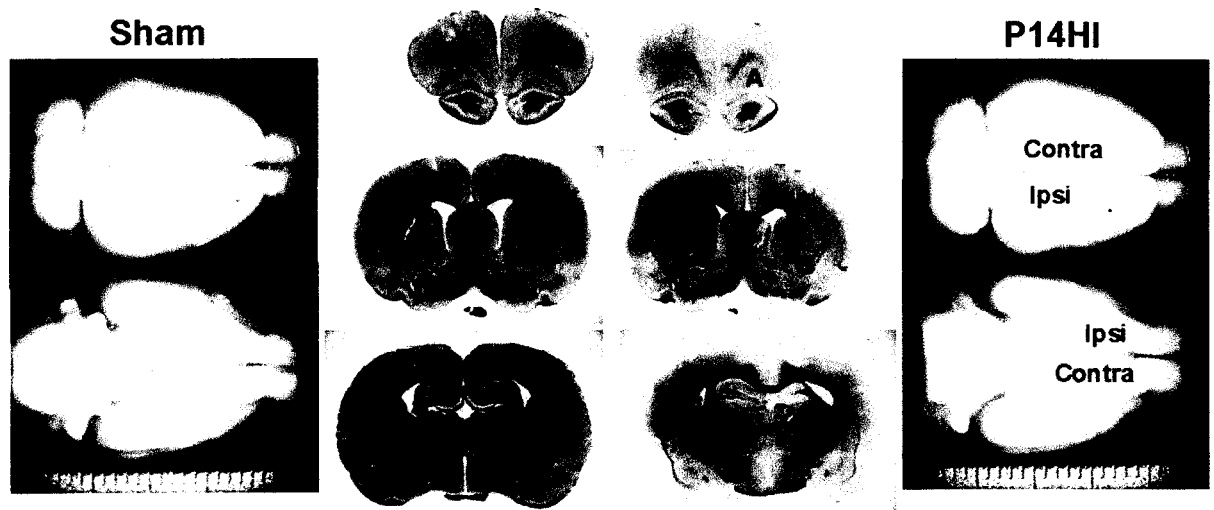


Figure 6.3. Lesion pathology in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment. General observations were made across groups in brain sections stained for myelin. There were no porencephalic cysts in any of the cases suggesting that the lesion was moderate. The ipsi-lesion hemisphere appeared smaller (A) with a notably diminished striatum (B) and hippocampus. The internal capsule in the ipsi-lesion hemisphere was stained with less intensity indicating fewer myelinated fibers than the contra-lesion hemisphere (C). There did not appear to be treatment effects on pathology.

Myelination

The ipsi-lesion internal capsule was stained with less intensity indicating fewer myelinated fibers compared to the contra-lesion hemisphere in the P14HI groups (Fig 6.3). There were no obvious treatment effects on lesion pathology.

Behavioural Assessment

Cylinder Task

The general activity level was not different between the groups. There were no differences between the groups for the number of rears in the Center of the cylinder or rears onto the cylinder Wall [Center: Lesion $F(1,35)=3.19$, Treatment $F(1,35)=0.69$, Interaction $F(1,35)=0.20$; Wall: Lesion $F(1,35)=0.53$, Treatment $F(1,35)=2.07$, Interaction $F(1,35)=1.48$, $P's > 0.05$]. There were no differences between the groups for the number of total touches with the forelimbs on the cylinder wall [Lesion $F(1,35)=0.01$, Treatment $F(1,35)=1.176$, Interaction $F(1,35)=0.01$, $P's > 0.05$, Fig 6.4 A]. Finally, there were no differences between the groups on First Simultaneous touches upon rearing, or Total Simultaneous touches with the forelimbs across bouts of rears [Total: Lesion $F(1,35)=0.52$, Treatment $F(1,35)=0.10$, Interaction $F(1,35)=0.10$; First: Lesion $F(1,35)=3.30$, Treatment $F(1,35)=0.02$, Interaction $F(1,35)=0.46$; $P's > 0.05$]. There was an interaction between the groups for the number of Simultaneous Landings revealing that Nicotine treatment increased the number of Simultaneous Landings in the Sham group but had no effect in the P14HI group [Landing: Lesion $F(1,35)=0.35$, Treatment $F(1,35)=3.93^*$, Interaction $F(1,35)=5.34^*$; $*P's < 0.05$].

There were differences between the Lesion groups in the preference to use the ipsi-lesion forelimb to touch the wall for support. The P14HI operates used the ipsi-lesion forelimb more often for the First touch upon rearing compared to Shams [Percent Ipsi-First: Lesion $F(1,35)=4.70^*$, Treatment $F(1,35)=0.02$, Interaction $F(1,35)=0.01$; $*P < 0.05$, Fig 6.4 B]. Similarly, the P14HI operates used the ipsi-lesion forelimb more often during

bouts of rears compared to Sham's [Percent Ipsi-Total: Lesion $F(1,35)=6.55^*$, Treatment $F(1,35)=0.13$, Interaction $F(1,35)=2.97$; $*P<0.05$, Fig 6.4 C].

Food Maneuvering Task

The rats picked up the pasta off the floor with their mouth, purchased the pasta from the mouth with a power grip using their digits, squared their shoulders, and then sat-back stabilizing their posture on their haunches until finished eating the item. The forelimbs were staggered on the pasta and brought an end of the pasta to their mouth. On occasion a rat would flip the item end-to-end before starting, but usually not after starting, to eat the item. The rats made small rapid forelimb and digit maneuvers to facilitate moving the item inward as intermittent trains of incisor-chomps broke small pieces off one end of the pasta into their mouth. There did not appear to be obvious deficits in use of the digits across groups and none of the pieces were dropped on the floor.

There were no differences between the groups for time to eat the item, total number of Maneuvers, or the percentage of Maneuvers with the ipsi-lesion forelimb (Fig 6.4 D). Mann-Whitney U tests found no group differences for the first forelimb to make Contact with the pasta, or type of grasp (majority power grip), nor which forelimb was staggered furthest from the mouth.

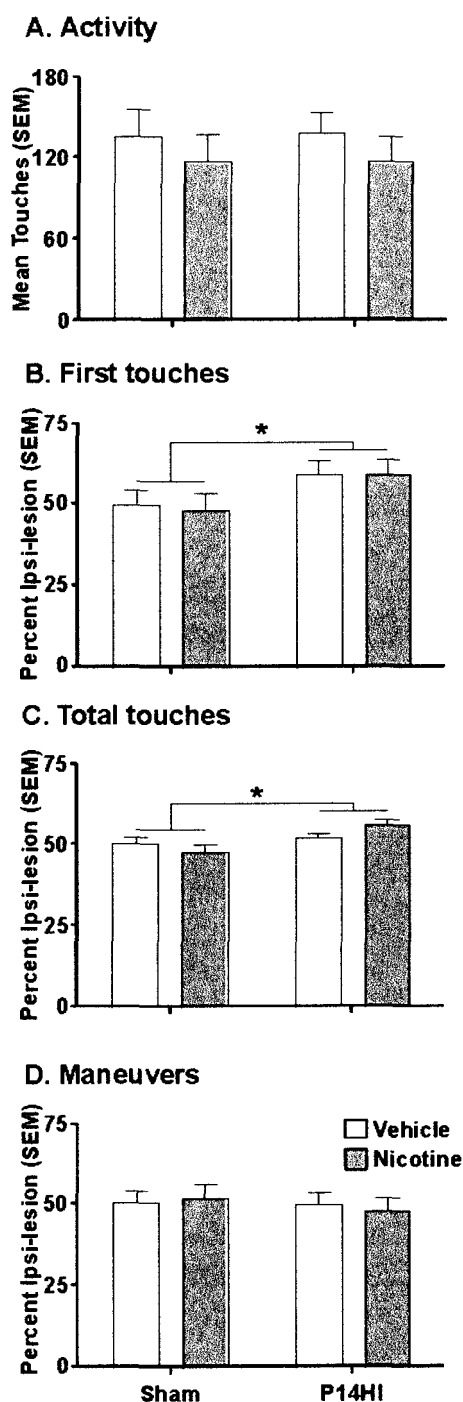


Figure 6.4. Juvenile motor performance following postnatal day 14 hypoxia ischemia (P14HI) and treatment. *A.* There was no difference between the groups for activity in the cylinder task. *B.* The P14HI groups used the ipsi-lesion forelimb more often upon rearing in the cylinder task (*P14HI differs from Sham). *C.* The P14HI group used the ipsi-lesion forelimb more often across bouts of rearing behaviour in the cylinder task (*P14HI differs from Sham). *D.* There were no differences between the groups for the number of maneuvers with the ipsi-lesion forelimb during pasta consumption.

Skilled Reaching Task

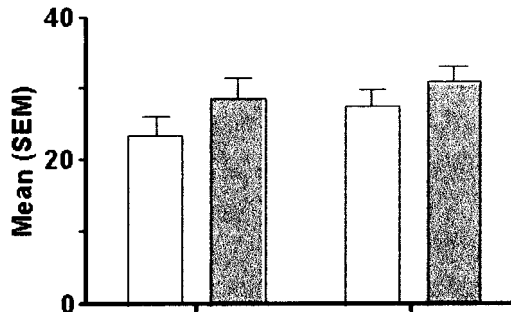
All rats learned to reach by the end of training although not all cases were reaching successfully for pellets within the first four sessions. The use of a bracelet to restrict the non-reaching forelimb was required in a subgroup of rats. The number of attempts decreased across testing sessions and the level of success increased. The P14HI group typically took longer to complete a session (data not shown).

There was a general decrease in the number of Attempts during testing but no group differences. Repeated measures two-way ANOVA indicated there was a decrease in the number of attempts during testing comparing Session 6,8,10,12, and 14 [Session $F(4,108)=2.51$, $P<0.05$]. The within-subject interactions were not significant [Session X Lesion $F(4,108)=1.20$; Session X Treatment $F(4,108)=0.74$; Session X Lesion X Treatment $F(4,108)=0.72$, $P's>0.05$]. There was a general increase in Success during testing. Repeated measures two-way ANOVA indicated there was a decrease in success during testing comparing Session 6,8,10,12, and 14 [$F(4,108)=3.04$, $P<0.05$]. The within-subject interactions were not significant [Session X Lesion $F(4,108)=1.05$; Session X Treatment $F(4,108)=0.09$; Session X Lesion X Treatment $F(4,108)=0.82$, $P's>0.05$].

Not all of the cases were able to complete a 20 trial session by Session 6 and thus were excluded from the previous analysis. To determine if there were group differences at the end of testing when every case completed a 20 trial session, a separate two-way ANOVA was conducted for Session 14. There were no differences in Attempts between the groups on the last day of testing [Lesion $F(1,35)=1.48$; Treatment $F(1,35)=2.73$; Interaction $F(1,35)=0.14$, $P's>0.05$, Fig 6.5 A]. However, analysis of Success on the last

day of testing indicated that the P14HI groups were less successful than Shams [Lesion $F(1,35)=6.97^*$; Treatment $F(1,35)=0.27$; Interaction $F(1,35)=0.12$, $*P<0.05$, Fig 6.5 B].

A. Attempts



B. Success

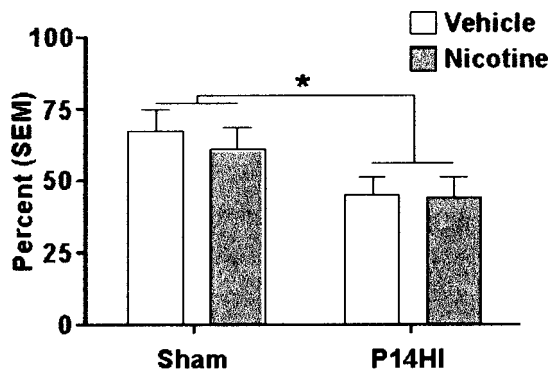


Figure 6.5. Reaching performance in adulthood following postnatal day 7 hypoxia ischemia (P7HI) and treatment. *A.* There were no differences between the groups for the number of attempts during the last day of testing (Session 14). *B.* The P14HI groups were less successful at reaching on the last day of testing and there were no treatment effects (*differs from Sham groups).

Motor Mapping

The mapping surgeries occurred after reach training in adulthood and ICMS in the ipsi-lesion hemisphere evoked forelimb movements in every case (Sham=6, Sham+Nicotine=6, P14HI=10, P14HI+Nicotine=10). The cortical localization of the

CFA and RFA motor maps in the ipsi-lesion hemisphere was consistent between the groups relative to Bregma. There was no difference in the number of microstimulation sites, microstimulation thresholds, or changes in the minimum threshold, nor electrode depth (approximately 1550 μm) amongst the groups.

Ipsi-lesion hemisphere: The Total area of forelimb representations in the ipsi-lesion hemisphere was abnormally small in the P14HI groups compared to the Sham groups [Lesion $F(1,29)=13.61^*$; Treatment $F(1,29)=0.06$; Interaction $F(1,29)=1.04$, $*P<0.05$, Fig 6.6 A and 6.7]. The reduction in forelimb representations in the P14HI groups was evident in a smaller CFA compared to the Sham groups [Lesion $F(1,29)=14.23^*$; Treatment $F(1,29)=0.04$; Interaction $F(1,29)=1.97$, $*P<0.05$, Fig 6.6 A and 6.7]. Further analysis within the CFA showed the decreased map size amongst the P14HI groups was attributable to reductions in the area of elbow and shoulder representations [Proximal: Lesion $F(1,29)=4.06$; Treatment $F(1,29)=2.83$; Interaction $F(1,29)=0.06$, $P's>0.05$]. Additionally, the P14HI groups also showed reductions in the amount of wrist and digit representations compared to the other groups [Distal: Lesion $F(1,29)=3.95^*$; Treatment $F(1,29)=3.69$; Interaction $F(1,29)=0.70$, $*P<0.05$, 6.6 B and 6.7]. Finally, the nicotine treated rats had a higher Percentage of wrist and digit representation compared to the vehicle treated rats [Lesion $F(1,29)=0.36$; Treatment $F(1,29)=5.82^*$; Interaction $F(1,29)=0.31$, $*P<0.05$]. There were no differences in the size of the RFA between the groups [RFA: Lesion $F(1,29)=0.12$; Treatment $F(1,29)=0.71$; Interaction $F(1,29)=0.02$, $P's>0.05$, Fig 6.6 A and 6.7].

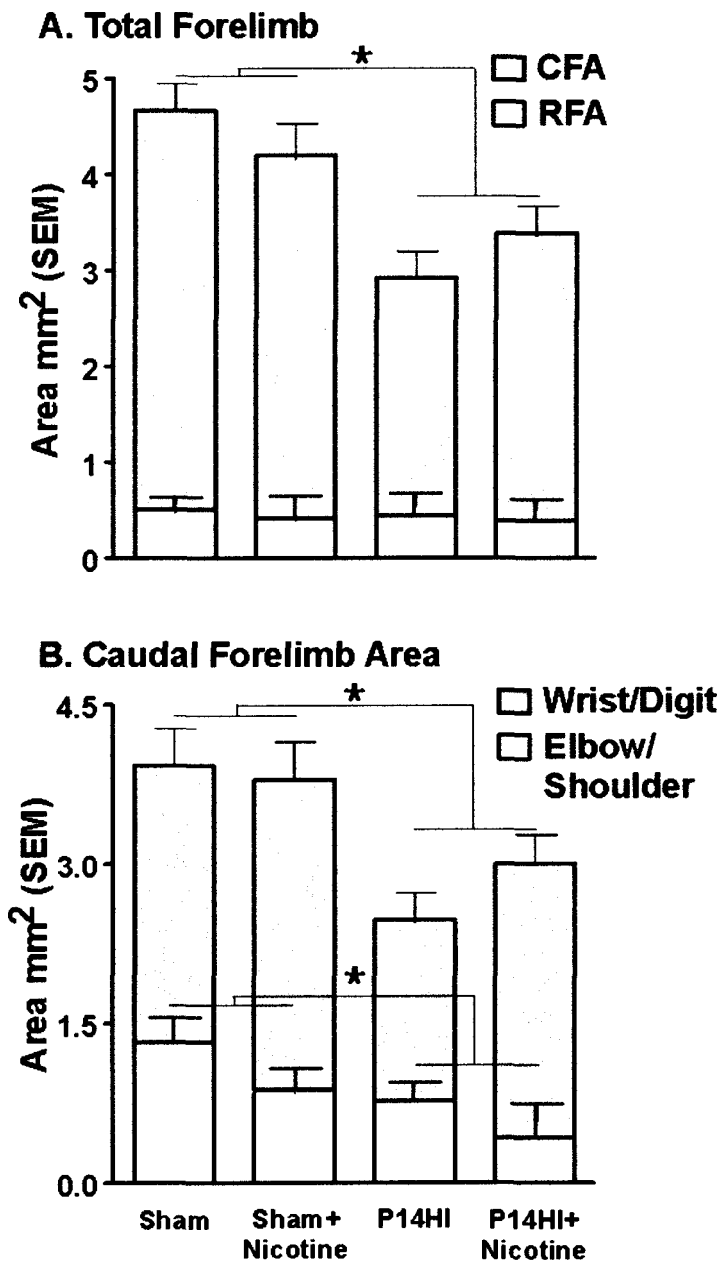


Figure 6.6. Motor map organization in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment. *A.* The total area of forelimb movements was reduced in the P14HI groups and was attributable to a decrease in size of the caudal forelimb area (CFA, differs from Sham), but not the rostral forelimb area (RFA). *B.* The smaller CFA in P14HI groups corresponded to less wrist and digit, as well as less elbow and shoulder representation (*differs from Sham). There were no nicotine treatment effects.

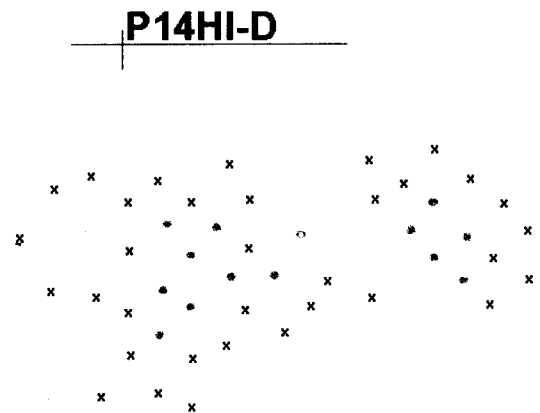
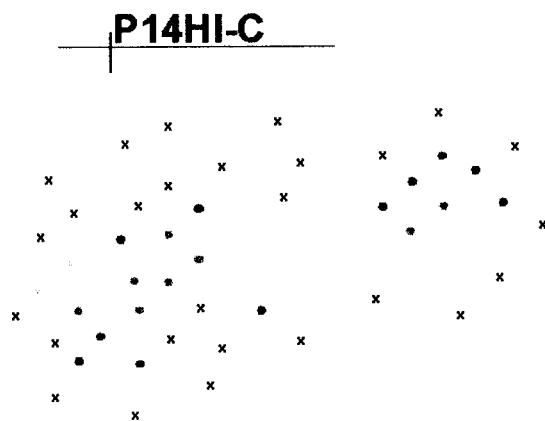
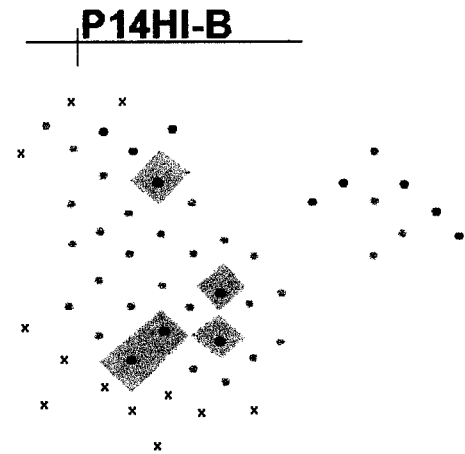
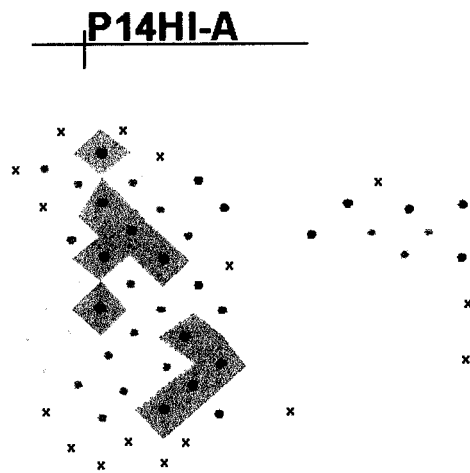
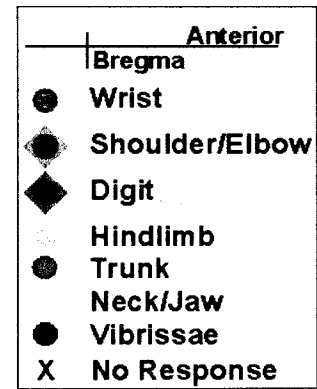
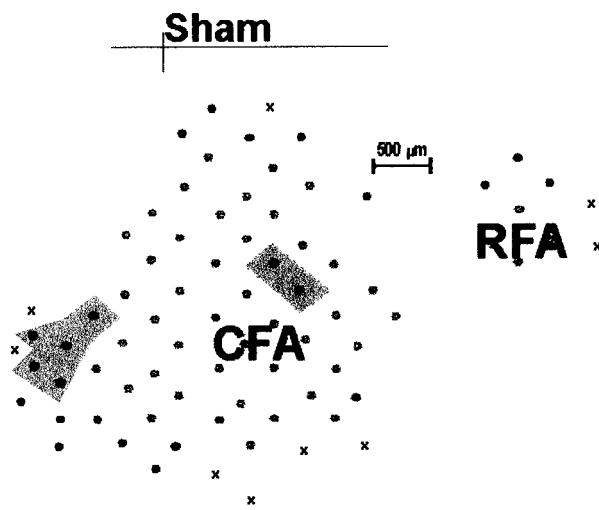


Figure 6.7. Reconstructed motor maps in adulthood following postnatal day 14 hypoxia ischemia (P14HI) and treatment. Shown are a typical caudal forelimb area (CFA) and rostral forelimb area (RFA) in a Sham case (*top left*). There was variability in the ipsi-lesion CFA map size among the P14HI groups although the CFA maps were smaller than Sham. Shown are large P14HI vehicle (P14HI-A) and nicotine (P14HI-B) maps, and large P14HI vehicle (P14HI-C) and nicotine (P14HI-D) maps. The RFA appeared normal in the P14HI groups and there were no treatment effects. Note the location of CFA and RFA is relatively consistent between the groups.

Contra-lesion hemisphere: Motor map organization was also investigated in the contra-lesion hemisphere in a subgroup of rats that did not include the Sham vehicle group (Sham+Nicotine 6, P14HI 6, P7HI+Nicotine 8). There were no differences between the Treatment groups in the contra-lesion hemisphere in size of Total forelimb representations [$F(2,19)=1.56$, $P>0.05$], or the CFA [$F(2,19)=1.89$, $P>0.05$], nor the RFA [$F(2,19)=0.05$, $P>0.05$].

Paired t-tests were performed to determine if the maps were different between hemispheres. The Sham+Nicotine group showed a smaller overall map in the ipsi-lesion (i.e. trained) map [Total $t(5)=2.89$, $P<0.05$] although there were no differences in size of the CFA or RFA [CFA $t(5)=2.18$; RFA $t(5)=0.69$, $P's>0.05$]. The P14HI group showed a smaller overall map in the ipsi-lesion hemisphere [Total $t(7)=5.05$, $P<0.05$]. The difference in the ipsi-lesion map for the P14HI group was attributable to a smaller CFA although there were no changes in the RFA [CFA $t(7)=4.24^*$; RFA $t(7)=0.12$, $*P<0.05$]. There were no differences between hemispheres in the P14HI+Nicotine group [Total $t(7)=1.18$; CFA $t(7)=1.18$; RFA $t(7)=0.26$, $P's>0.05$].

6.5. Discussion

The principal findings of this study are that: 1) hypoxia ischemia on postnatal day 14 impaired motor abilities with the contra-lesion forelimb and diminished the size of the corresponding motor maps in adulthood; and, 2) Nicotine administration following hypoxia ischemia did not ameliorate the motor deficits or the motor maps.

Motor behaviour following P14HI and treatment.

The brains vulnerability to hypoxia ischemia has been reported to increase with aging (Yager and Thornhill, 1997). In the current study there was zero mortality from the hypoxia ischemia surgeries although the P14HI groups had lower body weights than the Sham groups in adulthood. Interestingly, there were no differences in brain weight between the groups despite a noticeably smaller ipsi-lesion hemisphere. There was some variability in lesion pathology in the P14HI groups although there were no obvious nicotine treatment effects. There were no porencephalic cysts in any of the P14HI cases suggesting that the lesion was moderate. Typically, the ipsi-lesion hemisphere in the P14HI group appeared smaller with a notably diminished striatum and hippocampus. The ipsi-lesion internal capsule was stained with less intensity compared to the contra-lesion hemisphere indicating fewer myelinated fibers in the P14HI groups.

As juveniles, the P14HI groups showed some behavioural asymmetries later in maturity. The P14HI groups showed similar rates of activity although they favoured their ipsi-lesion forelimb for support during rearing in the cylinder task. In contrast, there were no forelimb preferences in food maneuvering. As adults, the P14HI group needed constraining bracelets on the ipsi-lesion forelimb during the reaching task. The P14HI

groups did learn the task and showed some improvements although they did not attain the same success levels as Shams at the end of training. Nicotine treatment following P14HI was unable to overcome the deficits. It is worth noting the Sham nicotine group did not show motor impairments. The long-term behavioural effects of children exposed to nicotine during development are unclear although there is evidence that early nicotine exposure is disruptive to the formation of cortico-thalamic connections that subserve sensory processing and attention later in maturity (Heath and Picciotto, 2009).

Motor map organization in adulthood following P14HI and treatment.

A novel finding in the current study is that P14HI disrupted motor map organization of the ipsi-lesion hemisphere in adulthood. The P14HI groups showed a diminished CFA that was attributable to decreases in the amount of wrist and digit (distal) as well as elbow and shoulder (proximal) movement representations. There were no differences between lesion or nicotine treatment groups in the size or organization of the ipsi-lesion RFA. There were no group effects in the contra-lesion hemisphere. There were no microstimulation sites evoking bilateral movements. Considering the maps were investigated in adulthood long after the neonatal injury, and after motor skill training in adulthood, it is unlikely that mapping at later ages, or with additional reach training, would result in a more 'recovered' map.

Motor map reorganization after learning following P14HI and treatment.

Motor learning is supported by changes in neural structure and organization. A hallmark of skilled motor learning in primates and rats trained to reach is they exhibit an increased

percentage of wrist and digit representation in the CFA opposite the trained limb compared to non-trained counterparts (Kleim et al., 1998; Plautz et al., 2000; Williams et al., 2006). There are few data to shed light on whether neonatal stroke disrupts the neural mechanisms that support motor map reorganization associated with motor skill learning in adulthood. A shortcoming of this study is that there were no hypoxia ischemia groups who did not receive reaching training. Nevertheless, the percentage of distal representation was not different after reach training between the P14HI and Sham groups and indirectly suggests that the P14HI group did show learning-induced reorganization of the ipsi-lesion (i.e., trained) maps. Previous studies have shown that hypoxia ischemia at earlier ages does spare similar learning-induced motor map reorganization (Ch 5). Interestingly, nicotine treatment increased the percentage of wrist and digit representation in the ipsi-lesion (i.e., trained) hemisphere. It has been shown that basal forebrain cholinergic lesion in adult rats disrupts map reorganization normally associated with learning (Conner et al., 2003).

6.6. Conclusion

There are motor deficits from neonatal stroke on postnatal day 14 that are attributable, at least in part, to abnormal ipsi-lesion motor maps in adulthood. Nicotine treatment was not able to reverse either the behavioural deficits or abnormalities in map size. It appears that more heroic efforts are needed for treatment of stroke occurring at late postnatal ages.

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Chapter 7

General Discussion

7.1. Overview

Stroke is a leading cause of mortality and neurological morbidity worldwide. There is a gap in understanding how stroke early in development affects behaviour-brain function later in life. The primary objective of the thesis was to determine if the nature of motor deficits ensuing after neonatal stroke are similar if age-at-stroke is varied. A second objective was to evaluate the use of drug treatments to promote compensation and recovery for the motor deficits. This thesis modeled high-risk pediatric stroke populations to understand the nature of the motor deficits in maturity using a multidisciplinary approach in rats. The rat neonatal stroke model was useful to examine the anatomical and neurophysiological changes in the lesion and non-lesion hemisphere that may account for neonatal stroke-induced impairments in behaviour later in life. The stroke model was also useful to determine if there are drug treatments, such as nicotine or fibroblast growth factor, that can at least partly reverse the motor deficits from neonatal stroke. The following discussion is structured to amalgamate the novel findings, propose mechanisms to account for the results, discuss caveats, and outline new directions for future stroke research.

7.2. Novel Findings

Perhaps the most significant finding from the thesis studies was that the details of motor deficits following neonatal stroke depended on the age that stroke occurred during development, even if the timing of the stroke was varied by only a few days. The age-at-stroke (postnatal day 3, 7, or 14) deficits arose from aberrant anatomical and neurophysiological organization. There was evidence of ischemia damage in the neonatal

stroke groups although the lesion pathology varied across experimental ages. Typically, each stroke age-group showed a ptotic eye and a smaller ipsi-lesion hemisphere with: increased ventricle size, decreased density of the internal capsule; and, reduced hippocampal and striatal size. Thickness in motor cortex in adulthood was not altered by neonatal stroke at any age. Remarkably, the nature of motor map reorganization can occur in opposite ways in the lesion hemisphere following stroke at different ages. For instance, the P7HI and P14HI stroke groups showed a decrease in map size whereas the P3HI stroke group showed an increase in map size. This thesis makes several novel contributions to advance an understanding of neonatal stroke. They are considered in turn.

The *first* study investigated the neurophysiological development of the motor maps in normal and in P7HI cases. The first experiment revealed the normal time course of motor map emergence did not evoke movements reliably until postnatal day 19. The second experiment demonstrated that P7HI did not affect the ontogeny of the forelimb motor map.

The *second* study assessed forelimb abilities in juveniles following P7HI. The lesion pathology was characterized post-mortem to account for variability in hemisphere damage noted in the first study, and the presence of cortical cysts was used to allocate cases as severe. In addition, the severe P7HI group showed a reduction in somatosensory cortical thickness in the ipsi-lesion hemisphere. The severe P7HI group indicated more deficits with the ipsi-lesion forelimb during cylinder exploration and food maneuvering compared to the moderate P7HI group. Remarkably, the extent of the decrease in size of

the ipsi-lesion motor maps among the Moderate and Severe P7HI groups was similar in adulthood, and did not appear to disrupt the rostral forelimb area.

The *third* study determined the benefit of nicotine or bFGF treatment following P7HI on forelimb abilities in juveniles and motor learning in adults. P7HI impaired the contra-lesion forelimb in tests of maneuvering and reaching and this was partially ameliorated by the treatments. The savings of motor learning and memory was robust in the P7HI treatment groups re-tested at reaching after a long interval without practice. The P7HI cases all showed cerebral pathology that was moderate although P7HI reduced the size of the cingulate cortex and this was partially ameliorated by the treatments. Finally, P7HI diminished the size of the ipsi-lesion hemisphere motor map replicating Study 2. The novel findings were that the post-stroke treatments partially ameliorated the abnormally small ipsi-lesion map in adulthood.

The *fourth* study assessed forelimb abilities and motor learning in adulthood following P3HI. The P3HI group was tested in adulthood and there was no evidence of limb preference during exploration in the cylinder task although they were less successful at reaching with the contra-lesion forelimb in adulthood. The study made the novel observations that the ipsi-lesion CFA and RFA motor maps in the P3HI group were abnormally large in adulthood. Reach training did not change the size of the motor maps in the P3HI groups although the CFA was reorganized from motor skill learning showing an increase in the percentage of wrist and digit representations.

The *fifth* study assessed forelimb abilities in juveniles and motor learning in adulthood following P14HI with or without nicotine treatment. P14HI impaired motor abilities with the contra-lesion forelimb and diminished the size of the corresponding

motor maps in adulthood. Nicotine administration following P14HI did not ameliorate the motor deficits or increase the size of the ipsi-lesion motor maps in adulthood.

7.3. Proposed Mechanisms

This thesis investigated the motor deficits and underlying brain changes following neonatal stroke using similar protocols for the different ages studied to allow direct comparisons. It is possible that the mechanisms supporting behaviour-brain plasticity varied in the different stroke age-groups although some similarities can be anticipated.

Hypoxia Ischemia

Stroke has many etiologies and may infarct the brain to a different extent via different mechanisms. In the rat model of neonatal hypoxia ischemia, an ischemic infarct is produced (following permanent common carotid artery occlusion) when the pup hyperventilates during low oxygen conditions and becomes hypoxic and hypocapnic (Yager and Ashwal, 2009). The rat brain is tolerable to hypoxia and hypocapnia although if a common carotid artery is experimentally occluded durations exceeding 60 minutes lead to ischemia. During an hypoxic-ischemic episode, blood pressure (systemic) declines approximately 25% and cerebral blood flow is reduced between 17-40% in the ipsi-lesion hemisphere (Vannucci et al., 1993). In addition, there is metabolic energy failure because of a decrease in glucose and a loss of high-energy phosphatases. Ischemia is halted when normoxic conditions are restored.

Recovery from hypoxia ischemia triggers a proinflammatory response upregulating chemokines and macrophage inflammatory protein within the first 24 hours.

Cerebral edema peaks at approximately 72 hours following the hypoxic-ischemic episode and evolves over a few days. Lymphocytes, microglia, macrophages, and astrocytes are reported to invade the lesion site over the next 42 days (Bona et al., 1998). The areas

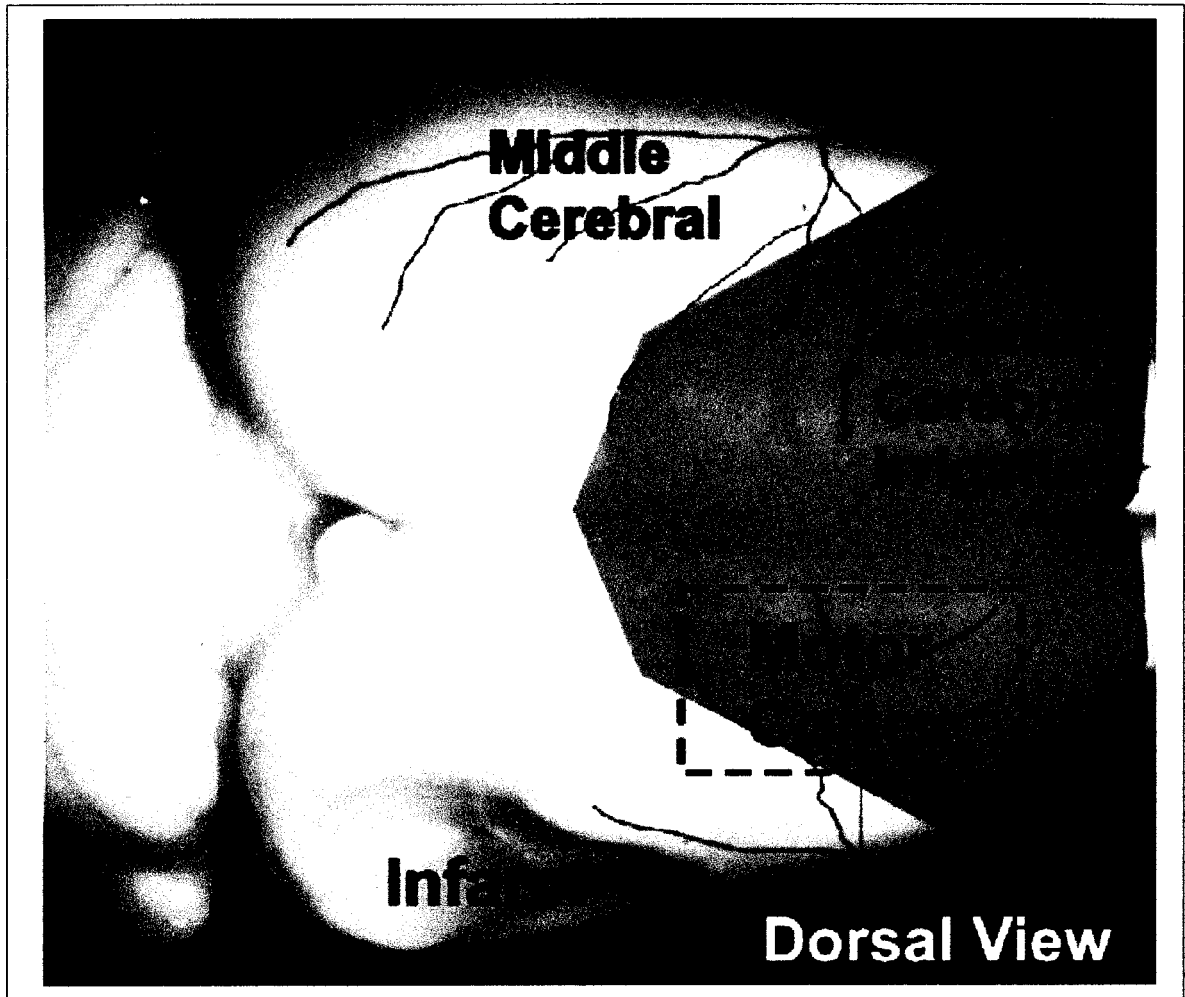


Figure 7.1. Model of stroke injury from neonatal hypoxia ischemia. Brain areas vulnerable to infarct from neonatal hypoxia ischemia are the distal branches of the middle cerebral artery in posterior brain regions because they receive the least anastomoses from the arterial network whereas anterior regions, including motor cortex, receive anastomoses from the anterior cerebral artery (shaded area) that is directly connected with the contra-lesion hemisphere via the azygomatic communicating artery (see also Fig 1.1). Subcortical damage may also be produced from ischemia to the lenticulostriate branch of the middle cerebral artery (not shown).

most susceptible to ischemia are those with the least anastomoses and are unable to cope when blood pressure decreases during hypoxia, such as the distal branches of the middle cerebral artery in posterior irrigation territories, leading to ischemic damage (Fig 7.1).

Behavioural Assessments

The behavioural results show that each stroke age group tested had at least some motor impairment, although the details do differ between studies. It is important to note that the neonatal stroke groups did show improvements in motor skill learning although they did not attain the same success levels at reaching compared to control groups. Taken together, no treatment group recovered and instead were compensating (Kolb and Whishaw, 1998). It is likely that the neonatal stroke groups used alternative movements to perform the success they did exhibit.

Drug Treatments

Nicotine

Nicotine treatment following neonatal stroke had little anatomical effect, if any, on body weight, brain weight, or cortical thickness so it is unlikely that nicotine reduced the size of the stroke infarct. Post-stroke nicotine treatment following P7HI, although not following P14HI, did partially ameliorate the motor impairments and organization of ipsi-lesion motor maps in adulthood.

The P7HI+Nicotine group showed partial amelioration of forelimb map size compared to the P7HI group that did not receive treatment. It is possible that the nicotine treatment following P7HI acted to facilitate excitatory thalamocortical and cortical

signalling important for building and maintaining motor maps in cortex during the second postnatal week. There was no benefit of nicotine administration following P14HI, perhaps because nicotine administration at the later timepoint was less effective to facilitate NMDA signalling in motor cortex. For instance, nicotine administration during the second postnatal week, but not before or after, can enhance the NMDA receptor-mediated component of excitatory postsynaptic signalling in thalamocortical neurons in auditory cortex *in vitro* (Armakis and Metherate, 1998; Armakis et al, 2000). There is evidence that nicotine treatment can also induce hypertrophy of synaptic connections in cortical pyramidal cells (Brown and Kolb, 2001; Gonzalez et al., 2006).

There is recent evidence that nicotine can indirectly stimulate angiogenesis that could help provide relief to the vasculature system following stroke. The activation of nAChRs can upregulate growth factors involved in angiogenesis, such as vascular endothelin growth factor (VEGF) (Costa and Soares, 2009). It has also been shown that nAChR activation upregulates bFGF which can also promote angiogenesis. Given the similarities in the benefits of nicotine or bFGF treatment to behavioural and neurophysiological outcomes following P7HI, it is possible that stimulating angiogenesis was common mechanism between the treatments.

bFGF

Treatment with bFGF following P7HI had little anatomical effect, if any, on body weight, brain weight, or cortical thickness so it is unlikely that bFGF reduced the size of the stroke infarct. However, the P7HI+bFGF group was not as impaired as the P7HI group

without treatment in the behavioural tasks and bFGF treatment did partially ameliorate the organization of the ipsi-lesion motor maps in adulthood.

Previous researchers using the P7HI model have shown that bFGF is neuroprotective if administered once 30 minutes before, or three times (30 min prior, during, and 30 min after) an hypoxic ischemic episode (Nozaki et al., 1993). The authors demonstrated that peripheral administration of bFGF did not produce hypothermia, at least within the first three hours following P7HI. However, the findings must be interpreted with some caution because in most cases it is not possible to predict a stroke will occur *before* it happens in order to begin bFGF treatment. In a subsequent study, the same authors did not find neuroprotective effects of bFGF if administered more than 2 hours after intrastriatal NMDA neurotoxic lesion on postnatal day 7 (Kirschner et al., 1995). It remains to be determined if bFGF is neuroprotective if administered after longer intervals following P7HI, although blocking bFGF can have a detrimental effect following motor cortex injury (Rowntree and Kolb, 1997).

bFGF may play a special role in supporting growth and function of the motor system. There is evidence that developmental knockout of bFGF diminishes the size and number of cortical cells in anterior, but not posterior, cortex (Vaccarino, 2002). Interestingly, the bFGF knockout effect on cortical cells was only observed in excitatory pyramidal cells and not in inhibitory cells. Treatment with bFGF following neonatal motor cortex lesions has been shown to spare function of the motor map, and may even spare and integrate fibers that would normally be pruned (Monfils et al., 2005; Monfils et al., 2006; Monfils et al., 2008). More detailed studies are needed to determine the precise role of bFGF on CST fibers during development.

Motor Map Organization

The representations of forelimbs evoked by microstimulation provide an index of motor system integrity and function. It is possible that motor map reorganization involved changes in CST function at cortical and/or spinal cord levels of synaptic circuitry.

From a cortical viewpoint, altering inputs to motor cortex from cortico-cortical connections with sensory areas and the basal ganglia via the thalamus may influence the organization of the motor maps. ICMS-evoked movements are the result of direct and indirect activation of CST neurons that then excite pools of motoneurons in order to activate or inhibit muscle groups for the production of movements. Direct CST activation via ICMS is achieved by electrically exciting dendritic synapses on local pyramidal cells, primarily the spines of the basilar tree within the perimeter of the electrode tip (Stoney et al., 1968). Indirect CST activation, which is the predominant mechanism via ICMS (Cheney et al., 2000), is due to temporal facilitation of stimulation pulses (Jankowska et al., 1975). That is, a single pulse delivered via a microelectrode into layer V of motor cortex stimulates local pyramidal cell bodies, traversing axons, and afferent terminals, as well as remote regions via local (<0.5 mm) physiological spread. Multiple pulses delivered in a train, as in the case of ICMS, acts to potentiate the temporal facilitation so that CST neurons are *trans*-synaptically activated, and recruit more synapses with each successive train (Cheney and Fetz, 1985; Cheney et al., 2000). The correlation between ICMS-pulses and the production of a movement is explicitly related to the organization of intracortical connectivity and the degree of synaptic recruitment (Cheney et al., 2000).

From a spinal cord viewpoint, altering the connectional specificity of the descending CST in spinal cord may influence the organization of motor maps. For

instance, the morphology of developing CST terminations in spinal cord matches their physiology. The initial organization of CST terminations with interneurons and motoneurons in the spinal cord is broad and diffuse (Li and Martin, 2000; Li and Martin, 2001; Li and Martin, 2002; Martin, 1993; Martin, 1996; Meng and Martin, 2003). CST terminations are restricted to the deep laminae of the dorsal horn, the intermediate zone, and the ventral horn where chemotropic factors induce axonal branching and synapse formation. Subsequently, CST terminations undergo refinement that leads to the formation of dense clusters of presynaptic boutons (Li and Martin, 2001). Electrical stimulation of the pyramid (containing all descending fibers) during the first postnatal month in cats produces weak synaptic responses in the spinal cord presumably because of the sparse terminals and lack of pre-synaptic vesicles (Meng et al., 2004). After transient CST projections are eliminated and terminal boutons form dense clusters restricted in dorsoventral lamina (10 weeks in cats), the same electrical stimulation produces much larger spinal responses. Importantly, the increased amplitude of CST spinal responses in maturity is achieved by synaptic facilitation in spinal motor circuits and is sufficient to evoke muscle responses (Meng et al., 2004).

It is possible that the aberrant maps in adulthood following neonatal hypoxic ischemia are attributable to pathology observed in striatum and/or hippocampus. However, there is evidence that motor impairments from unilateral striatal lesion (Whishaw et al., 1997) are not related to changes in the motor maps (Metz et al., 2004). Although there is no clear role for the hippocampus in movement production, bilateral lesion in adulthood does not disrupt the motor maps (Williams et al., 2009). Nevertheless,

postnatal damage to the hippocampus can alter the function of frontal cortex (Lipska et al., 1994).

Principles of motor map organization

A major thrust of the thesis was the investigation into the organization of normal motor maps and how they are pliable to experiences, such as skill learning or stroke injury. The nature of map organization and reorganization are exemplified in the following principles.

1) *The forelimb motor map is an emergent property of motor cortex.* The presence of motor maps is not innate and emerges as an epiphenomenon of motor cortex circuitry during postnatal development and maturity. In rats, cats, and primates, the motor map develops in the juvenile period and emerges concurrently with the onset of voluntary motor skills (Martin, 2005). Although CST fibers are splayed throughout the cortex during early development, it does not appear that they support the motor map because microstimulation evoked forelimb movements were found coalesced in motor cortex of rats (e.g. Ch 2) and cats (Bruce and Tatton, 1980a; Bruce and Tatton, 1980b; Chakrabarty and Martin, 2000) as well as primates (Eyre et al., 2001). In addition, it appears the caudal forelimb area precedes emergence of the rostral forelimb area in rats. This thesis provides the first demonstration in rat that the emergence of the motor maps is not disrupted by neonatal stroke, at least in the P7HI group. There is corroborating evidence using TMS following full-term infant human stroke (Eyre et al., 2007).

2) *The forelimb motor map is mosaic and contiguous.* Microstimulation in rat evokes forelimb movements in two separate cortical regions in primary motor cortex.

Although the intra-areal organization of distal and proximal forelimb movement representations may vary, the motor maps in each region are found in adjacent microstimulation sites. Lesions to input or output components of the motor system, such as the spinal cord, basal ganglia and thalamus, or cortex can disrupt motor map organization although such insults do not ‘break-up’ the CFA forelimb areas (Gharbawie et al., 2007; Gharbawie et al., 2008; Piecharka et al., 2005). Neonatal stroke aberrantly altered the size of the ipsi-lesion motor map but did not alter the contra-lesion map in adulthood. The size and proportion of proximal and distal forelimb movement representations was similar to Shams in the contra-lesion motor map. It is possible that diaschisis may produce dysfunctional peri-infarct regions acutely (Brown et al., 2007).

3) *The forelimb motor maps are same size between hemispheres.* Motor map size was shown to be similar between hemispheres in this thesis. Rats do not show unilateral dominance in forelimb use, although they do exhibit preferences (Whishaw and Kolb, 2005). This thesis demonstrates that training one forelimb to learn a new motor skill does not necessarily change the size of the corresponding motor map although the map shows an increase in the proportion of wrist and digit movements. The reorganization to bias wrist and digit movements after motor learning corresponds with the new skills using the distal forelimb for pronating, grasping, and supinating to successfully reach for a target (Kleim et al., 1998; Nudo et al., 1996; Whishaw and Pellis, 1990).

4) *The forelimb motor map size does not vary with sex.* There were no sex differences in the size of the motor maps in the thesis studies and there were no sex differences following neonatal stroke in adulthood. A previous study has shown that there are no sex differences in quantitative or qualitative abilities during reaching (Williams et

al., 2006). It is interesting that there are no sex differences in reaching ability or map size given females have smaller and lighter brains compared to males in adulthood.

5) *There is an optimal size and organization of the motor maps.* A common misconception about motor map organization is that bigger is better. The findings from this thesis lend further support to refute this concept. Neonatal stroke produced abnormal maps that were too small in the P7HI and P14HI groups and maps that were too large in the P3HI group. There is also evidence that seizures can impair motor skills and double the size of the motor map (Teskey et al., 2008). Motor map size is not the only factor because the intra-areal organization of forelimb movement representations is also pliable to experiences such as motor learning and practice. The proportions of wrist and digit (distal) in concert with elbow and shoulder (proximal) influence the networks malleability to reorganize in response to future learning. For instance, individual variability in distal and proximal movement representations in adults may impact subsequent motor learning (i.e, poor reacher's start with a high proportion of wrist and digit representation) (Kleim et al., 2004).

6) *The forelimb motor maps respond differently to experience.* Motor skill learning and practice reorganizes the neural networks to bias wrist and digit movements in the caudal forelimb area but does not have an obvious effect on the rostral forelimb area. Neonatal stroke can increase, decrease, or have no effect on the caudal and rostral forelimb areas. Drug treatments (e.g. Chapter 4) or adult rehabilitation (Williams et al., 2006) can partially restore neonatal lesion-induced aberrant organization of the maps in adulthood.

7.4. Caveats

Model of Stroke

The model of stroke in the thesis experiments may have somewhat limited generality to human infants because it does not exactly replicate human stroke. Nonetheless, the finding that in the absence of obvious infarcts there are significant behavioural and neurophysiological correlates of perinatal hypoxia ischemia is clearly relevant to understanding the effects of hypoxia ischemia in human infants. In addition, there was individual variability within and between stroke groups and it is not exactly clear what pathological aspects lead to the behavioural, anatomical, and neurophysiological deficits. A different approach would be to try and match lesion size and location among the age groups by adjusting the duration of hypoxia exposure.

Behavioural Assessment

The behavioural tasks were chosen to assess voluntary movements with the forelimbs. Each task has been previously used in stroke research to demonstrate motor deficits following cortical injury. The behavioural assessments were endpoint measures and do not clarify how the quality of motor performance was altered by the neonatal stroke. The assessments were made once so it is unknown how performance changes over time. Study 3 investigated savings of a learned motor skill and demonstrated that reaching performance with the contra-lesion forelimb did not drop over a long interval without practice and can be used as evidence that other behaviours were indicative throughout maturation as well. Future studies should address the nature of motor impairments with the ipsi-lesion forelimb following neonatal stroke.

Other behavioural tasks could have been used to assess forelimb abilities. It is important to note that developmental studies are restricted to test behaviours that are normally expressed at the age being tested and it was not possible to obtain pre-stroke scores. It is not trivial that voluntary motor skills develop relatively late in maturity although the motor system does not act alone. It is likely that abnormalities in other brain functions, such as cognitive, emotional, and sensory, were also affected and contributed to the motor deficits.

Gross Anatomy

Anatomical measures were brain weight and body weight although other measures could have been taken. The stroke age groups tested showed a ptotic eye and narrowing of face that should be considered more closely in future studies (Fig 7.2). Casual observation also indicated that the ipsi-lesion vibrissa were shorter in length and ectopically organized compared to the contra-lesion hemisphere (Fig 7.2). In addition, the length of the forepaw and hindpaw claws appeared longer suggesting cortical damage and requires further study (Whishaw et al., 1983).

Hormones are known to play an organizing role in CNS maturation (Kolb et al., 1998). It is unknown if neonatal stroke alters the expression of hormones or endocrine function. Measures of the reproductive organs and endocrine gland weights, or blood assays for hormone concentrations, may provide insights.

Histology

Histological assessments of cortical thickness and myelin were used to characterize the nature of ischemia pathology following developmental stroke. The measures do not definitively determine if there were changes in neural or glial elements, but they do provide a starting point for places to look in the future. There were gross changes in myelination following neonatal hypoxia ischemia although further study is required to assess the number of fibers and amount of myelin. It is also unclear where the axonal fibers originate from and where they are destined to, and if they make appropriate connections. It is worth noting that in a parallel study van Waes & Kolb (van Waes and Kolb, 2009) found that P7HI brains similar to those in this thesis showed changes in dendritic organization in the layer III pyramidal neurons in parietal cortex adjacent to the motor cortex. Specifically, they found increased dendritic complexity but decreased dendritic length, suggesting significant changes in the organization of cortical neuronal networks following P7HI.



Figure 7.2. Facial abnormalities following neonatal hypoxia ischemia. Neonatal hypoxia ischemia disrupts blood flow to the external carotid artery that supplies blood to the facial artery. Ischemia to territories of the facial artery decreased the size of the ipsi-lesion hemisphere (left of dotted line), eye (arrow), and vibrissae (pink lines). The ipsi-lesion vibrissae pad appears abnormal with ectopic whiskers (double arrow).

Motor Mapping

Motor mapping was done in a between-subjects design because of the complications of re-mapping rats. It is of great interest to see how the maps change over time. The results from Study 1 indicate that the ontogeny of the maps may not be affected by neonatal

hypoxia ischemia, but this was only confirmed in the P7HI group. It would be useful to know if map development was affected in the other age groups and to map the same rat throughout maturation. Other species such as primates and cats are more amenable to within-subject designs.

The presence of evoked movements is not the only indicator of cortical communication with the spinal cord and electromyography, stimulus-trigger averaging, or focal synaptic potentials could also provide insight into motor system organization (Porter and Lemon, 1995). Increasing the duration of the microstimulation train has been shown to evoke movements that reveal a different repertoire of movement in the cortex in primates (Graziano, 2006). There is evidence in adults rats that long-duration microstimulation demonstrated abnormal ipsi-lesion maps following stroke, and beneficial reorganization from behavioural therapy (Ramanathan et al., 2006).

7.5. Conclusions

The studies presented in the thesis are the first comprehensive examination of neonatal hypoxia ischemia on anatomy, behaviour, and neurophysiology. The experiments provide strong evidence showing that the nature of motor deficits is dependent on the age-of-stroke. These findings show that normal motor learning requires an optimal organization of the motor cortex to support motor behaviours; early experiences, such as a stroke, can impair motor skills and the organization and function of the motor system. The stroke groups did show learning in adulthood and the neural mechanisms reorganizing the map from learning were spared. The therapeutic effects of treatments in the P7HI groups are encouraging, but unfortunately, the effectiveness of the treatments may be age-

dependent. This possibility has important implications and requires more thorough study.

7.6. Future Directions

This thesis studies investigated if age-at-stroke alters motor behaviour-brain relationships. In effort to answer this profound problem, many more questions arose although they are beyond the scope of the thesis.

Are there other stroke models?

There is individual variability in the hypoxia ischemia model and it is unclear why. Future studies that could identify biological markers that can predict outcomes would enhance the development of new treatments. Direct stroke models that are considered to induce reproducible pathology also show individual variability (Gonzalez and Kolb, 2003). Nevertheless, although individuals may differ, there ought to be common threads that treatments can harness to ameliorate the deficits.

There are other stroke models that should be used to study the benefits of nicotine or bFGF treatments. Two other ischemia models, the MCA occlusion model (Ashwal et al., 2007) or direct administration of vasoconstrictors (i.e., endothelin) (Yager et al., 2005; Yager et al., 2006) have been recently adopted to study the influence of other aspects of neonatal stroke.

How does the quality of behaviour change following neonatal stroke?

The behavioural measures in the thesis are endpoint assessments. It is important to determine how the quality of movements changed. The performance of the rats in the thesis studies was videorecorded and movement analysis is ongoing. Future studies

should evaluate other types of behaviour, such as cognitive, emotional, and sensory systems and adopt a similar comprehensive approach that this thesis took.

How might other treatment regimes work: doses; timing; other candidates?

The behavioural and neurophysiological improvements from nicotine or bFGF in the P7HI group raise the question whether other psychomotor stimulants or growth factors may also influence behaviour-brain plasticity. It is possible that the drug treatment benefits could be attenuated if the drugs were administered immediately or delayed after stroke, for shorter or longer periods after stroke, or in some combination (Kolb et al., 2007). The best timing of drug administrations are details that still need to be worked out, and may help explain why nicotine was not effective in the day 14 stroke group. It is likely the case that more treatment is needed, not less.

It has yet to be determined if treatments would be beneficial following P3HI. The P3HI group showed abnormally large maps in the ipsi-lesion hemisphere posing a different treatment problem; how do you make the map smaller. Further study is needed to determine if the P3HI group maps are larger because of disruptive seizures. For instance, Teskey and colleagues have shown that specific types of seizures can impair motor skills and produce abnormally large maps (Henry et al., 2008). They further showed that low frequency stimulation could 'de-potentiate' the abnormally large maps (Ozen et al., 2008; Teskey et al., 2008).

One of the challenges is to redirect new growth in appropriate ways, especially axons that may have long distances to travel and make new connections. Promising drug treatments, such as inosine and antibodies to No-Go, administered following adult stroke

have been shown to promote behavioural compensation and axonal growth (Chen et al., 2002; Papadopoulos et al., 2006). Overall, the thesis demonstrates that the actions of nicotine or bFGF treatments after neonatal stroke *can* be beneficial following neonatal stroke and provides a good starting point to go further.

How does neural complexity change following neonatal stroke?

The changes observed in behaviour are mirrored in changes to the structure and connectivity of the brain (Kolb and Whishaw, 1998). Behaviour can be impaired if neurons are unable to pathfind, connect to the appropriate targets, or their synaptic activity is diminished. This thesis revealed abnormal motor map organization following neonatal stroke suggesting that the intrinsic connections of motor cortex have changed substantially, even though there were no changes in motor cortex thickness (Aroniadou and Keller, 1993; Weiss and Keller, 1994). Future study of dendritic complexity, in both hemispheres, is needed to elucidate the intrinsic synaptic organization of motor cortex following stroke. The hippocampus and striatum show pathology in the lesion hemisphere following neonatal stroke and provide other areas to look for changes in dendritic complexity.

Unilateral brain injuries during development can disrupt long-range projections, such as in the corpus callosum, external capsule, and CST. Axonal tract tracing studies would help determine the topography of cortical connectivity between hemispheres (Brus-Ramer et al., 2009), and to spinal cord (Martin and Lee, 1999).

Is it possible to avert dysfunction of the motor maps following neonatal stroke?

Identifying and restoring abnormal inputs in the motor system following injury is key to ameliorating motor disorders. Thus, preventing brain injury may not be possible *per se*, although preventing aberrant reorganization of the motor system that typically ensues brain injury may be possible. For example, CST function in hemiplegic cerebral palsy (CP) cases assessed with transcranial magnetic stimulation (TMS) shows normal CST activation from the affected hemisphere before three months of age, but abnormal CST activation after three months of age (Eyre et al., 2007). This thesis demonstrates that neonatal stroke on day 7 does not disrupt the development of the forelimb map although the maps do not appear normal in adulthood. Future studies should examine the organization of the maps at intermediate timepoints to determine the factors that limit the normal growth of the map (i.e., circuitry). It is possible that treatments delivered at the right time could spare motor circuitry from abnormal wiring and repeal the motor deficits.

A non-invasive strategy for treating motor disorders is to promote activity of the diminished neural pathway(s). A promising approach for treating neonatal movement disorders during development is constraint-induced-movement-therapy (CIMT) of the less-affected limb (Taub et al., 2002). Constraining the less-affected limb with a cast encourages the subject to engage behaviour using the impaired limb and has been shown to improve motor skills in children with CP (DeLuca et al., 2003; Taub et al., 2004). A major advantage of CIMT is that extensive training is not required for practitioners to administer CIMT (Lum et al., 2006). It is clinically useful to know that CIMT is effective to treat motor impairments, but it is still important to know why. It is likely that any

circuit re-wiring due to CIMT is shaped by ecologically-relevant patterns of neural activity driven by the subjects' own behaviour during activities of daily living. Thus, a new way of thinking about treatment for aberrant synaptic organization in the ipsi-lesion hemisphere (stroke) may be ameliorated by increasing the synaptic activity of the deprived pathway, or by restricting the synaptic activity of opposing pathways (Chakrabarty et al., 2009).

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