The Neuroanatomical Effect of Brain Injury during Early Development in a Rat Model

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Psychology

University of Manitoba

Winnipeg

Acknowledgements

First and foremost I would like to thank my parents for their endless support throughout this entire project. Without them none of this would be possible and I am eternally grateful. I would also like to thank my advisor Dr. Tammy Ivanco. Under her guidance, I have become a confident and skilled researcher. I will always be thankful for the valuable opportunities Dr. Ivanco has provided me with, allowing me to expand my knowledge and experience. Finally, I would like to thank my committee members for their indispensable feedback on earlier versions of this manuscript.

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Abstract

The brain responds to injury during early development with alterations in behaviour and dendritic morphology of motor cortex neurons. Rats were exposed to damage either prenatally or after the first postnatal week, using different models of damage and motor cortex was examined. Prenatal injury resulted in a decrease in length, complexity and volume in layer II neurons, but no differences in layer V neurons or behavioural tasks. Postnatal damage produced increases in length of basilar dendrites, but no differences in spine density at 2 months of age, whereas at 6 months of age, an overall decrease in apical and basilar spine density was observed. Findings demonstrate the maturational status of the brain at the time of injury play a crucial role in response to injury.

Chapter 1

Effects of Early Developmental Injury on Human Brain and Behaviour

Preterm birth, defined as birth before 37 weeks or 259 days gestation is complete, poses a major challenge for perinatal care worldwide and is one of the leading causes of infant morbidity and mortality. In the United States, there has been a steady increase in preterm births of 12-13% all of births (Allen, 2008). Over the past 25 years, the rate of preterm birth in the US has increased 36% (March of Dimes White Paper on Preterm Birth, 2009). The Canadian preterm birth rate has also increased in recent years, from 7.0 per 100 live births in 1995 to 8.2 per 100 live births in 2004 (Canadian Perinatal Health Report, 2008). In absolute terms, the increase in the overall preterm birth rate is largely due to an increase in late preterm birth (32-36 weeks), from 6.0 per 100 live births in 1995 to 7.0 per 100 live births in 2004. Preterm birth (less than 32 weeks) also increased from 1.0 per 100 live births in 1995 to 1.2 per 100 live births in 2004. Prematurity accounted for 75-85% of all perinatal mortality in Canada (Public Health Agency of Canada, 1999). Even mild and moderate preterm birth puts infants at increased risk of death during infancy (Kramer, Demissie, Hong, Plat, Sauve, & Liston, 2000). There are a host of different socioeconomic, biological, and environmental factors that contribute to a woman's risk for preterm delivery. Medical conditions can include chronic hypertension, diabetes, infections, and stress. Other contributing factors are the greater usage of assisted reproductive techniques which increased the rate of multiple gestations, a rise in the proportion of births to women over 35 years of age, and changes in clinical practice such as the early induction of labour or performance of Caesarean sections close to, but not at full term. Now that advances in medical technology have made survival of

premature infants possible, research has turned its focus to the impact prematurity has on the development of these children as they get older. One of the remaining questions, however, is how the brain and subsequent behaviour is affected by conditions associated with premature birth.

Improvements in the treatment of premature infants in Neonatal Intensive Care Units have greatly increased the survival rate of children born prematurely. Despite these improvements, premature infants remain vulnerable to many health issues, including, respiratory, gastrointestinal, immune system, central nervous system, hearing, and vision problems (March of Dimes White Paper on Preterm Birth, 2009). Some longer term health problems may include cerebral palsy, mental retardation, visual and hearing deficits, behaviour and social-emotional problems, learning difficulties, and poor health and growth. Babies born before 32 weeks gestation are at the greatest risk for death and poor health, however, infants born between 32 and 36 weeks, which make up the greatest number of preterm births, are still at a higher risk for health and development issues compared to full term infants (Kramer et al., 2000). The more premature the infant, the greater the extent of life support that is required and the longer the stay in intensive care. Overall, hospital stays for premature children are longer and the risks for rehospitalisation are significantly increased. The medical consequences of surviving premature birth often necessitate accessing a wide range of services and social supports.

The societal economic burden for long term care for surviving premature infants is staggering. In the United States, the cost associated with preterm birth was $26.2 billion in 2005, or $51, 600 per infant born. Nearly two thirds of this cost was for medical care. Medical care services contributed $16.9 billion ($33,200 per premature infant) to

the total cost, with over 85% of the services provided during early infancy. In addition, early intervention services for preterm children costs $611 million a year and special education costs $1.1 billion a year (Allen, 2008). Taken together, the total cost on society to provide children born prematurely with the services and health care they need to ensure the best quality of life possible is massive. As the children grow, they have other challenges, such as entering the school system and developing life skills.

Neurodevelopmental Outcomes of Premature Infants

Outcome of Children Born at Less Than 33 Weeks Gestation

Research has shown that children born preterm have more cognitive impairments and academic problems than full term controls. Sommer and colleagues (2007) assessed the neurologic and developmental outcome of children born at less than 27 weeks gestation. At 2 years of age, mental and psychomotor development was assessed by using the Griffith Mental Developmental Scales, which included locomotor, social, language, coordination and performance scales. A developmental quotient (DQ) of 100 is assumed as the mean in a normal distributed population with a standard deviation of 11 points. It was found that 54% of children born before 27 weeks gestation had a Griffith Mental Development Quotient greater than 2 standard deviations below the mean, indicating developmental delay. Only 40% of children born prematurely had normal cognitive abilities, suggesting that prematurity has profound and lasting effects on cognition. Premature birth has been shown to not only affects cognitive abilities, but motor abilities as well. Motor skill problems may make cognitive abilities appear even more impaired.

Motor problems that occur without overt signs of cerebral palsy tend to persist beyond infancy and are often considered under the umbrella terms of developmental

coordination disorder (DCD) or minor neurological dysfunction (MND). These terms encompass a wide variety of deficits in both gross and fine motor abilities that have been observed in older ex-preterm children that persist into adolescence.

Although many premature infants do demonstrate neuromotor abnormalities upon examination, many do not develop cerebral palsy (Fawke, 2007; Sommer, Urlesberger, & Maurer-Fellbaum, Kutschera, & Müller, 2007, Marlow, Hennessy, Bracewell, & Wolke, 2007). A study by Allin, Rooney, Griffiths, Cuddy, Wyatt, Rifkin and Murray (2006) examined children born at less than 33 weeks' gestation who were given a neuropsychological assessment at 18 years of age. The examination divided neurological signs into primary and integrative domains. Primary signs were those that could be elicited by traditional neurological examination such as cranial nerve abnormalities, asymmetry of limb reflexes, and eye movement abnormalities. The integrative signs required integration either within the motor system, or between motor and sensory systems, and are likely to depend on distributed processing involving more than one neural network compared to the localized primary signs. The integrative signs were sub-divided into three groups: sensory integration (e.g. stereognosis (perceiving and understanding the form and nature of objects by sense of touch), graphaesthesia (the ability to recognize writing on the skin purely by the sensation of touch), motor confusion (e.g. tandem walking, finger-thumb right hand, finger-thumb left hand alternating) and sequencing (e.g. fist-edge-palm right hand, fist-edge-palm left hand). Analysis revealed that young adults who were born prematurely showed an increase in total, primary and integrative neurological abnormalities compared to term-born controls. This finding suggests that, although the neurological dysfunction may be mild, it is strongly correlated

with reduced neuropsychological performance and may represent a hidden morbidity in children born prematurely (Allin, Rooney, Griffiths, Cuddy, Wyatt, Rifkin, & Murray, 2006). As with cerebral palsy, these types of motor abnormalities may exist in the absence of traditional neonatal neurological or imaging abnormalities. One longitudinal study examined high-risk "apparently normal" infants born less than 29 weeks gestation. Gross and fine motor tasks were assessed at 18 months, 3 and 5 years. Test items reflected typical motor tasks for each age, including ball skills, balance items, drawing and manual dexterity. High proportions of fine motor deficits and increasing proportions of gross motor deficits were found between 18 months and 5 years (Goyen and Lui, 2002). Fine motor deficits were found in 54% of children at 18 months, 47% of children at 3 years and 64% of children at 5 years. Gross motor deficits were found in 14% of children at 18 months, 33% of children at 3 years, and 81% of children at 8 years. Despite the fact that the infants examined did not exhibit major disability, a significant proportion were found to have continuing problems with fine motor skills and an increase in problems with gross motor skills from 18 months to 5 years, suggesting an underlying deficit. These children can be lost in the care system, and later in the school system, because they do not have gross disability, but do not function at their highest potential for of lack of support from parents and teachers.

Davis and colleagues (2007) examined the motor outcome preterm (less than 28 weeks) children compared with children born at term to assess motor performance at eight years of age, to determine the cognitive and behavioural consequences of developmental coordination disorder (DCD). Fine and gross motor abilities were assessed using the Movement Assessment Battery for Children (MABC) and cognitive ability was

assessed with the Wechsler Intelligence Scale for Children. The MABC is an assessment battery specifically designed to identify and evaluate movement problems that can determine a child's social integration at school. It yields both normative and qualitative measures of movement competence, manual dexterity, ball skills, and static and dynamic balance. Results indicated that children born preterm had significant impairments relative to term born children. The preterm group was substantially delayed on all scales of the MABC, indicating global motor impairments (Davis, Ford, Anderson, & Doyle, 2007). Most preterm children who had DCD were male and those who had DCD also exhibited poorer cognitive functioning, delayed academic progress, and more behavioural problems than preterm children without DCD. Based on this finding, the authors suggest that even minor deficits in motor functioning can have substantial influence over important aspects of adaptive functioning.

Marlow and colleagues (Marlow, Hennessy, Bracewell, and Wolke, 2007) examined motor and executive functioning in six year old children born preterm (less than 25 weeks' gestation) compared with their term born classmates. Motor ability was assessed using the Movement Assessment Battery for Children and cognitive ability was assessed using the Kaufman Assessment Battery for Children. Additional information about motor and executive functioning was collected through the administration of three domains of the neuropsychological battery NESPY. The three domains included tests of visuospatial, sensorimotor and attention-executive function. It was found that children born prematurely demonstrated a higher prevalence of deficits in visuospatial (design copying, line orientation and direction), perceptuomotor (finger tapping, imitating hand postures, visuomotor precision, finger discrimination), attention-executive (planning,

monitoring, self-regulation, problem solving, visual attention), and gross motor function at early school age, compared to their classmates. In each area examined, it was found that nearly half the deficits in motor skills or executive function was not accounted for by impairment in the cognitive score (Marlow et al., 2007). The authors suggest that it is possible that problems with motor and executive function abilities make an important contribution to the poor performance of children born prematurely, as rated by their teachers.

Outcome of Children Born Between 33 and 36 Weeks Gestation

Most of the research examining the neurodevelopmental outcome of premature infants has focused primarily on children born very prematurely (less than 32 weeks of gestation). This is most likely because the risk of impairment increases with decreasing gestational age at the time of birth, which puts pressure on researchers to accurately determine the short and long term consequences of very preterm birth. Conversely, the number of children born prematurely, but closer to term or within a birth weight considered closer to what is defined as normal, is much larger than children born very prematurely (Kirkegaard, Obel, Hedegaard, & Henriksen, 2006). There is little information on the developmental outcome of this group, due to the fact that these children are considered to be low risk of neurodevelopmental problems, since they often have an uncomplicated neonatal period and show no signs of brain abnormalities during early infancy. It is possible that subtle neurodevelopmental problems may not be evident until the child has reached school age, where more sophisticated and complex skills, such as reading and writing, are required.

The reading, spelling (writing words directly from dictation), and arithmetic skills were examined in 9 and 11 year old children who were born after 32 weeks gestation. Gestational age was categorized into four groups: 33 to 36, 37 to 38, 39 to 40, and greater than 41 completed weeks with the gestational age of 39 to 40 weeks acting as the reference category. Parents and teachers assessed the child's performance on a 4-point scale (none, minor, some, and severe). Results showed that children born between gestational weeks 33 to 36 had a nearly 50% increased risk of having reading difficulties compared with children born at term (Kirkegaard, Obel, Hedegaard, & Henriksen, 2006). Although, when adjustments were made for parental education, gender of the child, and breastfeeding, the association was no longer significant. Children born at gestational age 37 to 38 completed weeks also had a significantly higher risk of reading difficulties when compared with children born at 39 to 40 weeks. This difference remained significant even after adjustments were made for the potential confounders. The findings of this study suggest that not only extremely premature children, but also children born as late at 37 to 38, weeks may be at increased risk of learning disabilities compared with children born at 39 to 40 weeks.

In a French study, children born between 30 and 34 weeks gestation were examined at 5 years of age using the Kaufman Assessment Battery for Children (KABC), a standardized test that assesses intelligence and achievement in children aged 2-12 years (Kaufman, O'Neal, Avant & Long, 1987). The test yields four global test scores, including the Mental Processing Composite (MPC), which is a global measure of cognitive ability. The MPC score is standardized to a mean of 100. Scores of less than 70 define moderate or severe cognitive impairment, and scores between 70 and 85, mild

cognitive impairment. It was found that one fourth of the infants born at 33 or 34 weeks had mild to severe cognitive impairment, with MPC scores less than 85 at 5 years of age, which is more than a twofold rate in the general population (Marret, Ancel, Marpeau, Marchand, Pierrat, Larroque, et al., 2007). The authors recommend that additional research on children born after 33 to 36 completed weeks to further clarify the causes and outcomes of preterm birth.

In addition to these cognitive deficits, studies have shown that premature children born later in gestation also exhibit motor problems. The incidence of school and behaviour problems was investigated in 7 year old children born between 32 and 35 completed weeks gestation. Teachers were asked to rate the children on their level of function in six areas: speaking/listening, writing/composition, fine motor skills, mathematics, reading, and physical education, using a five point scale. On the scale, a score of 1 indicated good ability, a score of 3 indicated average ability, and a score of 5 indicated very poor ability. The teachers were also asked to complete the Strengths and Difficulties behaviour questionnaire (SQD). The questionnaire has five scales that cover conduct problems, hyperactivity, emotional symptoms, peer problems, and prosocial behaviour. All but the last were summed to generate a total problem score. Results showed that up to a third of children born between 32 and 35 weeks gestation had some form of school problem. Almost a quarter of the children in the study were receiving non-teaching assisted help at school and 4% were receiving help as a result of an educational statement (Huddy, Johnson & Hope, 2001). Male gender was found to be a risk factor, particularly for problems with speech, reading, fine motor skills, and writing/composition, but not mathematical skills. Males were also more likely to be

reported as hyperactive. The findings indicate that children born later in gestation may be at risk for developing motor, cognitive and behavioural problems at school age. The implications of these findings are important, as they suggest, although the likelihood of disability decreases as gestational age increases, the risk is still very real for children born closer to term.

Brain Injury in Premature Infants

The finding that premature birth often results in cognitive and motor problems led to the examination of the brains of premature infants to try to determine the underlying causes of these deficits. One of the most predominant injuries to the brains of premature infants was damage to the cerebral white matter. This condition, known as periventricular leukomalacia (PVL), has been found to be the major neuropathological substrate associated with the motor and cognitive deficits observed during childhood of premature infants (Volpe, 2001a; Volpe, 1991). PVL is typically comprised of two components: focal and diffuse. The focal component consists of localized injury deep in the white matter and can be macroscopic in size, evolving over several weeks to multiple cystic lesions, easily visible on cranial ultrasonography is known as "cystic PVL" (Volpe, 2009). In neonatal intensive care units, cystic PVL is observed in less than 5% of premature infants and therefore accounts for a very small percentage of PVL cases (Larroque, Marret, Ancel et al., 2003; Inder, Warfield, Wang, Huppi, & Volpe, 2005; Miller, Ferriero, Leonard, et al., 2005). Much more common is focal damage that is microscopic in size and evolves over several weeks to form glial scars, which are not easily visible using neuroimaging. This form of PVL, which accounts for the majority of cases, is called "non-cystic PVL" (Volpe, 2001b).

The diffuse component of PVL occurs throughout the cerebral white matter and is characterized by damage to glial cells as well as to immature oligodendrocytes, which ultimately become the cells responsible for producing myelin. It is this impairment in myelination that is responsible for the spastic motor deficits of cerebral palsy (Volpe, 2001a). There is evidence that damage to the white matter may in turn influence the development of the overlying cerebral grey matter. Marin-Padilla (1997) examined Golgi-Cox preparations of the overlying cerebral cortex of damaged white matter due to bronchopulmonary dysplasia (abnormal development of lung tissue). He found that the underlying white matter injury influenced the subsequent differentiation of the cerebral cortex through the destruction of afferent terminals from association areas and the axons of grey matter projection neurons, affecting their ability to reach their targets. Disturbances to the formation of the overlying cerebral cortex result in alterations in the structure (abnormalities in dendritic and axonal processes) and function of the mature grey matter, demonstrating that a primary source of injury (i.e. white matter damage) can influence surrounding tissue, resulting in a secondary source of injury (i.e. grey matter damage) . These findings led Marin-Padilla to propose that the neurological consequences (e.g. epilepsy and cerebral palsy) that occur after perinatal white matter injury are a direct result of post injury grey matter alterations. Consistent with Marin-Padilla's theory on secondary injury, Volpe (1996) suggested that white matter injury in the premature infant likely disrupts subplate neurons or their connections to cortical or subcortical regions, thereby interfering with their essential functions in cortical development, along with a massive influence on cortical neuronal development. The

neuroanatomical impact may explain many of the cognitive and motor deficits, as well as other types of brain abnormalities, not seen at the time of injury.

Until recently, work attempting to correlate the neuropathological findings of autopsied brains with the neurological consequences in surviving premature infants has focused on the role of white matter injury and hemorrhages, and not on grey matter injury. The general consensus was that cerebral white matter was particularly vulnerable to injury in the preterm brain, with relative sparing of the grey matter, and that grey matter injury occurs predominately in older infants, children, and adults (Kinney and Armstrong, 2002), despite evidence demonstrating that neuronal/axonal injury is common in the perinatal brain (Bell, Becher, Wyatt, Keeling, & McIntosh, 2005; Kinney, Panigrahy, Newburger, Jonas, & Sleeper, 2005). The long held idea that grey matter is largely spared from injury in the preterm brain is now being challenged by recent studies examining autopsied brain as well as by research using advanced MRI techniques to examine the brains of surviving preterm children.

The use of MRI technology has allowed researchers to investigate the possibility that PVL could lead to the subsequent impairment of cerebral cortical neuronal development in the living infant. Inder, Huppi, Warfield, Kikinis, Zientara, Barnes, Jolesz and Volpe (1999) used advanced quantitative volumetric three-dimensional magnetic resonance imaging (3D-MRI) techniques to assess the impact of PVL on subsequent cerebral development in three groups of infants at term: premature infants (less than 32 weeks, mean age 28 weeks gestation) with and without MRI evidence of PVL and a group of healthy term infants (40 weeks gestation). The major goal of the study was to determine whether PVL would result in an alteration of cerebral cortical volume at term.

It was found that premature infants who had evidence of PVL showed a marked reduction in cerebral cortical grey matter volume at term compared with both premature infants without PVL and control term infants. Cerebral cortical grey matter volume at term was reduced by 28% in the premature infants with PVL in comparison to the volume of control term infants. Subcortical grey matter volumes (i.e. basal ganglia, thalamus) were not found to be significantly different between the three groups. This study documented for the first time, a reduction in cerebral cortical grey matter at term in premature infants with PVL and also emphasized the value of the 3D-MRI technique for enabling the detection and quantification of these differences.

Inder and colleagues (Inder, Simon, Wang, Huppi & Volpe, 2005) examined the alterations in cerebral tissue volumes at a time point term equivalent in a large longitudinal cohort study of premature infants (born between 23 and 32 weeks gestation) in comparison with term infants. Assessment of development was done using the Denver Developmental Screening tool, which is a test for screening cognitive and behavioural problems in preschool children. Children were classified as exhibiting severe disability if they had clinical evidence of severe abnormality on neurologic motor examination (e.g. marked spasticity, weakness and developmental delay over than 6 months, moderate disability if they had clinical evidence of moderate abnormality on neurologic motor examination and developmental delay between 4 and 6 months, or mild disability if they had mild spasticity and/or motor deficit and developmental delay of 2 and 4 months). Compared to infants born at term, premature infants had significantly decreased volumes of cerebral tissue; cortical volumes were compared at a time during which premature infants would have been developmentally equivalent to an infant born at term. The

volumes of both cortical grey matter and deep nuclear grey matter were reduced by 22% and the volume of myelinated white matter was reduced by 35% for premature infants compared to term born infants. Neurodevelopmental outcome assessments later at 1 year of age showed that premature infants with moderate-severe disability had significant reductions in grey matter volumes, both cortical grey matter and deep nuclear grey matter volumes. The reduction in cerebral cortical grey matter and deep nuclear grey matter volumes appeared to be predictive as indicators for disability at 1 year (Inder, Simon, Wang, Huppi & Volpe, 2005). Since disability is not acute, it is likely that reductions in grey matter were predictive of longer term disability too.

Regional differences in brain volume and long term cognitive outcome in premature (26-33 weeks gestation) compared to term infants (37-42 weeks gestation) were investigated in a longitudinal study (Bradley, Peterson, Vohr, Staib, Cannistraci, Dolber, et al., 2000). At eight years of age, the neurodevelopmental outcome of the children was assessed (IQ was measured using the Wechsler Intelligence Scale for Children III; visuomotor functioning was assessed with the Developmental Test of Visual-Motor Integration, a test that involved having the children copy geometric forms that increase in difficulty; psychiatric diagnoses were based on the Kiddie Schedule for Affective Disorders and Schizophrenia, a semi-structured interview administered separately to parents about their children and to children about themselves; behavioural problems were assessed by parents using the Child Behaviour Checklist, a device through which the child's problem behaviours and competencies can be rated; MRI scans were also obtained for all children). For the neuroanatomical measurements, the brain was divided into hemispheres and each hemisphere was then divided into 8 anatomical

sectors. The 8 sectors were dorsal prefrontal, orbitofrontal, premotor, subgenual, sensorimotor, parieto-occipital, midtemporal, and inferior occipital cortices. Analysis revealed that at 8 years of age, children who were born prematurely had regional cortical volumes that were significantly smaller than in term controls. The abnormalities were focused in the sensorimotor cortex but also involved the adjacent premotor cortex, parietal-occipital, subgenual and midtemporal regions, and the cerebellum. Subcortical grey matter in the basal ganglia, amygdale and hippocampus were also greatly reduced compared to term controls. The volumes of these brain regions in the preterm group correlated significantly with IQ measures (Bradley et al., 2000). The authors suggest that taken together, these results indicate that perinatal events are capable of producing long term disturbances in cerebral development and that these disturbances in cortical development in turn are responsible for the cognitive deficits observed in premature infants. The involvement of motor regions of the cortex and basal ganglia could account for the tendency for cerebral palsy and other motor disturbances in premature children.

Although advancements in MRI technology have proven to be useful tools in gaining a better understanding of prematurity related brain damage, the findings would be further strengthened if histological examination of brain tissue showed structural changes in regions shown to have volumetric reductions on MRI scans. Relatively few systematic examinations of postmortem tissue have been done, despite the benefit they would provide. Pierson, Folkerth, Billiards, Trachtenberg, Drinkwater, Volpe, & Kinney (2007) conducted a histological examination of brain tissue from premature infants, 23-36 completed weeks gestation. The cases were separated into three groups: 1) Those with PVL, defined as diffused white matter injury combined with focal damage, 2) Those with

diffuse white matter injury (DWMI) but no focal damage, and 3) Those without diffuse or focal injury (Negative). It was hypothesized that PVL cases would have a significantly greater incidence and degree of grey matter injury than non-PVL groups and that the pattern of grey matter injury would mimic the pattern of volume reduction in the cerebral cortex detected by neuroimaging studies. The sections examined included cerebral cortex from all lobes, thalamus, hypothalamus, caudate, putamen, globus pallidus, hippocampus, amygdale, cerebellum, midbrain, pons and medulla. Analysis of the tissue revealed that neuronal loss and gliosis (scar tissue made up of microglia and astrocytes) were more prevalent and of greater severity in the brains of infants with PVL compared to those without. PVL cases also exhibited increased damage to the deep nuclear structures than was observed in the non-PVL cases. In brains with PVL, the thalamus and globus pallidus had a significantly higher incidence of neuronal loss and more severe neuronal loss compared to brains without PVL. The incidence of gliosis was also significantly higher in the thalamus (56%), caudate (60%), putamen (50%) and globus pallidus (60%) in PVL than in the DWMI (12-47%) and Negative cases (0-14%). The cerebellum showed a significantly higher occurrence of neuronal loss in PVL (29%) compared to the DWMI (6%) and Negative (14%) groups. The hippocampus also had considerable neuronal loss and gliosis. PVL cases exhibited relatively mild cerebral cortical neuronal loss and the incidence of gliosis ranged from 20% (temporal cortex) to 31% (frontal cortex). This neuropathological examination in multiple areas clearly demonstrated that grey matter abnormalities are more frequent in the presence of PVL than in its absence. Neuronal loss and gliosis in the cerebral cortex and deep nuclear structures were largely confined to infants with PVL. There was not a single infant with diffuse white matter

injury who exhibited neuronal loss in the cerebral cortex, hippocampus, and deep gray nuclei (Pierson et al., 2007). The neuroanatomic structures showing neuronal loss and/or gliosis correlates well with the neuroimaging data, which has demonstrated volumetric reductions in the thalamus and basal ganglia, and to a lesser extent, the hippocampus and the cerebral cortex in surviving premature infants.

Hypoxia as a Possible Underlying Mechanism for Brain Injury in Premature Infants

Neurons are critically dependent on a constant supply of oxygen to maintain proper function. Any systemic or central nervous system process that compromises the oxygen supply to part, or all, of the brain will interfere with its ability to function, and when severe enough, can cause the death of brain cells. The term hypoxia is used to describe a reduced concentration of oxygen. Premature infants are at an increased risk of hypoxia as lung maturation takes place during the third trimester of pregnancy. Thus, the smaller and gestationally younger the infant is at birth, the higher the probability of severe and chronic respiratory problems (Meisels, Plunkett, Pasick, Stiefel, & Roloff, 1987). Chronic respiratory problems may compromise the amount of oxygen the infant is able to take in, resulting in less than optimal oxygenation of the brain which, eventually leads to neuronal damage (Tina, Frigiola, Abella, Artale, Puleo, D'Angelo et al., 2009). In addition, infants may also be vulnerable to hypoxia in utero. Intrauterine asphyxia (impairment in the exchange of respiratory gases) has been shown to be a major influence on the origins of hypoxic insult in the immature brain (Volpe, 2001b). A challenge exists in knowing the level of hypoxia, how much damage has occurred, and the impact on cognitive, motor, and academic ability.

One of the problems with recognizing cortical damage in premature infants is that gross evidence of destructive processes (e.g. obvious neuronal death) is often lacking, except among children with PVL. Although the effects may be subtle, there are some risk factors associated with hypoxia. Hypoxic insult is often accompanied by acidosis, an abnormal increase in the acidity of the body's fluids, most importantly blood. The normal pH range for blood is between 7.35 and 7.45. Changes in arterial pH levels are considered to be the biochemical hallmark of the extent of hypoxic risk (Nelson, 1989; Carter, Havercamp, & Merenstein, 1993). Research has examined the neurodevelopmental outcome in children born prematurely who also had an increased risk of hypoxia. Stevens, Raz, and Sander (1999) examined the relationship between gestational age at birth, hypoxic risk and intellectual outcome at early school age. Premature children were born between 29-36 weeks gestation and term controls were born between 37-42 weeks gestation. The arterial pH of all children in the sample was obtained 3 hours after delivery and was between 7.3 (the lower end of the normal range) and 7.1 (the lower end of the moderately acidotic range). When the children were 5 years of age, they were given an abbreviated version of the Wechsler Preschool and Primary Scale of Intelligence-Revised. For analysis, outcomes on the Full Scale IQ (FSIQ), Performance IQ (PIQ) and Verbal IQ (VIQ) were assessed. The full scale IQ is based on scores on all of the subtests and is a reflection of both verbal IQ and performance IQ. The verbal IQ tests acquired knowledge, verbal reasoning and comprehension, and attention to verbal stimuli. The performance IQ tests fluid reasoning, spatial processing, attentiveness to detail, and visual-motor integration. The results showed a significant relationship between initial arterial pH, a general biochemical index of hypoxia, and developmental outcome in a

group of premature, at risk children. Specifically, it was found that in both premature and term children at slight to moderate hypoxic risk, an increase in the degree of risk was linked with a proportionate decline in cognitive performance between the ages of 4 and 7 years (Stevens, Raz, & Sander, 1999). Gestational maturity was not found to play a role in cognitive outcome measures. The authors suggest that this finding shows that term birth does not increase resilience to hypoxic insult. It was also found that arterial pH at birth was closely linked to VIQ and less so to PIQ, indicating a particular vulnerability of the left hemisphere.

Hopkins-Golightly, Sander, and Raz (2003) built on previous work by comparing cognitive and language function in premature children with a history of slight to moderate hypoxia and premature children not considered at risk for this complication. The psychological assessment involved administration of the Wechsler Preschool and Primary Scales of Intelligence –Revised. The Preschool Language Scale-3 was also administered to obtain an indicator of language function. It assesses receptive (Auditory Comprehension Scale) and expressive language (Expressive Communication Scale) skills in infants and young children. Results revealed that increasing acidosis was related to decreases in cognitive skills, particularly for verbal and visuospatial skills, indicating that in premature infants, even a minor risk for hypoxia may influence the course of cognitive development.

The relationship between hypoxia and neuropsychological outcome has been examined in premature children (Epsy, Senn, Charak, Tyler, and Wiebe, 2007). Neonates included in the study were born between 28 and 35 weeks and were considered at a low risk for severe sequelae. The mean blood pH was 7.297, which is in the moderately

acidotic range. The Picture Vocabulary subtest from the Woodcock-Johnson Psycho-Educational Battery-Revised was administered to assess verbal skills. Executive control, self-control, selective and sustained attention, and early mathematical abilities were also assessed. It was found that children with lower pH, that is whose arterial blood was more acidotic at birth, identified fewer words at 3 years of age than preschool aged children with higher initial pH values. In addition, initial arterial pH at birth was also related to performance on tests of visual attention and problem solving at 3 years of age. Preschool children born prematurely with lower initial arterial pH values at birth made a higher number of commission errors (responding where they should not) and identified fewer targets in the visual attention tasks, and were able to solve fewer simple mathematical problems at age 3 years than those with higher initial arterial pH values at birth. These findings extend those of Raz and colleagues (Golightly et al., 2003; Stevens, Raz, & Sander, 1999) into two additional cognitive domains—controlled attention and emergent mathematics. The results also provide more evidence that even premature infants at a low risk for hypoxia who show only mild acidosis exhibit cognitive deficits as enter into childhood.

Deficits in motor abilities have also been observed in premature infants at risk for hypoxic damage. Motor and cognitive deficits were examined at one year of age in 364 children who had been born prematurely (less than 37 weeks gestation) compared with those that had been born at term (40 weeks gestation). The Bayley Scales of Infant Development (the Physical and Mental Development Index) and the Uzgiris and Hunt Scale of cognitive development were administered. Motor and cognitive assessments were conducted when the children were six and 12 months of age. Motor and/or cognitive

deficits were detected at one year in 86 (24%) of the children included in the study. Motor deficits occurred in 79 children and cognitive deficits occurred in 38 children. It was found that probability of deficits in the absence of fetal complications (e.g. hypoxia, respiratory complications or major infections) in the children studied was 14 to 19%. When fetal hypoxia, moderate or severe respiratory complications or major infections occur separately, the probability of deficits increases to 25 to 45% (Low, Galbraith, Muir, Broekhoven, Wilkinson, and Karchmar, 1985), indicating that fetal hypoxia is independently associated with motor and cognitive deficits in premature infants at one year of age. In a recent study, Raz, Glogowski-Kawamoto, Yu, Kronenberg, Hopkins, Lauterbach et al. (1998) assessed whether differences in hypoxic risk between co-twins born prematurely (32 weeks gestation) adversely affects cognitive and motor abilities in early childhood compared with lower risk nondiscordant twins who were born slightly later (34 weeks gestation). Hypoxic risk status within each twin pair was determined on the basis of the Apgar score and the total time respiratory assistance was needed. Children were 6 years of age at the time of testing, and were administered the Wechsler Intelligence Scale for Children-Revised or the Wechsler Preschool and Primary Scale of Intelligence—Revised to assess general cognitive abilities and the McCarthy Motor Scale to assess motor abilities. The McCarthy Motor Scale is based on five tests, including Leg Coordination, Arm Coordination, Imitative Action, Draw-a-Design, and Draw-a-Child. The results revealed that moderate differences in hypoxic risk between co-twins were associated with significant discrepancy in motor abilities in childhood. The lower-risk twins significantly outperformed the higher-risk twins. Examination of the results of the individual subtests constituting the McCarthy Motor Scale, the Arm Coordination subtest

was the most sensitive to differences between the co-twins. The Arm Coordination test includes the following tasks: ball bouncing, beanbag catch game, and the beanbag target game. All of these tasks require ballistic movements (fast limb movements from one limb position to another) of the upper extremities to guide an object. In contrast, the tasks for the Leg Coordination subtests (walk backwards, stand on one foot, stand on the other foot, and skipping) require little or no ballistic movements. None of the tasks requires controlling the motion of an object, but instead demand sustained balance and gait. This type of movement involves not only the lower extremities, but also the upper extremities and the trunk. The authors suggest that the arm coordination skills show a greater vulnerability to hypoxic risk than the leg coordination because the upper extremities may be more extensively represented in parts of the brain motor system (e.g. basal ganglia) than are lower extremities (Raz et al., 1998). In addition, the tasks within the Arm Coordination subtest may require multiple functional resources that are unnecessary to successfully complete the Leg Coordination task. Therefore, among the range of motor abilities, it is possible that complex, visually guided motor performance (generally accomplished by the upper extremities), rather than maintenance of balance and gait (generally involving the lower extremities and trunk) is increasingly susceptible to perinatal hypoxic risk (Raz et al., 1998). The findings of this study provide evidence that increased hypoxic risk in premature infants may contribute to the development of motor deficits in childhood. In particular, motor tasks requiring visually guided arm control of the motion of an object seem to be especially vulnerable in premature infants.

Prematurity is not the only factor that can influence susceptibility to hypoxia and subsequent developmental outcome. Male sex is an acknowledged risk factor for stroke

and this sexually dimorphic sensitivity appears to be present until later in life, well beyond menopause (Vagnerova, Koerner, & Hurn, 2008). In a study of over 1200 genetically hypertensive and stroke-prone rats, life expectancy was longer in females than in males (Yamorie, Horie, Handa, Sato, & Fukase, 1976). There was also no evidence of cerebral hemorrhage and vascular lesions in females until an advanced age, a pattern that mimics what is observed in humans. In addition, genetically unaltered female rats and mice sustain smaller tissue damage and have improved functional outcome compared to their male counterparts after focal or global ischemia.

Examination of the male and female brain at the cellular level, using cultured cortical neurons, has shown that male neurons are more susceptible than female neurons to injury using pharmacological agents (e.g. glutamate, peroxynitrite) to simulate brain damage (Du, Bayir, Lai, Zhang, Kochanek, Watkins et al., 2004). Gender differences are not only observed in neurons, but cell death after oxygen-glucose deprivation is less widespread in female astrocytes (Liu, Hurn, Roselli, & Alkayed, 2007) and in female hippocampal slices (Li, Pin, Zeng, Wang, Andreasson, & McCullough, 2005). These findings suggest increased vulnerability of males to stroke may be partly due to the differential response of male and female brain cells. Therefore, in addition to gestational age, gender must also be taken into consideration in terms of preventative and therapeutic interventions.

Studies examining premature infants with an increased risk of hypoxia exposure have shown these children are more likely have deficits in verbal, visuospatial, visual attention, arithmetic problem solving, and motor abilities compared to premature children at low risk of hypoxia exposure and children born at term. These results indicate that

hypoxia could be an underlying cause, contributing to the deficits observed in premature children. The next step is to determine the impact that hypoxia has on the neurons in the developing brain. Changes in neurons correspond to changes in behavior, so it is important to determine how changes in neuronal structure influence subsequent behavior. Since it is impossible to use human infants to examine how hypoxia affects the immature brain, animal models, typically using rats have been developed. To ensure that the animal model is mimicking the human condition as accurately as possible, an understanding of the time line of human and rat brain development is essential.

Human and Rat Brain Development

The mammalian brain adheres to a common pattern of development. An initial stage of the development of the brain results from the formation of a hollow tube by folding of the neural plate, which is primitive neural tissue that occupies the outermost layer of embryonic cells. This tube surrounds a fluid filled space that will become a ventricle, and the surrounding tissues will eventually become the brain and spinal cord.

Cell Proliferation and Migration

In humans, shortly after the closure of the neural tube, two proliferation areas are formed in the ventricular and subventricular zones. The ventricular zone gives rise to neurons and glial cells, and the subventricular zone generates mainly neurons (Mrzljak, Uylings, Kostovic, & Vaneden, 1992). The majority of neuroblasts are formed during gestational weeks 5 to 25 and the majority of glial cells are produced during weeks 20 and 40 (Mrzljak, Uylings, Kostovic, & Vaneden, 1992). The period of major neocortical neuron formation is between approximately 6 and 18 weeks gestation (reviewed in Uylings, 2000). Once neurons have been generated, they travel from their place of origin,

to their mature positions. Most cortical neurons migrate to their destinations along specialized radial glial fibres, which extend over the entire thickness of the hemisphere (Rakic, 2002). These guiding glial cells are induced by the Cajal-Retizus cells, which are the first neurons to migrate into the marginal layer and play an important role in the establishment of cortical lamination. The settling of neurons into their mature positions occurs in an inside-out order, with the earlier generated neurons moving to the deepest cortical layers, and the later generated neurons assuming positions in the superficial layers of cortex. Evidence supporting this inside-out formation was provided in a series of studies by Marin-Padilla (1970a, 1970b) examining the sequential lamination of the motor cortex during development. It was discovered that by the fifth embryonic month, cortical layers V and VI were clearly visible, but not yet mature in appearance until birth. The superficial layers develop later, they are not visible until about 7 months, and are not mature in appearance until after birth.

In the human cerebral cortex, migration takes place during the early phases of brain development and peaks between the third and fifth month of gestation (Rakic, Cameron, & Komuro, 1994; Gressens, 2000). The point in time when migration stops is still under debate, but recent evidence indicates that it may be around 30 weeks gestation (Gupta, Hasan, Trivedi, Pradhan, Das, Parikh, et al., 2005) indicating that migration is still active later in gestation.

In the rat, the primary source of neurons exclusive to the nervous system (e.g. neurons, glia) is a proliferative germinal matrix that is referred to by several different names: the primitive ependymal layer, the ventricular zone, or the neuroepithelium (Bayer & Altman, 2004). In cerebral cortex, neurons are generated mainly from

embryonic day 10 until birth (reviewed in Uylings, 2000). Cells leaving the neuroepithelium have three possible fates: they can become postmitotic and differentiate into neurons, they can become glia or glial precursors, or they can maintain their proliferative capability and form secondary germinal matrices (Bayer & Altman, 2004). Migration in the cerebral cortex of the rat begins on about day 14 of gestation and soon accelerates so that by day 16, a definitive lamina of neurons has been formed. The pattern of migration in the rat is similar to that of the human. Studies using titrated thymidine established that laminar development in the rat cortex occurred in an inside-out manner (Berry and Rogers, 1965). Neurons that were formed up to day 16 of gestation were found to populate layer VI, those formed on about day 17 formed layer V, the neurons of layer IV were formed in the ventricular zone on about day 18, and the neurons of layers II and III were formed on days 19, 20, and 21 of gestation. The first formed neurons reached the cortex quite rapidly but later, as the migration path became longer, the migratory time became longer. Consequently, neurons formed early in the ventricular zone reached the marginal layer in about 1.5 days, but those formed later reached their definitive destination in about 6 days. In rats, the superficial layers (layers II and III) are thinner than those in the human neocortex, but layers VI and V are thick (Bayer and Altman, 1991). At birth, layers VI and V have migrated and occupy their mature positions, but layers IV, III, and II are still actively migrating and the six laminae of the neocortex do not become fully established until 7-10 days after birth.

Organization of Cortical Layers and Cell Types

The most characteristic form of the neocortex contains six layers, numbered from the outer cortical surface (pia mater) to the white matter. Layer I contains dendrites of the

cells located deeper in cortex and axons that travel through to form connections in this layer, as well as Cajal-Retzius cells. These cells possess long horizontal axons that form synaptic contacts with the dendritic shafts or spines of pyramidal neurons. They are also responsible for establishing early neuronal circuitry in the developing brain (Meyer, Goffinet, & Fairen, 1999). Layer II is made up of mostly small spherical cells called granule cells and therefore is called the extragranular cell layer. Layer III contains various cell types, many of which are pyramidally shaped; the neurons located deeper in layer III are typically larger than those located more superficially. Layer III is called the external pyramidal cell layer. Layer IV contains different types of stellate and pyramidal neurons and is called the internal granular cell layer. Layer V, called the internal pyramidal cell layer contains mainly large pyramidal neurons. In motor cortex, these large layer V neurons are called Betz cells. Finally, layer VI is a rather heterogeneous layer of neurons (including a few large pyramidal neurons and many small spindle-like neurons) and is therefore called the polymorphic or multiform layer. It merges with the white matter that forms that deepest limit of the cortex and carries axons to the cortex.

Martinotti cells, small neurons with short branching dendrites are scattered throughout the cortex, sending axons up to layer I where they form dendritic arborization. Arbors extend into layer IV and make contacts with the distal tuft dendrites of pyramidal neurons. Recent research has suggested that Martinotti cells are associated with a cortical dampening mechanism. When pyramidal neurons start getting overexcited, Martinotti cells start sending inhibitory signals to the surrounding neurons (Silberberg & Markram, 2007). Cortical basket cells are found in all layers except layer I. They are inhibitory interneurons and three types are found in cortex, the small, large, and nest type. The axon

of the small basket cell arborizes in the vicinity of that same cell's dendritic range. In contrast, large basket cells innervate somata in different cortical columns. The nest basket cells are an intermediate form of the small and large cells. Their axons are confined mainly to the same cortical layer as their somata (Marin-Padilla, 1983b).

The cortex also contains glial cells**,** which surround neurons providing support. Glial cells are the most abundant cell types in the central nervous system. There are three types of glial cells: astrocytes, oligodendrocytes, and microglia. Astrocytes are concerned with neurotransmission (e.g. maintaining environmental stability) and neuronal metabolism. Oligodendrocytes are involved in the production of myelin, the insulating material around neurons. Microglia are part of the immune system and comprise approximately 15% of the total cells of the central nervous system and multiply when the brain is damaged.

Even though each layer of the cerebral cortex is defined largely by the presence or absence of neuronal cell bodies, each layer also contains additional components. Layers I-III contain the apical dendrites of neurons that have their cell bodies in layers V and VI, whereas layers V and VI contain the basilar dendrites of neurons with cell bodies in layers III and IV. It appears then, that the profile of inputs to a particular cortical neuron depends more on the distribution of its dendrites than on the location of its cell body.

It should be noted that not all cortical regions have the exact same laminar organization. For instance, the primary motor cortex has essentially no layer IV and the primary visual cortex has a very pronounced layer IV. The size of layer IV within a given cortical region is proportional to that region's connections with the thalamus. Layer IV is the main target of sensory information from the thalamus. In extremely visual animals,

like humans, the lateral geniculate nucleus produces an extensive and highly organized input to layer IV of the primary visual cortex. In contrast, the motor cortex is principally an output region and therefore receives little sensory information directly from the thalamus, leading to a very small layer IV but very prominent output layers, such as layer V.

Development of Dendrites

Dendritic development in both humans and rats is studied primarily by using the Golgi-Cox stain to visualize neurons and their processes. The method involves immersing brains in a solution of potassium dichromate and then processing with ammonium hydroxide. Exposure to the ammonium hydroxide causes a precipitate to fill the neurons (Spacek, 1992, 1998). The impregnated neurons stand out against a relatively clear background, permitting direct visualization of the cell body and its processes under light microscopy for quantitative and qualitative analysis. The stain labels approximately 1-4% of neurons and has allowed for extensive investigation of development.

In humans, dendritic development of cortical neurons begins prenatally with the age of commencement varying with the cortical layer and location. Dendritic development of cortical neurons proceeds fairly slowly during the first two trimesters of gestation. Dendritic trees of the deeper cortical layers mature earlier than those of the superficial layers (Kostovic & Judas, 2002). The rate of growth accelerates from the third trimester of gestation onward and remains very active until the end of the first postnatal year (Super, Soriano, & Uylings; Eyre, Miller, Clowry, Conway, & Watts, 2000). The length of axons and dendrites increases five to ten times during the first six months of postnatally. This period is also marked by extensive dendritic elaboration (Rakic, 2002;

Becker, Armstrong, Chan, & Wood, 1984; Webb, Monk, & Nelson, 2001; Nimchinsky, Sabatini, & Svoboda, 2002). From then on dendritic growth of cortical neurons in the human continues until about 5 years of age (Koenderink & Uylings, 1995), at which point dendrites have achieved maturity.

In the rat, nearly all dendritic growth is postnatal. The peak of development is between postnatal days 8 and 20, with maximum growth accomplished by day 30 (Kolb, 1995). The regional differences in growth rate observed in humans are not nearly as extreme in the rat. The axon, extending from the bottom of the cell body and apical dendrites, extending from the top of the cell body of pyramidal neurons in the cortex begin to grow before the basilar dendrites, located on either side of the cell body appear (Berry, 1974). Thus, soon after migration, pyramidal neurons possess an axon and apical dendrite, but no basilar dendrites. It is not until postnatal day 30 that the structure of basilar dendritic fields approach adult dimensions. It is not easy to pinpoint comparative ages for humans and rats in terms of maximum dendritic growth or time at which dendritic growth is complete. The main reason for this difficulty is the striking disparity in dendritic growth in different brain regions.

Development of Synapses

The formation of synapses is generally studied using electron microscopy. Electron microscopes use a particle beam of electrons to illuminate a specimen and create a highly-magnified image. They have much greater resolving power than light microscopes and can obtain much higher magnifications of up to 1 million times, thus allowing for a much more detailed view of the tissue under examination. Sections of tissue are embedded in an epoxy resin and ultrathin sections are stained with uranyl

acetate or lead citrate. Both stains can be used to provide contrast for viewing tissue under an electron microscope. Once the tissue is stained and visible under the microscope, analyses of the structures of interest are conducted.

In parallel with the normal development and maturation of dendrites, is an increase in synaptic connections. In the human cerebral cortex, the very first synapses are found in the cerebral cortex at 9-10 weeks gestation (Molliver, Kostovic, & Van der Loos, 1973; Zecevic, 1998). Synaptic density then steadily increases at a rate of about 4% per week until 24-26 in nearly all cortical regions (Zecevic, 1998). Maximum synaptic density is reached in primary sensory areas such as the auditory and visual cortex at 3 months postnatally (Huttenlocher, 1997; Huttenlocher, 1984). In the visual cortex, synaptic density roughly doubles between the second and fourth month after birth and continues to increase until 1 year of age. After the first year, synaptic density begins to decline to adult levels, which take place around the age of 11 (Huttenlocher, 1990). Within the frontal cortex synaptic density also reaches peak levels at around 1 year, but in contrast to the visual cortex, the density is much higher and decline does not begin until about 5-7 years of age. Once the decline has begun, it takes until approximately 16 years of age to reach adult levels (Huttenlocher, 1984). It seems then, that the refinement of connections takes place much sooner in the visual cortex than in the frontal cortex, as synaptic density begins to decline later than in the visual cortex and adult levels are not reached until the mid-teens. This delay in synaptic pruning may be due to the fact that the frontal cortex is responsible for higher order processing, requiring an increased synaptic density and a longer time period to establish the connections robust enough to remain and eliminate the ones not required.

In the rat, the majority of neocortical synaptogenesis is postnatal. At birth most synapses that are present are axodendritic contacts. These axodendritic connections are immature and exhibit a small area of contact, few synaptic vesicles, and relatively little thickening of the opposing membranes (Berry, 1965). As the neuropil matures, axodendritic synapses gradually show increased thickening of the postsynaptic membrane, synaptic vesicles become more numerous; there was a greater area of contact, more distinct subsynaptic thickening, and uniformity of width of the synaptic cleft. By 22 postnatal days, the axosomatic synapses become more frequent and appear to reach adult frequencies 30 days postnatally. The first spines emerge between postnatal days 10 and 20 but they are not adult-like in appearance until postnatal day 30 (reviewed in Kolb, 1995). Synaptic density seems to peak at around day 35 in sensory and motor cortex, and then subsequently decline (Blue & Parnavelas, 1983a, 1983b). The elimination of excess synaptic connections during later childhood years in humans and what could be considered as later childhood in rats suggests that the exuberant connections that are established during infancy may form the anatomical substrate for neural plasticity and for certain types of early learning (Huttenlocher, 1990). This suggests similar mechanisms at work in both the human and rat brain during roughly equivalent time points in development.

Organization of Input and Output by Cortical Layers

The neocortex receives inputs from the thalamus, other cortical regions on both sides of the brain, and from numerous other sources. The output of the neocortex is directed to various brain regions including regions of the neocortex on both sides of the brain, the basal ganglia, the thalamus, the pontine nuclei and the spinal cord. Different

inputs to the cortex seem to be processed in different ways and the output from the neocortex arises from different neural populations. Projections to other parts of the neocortex (referred to as corticocortical or associational connections) emanate primarily from layers II and III. Projections to subcortical regions emanate primarily from layers V and VI. Thus, the arrangement of cells into layers creates an efficient means of organizing the input-output connections between cortical cells. Invasion of the cortex by thalamic fibers (e.g. Krubitzer and Huffman, 2000) may serve to influence the development of cortical layers through intrinsic mechanisms, such as genes.

In mammals, thalamic fibers reach the appropriate cortical regions before their definitive target neurons are born (Lund and Mustari, 1977; Rakic, 1976; Shatz and Luskin, 1986). They then have to wait for two to three days (E16-E19 in rodents) before continuing to grow and establish their final innervations pattern within the cortical plate (Lopez-Bendito and Molnar, 2003). Shortly before birth, most of the thalamic axons start to detach from the subplate and grow into the cortical plate, forming branches and synapses in the appropriate cortical layer. The majority of thalamic axons grow in deep layers (V and VI) than in superficial layers (II-IV) (Lopez-Bendito and Molnar, 2003). Given that the bulk of thalamic connections are with deep cortical layers, which are output layers, the thalamus then, is more involved in the execution of behavior.

One area of controversy involves how much influence thalamocortical axons have in terms of cortical patterning. Some studies (e.g. Rakic, 1988; Rubenstein, Anderson, Shi, Miyashita-Lin, Bulfone, & Hevner, 1999) have proposed that intrinsic molecular determinants of the proliferative zone play a part in the early regionalization of the developing cortex. Indeed, some genes are expressed in a region and lamina specific

manner before thalamic afferents have invaded the cortex (Stoykova and Gruss, 1994; Gulisano, Broccoli, Pardini, & Boncinelli, 1996; Nakagawa, Johnson, & O'Leary, 1999) suggesting that some aspects of early cortical regionalization are not dependent on extrinsic influences. Other research has shown that input from thalamocortical axons may influence the development of anatomical features that distinguish different cortical areas (Krubitzer and Huffman, 2000; Rakic, 1988). Some studies have shown that thalamocortical projections can influence the size and identity of specific cortical areas (Krubitzer and Huffman, 2000; Pallas, 2001; Kaas, Florence, & Jain, 1999). For example, decreased thalamic input to the cortex following removal of both eyes modifies the areal fate of cortical cells by creating a "hybrid" visual cortex in place of area 17, also called primary visual cortex (Rakic, Suner, & Williams, 1991; Dehay, Giroud, Berland, Killackey, & Kennedy, 1996). Similarly, early damage to thalamic nuclei produces alterations in the size and cell number in the corresponding region of the neocortex (Windrem and Findlay, 1991). Gaillard and Roger (2000) examined the thalamic connectivity formed by grafts of E16 parietal and occipital cortex placed into the corresponding cortex or into a different region of cortex (barrel cortex). It was found that although E16 parietal and occipital cortical grafts attracted thalamic projections, the cells did not have the ability to differentiate and maintain the organization of barrel cortex. This finding suggests cortical regionalization begins with graded expression of a variety of genes, and thalamic input controls the later stages of regional subdivision through activity-dependent or independent mechanisms (Lopenz-Bendito and Molnar, 2003). Taken together, the results of these studies indicate the remodeling of cortical circuitry during the invasion of thalamic fibers is a complex process, in which the expression of

molecules and growth factors, along with patterns of afferent and local activity, seem to play an important part.

In an effort to better understand the consequences of early developmental damage on the brain and behaviour, animal models have been utilized. The animal models are extremely useful given their comparable developmental time course and the fact that rats born at term are equivalent to a preterm human infant.

Chapter 2

Animal Models of Early Developmental Damage

One aspect within neuroscience that has been a source of debate is whether or not there is an "optimal" period during development to sustain brain damage and still experience behavioural and morphological recovery. The idea that the immature brain does not respond to damage in the same way as the mature brain was first suggested by the pioneering work by Margaret Kennard in the late 1930's and early 1940's. Kennard's task was to learn about the roles of the primate motor and premotor cortex. Her early work focused on the consequences of damage to the adult brain and then gradually expanded to include examining the impact of damage to the immature brain. She originally posited that early brain damage might actually produce more deleterious effects than injury during adulthood, which ultimately came to be known as the Kennard Principle. The results of her work, however, indicated that the opposite may be true.

Kennard's Research into Damage During Infancy

The first study Kennard (1936) conducted examining the effects of early brain damage involved the study of two monkeys (Macaca mulatta). In one of the monkeys, Kennard removed the left motor and premotor areas at 10 days of age and the other had the entire left hemisphere removed at 40 days of age. An examination of postoperative functioning revealed that the monkey operated on at 10 days was able to walk around using all four extremities. A slight slowness on the right side was present 24 hours after the surgery. The animal exhibited a deficit in purposeful movement, such as grasping or picking up an object, and used its right fingers and toes less frequently, and slightly more awkwardly than the left, but this deficit disappeared within ten days (Kennard, 1936).

Deficits in forced grasping also disappeared after several months and the animal developed normally with no signs of any lingering motor deficits. Examination of the infant monkey that had the entire left hemisphere removed at 40 days of age revealed deficits characteristic of adult animals that had received similar damage. Recovery of function in the infant monkey after twenty-four hours was a great as the recovery observed in adult monkeys after several weeks (Kennard, 1936). One month after surgery, the infant was able to move accurately and rapidly using both hands and feet, although the right hand and foot were found to be slightly less accurate than the left. After four months there still remained a slight awkwardness of the movements of the right side of the infant that distinguished this animal from normally developing infants. A second surgery removing the motor and premotor areas from the other hemisphere in the same animal at five months of age also resulted in a fairly rapid recovery (Kennard, 1936). When compared to normal animals, deficits in agility and walking movements were evident and persisted throughout the animal's life.

Kennard provided further evidence of an infant's capacity for recovery in another study in which chimpanzees were subjected to the unilateral removal of Brodmann's areas 4 (motor) and 6 (premotor) on the left side at either four or ten months of age (Kennard, 1940). Motor performance at four months is fairly simple. The animal lays on its back, as human infants do, waving its arms and legs, but is unable to right itself or to grasp "voluntarily". After the surgery, there were no noticeable differences in posture or in the types of behavior, except that less spontaneous movements were observed on the left. At six months of age, the animal began to show posture differences in addition to hyperreflexia (overreactive reflexes) and a Rossolimo response (tapping of the sole of the

foot causing abnormal flexion of the toes) developed on the right. There was no resistance to passive manipulation until the age of eight months. The other chimpanzee had areas 4 and 6 removed on the left side at the age of ten months, an age at which it was already engaging in behaviours such as walking, climbing and feeding itself with its hands. Almost immediately after surgery paresis appeared, but subsided slightly after eight months. Despite the small abatement in paresis, the chimpanzee continued to hold the arm and hand in a paretic position (Kennard, 1940). Kennard noted that position at which the chimpanzee held its hand and arm were similar to what has been seen in human brain injury in the same location. Walking movements were greatly disturbed, with the right knee raised high, and the toes on the right foot were no longer used for fine adjustments of balance. The findings of these studies lead Kennard to conclude that the rate of recovery and extent of motor impairment after unilateral and bilateral ablations of the motor and premotor areas was dependent on the age at which the injury occurred. Immature animals were found to recover more quickly and extensively than adults.

Recent Research into Consequences of Early Injury: Challenging the Kennard Principle

Work done since Kennard's experiments in the 1930's and 40's have also provided evidence that recovery from brain injury is maximal if the injury is sustained during infancy, rather than later in life. This research has also refined Kennard's theory to show that there are certain periods during infancy that allow for more functional recovery than others. Most of the research has used rats to examine the consequences of injury early in life and much of this rodent work has been conducted by Bryan Kolb and colleagues, but Kolb does note caveats to these changes.

In the late 1980s and early 1990s, Kolb and his group carried out a series of experiments specifically aimed at investigating the behavioural and anatomical effects of cortical damage in rats of different ages. The first experiment of the series set out to determine if there was an optimal age at which to recover from lesions to the motor cortex (Kolb, Cioe, & Whishaw, 2000a). Animals were given bilateral lesions (achieved through aspiration) of the motor cortex on Postnatal day 1 (P1), Postnatal day 10 (P10), or in adulthood (90 days) and then trained on several motor tasks (skilled forelimb reaching, beam traversing, tongue extension), general motor activity, and a test of spatial learning (Morris water maze). The Morris water maze involves placing animals into a pool of water that contains an escape platform hidden just below the water. Visual cues, such as colored shapes, are placed around the pool in plain sight of the animal. When the animal is released, it immediately swims around the pool, searching for an exit. Time spent in each quadrant of the pool, time taken to reach the platform (latency) and total distance travelled is recorded. With each subsequent trial, the animal's ability to find the platform improves because it has learned where the platform is located relative to the obvious visual cues. Anatomical results revealed that animals in the postnatal day1 and postnatal day 10 groups both had smaller brains than the other two groups, as well as a general decrease in cortical thickness (Kolb et al., 2000a). P1 animals were also found to have an abnormal pattern of corticospinal projections compared to the other two groups. Behavioral performance showed that all lesion groups were impaired at the skilled reaching, but the P10 animals were less impaired than those that had received lesions at P1 or adulthood. Additionally, on the other motor tasks animals were tested on, performance of the P10 group did not differ significantly from that of controls, whereas

both the P1 and adult groups were impaired. Only the P1 group was impaired at the Morris water maze. Animals in the P1 group were unable to learn to swim directly to the hidden platform, instead adopting a looping strategy in which they swam parallel to the tank wall with a trajectory that would guarantee they would eventually hit the platform. The results of this study suggest earlier is not always better as Kennard believed. Lesions at P1 resulted in anatomical abnormalities as well as obvious behavioral deficits that extended beyond those observed in animals with lesions at P10. In fact, in most instances, the performance of the P10 group reached control levels, indicating that for motor cortex lesions in the rat, injury at around 10 days of age may be the "best" time in terms of functional recovery.

Kolb and others have extended the findings from motor and frontal areas to other regions of the brain. For example, Kolb, Petrie, and Cioe (1996) examined the prefrontal cortex to determine its response to early brain damage. The medial prefrontal cortex of rats was lesioned at days 3, 6, 9, 15, or 30 and anatomical and behavioral comparisons were conducted with normal control littermates. Performance on the Morris water maze revealed that P9 animals faired the best, P3 and P30 animals did poorly and P6 and P15 animals fell in the middle. Results on the skilled reaching task revealed that control animals did significantly better on this task than all of the lesioned animals except for the P9 rats. Analysis was also done on claw cutting behavior. Rats will trim their claws by rapidly nibbling with their incisors. Animals with motor or prefrontal damage in adulthood are impaired at this behavior and have claws that are nearly twice as long as normal, which most likely reflects a deficit in fine motor control of movement (Whishaw, Kolb, Sutherland, & Becker, 1983). When the brains of animals were examined early in

the postoperative period, an obvious cavity was observed, which could account for the poor performance on the behavioural tasks. The brains from animals lesioned at P9 or P15 had no lesion cavity in adulthood, as a portion of the cortex appeared to have regrown (Kolb, Petrie, & Cioe, 1996a). In general, the study found that lesions in young rats produced milder behavioral deficits than rats with comparable lesions in adulthood; however the functional outcome was greatest for animals lesioned at 9 days of age, a timeframe similar to that observed in both motor and frontal areas.

Kolb and colleagues also investigated the impact that early developmental injury had on the temporal association cortex (Kolb & Cioe, 2003), which, like the posterior parietal cortex, has extrastriate visual functions. Animals were divided into four groups: control, temporal lesions at P4, P10, or at adulthood (90 days). Ninety days after surgery, the rats were trained on two visual tasks: the Morris water maze, a visual-spatial navigation task and a horizontal-vertical stripes discrimination task. Behavioral results revealed that lesions at P4 produced a larger deficit in performance on the Morris water maze than lesions at P10 or adult lesions. No deficits were found in any of the groups on the visual discrimination task. Analysis of the brains showed that lesions at day 4 resulted in a smaller brain and a thinner cortex than lesions at P10. The reduced cortical thickness was restricted to the posterior cortex, whereas there was little effect of the infant lesions on the anterior cortex (Kolb & Cioe, 2003). Lesions at day P10 produced hypertrophy in the dendritic arborization of pyramidal neurons in the parietal cortex that could account for the better behavioral performance observed in these rats. Once again, the findings of this study are consistent with those from other regions examined, that

cortical lesions between days 1 and 5 result in more severe behavioral and anatomical effects than similar lesions acquired at 10 days of age.

One cortical region that appears to not follow the trend seen in other areas is the occipital cortex. Extensive research has been done examining the effects of early damage to the visual cortex using cats (e.g. Cornwell, Herbein, Corso, Eskew, Warren, & Payne, 1989; Cornwell & Payne, 1989) showing sparing of function in animals that received lesions as infants as opposed to adults. In contrast, not much is known about the effects of selective lesions to the occipital cortex of the rat. Kolb, Ladowski, Gibb, & Gorny (1996b) conducted a study to investigate the effects of lesions to the occipital cortex given at postnatal days 4 or 10 and in adulthood. Ninety days after the surgery, animals were trained on a horizontal-vertical stripes discrimination task and a visual-spatial task (Morris water task). Behavioral results revealed that rats with occipital lesions at either 4 or 10 days of age showed no evidence of sparing of visually-guided function (Kolb et al., 1996b). Although their swimming was normal, all lesioned rats were impaired at learning the location of the platform in the Morris water task. The lesion groups were able to learn the general location of the platform, but when the platform was moved, the animals would take much longer to find it than normal controls. Similarly, none of the lesioned groups learned the visual discrimination task after 10 days of training, compared to the normal controls that were performing near 90% for the last half of the testing. Anatomical results followed the pattern observed in other cortical areas after damage; occipital lesions at day 4 led to a significantly lighter brain in adulthood compared to normal controls and lesions at day 10. Analysis of dendritic arborization of layer III pyramidal neurons of the somatosensory cortex revealed a decrease in dendritic

branching in the day 4 rats relative to controls. Conversely, the day 10 rats showed an increase in dendritic branches relative to controls. There were very little changes in dendritic branching in animals lesioned as adults. It was also found that rats given occipital lesions at 10 days exhibited increases in dendritic arborization, but not the subsequent improvement in behavioral performance that is seen in other brain regions damaged at this age. The authors speculate that the reason the occipital cortex seems to respond differently to early injury compared to other cortical areas could be because the resulting growth may have facilitated other somatosensory functions that did not include visually-guided behavior (Kolb et al., 1996b). Evidence to support this theory lies in the observation that animals lesioned at 10 days of age had whiskers that were up to 2cm longer than control animals indicating that although cortical areas may show similar changes in response to damage, these changes may influence adjacent cortical areas differently, leading to changes in behaviours that are not necessarily controlled by the damaged region.

The behavioural response in rats to visual cortex damage is different from the response observed in cats. There was an absence of sparing of visually-guided behavior in neonatal rats with occipital damage compared to some sparing of function in cats with a similar injury (Cornwell & Payne, 1989). This disparity brings to the forefront the issue of species differences when conducting research using animals. The organization of the visual system of the cat and the rat are quite different. For example, projections to the lateral geniculate nucleus and extrastriate visually-related cortical regions are more extensive in the cat than the rat (Kolb et al., 1996b). This latter difference may play an important role in recovery from damage as it seems that the lateral visual areas undergo

extensive reorganization after early lesions. This reorganization has been thought to serve in guiding visual behaviors that would be lost after similar injury in adulthood (Shupert, Cornwell, & Payne, 1993). In addition, the cat and the rat are born at differing levels of maturity. The rat is born more immature than the cat, so the visual cortices of the rat and cat are not developmentally equivalent in neonatal animals (Kolb et al., 1996b). The results of these studies indicate that the reorganizational response to damage observed in one cortical region cannot be generalized to all cortical regions and that the brain's response to injury also varies across species.

The best recovery from early cortical injury in rats appears to be roughly between postnatal days 6-10, with day 10 being the optimum (Kolb & Gibb, 2007), but not all regions of the brain follow this pattern. At least one exception is the occipital cortex in which damage at day 10 was just as detrimental to performance as damage at day 4, indicating that this region may be particularly vulnerable during development. The results of the series of studies conducted by Kolb and his colleagues led to a refinement of Kennard's findings by elucidating precisely when during infancy is the least bad time to sustain brain damage and experience functional recovery. It is also important to determine if there is a critical period for recovery in human infants who have suffered an injury to their brain. Gaining some insight into the possibility of whether such a critical period exists, and when during development it takes place, would be beneficial not only in terms of increasing the understanding of how the immature brain deals with insult, but also in terms of developing therapeutic interventions.

One issue not taken into consideration in Kennard's work is the notion that a poor functional outcome may not only occur after the neonatal period, but before it as well.

Based on Kolb's research, it is apparent that injury early in the neonatal period (within the first five days) produces the worst anatomical and behavioral outcomes (Kolb, 1995). It would then stand to reason if animals experienced damage before that period (i.e. prenatally), the outcome may be just as poor because the brain is even more immature, increasing its vulnerability to early injury and subsequently to poor behavioural outcome.

Much of the early work examining the influence that age has on the anatomical and behavioural response to injury used animals that were damaged within the first two weeks of life. This timeframe is popular, as it is roughly similar to the last trimester of human gestation (Avishai-Eliner, Brunson, Sandman, & Baram, 2002) a time during which the fetus is particularly vulnerable to injury. Studies using monkeys (Kennard, 1936, 1940), cats (Villablanca et al., 1993) and rodents (Kolb et al., 1996a, 2000) all provide evidence that cortical injury during the period of migration, but after neurogenesis is complete, allows for very little or no recovery of function, whereas cortical injury during the period of maximum dendrite and synapse formation allows for a much more extensive recovery of function. One experimental research question that arises from these studies relates to the behavioural and neuroanatomical response to brain injury incurred during the *prenatal* period when the brain is even less mature and neurogenesis is still underway. Some very early investigations into this question were conducted by Hicks and colleagues (Hicks, Brown, & D'Amato, 1957; Hicks & D'Amato, 1961) and involved exposing pregnant rats to radiation at various time points during gestation (11^{th}, 12^{th}, and 13^{th} days) and then examining the anatomical and behavioural effects. It was found that pups that were exposed to radiation on the eleventh day of gestation, a point in development during the early period of cerebral mitosis

exhibited remarkably good anatomical compensation with very little abnormalities and good functional recovery. Hicks et al., (1957) were able to show that the embryonic brain was capable of replacing lost neurons even after fairly extensive damage. Regeneration of neurons may be beneficial for restoring function.

Kolb, Cioe, and Muirhead, (1998) created focal damage by aspiration to the frontal cortex of rats on gestational day 18, because a thin cortical mantle has begun to form (Bayer & Altman, 1990; Berry, 1974) and since mitosis is still underway, it is possible that the brain may be capable of replacing lost neurons. In addition to examining the anatomical consequences of injury at gestational day 18, animals' performance on tests of spatial navigation (Morris water maze, radial arm maze), forelimb reaching, measures of sensitivity to somatosensory function (tactile sensitivity and hindlimb control) and measures of general activity were assessed. The brains of animals with prenatal frontal damage were found to be 10% lighter than control animals and had abnormal patches of white and/or grey matter. Other abnormalities observed in the brains of experimental animals included the growth of neuronal bridges between cerebral hemispheres and hydrocephalus (fluid on the brain) and hydrocephaly. These findings stand in contrast to studies in which frontal tissue was removed during the postnatal period, with ages ranging from postnatal day 1 to 30, and in adult animals (e.g. Kolb & Nonneman, 1978; Kolb, Petrie & Cioe, 1996a; Kolb & Whishaw, 1981). Similar to the prenatal injury, injury at the postnatal times examined produced a reduction in brain weight and a reduction in cortical thickness. Animals that received similar damage postnatally did not exhibit the same abnormalities as those found after prenatal injury. Behavioural outcome showed that focal cortical lesions on gestational day 18 had very

little effect on any measure tested, leading to the conclusion that if the cortex is injured during active mitosis of neurons, it is morphologically abnormal, but is still able to functionally reorganize itself to such as an extent that behavior remains normal (Kolb et al., 1998). The brain is capable, after damage at a particular point in gestation, to compensate for any anatomical abnormalities to such an extent that behavior remains relatively undisturbed.

The work done by Kolb and colleagues is consistent with that done by Hicks and colleagues in terms of correlating anatomical changes with behavioural changes, despite the fact that different models were used. Both models are similar in that the injury is acute. In the studies by Hicks and colleagues, animals were only exposed to radiation once and in Kolb's studies animals were lesioned once. Although these studies provided valuable information on brain response, generally the brain damage suffered by the fetus is the result of a chronic condition, which could change the way and the extent to which the brain is able to respond. One way to experimentally examine how the brain responds to a chronic injury is to raise animals in an environment in which the oxygen level has been reduced to between 9 or 10% (approximately half the normal 21%) resulting in hypoxia.

Hypoxia Exposure in Rats: A Model for Early Developmental Injury

The prevalence of infants being born prematurely is increasing and with more of these infants surviving the rates of cerebral palsy and behavioural problems are also on the rise. It has also been reported that premature infants exhibit structural and metabolic delays in postnatal brain development (Huppi, Maier, Zientara et al., 1998; Huppi, Warfield, Kikinis et al., 1998; Huppi & Lazeyras, 2001; Huppi, Murphy, Jaier et al.,

2001). These finding suggest it is essential to develop strategies for identifying and preventing the causes of disability in premature infants.

Oxygen deprivation is one of the main causes of neurodevelopmental disorders in premature infants (Pinto-Martin, Whitaker, Feldman, Rossem, & Paneth, 1999; Volpe, 1998; Volpe, 2001c; Paneth, 1999). Although intraventricular hemorrhage (IVH) and periventricular leukomalacia (PVL) and ventriculomegaly (enlarged lateral ventricles) are the most frequently recognized and best studied conditions in premature infants, hypoxia is particularly predominant among very low birth weight premature infants (Hack, Wright, Shankaren, et al., 1995; Vohr & Msall, 1997). In addition, PVL and ventriculomegaly are believed to be the result of the chronic hypoxia of pulmonary origin that is associated with preterm birth (Volpe, 1998; Volpe, 2001b; Paneth, 1999; Perlman, 1998; Hack & Fanaroff, 1999; Stewart, Rifkin, Amess, Kirkbride, Townsend, Miller, et al., 1999; Ment, Vohr, Allan, Westerveld, Katz, Schneider, et al., 1999; Peterson, Vohr, Cannistraci et al., 2000). Hypoxia may play a very important role in the anatomical and behavioural consequences of premature birth and a closer examination is required.

The newborn rat serves as a good model for the study of the preterm human infant because the two are similar in terms of brain development. Neurons are exceptionally vulnerable to hypoxic conditions occurring during the third trimester of gestation in the human fetus (Huttenlocher, de Courten, Garey, et al., 1982; Kostovic, & Rakic, 1990) occur during the first 20 postnatal days in the developing rat cerebral cortex. Such events include the elaboration of dendritic arbors and the creation and maintenance of synaptic connections (Dobbing, 1971; Rothblat & Hayes, 1982; Olavarria & Van Sluyters, 1985; Malinak & Silverstein, 1996). To mimic the effects of the chronic hypoxia often

associated with preterm birth, researchers expose rat pups to chronic sublethal hypoxia. This can achieved by placing a pregnant female inside a hypoxic chamber, where she is exposed to about 8-10% oxygen or pups are gestated in normal room air conditions and then subjected to hypoxia postnatally. Studies examining the anatomical consequences of oxygen deprivation have found that rat pups raised in chronic hypoxia for 30 days had a 25% reduction in total cortical cell number (Schwartz, Vaccarino, Chacon, Yan, Ment, & Stewart, 2004). Glia were reduced by 34% and 41% in number after 10 and 30 days of hypoxia, respectively, whereas neuronal numbers were only significantly reduced by 14% after 30 days of hypoxia. Curristin, Cao, Stewart, Zhang, Madri, Morrow, and Ment (2002) employed microarray techniques to determine the developmental pathways most severely affected by hypoxia. Analysis revealed a global disturbance in the coordination of the expression of genes required to construct mature synapses. Specifically, hypoxia appeared to accentuate genes subserving presynaptic function, and suppressed genes involved in glial maturation, vasculogenesis, and elements of the cortical and microtubular cytoskeleton (Curristin et al., 2002). Chronic sublethal hypoxia also results in features comparable to those observed in periventricular white matter injury, including ventriculomegaly (enlarged lateral ventricles), reduced white and gray matter, and reduced myelination (Ment, Schwartz, Makuch, & Stewart, 1998; Turner, Seli, Ment, Stewart, Yan, Johansson, Fredholm, Blackburn, & Rivkees, 2003). The effect on neuron populations specifically in the motor areas remains unknown, but deficits in motor behavior after hypoxia exposure have been observed.

Motor abilities of even very young rats after hypoxia exposure are impaired. Animals required more time to come back to a quadruped position in a test of the righting

reflex at P4 (Grojean, Schoeder, Pourie, Charriaut-Marlangue, Koziel, Desor, Vert, & Daval, 2003). The ability to turn until the head is facing forward when placed on an incline plane (negative geotaxis) was delayed in P9 rats subjected to prenatal or neonatal hypoxia (Grojean et al., 2003; Zhuravin, Dubrovskaya, & Tumanova, 2004). Hyperactivity in an open field task was observed in rat pups (P15) that had been subjected to prenatal hypoxia (Cai, Xiao, Lee, Paul, & Rhodes, 1999). Rod and beam walking (P20) was impaired in both mice and rats exposed to prenatal or neonatal hypoxia. Rodents were found to have lower scores (McQuillen, Sheldon, Shatz, & Ferriero, 2003), increased slipping during beam walking (Aden, Dahlberg, Fredholm, Lai, Chen & Bjelke, 2002), and required more time to climb a vertical pole (Grojean et al., 2003). Deficits in motor performance were also revealed as increased stumbling when climbing an inclined staircase (McQuillen et al., 2003). Additionally, exposure to hypoxia shortened the amount of time mice and rats could hang from a wire (P10), as compared to controls (Grojean et al., 2003; Golan, Kashtuzki, Hallak, Sorokin, & Huleihel, 2004). Research employing hypoxia exposure as a model for examining early developmental damage has shown that is produces changes in brain structure and subsequently behavior that endures into adulthood. These features have made hypoxia a popular model for trying to understand perinatal brain injury.

Originally it was assumed that damage early in development resulted in better functional recovery. Work done by Margaret Kennard using primates injured during infancy and as adults provided evidence that damage sustained early in life did in fact produce an improved behavioural outcome. Recent work by Kolb and colleagues using rats, refined Kennard's findings. Kolb's research demonstrated that a better functional

recovery was only possible when the injury occurred during the second week of life, a time of rapid dendritic growth and synaptogenesis, but not when the injury occurred during the first week, a period of neurogenesis and migration. The results of many studies brought to light the importance of the brain's maturational state at the time of the injury and the influence that it has in terms of the potential for recovery of function.

The aim of the first experiment was to examine the anatomical and behavioural consequences of exposing rat pups to hypoxia in utero. Although the project was conducted as one experiment, it has been separated into four chapters to make it easier to read and to help clarify the themes of the thesis. The second experiment is reported as one chapter.

Chapter 3

Methods For Studies Reported in Chapters 4-6

Subjects

Five female Long Evans rats were mated with five male Long Evans rats in hanging cages. Five females were bred to ensure that there was four successful pairings to be used in the actual study. The trays beneath the cages were monitored for evidence of mating. The day the sperm plug was evident in the tray was counted as Embryonic Day 0 (E0). Males and females were kept together for 7 days to check for a second mating time, indicating that pups may be expected up to a week later. At the end of the 7 days, males and females were placed in standard cages by themselves. At 18 days gestation, approximately 4 days before birth, four of the five females were transferred from the laboratory's breeding protocol to the hypoxia protocol; both protocols had received ethics approval from the University of Manitoba's Protocol Management Review Committee and conformed to Canadian Council for Animal Care Standards. There were 33 pups born in total, 19 control animals (12 males, 7 females) and 14 experimental animals (7 males and 7 females). Data from all 33 pups was used for the behavioural portion of the experiment. For the anatomical portion of the project, to make the number of experimental and control animals more evenly matched for the time consuming anatomical analysis, the tissue from 5 control animals was not used (leaving 14 in each group).

Hypoxia Induction

On E20, two of the pregnant females (called dams) were placed in a hypoxic chamber with 10% oxygen delivery. The chamber is acrylic and wood (approximately 20 x 24 x 24 inches), which can accommodate two standard laboratory shoebox cages. Two sides are acrylic and allow substantial light into the box. The box houses an extremely sensitive oxygen sensor (Alpha Omega Instruments) and valve arrangement to allow a nitrogen feed to wash out oxygen after cage changes, water bottle changes and food delivery. These changes were timed with animal care staff to ensure adequate oxygen and washout to maintain 10% oxygen. The washout procedure involved delivering a gentle flow of both oxygen and nitrogen into the chamber. At first, the flow of nitrogen was higher in order to reduce the amount of oxygen. The oxygen sensor was carefully monitored during this time and once levels started getting close to the desired level, the nitrogen and oxygen flows were adjusted until the sensor showed a stable 10% concentration of oxygen in the chamber. Control animals were housed in shoebox cages in the same room, nearby, with normal 21% oxygen provided by room air. Pups remained in the chamber for 5 days, 3 days before and 2 days after birth (60 hours) a time at which they are developmentally similar to a premature human infant (Romijn, Hofman, & Gramsbergen, 1991). Twenty-four hours after the pups were born, and for the last 2 days of the experiment, the dams were taken out of the chamber and allowed to remain in room air for several hours.

Behavioural Measures

All animals were tested on measures related to untrained behaviours. No learning is expected to have taken place by the animals, but they would have "experience" in some novel environments.

Righting Behaviour

Righting was tested at 5 time points: Postnatal day (PND) 5 (when taken out of the chamber), PND 6, PND 7, PND 10, and PND 15. The righting response was tested by gently placing the rats onto their backs on a cotton sheet. The time required to return to prone position was recorded. Duration of the test was limited to 2 minutes and was done in triplicate on each testing day.

Explorative Behaviour

Exploration was tested at 3 time points: PND 11, PND 16, and PND 21 (to not overlap with other tests). Animals were placed in the centre of an open wooden enclosure and allowed to explore freely for 20 minutes when being videotaped. Videotapes were analyzed by placing an acetate sheet over the television screen used for viewing the behaviour. The acetate sheet contained a grid, dividing the enclosure into 16 squares. The following behavioural measures were taken: (i) latency to start exploring: timing with a stopwatch commenced once the animal had been placed in the centre of the enclosure and ended as soon as the animal left the centre; (ii) Amount of time spent in a particular quadrant. As soon as the rat's nose crossed a line separating one quadrant from another, timing began; (iii) number and duration of rearings on the hindlegs and stationary episodes were recorded. At PND 11, the animals' eyes are not yet open and they are not as mobile. Rats at this age were put into a smaller box than the one that was used for

subsequent testing. The following behaviors were videotaped and scored for PND 11: 0 = immobility, 1 = lifting or turning the head, 2 = moving the head and one forelimb, 3 = moving both forelimbs, 4 = moving three or four legs (complete locomotion).

Strength Test

Forelimb suspension was tested at 5 time points: PND 15, PND 20, PND 30, PND 40, and PND 50. To assess forelimb strength, rats were allowed to grasp a wooden dowel (with a soft pillow placed underneath) with their forepaws. The apparatus was set up to accommodate the animal as it grows. There were three dowels of differing thicknesses and heights. Younger animals (e.g. PND 15 and 20) were placed on the lowest and thinnest dowel; animals that were a little older (e.g. PND 30) were placed on the next thickest and highest dowel, and the oldest animals (e.g. PND 40 and 50) were placed on the highest and thickest dowel. Timing with a stopwatch commenced as soon as the animal had a hold on the dowel and was released by the experimenter. The time of falling was recorded; the maximum time allotted was 2 minutes and the test was done in triplicate on each testing day.

Cylinder Exploration

The cylinder measures were taken twice: PND 25 and PND 35. To assess forepaw preference, rats were placed in a transparent cylinder for 5 minutes. In this situation, the natural response is to search for an escape route. The paw (right, left or both) it initially used to support its body against the cylinder was recorded.

Ladder Runway

The ladder runway (as per Derksen et al., 2007) was used on PND 60-65. Animals were pretrained for three days on a flat runway beginning at PND 57. The

purpose of pretraining was for the rats to learn that treat pellets were located in a goal box at the end of the runway. After the pretraining sessions animals traversed a runway that was basically a ladder that had been laid flat. Each animal received 10 trials per day for 5 days. The time required to traverse the task was measured with a stopwatch and recorded. Timing commenced when the animal's front paws made contact with the runway and ended once the hindlimbs left the runway. The 10 trials were averaged on each testing day as an indication of the animal's performance for that day.

Quantitative Morphology

At 65 days of age, animals were given a lethal dose of sodium pentobarbital (100mg/kg). The animals were then perfused through the heart with saline. The age of 65 days was chosen as the time point for sacrifice because at this age the rat is developmentally, roughly equivalent to a young adult human. They are adults, but still young enough that the effects of the aging process would not have begun and, thus, would not influence our anatomical findings. Once the perfusion was complete, brains were extracted and placed in Golgi – Cox solution to allow for the visualization of neurons. After 21 days the tissue was transferred to a sucrose solution for 7 days. The tissue was sectioned into thick slices (200μm) with a vibratome (Vibratome 1000 plus sectioning system) and placed on slides. After forty-eight hours, sections were processed using ammonium hydroxide, Kodak fix and alcohol baths (as per Gibb & Kolb 1998). Layer II and layer V pyramidal neurons from the motor cortex were drawn from each animal (Fig 1). Motor cortex was defined as being M1 or the cortical tissue +/- 3mm from bregma and 1.0-2.0mm lateral from the midline (as per Paxinos and Watson, 2005). Neurons that met the following criteria were chosen for data collection: i) the neuron was

located in the primary motor cortex, the brain region of interest; ii) the neuron was stained sufficiently to allow for accurate visualization of processes; iii) the neuron was not obscured by other material, such as glia, vasculature, and other neurons; and iv) the neuron was largely intact, with few truncated or cut processes. Neurons from layers II and V were chosen because research has shown that they exhibit the most change in response to damage (e.g. Kolb, Cioe, & Whishaw, 2000). These neurons were traced using a BX51 microscope (Olympus) equipped with NeuroLucida (Microbrightfield, Inc), a computer software program that enables the reproduction of the 3-dimensional neuron. NeuroExplorer (Microbrightfield, Inc) software was used to conduct a modified Sholl analysis to estimate dendritic length. Sholl analysis involves using a series of equidistant rings centered on the cell body of a drawing (Sholl, 1967). The space between each ring represents 10μm. The computer program counts intersections with dendrites at each ring and measures a total dendritic length (in μm) by calculating the length between intersections. NeuroLucida also allows the user to assess the volume of each dendritic process. The scroll wheel on the mouse enables the user to control the size of a circle, which can be made smaller or larger depending on the thickness of the dendrite. In this way the diameter of each dendrite can be monitored as it is being traced. NeuroExplorer does the calculations of a variety of measures once the drawing is complete. Branch order analysis was also conducted with NeuroExplorer. Numbers are assigned to branches to describe the hierarchy of the branching scheme. This numbering scheme is termed the branch order for the tree. Branch order can be used as a measure of the complexity of the neuron. Neurons that have an increased number of higher order

branches are considered to be more complex than neurons that have fewer or no higher order branches.

Statistical Analysis

A power analysis was conducted post hoc for previous studies to determine how many animals would be needed for each group to ensure significance. A similar study by Kolb, Cioe and Whishaw (2000) found a significant group x ring intersection effect with an F-value of 15.6. We used this F value along with the parameters $\alpha = 0.5$ and a confidence level of 90% to calculate power. The analysis revealed that an effect would be seen with 7 or greater animals per group if it were of the magnitude seen previously. This power analysis has been used to justify animal numbers for a series of similar studies. A new power analysis was not redone here.

Changes in dendritic length were assessed with 4-way ANOVAs using condition (2) x gender (2) x cell (10) x ring (15 rings for basilars, 24 rings for apicals) (as per Ivanco et al., 2000). Previous data indicates that cell will likely not be a significant variable, as it indicates variability in cell type that is not evident in experienced cell drawers or often reflects a rostral to caudal difference. We maintain this measure as a check, but also expect to not evaluate this variable as a main effect or a variable of interest in interactions. Dendritic complexity was also analyzed with 4-way ANOVAs using condition (2) x gender (2) x cell (10) x order (4 orders for basilars, 6 orders for apicals).

Changes in volume were assessed with 3-way ANOVAs using condition (2) x gender (2) x cell (10). In cases where significant ANOVAs were found for a particular measure, the percent change between control and experimental animals was calculated.

All behavioural data were analyzed using repeated measures 2-way ANOVAs (condition x day), with DAY as the repeated measure. Each behaviour was used as a dependent measure in the analyses. Sex was not used as a factor for the behavioural data because spontaneous, naturally occurring abilities were examined and it was not expected that sex differences would influence their performance. Fishers's post hocs were used to further investigate main effects and interactions found in the ANOVAs in this study. We assessed significance at the alpha level of 0.05. Spearman correlations were conducted to evaluate relationships between each of the behavioural and morphological measures. Given the wide variety of behavior and potential for anatomical change, we opted for 2-tailed tests. The common significance level of alpha=0.05 was used to evaluate significance of all effects.

Chapter 4

Experiment 1: Effects of Hypoxia Exposure on Spontaneous Motor Behaviour

Introduction

Preterm birth presents a major challenge for perinatal care around the world and is one of the leading causes of infant morbidity and mortality. In both Canada and the United States the rate of premature births has been steadily increasing. Over the past 25 years, the rate of preterm birth in the US has increased 36% (March of Dimes White Paper on Preterm Birth, 2009), and the Canadian preterm birth rate has also increased, from 7.0 per 100 live births in 1995 to 8.2 per 100 live births in 2004 (Canadian Perinatal Health Report, 2008). Although there have been major improvements in the treatment of premature infants in Neonatal Intensive Care Units, these infants still remain vulnerable to many health issues, including, respiratory, gastrointestinal, immune system, central nervous system, hearing, and vision problems. Longer term health problems include cerebral palsy, mental retardation, visual and hearing deficits, behaviour and social-emotional problems, learning difficulties, and poor health and growth. Babies born before 32 weeks gestation are at the greatest risk for death and poor health, however, infants born between 32 and 36 weeks, which make up the greatest number of preterm births, are still at a higher risk for health and development issues compared to full term infants. As the rate of premature infants born and surviving increases, so too does the need to understand the impact that prematurity has on the physical and neurological development of the child.

Premature infants have been found to experience more motor difficulties compared with infants born at term. Children born prematurely exhibit deficits in gross

and fine motor skills (e.g. Goyen & Lui, 2002; Allin, Rooney, Griffiths, Cuddy, Wyatt, Rifkin & Murray, 2006; Davis, Ford, Anderson, & Doyle, 2007) compared to children born at term. Although many premature infants do develop cerebral palsy, research has shown that in those infants that do not develop the condition many still demonstrate neuromotor abnormalities upon examination during childhood. One of the potential mechanisms underlying behaviour impairments is hypoxia-induced damage.

Hypoxia occurs when there is a reduction in the concentration of oxygen being supplied to the body's vital organs, such as the brain. Premature infants are at an increased risk of hypoxia because they are typically born before lung maturation is completed. Therefore, the earlier in gestation the infant is born, the higher the probability of severe and chronic respiratory problems (Meisels, Plunkett, Pasick, Stiefel, & Roloff, 1987). Chronic respiratory problems may compromise the amount of oxygen the infant is able to take in, resulting in less than optimal oxygenation of the brain, which eventually leads to neuronal damage. In addition, infants may also be vulnerable to hypoxia in utero. Intrauterine asphyxia (impairment in the exchange of respiratory gases) has been shown to be a major influence on the origins of hypoxic insult in the immature brain (Volpe, 2001b). Studies examining children born prematurely who were at an increased risk of perinatal hypoxia found that they exhibited deficits in cognitive performance (Stevens, Raz, & Sander, 1999; Hopkins-Golightly, Sander, & Raz, 2003; Epsy, Senn, Charak, Tyler, & Wiebe, 2007) and motor abilities (Low, Galbraith, Muir, Broekhoven, Wilkinson, & Karchmar, 1985; Raz, Glogowski-Kawamoto, Yu, Kronenberg, Hopkins, Lauterbach et al., 1998).

Research examining spontaneously occurring behaviours has shown that animals exposed to hypoxia exhibited deficits on tests of the righting reflex (Grojean, Schoeder, Pourie, Charriaut-Marlangue, Koziel, Desor, Vert, & Daval, 2003), hyperactivity in an open field task (Cai, Xiao, Lee, Paul, & Rhodes, 1999) and impaired rod and beam walking. Hypoxia exposure was also found to shorten the amount of time mice and rats could hang from a wire as compared to controls (Grojean et al., 2003; Golan, Kashtuzki, Hallak, Sorokin & Huleihel, 2004). The present study examined the impact that early hypoxia exposure and premature birth had on spontaneous motor behaviours. The reason for choosing to examine spontaneous behaviour is that tasks that require learning a motor skill (e.g. reaching tasks) produce changes in the brain. Therefore it becomes difficult to determine if any reorganization observed was in response to the hypoxic insult or to the acquisition of the motor skill. It was hypothesized that animals exposed to hypoxia prenatally would exhibit mild deficits in spontaneous motor abilities.

Results

A total of four litters of animals were born, two control litters and two hypoxic litters. All animals born in the control litters were used for analysis. For the hypoxia exposed (HE) animals, the experimenter culled one pup at the age of nine days, because she had stopped eating, lost a significant amount of weight and become lethargic. One of the experimental dams had killed a large portion of her litter while in the chamber, leaving 5 pups alive. The 14 animals mentioned in the Methods section represent the surviving pups from both experimental litters. All were used for analysis.

Righting

Results revealed no main effect of condition (Fig 14), $F(1, 31) = 2.67$, $p = 0.112$ (Fig 2). There was a main effect of days, $F(4, 124) = 23.67$, $p < 0.001$, indicating that there was a decrease in time to return to the quadruped position across days regardless of experimental condition. There was no day x condition interaction, $F(4, 124) = 1.13$, $p = 0.344$.

Explorative Behaviour

Analysis of start latency, exploration, stationary time and rearings at 16 days of age revealed a main effect of condition, $F(1, 31) = 5.4$, $p = 0.027$ and a main effect of behaviour, $F(3, 93) = 259.1$, $p<0.001$. There was also a significant behaviour x condition interaction, $F(3, 93) = 18.6$, $p<0.001$. There was a significant effect of exploration, $F(3, 93) = 575$, $p<0.001$, as well as a significant exploration x condition interaction, $F(3, 93) = 13$, $p<0.001$, a significant days x exploration interaction, $F(3, 93) = 3$, $p = 0.019$ and a significant days x exploration x condition interaction, $F(3, 93) = 9$, $p<0.001$ (Fig 3A). Analysis at 21 days of age revealed no main effect of condition, $F(1, 31) = 0.0$, $p = 0.882$. There was a significant effect of behaviour, $F(1, 31) = 411.6$, $p<0.001$ but no significant condition x behaviour interaction, $F(3, 93) = 1.2$, $p = 0.320$. Post hocs revealed that on PND 16 HE animals engaged in more exploratory behavior than control animals did and that control animals engaged in more stationary behavior. This difference was only present on PND 16, by the second testing day on PND 21 there was no difference between control and HE animals on any of the behavioural measures (Fig 3B). For the testing on PND 11, all animals were judged as exhibiting complete locomotion (a

score of 4 out of 4), resulting in a lack of variability, therefore statistical analysis was not conducted on this data.

Examination of the time spent in each quadrant at 16 days of age revealed that there was no main effect of condition, $F(1, 31) = 1.07$, $p = 0.309$. There was a significant effect of quadrant, $F(15, 465) = 19.53$, $p<0.001$ but no significant quadrant x condition interaction (Fig 3C), $F(15, 465) = 0.85$, $p = 0.623$. Post hocs showed that animals spent more time in quadrants 1 and 13. Analysis at 21 days of age revealed no main effect of condition, $F(1, 31) = 0.56$, $p = 0.458$. There was a significant effect of quadrant, $F(15, 456) = 30.28$, $p<0.001$ and a significant condition x quadrant interaction (Fig 3D), $F(15, 465) = 3.80$, $p<0.001$. Post hocs showed that control animals spent more time in quadrant 13 and HE animals in quadrant 4, both of which were corners of the box.

Strength

Analysis showed no main effect of condition, $F(1, 31) = 0.315$, $p = 0.579$ (Fig 4). There was an effect of days, $F(4, 124) = 4.795$, $p = 0.001$, showing a decrease in the amount of time animals were able to stay suspended. There was no days x condition interaction, $F(4, 124) = 0.664$, $p = 0.618$.

Cylinder Exploration

No main effect of condition, $F(1, 31) = 0.23$, $p = 0.631$ was found. There was also no main effect of days, $F(1, 31) = 0.21$, $p = 0.652$ and no days x condition interaction, $F(1, 31) = 2.01$, $p = 0.166$. There was a significant effect of paw, $F(2, 62) = 71.09$, $p<0.001$ (Fig 5A, B) There was no paw x condition interaction, $F(2, 62) = 0.73$, $p = 0.488$.

Ladder Runway

Analysis revealed no main effect of condition, $F(1, 31) = 0.00$, $p = 0.951$. There was a main effect of days $F(4, 124) = 38.05$, $p<0.001$ and a days x condition interaction, $F(4, 124) = 2.98$, $p = 0.022$ (Fig 6). Post hoc analysis showed that HE animals took longer to traverse the runway on training day 2 than control animals. There were no other differences across days between the two groups.

Discussion

No significant deficits in the behaviors studied were evident in HE animals, but some small differences from controls were observed. Analysis of explorative behavior showed that HE animals tested on PND 16 engaged in more exploratory behaviour compared to control animals, who engaged in more stationary behavior. When animals were tested again at 21 days of age, there was no long a difference in exploratory behavior between HE and control animals. Other studies have reported hyperactivity in open field tasks in animals exposed to hypoxia that diminished over time. Cai, Xiao, Lee, Paul and Rhodes (1999) found that locomotor activity increased with age until PND 15 in both animals exposed to hypoxia on gestational day 17 and sham controls. The hypoxia group showed a significant increase in locomotor activity between PND 13 and 15 compared to control animals. No difference between the two groups was observed after PND 15. It is possible that as the HE animals matured, they simply outgrew the hyperactive behavior. In terms of the time spent in each quadrant of the open field, a quadrant effect was found, revealing that animals spent more time in quadrants 1 and 13. Both of these quadrants were corners of the enclosure. It could be that despite attempts to

illuminate the entire open field that those corners remained slightly darker, potentially attracting the attention of the animals as a place to hide.

Analysis of the animals' ability to do the strength test revealed an effect of days. As animals got older they also got heavier, regardless of whether they were in the control or experimental condition, and this influenced their ability to maintain a hold on the dowel for any length of time. Analysis of behavior in the cylinder task showed a significant effect of paw that occurred because animals were more likely to reach out and touch the cylinder with either the right or left paw, but not both. HE animals took longer to cross the runway task on the second day of training compared to control animals. One postulate is that it took a little longer for HE animals to remember how to traverse the runway, whereas the control animals had no such difficulty. We did not look at the hippocampus in this study, but it is a brain region that is very vulnerable to hypoxia. The hippocampus is available for analysis by others.

Studies reported in the literature examining the influence of early hypoxia exposure on behavior (Grojean, Schroeder, Pourie, Charriaut-Marlangue, Koziel, Desor, Vert, & Daval, 2003; Cai, Lee, Paul, & Rhodes, 1999; Golan, Kashtuzki, Hallak, Sorokin, & Huleihel, 2004) typically exposed animals to hypoxia much earlier in gestation (often around gestational day 13) than in the present study. The fact that the animals in the present experiment were more advanced in their development may have enabled them to compensate more easily for any deficits in motor abilities. Support for this theory may be found in a study by Kolb et al., (1998) in which rats were given frontal lesions on gestational day 18 and subsequently examined anatomical and behavioural outcomes. The authors found that despite cortical abnormalities, lesioned animals were

indistinguishable from control animals in their performance on tests of spatial navigation, motor tasks, and locomotor activity. It is also possible that any behavioral problems may have been subtle and the tasks used were not sensitive enough to detect them.

In the present study, the tasks involved natural abilities that the animal should have at the various time points examined because we were interested in determining whether normal developmental milestones were delayed or if performance was abnormal. Given that HE rats showed no difference in any of the behavior measures in this study may be due to the fact that the damage was mild or that naturally occurring behaviors are hardwired in the brain and are not easily perturbed, though some studies examining spontaneous behaviour after damage have found differences (Gramatte & Schmidt, 1986; Nyakas, Markel, Schuurman & Luiten, 1991). Tasks requiring some motor skill to execute, such as reaching or traversing a series of upended dowels may be necessary to determine if there were any behavioral deficits. We were not interested in testing whether the animals could learn more complex motor skills, although, that type of investigation would be beneficial in the future in this model of developmental injury.

Although the majority of spontaneous behaviors examined did not show a significant difference between HE and control animals, several tasks did exhibit some small differences between the two groups. HE animals were found to take longer to complete the runway task on the second day of training compared to control animals, potentially indicating that animals exposed to hypoxia had some difficulty remembering what they had learned about the task on the first day of training. HE animals were also found to exhibit hyperactivity in the open field task when tested at 16 days of age, but were not significantly different from controls when tested at 21 days of age. This finding

indicates that hypoxia exposure may predispose animals to hyperactivity early in life, but with increased maturity, they become indistinguishable from controls. It is possible that since the animals in the present study were exposed to hypoxia later in gestation, the impact on their subsequent behavior was less than in other studies in which hypoxia exposure was much earlier in gestation. Therefore, the data indicate the developmental time point at which hypoxia was sustained may influence the impact on subsequent behavior.

Chapter 5

Experiment 2: Effects of Hypoxia Exposure on Layer II Pyramidal Neurons in Primary Motor Cortex

Introduction

Children born prematurely are at an increased risk of exhibiting motor deficits and abnormalities in brain regions involved in motor behaviour, such as layer II neurons of the primary motor cortex, compared to children born at full term. Layer II pyramidal neurons have extensive connections to other cortical areas and are the major input neurons in the cortex. Damage to these neurons could affect communication between the primary motor cortex and the other cortical regions (such as sensory cortex) that it shares information with. Children born prematurely have been found to have problems with tasks that involve the integration of both sensory and motor systems (Allin, Rooney, Griffiths, Cuddy, Wyatt, Rifkin, & Murray), suggesting that layer II neurons may be involved. Previous research (e.g. Kolb, Cioe, & Whishaw, 2000a) has shown that layer II neurons are capable of extensive reorganization after injury. Although these neurons are known to reorganize in other models of injury, there is little evidence of neuronal changes in layer II following chronic hypoxia.

The mounting evidence showing that premature birth often results in cognitive and motor problems led to the examination of the brains of premature infants to try to determine the underlying causes of these deficits. Most research has focused on damage to the cerebral white matter that is frequently observed in premature infants. This condition, known as periventricular leukomalacia (PVL), has been found to be the major neuropathological substrate associated with the motor and cognitive deficits observed

during childhood of premature infants (Volpe, 2001a; Volpe, 1991). Not much attention was paid to the impact immaturity may have had on cerebral grey matter. It has been a long held assumption that cerebral white matter was particularly vulnerable to injury in the preterm brain, with relative sparing of the grey matter, and that grey matter injury occurs predominately in older infants, children, and adults (Kinney and Armstrong, 2002), despite evidence demonstrating that neuronal/axonal injury is common in the perinatal brain (Bell, Becher, Wyatt, Keeling, & McIntosh, 2005; Kinney, Panigrahy, Newburger, Jonas, & Sleeper, 2005). The idea that grey matter is largely spared from injury in the preterm brain is now being challenged by recent studies using advanced MRI techniques to examine the brains of surviving preterm children. Studies employing advanced MRI techniques to examine children and adolescents who were born prematurely led to the important observation of decreased cerebral cortical cerebral grey matter volumes (Bradley, Peterson, Vohr, Staib, Cannistraci, Dolberg, et al., 2000; Nosarti, Al-Asady, Frangou, Stewart, Rifkin, & Murray, 2002, Kesler, Ment, Vohr, Pajot, Schneider, Katz, et al., 2004). Premature infants studied at term equivalent using MRI technology revealed a 28% reduction in cortical grey matter compared with healthy term infants (Inder, Huppi, Warfield, Kikinis, Zientara, Barnes, et al., 1999). The neuronal deficits observed in cerebral cortex and deep nuclear structures were correlated with moderate/severe neurodevelopmental disabilities at 1 year of age (Inder, Warfield, Wang, Huppi, & Volpe, 2005). Taken together, these findings suggest that cerebral grey matter is more vulnerable to damage in premature infants than was originally thought, and that abnormalities in cerebral grey matter may play a role in later neurodevelopmental deficits.

A potential mechanism for the grey matter damage observed in premature children is hypoxia exposure. Hypoxia occurs when the amount of oxygen being supplied to the body's vital organs is compromised and can lead cellular damage and death. Premature infants are at an increased risk for hypoxia since they are typically born before lung maturation is completed, therefore, the more prematurely the infant is born, the greater the risk of severe and chronic respiratory problems (Meisels, Plunkett, Pasick, Stiefel, & Roloff, 1987). Studies examining children born prematurely who were at an increased risk of perinatal hypoxia found that they exhibited deficits in motor abilities at one year of age compared to children who were born at term (Low et al., 1985). There is also evidence that certain motor abilities may be more vulnerable to hypoxia than others. Raz, Glowgowski-Kawamoto, Yu, Kronenberg, Hopkins, Lauterbach et al., (1998) showed that in premature infants with an increased risk of hypoxia demonstrated deficits in motor tasks that required use of the upper extremities to guide the motion of an object (e.g. throwing a ball) in contrast to tasks requiring the use of the lower extremities to maintain balance or gait, suggesting that the brain regions involved in controlling the upper extremities are more vulnerable to the effects of hypoxia than those that control the lower extremities.

Given the motor problems and loss of grey matter volume associated with prematurity, the aim of the present study was to examine the morphology of pyramidal neurons in layer II primary motor cortex after hypoxia exposure early in development to determine how these neurons would be affected. Layer II neurons are still actively migrating during the late prenatal period, potentially making them vulnerable to the effects of hypoxia exposure during this point in development. It would therefore, be of

interest to determine the types of plastic change layer II neurons undergo in response to an early hypoxic insult. It was hypothesized that neurons in layer II would exhibit reorganization in response to hypoxia in contrast to control animals raised in a normoxic environment.

Results

Brain Weights

Brains of all animals were weighed after extraction. No main effect of condition (Fig 7), $F (1, 31) = 1.650$, $p = 0.208$ was found between control and HE animals.

Dendritic Length

Analysis of basilar length revealed no main effect of condition, $F (1, 24) = 3.4$, $p = 0.077$, but there was a trend for HE animals to have shorter dendrites than controls. There was no main effect of gender, $F (1, 24) = 0.2$, $p = 0.692$. There was also no condition x gender interaction, $F (1, 24) = 0.2$, $p = 0.622$. There was a ring x condition interaction, $F (14, 336) = 4.8$, $p < 0.01$ (Fig 8A), indicating that there were differences in the number of intersections at certain rings between HE and control animals. Fisher's post hoc test revealed that HE and control animals differed significantly at ring numbers 6 – 15 ($p<0.05$).

Analysis of apical dendrites revealed a main effect of condition, $F (1, 24) = 16.2$, $p<0.01$, indicating HE animals have shorter apical dendrites than controls. HE animals had an 11% decrease in apical length compared control animals. The main effect of gender was not significant, $F (1, 24) = 3.5$, $p = 0.076$, but does suggest a trend towards males having longer apical dendrites than females. There was a condition x gender interaction, $F (1, 24) = 8.4$, $p = 0.008$ (Fig 8B). There was a ring x condition interaction,

F (23, 552) = 2.5, $p<0.01$ (Fig 8C) indicating that there were differences in the number of intersections at certain rings between HE and control animals. Fisher's post hoc test revealed that experimental and control animals differed significantly at ring numbers 12 – 24 ($p<0.01$).

Dendritic Volume

For basilar dendrites, there was a main effect of condition, F (1, 24) = 14.80, $p < 0.01$(Fig 9A) with HE animals showing a reduction in basilar volume compared to controls. HE animals had a 17% decrease in basilar volume compared to control animals. There was also a main effect of gender, F (1, 24) = 7.22, $p<0.05$ (Fig 9B) with males having thicker basilar dendrites than females. Female rats had 12% less basilar volume compared to male rats. There was no condition x gender interaction, F (1, 24) = 0.43, $p = 0.518$.

Analysis of apical volume revealed a main effect of condition, F (1, 24) = 28.57, $p<0.001$ showing that HE animals had a significant decrease in apical volume compared to controls. HE animals had a 20% decrease in apical volume compared to control animals. There was also a main effect of gender, F (1, 24) = 15.32, $p<0.01$, with males having greater apical volume than females. Female animals had 15% less apical volume than male animals. A condition x gender interaction, F (1, 24) = 11.43, $p<0.01$ (Fig 9C) was also found showing that control males had more apical volume than any of the other groups and HE males had less apical volume than any of the other groups. The biggest change was between control and HE males, in which there was a 29% decrease in apical volume in HE males compared to control males. There was a 9% decrease in HE females

compared with control females, a 2.5% decrease in HE females compared to HE males, and a 6% decrease in HE males compared to control females.

Dendritic Complexity

Basilar complexity showed no main effect of condition, $F(1, 24) = 1.1$, $p = 0.303$ and no main effect of gender, $F(1, 24) = 0.3$, $p = 0.593$. There was also no condition x gender interaction, $F(1, 24) = 0.5$, $p = 0.484$ (Fig 10A). There was a significant order x condition interaction, $F(4, 96) = 6.1$, $p < 0.001$. Post hoc revealed no significant differences in orders between HE and control animals.

Apical complexity revealed a main effect of condition, $F(1, 24) = 9.68$, $p<0.01$, showing that HE animals had less complex apical dendrites than controls. HE animals had an 11% decrease in apical complexity compared to control animals. There was also a main effect of gender, $F(1, 24) = 4.24$, $p = 0.050$, with males having more complex apicals than females. Female animals had 7% less apical complexity compared with male animals. There was no condition x gender interaction, $F(1, 24) = 2.03$, $p = 0.167$ (Fig 10B) and no order x condition, $F(4, 96) = 1.13$, $p = 0.348$ (Fig 10C).

Discussion

Prenatal hypoxia during the period of neural migration in a rat model had a profound effect on the morphology of neurons within the primary motor cortex when animals were examined as young adults. Analysis of dendritic length revealed a trend for the basilar dendrites of HE animals to be shorter than those of control animals. There were also significant differences in the number of intersections at ring numbers 6-15 of the Sholl analysis, with HE animals having fewer intersections at these rings than control animals, indicating that the largest changes between HE and control animals occurred at

regions of the dendrite that were distal to the cell body. HE animals were also found to have shorter apical dendrites than control animals. There was also a trend for males to have longer apical dendrites than females and for control males to have longer apical dendrites than the other three groups. Control females were found to have longer apical dendrites than HE females and HE males were found to have shorter apical dendrites than the other three groups, indicating that hypoxia exposure had a more significant effect on males than females. As with the basilar dendrites, apical dendrites showed significant differences in the number of intersection at specific rings. HE and control animals differed significantly at ring numbers 12 -24, indicating that the largest changes between the groups took place at regions of the dendrite that were distal to the cell body.

The volume of both basilar and apical dendrites was found to be significantly decreased in HE animals compared to controls. Males were also found to have increased basilar and apical volume compared to females. Analysis of apical volume also revealed a condition x gender interaction showing that control males had significantly thicker apical dendrites than the other three groups and that HE males had significantly thinner apical dendrites compared with the other groups. No differences were observed in the complexity of basilar dendrites between HE and control animals. Examination of apical dendrites revealed that HE animals had less complex apicals than controls and males had more complex apicals than females.

Hypoxia exposure in the present study occurred during the period of neuronal migration, which in the rat cerebral cortex begins on about day 14 of gestation and soon accelerates so that by day 16, a definitive lamina of neurons has been formed. Studies using titrated thymidine established that laminar development in the rat cortex occurred

in an inside-out manner just as is observed in humans (Berry and Rogers, 1965). Neurons formed up to day 16 of gestation were found to populate layer VI, those formed on about day 17 formed layer V, the neurons of layer IV were formed in the ventricular zone on about day 18, and the neurons of layers II and III were formed on days 19, 20, and 21 of gestation. At birth, layers VI and V have migrated and occupy their mature positions, but layers IV, III, and II are still actively migrating and the six laminae of the neocortex does not become fully established until between 7-10 days after birth (Berry and Rogers, 1965). In the present experiment, animals were subjected to hypoxia beginning at gestational day 20 and continuing until postnatal day 2, a time during which layer II neurons are being formed and migrating out to assume their mature positions, making them vulnerable to injury. In a series of studies conducted by Kolb and colleagues (e.g. Kolb, Cioe, & Whishaw, 2000b; Kolb, Gibb, & van der Kooy, 1994) it was consistently found that damage within the first five days of life resulted in abnormal dendritic growth and behavioral deficits, which coincides with the period of migration of layer II cortical neurons. HE animals in the present study exhibited observable morphological changes in layer II neurons. A possible explanation for why most changes occurred in this layer may be due to the fact that layer II neurons were still quite immature at the time animals were exposed to hypoxia, making them more vulnerable to the effects of oxygen deprivation.

HE animals were found to have significant morphological differences in layer II pyramidal neurons of the primary motor cortex compared to control animals in our study. Basilar and apical dendrites exhibited significant reductions in length and volume. Apical dendrites also showed a reduction in complexity compared to animals gestated under normoxic conditions. These findings indicate that layer II neurons that are still migrating

and quite immature at the time hypoxia was sustained were very vulnerable to the effects of the insult. The results also are consistent with the theory that the brain is more vulnerable to the effects of damage during the period of neural migration then it is later in development, specifically during the period of dendritic differentiation and synaptogenesis when neurons are mature enough to able to reorganize in response to an insult.

Chapter 6

Experiment 3: Effects of Hypoxia Exposure on Layer V Pyramidal Neurons in Primary Motor Cortex

Introduction

To further investigate the potential underlying causes of the motor difficulties exhibited by premature children, Experiment 2 examined the morphology of layer II neurons of the primary motor cortex after exposure to hypoxia in utero. It was found that the HE animals had smaller and less complex layer II neurons compared to control animals. Thus, it was of interest to examine the effects of prenatal hypoxia exposure on layer V pyramidal neurons of the primary motor cortex for several reasons. In terms of the circuitry of the motor cortex, the axons of layer II pyramidal neurons synapse with the apical dendrites of the layer V pyramidal neurons (Asanuma & Rosen, 1972). Since the results of Experiment 4 showed that layer II neurons were altered as a consequence of hypoxia exposure, it is possible that such changes would in turn mediate changes in layer V neurons. In contrast to layer II neurons that are still migrating out to their final positions, layer V neurons have already reached their mature positions and have begun to differentiate (Berry and Rogers, 1965). The increased maturity of the layer V neurons may influence their response to hypoxia exposure compared to the less mature layer II neurons.

Layer II neurons showed profound change with hypoxia. These neurons integrate sensory and motor information and send signals to layer V, which are involved in producing movements. Therefore, it would be of interest to determine the types of plastic change layer V neurons undergo in response to an early hypoxic insult. It was

hypothesized that neurons in layer V may exhibit reorganization in response to hypoxia in contrast to control animals raised in a normoxic environment. If any reorganization is present, it may not be as extensive as that potentially observed in layer II due to the increased maturity of layer V neurons at the time of injury.

Results

Dendritic Length

Analysis of basilar length revealed no main effect of condition, $F(1, 24) = 0.3$, $p = 0.584$ and no main effect of gender, $F(1, 24) = 3.4$, $p = 0.078$. There was also no condition x gender interaction, $F(1, 24) = 1.9$, $p = 0.182$. There was no ring x condition interaction, $F(17, 408) = 0.3$, $p = 0.999$ (Fig 11A), but there was a ring x gender interaction, $F(17, 408) = 2.4$, $p = 0.001$ (Fig 11B), indicating that there were differences in the number of intersections at certain rings between male and female animals. Fisher's post hoc test revealed that males and females differed significantly at ring numbers 7 – 14 ($p<0.05$).

Analysis of apical dendrites showed no main effect of condition, $F(1, 24) = 0.2$, $p = 0.652$. Although there was a main effect of gender, $F(1, 24) = 4.3$, $p = 0.050$, there was no gender x condition interaction, $F(9,216) = 0.2$, $p = 0.633$. There was a ring x gender interaction, $F(23,552) = 4.5$, $p <0.001$ (Fig 11C). Fisher's post hocs revealed that males and females differed significantly at ring numbers 10 – 19 ($p<0.05$). There was also a ring x condition interaction, $F(23, 552) = 2.1$, $p<0.01$ (Fig 11D). Fisher's post hocs showed no significant differences between HE and control animals across rings.

Dendritic Volume

For basilar dendrites, there was no main effect of condition, $F (1, 24) = 0.217$, $p = 0.646$ and no main effect of gender, $F (1, 24) = 1.071$, $p= 0.311$. There was also no gender x condition interaction, $F (1, 24) = 0.775$, $p = 0.387$ (Fig 12A).

Similarly, analysis of apical dendrites revealed no main effect of condition, $F (1, 24) = 0.014$, $p = 0.906$ and no main effect of gender, $F (1, 24) = 1.707$, $p = 0.204$. There was also no gender x condition interaction, $F (1, 24) = 0.283$, $p = 0.600$ (Fig 12B).

Dendritic Complexity

Analysis of basilar complexity revealed no main effect of condition, $F (1, 24) = 0.0$, $p = 0.859$ and no main effect of gender, $F (1, 24) = 1.6$, $p = 0.215$. There was no gender x condition interaction, $F (1, 24) = 1.2$, $p = 0.279$, no orders x gender interaction, $F (3, 72) = 0.2$, $p = 0.869$, no orders x condition interaction, $F (3, 72) = 0.4$, $p = 0.774$ and no orders x gender x condition interaction, $F (3, 72) = 0.5$, $p = 0.709$ (Fig 13A).

For apical dendrites, there was no main effect of condition, $F (1, 24) = 0.13$, $p = 0.720$ and no main effect of gender, $F (1, 24) = 1.95$, $p = 0.175$. There was no gender x condition interaction, $F (1, 24) = 1.19$, $p = 0.287$ and no orders x condition interaction, $F (5, 120) = 1.87$, $p = 0.105$. There was an orders x gender interaction, $F (5, 120) = 13.69$, $p<0.001$. Post hoc tests revealed that males and females differed significantly at first and second order branches ($p<0.05$) and at fifth and sixth order branches ($p<0.001$). There was also an orders x gender x condition interaction, $F (5, 120) = 4.13$, $p = 0.002$ (Fig 13B). Post hoc tests revealed that control males and females differed significantly at only fifth order branches ($p<0.01$), whereas HE males and females were found to differ significantly at first, second, sixth orders ($p<0.001$) and fifth order ($p<0.01$) branches. It

was also found that HE males had significantly fewer second order branches and significantly more sixth order branches than the other three groups.

Discussion

Prenatal hypoxia during the period of neural migration of superficial cortical layers in a rat model of early developmental damage had very little impact on the morphology of neurons in layer V, a less superficial layer with neurons that had already arrived in their final location when hypoxia was induced. Most of the effects that were found in this study were due to gender, which is not particularly surprising, as differences in the brains of male and female rats have been documented in other studies (e.g. Diamond, 1989, Juraska, 1984). Most of the gender effects observed involved apical dendrites, where males had longer and more complex dendrites than females. The only gender effect for basilar dendrites was a ring x gender interaction, where males were found to have longer dendrites than females. Only apical complexity produced a significant interaction involving condition, although significance was reached due to the combination of both condition and gender. Control males and females were found to differ only in the number of fifth order branches, with males having more than females. HE males and females were found to differ in the number of first and second orders (females having more than males) and fifth and sixth order branches (males having more than females). HE males had fewer second order branches and more sixth order branches compared to the other three groups. This finding suggests a potential difference in how the male and female brains respond to injury: males respond by generating more branches distal from the cell body, whereas females cope by generating more branches closer to the cell body. In contrast, layer II neurons of HE animals exhibited significantly shorter,

thinner and less complex basilar and apical dendrites than control animals. These findings suggest that layer V neurons may be less sensitive to the effects of hypoxia exposure during late gestation than layer II neurons.

A possible explanation for why layer V neurons were better able to tolerate hypoxia is that at birth, these neurons had an increased level of maturity making them less sensitive to the effects of the insult than the less mature layer II neurons. In the present experiment, animals were subjected to hypoxia beginning at gestational day 20 and continuing until postnatal day 2, a time during which layer II neurons are being formed and migrating out to assume their mature positions but layer V neurons have already migrated out to their mature positions and have begun to differentiate basilar and apical dendrites. Dendritic growth was found to be significantly compromised in animals that were exposed to hypoxia early in development. Nearly all dendritic growth in the rat takes place postnatally. The peak of development is between postnatal days 8 and 20, with maximum growth accomplished by day 30 (Kolb, 1995). Soon after migration is complete, pyramidal neurons first develop an apical dendrite, but no basilar dendrites (Berry, 1974). Basilar dendrites are poorly developed after birth in superficial layers, and it is not until postnatal day 30 that the structure of their dendritic fields approach adult dimensions. Research has shown that the most functional recovery occurs when the injury is sustained during the period of neural differentiation and synapse formation (reviewed in Kolb, 1995). Since layer V neurons were differentiating at the time the animals were exposed to hypoxia in the present study, it is reasonable to conclude that they were at a more advanced stage of development compared to layer II neurons, which better prepared them to deal with the effects of hypoxia.

Prenatal exposure to hypoxia was found to have very little influence on the development of layer V neurons of the primary motor cortex. The majority of the differences found were between males and females, in which males were found to have larger neurons than females. Overall, it seems that in comparison to layer II neurons, layer V neurons are much less at risk for exhibiting changes in dendritic structure after hypoxia exposure during early development. In predicting the impact of layer II versus layer V neuron morphology on behaviour, it would be expected that gross behaviour would not differ, but more complex behaviour requiring sensory integration would be impacted.

Chapter 7

Anatomical and Behavioural Correlations

Introduction

The study of brain and behaviour correlations involves one major assumption, specifically, that changes in brain structure will be reflected in changes in behaviour (Kolb and Whishaw, 1998). Therefore, any changes observed in neuronal morphology are assumed to influence the functioning of the brain and subsequently, behaviour. There are several lines of research that provide evidence to support this claim. One line of research involves training animals in specific tasks and then demonstrating specific changes in dendritic morphology in neurons in the regions believed to be involved in the ability to perform the task. One of the brain regions targeted for these types of studies is the motor cortex. A series of experiments involved teaching rats to reach though a slot to retrieve a treat pellet. Since cortical control of the forelimbs is mostly crossed, researchers are able to train one limb to reach for food and to compare neurons in the forelimb region of motor cortex in the trained and untrained hemispheres. Several studies have demonstrated dendritic changes in the neurons in the motor cortex of the trained hemisphere compared to the untrained hemisphere (Greenough, Larson, & Withers, 1985; Kolb, Forgie, Gibb, Gorny, & Rowntree, 1997a; Withers & Greenough, 1989), demonstrating that the experience of learning something new influences brain structure.

The finding that dendritic change can be influenced by experience is interesting, but does not really prove there is a relationship between the two. The important experiments are those that employ an experimental manipulation that changes both the behavior and the neuronal morphology in a significant way. One of the manipulations

used to reveal the relationship between behavior and morphological changes is damage. When the cortex is damaged, there are changes in the structure of the surviving neurons that can be correlated with functional recovery. For example, Kolb and Gibb (1991b) conducted a study in which the frontal cortex of adult rats was removed. Immediately after the injury, an initial drop in dendritic arborization was observed near the site of the damage. Over the next four months, the loss of dendritic material began to resolve and an increase in dendritic arborization was observed, which was correlated with a partial restoration of function. In contrast, Kolb, Gorny, Cote, Ribeiro-da-Silva, and Cuello (1997b) gave animals large, sensorimotor cortex lesions, which lead to only neuronal degeneration and no functional recovery. The results of these studies indicate that when there is proof of dendritic growth, it is accompanied by functional recovery, whereas when there is no proof of dendritic growth, there is no functional recovery. This principle can also be seen very clearly in the developing brain. When the developing cortex of the rat is damaged during the first few days of life, a time that corresponds to just after proliferation is complete, but neural migration and differentiation is still ongoing, there is an obvious atrophy of dendritic arborization (Kolb, Petrie & Cioe, 1996; Kolb, Cioe, & Whishaw, 2000a). This reduction of dendritic material has been correlated with dismal functional recovery. In contrast, when the cortex is damaged during the second week of life, a time point that corresponds to rapid dendritic growth, there is a significant increase in dendritic arborization throughout the surviving cortex (Kolb & Gibb, 1991). This increase in dendritic material was correlated with impressive recovery. Although changes in neuronal structure after damage sustained during infancy or adulthood are often associated with performance on behavioural tests (Kolb, Gibb & van der Kooy, 2004;

Kolb and Gibb, 1991), not all studies directly compare changes in anatomy to changes in behaviour. In the present study, it was of interest to determine if any of the structural changes observed in hypoxic animals were associated with their ability on any of the behavioural tasks. Correlational analyses were conducted on the outcome observed in brain anatomy and the performance on behavioural measures of control and hypoxic animals. By directly comparing anatomical and behavioural data, a better understanding of the nature of the relationship between brain damage, reorganization and functional recovery can be obtained.

It was hypothesized that changes in dendritic morphology would influence subsequent behavior. Since most of the morphological changes were observed in Layer II neurons, only the anatomical data from these neurons were used in the correlations with behaviour.

Results

Ladder Task

For basilar dendrites, no significant correlations were found between performance on the ladder task and length, $r = -0.103$, $p > 0.05$, volume, $r = -0.353$, $p > 0.05$, complexity, $r = 0.361$, $p > 0.05$.

Examination of apical dendrites also revealed no significant correlations between performance on the task and length, $r = -0.103$, $p > 0.05$, volume, $r = -0.103$, $p > 0.05$, complexity, $r = 0.361$, $p > 0.05$.

Exploratory Behaviour

Significant correlations did not emerge between exploratory behaviour in the open field and basilar length, $r = -0.304$, $p > 0.05$, volume, $r = -0.209$, $p > 0.05$, or complexity,

r = -0.221, p > 0.05. There were also no significant correlations between stationary behaviour in the open field and basilar length, r = 0.335, p > 0.05, volume, r = 0.228, p > 0.05, or complexity, r = 0.218, p > 0.05.

For apical dendrites, correlations with exploratory behaviour in the open field revealed a non significant relationship with length, r = -0.304, p > 0.05, a significant relationship with volume, r = -0.380, p< 0.05 (Fig 14A), and a non significant relationship with complexity,

r = -0.216, p> 0.05. For stationary behaviour in the open field, there was a non significant relationship with length, r = 0.335, p > 0.05, a significant relationship with volume, r = 0.452, p <0.05 (Fig 14B), and a non significant relationship with complexity, r = 0.265, p > 0.05.

Due to the strong correlations between exploratory and stationary behavior with volume, the analysis was redone comparing volume with exploratory and stationary behavior in control animals and HE animals separately. For control animals, non significant correlations were found between volume and exploratory behaviour in both basilar dendrites, r = 0.015, p > 0.05 and apical dendrites, r = -0.178, p > 0.05 (Fig 14C). Similarly non significant correlations were found between volume and stationary behaviour in both basilar dendrites,

r = -0.024, p > 0.05 and apical dendrites, r = 0.248, p > 0.05 (Fig 14D). For experimental animals, non significant correlations were found between volume and exploratory behaviour in both basilar dendrites, r = -0.235, p > 0.05 and apical dendrites, r = -0.314, p > 0.05 (Fig 14C). Non significant correlations were also found between volume and

stationary behaviour in both basilar dendrites, $r = 0.340$, $p > 0.05$ and apical dendrites, $r = 0.406$, $p > 0.05$ (Fig 14D).

Discussion

Correlations between neuronal morphology and behavior showed that layer II apical volume was significantly correlated with amount of time animals spent engaged in exploratory or stationary behavior in the open field task. This relationship was only significant in PND 16 animals, but not in PND 21 animals. This loss of volume, which would result in similar length, but less surface area for synapses, potentially most significantly near the cell body, may have compromised the ability of neurons to communicate with one another, leading to a decrease in inhibition, which subsequently resulted in hyperactivity in HE animals. Reductions in dendritic volume could be a contributing factor to reductions in cortical volume, which has been associated with behavioural deficits after early developmental injury (Kolb, Cioe, & Whishaw, 2000a). In addition, decreases in dendritic volume reduce the surface area available to establish connections with other neurons. By 21 days there were no significant correlations with any of the open field behaviors examined. There may have been slight increases in apical volume in experimental animals, possibly as a function of maturity. This increase in volume was not sufficient to reach control levels, but may have been adequate enough to enable the animals to more easily compensate so that their behavior became indistinguishable from controls. Although we did not examine morphological changes throughout development, as the Golgi technique only allows for one time point per animal, it is possible that the volume decreases were more severe earlier in postnatal life. A timecourse study would provide information on whether these dendritic changes are

there immediately, or develop over time. There were very few significant correlations between the behaviours examined and the anatomical changes that were observed in layer II neurons, possibly due to the fact that when the animals were divided by experimental condition group sizes were small. There is another potential theory for our results.

It is possible there were few correlations between layer II morphology and behaviour because the spontaneous behaviours that were examined involved purely the execution of motor behavior without a very large sensory component. Layer V neurons are more involved in the execution of motor behavior and layer II neurons are more involved with the integration of sensory and motor information. It could be that layer V neurons were largely responsible for the behaviours that were examined and the fact that no differences in morphology were found between HE and control animals contributed to the lack of difference in spontaneous behavior between the two groups. If the behaviours examined had more sensory requirements along with motor requirements, layer II neurons would have been highly involved and potentially significant differences in behaviour would have been seen. Only with larger behavioural differences would it be expected that significant correlations between behaviour and the morphological changes observed in layer II neurons would have been discovered.

Children with early developmental damage often do not appear to have any problems at first and do not seem to be any different than other children. As these children get older and are challenged to engage in more complex and fine motor movements, deficits start to become obvious. Complex movements may recruit more layer II neurons, leading to problems that were not noticeable before. Thus, the behaviours reported on within this study could be complemented with an investigation

using more complex tasks, learning tasks and tasks known to involve sensory motor integration.

Chapter 8

Discussion of Hypoxia Model: Revisiting Chapters 4-7

The experimental hypothesis of the studies discussed in chapters 4-7 was that hypoxia during migration would influence only those neurons that were migrating at the time of injury and the behaviour related to those neurons. Hypoxia exposure during the period of neural migration was found to have a differential effect on the morphology of cortical neurons depending on the layer they were in and did not seem to influence spontaneous motor behaviour. No significant deficits in the behaviors studied were evident in HE animals , but some small differences from controls were observed. Most notable among the behavioural differences was open field task and the ladder task. Analysis of explorative behavior in the open field revealed that at PND 16 HE animals were significantly more active than control animals, but that this difference was no longer present when animals were retested at PND 21. Examination of performance on the ladder task revealed that HE animals exposed to hypoxia took longer to cross the runway task on the second day of training compared to control animals. These findings are consistent with and supported by the literature.

Despite the small differences that were observed in some of the behaviours examined, the majority showed no significant deficits in HE animals compared to controls. Studies reported in the literature examining the influence of early hypoxia exposure on behavior have found deficits in righting behavior (Grojean, Schroeder, Pourie, Charriaut-Marlangue, Koziel, Desor, Vert, & Daval, 2003), hyperactivity (Cai, Lee, Paul, & Rhodes, 1999) and shortening of the time animals could hang from a wire (Golan, Kashtuzki, Hallak, Sorokin, & Huleihel, 2004). These studies exposed animals

to hypoxia much earlier in gestation (often around gestational day 13) than in the present study. The fact that the animals in the present experiment were exposed to hypoxia later in gestation and were more advanced in their development may have enabled them to compensate more easily for any deficits in motor abilities. Support for this theory may be found in a study by Kolb et al. (1998) in which rats were given frontal lesions on gestational day 18 and subsequently examined anatomical and behavioural outcomes. The authors found that despite neuronal abnormalities, lesioned animals were indistinguishable from control animals in their performance on tests of spatial navigation, motor tasks, and locomotor activity.

It is also possible that any behavioral problems may have been subtle and the tasks used in the present studies were not sensitive enough to detect them. The tasks we used involved natural abilities that the animal should have at the various time points. These types of behaviours were examined because we were interested in determining whether normal developmental milestones were delayed or if performance was abnormal. Given that HE rats exposed showed no difference in any of the behavior measures in this study may be due to the fact that the damage was mild or that naturally occurring behaviors are hardwired in the brain and are not easily perturbed. Tasks requiring some skill to execute, such as reaching or traversing a series of upended dowels may be necessary to determine if there were any behavioral deficits. Children born prematurely and at risk for hypoxia sometimes do not immediately show motor deficits - they learn to roll over, sit up and eventually walk. When these children are tested at school age, however, and the tasks become more complex, involving more coordination and fast movements, deficits become noticeable (Raz, Glogowski-Kawamoto, Yu, Kronenberg,

Hopkins, Lauterbach, et al., 1998), suggesting that testing of motor behaviour should extend past infancy into childhood and possibly adolescence. Although we were not interested in testing whether the animals were capable of performing complex skills, that type of investigation would be beneficial in the future in this model of developmental injury to determine whether deficits do arise when the animals are challenged with a more difficult task later in life.

One of the significant differences in the present study, between HE animals and control animals was increased exploratory behaviour of experimental in the open field task. This hyperactivity was transient in nature, as it was present when animals were tested at 16 days of age but not when animals were retested at 21 days of age. Similar findings were demonstrated in a study by Cai, Xiao, Lee, Paul and Rhodes (1999) in which it was found that locomotor activity increased with age until PND 15 in both animals exposed to hypoxia on gestational day 17 and sham controls. Unlike the control group, though, the hypoxia group showed a significant increase in locomotor activity between PND 13 and 15. Testing after PND 15 revealed no significant differences in locomotor activity between the two groups. In addition, a study conducted by Karasawa, Araki, and Otomo (1994) showed that the locomotor activity in gerbils exposed to hypoxia increased in an open field task 1 and 3 days after exposure, but had decreased to control levels by 14 days after exposure. One possible explanation for the transient nature of the hyperactivity could be maturation that the animals simply grew out of their hyperactivity. It is also possible that as animals got older they were better able to modulate their behaviour, because although locomotor activity in HE animals was still

higher than that of control animals at 21 days of age, it was reduced from previous measures.

The ladder task produced the other significant difference between HE and control groups. Specifically, HE animals took longer to cross the runway on the second day of training compared to control animals. The hippocampus is an important brain region for learning and memory (Phelps, 2007). Hippocampal neurons are also extremely vulnerable to the effects of hypoxia (Zola-Morgan, Squire, & Amaral, 1986; Hodges, Sowinski, Fleming, Kershaw, Sinden, Meldrum, et al., 1996). Although the hippocampus was not examined for any pathological changes associated with hypoxia exposure in the present study, it is possible that damage to hippocampal neurons may have impacted the rat's ability to either store or retrieve the memory of how the task was performed. Research has shown that the neurons in the region of the hippocampus responsible for storing and managing information are among the most sensitive to hypoxic insult and can lead to memory problems in both humans (Grubb, O'Carroll, Cobbe, Sirel, & Fox, 1996; Newman, Kirchner, Phillips-Bute, Gaver, Grocott, Jones, Mark, Reves, & Blumenthal, 2001) and rats (Sun, Xu, & Alkon, 2002; Volpe, Pulsinelli, Tribuna, & Davis, 1984). It is possible that this cell population was affected by the hypoxia exposure and subsequently influenced the ability of the experimental animals to remember how to perform a task they had done previously.

The results of the anatomical data showed that prenatal hypoxia during neural migration had a profound effect on the morphology of neurons within the primary motor cortex, particularly layer II neurons. There was a trend for the basilar dendrites of HE animals to be shorter than those of control animals. HE animals were also found to have

significantly shorter apical dendrites than control animals. The volume of both basilar and apical dendrites was found to be significantly decreased in HE animals compared to controls. Control males were found to have significantly thicker apical dendrites than the other three groups and HE males to have significantly thinner apical dendrites compared with the other groups. There were no significant differences in the complexity of basilar dendrites between HE and control animals, but analysis of apical complexity showed that HE animals had less complex apical dendrites than controls. The decrease in dendritic length, volume and complexity exhibited by HE animals may have compromised the neuron's ability to establish connections with other neurons or if connections were made, they may not have been as stable, which would affect communication between neurons in different cortical areas.

In contrast, the influence of prenatal hypoxia on the morphology of layer V neurons was much less dramatic in comparison to layer II neurons. The majority of the differences that were found were due to gender rather than experimental condition. The finding of morphological differences in the neurons of male and female brains is not particularly surprising, as gender differences in the brain have been documented in other studies (Diamond, 1989, Juraska, 1984) showing that males have larger cortical neurons than females.

Analyses of experiments 1-3 suggest that layer II neurons may be more vulnerable to the effects of hypoxia exposure during late gestation than layer V neurons are. In the present set of experiments, animals were subjected to hypoxia beginning at gestational day 20 and continuing until postnatal day 2. At this point, layers VI and V have migrated and occupy their mature positions, but layers IV, III, and II are still actively migrating

and the neocortex does not reach its mature six layered structure become until 7-10 days after birth (Berry and Rogers, 1965). It is possible that the immaturity of layer II neurons at the time of injury make them more susceptible to injury compared to the mature layer V neurons, a theory that is supported by work done by Kolb and colleagues. Rats given lesions at different ages were found to have a significant decrease in cortical dendritic arborization and to perform poorly on tests of learning and species specific behaviors if the injury was sustained within the first few days of life (Kolb & Gibb, 1990; Whishaw & Kolb, 1989; Kolb, Cioe, & Whishaw, 2000; Kolb, Gibb, & van der Kooy, 1994). The findings of these studies consistently demonstrated that animals damaged within the first five days of life resulted in abnormal dendritic growth and behavioral deficits, which coincides with the period of neural migration of layer II cortical neurons in particular. Increased vulnerability for changes in neuronal morphology and behavioural problems resulting from injury sustained during neural migration has been observed regardless of the model used to induce damage (Kolb, Petrie, & Cioe, 1996; Kolb & Cioe, 2003). This increased sensitivity has also been demonstrated using other species. For example, cats that underwent unilateral lesions of the frontal cortex showed gross morphological changes including shrinkage of the hemisphere ipsilateral to the damage and exhibited significant deficits in visual perception when the injury occurred prenatally, whereas animals that sustained the injury neonatally showed no such morphological or behavioral changes (Villablanca, Hovda, Jackson, & Infante, 1993). Thus, damage during early development is only beneficial in terms of functional recovery if the injury is sustained at a particular time point during development. An injury incurred earlier than the critical age results in a much poorer recovery of function.

The finding that injury early in life may not always lead to improved functional recovery is in conflict with Kennard's assertion that damage when young results in a faster and more extensive recovery than damage during adulthood. The results of the present study, along with other consistent literature (e.g. Kolb & Cioe, 2003; Villablanca et al., 1993) can be used to refute the Kennard Principle by demonstrating that injury early in development produces long lasting changes to the morphology of neurons that are still evident into adulthood. This finding is consistent with the research conducted by Kolb and colleagues (e.g. Kolb & Gibb, 1990; Whishaw & Kolb, 1989), in which it was shown that damage before postnatal day 10 in the rat results in extensive damage to neurons and very poor functional recovery. It appears that sustaining an injury early in development does not necessarily insure satisfactory functional outcome. Rather, there seems to be a small window during early life that allows for extensive recovery and damage sustained before and after this period is not easily compensated for.

Neuronal organization is highly dependent on the connections made between neurons and activity dependent processes. Although not examined in the present research, it is also possible that hypoxia exposure influenced the development of cortical connectivity, potentially affecting the ability of layer II neurons to send and receive information. Kolb, Gibb and van der Kooy (1994) gave rats frontal cortical lesions at 1 and 10 days of age. As adults, animals were either processed with Golgi-Cox to examine dendritic arborization, given injections of True Blue (a UV excitable dye that stains cytoplasm with blue fluorescence) into the parietal or visual cortex, or the substantia nigra. An additional group was given injections of fluorescent dye into the cortex at 4 and 10 days of age. The results of dendritic growth were the same as was found in previous

studies. Animals damaged at 10 days of age exhibited an increase in dendritic arborization, whereas those injured at 1 day of age showed a decrease in dendritic arborization. An additional finding was that animals with lesions at 10 days had no apparent abnormalities in cortical connections. Rats with lesions at 1 day, however, were found to have abnormal thalamo-cortical, amygdalo-cortical, and nigro-cortical connections. Of particular interest was the discovery that these abnormal connections were found in the brains of 4 day old normal rats, leading the authors to suggest that the early damage resulted in the failure of retraction or pruning of exuberant connections (Kolb et al., 1994). It is possible that the animals in our study may have also experienced similar abnormal connections and disruption of the pruning process. Disturbance of the brain's capacity to retract or prune back poorly formed or underused connections could potentially affect the efficiency of communication between neurons and may contribute to the morphological changes in neurons observed in the present study. It appears that changes in connectivity observed in a model of focal damage also extend to models of more global damage.

Alterations in cortical connectivity have been exhibited in studies of more global insults. For example, rats exposed to alcohol during gestational days 14-19 were found to have damage to thalamic terminals that increased in severity through sensorimotor areas (Granato, Santarelli, Sbriccoli, & Minciacchi, 1995). In addition to a reduction in thalamic-cortical afferents to the cerebral cortex, prenatal alcohol exposure has been found to reduce the dendritic arborization in layer II/III neurons of the sensorimotor cortex (Granato & Van Pelt, 2003). Although beyond the scope of the present study to examine, it is possible that prenatal exposure to hypoxia may also influence thalamo-

cortical development in a manner similar to prenatal alcohol exposure. Layer II neurons of the primary motor cortex have connections with other cortical regions including sensorimotor and somatosensory cortices both of which have extensive thalamic connections. Since thalamo-cortical input sent to layer IV is then passed on to layer II/III neurons (Lubke, Egger, Sakmann, & Feldmeyer, 2000) any compromise in the flow of information from the layer IV neurons may lead to a resultant decrease in the dendritic arborization of layer II/III neurons (Granato & Van Pelt, 2003). This reduction of information delivered to layer II/III neurons in the sensorimotor cortex may in turn affect the ability of those neurons to send out that information to other cortical areas. Therefore, it is possible that layer II neurons of the primary motor cortex in the present study may also suffer from a decrease in dendritic of arborization after exposure to hypoxia.

Before approximately gestational day 19-20 there are very few thalamic axons that have grown into the cortical plate. Then, beginning at approximately gestational day 19-20, a substantial invasion begins and by postnatal day 2, most axons have bifurcated near layer V and have arborized into the lamina that is differentiating into layer IV, becoming mature at P8 (Molnar, Adams, & Blakemore, 1998). Hypoxia exposure in Experiments 1-3 began at gestational day 20 and continued until postnatal day 2. Given the timeline of thalamo-cortical innervations and the period chosen for hypoxia exposure in the present experiment, it may very well be that those connections were disturbed, leading to inadequate information flow and the subsequent reduction of dendritic fields in layer II neurons. Research has shown that thalamocortical projections can influence that development and organization of cortical areas (Krubitzer and Huffman, 2000; Pallas, 2001; Kaas, Florence, Jain, 1999). Damage to developing thalamic nuclei was found to

result in alterations in the size and number of cells in the corresponding region of cortex (Windrem and Findlay, 1991). Hypoxia exposure in the present study could have impacted the development of thalamic nuclei, thereby affecting the thalamic innervation to the sensorimotor cortex, potentially resulting in fewer and smaller neurons in that region. Such changes in the sensorimotor cortex may have affected its ability to effectively communicate with other brain regions, thereby producing changes in the neurons in those regions as well.

Correlations between neuronal morphology and behavior showed that layer II apical volume at 65 days of age was significantly negatively correlated with exploratory and stationary behavior in the open field task at PND 16, indicating that as exploratory behaviour increased, apical volume decreased, specifically in HE animals. Early developmental damage has been found to lead to a thinner cortex throughout the entire brain and poorer behavioural performance (Kolb, Cioe, & Whishaw, 2000a, Kolb, Cioe, & Whishaw, 2000b). Although cortical volume was not examined in the present study, it is possible that loss of dendritic material (e.g. length and volume) may have contributed to a decrease in cortical volume, which in turn, influenced the development of behaviour. In addition, decreases in dendritic volume reduce the surface area available to make connections with other neurons, potentially leading fewer connections being made between neurons than would have been made otherwise. By 21 days there were no significant correlations with any of the open field behaviors examined. There may have been slight increases in apical volume, possibly as a function of maturity, not enough to be equivalent with control animals, but enough to enable the HE animals to more easily compensate so that their behavior became indistinguishable from controls. Although we

did not examine morphological changes throughout development, as the Golgi technique only allows for one time point per animal, it is possible that the volume decreases were more severe earlier in postnatal life. A timecourse study would provide information on whether these dendritic changes are there immediately, or develop over time.

In the present set of studies, male rats were consistently found to exhibit more dendritic material than female rats is consistent with the existing literature, making any gender effects that were observed of less interest than the effects of hypoxia or the hypoxia-gender interaction. The results indicate that hypoxia may have a more detrimental effect on the developing male brain than female brain. It has been known for decades that human male brains are generally larger than human female brains (Kretschmann, Soman, Takeoka, & Schaefer, 1979). With advances in imaging technology, it has now become possible to detect smaller, but significant structural differences (Allen, Damasio, & Grabowski, 2002; Carne, Vogrin, Litewka, & Cook, 2006). Research has also shown that male and female rats show similar sex differences in brain size (e.g. Diamond, 1989, Juraska, 1984). The trend for males to be more vulnerable to perinatal injury has also been observed in both the human (Lauterbach, Raz, & Sander, 2001) and rat (Nunez and McCarthy, 2003) fetus, leading to research into possible explanations for the increased susceptibility of males.

Investigation into potential reasons leading to increased susceptibility in males has shown that the pattern of cerebral maturation is different in the male brain compared to the female brain. Diamond (1989) examined the cortical thickness in samples from the frontal, somatosensory, and occipital cortex of male rats over successive intervals from the age of 6 to 900 days of age. It was found that all regions of the cortex developed

equally rapidly after birth until between the ages of 26 and 41 days. In contrast, the female brain was found to follow a very different trajectory, with more regional variation. For example, the frontal cortex in the female brain was found to be fairly well developed at birth and only grew by 2% during the second postnatal week (Diamond, 1989). The somatosensory cortex was also found to be more developed at birth than that of the male. The data suggests that various region of the female cortex developed at different rates from those of the male cortex. The fact that all cortical regions of the male brain seem to be at the same maturational state may serve to make their brains increasingly vulnerable to injury as they are all relatively immature and actively developing. The slower rate of maturation could have produced a longer period of developmental plasticity in males, leading to an increased vulnerability to environmental conditions (Juraska, 1984). In comparison, female rats are born with some cortical regions that are more highly developed. Diamond (1989) suggested that the more highly developed female cortex may serve an evolutionary purpose by ensuring a better reproductive start for the species. A possible additional advantage that a more developed female cortex may have could be to protect her from any perinatal insults that may occur. Having regions of the brain that are more mature may serve to make the female brain robust to unfavourable environments that impact the brain. In a recent study, Mayoral, Omar, and Penn (2009) examined sex differences in a neonatal rodent chronic hypoxia model involving placing mouse pups in a hypoxic chamber to mimic the persistent low-grade hypoxia related to chronic lung disease experienced in prematurity. The hippocampus, cortex and cerebellum were examined in male and female mice that had undergone normoxia (21% O_2) or hypoxia (10% O_2) from postnatal days 3-11. Although both genders experienced decreases in

volume of the regions examined, particularly in the hippocampus and cerebellum, male mice exhibited more extensive reductions in volume than female mice did. The model used by Mayoral and colleagues was similar to that used in the present study and in both instances male pups exposed to hypoxia were found to be more susceptible to the damaging effects than females, indicating that the results of the present study are consistent with other research being conducted using the same model.

Differential rats of cortical maturation in male and female human infants have also been suggested by different research teams (e.g. Taylor, 1969; Geschwind and Galaburda, 1985). Although examinations of these types of differences are more difficult to study in humans, it has been shown that males do fare worse after injury early in development (Lauterbach, Raz, & Sander, 2001). A slower maturation rate in males may be one of the underlying reasons for their increased vulnerability and subsequent poor outcome. A slow maturation would potentially result in differential impact of hypoxia for males than for females.

The results of the present study indicates that cortical layers that are still actively migrating (layer II) when an injury early in development is sustained are more vulnerable to the effects of that injury than cortical layers that have already migrated to their mature positions and have begun to differentiate (layer V). Normally, it is expected that as neurons find their location and become more mature, that behaviours will be seen paralleling these neuronal stages of maturation (reviewed in Kolb & Whishaw, 1998). Despite the changes in layer II, behavior was not significantly affected. It is possible that using tasks that were more difficult might have revealed subtle differences in performance. It was also found that early damage may also have a more detrimental

effect on males compared to females, a trend that has been revealed in other studies using both rats and humans. It appears then, that not only does the state of maturation of the brain at the time of injury play an important part in the brain's response, but gender may also have an additional influence, as well. The findings of these studies may provide evidence of the morphological changes that may underlie some of the conditions associated with hypoxia exposure, such as motor deficits. In addition, the finding that males were more susceptible to the repercussions of early developmental damage suggests that attention should be paid to the differential response of the male and female brain to injury and the potential biological basis of this difference, as well as to the influence that the model itself may have on the types of plastic changes observed.

The present set of experiments demonstrated the vulnerability of layer II neurons of the primary motor cortex to hypoxic damage in utero—particularly in males. The next study serves to extend these findings by using a different model of damage to examine the impact on the structure of layer II neurons of the primary motor cortex in male rats injured slightly later during infancy, when layer II neurons have already located to their final destination. An additional component to the following study is the examination of age as a factor in the evolution of the injury and how neuronal structure is influenced by the natural aging process.

Chapter 9

Consequences of Photochemical Stroke When Young

Introduction

One of the brain's most impressive and important features is its ability to continually change structure and, subsequently, function across the lifespan. This capability for change is known as brain plasticity and it enables the brain to respond to an extensive array of experiences. Examples of plastic change include the birth of new neurons, increases in the complexity of dendritic trees of existing neurons and increases in the size, number and shape of synapses (Whishaw, Alaverdashvili, & Kolb, 2008). Learning in the form of exposure to complex environments (Leggio, Mandolesi, Federico, Spirito, Ricci, Gelfo, & Petrosini, 2005; Pereira, Arteni, Peterson, da Rocha, Achaval, & Netto, 2007) and skill training (Greenough, Larson, & Withers, 1985; Withers and Greenough, 1989) has been used to examine brain plasticity. Although both methods have been useful and informative in the study of how the brain reorganizes, there are some difficulties with simply examining the changes the brain undergoes when learning something new. Not only are the changes often too subtle in the case of learning, it is also difficult to know precisely where to look for plastic change among billions of neurons as the effects are global in nature (Kolb, 1995). What is required is a situation in which the plastic changes are large and unmistakable, and where specific brain regions can be targeted and age of onset can be specified if necessary. The most effective way to achieve this is to damage the brain in some way. Typically, reorganization resulting from damage is more dramatic and widespread compared to learning induced changes. Thus, damage can be a useful tool for understanding brain

plasticity after cerebral injury. One factor that plays a role in the type and extent of plastic changes is the age at which the damage occurs. Although adults do retain some reorganizational capabilities (Buell and Coleman, 1981) the brain is at its most plastic early in life. Despite this knowledge, the response of the developing brain to injury remains poorly understood.

One of the leading causes of injury to the developing brain is stroke. The incidence of stroke in infants is estimated at between 1 in 4,000 and 1 in 10,000 (Ashwal and Pearce, 2001). Initially, most children with neonatal stroke appear to have a good short term outcome (Sran and Baumann, 1988). Examination after 3 years of age, however, revealed that most children who suffered neonatal stroke developed some form of hemiparesis, seizure disorder, cognitive difficulties or developmental delay (Balcom and Redmond, 1997). Other problems associated with neonatal injury include issues with concentration, speech, perception, intelligence, seizures and cerebral palsy (Koelfen, Freund, Konig, Varholt, Rohr, & Schultze, 1993; Sreenan, Bhargava, & Robertson, 2000). These deficits often persist throughout the lifespan.

Research using animal models of neonatal damage have found that the appearance of gross motor skills (e.g. head lifting, walking, righting) occurred at the same time in both experimental and control animals, but the development of complex motor skills (e.g. free fall, bar walking) and neurological reflexes (e.g. ear twitch, grasping, gait) were delayed in experimental animals (e.g. Young, Kolonich, Woods, & Yagel, 1986; Lubics, Reglodi, Tamas, Kiss, Szalai, Szalontay, & Lengvari 2004). Similarly, studies measuring degree of motor impairment at school age in children who had suffered neonatal injury found decreased function in the affected limb, spasticity and some neuromotor

abnormalities such as asymmetry (e.g. Wu, March, Croen, Grether, Escobar, & Newman, 2004; Mercuri, Barnett, Rutherford, Guzzetta, Haataja, Cioni, Cowan, & Dubowitz, 2004). Despite the prevalence of motor abnormalities after stroke during infancy as well as evidence of cellular change observed in animal models, not much has been done to investigate the kinds of changes exhibited after motor cortex stroke in the immature rat, and how these changes evolve as the animal ages. Gaining some insight into these issues is important as motor problems are a major issue after perinatal and neonatal injury.

In the motor cortex, Kolb et al., (2000a) found that despite the fact that animals lesioned at postnatal day (PND) 10 exhibited a better functional recovery compared to animals lesioned at PND 1, the brains of animals lesioned at PND 10 were 85% lighter and the cortex 12% thinner than control animals. Animals lesioned at PND 10 were also found to have reduced dendritic branching for both apical and basilar fields of layer III and V neurons. No difference in spine density was observed, in contrast to animals lesioned at PND 1 that showed an increase in spine density. Kolb's findings indicate there are differing plastic responses to damage depending on the age at which the injury was sustained. The data reported in Chapters 4-6 also confirm a unique change based on the age at injury, relative to the stage of neuronal migration.

The purpose of the current study was to examine the effects of perinatal stroke on dendritic arborisation in the primary motor cortex and how changes in morphology evolve across the lifespan. Damage was induced at postnatal day 10 and animals were allowed to survive to either 2 months (young adult) or 6 months of age (older adult). To examine the structure of neurons in the primary motor cortex, apical and basilar dendrites were traced and spine density evaluated for layer II in animals that had stroke induced

damage during infancy and sham controls. It was expected that a short ischemic episode would have lasting consequences to nearby neurons that would evolve as the natural demands of aging became evident.

Materials and Methods

Subjects

Male Long Evans rats (n = 30) underwent a photochemically-induced stroke within the right motor cortex at the age of 10 days or were sham controls. After being anesthetized with sodium pentobarbital (40 mg/kg), animals were given an intraperitoneal injection of light sensitive Rose Bengal dye (50 mg/kg), the skull was exposed and a laser beam (wavelength = 520 nm, intensity = 300 mW) directed through a probe 1.5mm in diameter, was positioned over the right motor cortex (Fig 15). The probe was positioned such that the lateral portion of the probe was lined up with the midline; the anterior edge of the probe was lined up with the skull sutures that traverse laterally from bregma. The result is that the core area of the probe sits over the bulk of primary motor cortex (M1) in an animal of this age and that when illuminated, activates the Rose Bengal in almost all of primary motor cortex (as per Paxinos et al., 1994). The laser was left on for 20 minutes. Control animals underwent the same surgical procedure except the laser was not turned on. Rose Bengal appears to cause no gross adverse effects in the central or peripheral nervous system after injection at this concentration.

Quantitative Morphology

Animals were sacrificed at either 2 months of age (young adult) or 6 months of age (mature adult) (N for each age group = 15, 7 control, 8 experimental). Animals were given a lethal dose of sodium pentobarbital (100 mg/kg) and were then perfused through

the heart with saline (0.9% physiological saline). Once the perfusion was complete, brains were extracted and placed in Golgi –Cox solution (Gibb and Kolb, 1998). After 21 days the tissue was transferred to a 30% sucrose solution for 7 days. The tissue was then sectioned into thick slices (200μm) with a vibratome (Vibratome 1000 Plus Sectioning System) and placed on slides. After forty-eight hours, sections were processed using ammonium hydroxide, Kodak fix and alcohol baths. This method allows the cell body, dendrites, and dendritic spines to be clearly visible with a light microscope. All of the animals used in the study had obvious infarcts. As soon as the skull was removed, a small divot could clearly be seen on the cortex. To ensure that we were inducing a lesion, several practice animals underwent the lesion procedure at 10 days of age, and were then sacrificed 24 hours later. Those animals had lesions. At sacrifice all the animals in this study had a lesion. Conducting this surgery on young animals was very effective, whereas older animals that have thicker skulls are more problematic.

Sections from animals with a visible lesion were used as a guide to determine stereotaxic coordinates for cell selection for all animals prior to drawing. The spatial coordinates were recorded by using an X, Y, Z system for virtual three-dimensional (3D) drawing. The distance from the midpoint of the lesion to the edge was determined using the graduated x, y locator markings on the mechanical portion of the microscope stage (see Fig 16A). This measurement was used to determine how far to move each slide to ensure that cells were drawn from the same area in both hemispheres for all animals. Cells selected were those approximately 1mm from the lesion site (Fig 16A, and were complete and free from obscuring vasculature. A motorized stage (Ludl) on a BX51

microscope (Olympus) was used to adjust the Z-axis on the screen when tracing. The data was collected by an individual blind to the condition of the animals.

Ten layer II cells were drawn from each of the right and left motor cortex for a total of 20 cells per animal. In a small percentage (1-4%) of cells the precipitate can completely fill the cell, allowing it to be easily visualized under a light microscope. Under the microscope, the cell body, dendrites, and dendritic spines are clearly visible. Neurons that met the following criteria were chosen for data collection: i) the cell was located in the primary motor cortex; ii) the cell was stained sufficiently to allow for accurate visualization of processes; iii) the cell was not obscured by other material, such as glia, vasculature, and other neurons; and iv) the cell was largely intact, with few truncated or cut processes. Neurons from this layer were chosen because research has shown that they exhibit the most change in response to damage (Kolb et al., 2000). The outline of the cell body and the routes of dendrites extending from the soma were traced using NeuroLucida software (MicroBrightField, Inc). The scroll wheel on the mouse allowed for concurrent adjustment of the diameter of the dendrites when drawing the path of the dendrites. As such, dendritic length and volume could be recorded and calculated. Any dendrites extending from the soma were traced and dendritic endings were marked with a normal ending or truncated ending; we did not analyze different ending types in this study. NeuroExplorer (MicroBrightField, Inc) rendered a 3D image of each neuron after the drawing was completed.

A morphometric analysis of dendritic length, volume and branching complexity was performed with the NeuroExplorer software to obtain measures for each of these dependent variables for both apical and basilar dendrites (as per Snow, Hartle, & Ivanco,

2008). For example, dendritic length was computed in NeuroExplorer by determining the length of dendrite between each sphere-shaped 'ring.' NeuroLucida also allows the user to reproduce the volume of each dendritic process. The scroll wheel on the mouse enables the user to control the size of a circle, which can be made smaller or larger depending on the thickness of the dendrite. Branch order analysis was also conducted (as per Snow et al., 2008) and used as a measure of the complexity of the neuron. Numbers are assigned to dendritic branches to describe the hierarchy of the branching scheme. This numbering scheme is termed the branch order for the tree. Neurons that have an increased number of higher order branches are considered to be more complex than neurons that have fewer or no higher order branches. NeuroExplorer uses branch orders as defined by the drawer for each dendritic tree giving the total number of branches in each order.

Spine Density Measurement

Dendritic spine counts were performed on apical and basilar dendrites of layer II cells from the same sections used in the neuronal tracing portion. Oil immersion microscope objective lenses were used in order for visualization of spines under high (100X) magnification. Spine counts were taken from two locations, one proximal and one distal to the soma, on the same cell for both apical and basilar dendrites. Dendrites were randomly selected from each chosen cell and followed from the cell body outward until the spines appeared to be approximately uniform in density. This was usually just after the first branch point in the dendrite. After this point a section of dendrite approximately 20μm in length was traced and each individual spine within this traced region was counted (see Fig 16B). The total number of each of these spines in the selected area was then noted. The dendrite was followed outward further, to a section

distal to the cell body, 1-2 branch points in the dendrite away. The counting procedure was then repeated for this section of dendrite as well. Tracings of twenty cells (10 from the ipsilateral and 10 from the contralateral hemisphere to the lesion) were analyzed from each animal. Using the "Scale Master Classic" (Calculated Industries), a device specifically designed for easy measurement of curved lines, the number of spines per micrometer of dendrite was determined by measuring the length of dendritic segment from which the spines were counted.

Statistical Analysis

Changes in dendritic length were assessed with 4-way ANOVAs using condition (2) x age (2) x side (2) x cell (8) x ring (15-basilars; 25-apicals) as factors. Fifteen rings were used for basilar dendrites as the majority of cells did not have dendrites that extended past this ring. Changes in complexity and volume were assessed with 4-way ANOVAs using condition (2) x age (2) x side (2) x cell (8) as factors. Changes in spine density were analyzed with repeated measures ANOVAs using condition (2) and age (2) as factors.

Results

Basilar Dendrites

Length

Results revealed no main effect of condition, $F(1, 52) = 1.2$, $p = 0.282$. There was no main effect of age, $F(1, 52) = 0.1$, $p = 0.718$. There was a ring x age interaction (Fig 17A),

$F(14, 728) = 3.3$, $p < 0.01$, indicating that there were differences in the number of intersections at certain rings between the 2 months and 6 months groups. Fisher's post

hoc test conducted on the interaction revealed no significant differences in the number of intersections per ring across groups. There was no condition x age interaction, F (1, 52) = 2.2, p = 0.143. Simple effects ANOVA (Fig 17B) removing side as a factor as there was no effect, showed that there was an overall increase in dendritic length in experimental animals at 2 months of age, F (1, 52) = 4.93, p = 0.043. Experimental animals had a 14% increase in basilar length compared to control animals at 2 months of age. The same simple effects ANOVA conducted on animals at 6 months of age revealed no difference between experimental and control animals, F (1, 52) = 0.07, p = 0.789.

Volume

Analysis of basilar volume showed no main effect of age, F (1, 21) = 0.003, p = 0.956 (Fig 17C); there was also no main effect of condition, F (1, 21) = 0.009, p = 0.923. There was no interaction between age and condition, F (1, 21) = 0.113, p = 0.740.

Complexity

Complexity measures revealed a main effect of age, F (1, 26) = 6.5, p = 0.017 (Fig 17D). Experimental animals at 2 months of age had a 10% increase in basilar complexity compared to experimental animals at 6 month old animals. There was no main effect of condition, F (1, 26) = 0.2, p = 0.697 and no interaction between age and condition, F (1, 26) = 0.1, p = 0.746. There was, however, a significant interaction between side and condition, F (1, 26) = 4.5, p = 0.044 (Fig 17E), indicating a difference in growth between the right and left hemisphere in control and experimental

animals. An 8% difference in basilar complexity was found for the damaged right hemisphere between experimental and control animals.

Apical Dendrites

Length

There was no main effect of condition, F (1, 46) = 0.0, p = 0.958 or of age, F (1, 46) = 0.0, p = 0.838. There was an interaction between rings and condition, F (24, 1104), = 6.1, p<0.001 (Fig 18A), indicating that the number of intersections at some rings differed between experimental and control animals. Fisher's post hoc test revealed that experimental and control animals differed significantly at ring numbers 4, 5, 6, 16, 17, 18, 19, and 20 (p<0.05). There was also no interaction between age and condition, F (1, 46) = 0.5, p = 0.464 (Fig 18B).

Volume

There was no main effect of condition, F (1, 21) = 0.645, p = 0.431 or of age, F (1, 21) = 0.049, p = 0.827 for apical volume. There was no interaction between age and condition, F (1, 21) = 0.256, p = 0.618 (Fig 18C).

Complexity

Complexity measures revealed no effect of condition, F (1, 26) = 0.12, p = 0.731 or of age, F (1, 26) = 0.13, p = 0.716 for apical dendrite complexity. There was no interaction between age and condition, F (1, 26) = 0.10, p = 0.758 (Fig 18D).

Spine Density

Assessment of basilar dendrite spine density showed a main effect of group, F (1, 9) = 5, p = 0.042, with experimental animals exhibiting a decrease in spine density compared to control animals. Experimental animals had a 6% decrease in basilar spine

density compared to control animals. A main effect of age was also found, $F(1, 9) = 5$, $p = 0.029$ with fewer spines seen in animals sacrificed at 6 months than those sacrificed at 2 months. Animals at 2 months of age had a 7 % increase in basilar spine density compared to 6 month old animals. There was also an interaction between condition and age, $F(1, 9) = 6$, $p = 0.020$, with experimental animals at the age of 6 months exhibiting a decrease in spine density compared with control animals at the same age (Fig 19A). Experimental animals at 6 months had a 13% decrease in basilar spine density compared with control animals at the same age.

Analysis of apical dendrite spine density revealed a main effect for group, $F(1, 9) = 66$, $p < 0.001$, with experimental animals showing a decrease in density compared to control animals. Experimental animals had a 7% decrease difference in apical spine density compared to control animals. There was also a main effect of age, $F(1,9) = 12$, $p = 0.002$, with experimental animals at the age of 6 months exhibiting a decrease in spine density compared with animals at 2 months of age. Experimental animals at 6 months of age had a 3% decrease in apical spine density compared to experimental animals at 2 months of age. An interaction between condition and age was also found to be significant, $F(1,9) = 47$, $p<0.001$, with experimental animals exhibiting a considerable decrease in spine density at 6 months of age compared to control animals of the same age (Fig 19B). A 13% difference in apical spine density was found between control and experimental animals at 6 months of age.

Discussion

Injury occurring early in brain development affects aspects of dendritic structure that persist well past infancy and others that remain unperturbed. Two month old animals that

sustained neonatal lesions had significant increases in basilar dendritic length compared to controls. No significant changes were observed in basilar volume, but analysis of basilar complexity revealed that two month old animals had more higher order branches, which then decrease as the animal ages. This may reflect an age-related loss of complexity, possibly due to a refinement of connections. There were no differences in the number of branches per order as a function of the experimental condition. There was also a difference in complexity between the hemispheres as a function of experimental condition. Control animals were found to have more higher order branches in cells in the right hemisphere compared to cells in the left hemisphere. The opposite was observed in experimental animals in which there were fewer higher order branches in the damaged right hemisphere compared to the intact left hemisphere. The difference between the right and left hemisphere in control rats is consistent with literature showing that that right hemisphere is larger than the left in male Long Evans rats. Diamond (1988, 1991) measured the cortical areas of male rats from infancy until late adulthood and found that 48 out of the 49 areas measured were thicker on the right side than on the left. It is possible that this observed difference in thickness between the two hemispheres was reflected in our date through the increased dendritic complexity observed in the right hemisphere compared to the left. In the present study, stroke was induced in the right motor cortex. The resulting damage could have influenced the growth of cells in the right motor cortex, causing them to be smaller compared to the cells in the left motor cortex of experimental animals.

Analysis of apical dendrites revealed no difference in length between experimental and control animals or between 2 month and 6 month old animals. There

was a significant ring x condition interaction, indicating that experimental animals had fewer intersections with certain rings compared to control animals. The majority of the rings that showed a significant difference between the two groups were those that were distal from the cell body. This is consistent with the literature suggesting dendritic growth and branching occur mostly at the outer ends of dendrites. Analysis of apical volume showed no difference in thickness between control and experimental animals or between animals at 2 or 6 months of age. There was also no interaction between age and experimental condition for apical volume. Similar to apical volume, analysis of apical complexity revealed no difference in branch order between control and experimental animals or between animals at 2 or 6 months of age. There was no interaction between age and condition for apical complexity.

At six months of age the increases in length observed at two months of age were gone and experimental animals were not significantly different from controls. These results suggest that the brain underwent extensive reorganization and plasticity in response to the damage and these changes were still evident into young adulthood (2 months). As the animals aged, these changes regressed so that by the age of 6 months experimental animals were indistinguishable from controls. Although the brain retains some of its reorganizational capabilities into older adulthood (reviewed in Kolb, 1995) the findings of the present study indicate that damage early in development may consume the remaining plastic abilities that remain as the animal ages. The result may be a kind of ceiling effect, so that as the animal ages any remaining plasticity that would have normally existed is gone and the growth exhibited when young cannot be maintained. Some evidence supporting this may be seen in human cases of traumatic brain injury. For

example, Corkin, Rosen, Sullivan, & Clegg (1989) re-administered cognitive tests given to World War II veterans 40 years earlier when they entered the armed forces as young men. Some of men subsequently suffered a penetrating head wound during the war and Corkin and colleagues were interested in learning whether the cognitive decline observed in normal aging would be exacerbated by a brain injury earlier in life. It was found while both the brain injured group and the age matched control group showed a decline in their scores on measures of cognition compared to scores collected when they were younger, the brain injured group exhibited a more significant decline in scores than the control group did. Similarly, another study tested the cognitive abilities of individuals who had suffered a brain injury as children twenty-three years earlier. Hessen, Nestvold, & Anderson, (2007) found that individuals who experienced pediatric head injury exhibited more neuropsychological deficits compared to those who experienced head injury as adults. These results suggest that sustaining a traumatic brain injury earlier in life makes one more vulnerable to neuropsychological problems later in life (Hessen et al., 2007). Taken together, the results of both studies indicate that when the brain is injured earlier in life it does not age as well as it would normally. One explanation that has been put forth to explain this kind of accelerated aging of the brain after injury is the "margin-of-safety" model (Teuber, 1974; Glassman, 1987). This theory posits that the organization of the brain is intrinsically redundant, allowing normal functioning to be maintained despite the loss of a considerable amount of tissue. Early brain injury then reduces some of this redundancy, as does the aging process. The double hit of injury and aging to the brain's reserves result in the emergence of new deficits or aggravation of pre-existing deficits long after the initial injury.

There were no differences in spine density at 2 months of age in our study, indicating that both groups may have achieved maximum spine density at this age. This finding is consistent with the developmental timeline presented by others (reviewed in Kolb, 1995) indicating that maximum synaptic density is reached by 35 days of age in the rat. Generally, by 6 months spine turnover rates have stabilized (Holtmaat, Trachtenberg, Wilbrecht, Shepherd, Zhang, Knott, & Svoboda, 2005). In our hands, overall spine density decreased in animals with lesions relative to controls. This reduction in spine density may indicate inefficient spine turnover, where spines are not being replaced at the same rate at which they are lost, or accelerated pruning. During normal development, the rate of spine elimination begins to decrease and the half-life of spines to increase after 4 months of age (Holtmaat et al., 2005; Grutzendler, Kasthuri, & Nan, 2002; Zuo, Lin, Chang, & Gan, 2005a, Zuo, Yang, Kwon, & Gan, 2005b). Our control animals were found to follow the expected trajectory, showing an increase in spine density at 6 months compared to 2 months. The fact that experimental animals do not show a similar increase in spine density as they age, suggests that damage early in life may disrupt the equilibrium between spine loss and maintenance that is achieved with maturity. The brains of animals subjected to early injury may also expend all their energy lengthening dendrites, leaving little left over for spine production, resulting in spines that were not properly formed and were ultimately eliminated.

Despite increases in overall length of basilar dendrites and specific apical length increases in experimental animals at 2 months of age, there was no concurrent increase in spine density. It would be reasonable to assume that with the additional surface area provided by longer processes, more spines would be formed to accommodate the

changes, however, this does not appear to be the case. In rats, the second week of life is a period of rapid dendritic growth and synaptic formation (De Lima, Merten & Voigt, 1997). Damage during this period has been shown to produce a generalized increase in dendritic arborisation and/or synaptic density throughout the surviving cortex (Kolb, Gibb, & van der Kooy, 1994; Kolb, Petrie, & Cioe, 1996a), but not always in both. In one study (Kolb and Gibb, 1993) the medial frontal cortex of rats were lesioned on postnatal day 1 or 10 and subsequently trained them on the Morris water task. Analysis of the anatomical data revealed that at day 22, dendritic branching in control and day 10 groups were virtually identical. In contrast, day 1 animals had less apical arborization than the other groups at day 22. At day 60, the day 10 animals showed a small increase in branching and a large increase in spine density compared to the other groups, which means that changes in dendritic length and spine density can occur independently of each other. A recent study found a similar dissociation between dendritic length and spine density (Whitcher and Klintsova, 2008). Rat pups were exposed to binge-like quantities of alcohol at PND 4-9 and the pyramidal cells of layer III of the medial prefrontal cortex examined in early adulthood. Changes in spine density, but not dendritic complexity were observed in animals exposed to alcohol, indicating that this differential response of the dendrites and spines to early injury is not exclusive to stroke.

The results of this study provide evidence for some of the morphological changes that may underlie the motor difficulties exhibited by children who suffer early developmental injury. Decreased spine density observed in 6 month old animals could possibly alter the functioning of motor cortex neurons, which subsequently influences behaviour, offering a potential explanation as to why initially children seem to be developing normally, but

start to experience difficulties as they get older (Balcom and Redmond, 1997). Our data also suggests that changes in spine density and dendritic length do not always occur in the same direction within the motor cortex, which causes us to suggest that plasticity is far more dynamic than often described. We also suggest that researchers investigate both length and spine density in Golgi impregnated sections to get a clear picture of brain plasticity.

Chapter 10

Overall Discussion

It was originally thought that if one was going to sustain a brain injury, doing so early in life would guarantee a better functional recovery. Recent research has shown the situation may actually be more complicated and that the developmental events occurring at the time of early injury play an important role in anatomical and functional recovery. The goal of the present experiments was to determine the outcome of injury early in brain development on brain anatomy and behavior. Two different rat models of early developmental injury were used. One model attempted to mimic the hypoxic conditions that are often associated with premature birth, specifically during period of neural migration and the other examined the consequences of stroke in the full term infant. The findings using both models, despite their own inherent differences, are consistent with research done previously (for a review see Kolb and Whishaw, 1989) showing animals that sustained damage before and during the first week of life exhibited more detrimental changes to cell morphology compared to animals damaged during the second week of life.

The results of the hypoxia model of early injury (Chapters 3-5) showed that damage sustained late in gestation and continuing into the early postnatal period, a time during which neural migration is still actively occurring, resulted in a loss of dendritic length, volume and complexity in layer II pyramidal neurons in animals exposed to hypoxia, compared with control animals. In contrast, the study using the photochemical model to selectively deprive the motor cortex of oxygen in 10 day old rats showed that experimental animals exhibited an increase in dendritic length compared to control

animals, whereas volume and complexity remained unaffected. During the second week of postnatal life, cortical migration is complete and neurons are beginning to grow dendrites and to form connections. Since the brain is in a period of active growth at 10 days of age, there is a greater likelihood that it is better able to compensate after damage than if it is in the process of setting up for growth (i.e. migration) or if growth has already been completed. The maturational state of the brain could explain why animals injured during the first week of life exhibit poorer anatomical and behavioural outcomes compared to animals injured during the second week of life. Human infants also show increased vulnerability during certain time points during development. Cortical injury sustained midway through the third trimester or through part of the first year of life have been found to have particularly detrimental effects on subsequent development compared to injury occurring later in childhood (e.g. Riva & Cazzaniga, 1986; Kolb & Fantie, 1989), suggesting that cortical maturation at the time of injury also plays a critical role in the development of subsequent behaviour in human infants.

In the study described in Chapter 6, it was found that increased exploratory behavior in 16 day old experimental animals was correlated with a decrease in dendritic volume. In contrast, control animals at the same age showed a correlation between an increase in stationary behavior and increased dendritic volume. No differences between experimental and control animals in exploratory behavior were evident when tested at 21 days of age. It is possible that the effects of aging were exerting an influence in this study and that subtle changes in morphology were occurring as the animals matured allowing their behavior to be more similar to that of controls. Previous research has shown a decrease in activity back to control levels in animals exposed to hypoxia as they age. Cai,

Xiao, Lee, Paul, and Rhodes (1999) found that animals exposed to hypoxia on gestational day 17 exhibited a significant increase in activity in open field compared to control animals until PND 15, at which time no difference was observed between the two groups. Similar findings were presented in a study by Karasawa, Araki and Otomo (1994) that examined spontaneous locomotor of gerbils exposed to hypoxia in an open field task. Motor activity was measured at 1, 3, 7, 14, or 28 days after hypoxia exposure. It was found that experimental animals exhibited a significant increase in motor activity 1 and 3 days after hypoxia exposure, compared to controls. There was no difference in activity level between experimental and control animals 14 or 28 days after hypoxia exposure. It is possible that the transient nature of the hyperactivity observed after hypoxia is due in part to changes within the brain as the animals mature that enable them to be able to eventually compensate and become indistinguishable from control animals. Unfortunately, not much research has been done examining changes in dendritic volume after injury, not even in laboratories equipped with the NeuroLucida system. Given the results of the present study, it may be worthwhile to include volume when examining morphological changes after injury.

The study described in Chapter 8 provides evidence that injury is followed by dramatic change, but that neuronal morphology is different later in life than it was shortly after the injury. Changes in dendritic length and spine density that were observed in animals at 2 months of age were found to be no longer present at 6 months of age. Although dendritic length regressed back to control levels, spine density was found to decrease dramatically in experimental animals between the ages of 2 and 6 months, compared to control animals that showed an increase in spine density as they matured.

The experiments conducted for this thesis have made some valuable contributions to the study and understanding of early developmental injury. The experiments using the hypoxia model exposed animals to oxygen deprivation at a unique time point, encompassing late gestation and the perinatal period. Human infants are most vulnerable to injury at these points in development, as many important neural events are occurring and any disruption could be extremely detrimental to subsequent brain development and behavior. Most studies examining the effects of hypoxia using animal models expose the animals at much earlier periods during gestation, but no studies have examined the effects of hypoxia exposure using the time point in development that was used in Experiments 1-3. Injury at the chosen period used in the first three studies showed distinct laminar differences in response to hypoxia, possibly explaining why initially infants exposed to hypoxia do not show any deficits in motor behavior until they are older and need to develop more complex motor skills.

The experiment using the photochemical model is unique in that very few studies use this model to examine injury in neonatal animals. The technique is predominantly used in adult animals to model adult cerebrovascular disease and thrombosis. The procedure for lesion induction is very straight forward, non-invasive and can be easily modified for use in neonatal animals. The results of this study prove that the photochemical model is effective for producing lesions in infant animals that endure throughout their lives, producing extensive reorganization of the surviving neurons. The study also shows how the aging process can influence lesion-induced reorganization, something that has not been well studied. Animals are typically only allowed to survive into early adulthood, so the effects of aging remains unknown. Examination of animals

that had been lesioned at 10 days of age showed extensive reorganization that was still evident at 2 months of age but that at 6 months these changes were no longer there and experimental animals had regressed to control levels and in some cases below control levels. These results suggest that the reorganizational capabilities of the brain are compromised by early developmental injury, leading to an inability to compensate for the effects of aging.

Using animal models that mimic conditions experienced by human infants provides a way for researchers to gain insight into how the brain is affected at a cellular level and how changes in neurons may play a role in the child's later development. Results of the present experiments employing two different models of early injury demonstrate that the period during development during which the damage was sustained played an important part in the response of the neurons. Damage occurring during the period of neuronal migration produced smaller and less complex neurons in the brains of experimental animals compared to control animals. In contrast, damage sustained later in development, during the period of dendritic differentiation and synapse formation lead to an increase of dendritic material. By having a basic understanding of the underlying mechanisms of brain damage and the influence that the maturational state the brain is in at the time of injury, better preventative and therapeutic interventions can be developed to improve the quality of life for these children.

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Figure Legends

Figure 1: Images of Neurons Traced from Experimental and Control Animals

A. On the left is a photograph of a Golgi-Cox stained section displaying a layer II cell from a control animal. On the right is a tracing of the neuron from the photograph done with NeuroLucida. The schematic is showing a flattened image of our three dimensional reconstructions of the neuron, as well as the location of basilar and apical dendrites.

B. On the left is a photograph of a Golgi-Cox stained section displaying a layer II cell from an experimental animal. On the right is the tracing of the neuron from the photograph done with NeuroLucida. The schematic is showing a flattened image of our three dimensional reconstructions of the neuron.

Figure 2: Righting

Mean number (±SEM) seconds taken to complete the task as a function of experimental condition. The x-axis is the age at which the animals were tested and the y-axis is the average time taken to complete the task. No significant difference was found in the time required to complete the task across days between experimental and control animals.

Figure 3: Open Field

A. Explorative Behaviours 16 Days: Amount of time spent (±SEM) carrying out each behaviour of interest. The x-axis is the four behaviours measured and the y-axis is the percentage of time the animals spent engaged in each behaviour. It was found that experimental animals at PND 16 engaged in more exploratory behaviour than control animals did and that control animals engaged in more stationary behaviour.

B. Explorative Behaviours 21 Days: Amount of time spent (±SEM) carrying out each behaviour of interest. The x-axis is the four behaviours measured and the y-axis is the

percentage of time the animals spent engaged in each behaviour. When the animals were retested at PND 21 there was no difference between control and experimental animals on any of the behavioural measures.

C. Quadrant 16 Days: Mean number (±SEM) seconds spent in each quadrant. The x-axis is the quadrant number and the y-axis is the average time spent in each quadrant. It was found that animals spent more time in quadrants 1, 4 and 13 all of which were corners, indicating desire to hide.

D. Quadrant 21 Days: Mean number (±SEM) seconds spent in each quadrant. It was found that animals spent more time in quadrants 4 and 13, both of which were corners, indicating a desire to hide.

Figure 4: Strength

Mean number (±SEM) seconds the animal was able to hang from the dowel. The x-axis is the ages at which the animals were tested and the y-axis is the average length of time the animal was able to stay suspended. The only significant finding was a decrease in the length of time the animal was able to hang on to the dowel across days. This may be due to the increase in size the animal experiences as it gets older making it harder to hang on.

Figure 5: Cylinder Exploration

A. Exploration at 25 Days. Mean number (±SEM) of reaches the animal made with one or both paws. The x-axis is the three paw preferences and the y-axis is the mean number of reaches the animals made with each preference.

B. Exploration at 35 Days. Mean number (±SEM) of reaches the animal made with one or both paws. The x-axis is the three paw preferences and the y-axis is the mean number of reaches the animals made with each preference. The only significant difference was an

effect of paw, which likely occurred because animals were inclined to reach out with either the right or left paw, but not with both.

Figure 6. Ladder Runway.

Mean number (±SEM) of time in seconds taken to traverse a runway. The x-axis is the training day and the y-axis is the average time taken to negotiate the runway across days. A significant days X condition interaction revealed that experimental animals took longer to traverse the runway on training day 2 than control animals. There were no other differences across days between the two groups.

Figure 7: Brain Weights

Mean weights (±SEM) of brains from experimental and control animals. The x-axis is the experimental condition and the y-axis is the mean weight in grams. There was no significant difference in brain weight between experimental and control animals.

Figure 8: Layer II Basilar And Apical Length

A. Ring X Condition Interaction. Mean number (±SEM) of intersections of basilar dendrites at each ring number as a function of experimental condition. The x-axis is ring number and the y-axis is the mean number of intersections with each ring. There was a significant ring X condition interaction indicating that there were differences in the number of intersections at certain rings between experimental and control animals. Asterisk (*) indicate significant differences at $p<0.05$.

B. Condition X Gender Interaction. Mean number (±SEM) total intersections of apical dendrites as a function of condition and gender. The x-axis is condition and the y-axis is the average number of total intersections. Asterisk (*) indicate significant differences at $p<0.05$.

C. Ring X Condition Interaction. Mean number (±SEM) of intersections of basilar dendrites at each ring number as a function of experimental condition. The x-axis is ring number and the y-axis is the mean number of intersections with each ring. There was a significant ring X condition interaction indicating that there were differences in the number of intersections at certain rings between experimental and control animals. Asterisk (*) indicate significant differences at $p<0.05$.

Figure 9: Layer II Basilar and Apical Volume

A. Effect of Condition. Mean volume (±SEM) of basilar dendrites as a function of experimental condition. Condition is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. Animals exposed to hypoxia early in development were found to show a significant reduction in basilar dendrite volume compared to control animals.

B. Effect of Gender. Mean volume (±SEM) of basilar dendrites as a function of gender. Gender is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. There was a significant difference between males and females, with males exhibiting increased basilar volume compared to females.

C. Condition X Gender Interaction. Mean volume (±SEM) of apical dendrite as a function of gender and experimental condition. Condition is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. Asterisk (*) indicate significant differences at $p<0.05$.

Figure 10: Layer II Basilar and Apical Complexity

A. Condition X Gender Interaction. Mean number (±SEM) of branch orders of basilar dendrites as a function of gender and experimental condition. The x-axis is experimental

condition and the y-axis is the average total number of branch orders. Although there was a tendency for control males to have an increased number of higher order branches the difference was not statistically significant compared to the other groups.

B. Condition X Gender Interaction. Mean number (±SEM) of branch orders of apical dendrite as a function of condition and gender. The x-axis is condition and the y-axis is the average total number of branch orders per cell. No significant difference in apical branch order was found between experimental and control males and females.

C. Order X Condition Interaction. Mean number (±SEM) of branch orders of apical dendrite as a function of order and condition. The x-axis is branch order and the y-axis is the average total number of branch orders per cell. No significant differences at specific branch orders were found between experimental and control animals.

Figure 11: Layer V Basilar and Apical Length

A. Ring X Condition Interaction. Mean number (±SEM) of intersections of basilar dendrites at each ring number as a function of experimental condition. The x-axis is ring number and the y-axis is the mean number of intersections with each ring. There was no significant difference at any ring between experimental and control animals.

B. Ring X Gender Interaction. Mean number (±SEM) of intersections of basilar dendrites at each ring number as a function of gender. The x-axis is ring number and the y-axis is the mean number of intersections with each ring. Differences were found in the number of intersections at certain rings between male and female animals. Asterisk (*) indicate significant differences at $p<0.05$.

C. Rings X Condition Interaction. Mean number (±SEM) of intersections of apical dendrite at each ring number as a function of experimental condition. The x-axis is ring

number and the y-axis is the mean number of intersections with each ring. Although the analysis revealed a difference at certain rings between experimental and control animals, post hocs showed that there was actually no significant differences between experimental and control animals across rings.

D. Rings X Gender Interaction. Mean number (±SEM) of intersections of apical dendrite at each ring number as a function of gender. The x-axis is ring number and the y-axis is the mean number of intersections with each ring. There was a significant ring X gender interaction indicating that there were differences in the number of intersections at certain rings between male and female animals. Asterisk (*) indicate significant differences at $p<0.05$.

Figure 12: Layer V Basilar and Apical Volume

A. Condition X Gender Interaction. Mean volume (±SEM) of basilar dendrite as a function of gender and experimental condition. Condition is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. No significant differences in basilar volume were observed between experimental and control males and females.

B. Condition X Gender Interaction. Mean volume (±SEM) of apical dendrite as a function of gender and experimental condition. Condition is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. No significant differences in apical volume were observed between experimental and control males and females.

Figure 13: Layer V Basilar and Apical Complexity

A. Orders X Gender X Condition Interaction. Mean number (±SEM) of branch orders of apical dendrite as a function of branch order and gender. The x-axis is branch order and

the y-axis is the mean number of branch orders per cell. No significant differences were found in branch order between experimental and control males and females.

B. Orders X Gender X Condition Interaction. Mean number (±SEM) of branch orders of apical dendrite as a function of branch order and gender. The x-axis is branch order and the y-axis is the mean number of branch orders per cell. Significant differences across orders were found in control males and females and experimental males and females. Asterisk (*) indicate significant differences at $p<0.05$.

Figure 14: Layer II Apical Volume and Open Field Behaviour

A. Scatter plot of apical volume and exploratory behaviour in the open field task. The average time spent exploring is plotted on the x-axis and the mean apical volume is plotted on the y-axis. A significant inverse correlation was found, showing that as time spent exploring increased, apical volume increased.

B. Scatter plot of apical volume and stationary behaviour in the open field task. The average time spent in stationary position is plotted on the x-axis and the mean apical volume is plotted on the y-axis. A significant positive correlation was found, showing that as time spent remaining stationary increased, apical volume increased.

C. Scatter plot comparison of apical volume and exploratory behaviour in the open field task between control and experimental groups. When animals were divided into their respective experimental condition to evaluate whether one group was driving the effect, no significant correlations were found.

D. Scatter plot comparison of apical volume and stationary behaviour in the open field task between control and experimental groups. When animals were divided into their

respective experimental condition to evaluate whether one group was driving the effect, no significant correlations were found.

Figure 15: Landmarks used to Position Laser Probe

Drawing of a rat skull, showing the cranial sutures. Bregma, the point on the skull at which the coronal suture is intersected perpendicularly by the sagittal suture, was located. The laser was then positioned so that the outer edge of the probe was along the sutures on one side, ensuring the beam itself was over motor cortex.

Figure 16: Location of Neurons Traced

A. Photograph of a Golgi-Cox stained section. Starting at the centre of the lesion, as indicated by the line, the stage was moved approximately 1mm out on either side of the lesion. This created a standard reference frame for drawing and was completed for a series of easily seen lesions. Neurons that were selected for tracing were in close proximity to the 1mm mark. The same procedure was carried out for the undamaged hemisphere with the same standard reference used for lesioned sections.

B. On the left is a tracing of a neuron from NeuroLucida. The schematic is showing a flattened image of our three dimensional reconstructions of neurons, as well as demonstrating the proximal and distal locations on basilar and apical dendrites from which spines were traced. On the right is a photograph of the actual neuron that was traced.

Figure 17: Basilar Dendrites

A. Mean number (±SEM) of intersections of basilar dendrites at each ring number as a function of age. The x-axis shows ring number and the y-axis shows the mean number of intersections with each ring. The 2 month old animals were found to have fewer

intersections compared to 6 month old animals. Post hocs indicated no significant difference in the number of intersections at each ring.

B. Mean number (±SEM) total intersections of basilar dendrites as a function of age and condition. The x-axis shows age in months and the y-axis shows the average number of total intersections. At 2 months, animals exposed to stroke early in development were found to have fewer total intersections compared with control animals of the same age. At 6 months of age animals exposed to infant stroke are similar to control animals.

C. Mean volume (±SEM) of basilar dendrites as a function of age and experimental condition. Condition is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. No difference in volume was observed between 2 or 6 month experimental or control animals.

D. Mean number (±SEM) of branch orders of basilar dendrites as a function of age and experimental condition. The x-axis shows age in months and the y-axis shows the mean number of branch orders. No difference in branch order was observed between 2 or 6 month experimental and control animals.

E. Mean number (±SEM) of branch orders of basilar dendrites as a function of hemisphere and experimental condition. Hemisphere is along the x-axis; 1= left hemisphere, 2= right hemisphere. Mean number of branch orders is along the y-axis. Control animals have an increased number of branches in the right hemisphere compared to the left. Experimental animals have fewer branches in the right stroke damage hemisphere compared to the left undamaged hemisphere.

Figure 18: Apical Dendrites

A. Mean number (±SEM) of intersections of apical dendrites at each ring number as a function of experimental condition. The x-axis shows ring number and the y-axis shows the mean number of intersections with each ring. Experimental animals had fewer intersections closer to the soma and increased intersections further from the soma compared to control animals. Asterisk (*) indicate significant differences at $p<0.05$.

B. Mean number (±SEM) total intersections of apical dendrites as a function of age and condition. The x-axis shows age in months and the y-axis shows the average number of total intersections. No significant differences were observed between experimental and control animals at 2 or 6 months of age.

C. Mean volume (±SEM) of apical dendrites as a function of age and experimental condition. Condition is along the x-axis and mean dendritic volume in microns cubed is along the y-axis. No differences in volume were observed 2 or 6 month animals in either condition.

D. Mean number (±SEM) of branch orders of apical dendrites as a function of age and experimental condition. The x-axis shows age in months and the y-axis shows the mean number of branch orders. No difference in branch order was observed between 2 or 6 month experimental and control animals.

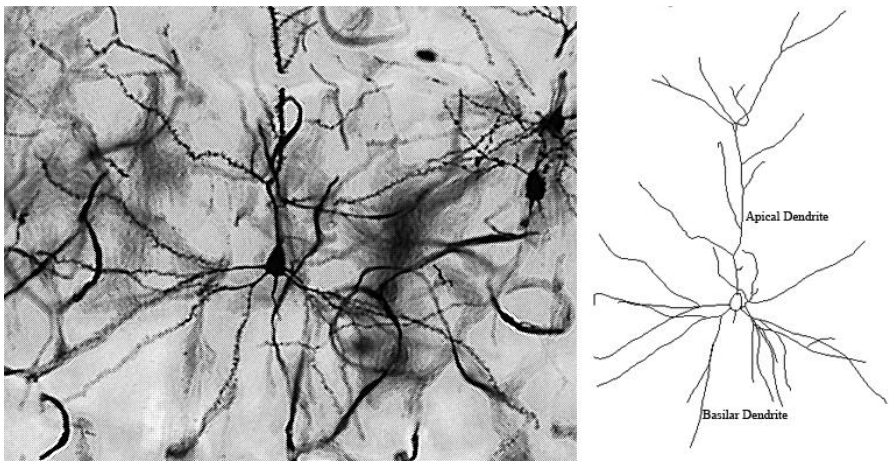
Figure 19: Spine Density

A. Average (±SEM) overall density of basilar dendritic spines. The age in months is along the x-axis and spine density is along the y-axis. No difference was observed between experimental conditions at 2 months of age in overall spine density. At 6 months

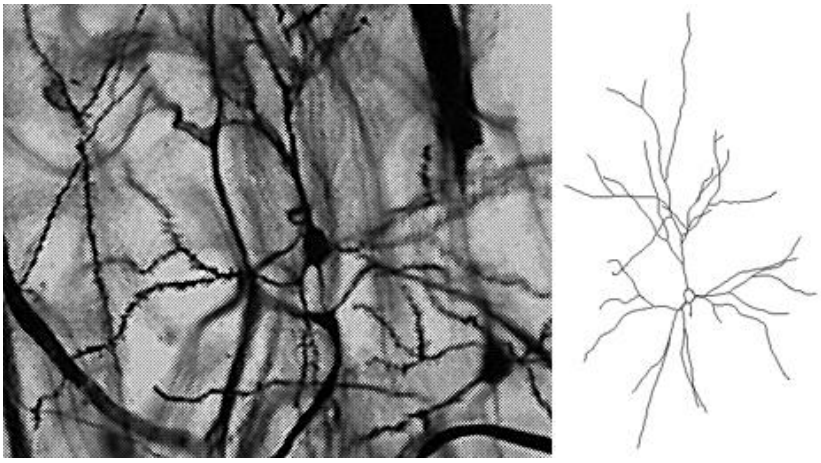
of age experimental animals show a decrease in overall spine density compared to controls, indicating a loss of spines between the ages of 2 and 6 months.

B. Average (±SEM) overall density of apical dendritic spines. The age in months is along the x-axis and spine density is along the y-axis. No difference in overall spine density was observed between experimental and control animals at 2 month of age. At 6 months of age experimental animals show a decrease in overall spine density compared to controls, indicating a loss of spines between the ages of 2 and 6 months.

Figure 1



A



B

Figure 2

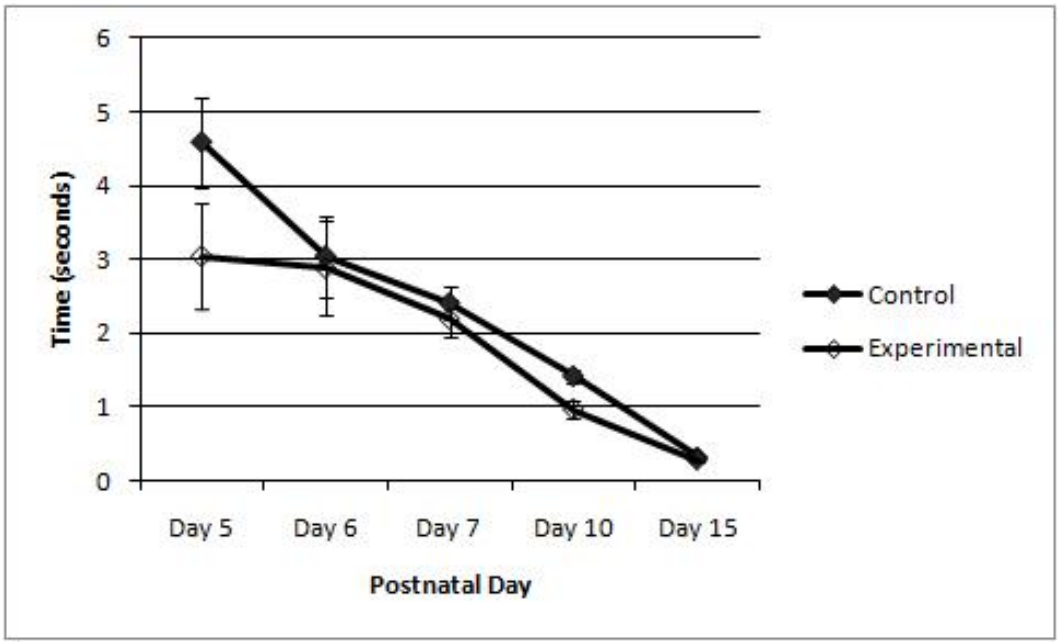
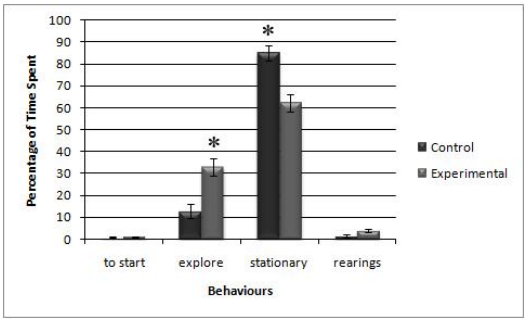
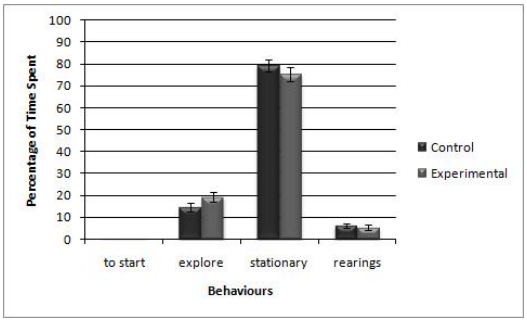

6
5
4
3
2
1
0
Time (seconds)
Day 5
Day 6
Day 7
Day 10
Day 15
Postnatal Day
Control
Experimental

Figure 3

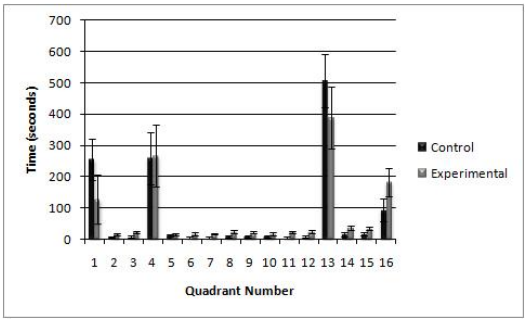
A



B



C



D

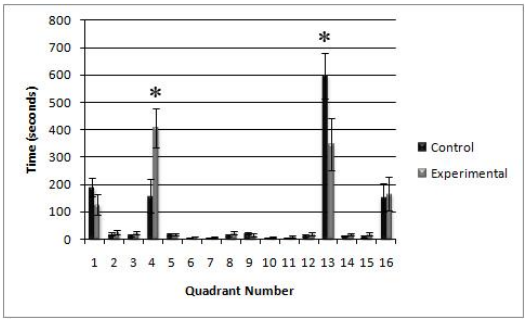


Figure 4

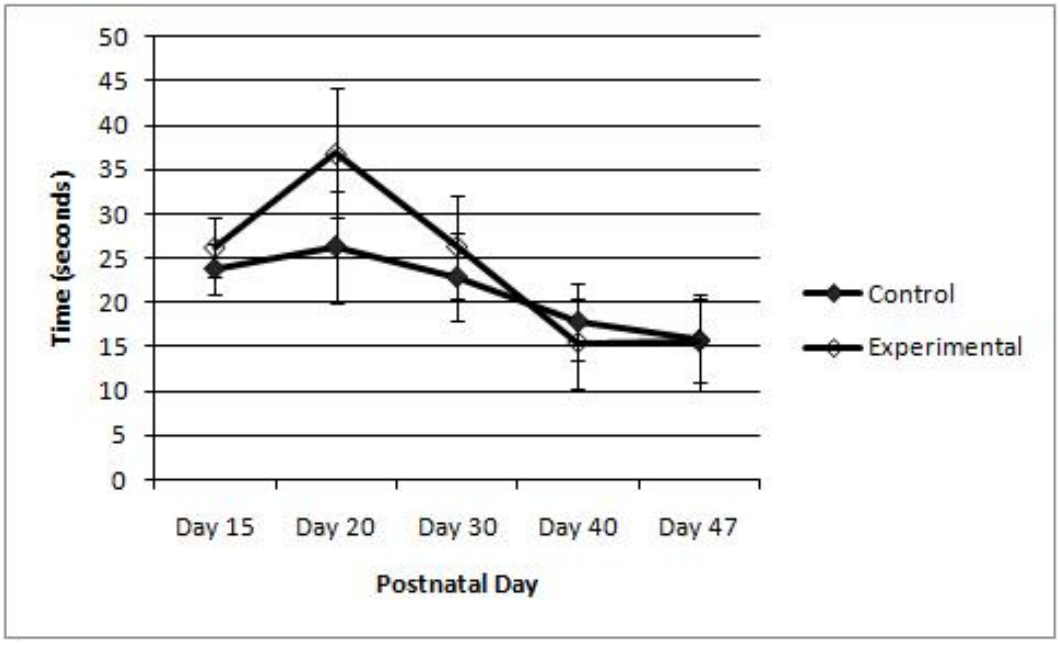
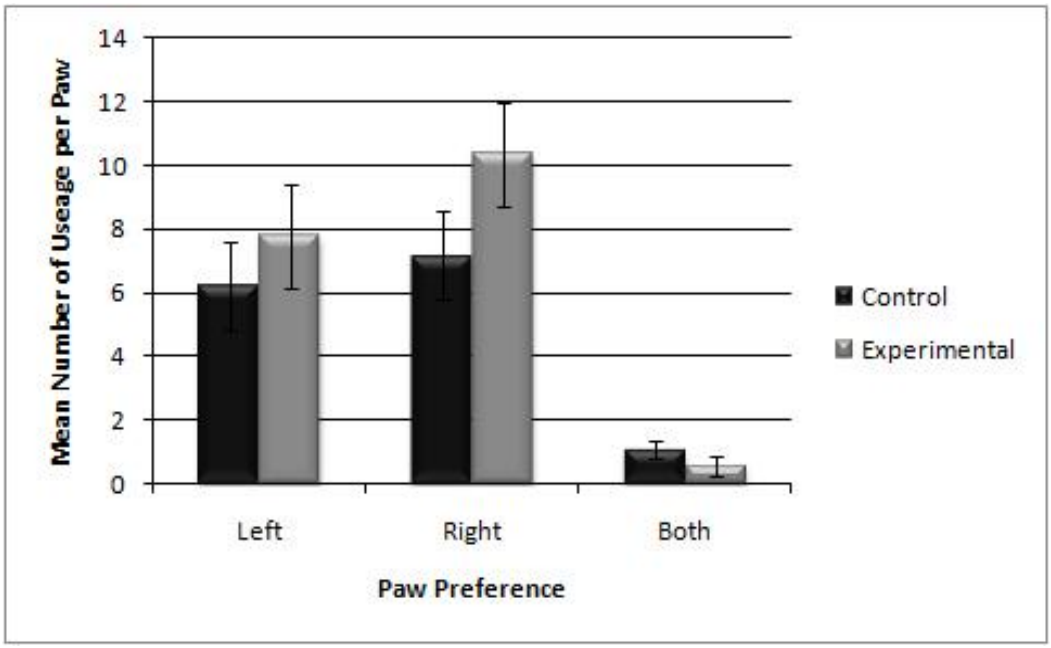
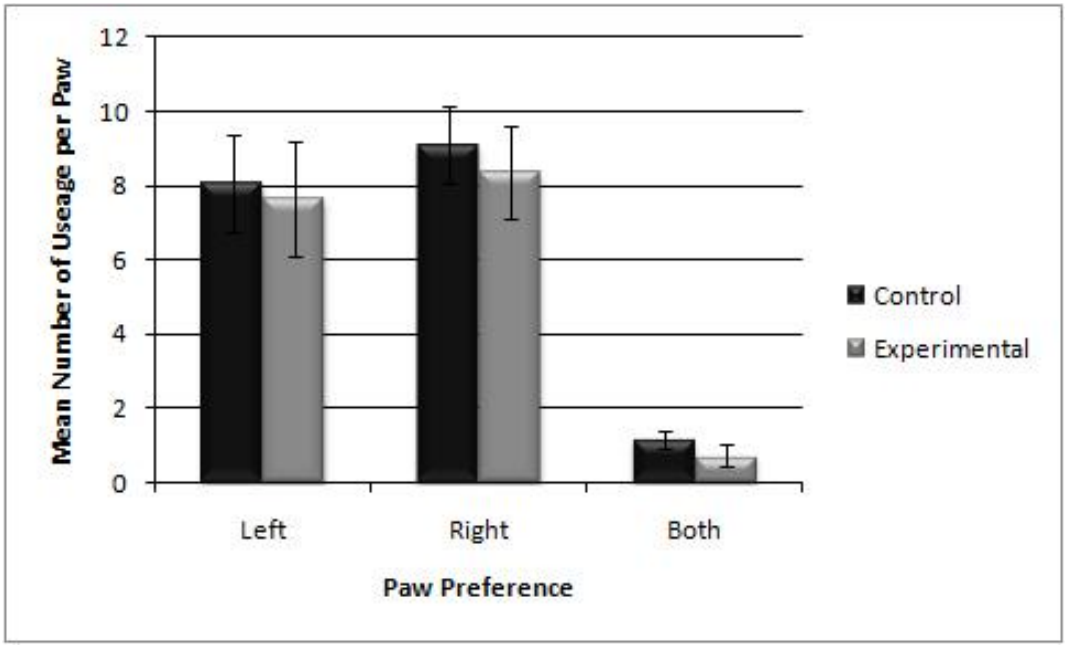

50
45
40
35
30
25
20
15
10
5
0
Time (seconds)
Day 15
Day 20
Day 30
Day 40
Day 47
Postnatal Day
Control
Experimental

Figure 5


14
12
10
8
6
4
2
0
Mean Number of Useage per Paw
Left
Right
Both
Paw Preference
Control
Experimental

A


12
10
8
6
4
2
0
Mean Number of Useage per Paw
Left
Right
Both
Paw Preference
Control
Experimental

B

Figure 6

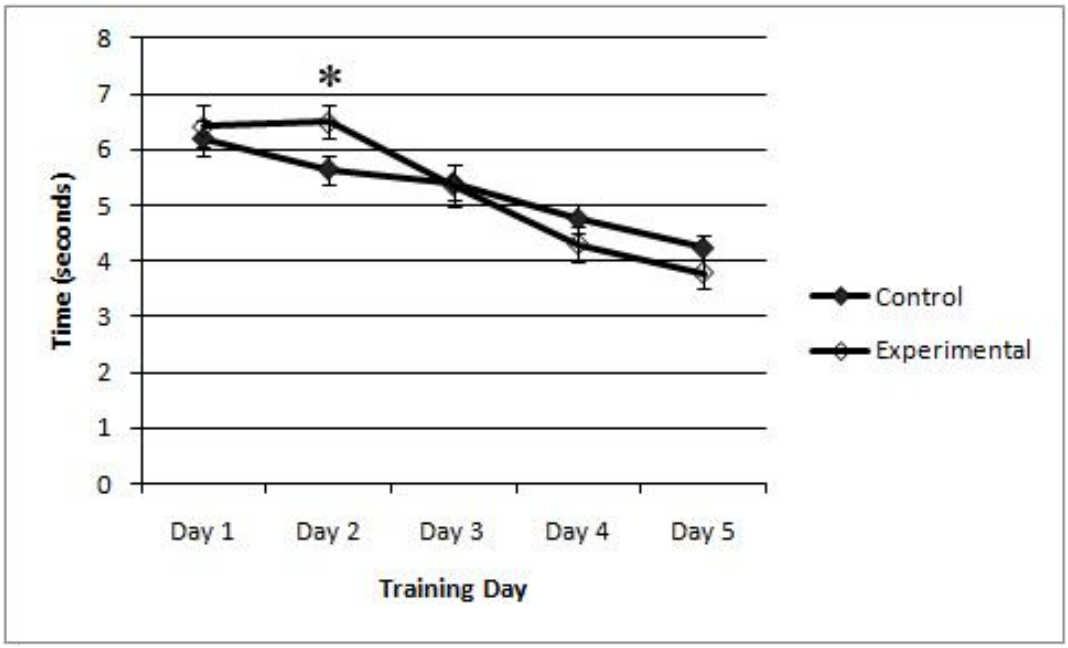

8
7
6
5
4
3
2
1
0
*
Time (seconds)
Day 1
Day 2
Day 3
Day 4
Day 5
Training Day
Control
Experimental

Figure 7

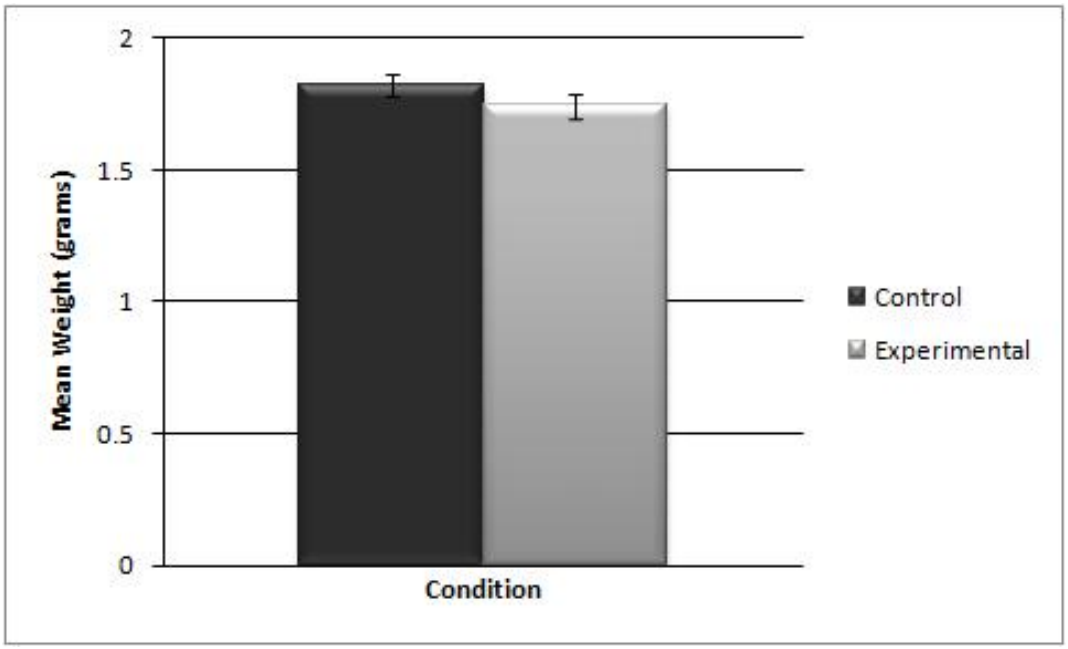
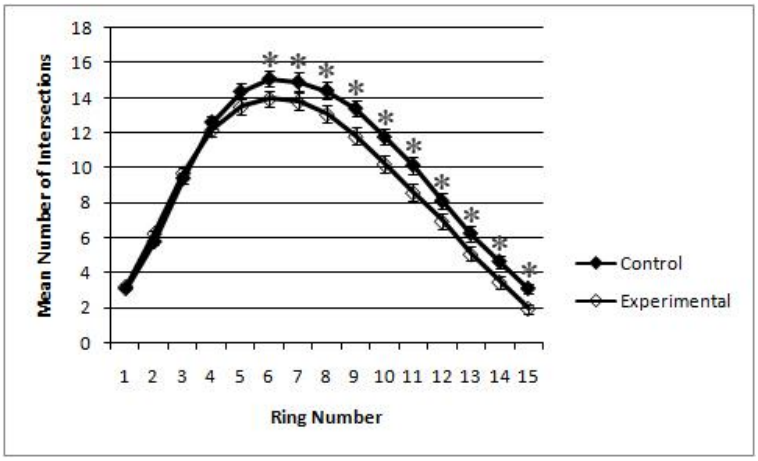
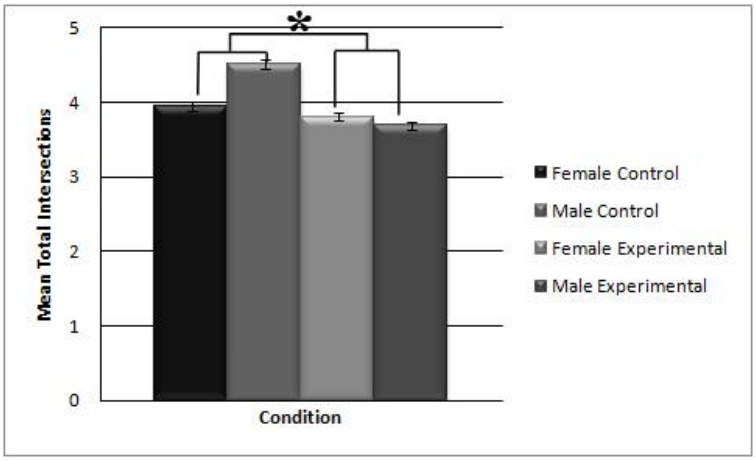

2
1.5
1
0.5
0
Mean Weight (grams)
Condition
Control
Experimental

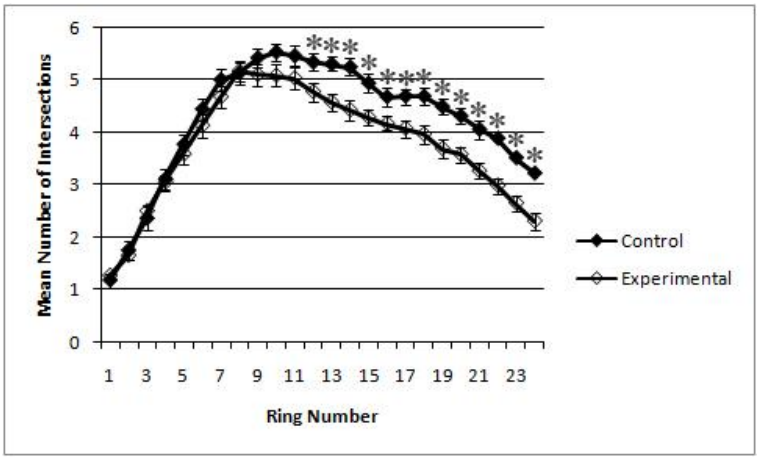
Figure 8


Mean Number of Intersections
18
16
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Ring Number
Control
Experimental

A

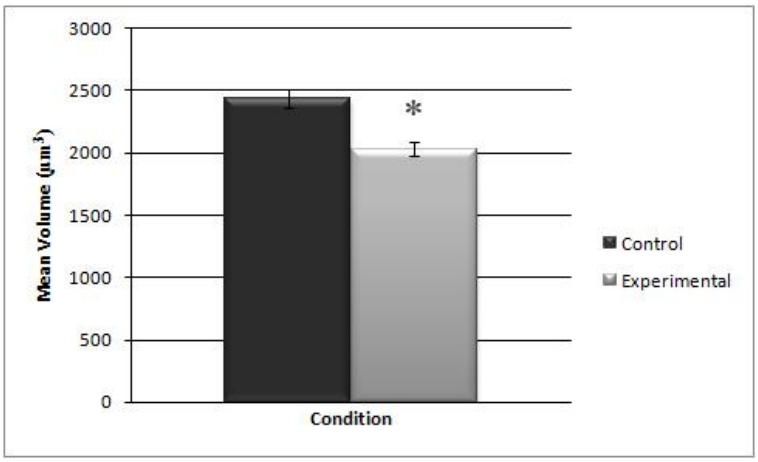

Mean Total Intersections
5
4
3
2
1
0
Condition
Female Control
Male Control
Female Experimental
Male Experimental

B

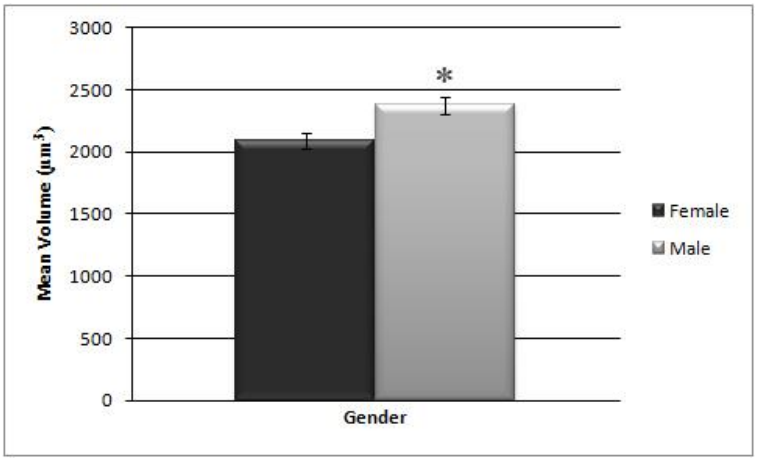

Mean Number of Intersections
6
5
4
3
2
1
0
1 3 5 7 9 11 13 15 17 19 21 23
Ring Number
Control
Experimental

C

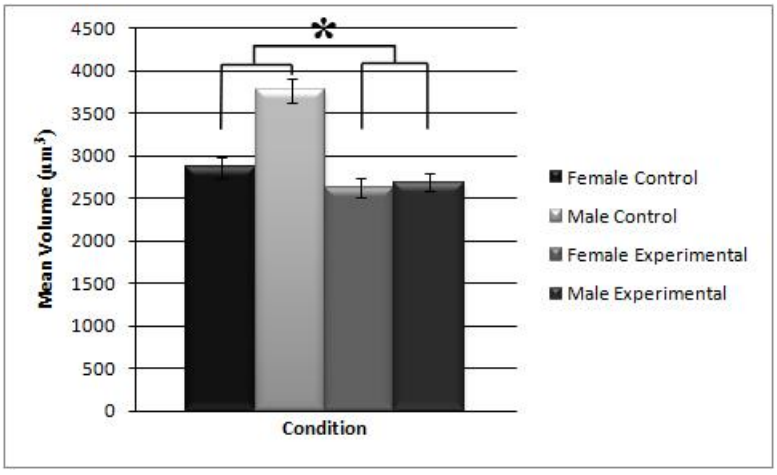
Figure 9


3000
2500
2000
1500
1000
500
0
Mean Volume (µm³)
*
Condition
Control
Experimental

A

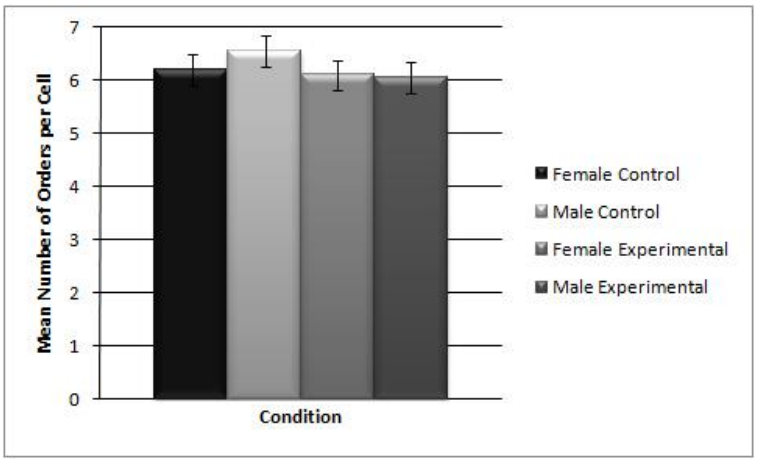

3000
2500
2000
1500
1000
500
0
Mean Volume (µm³)
*
Gender
Female
Male

B

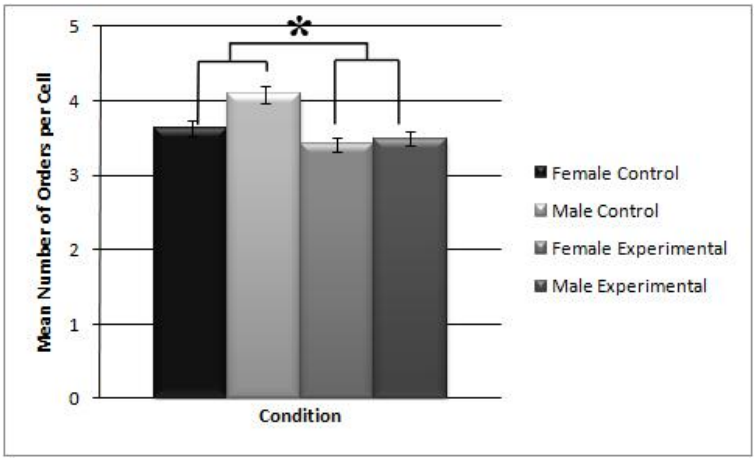

4500
4000
3500
3000
2500
2000
1500
1000
500
0
Mean Volume (µm³)
*
Condition
Female Control
Male Control
Female Experimental
Male Experimental

C

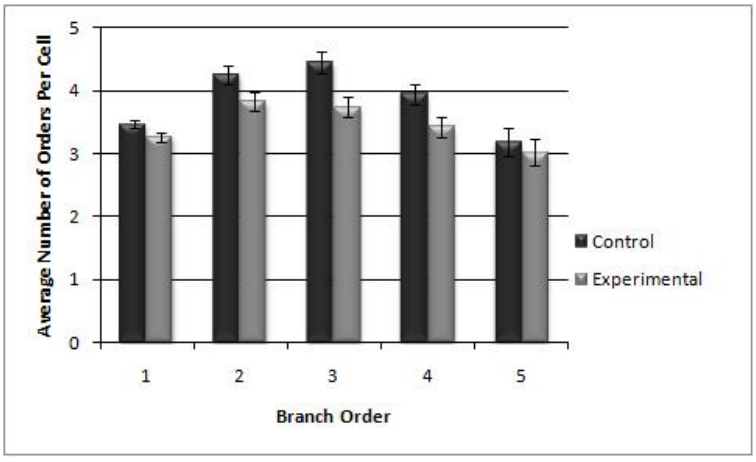
Figure 10



A

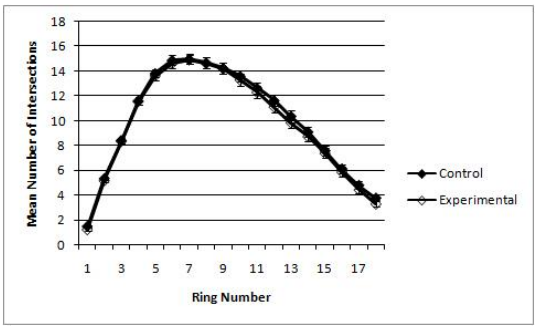


B

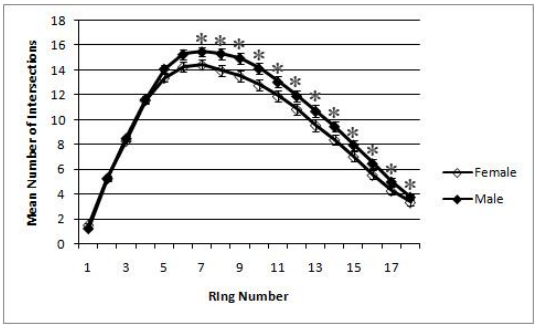


C

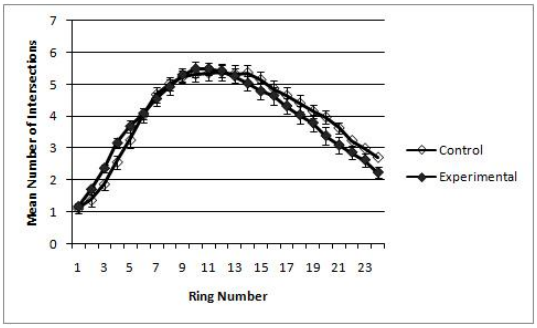
Figure 11



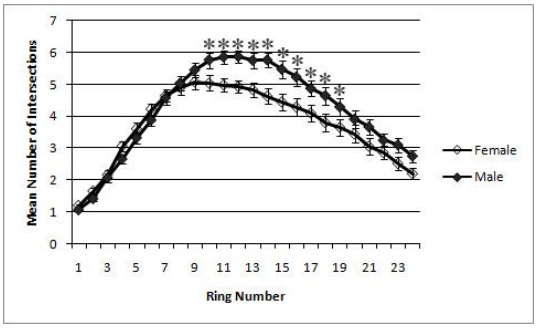
A



B

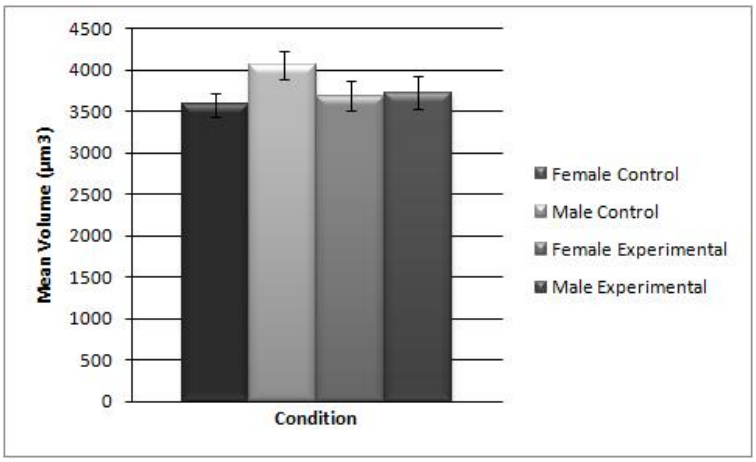


C

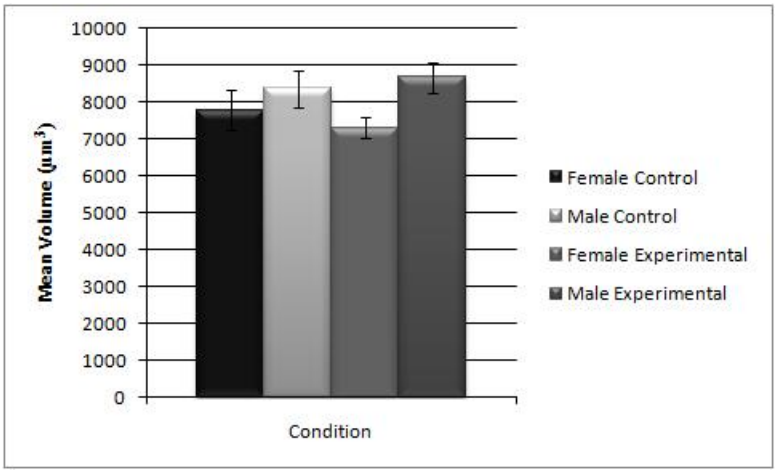


D

Figure 12

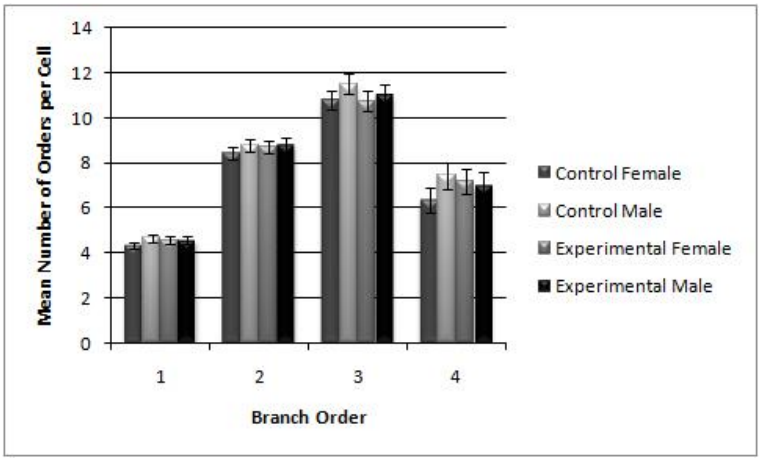

4500
4000
3500
3000
2500
2000
1500
1000
500
0
Mean Volume (µm3)
Condition
Female Control
Male Control
Female Experimental
Male Experimental

A

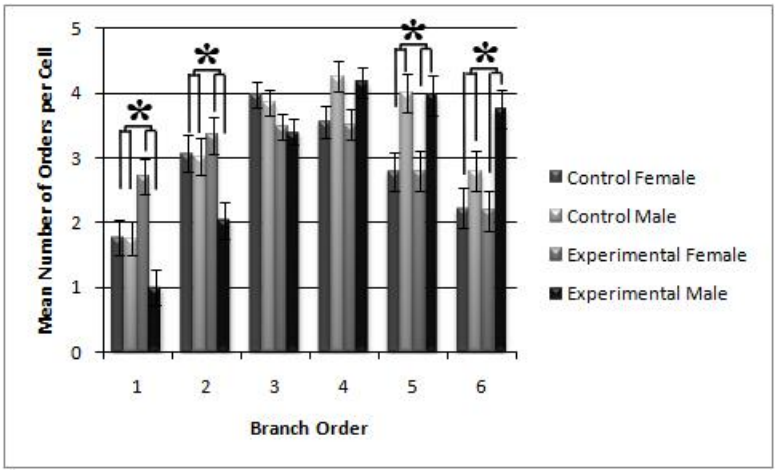

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Mean Volume (µm³)
Condition
Female Control
Male Control
Female Experimental
Male Experimental

B

Figure 13


Mean Number of Orders per Cell
14
12
10
8
6
4
2
0
1
2
3
4
Branch Order
Control Female
Control Male
Experimental Female
Experimental Male

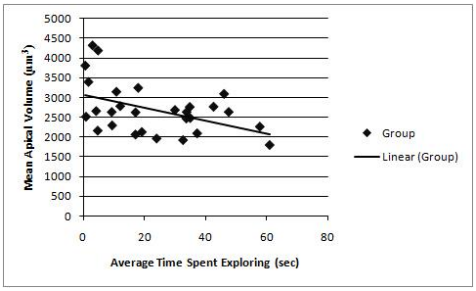
A


Mean Number of Orders per Cell
5
4
3
2
1
0
1
2
3
4
5
6
Branch Order
Control Female
Control Male
Experimental Female
Experimental Male

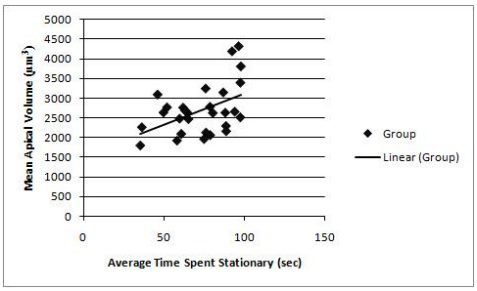
B

Figure 14

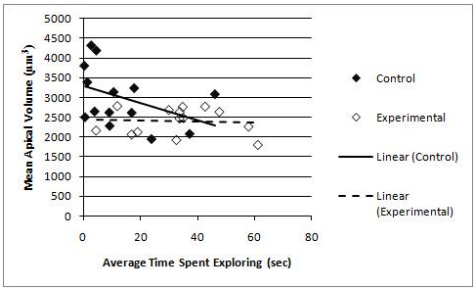
A



B



C



D

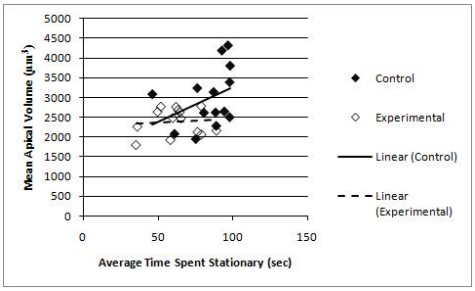


Figure 15

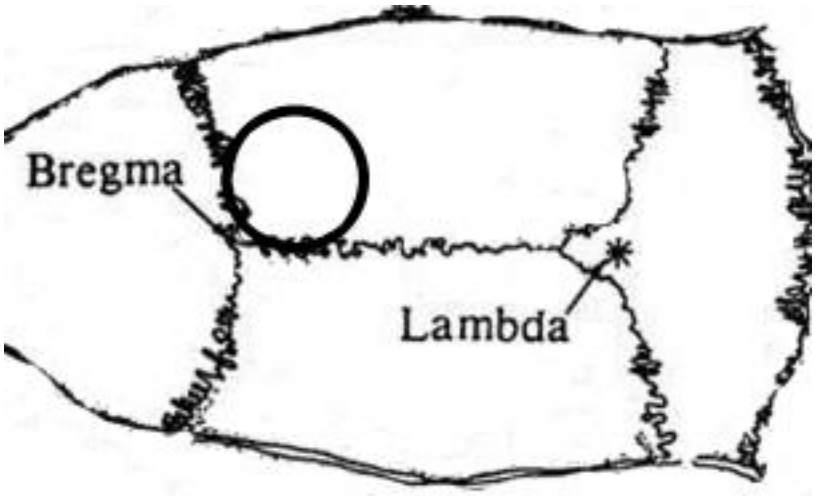
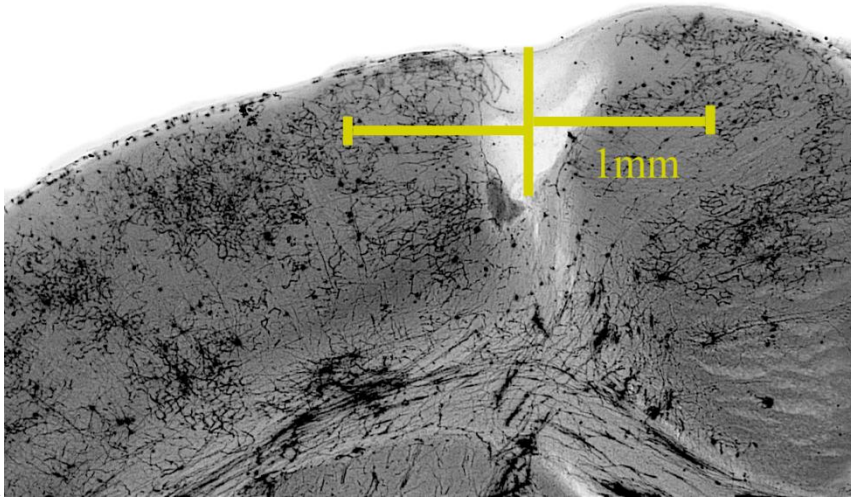
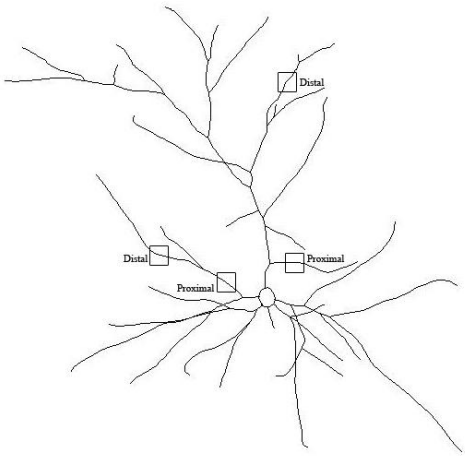
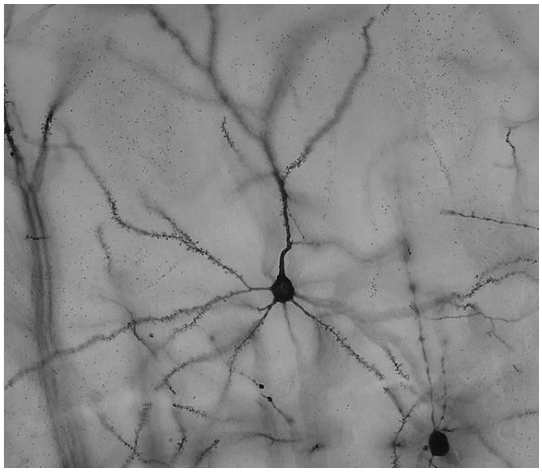


Figure 16



A

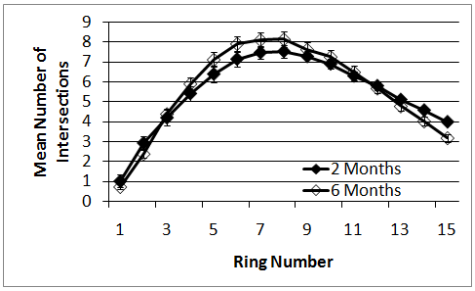




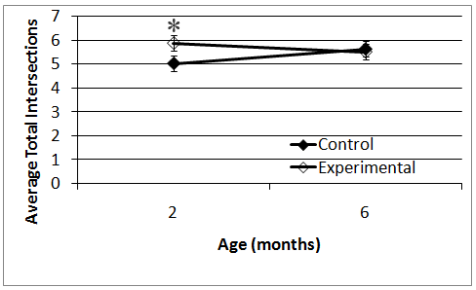
B

Figure 17

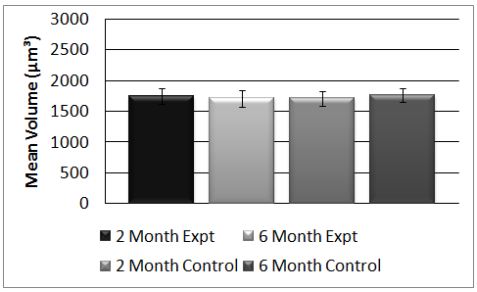
A



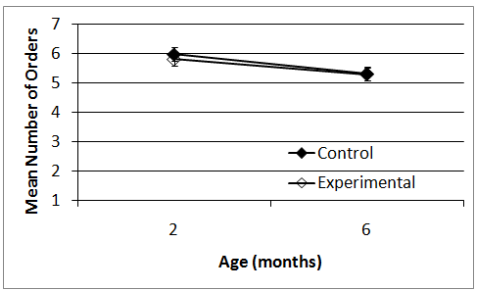
B



C



D



E

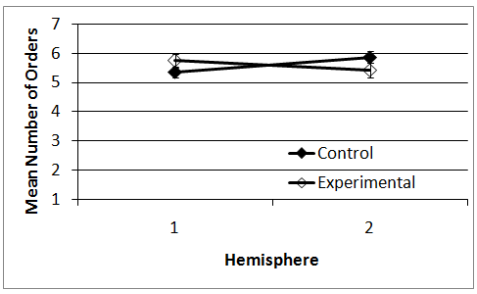
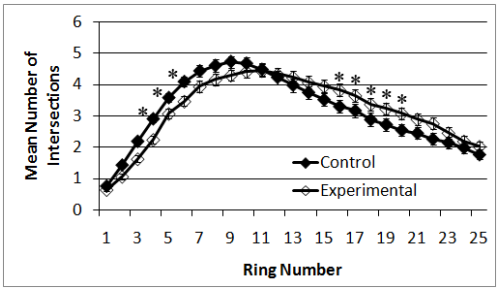
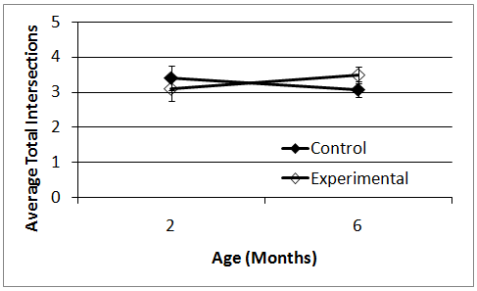


Figure 18

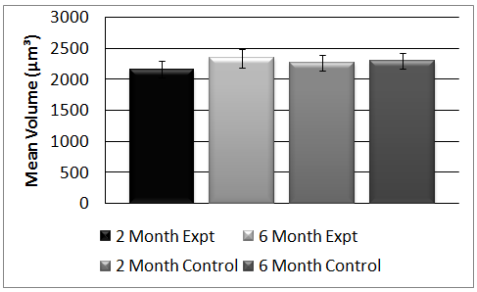
A



B



C



D

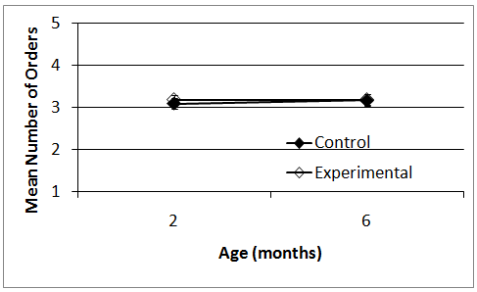
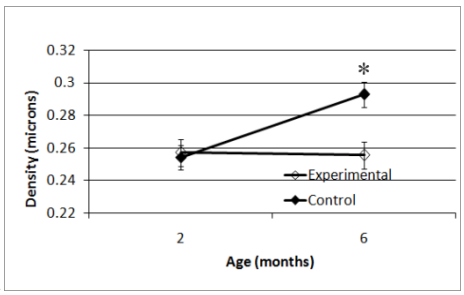
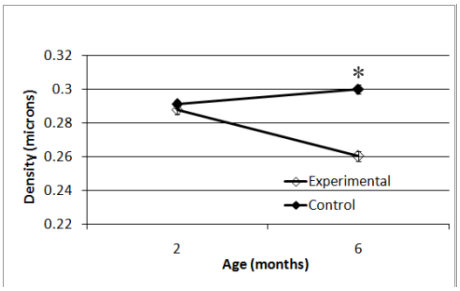


Figure 19

A


0.32
0.3
0.28
0.26
0.24
0.22
Density (microns)
*
Experimental
Control
2
6
Age (months)

B


0.32
0.3
0.28
0.26
0.24
0.22
Density (microns)
*
Experimental
Control
2
6
Age (months)