ENVIRONMENTAL AND PHARMACOLOGICAL INTERVENTION FOLLOWING CORTICAL BRAIN INJURY

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DEDICATION

To my husband, Timothy Hastings who has provided me with constant support and encouragement at times when I have needed it the most.

ABSTRACT

This thesis focuses on the effects of pharmacological and environmental interventions following perinatal prefrontal cortex lesions. Rats given postnatal day 3 medial prefrontal cortex lesions were provided with one of the following treatments: basic fibroblast growth factor (bFGF), complex-housing, tactile stimulation, or a combined treatment of both bFGF and tactile stimulation or bFGF and complex-housing. Rats given postnatal day 3 orbital prefrontal cortex lesions were housed in a complex environment. The findings of these studies suggest that bFGF, complex-housing or tactile stimulation are beneficial after early brain injury. The combined treatment of bFGF with complex-housing provides a synergistic effect, as the combined condition is more advantageous than bFGF alone. In contrast, the combined treatment of bFGF with tactile stimulation produced adverse effects. These results suggest that pharmacological and environmental manipulations change cortical plasticity and therefore functional recovery after neonatal cortical injury.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
C	Control
Cg	Cingulate cortex
E	Embryonic day
FGF-2	Basic fibroblast growth factor
Fr	Frontal cortex
IL	Infralimbic cortex
LO	Lateral orbital cortex
LSD	Least significant difference
LTP	Long term potentiation
MF	Medial prefrontal cortex
mg	Milligrams
ml	Milliliters
MO	Medial orbital cortex
NA	Not applicable
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NT	No-treatment
OF	Orbital prefrontal cortex
P	Postnatal day

PRh   Perirhinal cortex

SE   Standard error

TS   Tactile stimulation

VO   Ventral orbital cortex

ENVIRONMENTAL AND PHARMACOLOGICAL INTERVENTION FOLLOWING CORTICAL BRAIN INJURY

1. GENERAL INTRODUCTION

It is difficult to assess the exact number of people who acquire brain injury, but a conservative estimation suggests that every year approximately one million Americans, will sustain a disability due to traumatic brain injury. Although a substantial number, this statistic is likely an underestimation of the actual number, as it does not include brain injury acquired through stroke, genetic or insult during development. Impairments produced by injury may be minor, but may also be very debilitating, affecting language, cognitive, and motor skills all of which may incapacitate the individual. Not only is brain injury devastating to the individual, but family and society in general are also affected. For example, hospitalization is costly to government funding, as well as insurance and private funds. For traumatic injury alone, 48.3 billion dollars is spent each year in the United States to cover costs for neurosurgery, hospitalization, therapy, and the patient's loss of employment (International Brain Injury Association, 2001).

Although it would seem logical to provide a treatment that would reduce the symptoms in brain-injured individuals, this is difficult to accomplish as each individual has unique symptoms based on personal circumstances. The location and extent of injury, age at the time of injury, sex, as well as other factors such as experience prior to the injury all contribute to the functional outcome. In order to tailor a specific rehabilitative program, the influence of these factors must be taken into consideration. Although much is known in regards to the 'rules' governing spontaneous functional recovery after brain injury, less is known of the influential factors that promote recovery of function. The goal of this thesis is to examine the role of pharmacological intervention and experience in influencing recovery from perinatal prefrontal cortex injury. I begin with a general discussion of the brain's capacity for change in response to such events (a

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process referred to as brain plasticity) and the possibility of using this knowledge to enhance recovery following prefrontal cortex injury using the rat as a model.

1.1. QUESTIONS ADDRESSED IN THIS THESIS

This thesis is comprised of four experiments that examine the effects of enriched environment and basic fibroblast growth factor (bFGF) following either perinatal medial or perinatal orbital prefrontal cortex lesions. Experiment 1 was designed to study the effect of bFGF in conjunction with tactile stimulation after early medial frontal cortex lesions to determine whether the combined treatment would have greater benefits than either administered alone. Experiment 2 examined the effects of a high dose of bFGF following postnatal day 3 (P3) medial frontal cortex lesions. Experiment 3 investigated the synergistic effect of complex-housing and bFGF administration relative to either condition alone after P3 medial frontal cortex lesions. Finally, Experiment 4 looked at the effects complex housing following P3 orbital frontal cortex lesions.

Understanding how possible treatments, such as pharmacological manipulations or behavioral therapies, influence recovery of function following injury, will aid in designing therapies appropriate to the individuals needs, thus maximizing recovery of function.

1.2. BRAIN PLASTICITY

Underlying the principles of neuroscience is the assumption that the connections and functioning of the brain reflect behavior. The capacity of the brain to structurally change is known as plasticity, and occurs in response to experience. Experience is a general term encompassing a number of events including sensory stimulation, stress gonadal hormones, neurotrophins, drugs and brain injury, each of which can modulate brain morphology (Kolb, 1999; Johansson, 2000; Kolb & Whishaw, 2001). Changes involved in plasticity include synaptic change through the modulation of neuropil and

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spine density, changes in chemical messengers, and increased cell production such as neurogenesis and gliogenesis. The greater the availability of mechanisms associated with plasticity, the greater the capacity for alterations in connectivity, thereby facilitating recovery of function. Exactly how the brain responds and changes with such experience depends on a variety of factors, such as the age and sex of the individual as well as the location of the acquired brain injury.

According to the Kennard principle, the earlier in life brain injury is incurred the better the behavioral recovery. This principle was based on findings that infant motor cortex lesions in rhesus monkeys did not produce as debilitating behavioral deficits as similar lesions acquired in adulthood (Kennard, 1936). Although the Kennard principle accounts for a large proportion of cases of early brain injury in children, it is clear that there are caveats to this general concept (e.g. Nonneman and Isaacson, 1972).

Kolb and his colleagues have shown that injury to the developing brain has functional and anatomical consequences that are tightly linked to precise developmental stages. For example, lesions administered prenatally during the period of neurogenesis, E18 in the rat, will lead to sparing of function when later tested on both cognitive and motor tasks. Yet, morphological irregularities that included abnormal clusters of cortical neurons and atypical patterns of white matter were present. Therefore, although development was distorted in the brain, retained functions suggest that the brain was able to compensate for the injury (Kolb et al, 1998a).

Furthermore, lesions acquired around postnatal day 10, do not lead to as severe behavioral impairments as compared to the effects of similar lesions acquired immediately after birth or later on in adulthood. Subjects with day 10 lesions thus display spontaneous recovery of function that is correlated with anatomical changes within the remaining cerebral cortex. These changes include increased dendritic arborization and spine density in tissue neighboring the lesion reflecting a change in

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cortical circuitry and in the number of synapses (Kolb & Gibb, 1991b; Kolb et al, 1998b; Kolb et al, 2000).

Lesions acquired earlier in development such as on postnatal day 3, however, do not show spontaneous functional recovery, and instead have worse functional outcome than similar lesions given later in development (i.e. on day 10). This poor functional outcome is correlated with cellular atrophy in both dendritic arborization and spine density, a shrunken thalamus due to lost connections with the cortex, and a greater reduction in brain weight and cortical thickness than is typically found after P10 or adult lesions (Kolb & Gibb, 1990; Kolb, 1987; Kolb & Cioe, 2000).

Therefore, although it was initially thought that earlier brain injury is associated with better functional recovery, this is not always the case. Instead, behavioral recovery following injury depends on the developmental stage of the brain at the time of lesion, rather than the actual postnatal age of the animal (Figure 1. 1). The developmental stage of the brain at the time of injury will dictate the mechanisms available for brain plasticity and ultimately the behavioral outcome. This critical maturational period occurs at around E18 and between P7-12 in rats, prenatally in monkeys and around birth in cats. Injury acquired outside of the critical maturational period will lead to poor behavioral outcome and neuronal atrophy (reviewed in Villablanca & Hovda, 2000).

In order to fully understand what periods of development are considered a time of low plasticity and at what time the brain expresses high plasticity, a background regarding brain development is required.

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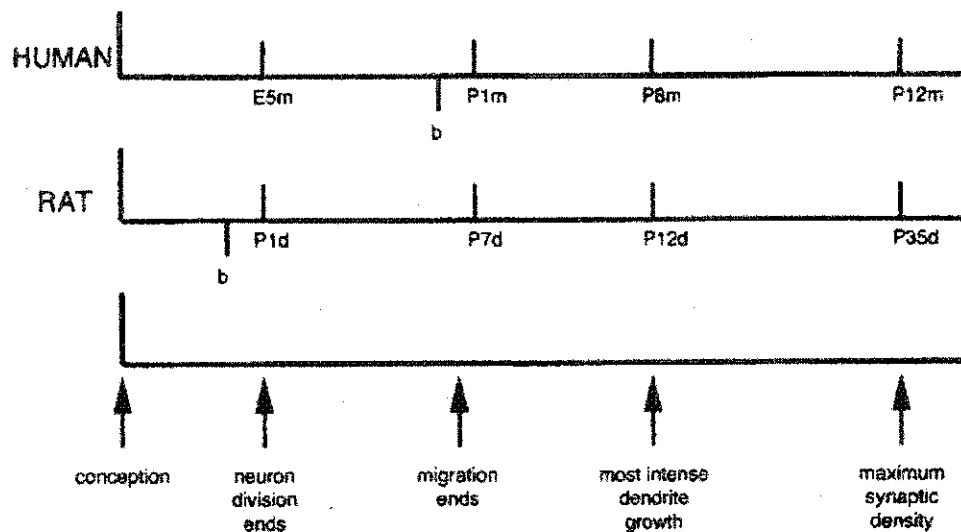
Figure 1. 1. A comparison of the stages in brain development of the human and rat. E = embryonic day; P = postnatal day; and b = day of birth (From Kolb et al, 2000).

1. 2. 1. Brain development and cortical plasticity

Initially, the central nervous system is a flat sheet of cells that folds over to form the neural tube. Following closure of the neural tube, cell mitosis, cell migration, differentiation, dendritic and axonal growth, synaptogenesis and cell death of excess synapses take place in sequence. In the rat, cortical neurogenesis is primarily a prenatal event that occurs between embryonic day (E) 14-20. Following mitosis, undifferentiated progenitor cells migrate from the subventricular zone to the destined location within the developing cortex in an inside-out fashion, such that new migrating cells must travel through layers of previously migrated cells that were generated earlier in development (Bayer & Altman, 1991; Uylings et al, 1990). Migration that ends at approximately P7 also occurs in a transverse manner as ventral areas of the cortex receive migrating cells earlier than dorsal areas of the cortex (Bayer & Altman, 1991).

Although neurons and some glia are present at birth, astrocytes, which play an important role in synaptic plasticity, are not generated until the second postnatal week (see Figure 1. 2). Aside from astrocytic proliferation, dendritic proliferation in the cortex is also peaking between P10-15. As this is a time of optimal functional recovery and brain plasticity in the rat it suggests that astrocytosis and synaptic formation may play a crucial role with this recovery. The period directly following, P15-P30 is also highly plastic corresponding to synaptic pruning and cell death that are modified by experience. Astrocytic and dendritic growth promotes an exuberant overproduction of synaptic contacts that are molded by internal or external factors (Uylings et al, 1990; Kolb, 1995).

In summary, E18, a time of neurogenesis is associated with high cortical plasticity. Whereas P1-P7 is associated with low cortical plasticity and is correlated with no neurogenesis, peak cell migration and the absence of cortical astrocytes. A second period of high cortical plasticity is found from P7-12 and is correlated with synaptogenesis, apoptosis, and gliogenesis. Once these developmental processes are complete, cortical plasticity gradually declines. The level of spontaneous plasticity declines in adulthood, and even more so in senescence.

1. 2. 2. Sex differences in brain plasticity

Perhaps one of the earliest prenatal experiences the developing brain is exposed to are gonadal hormones. Around the time of birth and shortly after, gonadal hormones play an active role in brain development. These hormones strongly influence brain morphology in a variety of brain structures and has been attributed to the underlying behavioral differences between males and females. For example, in the prefrontal cortex males have greater dendritic branching in the medial subregion, whereas females have an increase in dendritic branching in the orbital subregion. Elimination of testosterone by castration abolished the sex differences in cell morphology (Kolb & Stewart, 1991).

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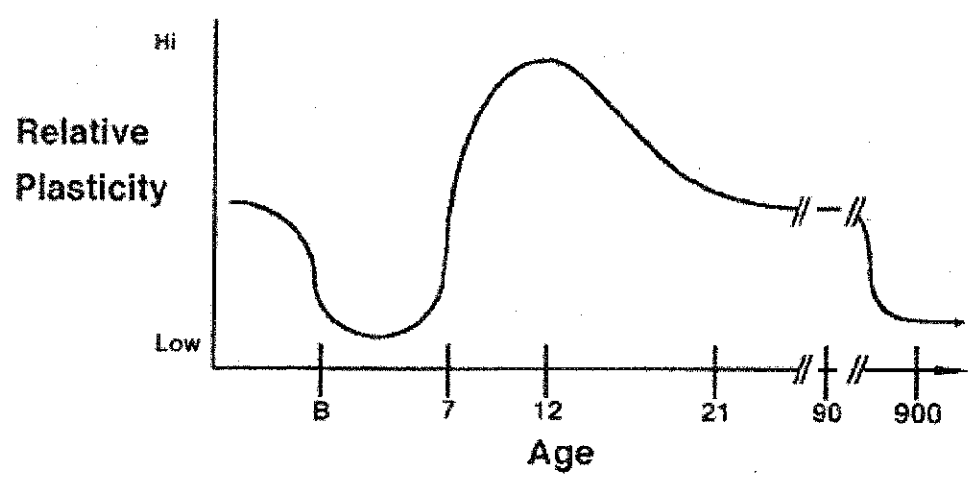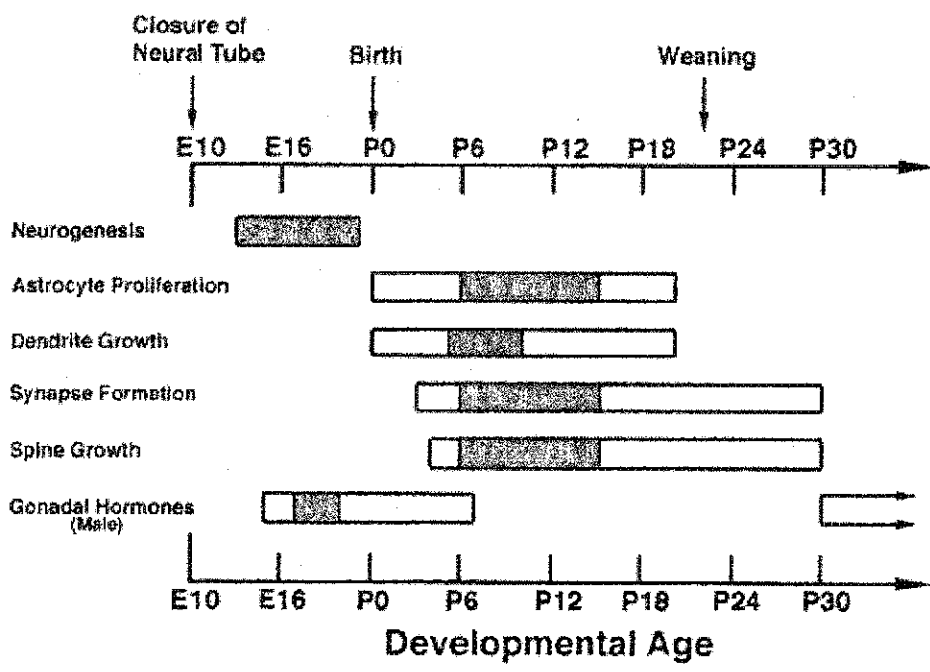
Figure 1. 2. Developmental plasticity of the rat brain. Top: Cellular events associated with cortical plasticity. Bars represent developmental period of each cellular event. Shaded regions on bars represent peak periods of the developmental events. Bottom: Optimal postnatal plasticity is found between postnatal day 7 and 15. Periods earlier and later in development have lower levels of plasticity (From Kolb, 1999).

The anatomical differences acquired prenatally have a later affect in responsiveness to other experiences. Indeed, research by Juraska and colleagues (e.g. Juraska et al, 1985; Juraska, 1984) demonstrated that males and females respond differently to specific experiences. Generally, males are more vulnerable to circumstances such as malnutrition, isolation, stimulation and parental care. Regardless of whether particular experiences are to their advantage or disadvantage, male brains are typically influenced by these experiences more than females, such that Sackett has termed females as the "buffered sex" (for review see Juraska, 1986). Therefore to examine experiential effects on brain structure and function sex is a consideration.

1. 2. 3. The rat as a model of cortical plasticity

Work by a variety of investigators over the past 25 years has shown that the rat is an excellent model for investigating brain plasticity during development as the rat has the advantage that it is born very immature relative to monkeys or humans. For instance, P3 in the rat pup corresponds to a developmental stage in the third trimester in humans correlated with poor functional recovery (Figure 1.1). Because brain development advances through the same phases and in the same sequential order in all species, the P3 rat allows access to perturb or stimulate the developing brain ex-utero. Also, the underlying mechanisms of plasticity are identical across species and therefore mechanisms found to enhance or disrupt cortical plasticity in the rat brain may also be applied to the human brain (Rakic, 1991).

1. 3. PHARMACOLOGICAL INTERVENTION

1. 3. 1. The neurotrophin hypothesis

One way of promoting an increase in brain plasticity is through pharmacological intervention, such that the chemical environment may be optimized to allow for maximum recovery of function following brain injury. Neurotrophins such as nerve

growth factor (NGF), brain derived neurotrophic factor (BDNF), NT-3 and NT-4/5, and neurotrophic factors such as basic fibroblast growth factor (bFGF), may be possible candidates in modifying brain plasticity, and therefore facilitate recovery of function after brain injury. The underlying principle of this assumption is known as the neurotrophin hypothesis (reviewed in Thoenen, 1995; Schinder & Poo, 2000). The neurotrophin hypothesis suggests that neuronal activity is the underlying factor to the expression; secretion and action of neurotrophins in modifying connectivity within the brain (Figure 1.3).

The mechanism by which neuronal activity translates into synaptic changes within the brain is still unclear, however, there are two general ways that neurotrophins may act. First, neurotrophins may directly influence synaptic morphology by producing changes in the synapse itself. Second, there may be other factors that modify the synapse following neuronal activity, and the role of the neurotrophin is to provide maintenance functions in order for synaptic plasticity to occur. Although both prospects are possible, we are unsure at this time of how each neurotrophin responds to different regions of the brain, at different ages, with different levels of activity and so on (Schinder & Poo, 2000). For example, whereas NGF is found in striatal and basal forebrain cholinergic neurons, other neurotrophins such as BDNF and bFGF (although not classified as a neurotrophin per se, has many neurotrophic properties), are widely distributed throughout the brain, especially in regions of synaptic plasticity (McAllister et al, 1999, Riva & Mocchetti, 1991).
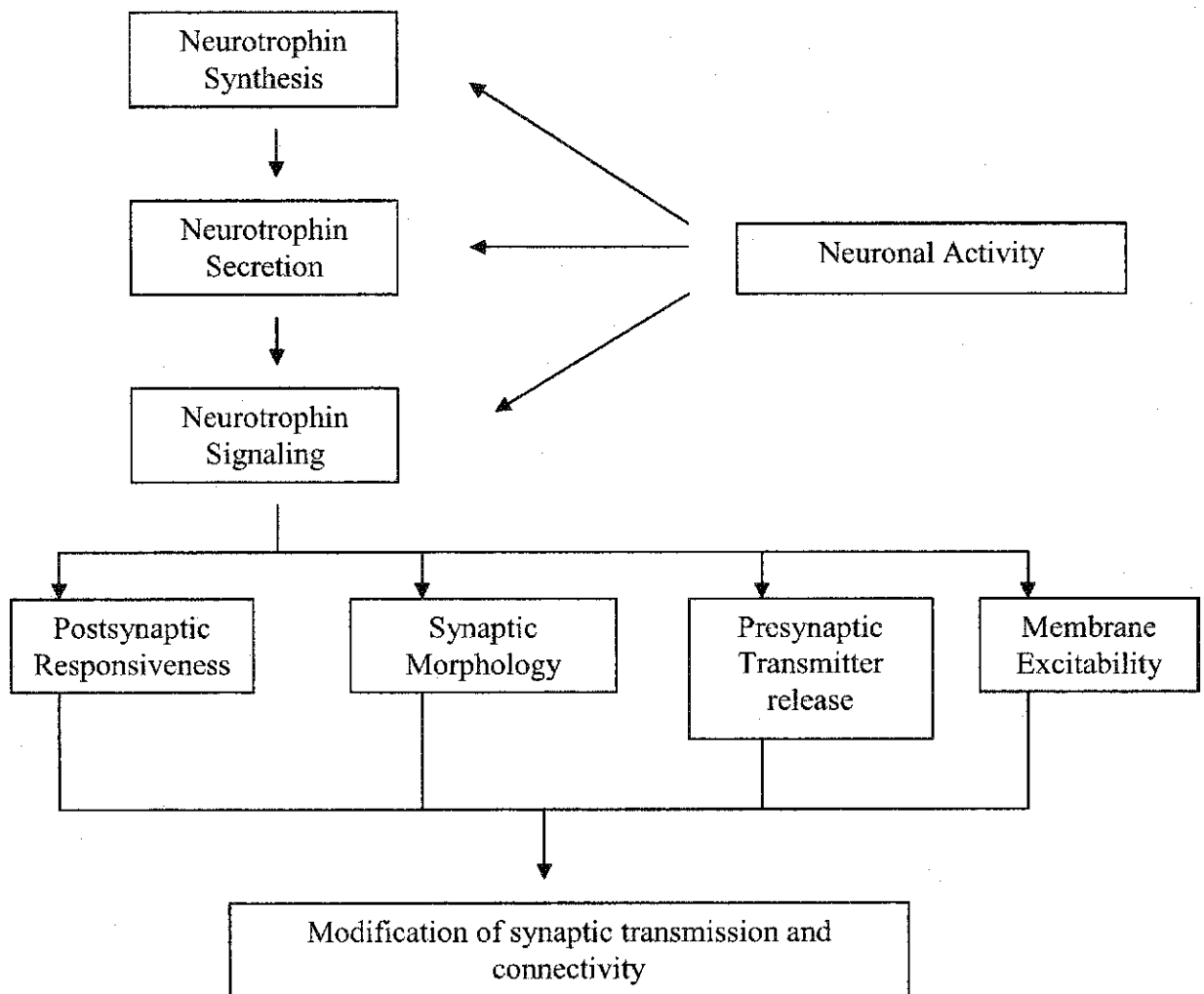
Figure 1. 3. The neurotrophin hypothesis, demonstrating how neuronal activity may influence synaptic transmission and connectivity (From Schinder & Poo, 2000).

1. 3. 2. Basic fibroblast growth factor (bFGF)

Known for its broad spectrum of functions, bFGF (or FGF-2) is an endogenous growth factor located throughout the peripheral and central nervous system. bFGF has been found in neurons, glia, membranes of blood vessels, and in ventricular ependymal cells. Unlike some growth factors such as NGF, bFGF in the mature organism is widely

distributed throughout the brain and found in high levels in the hippocampus, cerebral cortex, striatum and spinal cord, intermediate levels in the hypothalamus, olfactory bulb, and brain stem, and low levels are detected in the cerebellum (Riva & Mocchetti, 1991; Finklestein et al, 1988).

bFGF binds to two main types of receptors within the nervous system. The first is a high-affinity tyrosine kinase receptor. Binding to this receptor will induce changes in gene expression in the cell, producing proteins that may ultimately contribute to synaptic plasticity. A second, low-affinity receptor is the heparin sulphate proteoglycan receptor located in the extracellular matrix (ECM). When bFGF is released into the ECM the growth factor is in an inactive form. As bFGF binds to the heparin sulphate proteoglycan receptor, the bFGF is transformed into an active state and presented to the high-affinity tyrosine kinase receptor (Ay et al, 1999; Eckenstein, 1994).

The actions of bFGF are pleiotrophic, promoting cell proliferation and differentiation, survival of cells, and neurite outgrowth of hippocampal and cortical neurons (e.g. Morrison et al, 1986; Walicke et al, 1986). Angiogenesis (formation of blood vessels) and fibroblast proliferation also occurs, and has been linked to the repair of wound healing in peripheral tissue and in the central nervous system (Folkmar & Klagsburn, 1987).

1. 3. 3. Possible mechanisms of action for bFGF in the injured adult brain

Following injury in the adult brain, astrocytes and microglia invade the lesion cavity. As the astrocytes become hypertrophied and adapt a fibrous appearance with thicker and longer processes, these astrocytes become "reactive" and form a glial scar along the periphery of the lesion cavity. Two days after injury, an increase in bFGF immunoreactivity is located within the lesion cavity and this increase peaks seven days after injury (Rowntree & Kolb, 1997; Eclancher et al, 1996). The abundance of bFGF is

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produced by the reactive astrocytes, or according to the "leakage hypothesis", bFGF leaks out of the damaged neurons into the extracellular space (e.g. Eclancher et al, 1996).

A cascade of actions occurs once bFGF binds to the receptors as described above. Following brain injury, a homeostatic imbalance occurs within the brain causing an increase in apoptotic proteins, free radicals, intracellular calcium, and glutamate. All of these events are toxic to the brain and will cause further damage to the tissue. The general effects of bFGF works by counteracting these changes, and induces RNA transcription or protein synthesis in the cell to produce antiapoptotic proteins, antioxidant enzymes, calcium binding proteins, and reduces the NMDA receptor protein, all of which are considered neuroprotective actions (Ay et al, 1999; Mattson & Cheng, 1993).

Studies analyzing the effects of exogenous bFGF administration further support the neuroprotective role of bFGF. For instance, following a fimbria-fornix transaction, the exogenous administration of bFGF prevents cholinergic neurons from dying (Anderson et al, 1988). A reduction in infarct size if bFGF is given immediately following the injury has also been found, and is thought to be due to bFGF saving neurons along the periphery of the lesion site that would normally die within a short time following injury (Fisher et al, 1995; Kawamata et al, 1996; Bethel et al, 1997). Angiogenic effects also contribute to this effect, as bFGF enhances the formation of new blood vessels into the lesion area thus providing oxygen and nutrients to the cells (Kawamata et al, 1996). In order for bFGF to save these neurons, it is crucial that this growth factor is present immediately after injury as waiting twenty-four hours before bFGF administration has not shown a reduction in infarct size and therefore injured neurons found along the periphery of the lesion cavity have already died. Importantly, although there appears to be a critical twenty-four hour period (or less) for bFGF to preserve neurons in the penumbra, the secondary effects of stroke such as atrophy of thalamic inputs, may be hindered by exogenous bFGF administered past twenty-four hours (Yamada et al, 1991).

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Behavioral changes are also associated with both endogenous and exogenous administration of bFGF. For example, recovery of function has occurred after bFGF administration; however, the effects are most prominent on sensorimotor tasks and less prominent on reflex or postural function (Kawamata et al, 1996). Gomez-Pinilla and Kesslak (1998) quantified the amount of endogenous bFGF following learning (e.g. Morris water task) and exercise within the hippocampus, cerebellum and cerebral cortex. In both groups an increase in bFGF was detected, and was especially pronounced in the learning group, suggesting a role of bFGF for circuit modification required for memory formation. Rowntree and Kolb (1997) also studied the effects of blocking endogenous levels of bFGF with neutralizing antibodies following unilateral motor cortex injury. With this type of injury rats typically express an endogenous increase of bFGF followed by a slow recovery of forelimb use. With bFGF neutralizing antibodies, however, there was not an increase in bFGF, nor was there recovery of forelimb use. Atrophy of layer V pyramidal cells in the remaining motor cortex was found in both dendritic branching and a decline in spine density when compared to lesion subjects that did not receive neutralizing antibodies to bFGF. Therefore, an increase of bFGF is important in facilitating both cellular and behavioral modifications associated with learning, memory and behavioral recovery.

The actions of bFGF may directly affect synaptic plasticity or may work indirectly by regulating NGF, as studies have found that bFGF modulates and enhances NGF mRNA expression in both glia and neurons (Ferhat et al, 1997; Yoshida and Gage, 1991). Nerve growth factor is a well characterized neurotrophin that plays a role in survival and differentiation of cells in the developing and adult brain. In particular, the cholinergic system originating in the basal forebrain is regulated and develops with the help of NGF (Calamandrei and Alleva, 1995; Hefti et al, 1990).

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1. 3. 4. bFGF in the injured developing brain

Although the postnatal expression of bFGF is higher in the adult brain, in the developing brain, bFGF has been detected in the telencephalon as early as embryonic day 9.5 and in the cortex during the developmental period of neurogenesis (Temple and Qian, 1995). Depending on the developmental stage, bFGF plays diverse roles in supporting brain function. In the developing brain, bFGF is considered important for regulating neuronal production (Tao et al, 1996; Gomez-Pinilla et al, 1994). Unlike adulthood, injury in the newborn rat does not lead to the production of a glia scar as the immature cortex lacks the presence of astrocytes until postnatal day 7. Therefore, following injury bFGF that is present in the lesion cavity is usually attributed to microglia or neuronal production of bFGF (Gremo & Presta, 2000).

The behavioral and anatomical consequences of bFGF following perinatal injury in the rat are poorly understood and lack evidential support. Currently, unpublished studies by Kolb, Gibb, Schimanski and West (2001) have found that bFGF facilitates functional recovery following P3 medial prefrontal cortex injury. Subjects treated with bFGF for seven consecutive days beginning twenty-four hours after injury significantly improved water task performance, and in some circumstances forelimb reaching skills were improved. There appears even during development to be an age-dependent effect of bFGF as the beneficial effects of bFGF was found following P3 lesions a time of low brain plasticity. There was little or no benefit of bFGF following P10 lesions, a time of high brain plasticity.

1. 4. ENVIRONMENTAL ENRICHMENT

Typically, lab animals are housed in a relatively impoverished environment. For example, a rat may be housed with one or two other subjects in a 46 X 23 cm cage, with little inside the cage other than food and access to a water spout. In an enriched or complex environment, however, not only is there more vertical and horizontal space for

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mobility, there is also an enhanced social environment, and increased sensory

stimulation. Auditory stimuli provided by a radio playing, and interactions with novel

objects such as ropes, beams, tunnels and other objects are common. A key feature to a

complex environment is novelty, as changing stimuli weekly will encourage curiosity and

therefore increase interactions with the environment (Figure 1.4).
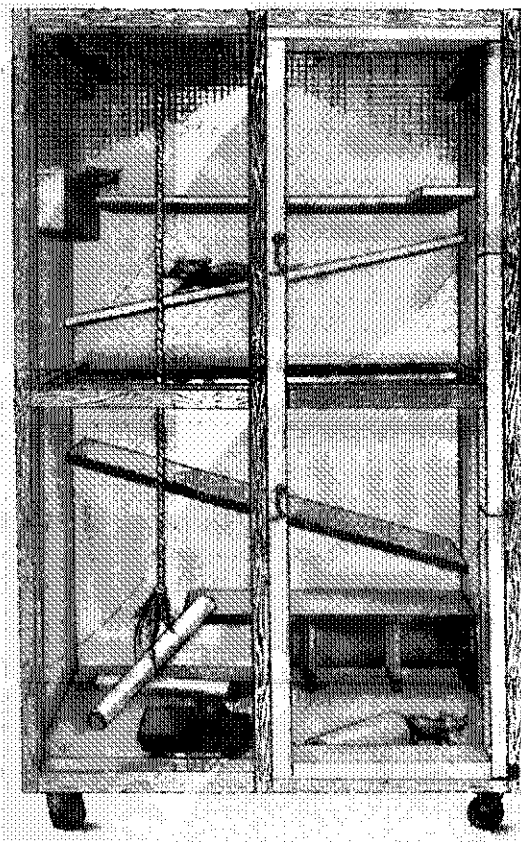
Figure 1. 4. Complex-housing provides enhanced sensory stimulation through several sensory modalities. This stimulation has been associated with increasing synaptic plasticity (Kolb & Whishaw, 2001).

The importance of an enriched environment to the developing and adult brain

cannot be overstated. In 1928, Ramon y Cajal first presented the idea that experience

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has the ability to modify the brain. However, it wasn't until 1947 that this hypothesis was tested by Donald Hebb, who brought home rat pups for his children as pets. Throughout their duration in his home, the rats roamed freely and when later returned to the lab, Hebb found that these rats performed better at maze learning than rats living in standard laboratory cages. This suggested that living in an enriched environment promoted an improved performance of cognitive tasks. Later, studies by Rosenzweig and colleagues found that complex/enriched environments as well as training facilitate behavioral changes as well as chemical and anatomical alterations in the brain. Since these early studies, there has been a massive body of research demonstrating the behavioral and anatomical affects of an enriched environment in both non-lesion and lesion subjects.

1. 4. 1. Environmental enrichment in the non-lesion subject

Initial studies using enriched environments studied subjects with intact brains. When tested on learning and memory tasks such as the Morris water task or radial arm maze, complex-housed rats excelled in performance when compared to lab-reared subjects (e.g. Mohammed et al, 1990; Parks et al, 1992). Performance on motor tasks such as traversing a narrow beam is also completed quicker and with greater agility in rats housed in enriched environments than lab-reared subjects (Gentile et al, 1987).

Because an enriched environment influences performance on a variety of both cognitive and motor tasks, Greenough and colleagues (1976) initially suggested that aspects of an enriched environment can act as a training ground for the specific behavior to solve problems. Others have a different perspective on the mechanisms underlying the benefits of an enriched environment. For instance Goldman and Lewis (1978) suggest that an enriched environment affects performance in a non-specific manner. For example, monkeys with orbital prefrontal cortex lesions were pretrained on a non-spatial task. Later, the same subjects were retested on the non-spatial and a novel spatial task. Performance was compared to naïve orbital frontal subjects and it was anticipated that an

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apparent training effect would occur for the non-spatial task. However, subjects were instead still impaired at the non-spatial task whereas an enrichment effect was found on the spatial task. This result suggests that the enrichment effect is non-specific in nature, and is most likely due to an overall general increase in neuronal activity of the brain (Goldman and Medelson, 1977). Further studies have also found an upregulation of metabolic activity via increased mitochondria following enrichment, again supporting the idea of increased activity playing an important role in this process (Sirevaag & Greenough, 1987).

1. 4. 2. Environmental enrichment in the brain injured subject

The establishment of cognitive and motor benefits through enhanced sensory stimulation initiated a series of studies that looked at the benefits of similar stimulation following brain injury. An enriched environment attenuates recovery of function and decreases the deficits in the subjects. For instance, when tested on a battery of behavioral tasks, complex-housing enhanced motor performance in both general motor patterns such as traversing a beam and fine digit movements as found using the Whishaw reaching task (Gentile et al, 1987; Gibb, 2001). Cognitive performance is also enhanced in lesion subjects receiving environmental enrichment regardless of the precise location of injury. For example, ameliorating cognitive deficits by complex-housing has been found following hemidecortication (Whishaw et al, 1984), prefrontal cortex lesions (Kolb & Gibb, 1991a), fimbria fornix lesions (Van Rijzingen et al, 1997), hippocampal lesions (Galani et al, 1997), and occipital lesions (Rose et al, 1993).

1. 4. 3. Anatomical correlates with environmental enrichment

Anatomical changes associated with complex-housing are numerous and correlate with functional enhancement on tasks. An increase in brain size such as heavier brains and thicker cortices are found after exposure to an enriched environment (Diamond et al,

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1966; Rosenzweig & Bennet, 1996; Rosenzweig et al, 1968). Cellular changes such as increases in neuron size, decreased apoptosis and enhanced neurogenesis (Young et al, 1999; Kempermann et al, 1997), gliogenesis (both astrocytes and oligodendrocytes) (Diamond et al, 1966), as well as an increase in the nuclear size of astrocytes, synaptic vesicles in the synapse and an increase in dendritic spines and arborization has been documented (Greenough et al, 1973a; 1973b; Nakamura et al, 1999; Greenough et al, 1985; Kolb et al, 2003). Enhanced levels of neurotransmitters such as noradrenaline (Naka et al, 2002) as well as neurotrophins such as NGF and bFGF have also been found (e.g. Pham et al, 1999; Torasdotter et al, 1998; Rowntree, 1995). The anatomical changes described above act throughout the brain as many different structures both cortical and subcortical are influenced by an enriched environment, including but not limited to the superior colliculus (Fuchs et al, 1990), hippocampus (Diamond et al, 1976; Kempermann et al, 1997; 1998), and cerebral cortex (e.g. Kolb & Gibb, 1991a, Diamond et al, 1964).

1. 4. 4. Factors influencing functional recovery following environmental enrichment

Although enrichment effects are less understood in lesion animals, there is a general consensus that this type of treatment is beneficial to some forms of functional recovery. The degree to which functional recovery is observed is influenced by factors such as the age of injury, the location and extent of injury, and the temporal proximity of complex-housing to the time of lesion (Goldman & Lewis, 1978). Each of the following will be discussed in turn.

The age of injury affects the impact that the complex-housing will have on functional recovery. For instance, rats with P5 medial frontal cortex lesions benefit to a greater extent from an enriched environment than P1 lesion animals (Kolb & Elliot, 1987). As previously discussed, P7-P12 is a time of high plasticity and spontaneous recovery of function. The spontaneous recovery appears to be at an optimal level, however, as there is little or no functional improvement following complex-housing.

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Not only does the age of acquired injury affect possible recovery, but also the age when first exposed to an enriched environment. Kolb and colleagues (2002) found that offspring from mothers housed in a complex environment during pregnancy demonstrated better recovery of function following perinatal lesions than offspring from lab-reared mothers. Environmental enrichment appears to be more advantageous when experienced at a young age rather than in adulthood or in senescence (Gibb, 2001). Although the reason for this phenomenon is unclear, it has been proposed that the immature nervous system is less restricted in terms of neuronal connectivity organization and therefore may be influenced to a greater extent by the early experience. With the earlier exposure, not only are subjects more active and interact to a greater extent with the complex-housing environment, thus promoting better functional recovery after injury, there are also anatomical changes that are different in these cases than the changes found in adults facing similar experiences. For example, complex-housing as juveniles causes a decrease in spine density, whereas in adults the spine density increases (Kolb et al, 2003).

The location of injury also influences the brain's ability to compensate in response to enhanced sensory stimuli. For example, animals receiving lesions of the motor cortex that sever cortico-spinal projections continue to show reaching and other fine digit motor impairments despite environmental enrichment. In contrast, lesions of the medial prefrontal cortex benefit from complex-housing as there are improvements on cognitive tasks (e.g., Kolb, 1995; Gibb, 2001).

Similarly, if lesions are too extensive such that critical areas normally compensating for the lesion tissue are injured, little functional recovery will occur (Kolb & Gibb, 1990). For example, extensive postoperative training administered to monkeys following early orbital frontal lesions produce functional recovery on a spatial task. Removal of the dorsolateral prefrontal cortex (equivalent to medial prefrontal cortex in the rat) and orbital prefrontal cortex impedes recovery (Miller et al, 1973; Goldman, 1976).

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1. 4. 5. Tactile stimulation as a form of environmental enrichment

In addition to complex-housing as a means of providing an enriched environment, tactile stimulation may also be beneficial. First used on premature human neonates, it was recognized that tactile stimulation on infants promoted an increase in body weight, alertness and orientating behavior, such that they were released from the hospital earlier than premature neonates that did not receive tactile stimulation (e.g. Field et al, 1986).

In rats, early postnatal tactile stimulation is produced through the stroking of rat pups with a soft camel hair paintbrush beginning on postoperative day 1 until weaning (Figure 1. 5). The advantage of tactile stimulation over complex-housing at this age is that it provides additional sensory stimulation earlier in development. Pups primary senses used at this age include tactile sensation, olfaction and audition. It is believed that by applying tactile stimulation to the pup there is an increase in sensory information and processing in the brain, and thus an increase in general neuronal activity, much like complex-housing in older rats. In addition, Gibb (2001) proposed that stimulating the skin increases the skin's production of bFGF that influences the brain by enhancing cortical plasticity. Benefits of tactile stimulation reported by Gibb include enhanced cognitive as well as motor performance.
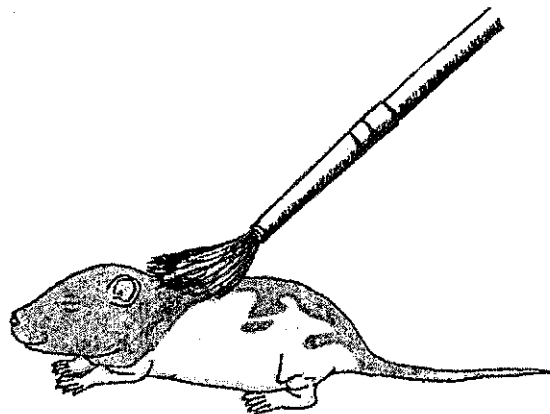
Figure 1. 5. Tactile stimulation is a sensory stimulating treatment administered prior to weaning in rat pups (Gibb, 2001).

1. 5. PREFRONTAL CORTEX

The prefrontal cortex is defined as tissue that receives projections from the mediodorsal thalamic nuclei. Composed of two distinct regions, namely the dorsolateral prefrontal cortex, also known as the medial prefrontal cortex in rats, and the orbital/ventral frontal cortex (see Figure 1. 6). Although both subregions are considered prefrontal cortex, they differ in terms of their development as the orbital cortex matures approximately 1 day earlier than the medial cortex (Bayer & Altman, 1991). In addition to connections between the two regions, each region has unique and specific connections with other structures. These differences in terms of connectivity influence the overall function of each subregion (Kolb, 1984; Carmichael & Price, 1994; 1995). As the focus of this thesis includes both the medial and orbital prefrontal cortex, each region shall be described below.

1. 5. 1. Medial Prefrontal Cortex

The medial prefrontal cortex in the rat is comprised of infralimbic and anterior cingulate cortex. In addition to receiving projections from the anterior medial, mediodorsal and ventromedial thalamic nuclei, there are a number of other afferents from both cortical and subcortical structures. For instance, the basolateral amygdala sends information to the medial subregion. The parietal (primary and secondary somatosensory cortex), occipital, and temporal cortex also provide the prefrontal cortex with sensory information (i.e. somatosensory, visual, and auditory). The medial frontal cortex, in turn, projects to the posterior cingulate and retrosplenial cortex, medial striatum, pretectum and superior colliculus (for the control of eye movements) (reviewed in Kolb, 1984). As a result, the medial prefrontal cortex in the rat plays a role in a wide variety of behaviors (see Table 1.1). For example, lesions often lead to impairments on tasks of spatial navigation and fine motor skills.

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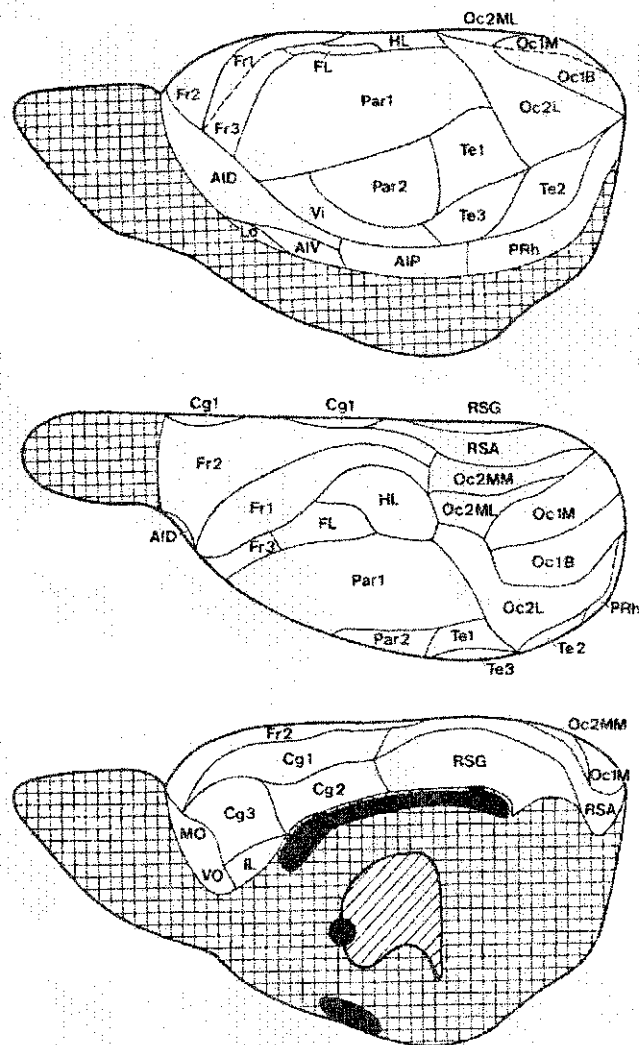
Figure 1. 6. Map of the rat cortex from the lateral (top), dorsal (middle) and medial (bottom) perspective. Abbreviations: Fr1-Fr3 are frontal areas; Cg1-Cg3 are medial prefrontal cortex areas; IL is infralimbic cortex; MO, LO, VO represent orbital frontal cortex; AID, AIP, AIV are insular cortex areas; PRh is perirhinal cortex (From Zilles, 1985).

Table 1. 1. Summary of Deficits Associated With Prefrontal Cortex Lesions

Function	Behavioral symptoms	Area damaged
Motor	Poor reaching	Motor; MF
	Poor execution of mvt chains	MF
	Restricted tongue mobility	OF
Response inhibition	Impaired on tasks requiring changes in behavior	MF; OF
Temporal ordering	Difficulty in ordering mvts	MF
Spatial orientation	Poor learning of spatial tasks	MF
Social; affective	Abnormal social interaction	MF; OF
	Abnormal male sexual behavior	MF
Behavioral spontaneity	Inability to initiate new response strategies	OF
Olfaction	Defective odor identification	OF
Habituation	Impaired habituation	MF
Activity	Hyperactivity	OF
	Exaggerated response to starvation and drugs	OF
	Abnormal circadian rhythms	OF
Homeostasis	Transient anorexia	OF
	Reduced chronic body weight	OF
Contralateral neglect	No response to sensory stimuli	MF

MF, medial prefrontal cortex; OF, orbital prefrontal cortex (From Kolb, 1984).

Dysfunction in spatially-guided tasks was first described by Mishkin and Pribram (in Fuster, 1980), in their studies of monkeys trained on a delayed response task. Initially a reward such as a piece of food was placed under one of two objects in full view of the subject. The monkeys were forced to remember the location of the food for a brief interval before being allowed to respond. Mishkin and Pribram found that if the delay was too long then medial frontal lesion subjects were unable to remember under which object the reward was hidden. Spatial deficits have also been found in rats after receiving prefrontal cortex lesions, on tasks similar to the delayed response (e.g., Aggleton nonmatching-to-sample task) and spatial tasks that do not require a delay (e.g., Morris water task). Underlying the spatial deficits on these tasks may be a deficit that is non-spatial in nature. Therefore rather than an inability to form a cognitive map of the platform location in the Morris water task, subjects may be unable to produce a prosperous search strategy when solving the task. It is also possible that the problem may be attributed to poor temporal memory (Kolb et al, 1994).

A motor deficit such as producing accurate distal movements is common following medial frontal cortex lesions. In rats, this is apparent as there is difficulty in manipulating objects such as food pellets. The primary reason for this deficit is due to lost cortico-spinal projections in motor cortex adjacent to the lesion (Kolb & Whishaw, 1985).

1. 5. 2. Orbital Prefrontal Cortex

The rat orbital frontal cortex is comprised of the orbital and insular areas (Figure 1.6). In addition to receiving projections from the mediodorsal thalamic nucleus, projections from the anteromedial and ventromedial nucleus also occur (e.g. Kolb, 1984). The orbital frontal cortex receives highly processed sensory information from many different cortical areas in the brain. For instance, olfactory information is received via

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the anterior olfactory nuclei and pyriform cortex (Zald & Kim, 1996). Somatosensory information regarding facial and digit information is received from area SII and the insula (Whishaw & Kolb, 1983; Carmichael and Price, 1996), whereas visual information regarding object and pattern recognition via the ventral stream is projected from the inferior temporal region TE (Barbas, 1988). Reciprocal connections are also found with limbic structures such as the amygdala, entorhinal cortex, perirhinal cortex and subiculum (Price, 1999). Autonomic responses such as eating, drinking, heart rate, and respiratory rate are associated with projections from the orbital frontal cortex to the hippocampus, amygdala and hypothalamus (Kolb & Nonneman, 1976).

The orbital subregion in rats is involved in behaviors unique from the medial subregion, and lesions to the orbital prefrontal cortex will produce a variety of deficits on these behaviors (Table 1.1). For instance, impairment in social behavior is found as subjects tend to be overly aggressive with one another, and demonstrate an increase in emotionality. Furthermore, lesions administered in adulthood produce lethal disruptions in eating and drinking ability such that many subjects will die if special care is not taken to ensure that they are able to eat (Kolb & Nonneman, 1976).

A cognitive impairment that is found following orbital frontal lesions is impairment in extinguishing a previously rewarding behavior. For example, Kolb and colleagues (1974) trained rats on an extinction task that required pressing a lever to receive a food pellet for reward. After ten days of training, the reward was removed. Control rats learned that pellets were no longer associated with lever pressing, therefore a decrease in the number of lever presses was found over the next few days. Adult rats with orbital frontal lesions, however, did not learn this and continued to press at a much higher rate.

Motor deficits are also found following orbital prefrontal cortex lesions, and the results are again age-dependent. In adults, while there is no impairment in forelimb manipulating abilities, tongue extension ability is hindered. After early brain injury,

difficulties in tongue extension are found although there is partial sparing as early lesions do not produce as severe a deficit as a similar lesion in adulthood. Interestingly, a new deficit emerges after early lesions, which is the inability to use forepaws in food manipulation. The mechanism for this impairment is unknown because the orbital frontal cortex does not have direct cortico-spinal projections important for forelimb use. The impairment therefore may be non-motor in nature (Whishaw & Kolb, 1983).

1. 6. SUMMARY

Cortical plasticity following injury is influenced by a variety of factors including age of injury, location of injury, and intervention following injury. The focus of this thesis will be to analyze the effect of both environmental (i.e., TS; complex housing) and pharmacological (i.e., bFGF) manipulations, or a combination of both, following postnatal Day 3 medial or orbital frontal cortical injury.

1.7. GENERAL METHODS AND MATERIALS

The following section describes the behavioral and anatomical analyses used throughout this thesis as well as the rationale for choosing each measure.

1. 7. 1. Behavioral Measures

1. 7. 1. 1. Open Field

In order to measure general activity, movements and response to a novel environment, rats are placed in an open field activity box. There are sensors within this box that measures a variety of movements made in a ten-minute period. Measuring activity in this task is a measure of emotionality and the subject's response through activity to a novel environment. Although rats with medial prefrontal cortex lesions do

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not usually differ from controls on this task, treatments such as an enriched environment have shown to affect performance on this task.

1. 7. 1. 2. Morris Water Task

Created by Richard Morris (1981), the water maze is a cognitive task that requires the use of spatial navigational skills (Figure 1. 7). A large pool is set in the middle of a room surrounded by extramaze cues. The pool is filled with water and skim milk powder is added to make the water opaque. A Plexiglas platform is placed just below the water's surface in a constant position. The rat is then placed in one of four random positions of the pool (north, south, east or west) and the latency to reach the hidden platform is recorded. To date, perinatal lesions of several different cortical areas, such as the medial prefrontal cortex, parietal cortex, motor cortex and visual cortex will result in deficits on this task even if similar deficits are not detected after adult lesions of the same area.
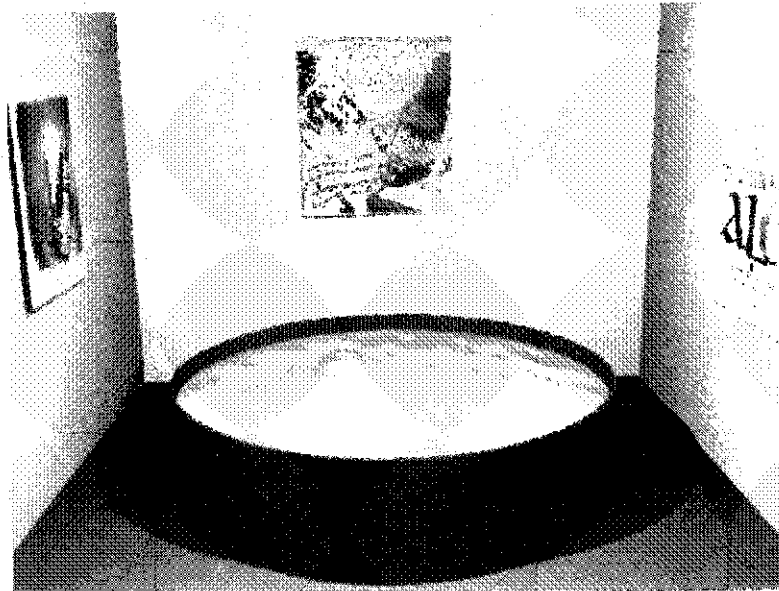
Figure 1.7. The Morris water task. A cognitive task testing spatial navigation.

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1. 7. 1. 3. Whishaw Reaching Task

The Whishaw reaching task has been designed to study the grasping ability of the rat's forepaws (Figure 1. 8). First devised by Whishaw and colleagues (e.g. Whishaw et al, 1986), it consists of a Plexiglas box with vertical bars on one side. In the front is a tray filled with chicken feed. In order to gain access to this food, rats are food deprived to 85% their own body weight to motivate them to reach through the vertical bars into the tray and grab the feed with their forepaws. To readily obtain feed, subjects must learn to reach through the bars, grab the food and bring it back through the bars to successfully eat it. Rats with lesions (e.g. medial prefrontal cortex, motor cortex) generally have difficulty manipulating food with their forepaws. Severing of direct cortico-spinal projections is responsible for this impairment. Improvement on this task is possible with such manipulations as exposure to an enriched environment, yet this is attributed to compensatory mechanisms as lost cortico-spinal connections cannot be replaced.
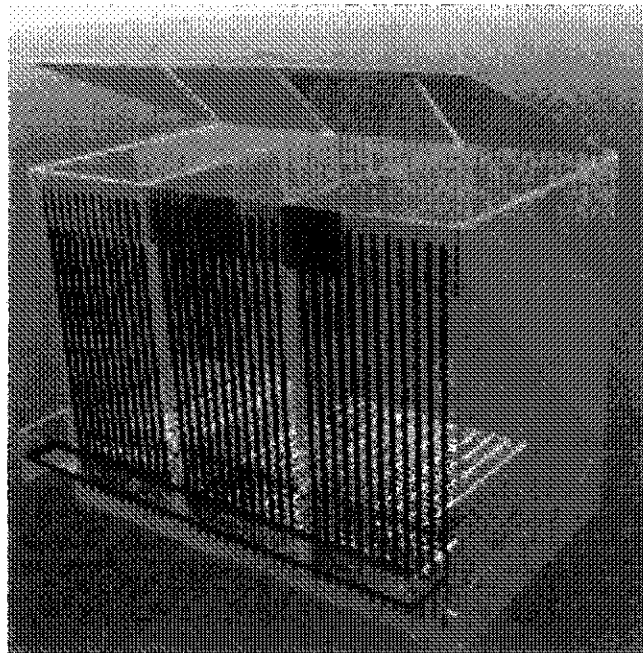
Figure 1. 8. The Whishaw reaching task. Designed to assess the manipulatory skills of the forepaws.

1. 7. 1. 4. Extinction Task

Typically following adult orbital frontal lesions there is a deficit in inhibiting behavior that is no longer rewarding to the organism. This task is designed to extract this deficit as rats are trained to press a lever within an operant conditioning chamber in order to gain access to a small food pellet. As with the Whishaw reaching task, all subjects are food deprived to no more than 85% of the initial body weight, as a motivating factor to learn this task. Once the association has been learned, subjects continue to execute the task for several days in order to reinforce the association between pressing the lever and receiving a pellet. The extinction phase of the task consists of no longer reinforcing the lever press behavior with a pellet. Typically, when tested over a period of five days, control rats learn that pressing the lever is not associated with receiving the reward and thus as each day progresses, presses the lever less and less. Orbital frontal lesion animals, however, do not learn that the 'rules' of the task has changed and therefore continue to press at a significantly higher rate than controls despite the lack of reward when pressing the lever (Kolb et al, 1974).

1. 7. 1. 5. Tongue Extension

Adjacent to the orbital frontal cortex in the rat is motor cortex responsible for facial and tongue movements. Lesions to the orbital frontal cortex therefore will often infringe on this tissue causing a deficit in tongue extension. To measure how far a rat can extend its tongue, a slurry of cookie mash is spread along a clear plastic ruler that is placed against the bars of the reaching task apparatus. The rat within the reaching box learns to extend its tongue in order to lick off the cookie mash. The distance of tongue extension is measured according to the amount of missing cookie mash along the length of the ruler. Although control rats can typically extend the tongue approximately 15mm, a rat with an orbital frontal lesion has difficulty doing so and thus extends a much shorter distance (Kolb & Whishaw, 1985; Whishaw & Kolb, 1983).

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1. 7. 2. Anatomical Measures

1. 7. 2. 1. Brain Weight

Following a lesion, a reduction in brain weight will become evident. The smaller brains result not only from missing cortical tissue, but are also a reflection of atrophy in measures such as cortical thickness, thalamic size, decrease in neuron number and dendritic branching. The reduction of brain weight is age dependent, as the earlier the administration of the brain lesion, the greater the reduction in brain weight. Therefore following postnatal day 3 lesions there is a greater reduction in brain weight than similar lesions administered in adulthood. Housing in a complex environment has shown to increase brain weight after injury.

1. 7. 2. 2. Body Weight

Disruption to the brain will ultimately cause a disruption in other behaviors including eating and metabolic changes. Body weight therefore is a way to measure any changes that the lesion may affect. Furthermore, treatments following injury may also affect body weight whether it is through changes within the brain, or enhanced exercise or training that may influence body weight. For example, complex-housing in an enriched environment will cause subjects to have a decrease in body weight due to the exercise component of the treatment. In addition, following adult orbital frontal lesions, there is a chronic drop in body weight, due to influences the lesion had on connectivity with the hypothalamus.

1. 7. 2. 3. Cortical Thickness

As found in brain weight following cortical injury, a reduction in cortical thickness occurs after injury. Again, this is likely due to atrophy in neuronal morphology, neuron number or glia number. Treatments such as complex-housing

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typically increase cortical thickness while tactile stimulation and bFGF treated subjects demonstrate a decrease in cortical thickness.

1. 7. 2. 4. Thalamic Cross-Sectional Area

Following lesions, atrophy in projecting thalamic nuclei occurs. Measuring the thalamic cross-sectional area that correlates with a section in which the appropriate thalamic nuclei would normally be present will help quantify the amount of degradation. Following medial prefrontal cortex lesions, atrophy occurs in the anteromedial and lateral portion of mediodorsal thalamic nuclei. Orbital frontal lesions will cause degradation of the medial portion of the mediodorsal thalamic nuclei and the medial ventral nucleus.

1. 7. 2. 5. Lesion Size

Because studies analyzing the effects of bFGF following injury have found that bFGF reduces the infarct size after administration, the effects of bFGF following perinatal medial frontal cortex lesions was analyzed. Dorsal photographs of lesion brains were measured and the total dorsal surface areas of the brain and lesion site were calculated. From these measurements, the percentage of total cortical surface area preoccupied by the lesion site was calculated to determine whether bFGF did affect infarct size in our experiments.

2. EXPERIMENT 1: The Effects of Basic Fibroblast Growth Factor Combined With Tactile Stimulation Following Perinatal Medial Frontal Cortex Lesions

2. 1. ABSTRACT

The effects of treatment with basic fibroblast growth factor (bFGF), tactile stimulation, or a combination of both, after postnatal day 3 medial frontal cortex lesions were determined. Although either treatment administered alone was beneficial on the performance of a spatial navigation task, the combined treatment proved to be detrimental. Not only was performance worse than bFGF or tactile stimulation groups, the combined treatment group performed worse than untreated lesion subjects. Similarly, tactile stimulation was beneficial to improving fine motor skills as assessed by successful reaching on a skilled reaching task. bFGF and tactile stimulation-treated animals, however, did not improve fine motor skills. In addition to behavioral consequences, anatomical measures were also influenced by the treatment. Females with frontal cortex lesions that received the combined treatment had a reduction in brain weight in comparison to untreated females with frontal cortex lesions. Furthermore, lesion subjects that received the combined treatment demonstrated a reduction in cortical thickness. These results suggest that there may be limits to functional recovery after perinatal cortical injury and that it may be possible to worsen functional outcome by overstimulating the animals.

2. 2. INTRODUCTION

The type, location, and age of acquired brain injury are all contributing factors to the sparing, recovery or loss of behaviors (Kolb, 1987; Kolb and Whishaw, 1985). In particular, perinatal medial prefrontal cortex lesions in the rat produce multiple chronic dysfunctions on both cognitive and motor tasks, dysfunctions that often are more severe than after similar injuries later during development or in adulthood. This poor behavioral outcome is correlated with a variety of anatomical events including a relative decrease in brain weight, decreased cortical thickness, and a shrunken thalamus (e.g. Kolb, 1987).

The poor behavioral outcome can be modified, however, as partial recovery of function on cognitive tasks has been found in response to the administration of various treatments. Two treatments that may be beneficial after early lesions include pharmacological manipulation through the addition of exogenous basic fibroblast growth factor (bFGF); and sensory manipulation through tactile stimulation achieved by stroking rat pups with a soft paint brush.

bFGF is a growth factor located within the peripheral and central nervous system, which is up-regulated following injury (Finklestein et al, 1988; Kiyota et al, 1991). bFGF typically provides aid to the injured brain as it acts by enhancing neuronal plasticity. In the injured adult brain, bFGF facilitates functional recovery through a variety of means such as increasing mitosis (Murphy et al, 1990) and angiogenesis (Folkman & Klagsburn, 1987), enhancing survival of cells that would typically die following injury, as well as increasing the number of synapses following injury (Walicke et al, 1986).

Although found in high quantities within the adult brain, in the perinatal brain the presence of bFGF is scarce (Gomez-Pinilla et al, 1994). Unfortunately for the developing brain, the lack of astrocytes that normally provide bFGF to the injured brain, negatively affects the functional outcome following perinatal injury. Therefore one hypothesis that

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has been researched in our laboratory is that exogenous administration of bFGF following

perinatal frontal cortex lesions will amplify functional recovery due to the neurotrophic

properties of bFGF. Indeed, unpublished observations by Kolb and colleagues (2001)

have found this to be the case. In addition, anatomical changes had been found such as

an increase in spine density and in female subjects an increase in cortical thickness.

The other treatment that has been beneficial following early frontal cortex injury

is the administration of tactile stimulation. First used on preterm infants, research

demonstrated that stimulated infants gained weight faster, were more alert and attentive,

and left the hospital earlier than unstimulated infants. Since then studies in our

laboratory (e.g. Gibb, 2001) had demonstrated that tactile stimulation applied to rat pups

with perinatal injury improved performance on both cognitive and motor tasks when

tested as adults. Again anatomical correlates such as enhanced acetylcholine production,

and changes in cortical thickness has been found following this treatment (Gibb, 2001;

Kolb et al, unpublished observations, 2001).

Recently, Witt-Lajeunesse (2001) found that bFGF administered to adult rats after

a motor cortex lesion was beneficial only when paired with behavioral training. Neither

bFGF nor behavioral training alone was effective in improving function after injury.

Thus, it appears that both pharmacological and behavioral modifications working

synergistically may have greater benefits than either treatment alone. Therefore, the

purpose of this study was to determine whether simultaneous administration of bFGF and

tactile stimulation following P3 medial frontal lesions would produce a greater benefit

than if each treatment was administered alone.

2. 3. MATERIALS AND METHODS

2. 3. 1. Subjects

Eighty-five Long-Evans rats were used from 8 litters of animals. All rats were

born and raised in the Canadian Center for Behavioural Neuroscience (CCBN) rat colony

at the University of Lethbridge. Using a cross-litter design, the subjects were divided into 8 groups (Table 2. 1) and each group consisted of both male and female subjects. The groups included: control-no treatment (C-NT) (6 males, 6 females), frontal-no treatment (F-NT) (3 males, 4 females), control- bFGF (C-bFGF) (6 males, 8 females), frontal-bFGF (F-bFGF) (7 males, 5 females), control-tactile stimulation (C-TS) (4 males, 1 female), frontal-tactile stimulation (F-TS) (2 males, 4 females), control- tactile stimulation with bFGF (C-TS+bFGF) (5 males, 6 females), and frontal-tactile stimulation with bFGF (F-TS+bFGF) (5 males, 6 females). For the water maze task, an additional 4 males and 3 females were added to the no treatment group. In the reaching task, an additional 4 males and 4 females were added to the TS control group to minimize variance within the groups. All subjects had access to food and water ad labium, except during the reaching task when each animal was placed on food deprivation. During this period, rats maintained a minimum 85% of total body weight. Room temperature was kept constant at 22°C, and the lights were maintained at a 12 hour day: night cycle (lights on at 7:30 pm). At weaning (postnatal day 23) all subjects were housed in groups with a maximum of 2 males or 3 females in 46 x 23.5 x 20 cm high standard lab cages. The cage walls were clear plastic with corn cob bedding. Experimentation conformed to Canadian Council of Animal Care (CCAC) guidelines.

2. 3. 2. Cortical Lesions

Pups were anesthetized on postnatal day 3 by cooling in a Thermatron cooling apparatus until a rectal temperature between 18-20° C was reached. An incision was made in the skin, the frontal bone was removed using iris scissors, and lesions were administered by aspiration. All medial frontal subfields including Zilles' regions of Cing 1, Cing 3, and prelimbic cortex were extracted (Zilles, 1985). Using silk thread, the incision was sutured. Sham operated controls were anesthetized by cooling, received an

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incision, followed by suturing of the skin. Pups were hand-held until their body felt warm and were subsequently placed under a desk lamp until returned to the dam.

Table 2. 1. Experimental Design

Group	Sex	NT	TS+bFGF	bFGF	TS
Control	Male	6 (10)	5	6	4 [8]
	Female	6 (9)	6	8	1 [5]
	Total	12 (19)	11	14	5 [13]
Frontal	Male	3	5	7	2
	Female	4	6	5	4
	Total	7	11	12	6

Note: (#) indicate number of rats participating in water maze task.
[#] indicate number of rats participating in reaching task.

2. 3. 3. Preparation of bFGF

A 10 ml solution of 0.1 M phosphate buffered saline was combined with 10 mg of BSA and 10 µg of basic fibroblast growth factor. The final concentration of the bFGF solution was 1 µg/ml. The bFGF solution for all injections was made on or before injection Day 1, and stored in a refrigerator at 4°C.

2. 3. 4. bFGF administration

Starting on post-operative day 1, bFGF was administered (0.01 cc/g body weight) daily for seven consecutive days at approximately 9 am. The dosage was administered subcutaneously between the shoulder blades with a 30 gauge needle and a 1cc syringe. For the first three days, pups were injected in an isolated room after which some subjects from the litter received tactile stimulation (TS+bFGF group). After three days, some

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pups appeared stressed following the injection and this stress (characterized by wriggling and squeaking) continued throughout the following session of tactile stimulation. Therefore, for the remainder of the injections, administration occurred within the animal colony room at 8:40 am, and pups were immediately returned to the dam. A minimum of 20 minutes elapsed before the combined treatment group was removed for tactile stimulation in order to reduce the likelihood of an association forming between the stress of the injection and the tactile stimulation procedure.

2. 3. 5. Tactile Stimulation

Tactile stimulation began at post-operative day 1. Following bFGF injections for the TS+bFGF treatment group or starting first thing in the morning for the TS group, each rat pup was stroked with a fine haired paint brush for 15 minutes, three times a day (9 am, 1 pm, 5 pm) for three weeks. Pups were stroked a litter at a time in a room away from the colony. Tactile stimulation ended at weaning (P23), and the rats were separated into standard size cages for the remainder of the experiment.

2. 3. 6. Behavioral Methods

All subjects began behavioral testing as adults (P60). Tasks were performed in the following order: Open field, Morris water maze, and the Whishaw reaching task.

2. 3. 6. 1. Open Field

Testing occurred in a small testing room in the presence of the experimenter. Each animal was placed individually in a Digiscan animal activity monitor (42 x 42 x 31 cm high) for 10 minutes. Infrared motion detectors located along the sides of the box recorded the distance traveled (cm). Movements were recorded in five, two minute intervals. The average of the five trials was calculated.

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2. 3. 6. 2. Morris Water Task

A pool with a white interior (1.5 m diameter, 45 cm height) was placed in the center of a testing room and filled with water at a temperature of 18-20°C. To create opaque water, 1L of skim milk powder was added. A white circular plastic platform (30 cm high, 12 cm diameter), was emerged 1 cm below water level, in one quadrant of the pool and remained in a fixed location throughout testing. The experimenter and holding chambers were located at the opposite quadrants of the pool.

Each subject was given one trial block a day for seven consecutive days. Each trial block consisted of four trials, such that for each trial the subject started in a different quadrant of the pool. Subjects were placed into the pool facing the pool wall of the tested location. Ninety seconds was allotted to search the pool for the escape platform. At the end of the 90 seconds if the platform was not found the subjects were placed on the platform for 10 seconds before being returned to a holding chamber until the next trial. If the platform was found the rat was left on the platform for 10 seconds and then returned to a holding chamber. A minimum of 20 minutes elapsed between trials to ensure each subject waited an equivalent amount of time before the next trial. The mean latency for each day was calculated (seconds) as well as the overall swim speed (cm/sec).

2. 3. 6. 3. Whishaw Reaching Task

Each reaching box (10 x 18 x 10 cm high) had sides and top made of Plexiglas with the front of the box made of 2 mm vertical bars that enabled subjects to reach through and grasp chicken feed located in a metal food tray. The cages had a mesh floor, to prevent subjects from raking the food into the cage. To encourage reaching, animals were food deprived to a minimum of 85% of the original body weight. Each rat was trained for 10 days and then scored on day 11 of the task. On the test day all animals were videotaped for 5 minutes, commencing at the first grasp for food. A successful reach was scored if the rat was able to grab the food and bring it to the mouth to eat. An

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attempt was scored if the subject extended the wrist past the vertical bars but was unsuccessful in obtaining food. If the subject reached partially through the bars no attempt was scored.

2. 3. 7. Anatomical Methods

At the completion of the experiment, the rats were weighed and then injected with Euthansol (1 cc/kg body weight). Subjects were intracardially perfused with 0.9% saline, followed by unbuffered para-formaldehyde (PFA). Brains were extracted, and stored in PFA in 30% sucrose for a minimum of one week before being blocked (olfactory bulbs and spinal cord removed), weighed, and sliced frozen on a Cryostat at 50 μm. Every tenth section was mounted on 1% gel and 0.2% chromium potassium sulphate slides. Sections were stained with crestyl violet.

2. 3. 7. 1. Cortical Thickness

A Zeiss petrographic viewer was used to view sections. Cortical thickness was measured in five different planes according to a set landmark (Figure 2. 1). The plane 1 measurement was taken from the first section containing striatum, plane 2 was characterized by the presence of the anterior commissure, plane 3 by the first section of the hippocampal formation, plane 4 by the posterior commissure, and plane 5 by the last section of the hippocampus. For each plane the lateral cortex from both hemispheres were measured (20X magnification) in lesion subjects. In control subjects the average of the medial, central and lateral measures was made for each plane. Because the lesions were bilateral, the average of both hemispheres for each plane was calculated. A two-tailed analysis of correlation was also performed for lesion subjects, comparing the overall performance on the water task with the plane by plane measurements on cortical thickness.
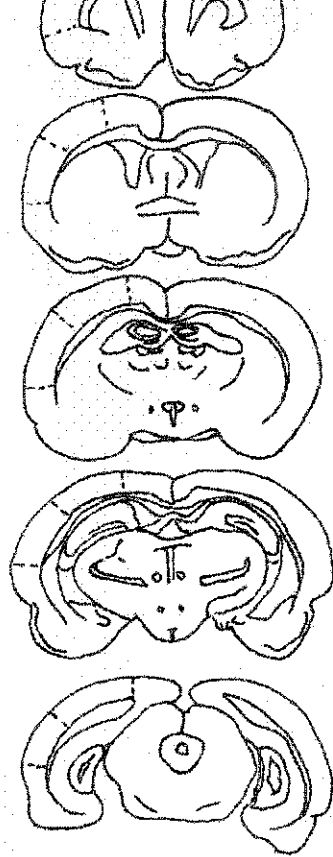
Figure 2. 1. Drawings of representative sections used for cortical thickness. The dashed lines on the cortex represent the medial, central and lateral measurements.

2. 3. 7. 2. Thalamic and Brainstem Cross-sectional Area

Two sections of the thalamus were photographed with a digital camera for analysis. The first section included a visible anteromedial thalamic nucleus and mediodorsal thalamic nucleus as well as the paraventricular hypothalamic nucleus. The second section included a visible posterior commissure and represented the thalamus and brainstem (Figure 2. 2). Scion image was used to determine the total cross-sectional area. The average thalamic cross-sectional area and thalamic/brainstem cross-sectional area (mm^2) was determined.
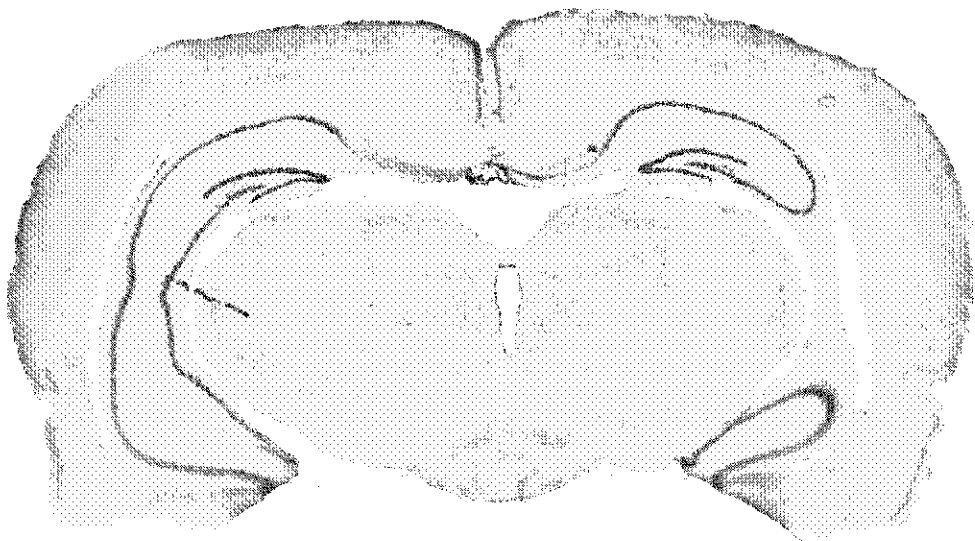
Figure 2. 2. Photographs of representative sections used for thalamic and brainstem cross-sectional area respectively.

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2. 3. 7. 3. Lesion Size

The entire brain was photographed from a dorsal view. Scion image was used to determine the total cortical surface area from the dorsal perspective as well as the surface area of the lesion. The percentage of missing cortical tissue relative to total surface area was calculated. Depth of the lesion was not considered.

2. 3. 8. Statistical Analysis

Either a two or three-way analysis of variance (ANOVA) or repeated measures ANOVA and the standard errors (SE) were used for each analysis. For post hoc tests a Fisher's LSD was used.The Levene's test of homogeneity of variance was also used. In the latter case, the results were only reported when a violation of variance was found.

2. 4. RESULTS

2. 4. 1. Behavior

2. 4. 1. 1. Open Field

Medial prefrontal lesions did not affect the subjects' response to novel environments. Because the average distance traveled between treatment groups was dependent upon the sex of the subjects, $(F(1,61) = 0.034, p = .854)$, males and females were analyzed separately (Figure 2.3a and b).

Tactile stimulation and TS+bFGF males showed an overall decline in activity when compared to the no-treatment group whereas bFGF treated males did not differ in activity from untreated males (Figure 2.3a).

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a.
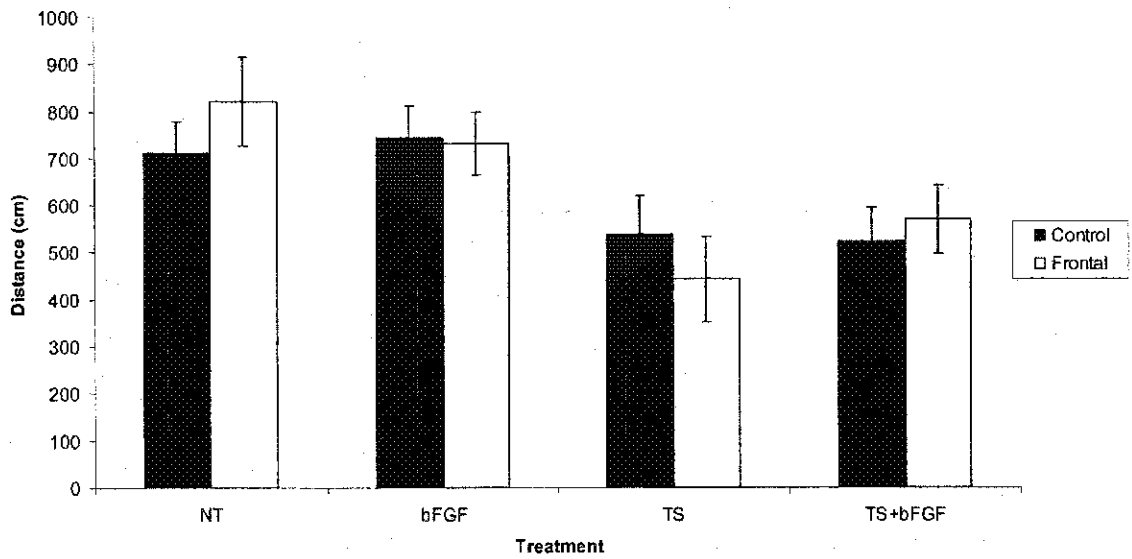
b.
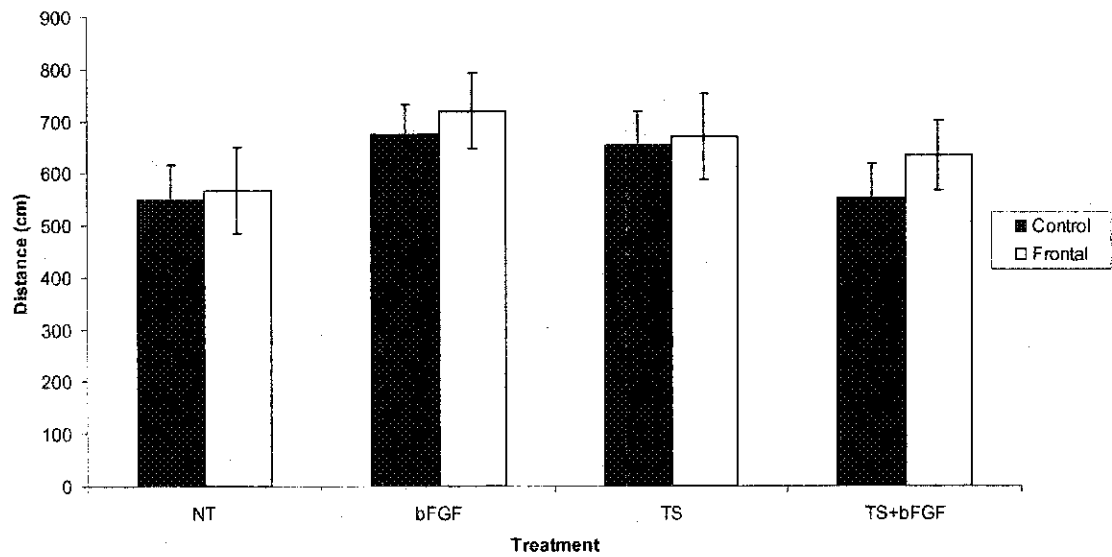
Figure 2. 3. Distance covered as measured by open field activity. a) In males, TS and TS+bFGF treated subjects demonstrated a decline in distance traveled. b) In females, distance traveled did not differ regardless of lesion group or treatment.

In males, each treatment group was compared separately to the no-treatment group using a two-way ANOVA (group X treatment) because statistical analysis of all groups violated homogeneity of variance. The tactile stimulation group when compared

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to the no-treatment group revealed a significant treatment effect ($F(1,11) = 6.67$, $p < .05$) but not an overall group effect ($F(1,11) = 0.003$, $p = .96$), nor a group X treatment interaction ($F(1,11) = 0.93$, $p = .36$). A similar finding occurred for the bFGF + TS group when compared to the no-treatment group as an overall treatment effect was found ($F(1,15) = 6.92$, $p < .05$) but not a group effect ($F(1,15) = 0.87$, $p = .37$) nor a significant group X treatment interaction ($F(1,15) = 0.14$, $p = .72$). Males treated with bFGF did not differ significantly from untreated males in group ($F(1,17) = 0.37$, $p = .55$), treatment ($F(1,17) = 0.12$, $p = .74$) or group X treatment interaction ($F(1,17) = 0.54$, $p = .47$).

Unlike males, female subjects regardless of group or treatment did not differ in distance on the open field task (Figure 2. 3b).

A two-way ANOVA (group X treatment) did not reveal a significant group effect ($F(1,32) = 0.38$, $p = .54$), treatment effect ($F(3,32) = 1.40$, $p = .26$) nor a significant group X treatment interaction ($F(3,32) = 0.08$, $p = .97$).

2. 4. 1. 2. Morris Water Task

Lesion animals performed worse on this task than controls (Figure 2.4). The results also showed that while bFGF or tactile stimulation facilitates recovery on this task, a combined treatment of bFGF and tactile stimulation actually produced adverse effects on the performance of lesion subjects. Overall, TS+bFGF treated subjects had slower latencies than bFGF, TS and NT subjects (Figure 2.5).

A repeated-measures ANOVA of daily performance revealed that all lesion subjects regardless of treatment took longer to locate the platform than untreated controls on the first day of training. By the second day, bFGF and TS-treated subjects were

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performing as well as untreated controls while untreated lesion subjects or combined treatment lesion subjects were still impaired. TS+bFGF lesion subjects continued to be impaired at this task until the last day of testing (day 7). Not only were these subjects impaired at the task relative to controls, but the F-TS+bFGF group performed worse than the F-NT group (days 4,5, and 6), F-bFGF group (days 3,4,5,6) and the F-TS group (day 5). By the last day of testing, C-bFGF and F-TS subjects had lower latencies than C-NT subjects.

A repeated-measures ANOVA of daily means showed an overall group effect $(F(1,77) = 27.20, p < .001)$ and a main treatment effect $(F(3,77) = 4.70, p < .01)$ but no group by treatment interaction $(F(3,77) = 1.48, p = .23)$. Post hoc analysis of treatments determined that the combined treatment group was significantly different from the NT group $(p < .05)$, bFGF group $(p < .001)$, and the TS group $(p < .05)$. Analysis on each day showed that C-NT subjects differed significantly from all lesion subjects $(p's < .05)$ on day one. On day two F-NT and F-TS+bFGF subjects were still significantly impaired relative to C-NT subjects $(p's < .05)$ while the other lesion subjects did not significantly differ from controls $(p's > .05)$. F-TS+bFGF subjects continued to be significantly impaired compared to C-NT subjects from days 3-6 $(p's < .05)$, F-NT subjects from days 4-6 $(p's < .05)$, F-bFGF subjects from days 4-6 $(p's < .05)$ and F-TS subjects on day 5 $(p = .05)$. Although on day four C-NT subjects were significantly better than F-TS $(p < .05)$, on day seven F-TS performed significantly better on the task than C-NT $(p < .05)$.

Because it is possible that the swimming abilities of the subjects may have been compromised thus affecting the latency scores, the lesion subjects receiving treatments were compared to untreated control subjects with regards to swim speed. bFGF (21.36

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cm/sec) and tactile stimulation lesion subjects (22.24 cm/sec) did not differ in speed from

the untreated control subjects (19.75 cm/sec), whereas the combined treatment of

TS+bFGF in lesion subjects (23.39 cm/sec) swam faster than untreated control subjects.

Therefore despite the faster swim speed of the F-TS+bFGF group, this group still took

longer to locate the hidden platform. Post hoc comparisons using Fisher's LSD found

that the C-NT subjects did not significantly differ from the F-bFGF subjects (p = .30), or

the F-TS subjects (p = .16) but did significantly differ from F-bFGF + TS subjects (p <

.05).
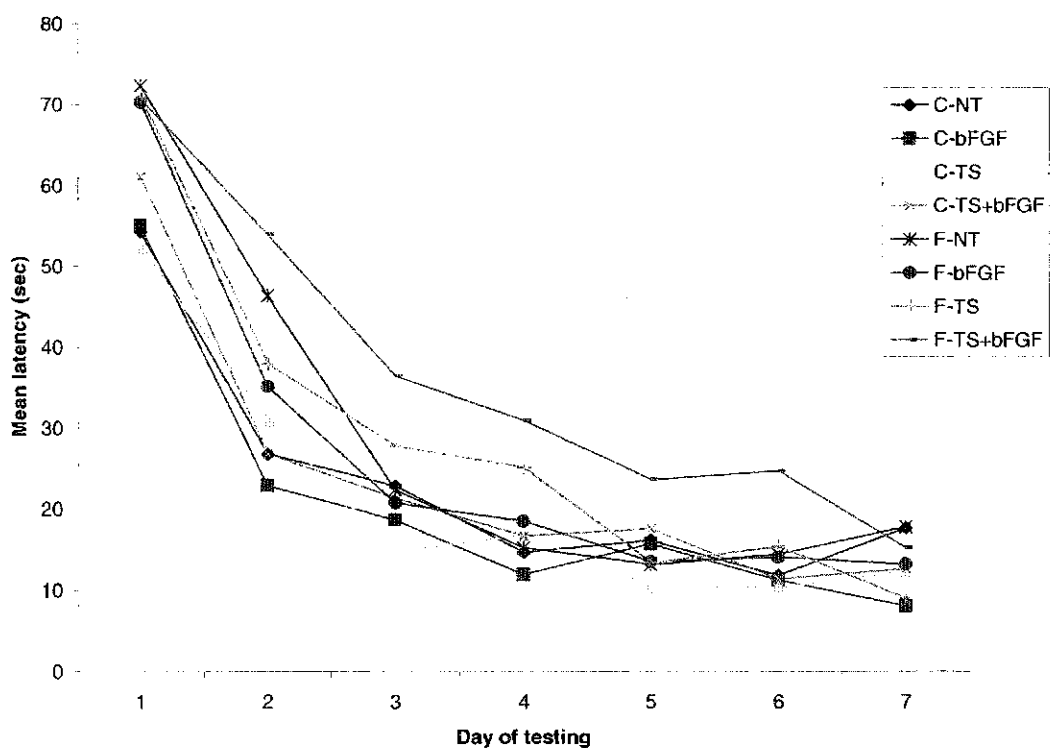
Figure 2. 4. Mean latency in the water task for each day. C represents control subjects; F represents frontal lesion subjects. TS+bFGF was detrimental on performance of this task, while bFGF or TS facilitated functional recovery in lesion subjects.

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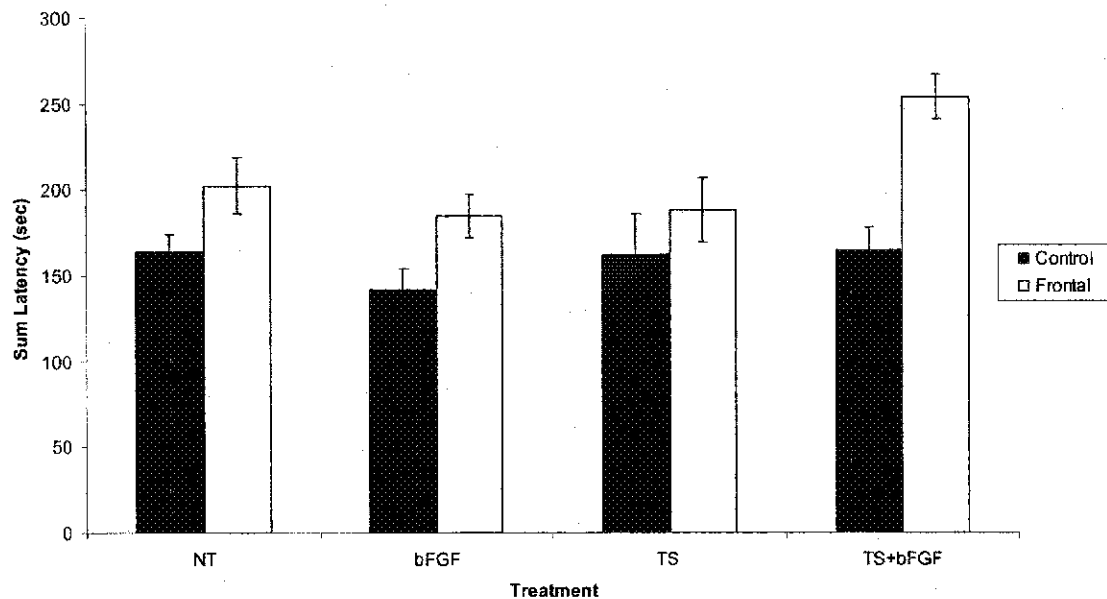
Figure 2. 5. Sum latency in the water task. TS+bFGF lesion subjects overall took longer than all other groups.

2. 4. 1. 3. Whishaw Reaching Task

The cortical lesions produced a decrease in success rate on the reaching task

(control: $65.99 \pm 4.65\%$ vs. lesion: $45.11 \pm 6.98\%$). The combined treatment did not

prove beneficial for this task as the frontal combined treatment group had the lowest

success rate when reaching for food ($34.10 \pm 4.85\%$) and, although not significant, the

success rate was 11% lower than frontals that did not receive treatment. bFGF did not

hinder nor improve reaching performance ($38.21 \pm 4.65\%$), yet lesion subjects that

received tactile stimulation ($50.56 \pm 4.85\%$) improved at skilled reaching (Figure 2. 6).

A two-way ANOVA (group X treatment) found a significant group effect ($F(1,78)$

$= 27.90$, $p < .001$) but no treatment ($F(3,78) = 0.82$, $p = .49$) or group X treatment

interaction ($F(3,78) = 1.61$, $p = .19$). Post Hoc comparisons (Fishers LSD) determined

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that unlike the other lesion subjects, lesion subjects that received tactile stimulation did not significantly differ from control subjects (p > .05).
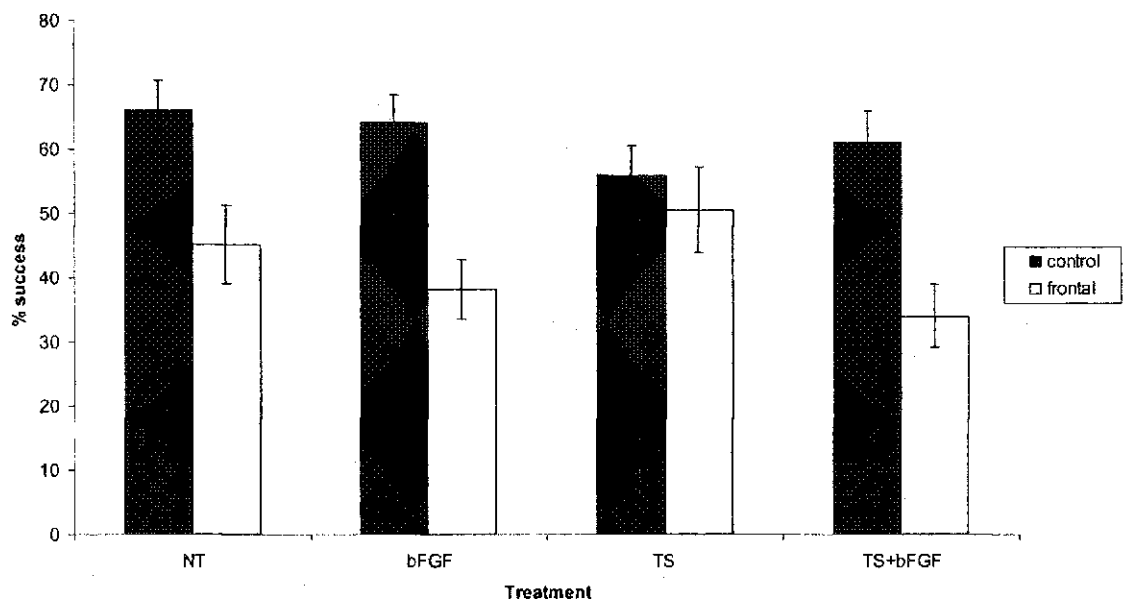
Figure 2. 6. Reaching performance expressed as a percent success of the total number of reaches. Tactile stimulation improved reaching in lesion subjects.

2. 4. 2. Anatomy

2. 4. 2. 1. Brain Weight

When compared to control rats, lesion rats show a reduction in brain weight by approximately 10.5% in males and 8% in females (Table 2. 2). Although the type of treatment did not influence brain weight in males, there was a treatment effect found in females. bFGF or tactile stimulation did not influence brain weight, but lesion females receiving TS + bFGF (1.39 ± .03g) had smaller brains (~ 7 %) than lesion females without treatment (1.50 ± .04g).

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Because male rats typically have heavier brains than females, each sex was analyzed separately. A two-way ANOVA (group X treatment) in males found a significant group effect $(F(1,30) = 71.33, p < .001)$. Brain weight in males did not differ significantly with the type of treatment received $(F(3,30) = 0.29, p = .83)$ and there was no group X treatment interaction $(F(3,30) = 0.66, p = .58)$.

A two-way ANOVA (group X treatment) in females found an overall group effect $(F(1,32) = 42.24, p < .001)$. There was not a significant treatment effect $(F(3,32) = 1.22, p = .32)$, nor a significant group x treatment interaction $(F(3,32) = 1.97, p = .14)$. Post hoc comparisons (Fishers LSD) reveal that the reduction in brain weight in the F- TS + bFGF group was significantly smaller than the F-NT group $(p < .05)$.

Table 2. 2. Mean Brain Weight

Group	Treatment	Brain Weight (g ± SE)	
		Male	Female
Control	NT	1.71 ± .03	1.63 ± .03
	bFGF	1.70 ± .03	1.60 ± .03
	TS	1.75 ± .04	1.59 ± .07
	TS+bFGF	1.74 ± .03	1.64 ± .03
Frontal	NT	1.53 ± .04	1.50 ± .04
	bFGF	1.51 ± .03	1.42 ± .03
	TS	1.47 ± .05	1.48 ± .04
	TS+bFGF	1.53 ± .03	1.39 ± .03 *

* Significantly different from F-NT group $(p < .05)$.

2. 4. 2. 2. Body weight

Male rats with lesions had a reduced body weight relative to controls. Although body weight was unaffected by tactile stimulation or the combined treatment of bFGF+TS, males receiving bFGF alone demonstrated a lower body weight in comparison to untreated subjects (Table 2.3). A two- factor ANOVA (group X treatment) found an overall group effect ($F(1,29) = 8.55$, $p = .007$), but no treatment effect ($F(3,29) = 1.65$, $p = .20$) nor interaction ($F(3,29) = 0.55$, $p = .65$). Fishers LSD post hoc analysis determined a significant difference between the no-treatment and bFGF treated subjects ($p < .05$).

In female subjects, body weight was unaffected by the injury. In contrast, the treatments selectively affected brain injured subjects (Table 2. 3). TS + bFGF treated subjects weighed less than untreated subjects. A two-way ANOVA (group X treatment) did not find a significant main effect of group ($F(1,32) = 0.76$, $p = .39$), treatment ($F(3,32) = 1.93$, $p = .14$) nor an interaction ($F(3,32) = 0.28$, $p = .84$). Post Hoc analysis determined a significant difference between the no-treatment and combined treatment females ($p < .05$).

2. 4. 2. 3. Lesion size

Lesion size, expressed as a percent of the total cortical surface area was consistent across sex so the data were collapsed across sex. The lesion area was larger in bFGF treated animals ($16.1 \pm 1.6\%$) than untreated animals ($10.2 \pm 1.5\%$) but tactile stimulation ($11.1 \pm 2.0\%$) and the combined treatment ($14.11 \pm 1.8\%$) did not affect the lesion size (table 2.4). A one-way ANOVA found an overall treatment effect ($F(3,30) = 2.93$, $p <$

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.05). Fishers LSD determined a significant difference between the no-treatment and bFGF group (p < .05).

Table 2. 3. Mean Body Weight

Group	Treatment	Weight (g ± SE)	
		Male *	Female **
Control	NT	554.00 ± 20.92	336.17 ± 12.73
	bFGF	521.50 ± 19.10	321.50 ± 11.03
	TS	553.75 ± 23.39	306.00 ± 31.19
	TS+bFGF	514.80 ± 20.92	315.33 ± 12.73
Frontal	NT	525.33 ± 27.00	336.50 ± 15.60
	bFGF	465.00 ± 17.68	298.20 ± 13.95
	TS	472.00 ± 33.07	302.00 + 15.60
	TS+bFGF	488.60 ± 20.92	300.83 ± 12.73

* Significant difference between NT and bFGF control and frontal subjects (p < .05).
** Significant difference between NT and TS+bFGF control and frontal subjects (p < .05).

Table 2. 4. Lesion Size as a Percentage of Total Dorsal Cortex

Treatment	NT	bFGF	TS	TS+bFGF
% lesion	10.2 ± 1.5	16.1 ± 1.6 *	11.1 ± 2.0	14.11 ± 1.8

* Significantly different from NT group (p < .05)

2. 4. 2. 4. Cortical Thickness

Following medial frontal cortex lesions there was an overall reduction of cortical thickness in lesion subjects. Although no single treatment influenced the overall

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thickness of the cortex, there were treatment effects at particular planes of measurement. In lesion subjects, a treatment effect was evident on plane 4, as the lesion subjects in the combined treatment group had a reduction in lateral cortical thickness when compared to both lesion subjects in the bFGF group or no-treatment group (Figure 2. 7).

Because there was not a sex difference, $(F(1,56) = 0.001, p = .974)$ data from both sexes were pooled. A repeated measures by plane did not find an overall treatment effect in lesion subjects $(F(1,31) = 0.09, p = .97)$. Post hoc analysis using Fisher's LSD found a significant difference on plane 4 of lateral cortical thickness. The combined treatment group was significantly different from both the no-treatment group and bFGF group (p's < .05).

In lesion subjects, there was a significant correlation between performance on the water task and lateral cortical thickness on planes 4 and 5. This indicated that the reduced cortical thickness found in lesion subjects may be responsible for the poor performance on the water task. A two-tailed Pearson's correlation was found to be significant for both plane 4 $(r = -0.45, p < .01)$ and plane 5 $(r = -0.36, p < .05)$.

In control subjects, despite the lack of behavioral differences between the treatment groups, there was a difference in cortical thickness due to the treatments found on planes 1-4. In particular, control subjects that received tactile stimulation had a thicker cortex than subjects that received bFGF (planes 1 and 3), or no-treatment (plane 3). Also, the combined treatment group of tactile stimulation and bFGF showed a thicker cortex than the bFGF group (planes 1, 2 and 4), or the no-treatment group (plane 4) (Figure 2. 8).

Repeated measures by plane of the total cortical thickness (including medial, central and lateral measurements) found a significant main effect of treatment ($F(1,36)$ = 3.18, $p < .05$). Fisher's LSD found a significant difference between the combined treatment group and the bFGF group on planes 1, 2 and 4 (p's $\leq .01$). The combined treatment group also had a significantly thicker cortex than the no-treatment group on plane 4 ($p = .05$). Tactile stimulation also significantly increased cortical thickness when compared to bFGF on plane 1 ($p < .05$) and 3 ($p < .01$), or when compared to no treatment on plane 3 ($p < .05$).
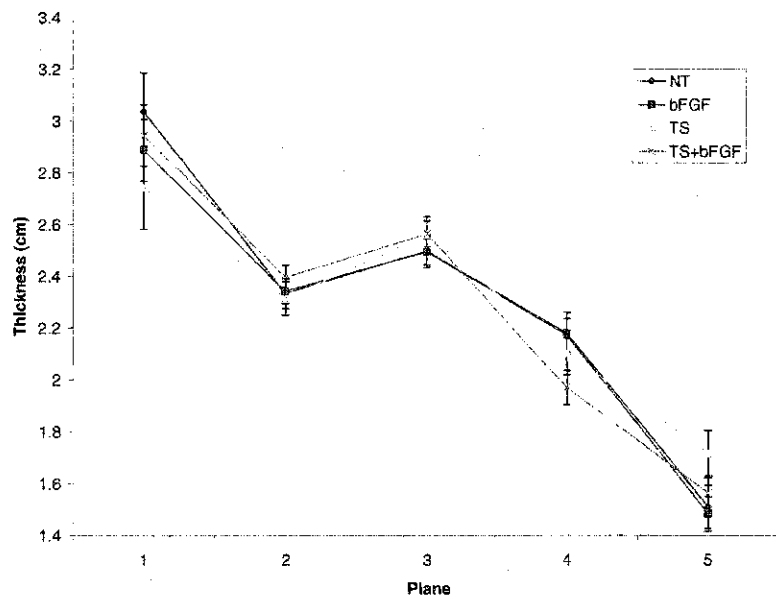
Figure 2. 7. Mean cortical thickness (20X magnification) of the lateral cortex in lesion subjects. On plane 4 the combined treatment group had a reduced cortical thickness.

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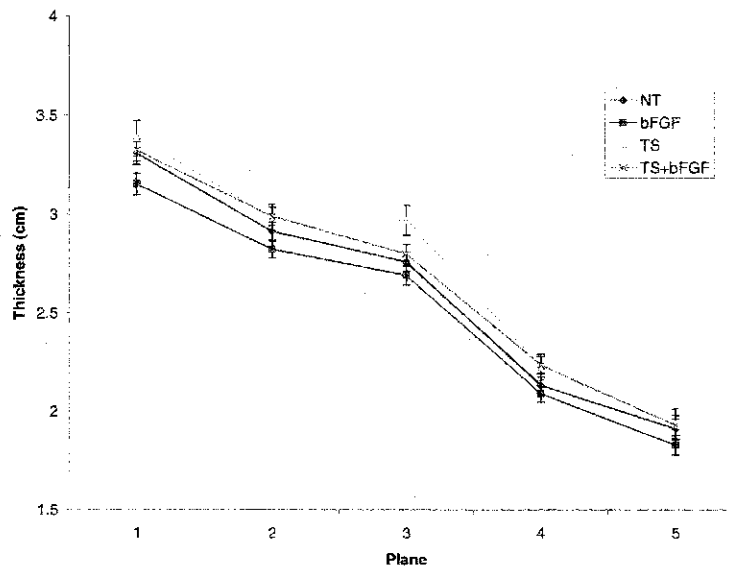
Figure 2. 8. Mean cortical thickness (magnification 20X) in control subjects. The combined treatment group demonstrated a thicker cortex than the bFGF group (planes 1, 2, and 4) or the no-treatment group (plane 4). Tactile stimulation increased cortical thickness on plane 3.

2. 4. 2. 5. Thalamic Cross-sectional Area

As shown in previous studies there was significant atrophy of the thalamus after perinatal medial frontal lesions (Table 2. 6). None of the treatments influenced thalamic size.

A two-way ANOVA (group X treatment) found an overall group effect ($F(1,69) = 38.32$, $p < .001$) but not an overall treatment ($F(3,69) = 0.78$, $p = .57$) nor interaction ($F(3,69) = 1.68$, $p = .18$).

2. 4. 2. 6. Brainstem Cross-Sectional Area

Lesion subjects displayed a shrunken brainstem compared to controls (Table 2. 7). A treatment effect was not found. A two-way ANOVA (group X treatment) found a

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significant group effect ($F(1,70) = 37.83$, $p < .001$) but no treatment effect ($F(3,70) = 0.28$, $p = .84$), nor interaction ($F(3,70) = 1.13$, $p = .34$).

Table 2. 6. Thalamic Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean $\pm$ SE
Control	NT	18.77 $\pm$ .71
	bFGF	17.19 $\pm$ .63
	TS	18.93 $\pm$ 1.05
	TS+bFGF	18.96 $\pm$ .71
Frontal	NT	15.77 $\pm$ .89
	bFGF	15.18 $\pm$ .68
	TS	14.77 $\pm$ .96
	TS+bFGF	14.02 $\pm$ .71

Table 2. 7. Brainstem Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean $\pm$ SE
Control	NT	31.88 $\pm$ .58
	bFGF	31.24 $\pm$ .54
	TS	32.59 $\pm$ .90
	TS+bFGF	31.97 $\pm$ .60
Frontal	NT	29.68 $\pm$ .76
	bFGF	29.23 $\pm$ .58
	TS	28.22 $\pm$ .82
	TS+bFGF	28.69 $\pm$ .60

2. 5. DISCUSSION

The main finding of this study was that although both tactile stimulation and bFGF treatments improved functional outcome after early medial frontal lesions, the combined treatment made the animals worse.

2. 5. 1. Absence of functional recovery associated with the combined treatment

The benefit of both basic fibroblast growth factor and tactile stimulation following injury has been well researched within our laboratory. Both have improved functional recovery after perinatal brain injury on cognitive tasks, and tactile stimulation is beneficial on the reaching task. Therefore the results of the present study were quite surprising. Although both bFGF and tactile stimulation were beneficial to performance on the spatial navigation task, and tactile stimulation on the reaching task, the combined treatment in lesion subjects resulted in worse functional outcome on spatial navigation than lesion subjects receiving no treatment at all.

Presently, the mechanism for the negative effect of the combined treatments is unknown. Although our hypothesis was that the combined treatment of bFGF and tactile stimulation would be beneficial following brain injury, an alternative hypothesis could be that each treatment alone has a maximal effect on recovery. If true, an additive effect of the two treatments would not occur and instead, behavioral performance of the TS+bFGF group would be equivalent to the two treatments administered alone. If this was the case, it would be reasonable to presume that a ceiling effect had been reached in which the cognitive performance of these subjects could only reach a certain level and once achieved, would not be able to perform any better. Indeed, this was the finding of a study

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by Yan and colleagues (2000) that recognized that both bFGF and hypothermia are beneficial treatments following brain injury. To determine if the combined treatment of bFGF and hypothermia would further enhance functional recovery on the performance of the Morris water task, both treatments were administered immediately after injury, followed by behavioral testing. Although there was a treatment effect, the functional recovery on this particular task did not improve more than if each treatment was administered alone, demonstrating a ceiling effect in brain plasticity. Therefore, it is possible that the combination of bFGF and tactile stimulation may not be expected to have additive effects on recovery. This does not explain, however, why the animals with combined treatments were worse.

A study by Blum and colleagues (2001) examined the effects of bFGF administration on the sensory perception of mechanical stimulation. bFGF was administered into the dorsal root ganglion of the spinal cord, and single cell recordings measured these cells electrical activity in response to mechanical stimulation. These cells had a decreased electrical response to the mechanical stimulation following bFGF administration, and thus there was a decline in sensory perception of the stimulation. In the present study, bFGF may have influenced electrical activity of sensory neurons activated during sessions of tactile stimulation but then we would still expect to see benefits of bFGF alone on the brain, which did not occur. Thus, an alternative explanation may be that instead of blocking stimulation as just described, the combined treatment may have had an overstimulating effect that was detrimental after injury. Numerous studies on preterm neonates (roughly equivalent to the rat pups at time of lesion), have described the negative effects of sensory overstimulation through many

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sensory modalities. Negative consequences such as increasing heart and respiratory rates have been recorded following noise and tactile manipulations (Allen, 1995; Zahr & Balian, 1995). Sensory overstimulation of the preterm neonate is very stressful to the infant and thus may have effects on the ability to promote growth and homeostasis with the immature nervous system (Blackburn, 1998). Perhaps a similar mechanism is working here, whereby if either treatment is administered alone, then it is beneficial, but if both are given, then it results in overstimulation of the brain either by increased glucocorticoids in response to stress, or increased neuronal activity.

Finally, although poor functional outcome may be related to stress, the open field analysis did not support this notion. Initially during the treatment period, rat pups were injected with bFGF and immediately after in the same testing room were administered tactile stimulation. As noted earlier, the pups were not as relaxed during the tactile stimulation period, as they did not show the typical REM sleep-related behaviors described in past research (e.g. Gibb, 2001). Thus it was possible that an association was made between the bFGF injections and tactile stimulation, such that tactile stimulation itself became a stressful experience. Because at four days of age the amygdala (responsible for fear conditioning) is fully developed, this association may have been possible, in spite of the procedural change of separating the bFGF and tactile stimulation experiences. Therefore, one may argue that the acute injection stress persisted throughout tactile stimulation sessions, producing a chronic stressor. Stressed subjects are impaired on cognitive tasks and demonstrate an increase in emotionality as demonstrated by enhanced locomotion in an open field apparatus (e.g. Roozendaal, 2002). Note, however, that in the current study the TS and TS+bFGF groups

demonstrated a decline in open field activity, thus suggesting a decrease in emotionality of these subjects.

2. 5. 2. Anatomical changes associated with the combined treatment

The mechanism for the enhanced outcomes of the bFGF and tactile stimulation animals and the worsened outcome for the animals with combined treatments is unknown. In previous research conducted in our laboratory (unpublished observations, 2001) there was an overall decrease in cortical thickness following bFGF treatment. In this study we did not find an effect of bFGF relative to untreated subjects on cortical thickness. Similarly, in male subjects but not female subjects, following the administration of tactile stimulation there was a decrease in cortical thickness in both control and lesion subjects (Gibb, 2001). In this study, there was not an overall sex difference in cortical thickness, or a sex X treatment interaction. Although tactile stimulation did not have an effect on lesion subjects, control subjects that received tactile stimulation had a thicker cortical mantle than untreated control subjects on plane 3. Interestingly, although the underlying mechanism for the poor performance of the combined treatment group is unclear, there was a significant correlation between overall water maze performance and lateral cortical thickness on planes 4 and 5 in lesion subjects. This suggests that the reduced cortical thickness found in lesion subjects receiving the combined treatment plays a role in the poor performance on the water task. Furthermore, in females but not males, there was a reduction in brain weight when compared to untreated lesion subjects. These findings may suggest that anatomical

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changes such as a decrease in the number of synapses may be an underlying mechanism

of the poor behavioral performance on the spatial navigation task.

2. 5. 3. Caveats

There were two distinct problems with this study, namely the storage and

preparation of bFGF and the methods used in the water task. Both of these problems may

have influenced these findings and thus shall be discussed in turn.

Unbeknownst to us, bFGF is a protein that degrades quite rapidly if not stored

properly between uses. In this study, the bFGF solution was prepared at the beginning of

administration and stored at 4° C in a refrigerator between each injection. Therefore,

although we thought that $1\mu g/ml$ of bFGF was given to each subject it is likely that the

dose is much less. This concern will be addressed in experiment 2.

Another issue with this study is the methods used on the Morris water task. The

testing room used as well as the computer equipment and platform were different in this

experiment from previous studies in our laboratory. As a consequence, not only did

lesion subjects have difficulty with this task, but control subjects were also affected as

they took longer than usual to locate the hidden platform and there was greater variance

among the scores on this task. A pilot study discussed in Appendix I analyzed the effects

of some of the factors that may have contributed to these findings.

3. EXPERIMENT 2: The Effects of bFGF Following Perinatal Medial Frontal Cortex Lesions

3. 1. ABSTRACT

The purpose of this study was to examine the effect of subcutaneous administration of bFGF on functional recovery from frontal cortex lesions on postnatal day 3. bFGF treatment promoted functional recovery on both a spatial navigation task and a skilled reaching task relative to untreated lesion subjects. In addition to behavioral modifications, bFGF treatment was correlated with anatomical changes that included proliferation of brain tissue within the midline region of the cortex. These results contrast with our previous study in which premixed bFGF was less beneficial. The differences between the studies may be a result of lowered biological activity in the premixed, but not fresh-mixed, bFGF.

3. 2. INTRODUCTION

Previous studies in our laboratory, including experiment 1, demonstrated beneficial effects of basic fibroblast growth factor on cognitive tasks following perinatal brain injury. In experiment 1, the bFGF was prepared at the beginning of the administration period and subsequently given for the following 7 days. Between injections, the compound was stored in a refrigerator. Later, it came to our attention that premixing this protein may cause a lowering of bFGF biological activity due to changes in structural confirmation. Therefore, although the same dose of bFGF was used in each experiment, premixed bFGF may not have recognized or been recognized by receptor sites. Preliminary evidence suggested that if prepared daily, prior to injection time, there is filling in of the lesion cavity following medial frontal lesions on postnatal day 3 (R. Diaz-Heijtz & R. Gibb, unpublished observations, June 2002). This result had not been observed in our previous studies. Therefore, the purpose of this study was to administer bFGF that had been prepared just prior to injection time in animals receiving postnatal day 3 medial frontal cortex lesions to determine if indeed there were differences in anatomical and behavioral consequences.

3. 3. MATERIALS AND METHODS

3. 3. 1. Subjects

Long-Evans hooded rats were selected in a cross-litter design from 11 litters of animals. All rats were born and raised in the Canadian Centre for Behavioural Neuroscience (CCBN) rat colony at the University of Lethbridge. The fifty-eight subjects were divided into 4 groups and each group consisted of both male and female

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subjects. The groups included: control-no treatment (C-NT) (5 males, 8 females), frontal lesion-no treatment (F-NT) (6 males, 9 females), control- bFGF (C-bFGF) (6 males, 6 females), and frontal lesion-bFGF (F-bFGF) (8 males, 8 females). All subjects had access to food and water ad labium, except during the Whishaw reaching task as each animal was placed on food deprivation. During this period, rats maintained a minimum 85% of total body weight. Room temperature was kept constant at 22°C, and the lights were maintained at a 12 hour day: night cycle (Lights on at 7:30 pm). At weaning (postnatal day 23) all subjects were housed in groups with a maximum of 2 males or 3 females in 46 x 23.5 x 20 cm high standard lab cages. The cage walls were clear plastic with corn cob bedding. Experimentation conformed to CCAC guidelines.

3. 3. 2. Surgical Procedures

On postnatal day 3, pups were anesthetized by cooling in a Thermatron cooling apparatus until a rectal temperature between 18-20°C was reached. An incision was made in the skin, and the frontal bone was removed using iris scissors. Lesions were administered by aspiration and included all medial frontal subfields including Zilles' regions of Cing 1, Cing 3, and prelimbic cortex (Zilles, 1985). Using silk thread, the incision was sutured. Sham operated controls were also anesthetized by cooling, received an incision, followed by suturing of the skin. Pups were hand held until their body felt warm and were subsequently placed under a desk lamp until returned to the dam.

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3. 3. 3. Preparation of basic Fibroblast Growth Factor (bFGF)

Preparation of bFGF occurred on the same day as administration for each of the seven days of growth factor administration. To prepare the bFGF solution, 10 ml of 0.01 M phosphate buffered saline was combined with 10 mg of Albumin (Bovine). The solution was then vortexed with 10 μg of basic fibroblast growth factor. The final concentration of the bFGF solution was 1 μg/ml.

3. 3. 4. bFGF administration

Starting on post-operative day 1, bFGF was administered (0.01 cc/g body weight) every 24 hours for seven consecutive days. The dosage was administered subcutaneously between the shoulder blades with a 30 gauge needle and a 1 cc syringe. Administration occurred within the animal colony room and pups were immediately returned to the dam.

3. 3. 4. Behavioral Methods

Starting on postnatal day 60, behavioral tasks were administered in the following order: open field, Morris water task, and Whishaw reaching task.

3. 3. 4. 1. Open Field

Testing occurred in a small room in the presence of the experimenter. Each animal was placed individually in a Digiscan animal activity monitor (42 x 42 x 31 cm high) for 10 minutes. Motion detectors located along the sides of the box recorded the distance made in this novel environment, as a measure of emotionality. Movements were recorded in five, 2 minute intervals. The average of the five trials was calculated.

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3. 3. 4. 2. Morris Water Task

A circular pool with white interior was filled with 24°C water and 1L of skim milk powder. A square platform (11 x 12 cm) was submerged 15 mm below water level in the southeast quadrant of the pool. The experimenter and rat holding tubs were located in the southern portion of the cue filled room. Each rat was placed in the pool, for four trials a day from a random starting location (north, south, east, and west). Testing occurred for seven consecutive days. Once immersed in the water, initially facing the pool's edge, each subject had 90 seconds to locate the hidden platform using the extramaze cues. Once the platform was found, 10 seconds elapsed before the animal was returned to the holding tub. If the platform was not found in 90 seconds, the subject was placed on the platform for 10 seconds. A maximum of 5 minutes elapsed between trials. The average latency (seconds) for each day was calculated as well as the sum total latency of all days. To determine whether swim speed was a factor in performance the overall swim speed was compared between groups.

3. 3. 4. 3. Whishaw Reaching Task

Each reaching box (10 x 18 x 10 cm high) was composed of Plexiglas, except for the front and bottom of the box. The front of each box was open with 2 mm vertical bars across the front, enabling subjects to reach through the bars and grasp chicken feed located in a metal food tray. The cages had a mesh floor, to prevent subjects from raking the food into the cage. To encourage subjects to reach for the pellets, all subjects are food deprived to no more than 85% of their own body weight and thus provided with 15 mg of rat chow in addition to the chicken feed obtained during the task. Each rat was trained

for 10 days and then scored on day 11 of this task. On the test day all animals were videotaped for 5 minutes, commencing at the first grasp for food. A successful reach was scored if the rat was able to grab the food with its forepaws and present the pellet to the mouth. An attempt was scored if they were unsuccessful, yet made a reach by extending the wrist past the vertical bars. If the subject only reached partially through the bars no attempt was scored. The total percentage of successful reaches was calculated by determining the total number of successful reaches out of the total number of attempts.

3. 3. 5. Anatomical Methods

At the completion of the experiment, the rats were weighed and then injected with Euthansol (1 cc/kg body weight). Subjects were intracardially perfused with 0.9% saline, followed by unbuffered para-formaldehyde (PFA). Brains were extracted, and stored in PFA in 30% sucrose for a minimum of one week before being blocked (olfactory bulbs and spinal cord removed), and weighed. Tissue was sliced frozen on a Cryostat at 50 µm. Every tenth section was mounted on 1% gel and 0.2% chromium potassium sulphate slides. Sections were stained with crestyl violet.

3. 3. 5. 1. Lesion size

The entire brain was photographed from a dorsal view. Scion image was used to determine the total cortical surface area from the dorsal perspective as well as the surface area of the lesion. The percentage of missing cortical tissue relative to total surface area was calculated. Depth of the lesion was not considered.

3. 3. 5. 2. Cortical Thickness

A Zeiss petrographic viewer was used to view sections. Cortical thickness was measured in five different planes, according to a set landmark (see Experiment 1, Figure 2. 1 for details). The first measure was from the first section containing striatum; plane 2 was characterized by the presence of the anterior commissure; plane 3 by the first section of the hippocampal formation; plane 4 by the posterior commissure; and plane 5 by the last section of the hippocampus. For each plane lateral measurements were taken for each hemisphere. Because the lesions were bilateral, the overall mean for each plane was calculated.

3. 3. 5. 3. Thalamic and Brainstem Cross-sectional Area

Two sections of the thalamus were photographed with a digital camera for analysis (see Experiment 1, Figure 2. 2 for details). The first section included a visible anteromedial thalamic nucleus, mediodorsal thalamic nucleus, and paraventricular hypothalamic nucleus. The second section included a visible posterior commissure and represented the thalamus and brainstem. Scion image was used to measure the total surface area. The average thalamic and thalamic/brainstem cross-sectional area was determined in square millimeters.

3.3.6. Statistical Analysis

An analysis of variance (ANOVA) or a repeated-measures ANOVA, with it's standard error (SE), was used for the statistical analyses and reporting of each measure.

All post-hoc comparisons were performed with Fisher's LSD. Levene's test of homogeneity of variance was also utililized.

3. 4. RESULTS

3. 4. 1. Behavior

3. 4. 1. 1. Open Field

Neither medial frontal cortex lesions, nor bFGF treatment influenced open field activity (Figure 3. 1). There was a sex difference, however, as females covered more distance than males.

A three-way ANOVA (group X treatment X sex) did not find a significant group effect ($F(1,34) = 0.19$, p = .66), nor a significant treatment effect ($F(1,34) = 0.19$, p = .67). A main effect of sex was found ($F(1,34) = 14.68$, p = .001). None of the interactions were significant (F's < 0.27, p's ≥ .60).
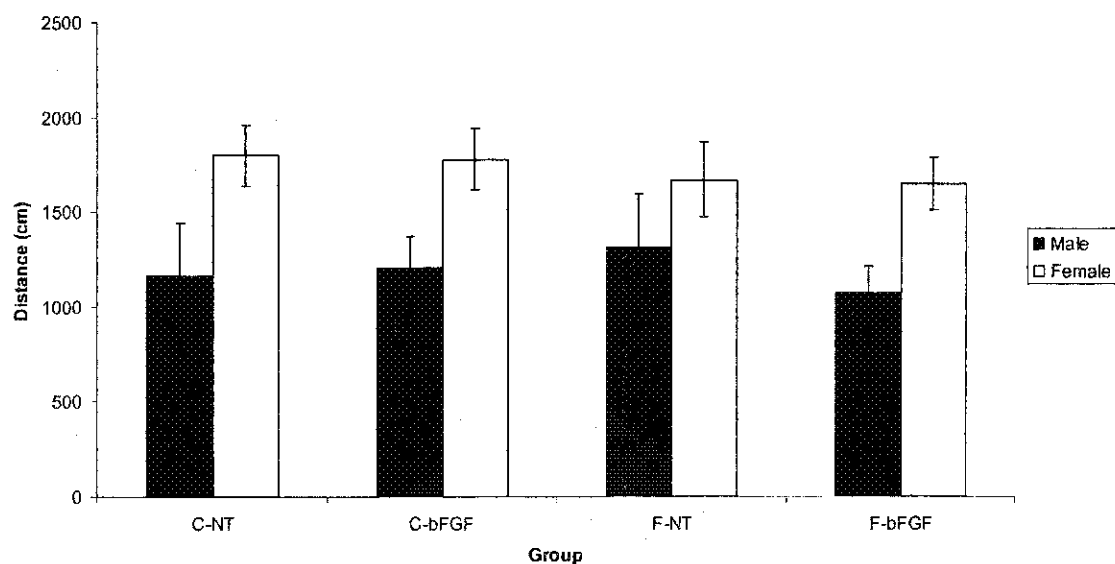
Figure 3. 1. Average distance in the open field apparatus. Males traveled less distance than females. There was not a lesion nor treatment effect.

3. 4. 1. 2. Morris Water Task

Lesion subjects were impaired at locating the hidden platform, and although they improved with time, the sum latency across 7 days was significantly longer than control subjects. If lesion subjects were treated with bFGF, enhanced performance on this task was seen such that lesion subjects treated with bFGF were 20% faster at locating the platform than untreated lesion subjects (Figure 3. 2). In addition, analyzing data on a day by day measure found that whereas the F-bFGF group was significantly impaired when compared to the C-NT group on the first day of testing, this impairment ceased on the following days as they performed as well as controls (Figure 3. 3). Untreated lesion subjects, however, continued to perform poorly on virtually every day of testing and asymptoted at a higher latency than the other groups. After seven days of testing, the F-NT subjects were consistently worse in performance than the C-NT subjects. It is possible that the improved latencies of the bFGF treated lesion subjects reflected an artifact of faster swimming in the treated than untreated subjects. An analysis of swim speed, however, found that this was not the case as there was not a difference in swim speed between the groups.

A two-way ANOVA (group X treatment) on the sum latency measure found a main effect of group ($F(1,52) = 16.01$, $p < .001$) but no overall treatment effect ($F(1,52) = 2.76$, $p = .10$) nor an interaction ($F(1, 52) = 2.17$, $p = .15$). Post hoc analysis using Fisher's LSD determined that while the C-NT group differed significantly from the F-NT

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group (p < .001), the latency achieved by the F-bFGF group did not differ significantly from the C-NT group (p > .05).

Analysis on each individual day of performance using Fisher's LSD determined a significant difference between C-NT subjects and F-NT subjects on all days (p's < .05) except for day 3 (p > .05). Untreated lesion subjects were also significantly different from all of the other groups on days 2 (p's < .01) and 4 (p's ≤ .02). bFGF-treated lesion subjects were significantly different from untreated control subjects only on day 1 (p < .05).

A two-way ANOVA on swim speed (group X treatment) did not detect a significant group effect (F(1,52) = 0.002, p = .97), treatment effect (F(1,52) = 0.03, p = .86), nor an interaction (F(1,52) = 0.74, p = .39).
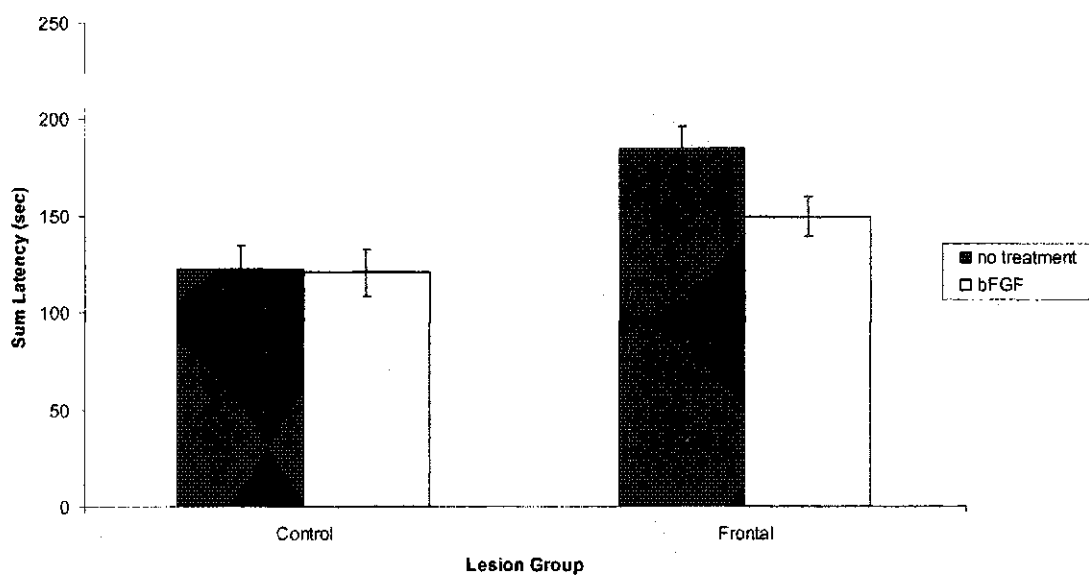
Figure 3. 2. Sum latency to locate the hidden platform in the Morris water task. bFGF significantly improved performance in lesion subjects.
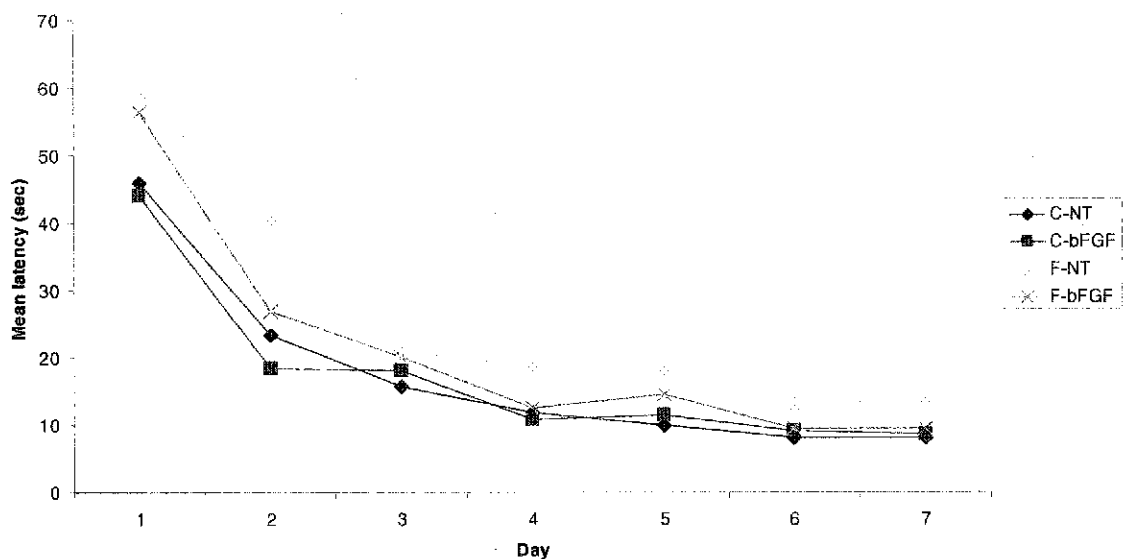
Figure 3. 3. Mean latency by day on the Morris water task. F-bFGF subjects were initially impaired at the task (day 1) relative to controls. Subsequent days found this group comparable to untreated controls in performance.

3. 4. 1. 3. Whishaw Reaching Task

bFGF enhanced reaching performance in lesion subjects but not in controls animals. Thus, whereas F-NT subjects were reaching at an accuracy of 36.2 ± 4.7 %, F-bFGF subjects had an enhanced reaching score of 50.2 ± 4.9 %, comparable to control subjects (60.9 ± 5.1 %). bFGF in control subjects did not affect performance (62.9 ± 5.5 %) (Figure 3.4). At the end of training, one C-bFGF and two F-bFGF subjects did not learn the task and thus were excluded from the analysis.

A two-way ANOVA (group X treatment) found a main effect of group ($F(1,49) = 20.43$, $p < .001$) but not treatment ($F(1,49) = 0.53$, $p = .47$). A significant group X treatment interaction ($F(1,49) = 4.08$, $p < .05$) reflects the enhanced performance of the

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F-bFGF group. The C-bFGF group was unaffected by the treatment. Post hoc

comparisons (Fisher's LSD) determined that whereas F-bFGF subjects were still

impaired at reaching relative to C-NT subjects ($p < .01$), the significant difference

between F-NT and F-bFGF subjects reflect the improved reaching performance of the

bFGF treated subjects ($p < .05$).
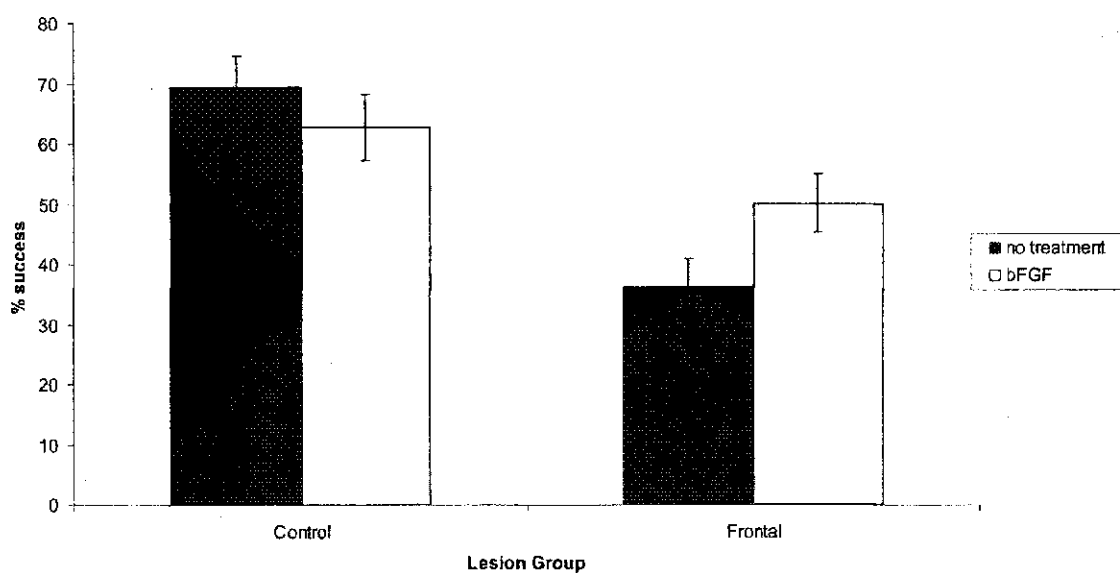
Figure 3. 4. Performance on the Whishaw reaching task. bFGF significantly improved reaching abilities in lesion subjects.

3. 4. 2. Anatomy

3. 4. 2. 1. General Observations

In this experiment 25% of the subjects show partial filling in of the lesion (figure

3.5). The new tissue is abnormal, however. Not only is the subventricular zone wider

than usual, but there are anomalies in the cytoarchitecture of the midline tissue.

Especially apparent along the midline of the cortex, there are bands of white matter that

appear to separate the intact tissue from the new tissue. The layers within the cortex are

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also abnormal and do not appear to line up with the intact tissue. At the present time, it remains unknown as to the constituents of the new tissue.
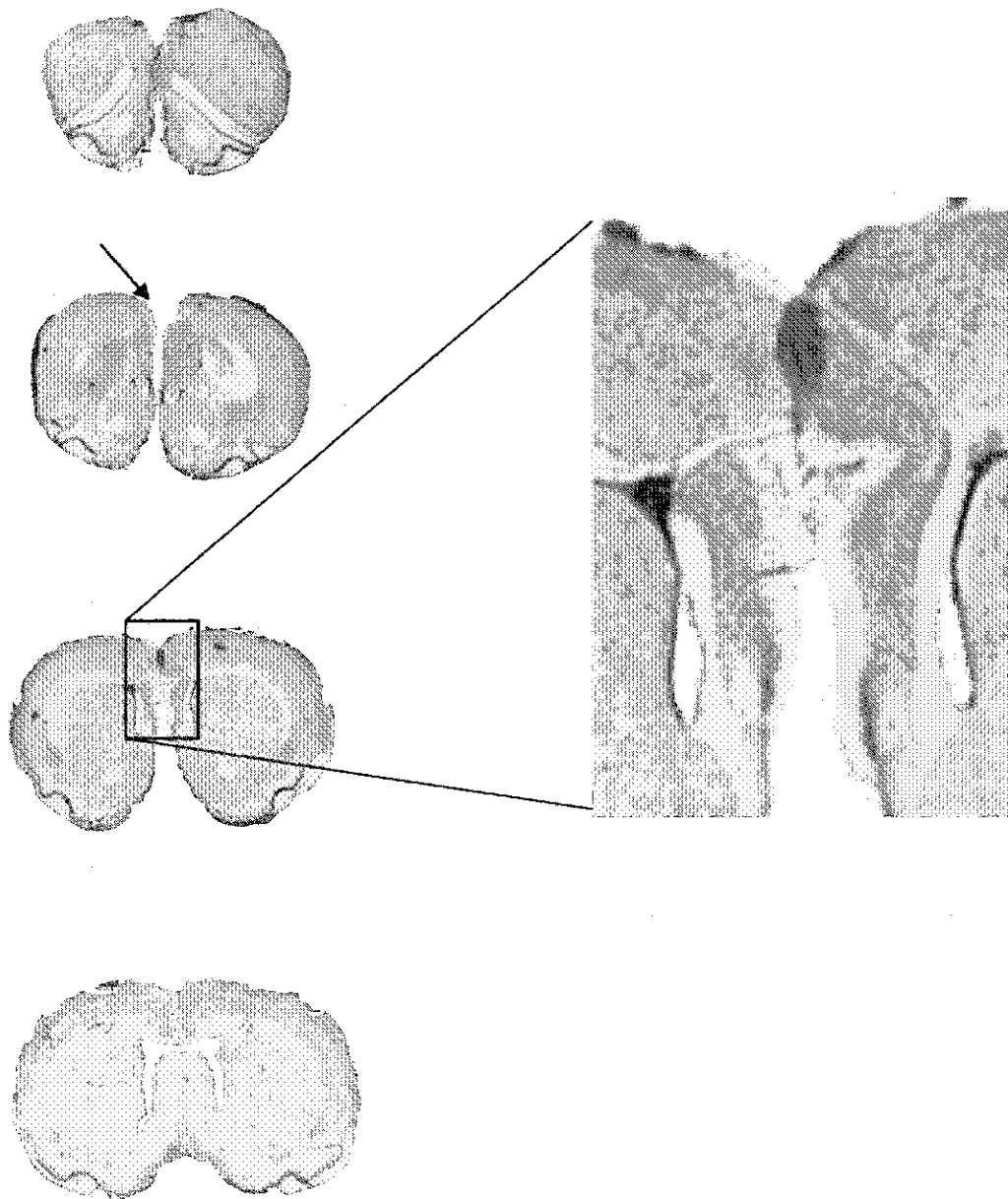
Figure 3. 5. Coronal sections of the F-bFGF group demonstrating the optimal regrowth of tissue found. Each section represents locations of abnormal tissue growth. The arrow indicates a lesion site that may have filled in with new tissue.

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3. 4. 2. 2. Lesion Size

bFGF did not affect the size of the lesion in this study. Despite the presence of new tissue in some animals, there still remained a large lesion cavity as measured from a dorsal perspective in the majority of animals. Importantly, a sign of tissue regrowth was often located near deeper layers of the cortex and therefore this measurement may not be an accurate representation of the lesion size. Untreated-lesion subjects had a lesion 10.87 ± 2.16 % of the total cortical surface area, whereas bFGF-treated lesion subjects had a comparable lesion of 10.97 ± 1.51 % of the total cortical surface area. A one-way ANOVA did not find a significant treatment effect ($F(1,20) = 0.001, p = .97$).

3. 4. 2. 3. Brain Weight

A reduction in brain weight was found in both male (15%) and female (7%) lesion subjects relative to controls. bFGF caused a further reduction in brain weight for males only. Both lesion and control males treated with bFGF had smaller brains than untreated control males. The treatment did not affect brain weight in females (Table 3. 1).

A two-way ANOVA in males determined both a significant group effect ($F(1,21) = 99.81, p < .001$) and treatment effect ($F(1,21) = 27.90, p < .001$). The group X treatment interaction was not significant ($F(1,21) = 2.81, p = .12$). Fisher's LSD revealed that the C-bFGF group was significantly smaller than the C-NT group ($p < .001$) and the F-bFGF group had smaller brains than the F-NT group ($p < .05$).

A two-way ANOVA in females revealed a main effect of group ($F(1,17) = 14.99, p = .001$), but no treatment effect ($F(1,17) = 2.98, p = .10$), nor an interaction ($F(1,17) = 0.36, p = .56$).

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Table 3. 1. Mean Brain Weight

Group	Treatment	Weight (g ± SE)	
		Male	Female
Control	NT	1.76 ± .03	1.48 ± .04
	bFGF	1.60 ± .02 *	1.56 ± .03
Frontal	NT	1.49 ± .02	1.37 ± .04
	bFGF	1.41 ± .02 **	1.41 ± .03

* Significantly different (p < .001) from C-NT
** Significantly different (p < .05) from F-NT

3. 4. 2. 4. Body Weight

Lesion males treated with bFGF showed a reduction in body weight relative to untreated controls. In addition, there was also a group effect as lesion subjects weighed less than control subjects (Table 3.2).

A two-way ANOVA for males (group X treatment) found a main effect of group $(F(1,20) = 4.70, p < .05)$, but no effect of treatment $(F(1,20) = 1.95, p = .18)$, nor an interaction $(F(1,20) = .09, p = .77)$. Post hoc analysis via Fisher's LSD determined a significant difference between the C-NT group and the F-bFGF group reflecting the decline in body weight of the bFGF treated lesion subjects $(p < .05)$. There was not a significant difference between C-NT subjects and F-NT subjects $(p = .11)$.

In females, lesion subjects treated with a higher dose of bFGF also had a decrease in body weight in comparison to untreated control and lesion subjects. A two-way ANOVA (group X treatment found a main effect of treatment $(F(1,26) = 5.92, p < .05)$. There was not a group effect $(F(1,26) = 0.78, p = .38)$, nor an interaction $(F(1,26) = 0.24,$

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p = .63). Fisher's LSD determined that F-bFGF females weighed significantly less than both C-NT and F-NT females (p's < .05).

Table 3. 2. Mean Body Weight

Group	Treatment	Weight (g $\pm$ SE)	
		Male	Female
Control	NT	436.5 $\pm$ 23.5	302.9 $\pm$ 12.2
	bFGF	399.4 $\pm$ 23.6	278.2 $\pm$ 14.1
Frontal	NT	382.5 $\pm$ 21.5	297.9 $\pm$ 12.2
	bFGF	358.4 $\pm$ 18.6 *	260.7 $\pm$ 12.2 * †

* Significantly different (p < .05) from C-NT group
† Significantly different (p < .05) from F-NT group

3. 4. 2. 5. Cortical Thickness

There was a treatment effect as bFGF-treated subjects had thicker cortices than untreated subjects. Comparing thickness on a plane by plane basis revealed that there was not a treatment effect on plane 1. On plane 2 the F-NT group had a significantly thinner cortex than the C-NT, whereas there was not a difference between the F-bFGF group and C-NT group. There was not a significant difference between the C-NT group and any of the other groups on plane 3. Interestingly, on plane 4 the C-bFGF group had a significantly thicker cortex than the other control group and both lesion groups. On plane 5, the C-bFGF group continued to have a thicker cortex than both the F-NT and F-bFGF group (Figure 3. 6).

Repeated measures by plane (group X treatment) found a significant group effect $(F(1,34) = 19.39, p < .001)$, but no overall treatment effect $(F(1,34) = 0.22, p = .65)$, nor

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an interaction (F(1,34) = 0.30, p = .59). Post hoc analysis via Fisher's LSD showed a significant difference between the C-NT group and F-NT and F-bFGF group on plane 1 (p's < .05). A significant difference between untreated control subjects and untreated lesion subjects was detected on plane 2 (p < .05), but there was not a significant difference between untreated control subjects and bFGF lesion subjects on this plane (p > .05). Because the C-bFGF group had a slight decline in cortical thickness at this plane it was not significantly different from the F-NT and F-bFGF group (p's > .05). Plane 3 did not differ between untreated controls and the other groups (p's > .05). On plane 4 the C-bFGF group was thicker than both lesion groups (p < .01) as well as the C-NT group (p < .05). On plane 5, the C-bFGF group continued to have a thicker cortex than both F-NT and the F-bFGF group (p < .01).
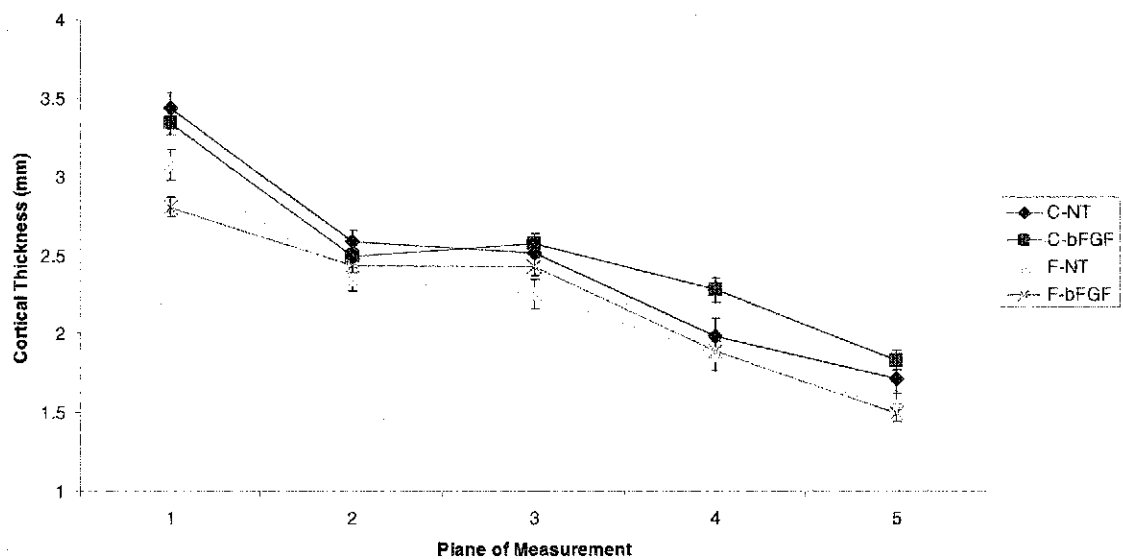
Figure 3. 6. Mean lateral cortical thickness across planes (20X magnification). bFGF increased cortical thickness at plane 4 and 5 for control subjects. On plane 2 the F-bFGF subjects did not significantly differ from C-NT subjects.

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3. 4. 2. 6. Thalamic Cross-sectional Area

Lesion-induced atrophy was present in the current study and bFGF did not enhance survival of thalamic projections. Curiously, control subjects treated with bFGF had a reduced thalamic cross-sectional area relative to untreated controls (Table 3. 3).

A two-way ANOVA (group X treatment) revealed an overall group effect $(F(1,33) = 76.23, p < .001)$. There was not a significant difference on the effect of treatment $(F(1,33) = 1.76, p = .19)$, nor an interaction $(F(1,33) = 2.88, p = .10)$. Post hoc analysis via Fisher's LSD found a significant difference between the C-NT and C-bFGF group $(p = .05)$.

Table 3. 3. Mean Thalamic Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean ± SE
Control	NT	18.37 ± .47
	bFGF	17.22 ± .32 *
Frontal	NT	14.39 ± .43
	bFGF	14.53 ± .27

* significantly different (p = .05) from untreated controls

3. 4. 2. 7. Brainstem Cross-sectional Area

A reduction in size of the brainstem was found following medial prefrontal cortex injury. The administration of bFGF following injury or in control subjects, did not affect the size of the brainstem at the measured cross-section (Table 3. 4).

A two-way ANOVA (group X treatment) determined a significant group effect $(F(1,34) = 11.33, p < .01)$. There was not a main effect of treatment $(F(1,34) = 1.16, p = .29)$, nor a group by treatment interaction $(F(1,34) = 0.04, p = .84)$.

Table 3. 4. Mean Brainstem Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean $\pm$ SE
Control	NT	30.11 $\pm$ .91
	bFGF	29.62 $\pm$ .62
Frontal	NT	27.48 $\pm$ .83
	bFGF	26.84 $\pm$ .51

3. 5. DISCUSSION

In this experiment there were two main findings. First, bFGF improved performance on a spatial navigation task, and a skilled reaching task. Second, anatomical changes associated with bFGF included an increase in cortical thickness in plane 2, such that bFGF-treated lesion subjects did not differ from controls, and in some cases filling in of the lesion cavity with new tissue. Both of these main findings will be addressed in turn.

3. 5. 1. Functional Recovery with bFGF administration

The behavioral effects of bFGF treatment following brain injury have been well documented in the adult brain (e.g. Rowntree & Kolb, 1997; McDermott et al, 1997; Gomez-Pinilla et al, 1998). Less is known, however, on the effects of bFGF following

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perinatal lesions of the medial prefrontal cortex. The results of both Experiment 1 and the current study show that although bFGF administration did not affect cognitive performance in control subjects, lesion subjects that received the treatment showed a marked improvement on spatial navigation. In contrast, bFGF treatment affected skilled reaching in the current experiment only. As mentioned earlier, the differences in the effect of bFGF in Experiment 1 and 2 were likely due to changes in the biological activity of the protein as a result of premixing the compound.

The exact mechanism of how bFGF facilitates functional recovery is currently unknown. Although an increase in dendritic branching and axonal sprouting has been found after adult lesions (Abe & Saito, 2001; Ramirez et al, 1999), similar increases in branching have not been found following early lesions of the medial frontal cortex. One hypothesis, yet to be tested, may be that bFGF influences the cholinergic system projecting to the cerebral cortex (R. Gibb, unpublished observations, 2002). It has been found in adult studies that bFGF preserves cholinergic neurons from cell death following injury (Ramirez et al, 1999; Anderson et al, 1988), an effect that may work indirectly through the action of bFGF on NGF. An increase in bFGF enhances NGF production, and NGF, in turn, enhances the cholinergic neurons both with regards to cell survival, and acetylcholine production (Yoshida & Gage, 1991; Hefti et al, 1990). Because an upregulation of acetylcholine is associated with an increase in plasticity, and ultimately with enhanced learning and memory, it may be possible that bFGF indirectly influences this system to facilitate the improved performance on the Morris water task. One way to test this possibility would be to inhibit acetylcholine production in the brain while administering bFGF.

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3. 5. 2. Anatomical Changes Associated with bFGF

Medial prefrontal cortex lesions administered at any developmental period except during postnatal day 7-12, will result in a lesion cavity. Removal of the medial frontal cortex on days 7-12 leads to a spontaneous regrowth of the lost tissue (Kolb et al, 1998c) but it has proven difficult to stimulate this neuronal growth at other lesion ages. Thus, for example, treatments such as complex housing, tactile stimulation, behavioral training, acetylcholine (acquired through a high choline diet), or low doses of bFGF will not trigger filling in of the lesion cavity with new tissue (R. Gibb & B. Kolb, unpublished observations, 2001). One hypothesis to explain this age-dependent phenomenon is that the regeneration is dependent upon rapid astrocytosis that is observed at days 7-12. Astrocytes are believed to produce growth factors such as bFGF. It is possible that through the administration of exogenous bFGF during a period in development that typically lacks astrocytes and therefore their plasticity inducing properties, the bFGF itself induces plasticity within the brain to produce new brain tissue that fills the lesion cavity (Gibb et al, 2002).

Importantly, the initial finding of regrown brain tissue following the administration of bFGF was found when pups were sacrificed 21 days following bFGF injections. In the present study, all subjects were sacrificed in adulthood (> P60). The age of the animal may have influenced the anatomical findings as the preliminary study demonstrated regrowth of tissue in nearly every animal, whereas in this study only 25% of the subjects in the anatomical analyses showed signs of new tissue. The remaining 75% of the subjects had lesion cavities that resembled those of the untreated lesion

subjects. One possible explanation for this difference is that in the time between the regrowth of tissue and the harvesting of the brains in adulthood, the new tissue died because of an inability to make connections with the surrounding tissue. Similar results were found in a study by Kolb and colleagues in which a cocktail of growth factors was administered to rats with cortical lesions in adulthood (Kolb, 1999). Although new tissue was initially produced, with time this tissue died as it was unable to form connections with other cells for continued survival. It is possible that in order to help the tissue survive, continued neuronal activity may be required.

One primary function of bFGF is to promote cell survival. Thus, it was initially hypothesized that bFGF administration might help to reduce lesion size in lesion subjects, but this was not observed in either Experiment 1 or 2. Kawamata and colleagues (1996) found that a reduction in lesion size occurred following bFGF administration only if bFGF was given immediately after injury. If a waiting period of twenty-four hours elapsed, however, the reduction in lesion size did not occur. Therefore, if bFGF had been administered immediately following injury, rather than 24 hours later, we may have found a decrease in lesion size.

Regardless of the presence of a lesion cavity, bFGF treated lesion subjects did not differ from controls in lateral cortical thickness on plane 2 of the brain, proximal to the lesion. This finding may represent increased dendritic branching in the remaining tissue or an increase in other factors such as blood vessels, glia, or neuron number. Although an increase in dendritic branching was not found following bFGF administration in earlier studies (R. Gibb & B. Kolb, unpublished observations, 2002), this finding was also based on the low-dose of bFGF. Interestingly, although control subjects that

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received bFGF did not improve in performance on the spatial navigation task, this group also demonstrated an increase in cortical thickness in posterior regions of the brain.

Yamada (1991) found that bFGF prevented thalamic retrograde degeneration and cell atrophy after injury in the adult brain. bFGF not only preserved neurons in the injured hemisphere, but an increase in cell number was also found. In this study, we did not find that bFGF was beneficial in preventing the typical atrophy associated with early brain injury, and thus there may be age-related effects of the role of bFGF.

4. EXPERIMENT 3: The Effects of a Combined Treatment of bFGF and Complex-Housing Following Perinatal Medial Prefrontal Cortex Injury

4. 1. ABSTRACT

This study analyzed the behavioral and anatomical effects of a combined treatment of bFGF (basic fibroblast growth factor) with complex-housing following postnatal day 3 medial frontal cortex lesions. Although either treatment alone was beneficial on a spatial navigation task, better performance was found in subjects that received the combined treatment. The combined treatment group demonstrated a lower latency on the water task than both the no-treatment and bFGF group. This improved functional outcome in the combined treatment was correlated with increased cortical thickness.

4. 2. INTRODUCTION

The benefits of basic fibroblast growth factor administration following perinatal brain injury have been demonstrated repeatedly by Kolb and colleagues (2000) and in experiment 1 and 2. The recovery is still incomplete, however, and therefore it is possible that the effects of bFGF might be enhanced when combined with experience. For example, following adult motor cortex lesions, bFGF is only beneficial if paired with behavioral training (Witt-Lajeunesse, 2001; Kolb et al, 2001). Experience may not always be advantageous, however, because experiment 1 showed that following perinatal medial frontal cortex injury the combination of bFGF with tactile stimulation produced a worse functional outcome than no treatment at all. Taken together these results suggest that experience may interact differently with bFGF at different times of brain development. One purpose of the current study is to determine the behavioral and anatomical effects of bFGF in conjunction with complex-housing following postnatal day 3 medial frontal cortex lesions. Unlike tactile stimulation, enhanced sensory stimulation provided by complex-housing will commence at weaning after the neurotrophic factor bFGF has already been provided rather than simultaneously.

The other purpose of this study stems from the peculiar anatomical results found in the previous experiment. Although unpublished preliminary data by R. Diaz-Heijtz and R. Gibb (June 2002) had found total filling in of the lesion cavity when animals were sacrificed at an early age (i.e. postnatal day 21), similar results were not found in experiment 2 when subjects were sacrificed in adulthood. Instead, partial regrowth was found in some, but not all brains analyzed, and for the most part a large lesion cavity was present. Also the extent of regrowth varied among animals, and currently the mechanism

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underlying these differences are unknown. Because anatomical analysis at postnatal day 21 found complete regrowth of tissue, whereas analysis of adult brains demonstrates only partial or no regrowth, it is possible that following postnatal day 21, the new tissue died.

As described by the neurotrophin hypothesis, cell survival is dependent upon neuronal activity. With increasing levels of activity between cells, a strengthening of synaptic connections occurs, thus increasing the chance for tissue survival (e.g. McAllister et al, 1999; Schinder & Poo, 2000). An effective way to enhance general neuronal activity is through complex-housing. Therefore a possible way to keep the newly generated tissue from dying within the adult brain may be through the use of complex-housing. The final purpose is therefore to determine whether the addition of complex-housing to bFGF-treated lesion subjects will influence the survival of the new tissue.

4. 3. MATERIALS AND METHODS

4. 3. 1. Subjects

Eighty-nine Long-Evans rats were selected using a cross-litter design from 11 litters of animals. All rats were born and raised in the Canadian Center for Behavioral Neuroscience (CCBN) rat colony at the University of Lethbridge. The subjects were divided into 8 groups (Table 1) including both male and female subjects. The groups included: control-no treatment (C-NT) (5 males, 8 females), frontal-no treatment (F-NT) (6 males, 9 females), control- bFGF (C-bFGF) (6 males, 6 females), frontal-bFGF (F-bFGF) (8 males, 8 females), control-complex-housing (C-complex) (4 males, 4 female), frontal-complex-housing (F-complex) (4 males, 4 females), control-complex-housing

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with bFGF (C-complex+bFGF) (5 males, 3 females), and frontal-complex-housing with

bFGF (F-complex+bFGF) (5 males, 4 females). All subjects had access to food and

water ad labium, except during the reaching task when each animal was placed on food

deprivation. During this period, approximately 15g of rat chow was provided to each

subject in addition to food acquired on the reaching task, such that rats maintained a

minimum 85% of total body weight. Room temperature was kept constant at 22°C, and

the lights were maintained at a 12 hour day: night cycle (lights on at 7:30 pm). At

weaning (postnatal day 23) all subjects were housed in groups with a maximum of 2

males or 3 females in 46 x 23.5 x 20 cm high standard lab cages. The cage walls were

clear plastic with corn cob bedding. Experimentation conformed to CCAC guidelines.

Table 4. 1. Experimental Design

Group	Sex	NT	complex	bFGF	complex+bFGF
Control	Male	5	4	6	5
	Female	8	4	6	3
	Total	**13**	**8**	**12**	**8**
Frontal	Male	6	4	8	5
	Female	9	4	8	4
	Total	**15**	**8**	**16**	**9**

4. 3. 2. Surgical Procedures

On postnatal day 3, pups were anesthetized by cooling in a Thermatron cooling

apparatus until a rectal temperature between 18-20°C was reached. An incision was

made in the skin, and the frontal bone was removed using iris scissors. Lesions were

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administered by aspiration and included all medial frontal subfields including Zilles' regions of Cing 1, Cing 3, and prelimbic cortex (Zilles, 1985). Using silk thread, the incision was sutured. Sham operated controls were also anesthetized by cooling, received an incision, followed by suturing of the skin. Pups were hand held until their body felt warm and were subsequently placed under a desk lamp until returned to the dam.

4. 3. 3. Preparation of basic Fibroblast Growth Factor (bFGF)

Preparation of bFGF occurred daily on the same day as administration for each of the seven days of growth factor administration. To prepare the bFGF solution, 10 ml of .01 M phosphate buffered saline was combined with 10mg of Albumin (Bovine). The solution was then vortexed with 10 μg basic fibroblast growth factor. The final concentration of the bFGF solution was 1 μg/ml.

4. 3. 4. bFGF administration

Starting on post-operative day 1, bFGF was administered (0.01 cc/g body weight) every 24 hours for seven consecutive days. The dosage was administered subcutaneously between the shoulder blades with a 30 gauge needle and a 1 cc syringe. Administration occurred within the animal colony room and pups were immediately returned to the dam.

4. 3. 5. Housing

The no treatment groups were housed in a large room with approximately 700 other rats. Rats were housed in groups of 2-3 in clear Plexiglas cages (46 x 23.5 x 20 cm high). Subjects housed in the complex environment were housed in groups of 10 in large

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stainless steel cages (63 x 148 x 187 cm). The top, bottom and back walls were metal, whereas the other three sides had mesh wiring to allow for visibility and climbing. There were many levels and ramps to climb on, as well as objects (changed weekly) to manipulate or chew. A radio played 24 hours/day. In contrast to the no treatment group, complex-housed subjects were located in a small secluded room with very little traffic due to a lack of space in the main animal colony. Each sex was housed separately.

4. 3. 6. Behavioral Methods

Starting on postnatal day 60, behavioral tasks were administered in the following order: open field, Morris water task, and Whishaw reaching task.

4. 3. 6. 1. Open Field

Testing occurred in a small room in the presence of the experimenter. Each animal was placed individually in a Digiscan animal activity monitor (42 x 42 x 31 cm high) for 10 minutes. Infrared motion detectors located along the sides of the box recorded the average distance tracked in this novel environment, as a measure of emotionality. Movements were recorded in five, 2 minute intervals. The average of the five trials was calculated.

4. 3. 6. 2. Morris Water Task

A circular pool with white interior was filled with 24°C water and 1L of skim milk powder. A square platform (11x 12 cm) was submerged 15 mm below water level in the southeast quadrant of the pool. The experimenter and rat holding tubs were located

in the southern portion of the cue filled room. Each rat was placed in the pool, for four trials a day from a random starting location (north, south, east, and west). Testing occurred for seven consecutive days. Once emerged in the water, initially facing the pool's edge, each subject had 90 seconds to locate the hidden platform using the extramaze cues. Once found, 10 seconds elapsed before returned to the holding tub. If the platform was not found, the subject was placed on the platform for 10 seconds. A maximum of 5 minutes elapsed between trials. The total latency of all days was calculated as well as the average latency (seconds) for day one. To determine whether swim speed was a factor in platform location, the overall speed was compared between groups.

4. 3. 6. 3. Whishaw Reaching Task

Each reaching box (10x18x10cm high) was composed of Plexiglas, except for the front and bottom of the box. The front of each box was open with 2mm vertical bars across the front, enabling subjects to reach through the bars and grasp chicken feed located in a metal food tray. The cages had a mesh floor, to prevent subjects from raking the food into the cage. To encourage subjects to reach for the pellets, all subjects are food deprived to no more than 85% of their own body weight and thus provided with 15mg of rat chow in addition to the chicken feed obtained during the task. Each rat was trained for 10 days and then scored on day 11 of this task. On the test day all animals were videotaped for 5 minutes, commencing at the first grasp for food. A successful reach was scored if the rat was able to grab the food with its forepaws and present the pellet to the mouth. An attempt was scored if they were unsuccessful, yet made a reach by extending

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the wrist past the vertical bars. If the subject only reached partially through the bars no attempt was scored. The total percentage of successful reaches was calculated by determining the total number of successful reaches out of the total number of attempts.

4. 3. 7. Anatomical Methods

At the completion of the experiment, the rats were weighed and then injected with Euthansol (1 cc/kg body weight). Subjects were intracardially perfused with 0.9% saline, followed by unbuffered para-formaldehyde (PFA). Brains were extracted, and stored in PFA in 30% sucrose for a minimum of one week before being blocked (olfactory bulbs and spinal cord removed), and weighed. Tissue was sliced frozen on a Cryostat at 50 μm. Every tenth section was mounted on 1% gel and 0.2% chromium potassium sulphate slides. Sections were stained with crestyl violet.

4. 3. 7. 1. Lesion Size

The entire brain was photographed from a dorsal view. Scion image was used to determine the total cortical surface area from the dorsal perspective as well as the surface area of the lesion. The percentage of missing cortical tissue relative to total surface area was calculated. Depth of the lesion was not considered.

4. 3. 7. 2. Cortical Thickness

A Zeiss petrographic viewer was used to view sections. Cortical thickness was measured in five different planes, according to a set landmark (see experiment 1, Figure 2. 1 for details). The first measure was from the first section containing striatum; plane 2

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was characterized by the presence of the anterior commissure; plane 3 by the first section of the hippocampal formation; plane 4 by the posterior commissure; and plane 5 by the last section of the hippocampus. For each plane lateral measurements were taken for each hemisphere. Because the lesions were bilateral, the overall mean for each plane was calculated. In addition the average cortical thickness including medial, central and lateral cortex was measured for planes 4 and 5.

4. 3. 7. 3. Thalamic and Brainstem Cross-sectional Area

Two sections of the thalamus were photographed with a digital camera for analysis (see Experiment 1, Figure 2. 2 for details). The first section included a visible anteromedial thalamic nucleus, mediodorsal thalamic nucleus, and paraventricular hypothalamic nucleus. The second section included a visible posterior commissure and represented the thalamus and brainstem. Scion image was used to measure the total surface area. The average thalamic and thalamic/brainstem cross-sectional area was determined in square millimeters.

4. 3. 8. Statistical Analysis

A two or three-way analysis of variance (ANOVA) or repeated-measures ANOVA were used for statistical comparisons with the standard error (SE) reported for each. Post hoc comparisons were made using a Fisher's LSD. Homogeneity of variance was determined with Levene's test of variance. Unless otherwise stated, homogeneity of variance was not violated.

4. 4. RESULTS

4. 4. 1. Behavior

4. 4. 1. 1. General Observations

In contrast to previous studies conducted in this laboratory, subjects in the current study were housed in the complex environments that were not located in the same room as the no-treatment groups. Instead, complex-housed subjects were alone in separate quarters. This may have had an effect on their overall activity and curiosity within the complex environments because unlike previous studies in which subjects were noted to explore the cage all day long, subjects of this experiment spent a lot of time in nests made on the floor of the complex environment. Although subjects did engage in vertical activity such as climbing the sides of the cage or following the ramps, most of this activity occurred at night. An increase in activity within the complex cages appeared when people entered the room either for testing, providing food and water, or cleaning of the cages. Although this difference in activity did not appear to affect behavioral performance on tasks, it may have influenced other measures such as brain weight and cortical thickness.

4. 4. 1. 2. Open Field

Females traveled a greater distance than males. Also, the early medial frontal cortex lesions did not change the number of movements made in the open field apparatus from control subjects. There was an effect of treatment, however, as the combined treatment subjects demonstrated an overall decrease in open field activity in comparison to all other groups (Figure 4. 1).

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Using a three-way ANOVA, a main effect of sex was found, $(F(1,58) = 9.08, p <$

.005) and treatment $(F(3,58) = 3.98, p < .05)$. There was not an overall group effect

$(F(1,58) = 2.52, p = .20)$. None of the interactions were significant, $(F$'s $\leq 1.87;$ p's $>$

.15). Fisher's LSD determined a significant difference between the complex + bFGF

group when compared to all other treatment groups $(p \leq .02)$.
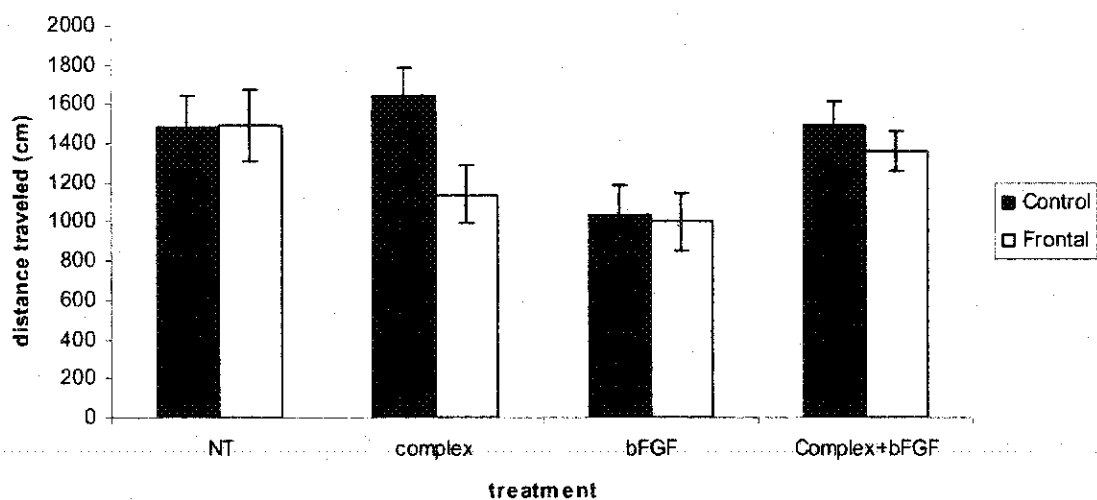
Figure 4. 1. The average distance traveled in the open field apparatus.
The complex+bFGF group traveled less than all other groups.

4. 4. 1. 3. Morris Water Task

Lesion subjects had difficulty in locating the hidden platform in the pool, and

therefore had an overall higher latency than control subjects on this task. If treated with

bFGF, complex-housing or the combination of both, the overall deficit in spatial

navigation was relieved. Furthermore, although all three treatments were helpful in

improving task performance, the lesion subjects receiving the combined treatment had the

lowest overall latency when compared to the other groups (Figure 4. 2). The combined

treatment group and the complex-housing group were comparable in spatial navigation

performance, whereas the combined treatment was more beneficial than bFGF given on its own.

A one-way ANOVA of lesion subjects found an overall treatment effect ($F(3,44)$ = 8.04, $p < .001$). A Fisher's post hoc analysis revealed a significant difference between untreated lesion subjects and lesion subjects receiving bFGF ($p < .05$), complex-housing ($p = .001$) or the combined treatment ($p < .001$). Although the bFGF-treated subjects did not differ from the complex-housing subjects ($p = .08$), there was a significant difference between those receiving bFGF and the combined treatment ($p < .05$).

The combined treatment was especially beneficial for lesion subjects on the initial day of learning the task (day 1). bFGF or complex-housing did not improve latency on this first day, whereas the combined treatment group performed better than both untreated or bFGF treated lesion subjects (Figure 4. 3). A one-way ANOVA found an overall treatment effect ($F(3,44) = 4.02$, $p < .05$). Fisher's LSD post hoc analysis revealed a significant difference between the combined treatment group and no-treatment or bFGF treatment group (p's $< .01$).

In control subjects, complex-housing with or without bFGF enhanced performance on the water task in comparison to receiving bFGF or no treatment at all (Figure 4. 2). A one-way ANOVA in control subjects found that untreated controls were significantly slower at locating the platform than the combined treatment group ($p < .01$) and those housed in a complex environment ($p < .01$). There was not a significant difference between untreated subjects and bFGF treated subjects ($p = .85$). Both groups that experienced the complex-housing were also significantly faster than the group receiving bFGF (p's $< .01$).

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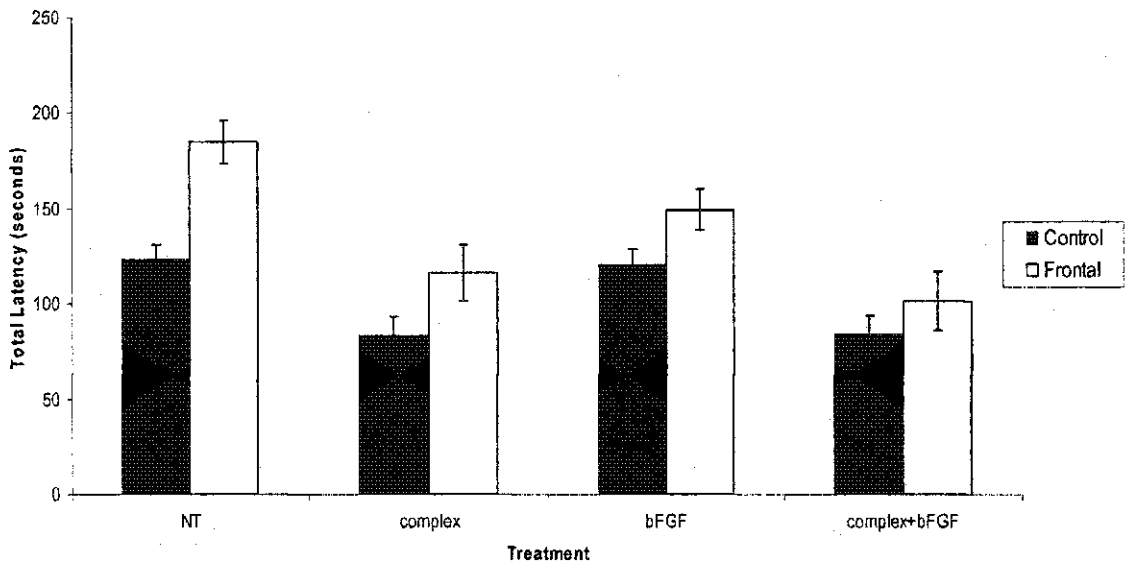
Figure 4. 2. Total latency on the water task. Complex-housing, bFGF, or the combined treatment was beneficial on this task. The combined treatment lesion subjects also performed better than bFGF treated subjects. In controls, the complex-housing or combined treatment group performed better than the bFGF group.
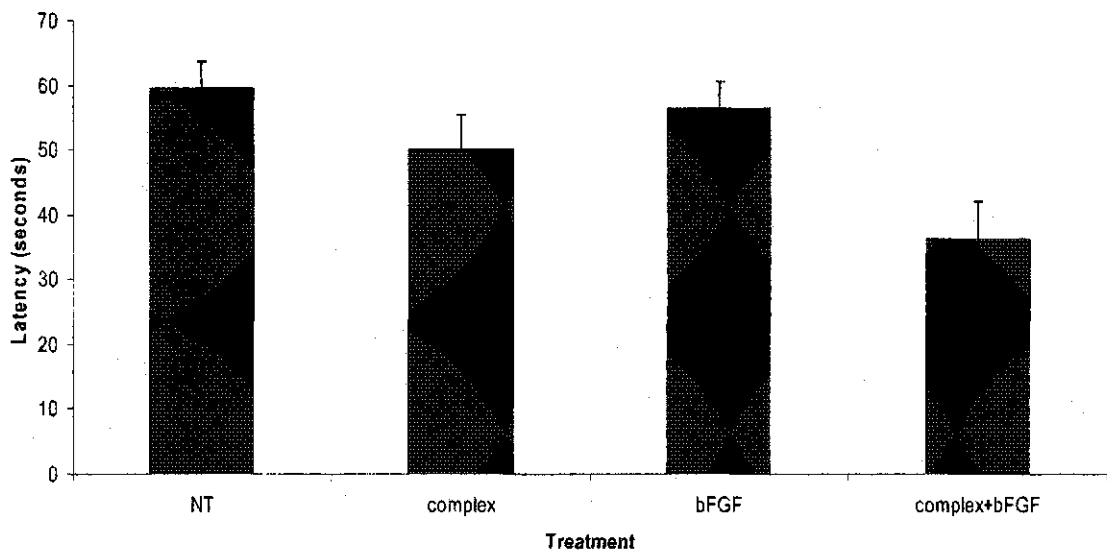
Figure 4. 3. Average latency of lesion subjects on day 1 of the water task. The combined treatment enhanced performance on this day.

Because the improved performance may be due to a difference in swim speed as opposed to enhanced learning, the swim speed was compared between groups. In control subjects, there was an effect of swim speed as the rats housed in a complex environment either on its own or in conjunction with bFGF swam faster than untreated or bFGF-treated control subjects, suggesting that these subjects were more fit than lab-reared subjects. In lesion subjects, however, there was not a treatment effect on swim speed. Therefore the improved performance on the task was not because the subjects were more physically fit, but rather because of improved cognitive functioning provided by the treatments (Figure 4. 4).
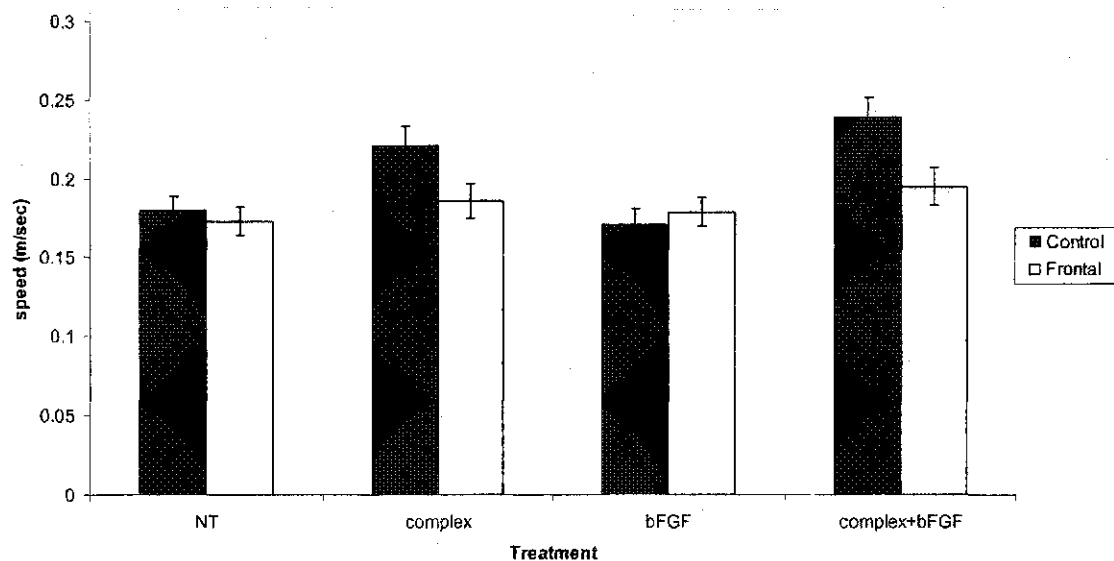
Figure 4. 4. Average swim speed in the water task. Lesion subjects did not differ in swim speed, whereas complex-housing or complex+bFGF treated control subjects swam faster than control counterparts.

An analysis of variance (group X treatment) found an overall group effect $(F_{(1,81)} = 6.83, p < .05)$ and treatment effect $(F_{(3,81)} = 7.33, p < .001)$. A trend towards a significant group by treatment interaction was also found $(F_{(3,81)} = 2.60, p = .06)$. Post hoc analysis found that control rats housed in the complex environment, with or without bFGF, swam significantly faster than control rats housed in standard lab cages (p's < .01).

4. 4. 1. 4. Whishaw Reaching Task

Medial frontal lesion subjects had difficulty at this task. Because the lesion affected fine motor skills of the forelimb, there was a decline in successful reaching when compared to control subjects (Figure 4. 5). Typically, poor performance was attributed to the inability to grasp the chicken feed with the digits influencing the reaching success rate of F-NT subjects (36.2 ± 4.4 %). In contrast, untreated control subjects had a much higher accuracy of approximately 69.5 ± 4.8 % success rate. Although the treatments did not influence the reaching performance of control animals, there was a benefit of the treatments in lesion animals. Lesion subjects treated with bFGF (50.2 ± 4.6%) were still impaired relative to untreated controls, but performed at a much higher rate than untreated lesion subjects, and were equivalent in performance to control subjects treated with bFGF or complex-housing. Although complex-housing (46.5 ± 6.5%) and the combined treatment (43.5 ± 6.1%) did not significantly improve in performance when compared to untreated lesion subjects, there was an improvement in reaching performance as they did not differ from control subjects housed in the complex environment (58.7 ± 6.1%).

A two-way ANOVA (group X treatment) found a main effect of group $(F(1,76) = 32.00, p < .001)$, but no overall treatment effect $(F(3,76) = 0.52, p = .67)$ nor a group by treatment interaction $(F(3,76) = 2.29, p = .09)$. Follow up tests using Fisher's LSD found a significant difference between F-NT subjects and F-bFGF subjects $(p < .05)$, and no significant difference between C-bFGF subjects and F-bFGF subjects $(p = .07)$. Although the C-complex group was significantly different from the F-NT group $(p < .05)$, there was not a significant difference when compared to the F-bFGF group $(p = .27)$, F-complex+bFGF $(p = .08)$ or F-complex group $(p = .18)$.
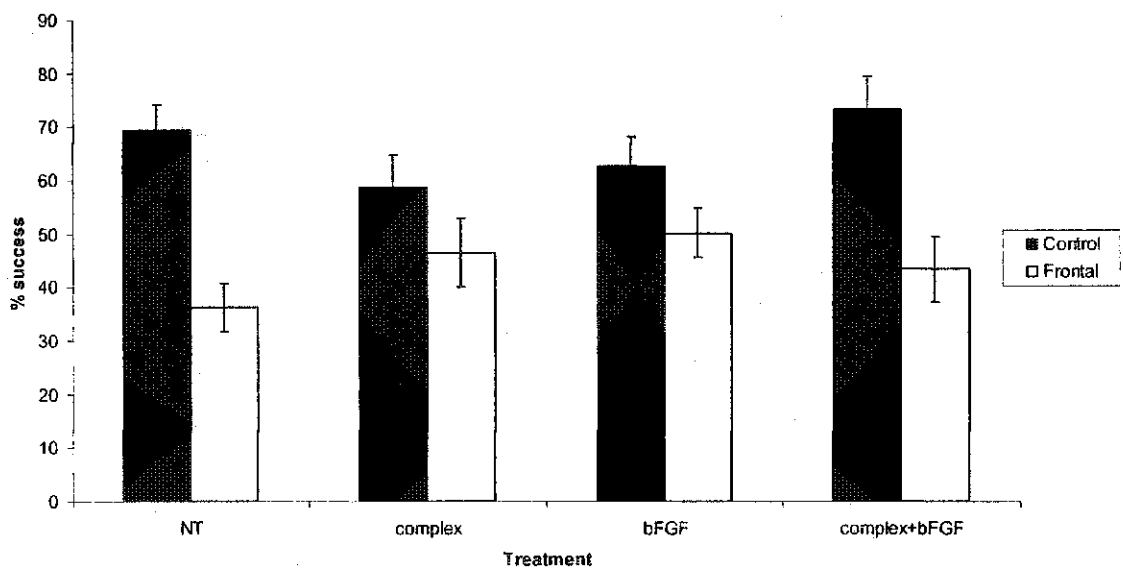
Figure 4. 5. Average reaching performance. bFGF enhanced reaching performance in lesion subjects.

4. 4. 2. Anatomy

4. 4. 2. 1. General Observations

Evidence of new tissue was found in approximately 56% of subjects exposed to

the combined treatment of bFGF and complex-housing. This is a dramatic increase in

tissue survival, as only 25% of subjects that received bFGF alone had new tissue present

in adulthood. The filling in of tissue was not complete in the majority of cases, and a

lesion cavity still remained, suggesting that some of the new tissue died with time.

4. 4. 2. 2. Lesion Size

In the previous experiment, bFGF did not reduce the lesion size as measured from

a dorsal perspective (depth of lesion not considered). Although it was hypothesized that

the addition of complex-housing may aid in this reduction typically found following adult

lesions, this was not the case. Regardless of treatment type there was no difference in

lesion size (Table 4. 2).

A one-way ANOVA did not find a significant treatment effect ($F(3,33) = .33$, $p =$

.80). Post hoc analysis via Fisher's LSD did not reveal any significant differences.

Table 4. 2. Lesion Size as a Percentage of Total Dorsal Cortex

Treatment	NT	complex	bFGF	complex+bFGF
% Lesion	12.19 ± 2.10	10.29 ± 1.60	10.97 ± 1.51	9.40 ± 1.45

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4. 4. 2. 3. Brain Weight

As males typically have larger brains than females, each of the sexes was analyzed separately. In males there was the usual lesion-induced atrophy of the brain causing an overall decline in total brain weight. While past research demonstrates an increase in brain weight with subjects housed in a complex environment, this was not found in this study. Instead subjects housed in a complex environment did not differ from subjects housed in a standard lab cage. This was also true for subjects that received the combined treatment. If bFGF was administered on its own than a further decline in brain weight was found in both lesion and intact subjects, whereas those subjects that received the combined treatment did not show this bFGF treatment effect, suggesting that the complex-housing may have reversed the reduced brain weight attributed to the bFGF treatment (Table 4. 3).

Analysis using a two-way ANOVA determined both an overall group effect ($F(1,35) = 120.43$, $p < .001$) and treatment effect ($F(3,35) = 7.02$, $p < .001$). A significant group by treatment interaction was not found ($F(3,35) = 1.34$, $p = .28$). The overall treatment effect was influenced by differences found between the bFGF group and all other treatment groups (p's $< .02$). Further post hoc analyses revealed that in particular the bFGF-treated controls were significantly different from both no-treatment controls ($p< .001$) and combined treatment controls ($p < .01$). In lesion subjects, the only significant difference was between bFGF treated subjects and untreated subjects ($p < .05$).

Females also demonstrated a reduced brain weight in lesion subjects. Unlike males, there was an effect of complex-housing on brain weight; however, this effect was

limited to lesion subjects. Control subjects housed in a complex environment did not

demonstrate an increase in brain weight. bFGF and the combined treatment did not

influence brain weight (Table 4. 3).

A two-way ANOVA (group X treatment) found a main effect of group $(F(1,34) = 45.57, p < .001)$. There was not a main effect of treatment $(F(3,34) = 1.08, p = .37)$ nor a significant interaction $(F(3,34) = 0.65, p = .59)$. Post hoc analysis using Fisher's LSD revealed that there was a significant increase in brain weight in lesion subjects housed in a complex environment such that they were not statistically different from untreated controls $(p > .05)$.

Table 4. 3. Mean Brain Weight

Group	Treatment	Weight (g $\pm$ SE)	
		Male	Female
Control	NT	1.76 $\pm$ .03	1.54 $\pm$ .04
	Complex	1.67 $\pm$ .03	1.62 $\pm$ .05
	bFGF	1.60 $\pm$ .03 *	1.56 $\pm$ .03
	complex+bFGF	1.71 $\pm$ .03	1.62 $\pm$ .05
Frontal	NT	1.49 $\pm$ .03	1.41 $\pm$ .03
	Complex	1.49 $\pm$ .03	1.44 $\pm$ .04 •
	bFGF	1.41 $\pm$ .02 †	1.41 $\pm$ .03
	complex+bFGF	1.44 $\pm$ .03	1.39 $\pm$ .04

* Significantly different from C-NT $(p < .001)$ and C-complex+bFGF $(p < .01)$
† Significantly different from F-NT $(p < .05)$
• Not significantly different from C-NT $(p > .05)$

4. 4. 2. 4. Body Weight

Because males are heavier than females each sex was analyzed separately. In males, untreated lesion subjects did not differ in body weight from their control counterparts. While the various treatments did not influence body weight in control subjects, there was a reduction in body weight in lesion subjects that received bFGF, or complex-housing. Not only were they smaller than untreated controls, but these groups also weighed less than complex-housed controls. In particular, lesion subjects receiving the combined treatment were also smaller than untreated lesion subjects as well as all control groups (Table 4. 4).

Analysis using a two-way ANOVA (group X treatment) found an overall group effect ($F(1,34) = 17.39$, $p < .001$), but not a treatment effect ($F(3,34) = 2.67$, $p = .06$), nor a group by treatment interaction ($F(3,34) = 0.31$, $p = .82$). Fisher's LSD found a significant difference between the C-NT and C-complex group when compared to the F-bFGF group, and the F-complex group (p's $\leq .007$). The F-complex+bFGF group differed significantly from both the F-NT group ($p < .05$) and all control groups (p's $\leq .01$).

As found in males, lesion females that received bFGF, complex-housing, or both, had a reduced body weight when compared to control subjects. Again, untreated lesion subjects did not differ from untreated control subjects. In controls, although complex-housing did not change body weight in comparison to untreated controls, there was a difference between subjects receiving bFGF, either alone or with complex-housing when compared to the C-complex group (Table 4. 4).

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A two-way ANOVA found an overall group effect (F(1,39) = 13.12, p = .001). A significant treatment effect (F(3,39) = 2.03, p = .13) or group by treatment interaction (F(3,39) = 2.69, p = .06) was not found. Post hoc analysis revealed a significant difference between untreated control subjects and treated lesion subjects (p's < .02). Although there was not a significant difference between the C-complex group and C-NT group (p = .13), all other control groups differed from the C-complex group (p's ≤ .008).

Table 4. 4. Mean Body Weight

Group	Treatment	Weight (g + SE)	
		Male	Female
Control	NT	436.48 ± 20.04	302.91 ± 10.75
	Complex	414.00 ± 22.41	328.33 ± 12.42
	bFGF	399.36 ± 20.04	278.17 ± 12.42 †
	complex+bFGF	392.60 ± 20.04	268.67 ± 17.56 †
Frontal	NT	382.48 ± 18.30	276.83 ± 10.75
	Complex	350.00 ± 22.41 * †	246.75 ± 15.21 *
	bFGF	358.38 ± 15.84 * †	260.70 ± 10.75 *
	complex+bFGF	315.80 ± 20.04 * † •	257.00 ± 15.21 *

* Significantly different from C-NT (p < .01)
† Significantly different from C-complex (p < .01)
• Significantly different from F-NT (p < .05)

4. 4. 2. 5. Cortical Thickness

After receiving an early frontal cortex lesion, there was an overall reduction in cortical thickness. Analyzing the lateral cortical thickness across all five planes in lesion animals revealed a treatment effect on an anterior plane of measure. In particular

subjects that were housed in the complex environment (with or without bFGF) demonstrated a thicker cortex adjacent to the lesion site on plane 2. The administration of bFGF on its own did not have an effect on the lateral cortex when compared to untreated lesion subjects (Figure 4. 6). Comparisons of the lateral cortex on all other planes did not show any further treatment effects, therefore the total cortical thickness (including medial, central and lateral measures) of the posterior cortex (planes 4 and 5) were compared between lesion subjects. Although there was not a treatment effect on plane 4, lesion subjects treated with complex-housing or the combined treatment had much thicker cortices than both untreated or bFGF-treated subjects on plane 5 (Figure 4. 7).

Repeated measures on the analysis of the lateral cortex did not find an overall treatment effect ($F(3,34) = 0.85$, $p = .48$). Post hoc analysis by plane, however, found significant differences on plane 2. For instance the subjects housed in the complex environment had a significantly thicker cortex on this plane than untreated lesion subjects ($p < .05$). In addition, the combined treatment produced an even greater increase in cortical thickness as this group had a thicker cortex than both untreated and bFGF treated subjects (p's $< .05$).

Repeated measures on the total cortical thickness including medial, central and lateral cortex for planes 4 and 5 did not find an overall treatment effect ($F(3,34) = 2.37$, $p = .09$). Post hoc analysis determined that although there was not a significant treatment effect on plane 4, on plane 5 complex-housing or complex+bFGF treated subjects demonstrated an increase in cortical thickness in comparison to no-treatment or bFGF (p's $\leq .05$).

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In control subjects, an analysis of the lateral measures for each plane revealed a different trend. Instead of affecting anterior cortex, the primary cortex that was affected was posterior cortex, especially plane 4. On this plane the cortex of the bFGF or the combined treatment group was significantly thicker than the other two treatment groups (Figure 4. 8). When comparing the total cortical thickness (including medial, central and lateral measurements), similar findings occurred, as the combined treatment group and bFGF group had thicker cortices than the no-treatment group on both planes 4 and 5 (Figure 4. 9).

Repeated measures for all five planes of the lateral cortex did not find an overall treatment effect (F(3,26) = 1.54, p = .23). Post hoc analysis via Fisher's LSD detected a treatment effect on plane 4, as both the combined treatment group and bFGF group were significantly thicker than the no-treatment group (p's $\leq$ .01) or complex-housing group (p's < .05).

Repeated measures on the total cortical thickness of planes 4 and 5, revealed a main treatment effect (F(3,26) = 4.34, p < .05). Post hoc analysis determined that for both of these planes bFGF (p's < .05) and the combined treatment groups (p's $\leq$ .005) showed a significant increase in cortical thickness when compared to the no-treatment group.
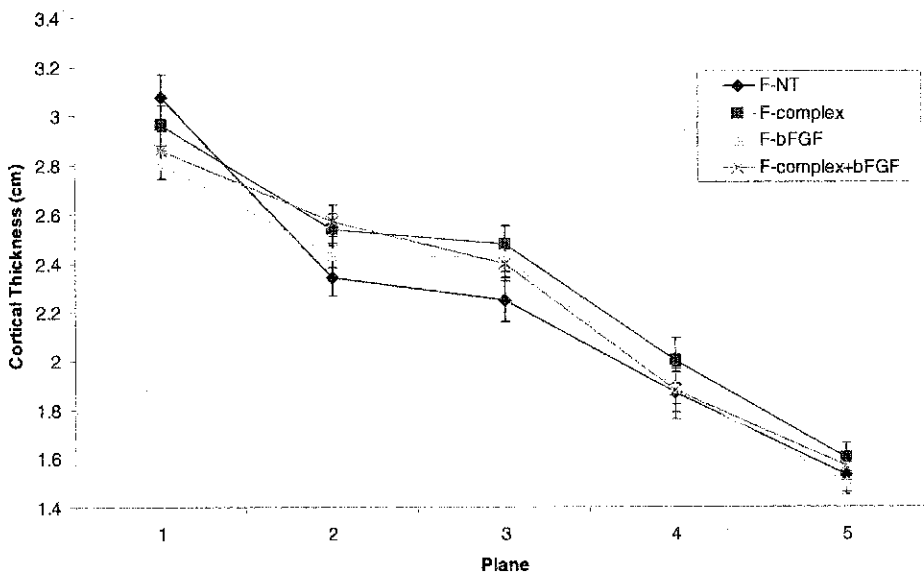
Figure 4. 6. Mean lateral cortical thickness (magnification 20X) in lesion subjects. Complex-housing and the combined treatment increased cortical thickness on plane 2.
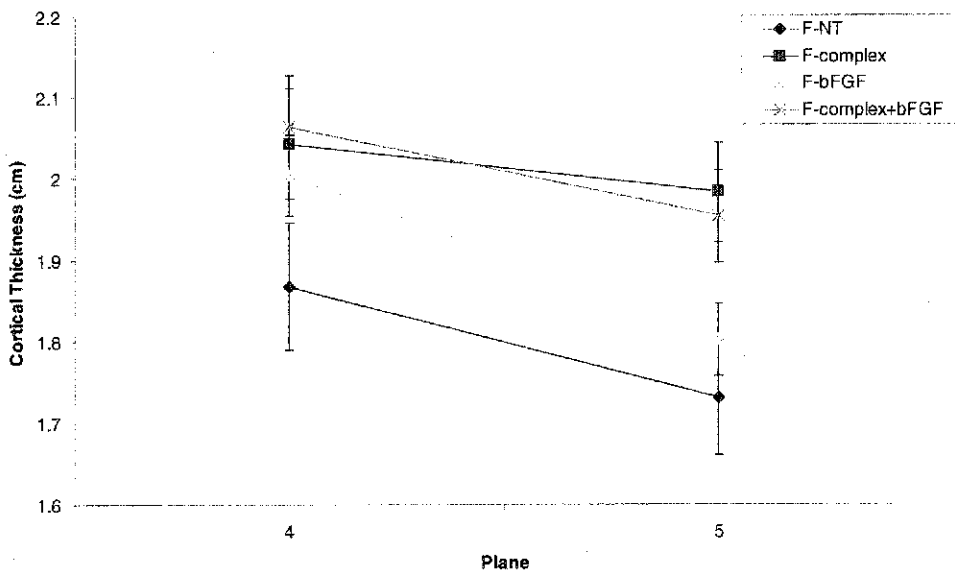
Figure 4. 7. Mean cortical thickness (magnification 20X) including medial, central and lateral cortex in lesion subjects. Complex-housing or the combined treatment increased cortical thickness on plane 5.

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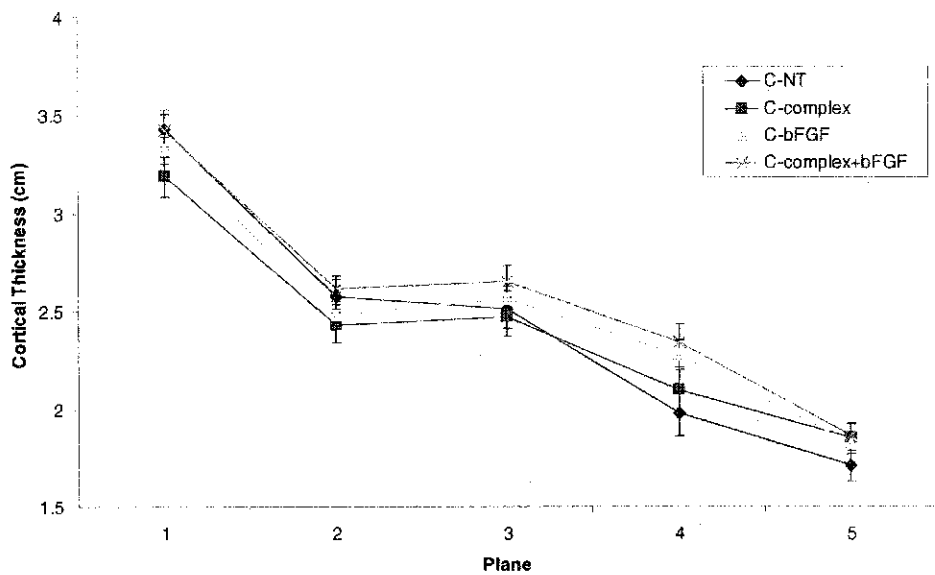
Figure 4. 8. Mean lateral cortical thickness (magnification 20X) in control subjects. bFGF or the combined treatment increased cortical thickness on plane 4.
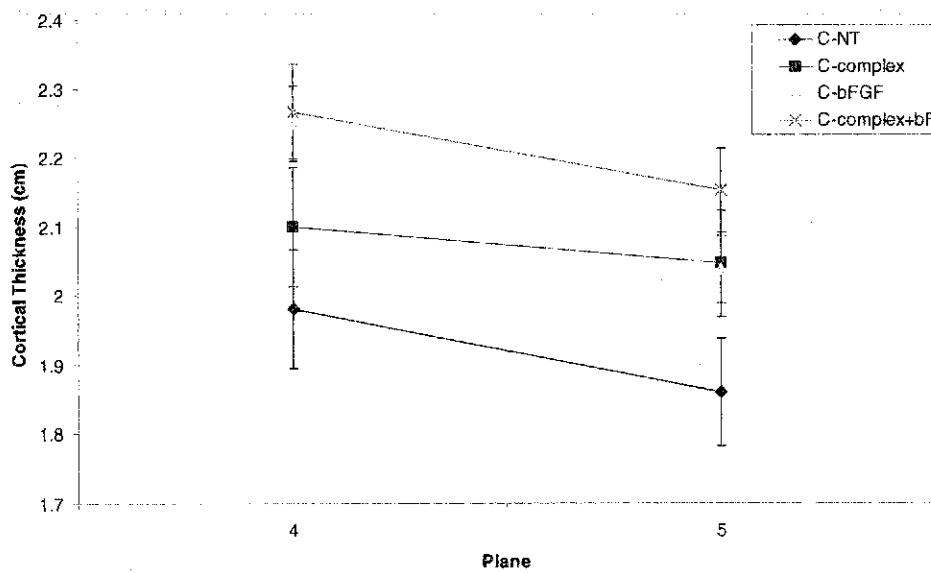
Figure 4. 9. Mean cortical thickness (magnification 20X) including medial, central and lateral cortex in control subjects. bFGF or the combined treatment increased cortical thickness in control subjects on both planes 4 and 5.

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4. 4. 2. 6. Thalamic Cross-sectional Area

Subjects receiving a frontal cortex lesion had a reduction in thalamic size. The administration of bFGF, complex-housing or the combined treatment did not influence the thalamic size in lesion subjects. In control subjects, however, although the complex-housing group did not significantly differ in size from the no-treatment group, it did increase in size slightly such that it was significantly larger than the bFGF group and the complex+bFGF group (Table 4. 5). This effect is also attributed to a slight decrease in thalamic size of subjects receiving bFGF either alone or in the combined treatment.

Table 4. 5. Mean Thalamic Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean ± SE
Control	NT	18.37 ± .59
	Complex	19.10 ± .59
	bFGF	17.22 ± .40 *
	complex+bFGF	16.89 ± .47 *
Frontal	NT	14.39 ± .54
	Complex	14.73 ± .47
	bFGF	14.53 ± .34
	complex+bFGF	13.89 + .44

* Significantly different from C-complex group (p < .05)

A two-way ANOVA (group X treatment) found an overall group effect (F(1,59) = 102.78, p < .001) and treatment effect (F(3,59) = 3.49, p < .05). There was not a significant group by treatment interaction (F(3,59) = 1.44, p = .24). Fisher's LSD

determined that the treatment effect was primarily due to a significant difference between the C-complex group and the C-bFGF group (p < .05) or C-complex+bFGF group (p = .005).

4. 4. 2. 7. Brainstem Cross-sectional Area

Untreated lesion subjects had a reduction in brainstem size in comparison to control subjects. As found with thalamic cross-sectional area, there was a slight increase in size of the C-complex group and a slight decrease in size of the C-bFGF and C-complex-bFGF group so that there was a significant difference between the two groups treated with bFGF and the complex-housing group. Unlike the thalamic measurement, in the brainstem lesion subjects housed in the complex environment alone, had a sparing of brainstem connections so that this group did not differ from the control groups (except for C-complex group) (Table 4. 6).

A two-way ANOVA (group X treatment) found a main effect of group ($F(1,59) =$ 30.39, $p < .001$) and a trend for a significant overall treatment effect ($F(3,59) = 2.67$, $p = .06$). There was not a significant group by treatment interaction ($F(3,59) = 0.62$, $p = .61$). Post hoc analysis via Fisher's LSD determined that there was a significant difference between the C-complex group and the C-bFGF and C-complex+bFGF groups (p's < .05). Also the F-complex group did not significantly differ from the control groups (p > .05), except for the C-complex group (p = .001).

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Table 4. 6. Mean Brainstem Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean + SE
Control	NT	30.11 + .91
	Complex	32.10 ± .91
	bFGF	29.17 ± .61 *
	complex+bFGF	29.63 ± .72 *
Frontal	NT	27.48 ± .83
	Complex	27.87 ± .77 **
	bFGF	26.84 ± .51
	complex+bFGF	27.05 ± .68

* Significantly different from C-complex group (p < .05)
** Not significantly different from controls (except C-complex group) (p > .05)

4. 5. DISCUSSION

The current experiment demonstrated two main findings. First, the combined treatment of bFGF and complex-housing improved performance in lesion subjects on a spatial navigation task, more so than bFGF alone. Second, although the addition of complex-housing to the bFGF treatment did not promote complete survival of tissue as there was still a lesion cavity present, a greater proportion of subjects in this group showed signs of new midline tissue. Also, the combined treatment increased cortical thickness in both control and lesion subjects to a greater degree than either treatment alone. Both of these findings will be discussed in turn (see Table 4. 7 for a summary of results).

4. 5. 1. Functional recovery following a combined treatment of bFGF and complex-housing

The combined treatment of bFGF and complex-housing was extremely beneficial to performance on a spatial navigation task. Although bFGF or complex-housing also helped improve performance in lesion subjects, an advantage of the combined treatment as opposed to either treatment alone was evident on the initial learning of the task. On day one of the water task, lesion subjects show an immediate impairment on the task, taking longer to locate the platform. Even though bFGF or complex-housing did not benefit performance on the initial learning phase, a significant improvement in performance was found in the combined treatment group. Analysis of the overall performance determined that although bFGF, complex-housing or a combination of both were ultimately influential in enhanced performance of the task, it appeared that subjects receiving the combined or complex-housing treatment performed better than subjects receiving no treatment or bFGF alone on the spatial navigation task. This finding parallels results found by Witt-Lajeunesse (2001) as the combined treatment of bFGF with training following adult motor cortex lesions was more beneficial than no treatment or bFGF alone.

The primary influence for the beneficial effect of the combined treatment therefore appears to be the experience gained from complex-housing. This is not to disregard the benefits of bFGF following injury as subjects receiving this treatment did show an improvement relative to untreated lesion subjects, but rather to bring forward the fact that there was not a significant difference between the combined treatment group and complex-housing treatment group, whereas the combined treatment group performed

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better than the bFGF treatment group. Furthermore, only lesion subjects demonstrated any benefit from the bFGF treatment, whereas it appears that the complex-housing has a much greater effect on subjects in general as both control and lesion subjects housed in the complex environment, with or without bFGF had lower latencies on the task. The effects of complex-housing, however, are different depending on whether or not the subject has a lesion. In control subjects, improved performance may be associated with a faster swim speed, indicating that these subjects were more physically fit that controls housed in a standard lab cage. In contrast, swim speed was unaffected in lesion subjects, suggesting that the benefit of complex-housing was targeted towards enhanced learning of the task.

A variety of factors may be credited to the greater benefit provided by complex housing. Although the role of bFGF has been suggested to enhance cortical plasticity and thus behavioral recovery, the actions of complex-housing may have a greater effect on the brain as a whole. Even though both bFGF and complex-housing promote increased levels of NGF, and acetylcholine, in both direct and indirect ways, complex-housing increases additional growth factors such as NT-3, GDNF, BDNF, and bFGF as well as neurotransmitters such as norepinephrine (Yoshida & Gage, 1991; Belluardo et al, 1999; Vige et al, 1991; Naka et al, 2002; Torasdotter et al, 1998; Parks et al, 1992).

Another possibility may be that complex-housing provides general "training" of the subjects as they experience enhanced sensory stimuli from a variety of sensory modalities as well as an enhanced social environment. It is the combination of both interacting with the environment and with other rats that facilitates the effectiveness of complex-housing (Johansson & Ohlsson, 1996). Also, as suggested by Juraska and

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colleagues (1984), complex-housing may facilitate the use of different problem solving strategies on the task. Similarly, Finger (1978) suggested that after brain injury, cues that would normally be salient or used for successful performance on a task may be unavailable after the injury. If so, it is likely that subjects will switch to using other modalities to solve the task and that the complex-housing may facilitate this form of compensation.

Interestingly, the effect of a combined treatment that includes both the pharmacological manipulation of bFGF as well as an enhanced sensory stimulation seems to depend on the time or age of treatment administration. As demonstrated in experiment 1, the combined treatment of tactile stimulation and bFGF produced adverse effects on the behavioral outcome in adulthood. Studies conducted by Witt-Lajeunnesse (2001) found that following adult motor cortex injury the combination of both bFGF and complex-housing had a synergistic effect as the combination provided greater benefits than either treatment alone (see also Kolb et al, 2001). This current experiment suggests that although the combined treatment is beneficial following postnatal day 3 injury, it remains unclear as to whether this is due to a difference in the procedure of applied sensory stimulation or because of a timing difference of the sensory stimulation.

4. 5. 2. Anatomical changes associated with the combined treatment

Unpublished observations had initially discovered that if bFGF was prepared daily prior to administration than a complete filling in of the lesion cavity occurs by postnatal day 21 (R. Diaz-Heijtz & r. Gibb, unpublished observations, June 2002). In experiment 2, we found that if the subjects were sacrificed in adulthood (~ postnatal day

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90) than in most cases a large lesion cavity was evident. There were, however, signs of partial filling in of the lesion cavity in some subjects. We proposed that between postnatal day 21 and postnatal day 90, the filling in of the lesion cavity with new tissue ultimately died between this time, and that perhaps with enhanced neuronal activity provided by complex housing starting at weaning (postnatal day 23) throughout adulthood this may promote survival of the new tissue. Current findings suggest that although a greater number of animals that received the combined treatment showed signs of partial filling in, the lesion cavity was still present.

Analysis of cortical thickness revealed that the combined treatment group demonstrated an increase in cortical thickness in both lesion and control subjects. The effect of the combined treatment was more pronounced than either treatment given alone. For instance, in control subjects, there was an increase in cortical thickness in bFGF treated subjects but not with complex-housing alone. In contrast, in lesion subjects there was a greater increase in subjects housed in a complex environment than subjects that received bFGF. Analysis of the combined treatment group revealed that administration of both of these treatments influenced cortical thickness so that lesion and control subjects demonstrated a thicker cortex. Currently, we are unable to determine the cause for the thicker cortex. Underlying mechanisms may include increased blood vessels, neuron and/or glia number, or increased synapse number (Rosenzweig & Bennett, 1996; Kolb et al, 2003; Sandeman & Sandeman, 2000; Diamond et al, 1964; 1966).

Table 4. 7. Summary of Results in Comparison to Untreated Subjects

	Complex+bFGF		Complex		bFGF	
	C	F	C	F	C	F
Open Field	↓	↓	---	---	---	---
Water Task	↑	↑	↑	↑	---	↑
Reaching	---	↑*	---	↑*	---	↑
Brain Weight	---	---	---	↑(♀)	↓(♂)	↓(♂)
Body Weight	↓*	↓	---	↓	↓*	↓
Cortical Thickness: Lateral	↑(4)	↑(2)	---	↑(2)	↑(4)	---
Cortical Thickness: planes 4 & 5	↑	↑(5)	---	↑(5)	↑	---
Thalamus	↓*	---	---	---	↓*	---
Brainstem	↓*	---	---	↑	↓*	---
Lesion Size	NA	---	NA	---	NA	---

--- relative to no-treatment group
↑ Increase/improvement relative to no treatment
↓ Decrease relative to no treatment
* When compared to C-complex group only
(#) indicates plane of cortical thickness result

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5. EXPERIMENT 4: The Effects of Environmental Enrichment Following Perinatal Orbital Frontal Cortex Lesions

5. 1. ABSTRACT

The current experiment studied the effects of environmental enrichment following postnatal day 3 (P3) orbital frontal cortex lesions on a battery of cognitive and motor tasks. P3 orbital frontal lesions produced motor deficits (skilled reaching; tongue extension) but the performance on cognitive tasks (the Morris water task; bar press extinction) was unaffected by the early orbital frontal lesions. Complex housing abolished motor impairments in both reaching and tongue extension in orbital frontal subjects. Furthermore, living in complex-housing improved the functional outcome on the spatial navigation task regardless of lesion group. Although the lesions produced small brains with a thin cortex, complex housing partly reversed these effects: There was an increase in brain weight as well as an increase of cortical thickness in posterior cortical regions. Complex housing therefore can reverse at least some effects of perinatal orbital frontal injury, likely by reducing some of the lesion-induced pathology in cerebral organization.

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5. 2. INTRODUCTION

Broadly defined, the prefrontal cortex may be subdivided into a medial and orbital frontal subregion. Both regions are associated with "higher cognitive functioning", yet differ in terms of connectivity with other areas of the brain, rate of development (medial region is developmentally delayed in comparison to the orbital region), and functional contributions (Carmichael & Price, 1994; Bayer & Altman, 1991). Although the function and deficits associated with injury has been extensively analyzed in the medial frontal region of the rat prefrontal cortex, less is known about the orbital region.

Early studies conducted by Kolb and colleagues analyzed the effects of adult orbital frontal lesions on a battery of behavioral tasks. Several impairments were found, including impairment in tongue extension (Whishaw & Kolb, 1983) and cognitive deficits such as poor spatial memory as measured by the water maze task and radial arm maze (Kolb et al, 1983), as well as impaired bar-press extinction (Kolb et al, 1974). Later studies analyzed the effects of orbital frontal lesions on postnatal day 7 and showed somewhat different results. Unlike adult operates, rats with early orbital frontal lesions showed sparing of function on the spatial navigation tasks. Like adult lesions, subjects were also impaired, although to a lesser degree, at tongue extension. Interestingly, early orbital frontal lesions also produced a new motor deficit as rats had difficulty in food manipulation using the forelimbs (Kolb & Whishaw, 1985).

One purpose of the current study was to determine the effects of early postnatal day 3 orbital frontal cortex lesions on a battery of behavioral tasks similar to the Kolb & Whishaw study (Kolb & Whishaw, 1985), in order to determine if there is a functional difference between the effects of postnatal day 3 and postnatal day 7 orbital frontal

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lesions, as has been demonstrated after medial frontal lesions (Kolb & Cioe, 2000; Kolb, 1987; Kolb et al, 1998c). Thus, rats with earlier (P3) medial frontal lesions have much more severe deficits than animals with day 7 lesions. Second, the beneficial effects of a complex environment in both lesion and non-lesion subjects have been well documented (e.g., Rosenzweig & Bennett, 1972; Will et al, 1977; Johansson, 2000). Such an environment not only provides enhanced motor training, but also an enhanced sensory and social environment. Complex-housing is therefore beneficial in many ways as it supplies a multi-sensory-stimulating atmosphere, which in turn has many positive effects on the brain such as increased blood vessels, gliogenesis, synaptogenesis, and enhanced neurotransmitter and growth factor production (e.g., Globus et al, 1973; Rosenzweig & Bennett, 1972; Diamond et al, 1966; Mohammed et al, 1990). Because complex-housing has been found beneficial after injury to many different cortical regions, the main purpose of this study was to determine whether a complex environment is beneficial at ameliorating orbital frontal lesions deficits after injury, and if so, to what extent.

5. 3. MATERIALS AND METHODS

5. 3. 1. Subjects

A total of 35 Long-Evans rats were used for this study. The groups were formed across several litters and were divided as follows: control-no treatment (C-NT) (n = 10; 4 males, 6 females); orbital frontal lesion- no treatment (OF-NT) (n = 10; 6 males, 4 females); control-complex environment housed (C-complex)(n= 8; 4 males, 4 females); and orbital frontal lesion- complex environment housed (OF-complex)(n = 7; 3 males, 4 females). All subjects received food and water *ad libitum* and were set on a 12 hour day:

night light cycle starting with lights on at 7:30 am. Experimentation conformed to CCAC guidelines.

5. 3. 2. Housing

The no treatment groups were housed in a large room with approximately 700 other rats. Rats were housed in groups of 2-3 in clear Plexiglas cages (46 x 23.5 x 20 cm high). Subjects housed in the complex environment were housed in groups of 10 in large stainless steel cages (63 x 148 x 187 cm). The top, bottom and back walls were metal, whereas the other three sides had mesh wiring to allow for visibility and climbing. There were many levels and ramps to climb on, as well as objects (changed weekly) to manipulate or chew. A radio played 24 hours/day. In contrast to the no treatment group, complex-housed subjects were located in a small secluded room with very little traffic due to a lack of space in the main animal colony. Each sex was housed separately.

5. 3. 3. Surgical Procedures

The orbital frontal cortex was removed in rat pups on postnatal day 3. In order to accomplish this, pups were first cooled in a Thermatron cooling chamber (-5°C) until anesthetized. A scalpel blade incised the skull and an opening was made using iris scissors along the lateral surface of the skull. The orbital cortex, including Zilles AID and LO was removed through aspiration (Zilles, 1985). Silk thread was used to suture the incision. For control subjects, an incision in the skin was made followed by suturing the tissue. Pups were hand-held until warm, and they were subsequently placed under a heat lamp until returned to the nest.

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5. 3. 4. Behavioral Methods

5. 3. 4. 1. Open Field

Testing occurred in a small room in the presence of the experimenter. Each animal was placed individually in a Digiscan animal activity monitor (42 x 42 x 31 cm high) for 10 minutes. Motion detectors located along the sides of the box recorded the total number of movements made in this novel environment, as a measure of emotionality. Movements were recorded in five, two minute intervals. The average of the five trials was calculated.

5. 3. 4. 2. Morris Water Task

A circular pool with white interior was filled with 24°C water and 1 L of skim milk powder. A square platform (11 x 12 cm) was submerged 15 mm below water level in the southeast quadrant of the pool. The experimenter and rat holding tubs were located in the southern portion of the cue filled room. Each rat was placed in the pool, for four trials a day from a random starting location (north, south, east, and west). Once emerged in the water, initially facing the pool's edge, each subject had 90 seconds to locate the hidden platform using the extramaze cues. Once found, 10 seconds elapsed before returned to the holding tub. If the platform was not found, the subject was placed on the platform for 10 seconds. A maximum of 5 minutes elapsed between trials. Testing occurred for seven consecutive days. The sum latency to locate the platform was calculated, as well as the average time spent around the edge of the pool.

5. 3. 4. 3. Whishaw Reaching Task

Each reaching box (10 x 18 x 10 cm high) was composed of Plexiglas, except for the front and bottom of the box. The front of each box was open with 2 mm vertical bars across the front, enabling subjects to reach through the bars and grasp chicken feed located in a metal food tray. The cages have a mesh floor, to prevent subjects from raking the food into the cage. To encourage subjects to reach for the pellets, they are all food deprived to 85% of their own body weight. Each rat was trained for 10 days and later tested on day 11 of this task. All animals were videotaped for 5 minutes, commencing at the first grasp for food, and the tapes were scored. A successful reach was scored if the rat was able to grab the food and bring it up to the mouth to eat the pellet. An attempt was scored if they were unsuccessful, yet made a reach by extending the wrist past the vertical bars. If the subject only reached partially through the bars no attempt was scored. An overall score expressed as a percent (number of successful reaches/ total number of reaches X 100%) was calculated.

5. 3. 4. 4. Tongue Extension

Subjects were placed in the tray reaching boxes for this task. Chocolate chip cookies were crumbled and mixed with water to produce a mash. The mash was spread along a clear plastic ruler that was placed against the bars of the reaching chamber. Each subject had an initial two days to acquire a taste for the mash and to learn how to lick it off of the ruler. For the next three days, tongue extension was measured by recording how much cookie mash was obtained from the ruler. The mean length (mm) of the three trials was analyzed.

5. 3. 4. 5. Extinction Task

Each subject was individually placed in an operant-conditioning chamber. The chamber was made of clear Plexiglas on the top and two sides, with the other two sides (opposite from one another) containing one lever on one side, and two levers and food dispenser on the other. The floor had 2 mm wide bars, 5 mm apart. Initially, each rat was placed in the box for 30 minutes each day. Two-three pellets (45 mg dustless precision pellets) were left in the food dispenser and more could be acquired by pressing the lever to the right of the dispenser. MED-PC IV software kept count of the number of right lever presses. Once a minimum of 50 responses were made in 30 minutes, subjects were placed on continuous reinforcement for 10 days at 15 min/day. On day 11 to day 15, food pellets were not available and the number of right bar presses was counted (30 minute sessions/day) to obtain a measure of preservative tendencies.

5. 3. 5. Statistical Analysis

There were no sex differences on any behavioral measures so the data were pooled for analysis. Statistical analysis was conducted by two-way ANOVA's (± standard error) and follow-up (Tukey's) tests. The Tukey's post hoc analysis was chosen because not all of the comparisons were planned or results expected.

5. 3. 6. Anatomical Methods

At the completion of the experiment, rats were weighed, and then injected with Euthansol (1cc/kg body weight). Male subjects were intracardially perfused with 0.9% saline, followed by unbuffered para-formaldehyde. Brains were extracted, and stored in

PFA in 30% sucrose for a minimum of one week before being blocked (olfactory bulbs

and spinal cord removed), and then weighed. Sliced frozen on a Cryostat at 50 μm, every

tenth section was mounted on 1% gel and 0.2% chromium potassium sulphate slides.

These sections were stained with crestyl violet. Female subjects were intracardially

perfused with 0.9% saline and the brains were extracted and weighed in a similar fashion

to male subjects. The brains were stored in 20 ml of golgi-cox solution for 14 days,

followed by 30% sucrose for 2-5 days. Tissue was sliced on a Vibrotome at 200 μm, and

processed according to procedures outlined by Gibb and Kolb (1998).

5. 3 .6. 1. Cortical Thickness

A Zeiss Petrographic viewer was used to view sections. Cortical thickness was

measured in five different planes, according to a set landmark (see experiment 1, Figure

2. 1). The first measure was taken from the first section containing striatum; plane 2 was

characterized by the presence of the anterior commissure; plane 3 by the first section of

the hippocampal formation; plane 4 by the posterior commissure; and plane 5 by the last

section of the hippocampus. For each plane, three measurements were taken for each

hemisphere. The overall mean for each plane was calculated.

5. 3. 6. 2. Thalamic Cross-sectional Area

The thalamic section chosen for analysis included the mediodorsal nuclei, as well

as the ventral lateral thalamic nuclei. This section was chosen because past research

demonstrated that early orbital frontal lesions will cause atrophy of the ventral lateral

nuclei (Kolb and Nonneman, 1978). The sections were photographed with a digital

camera, and scion image was used to determine the total cross-sectional area. This measure was taken for males only, as Golgi staining of the females' brain tissue made it difficult to locate individual thalamic nuclei.

5. 4. RESULTS

5. 4. 1. Behavior

5. 4. 1. 1. General Observations

Subjects housed in the complex environments were not located in the same room as the no-treatment groups. Instead, complex-housed subjects were alone in separate quarters. This may have had an effect on their overall activity and curiosity within the complex environments because unlike previous studies in which subjects were noted to explore the cage all day long, subjects of this experiment spent a lot of time in nests made on the floor of the complex environment. Although subjects did engage in vertical activity such as climbing the sides of the cage or following the ramps, most of this activity occurred at night. An increase in activity within the complex cages appeared when people entered the room either for testing, providing food and water, or cleaning of the cages. Although this difference in activity did not appear to affect behavioral performance on tasks, it may have influenced other measures such as brain weight and cortical thickness.

5. 4. 1. 2. Open Field

Within the open field apparatus, lesion rats made the same number of overall movements as control subjects. Both control and lesion subjects housed in the enriched

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environment displayed an overall decline in the number of movements. Decreased

activity was especially pronounced in control rats housed the complex environments

(Figure 5.1).

A two-factor ANOVA of group X treatment was performed. There was a main

effect of treatment (F(1,31) = 13.05, p = .001), but no significant group (F(1,31) = 1.64, p

= .21) nor group X treatment interaction (F(1.31) = 1.92, p = .18). Post hoc analysis

(Tukey HSD) found a significant decline in activity in the C-complex group when

compared to C-NT (p < .01) or OF-NT (p< .05). No other comparisons were significant.
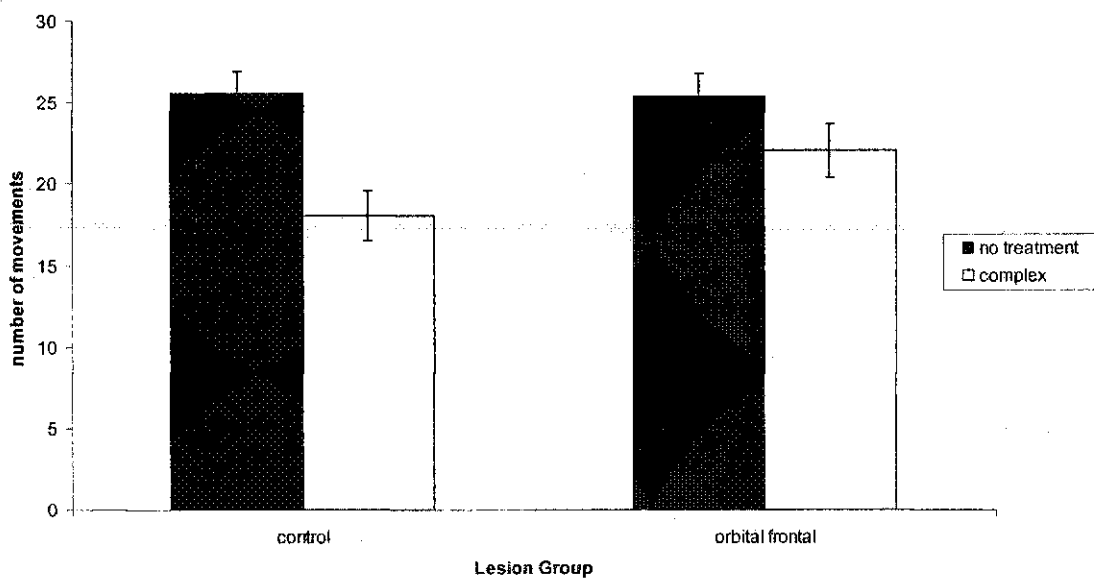
Figure 5. 1. Total number of movements in the open field (10 minutes). Orbital
frontal subjects did not differ in activity from controls.

5. 4. 1. 3. Morris Water Task

Both control and orbital frontal subjects performed equally well on this task as

they learned to quickly find the hidden platform. Subjects housed in the complex

environment demonstrated an even better performance, as shown by the decline in total

latency to find the platform (Figure 5. 2).

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A two-factor ANOVA (group X treatment) found an overall treatment effect

$(F(1,31) = 7.13, p < .05)$ reflecting the better performance of complex-housed subjects,

but there was not a significant group effect $(F(1,31) = 1.06, p = .31)$ nor interaction

$(F(1,31) = 0.001, p = .98)$.
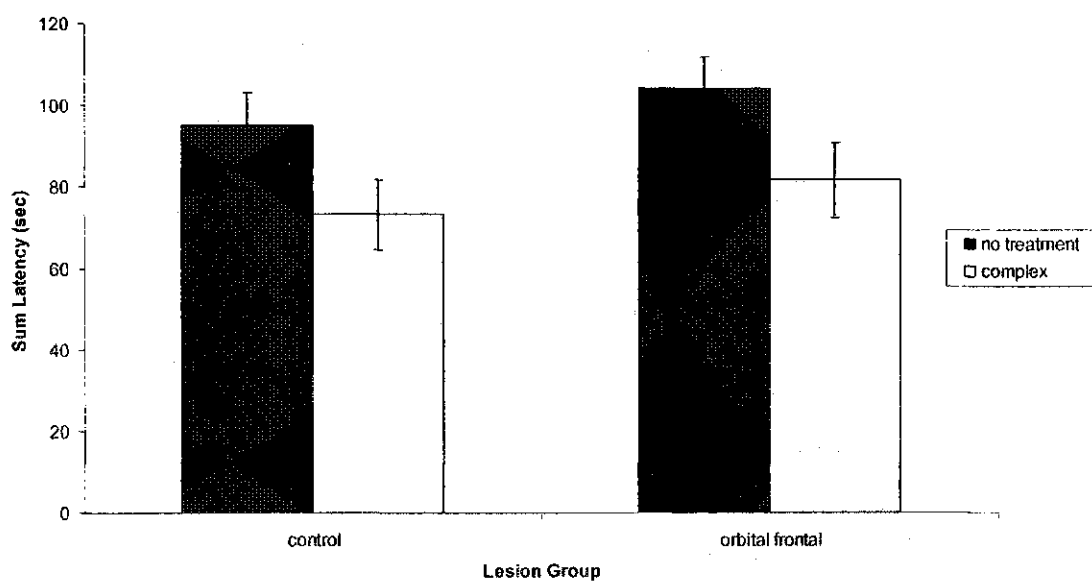
Figure 5. 2. Water maze performance expressed as the sum latency across all days. Environmental enrichment improved performance of this task.

Analyzing the average time spent around the edge of the pool walls found that

subjects housed in a complex environment spent less time swimming around the edge of

the pool than subjects housed in a standard lab environment (Figure 5. 3). This suggests

that complex-housed rats had an improved search strategy for the platform located away

from the pool's edge.

Because there was a high variance in the no-treatment group, each treatment

group was analyzed using one way ANOVA's while comparisons across treatment

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groups were made using independent t-tests (equal variance not assumed). A one-way

ANOVA between untreated subjects did not show a significant difference in the time

spent around the pool's edge ($F(1,16) = 0.55$, $p = .47$). A similar result was found using a

one-way ANOVA between complex-housed subjects ($F(1,13) = 2.31$, $p = .15$).

Independent t-tests found significant differences between untreated controls and

complex-housed subjects (C-complex: $t = 3.20$, $p < .05$; OF-complex: $t = 2.58$, $p < .05$).

Significant differences were also found between untreated orbital frontal subjects and

complex housed subjects (C-complex: $t = 3.41$, $p < .01$; OF-complex: $t = 2.48$, $p < .05$).

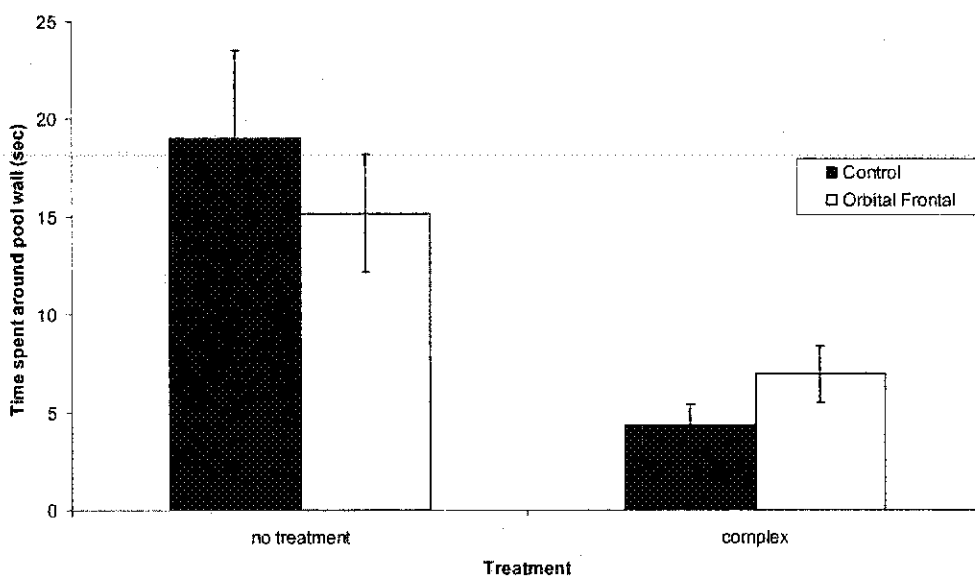**Figure 5. 3.** Average time spent around the edge of the pool. Complex-housing improved the search strategy for the hidden platform as this group spent less time along the edge of the pool.

5. 4. 1. 4. Whishaw Reaching Task

Control rats were able to reach with an accuracy of 61%, regardless of the

treatment group. Controls were generally successful in obtaining chicken feed, and in

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instances when they were not successful, it was generally a result of trying to grab too much feed at once. In contrast, OF-NT subjects were impaired at the task, with a reaching accuracy of only $29.7 \pm 6.6\%$. In these subjects, there appeared to be difficulties in grasping the food. In some cases subjects reached through the bars of the reaching box over 200 times within the ten minute taping session, yet had little success obtaining food. Interestingly, OF-complex rats were not impaired at reaching, but rather reached with an accuracy of $60.1 \pm 7.8\%$ that is comparable to controls (Figure 5. 4). At the end of training, three OF-NT and two OF- complex rats did not reach and thus were excluded from the analysis.

A two-way ANOVA analyzing group X treatment determined a main effect of lesion group ($F(1,26) = 6.26$, $p < .05$) and a significant group X treatment interaction ($F(1,26) = 7.38$, $p < .05$), but no main effect of treatment ($F(1,26) = 3.65$, $p = .07$). Tukey HSD post hoc analysis determined that the interaction is mainly due to the treatment being beneficial for lesion rats, yet had no effect on improving reaching performance in the control subjects. OF-NT rats were significantly impaired at reaching when compared to all other groups (p's $< .05$).
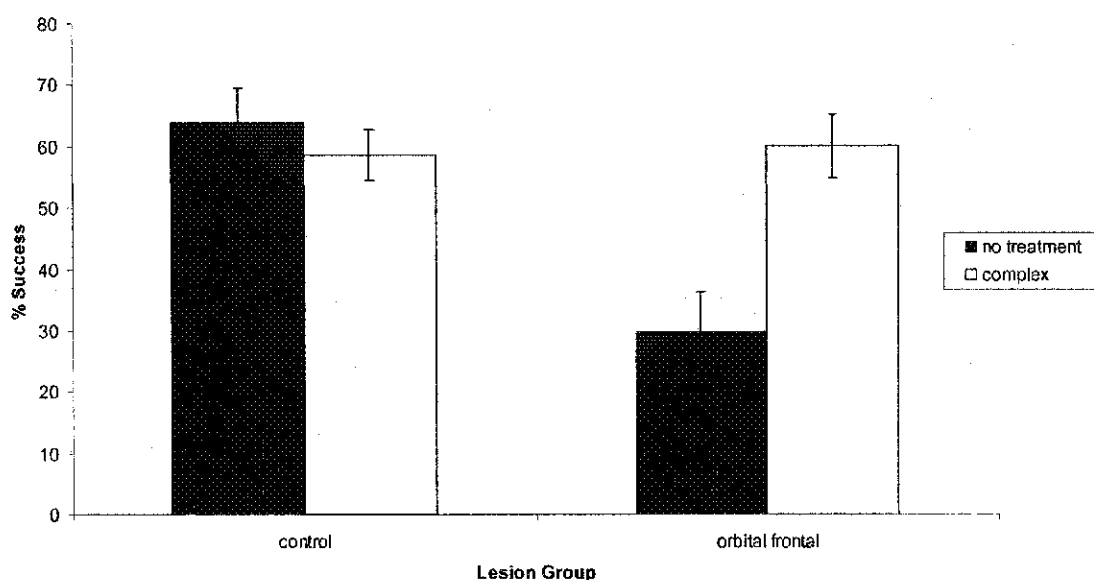
Figure 5. 4. Reaching performance expressed as a percent success of the total number of reaches. Environmental enrichment significantly improved reaching in lesion subjects.

5. 4. 1. 5. Tongue Extension

Subjects quickly learned to obtain cookie mash from the ruler, extending the tongue as far as possible. Lesion rats were impaired at this task, as the mean length of tongue extension (9.3 ± 0.5mm) was not as long as a control subject (13.4 ± 0.6mm). Lesion subjects housed in an enriched environment, however, were able to extend the tongue significantly farther than lesion rats without treatment ($11.6 \pm .6$mm) (Figure 5. 5).

A two-factor ANOVA (group X treatment) was used. A main effect of group ($F(1,26) = 17.48, p < .01$) and group X treatment interaction ($F(1,26) = 11.55, p < .01$) were significant, but there was no main effect of treatment ($F(1,26) = 0.71, p = .41$). The interaction reflected the effect of enriched housing on lesion subjects, but not controls. Post Hoc analysis via Tukey HSD determined that lesion rats without treatment extended

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their tongues less far than all other groups (p's < .05). Lesion subjects housed in an

enriched environment also differed significantly from the control-NT group (p < .05).
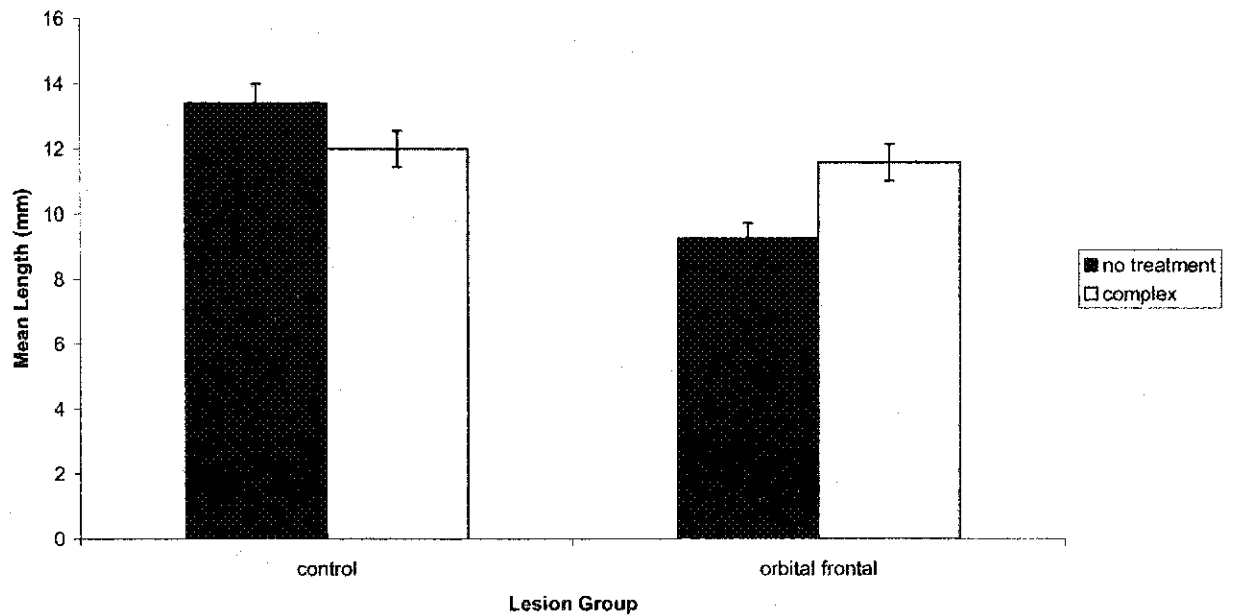
Figure 5. 5. Mean length of tongue extension. Environmental enrichment significantly improved the ability to extend the tongue.

5. 4. 1. 6. Extinction Task

By day 10 of the acquisition phase, all subjects were bar-pressing at about the

same rate. From day 1-5 of the extinction phase there was a gradual decline in bar

pressing as subjects pressed the bar less and less each day in the absence of a reward.

Unlike orbital frontal rats with adult lesions (see Kolb et al, 1974), early orbital frontal

lesions did not impair subjects on this task. Furthermore, exposure to an enriched

environment did not affect performance on this task (Figure 5. 6).

Repeated measures of day 1-5 of the extinction phase revealed no significant

difference of group, treatment, or an interaction (F's < .19, p's > .65).

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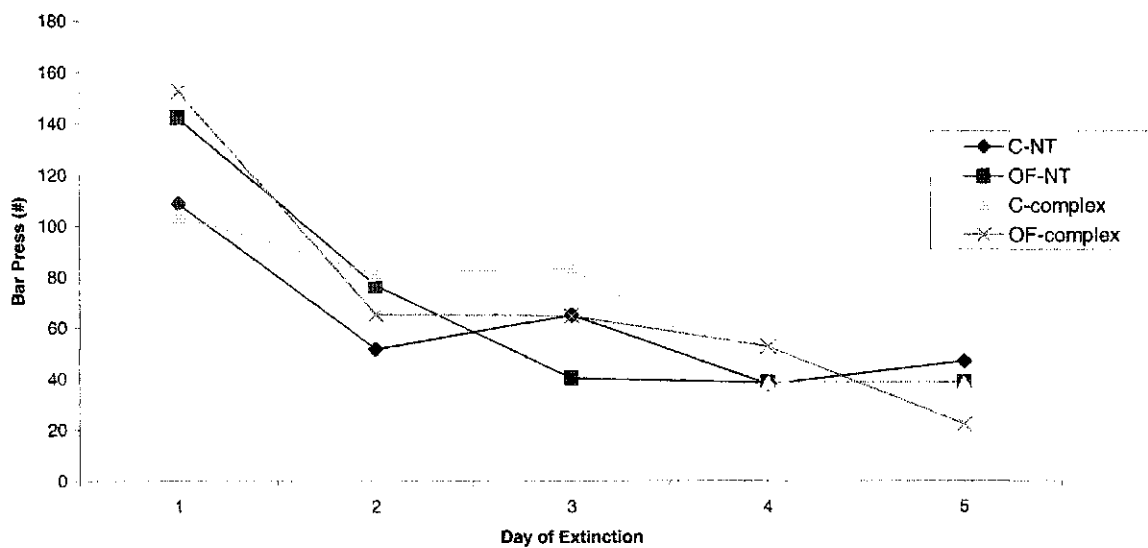
Figure 5. 6. Mean number of bar presses from day 1-5 of the extinction task. Performance is unaffected by lesion or treatment.

5. 4. 2. Anatomy

5. 4. 2. 1. General Observations

Upon initial observation of the orbital frontal lesion cavities, there was concern that the lesions were actually dorsal to the intended region. A similar pattern had been noticed in the medial frontal lesions but was found to reflect a movement of the lesion cavity (Kolb et al, 1996). To determine whether this was also the case in this current study, one litter of animals was given P3 orbital frontal lesions, and 2-3 subjects were sacrificed on post-surgery (PS) day 1, 3, 7, 15 and 21. As demonstrated by figure 5. 7, the lesions were placed correctly but the cavity moved dorsally with development.

Coronal views demonstrate other abnormalities or anatomical differences from controls (see Figure 5. 8). For instance, in lesion subjects there appears to be a much larger ventral portion of the brain in comparison to the remaining cortex when compared to controls. Within the ventricles, there is also darker staining evident suggesting that

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following the lesion there was an increase in proliferation of cells in the subventricular

zone.
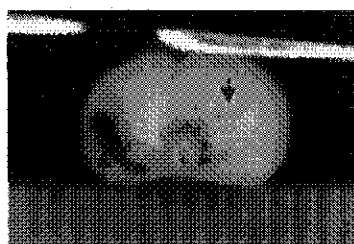
PS day 1

PS day 3
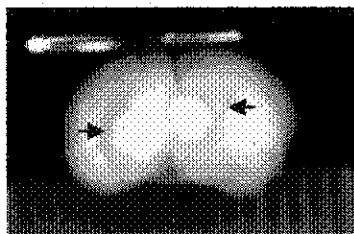
PS day 7

PS day 15

PS day 21

Figure 5. 7. Rostral view of orbital frontal cortex lesions on post-surgery day 1, 3, 7, 15, and 21. Arrows indicate boundary of the lesion. Photographs are not at the same scale. Ruler in background is measured in mm.

PS day 1:
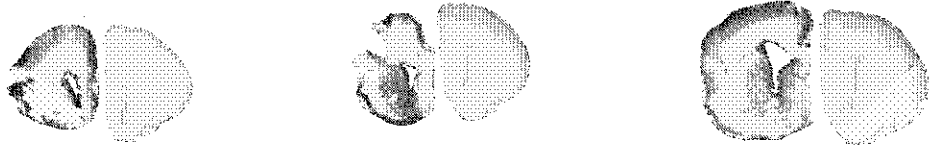
PS day 3:

PS day 7:
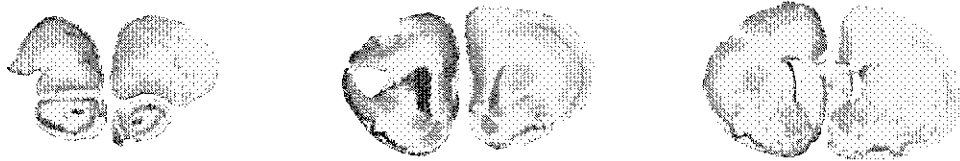
PS 15:
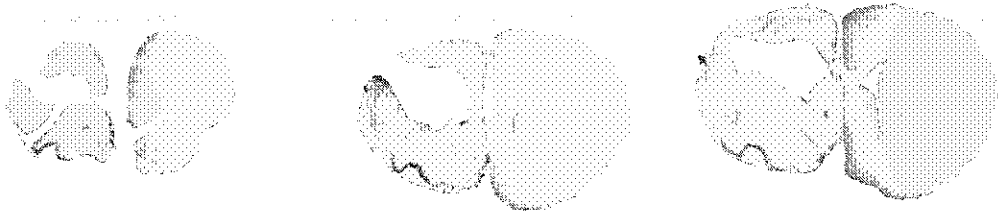
PS 21:
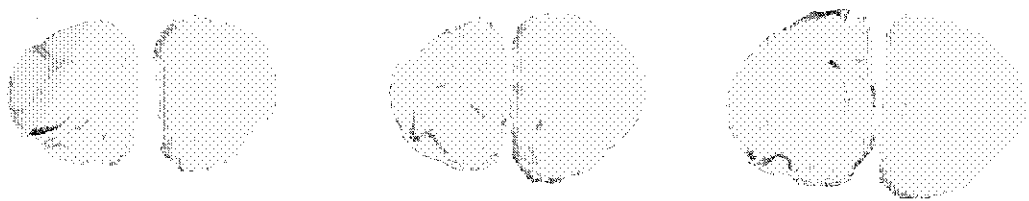
Figure 5. 8. Serial sections of orbital frontal cortex lesions (left hemisphere) and age-matched controls (right hemisphere).

5. 4. 2. 2. Brain Weight

Lesion subjects had a reduction in brain weight when compared to control subjects. Overall, although complex-housing did not increase brain weight of control subjects or female lesion subjects, there was a 5.5% increase in brain weight of male orbital frontal subjects (Table 5. 1).

Males and females were analyzed separately as the brain tissue of each sex was prepared differently. A two-factor ANOVA (group X treatment) in males revealed a main effect of group (F (1, 13) = 7.93, p < .05) but no overall treatment (F (1, 13) = 0.21, p = .68) nor interaction (F (1, 13) = 1.00, p = .34). Post hoc analysis using Tukey HSD determined a significant difference between both control groups when compared to OF-NT group. There was not a difference in brain weight between controls and the OF-complex group, due to the increase in brain weight of the OF-complex subjects.

In females, as with males, there was a main effect of group (F (1, 10) = 63.64, p <.001) but no treatment effect (F (1, 10) = 0.03, p = .87), and no group by treatment interaction (F (1, 10) = 1.04, p = .33). Post hoc analysis (Tukey HSD) found significant differences between control subjects of both treatments with lesion subjects of both treatments (p's ≤ .003).

Table 5. 1. Mean Brain Weight

Group	Treatment	Weight (g ± SE)	
		Male	Female
Control	NT	1.68 ± .06	2.56 ± .05
	Complex	1.65 ± .06	2.52 ± .05
Orbital Frontal	NT	1.47 ± .05 *	2.16 ± .04 *
	Complex	1.55 ± .06	2.22 ± .04 *

Mean weight (grams) ± standard error.
* differed from controls p < .05

Note: Owing to different fixation procedures male and female brain weights cannot be compared directly.

5. 4. 2. 3. Body Weight

Animals housed in complex-housing had a reduced body weight in comparison to lab-reared subjects. Although female lesion rats did not differ in body weight from controls, orbital frontal males weighed less than control males (Table 5. 2).

Because males are heavier than females, each sex was analyzed separately. A two factor ANOVA (group X treatment) for males determined a main effect of group (F (1, 13) = 19.43, p = .001) and treatment (F (1, 13) = 60.48, p <.001) but no interaction (F (1, 13) = 2.01, p = .15). A two factor ANOVA for females revealed no significant differences (F's < .3, p's > .60).

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Table 5. 2. Mean Body Weight

Group	Treatment	Weight (g ± SE)	
		Male	Female
Control	NT	555.50 ± 16.02	315.67 ± 17.89
	Complex	414.00 ± 16.02 †	331.00 ± 21.92
Orbital Frontal	NT	465.83 ± 13.08 *	325.25 ± 14.15
	Complex	368.00 ± 18.49 * †	318.00 ± 21.92

Mean weight (grams) ± standard error
* differed from controls p = .001
† differed from NT group p< .001

5. 4. 2. 4. Cortical Thickness

Males and females were analyzed separately for this analysis because of the differences in tissue preparation. In male subjects the effect of complex-housing on cortical thickness was different for control and lesion subjects. Although control subjects housed in a complex environment did not demonstrate a thicker cortex, lesion subjects showed an increase at particular planes. For instance OF-complex subjects showed a significant increase at plane 1 when compared to OF-NT subjects (Figure 5. 9a).

Repeated measures by plane found an overall group effect ($F(1,10) = 32.94$, $p <$ .001) as lesion subjects had a reduced cortical thickness relative to control subjects. There was no main treatment effect ($F(1,10) = 1.09$, $p = .32$) nor a group by treatment interaction ($F(1,10) = 1.37$, $p = .27$). Tukey's HSD found a significant difference on plane 1 between OF-NT and OF-complex subjects ($p < .01$) but not a significant difference between C-NT and OF-complex subjects ($p = .28$). On planes 4 and 5 there was not a significant group difference ($p > .05$).

Orbital frontal females housed in a complex environment also demonstrated an increase in cortical thickness on plane 5. Although OF-NT subjects had a significantly thinner cortex than C-NT subjects on plane 5, this was not the case with OF-complex subjects as this group was statistically equivalent to C-NT subjects on this plane (Figure 5. 9b).

Repeated measures by plane found an overall group effect ($F(1,8) = 46.77$, $p < .001$) but no treatment effect ($F(1,8) = 2.78$, $p = .13$) nor group by treatment interaction ($F(1,8) = 1.31$, $p = .29$). Post hoc analysis via Tukey HSD did not find a significant difference between C-NT and OF-complex subjects on plane 5 ($p = .48$), but did find a significant difference between C-NT and OF-NT subjects on the same plane ($p < .05$).

5. 4. 2. 5. Thalamic Cross-sectional Area

Only males were used for this analysis because the thalamic boundaries were difficult to establish in the golgi-stained tissue of the females. After an orbital frontal lesion, there is shrinkage of the ventral lateral thalamic nuclei, which is reflected by the general reduction of the thalamic cross-sectional area. Placing rats in an enriched environment did not prevent this reduction (Table 5. 4).

An overall group effect ($F (1, 13) = 81.44$, $p < .001$) was found. There was no effect of treatment ($F (1, 13) = 0.230$, $p = .64$) nor an interaction ($F (1, 13) = 0.23$, $p = .37$).

a)
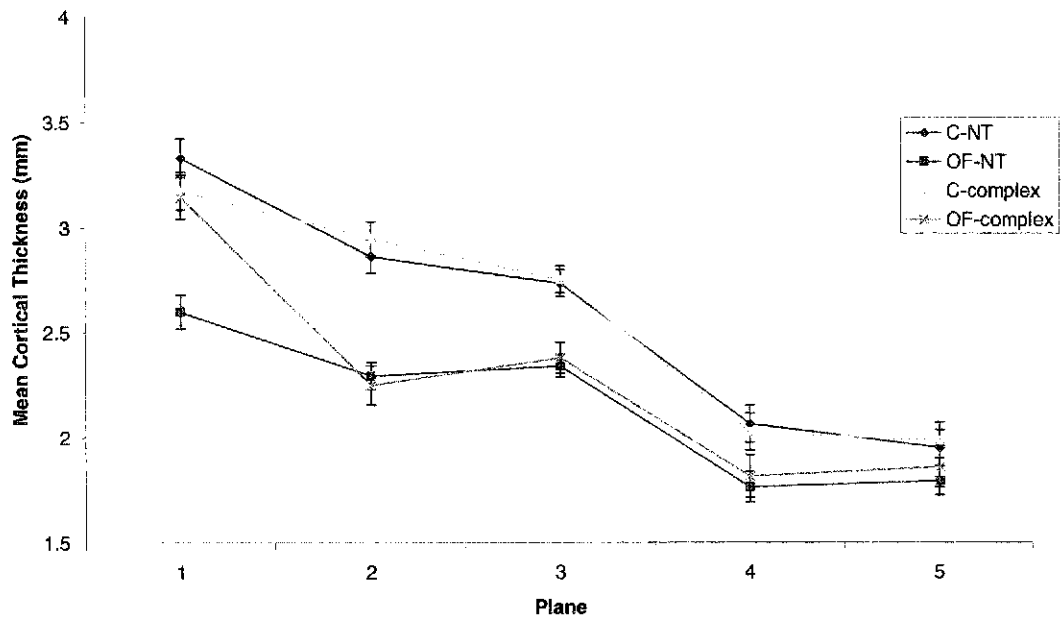
b)
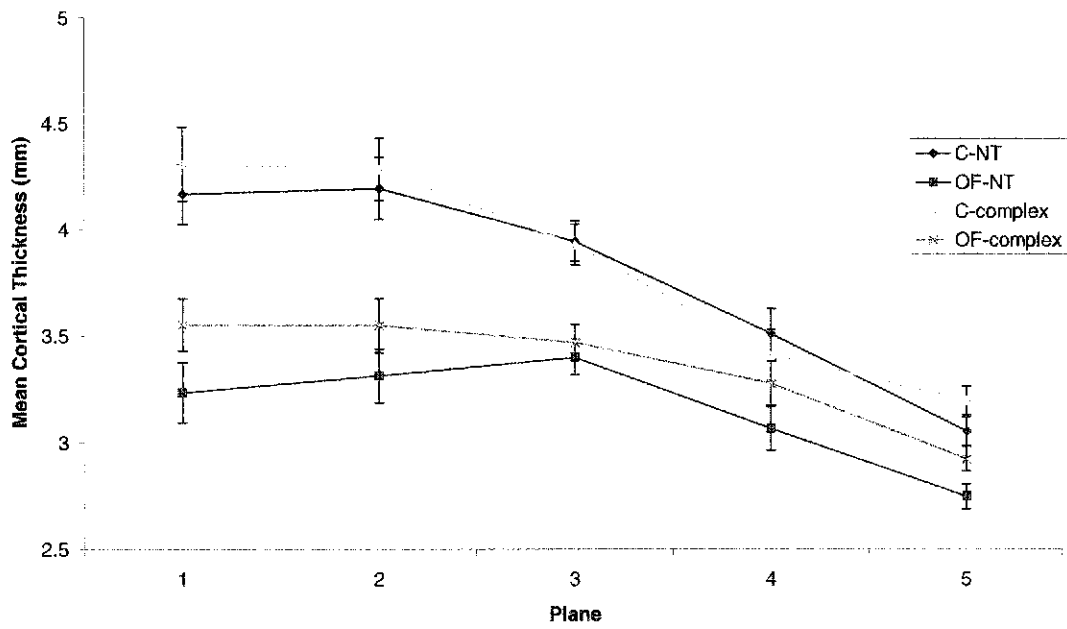
Figure 5. 9. Mean cortical thickness at each plane of measurement (20 X magnification). Orbital frontal lesions produced thinner cortices. Complex-housing partially reversed the decrease in cortical thickness in both a) males (plane 1) and b) females (plane 5).

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Table 5. 4. Thalamic Cross-sectional Area

Group	Treatment	Area (mm^2)
		Mean ± SE
Control	NT	18.98 ± .61
	Complex	19.25 ± .61
Orbital Frontal	NT	14.06 ± .49 *
	Complex	13.21 ± .70 *

Mean area (mm^2) ± standard error
* differed from controls p < .001

5. 5. DISCUSSION

This experiment demonstrated three main findings 1) Unlike adult injury,

perinatal orbital frontal lesions do *not* produce cognitive impairments, but *do* produce

deficits on a skilled reaching task; 2) Housing in a complex environment enhances

functional outcome on both cognitive and motor tasks; and, 3) Early frontal lesions

produce small brains and complex housing reduces this effect. Each of these findings

will be discussed in turn (see Table 5. 5 for summary).

5. 5. 1. Behavioral effects of early orbital frontal lesions

Previous research has demonstrated that following adult orbital frontal lesions,

there is a deficit in cognitive tasks such as spatial navigation, and response extinction

tasks (Kolb et al, 1974; Kolb & Whishaw, 1985) but do not produce deficits in skilled

forelimb tasks (Whishaw & Kolb, 1983). In contrast, the current study demonstrates that

following day 3 orbital frontal lesions there is not a performance deficit in cognitive

tasks, whereas such lesions do produce motor impairments on both skilled reaching as well as tongue extension. These results generally replicate the findings of Kolb & Whishaw (1985) who showed no cognitive deficits on two different spatial navigation tasks as well as demonstrating deficits in food handling and tongue extension following day 7 orbital frontal lesions.

The inability to detect cognitive deficits in the Morris water task after early orbital frontal cortex lesions is unique as previous studies have demonstrated cognitive deficits following lesions to virtually every other cortical region (including medial frontal, posterior cingulate, motor, parietal, visual and temporal) even if similar deficits are not found after adult lesions (e.g., Kolb et al, 2000). One possibility for this resilience after early orbital frontal lesions may include functional compensation of the orbital tissue by the medial frontal cortex. Although both the orbital and medial subregions receive projections from the mediodorsal thalamic nuclei during development, the orbital cortex develops about one day faster than the medial cortex (Bayer and Altman, 1991). As first suggested by Goldman (1976) to account for similar behavioral results in monkeys with early orbital lesions, it is possible that in the absence of the orbital frontal tissue, the less mature medial frontal cortex (dorsolateral in monkeys) is able to compensate for the lost orbital tissue. In contrast, if the tissue removed was medial frontal cortex, the orbital region would not be able to compensate for the medial frontal tissue because it is developmentally advanced, and therefore would already be committed to orbital frontal functions and unable to compensate for lost medial cortex. In addition, it would appear that there is no parallel mechanism for recovery from other early cortical injuries. An example of this is following motor cortex injury, which in principle should not produce

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cognitive deficits on nonmotor tasks. Indeed, no such deficits are shown after motor

cortex lesions in adulthood but are found after neonatal motor cortex lesions (Kolb et al,

2000). One possible explanation is that the motor cortex injury disrupts development of

nearby cortex such as the medial frontal or posterior parietal cortex, causing cognitive

deficits.

The motor deficits found after early orbital frontal lesions may be due to the

reorganization of the medial frontal cortex compensating for the cognitive deficits and/or

from disturbing the development of neighboring motor cortex. There are direct

corticospinal projections within both medial frontal and motor cortex. If these

projections are severed this produces chronic deficits in the performance of the tray

reaching task (e.g., Kolb, 1995; Kolb et al, 2000). Because the reaching deficits found in

this study were ameliorated by complex housing (see below) it seems likely that the

initial deficits are not due to severed corticospinal projections because these are not likely

to be regenerated after complex housing. Instead, the complex housing may have

produced a change in the intrinsic circuitry of the medial frontal and/or motor cortex.

One curious result of the current study was that like early medial frontal lesions,

the area of injury appears to migrate after the orbital frontal injury. There are two

possibilities of this finding. First, it is possible that just as there is neurogenesis after

medial frontal lesions in rats (Kolb et al, 1998b) there may be a similar cell proliferation

after neonatal orbital frontal lesions. Such proliferation could only be demonstrated by

studies using mitotic markers. We note here that if this were true, the time course of the

lesion induced neurogenesis appears to be different following medial and orbital lesions.

That is, cell proliferation is found only after lesions of the medial frontal cortex on days

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7-12, but found as early as day 3 following injury of the orbital frontal cortex. Second, it is possible that the orbital region normally migrates laterally during development. If true, then the lesions in the current study are not equivalent to lesions in previous studies by Kolb and colleagues. This conclusion, however, is not supported by the developmental studies of Kolb and Altman.

5. 5. 2. Complex housing and functional recovery

The novel contribution of the current study was to show that complex housing completely abolished the motor deficits as well as significantly enhanced spatial navigation performance. These functional improvements occurred in spite of the significantly smaller brain in the lesion animals.

The benefits of complex housing have been demonstrated both in animals with intact brains (e.g., Diamond et al., 1966; Kolb et al., 2003) as well as animals with early medial frontal lesions (Kolb & Elliot, 1987; Gibb, 2001; Kolb et al., 2003). We are unaware of previous studies demonstrating the effects of complex housing after early orbital frontal lesions, however, studies in adult rats with large frontal lesions including orbital cortex showed very little functional improvement (Kolb & Gibb, 1991). Curiously, the one behavior that did show benefit in the Kolb and Gibb study was tongue extension, which again showed a benefit of housing in the current study. Because it seems unlikely that complex housing increases tongue use, it seems reasonable that the experience may have more general effects on the brain. Such effects could be an enhanced production of growth factors such as basic fibroblast growth factor, or by more general mechanisms such as increased neuronal activity.

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As discussed above, the improved reaching performance of the complex-housed rats with early orbital frontal lesions is possibly due to reorganization of intrinsic circuitry of the medial frontal or motor cortex. Previous studies have shown that motor experience alone increases the number of synapses per neuron in layer V pyramidal cells in the caudal forelimb area (Kleim et al, 2002), and a similar effect is possible in this study. It is possible that the motor experience also altered the organization in the medial frontal cortex but this has not been demonstrated even in non-lesion animals.

5. 5. 3. Anatomical correlates of early injury and recovery

Early orbital frontal lesions produced smaller brains, thinner cortical mantles, and a shrunken thalamus, a result that consistently occurs after early injury to every cortical region studied in the rat (e.g., Kolb, 1995). Although the reason for this decrease in brain size and cortical thickness is unknown, it may be related to changes in normally occurring apoptosis, gliogenesis, or synaptogenesis. Housing subjects with early medial frontal cortex lesions in a complex environment has consistently demonstrated an increase in brain weight and cortical thickness (e.g., Kolb & Elliot, 1987; Gibb, 2001) and a similar result was expected in the current study. Indeed, there was an increase in brain weight and thicker cortices in the posterior cortex of female lesion rats, but in the anterior cortex of male lesion rats. Possible reasons for the increased brain weight and cortical thickness are unknown but could have been due to a variety of experience-dependent changes such as increase numbers of glia or blood vessels, increase numbers of synapses, or increased white matter, either in cortical or subcortical regions (Reviewed in Schallert et al, 2000). Surprisingly, there was not an increase in brain weight or

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cortical thickness in control subjects. The reason for the failure to see a change in cortical thickness is not immediately apparent, especially given that studies in the same laboratory and with the same strain of rats has consistently shown such increases. We note that in contrast to previous studies in which the complex housing cages were in the same animal room as the cage-housed rats, in the current study the complex environments were in a separate room. This arrangement made the surrounding activity outside of the complex environments more sterile than in the previous studies and this could have contributed to the reduced effect on cortical thickness in the current study.

Table 5. 5. Summary of Results In Comparison to Past Research of Adult Orbital Frontal Lesions

	Adult Lesion	P3 Lesion	P3 + Complex
Behavior			
Open Field Activity	--- [a]	---	↓
Cognitive Tasks			
Water Task	↓ [b]	---	↑
Extinction Task	↓ [c]	---	---
Motor Tasks			
Tray Reaching	--- [d]	↓	---
Tongue Extension	↓ [d]	↓	---
Anatomy			
Brain Weight	↓ [e]	↓	*♂
Cortical Thickness	--- [e]	↓	*
Thalamic area	↓ [f]	↓	↓

a = Kolb, (1974b); b = Kolb, Sutherland & Whishaw (1983); c = Kolb, Nonneman & Singh (1974); d = Whishaw and Kolb (1983); e = Kolb & Whishaw (1985); f = Kolb (1974a).
↓ symbolizes decline/impairment, ↑symbolizes increase/improvement, --- symbolizes no difference from controls, * reduced effect relative to standard cage-housed lesion animals.

6. GENERAL DISCUSSION

Although much is known about the organization of the brain and the 'rules' that govern it, the manner in which external factors influence organization both in the intact and injured infant brain is still largely a mystery. Periods of high cortical plasticity are associated with anatomical changes such as synaptogenesis, gliogenesis, neurogenesis, and apoptosis. In contrast, periods of low cortical plasticity coincide with cell migration, an absence of astrocytes and synaptogenesis. In this thesis, injury of the prefrontal cortex was incurred at a time of low plasticity thus providing a model with a high potential of functional recovery.

The goal of this thesis was to examine the role of both bFGF and experience in the enhancement of functional recovery following perinatal prefrontal cortical injury. Both of these treatments have the potential to ameliorate deficits associated with brain injury through mechanisms associated with plasticity. Four main findings from these studies include: 1) Pharmacological interventions through the exogenous application of bFGF facilitate functional recovery. 2) Environmental enrichment through complex-housing or tactile stimulation is beneficial after injury. 3) The combined treatment of tactile stimulation with bFGF lead to worse functional recovery, but complex housing with bFGF provided greater functional benefits than bFGF alone. 4) The developmental stage of the injured area (i.e. orbital vs. medial frontal cortex) plays an important role in the sparing of functions.

6. 1. PHARMACOLOGICAL INTERVENTION AND FUNCTIONAL RECOVERY

6. 1. 1. Basic fibroblast growth factor

An accumulation of evidence has found that following adult brain injury the exogenous administration of bFGF improves functional recovery (McDermott et al, 1997; Kawamata et al, 1996). The current experiments provide evidence that bFGF is beneficial at other developmental ages such as following postnatal day 3 frontal cortex injury. Our findings demonstrate a dose-dependent effect bFGF as our high dose was more effective in promoting functional recovery than our lower dose. That is, a higher dose of bFGF improved fine motor movements in addition to improved spatial navigation performance found in previous studies.

The cellular effects of bFGF following brain injury appear to involve a number of different mechanisms. In particular, two measures were used to analyze the role of bFGF in the reduction of infarct size and prevention of thalamic retrograde degeneration associated with injury. Past research demonstrated that bFGF following injury promotes neuronal survival of cells bordering the lesion site (Fisher et al, 1995; Kawamata et al, 1996; Bethel et al, 1997). Therefore it was hypothesized in the current study that a reduction in lesion size would be found. Although the dorsal cortex did not show signs of a reduced infarction, in some animals there were signs of cellular proliferation within the midline, despite its peculiar appearance. Currently, the type of cells found in this new tissue is unknown but may be any number of different cells such as glia or neurons as bFGF promotes mitosis of these cells (Murphy et al, 1990; Santa-Olalla & Covarrubias, 1995).

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6. 1. 1. 1. Possible mechanisms mediating the effects of bFGF

i) Neuronal activity and synaptic change. Molecular signals such as neutrophins are

currently being suggested as mediators between neuronal activity and synaptic

connectivity. Not only is bFGF located in areas of the brain associated with high levels

of plasticity (e.g. hippocampus and cerebral cortex) but bFGF acts in an activity-

dependent manner. For instance, following high-frequency stimulation of rat

hippocampal slices, the administration of bFGF promoted the induction of long-term

potentiation (LTP). Because LTP is considered a model of learning and memory, this

suggests that bFGF plays a role in these processes (Abe & Saito, 2001).

ii) Angiogenesis. bFGF enhances the formation of new blood vessels and capillaries.

This formation is accomplished by bFGF directly stimulating locomotion or mitosis of

endothelial cells to the appropriate location in the brain. By doing so, oxygen and vital

nutrients may reach the injured site (Folkman & Klagsbrun, 1987).

iii) NGF and acetylcholine production. NGF is a neurotrophin specifically located in

cholinergic neurons found in the basal forebrain, cortex and hippocampus. It has been

shown that NGF promotes the survival and differentiation of cholinergic neurons during

development, and promotes plasticity in adulthood (e.g. Hefti et al, 1990; Calamandrei &

Alleva, 1995). Kolb and colleagues (1997) demonstrated that following cortical injury,

NGF improved performance on motor and cognitive tasks. Atrophy of dendrites in

neighboring cells was also reversed with NGF administration. Because bFGF regulates

the production of NGF in both the hippocampus and in cultured glia cells it is possible

that in our study bFGF enhanced NGF that in turn would act on cholinergic neurons to

facilitate functional recovery (Ramirez et al, 1999; Vige et al, 1991; Yoshida & Gage, 1991; Yoshida et al, 1992).

iv) Preventing neurotoxicity. Following brain injury there are secondary events that cause further damage to the brain. Toxic levels of free radicals, intracellular calcium, and glutamate levels are examples of compounds that kill cells. bFGF prevents this damage by stimulating the production of antioxidant enzymes and calcium-binding proteins such as calbindin, and reducing the number of NMDA receptors (Ay et al, 1999; Eckenstein, 1994).

6. 1. 1. 2. Exogenous bFGF influencing the intact brain

Unlike lesion subjects that received bFGF, control subjects were unaffected behaviorally by this treatment. Despite this finding, bFGF-treated controls demonstrated a decrease in brain weight (males), increase in posterior cortical thickness, and a reduction in thalamic and brainstem cross-sectional surface area (when compared to C-complex subjects). The mechanism(s) mediating these changes are currently unknown. One possibility is that by increasing bFGF levels in the brain this may have interfered with the functioning of other growth factors or chemical messengers. For example, a study by Heuer and colleagues (1990) found that levels of FGF and NGF receptors in the central nervous system fluctuate during development in a reciprocal manner. In other words, periods of development associated with low levels of FGF receptors have high levels of NGF receptors. Alternately, periods associated with low levels of NGF receptors were found to express high levels of FGF receptors. It is possible that by increasing bFGF levels for a prolonged period of time during development, this may have

influenced the expression and function of other chemical messengers such as NGF in fulfilling their role. As a consequence this may have influenced the anatomical results found in our study.

6. 2. ENVIRONMENTAL ENRICHMENT AND FUNCTIONAL RECOVERY

6. 2. 1. Complex-housing

An abundance of literature has reported the beneficial effects of complex housing following acquired brain injury. Changes caused by complex-housing have been found after injury to the cortex, hippocampus, cerebellum, and septum (e.g. Kolb & Gibb, 1991a; Diamond et al, 1964; Will et al, 1977, Whishaw et al, 1984, Galani et al, 1997; Globus et al, 1973; Donovick et al, 1973). The current findings replicate this phenomenon as deficits associated with brain injury to either the medial or orbital prefrontal cortex were diminished following over one month of complex-housing. As reported in other studies, an improvement on the spatial navigation task and reaching task was found following prefrontal cortex injury.

Improved performance on cognitive tasks has often been attributed to the reorganization of the remaining cortex in order to compensate for the lost connections caused by injury. For instance, Kolb and colleagues (Kolb & Gibb, 1991a; Kolb et al, 2003; Kolb, 1999) have demonstrated that complex-housing after perinatal brain injury caused an increase in dendritic branching and spine density of layer III pyramidal cells of the adjacent parietal cortex (Par 1). Reorganization of the remaining cortex is a way for the brain to compensate for the lost tissue and thus improve cognitive functioning.

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Although the effects of complex-housing are remarkable for the improvement of cognitive processing, the benefits on fine forelimb movements have proven to be more specific. The resistance of circuit modification is likely attributed to the type of connections lost that are required for fine motor movements. Unlike cognitive skills that rely on connections between cortical and hippocampal structures, fine motor skills depend on direct connections from the cortex to the spinal cord. Once lost, these connections cannot be regenerated nor can other means of compensation occur within the remaining cortex. Complex-housing following early orbital frontal cortex lesions led to an improvement in reaching performance. The underlying mechanism for this finding is most likely compensatory in nature and does not involve cortico-spinal projections. Rather, as lesions to this area of the brain does not remove cortico-spinal projections the deficits on this task may be attributed to a sensory deficit that was ameliorated by complex-housing.

6. 2. 1. 1. Possible mechanisms mediating the effects of complex-housing

i) Neuronal activity and synaptic change. Unlike some treatments that target specific structures or cell populations, complex-housing influences the entire brain. Whereas some authors report that the enriched housing conditions inadvertently train the subjects on skills required for specific behavioral tasks, others suggest that the complex housing provides a general activation of all sensory and cognitive systems (Goldman & Lewis, 1978). The enhanced sensory stimulation acts directly on sensory receptors that in turn project to various cortical and subcortical regions of the brain. It is this up-regulation of neuronal activity that triggers a cascade of events within the brain, ultimately increasing

plasticity and producing anatomical changes. Therefore by enhancing the general activity

levels within the brain, complex-housing triggers a cascade of events that influence

behavior. Anatomical changes that have been proposed to support the improved

functional outcome include increases in the number of synapses, neuron number, glia

number, inhibition of apoptosis, angiogenesis, and increased dendritic length (Kolb,

1999; Soffie et al, 1999; Rosenzweig & Bennett, 1996; Young et al, 1999; Sirevaag &

Greenough, 1987; 1991; Black et al, 1990).

ii) Social environment. Studies have demonstrated that housing rats in a complex

environment alone is not sufficient for producing a complex-housing effect. Rather, a

socially-rich environment is required. The reasons for this may not be because of the

social interactions per se, but instead the presence of conspecifics may influence how

each subject interacts with the environment. For example, more activity and exploration

within the complex environment occurs (Rosenzweig, 1971; Rosenzweig & Bennett,

1972).

iii) Exercise. Complex-housing also provides an increase in exercise that would typically

not be present in the impoverished condition. Exercise alone has shown to have

beneficial effects on the brain, as it enhances angiogenesis and the production of growth

factors such as bFGF, BDNF, NT-3, and IGF-1 (Van Praag et al, 2000; Black et al, 1990;

Rowntree, 1995).

iv) Growth factor production. Complex-housing has shown to increase endogenous

levels of growth factors in the brain, above and beyond those provided by exercise alone.

Stimulation of growth factors such as BDNF, NGF, bFGF, GDNF and NT-3 has been

found (Van Praag et al, 2000; Torasdotter et al, 1996; Pham et al, 1999; Gomez-Pinilla &

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Cotman, 1992; Kolb et al, 2001). It is likely that these growth factors in turn influence the plasticity of the brain and its ability for change.

v) Neurotransmitter production. Neurotransmitters have also been implicated in cortical plasticity and recovery of function following brain injury. Depletion of endogenous norepinephrine prior to P7 medial frontal cortex lesions prevents spontaneous sparing of function on a spatial navigation task, suggesting that norepinephrine is important for plastic changes to occur that influences functional recovery (Sutherland et al, 1982). Furthermore, increased norepinephrine concentrations had been found following complex-housing (Naka et al, 2002).

Acetylcholine is also associated with cognitive performance, and is up-regulated in the cerebral cortex following exposure to a complex environment (Rosenzweig & Bennett, 1996). Enzymes that catalyze the synthesis of acetylcholine such as choline acetyltransferase is also up-regulated following complex-housing in the caudate nucleus, frontal cortex and hippocampus (Parks et al, 1992).

6. 2. 2. Tactile stimulation

Tactile stimulation in preterm neonates has been shown to aid in their growth and development, promoting an increase in body weight, attention and movements made by the infant (Field et al, 1986; Solkoff & Matuszak, 1975). The beneficial effects of tactile stimulation following early injury of the motor, parietal and prefrontal cortex in the rat have also been demonstrated (Kolb et al, 2001). The findings of this thesis replicate the behavioral findings of past research. Not only is there an improvement in a spatial navigation task, but subjects that received tactile stimulation improved in reaching

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performance. The reason for the improved behavioral recovery is not known although presumable the tactile stimulation has some nonspecific action that provides a general benefit.

6. 2. 2. 1. Possible mechanisms mediating the effects of tactile stimulation

i) Neuronal activity and synaptic change. Stimulation of the skin's mechanoreceptors will in turn activate sensory cortex and enhance neuronal activity. Structural changes within the brain following tactile stimulation include increases in dendritic length and changes in spine density (Gibb, 2001).

ii) basic fibroblast growth factor production. bFGF is a growth factor located not only in the nervous system but in organs and the skin. It is possible that tactile stimulation promotes the production of bFGF within the skin that ultimately influences the brain. Using Western blot analysis an up-regulation of bFGF has been found in the skin following tactile stimulation, supporting this hypothesis (R. Gibb, unpublished observations, 2003).

iii) Ornithine decarboxylase (ODC). ODC is an enzyme required for protein synthesis, and is therefore used as a measure of tissue growth. Unlike vestibular stimulation or kinesthesis, tactile stimulation has been shown to increase ODC (Schanberg & Field, 1987). The up-regulation of ODC may be partly responsible for protein synthesis required for cortical plasticity.

iv) Acetylcholine. Immediately following tactile stimulation the levels of acetylcholine increase in the hippocampus, medial frontal and fronto-parietal cortex by up to 140% (Himmelheber et al, 1998; Steinberg et al, 1998). Furthermore, Gibb & Kolb (2000)

found that tactile stimulation led to a chronic increase in acetylcholinesterase immunoreactivity in the cortex, a result that suggests the acute increase in acetylcholine may be permanent. Given the role of acetylcholine in brain plasticity it is reasonable to suppose that tactile stimulation aids via the increased acetylcholine.

6. 3. COMBINED TREATMENTS AND FUNCTIONAL RECOVERY

6. 3. 1. Tactile Stimulation with bFGF

Preliminary data had suggested that the combined treatment of bFGF and enhanced sensory stimulation is more productive than either treatment alone in acquiring functional recovery after adult motor cortex injury (Kolb et al, 2001). Thus our original hypothesis was that the combined treatment of tactile stimulation with bFGF would be more productive than either of the two treatments administered alone. Surprisingly, the combined treatment had adverse results, producing a worse functional outcome on a spatial navigation task. Correlated with this was a reduction in thickness of the cortical mantle, and in females a reduction of brain weight. An explanation for the adverse reaction to the combined treatment is that the administration of tactile stimulation immediately after the bFGF injections was unusually stressful. Previous studies had reported that tactile stimulation relaxed rat pups. In our study, pups remained active throughout stimulation and even displayed signs of stress through crying. This behavior persisted even after the change in methodology.

The open field task measures general activity in response to a novel environment, a result that is often termed as emotionality. It is hypothesized that the greater the stress, the higher the emotionality and therefore the greater the number of movements made

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within the open field (Levine et al, 1967). In our study we found that the subjects receiving the combined treatment demonstrated a decline in activity- opposite of what you would predict if the animals had felt extreme stress. Yet, although stress may have been a factor, results from the open field task do not support this hypothesis.

Another explanation may be that the pups were over-stimulated with the combined treatment. As demonstrated in the human literature (Allen, 1995; Zahr & Balian, 1995), over-stimulation of preterm neonates produces adverse effects and is detrimental to the growth and development of the neonate. It is therefore possible that the same sort of effect occurred with the combined treatment.

6. 3. 2. Complex-housing with bFGF

The combined treatment of complex-housing following bFGF administration facilitated functional recovery after injury. In particular, the combined treatment improved performance in the initial learning of the water task. A reason for this may be that complex-housing promoted subjects to use a compensatory mechanism in solving the cognitive task, in addition to the plasticity-inducing effects of bFGF. For example, it has been shown that following bilateral occipital lesions complex-housing produced an increase in performance on the water task relative to standard lab-housing (Rose et al, 1993; Finger, 1978). The authors suggested that rather than regaining sight, complex-housing promoted the use of different sensory information to solve the task more effectively.

It was anticipated that by providing the combined treatment, the survival of new tissue growth that was stimulated by bFGF may be promoted. An increase in the number

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of subjects demonstrating partial filling of the lesion cavity was found, but complex-housing did not promote the survival of the new tissue so that a complete filling in of the lesion cavity occurred. A similar finding occurred by Kelche and colleagues (1995) who determined that complex-housing for 2 months did not promote the functional use of grafted tissue on behavioral tasks.

6. 4. ORBITAL FRONTAL CORTEX AND SPARING OF FUNCTION

Studies by Kolb and colleagues (e.g. Kolb & Elliot, 1987; Kolb et al, 2000) have repeatedly demonstrated an age-dependent effect on recovery of function following medial prefrontal cortex lesions. Generally, a time of spontaneous recovery is found following lesions at P7-12, and correlative filling in of the lesion cavity and increased dendritic branching of adjacent parietal cortex is found. In contrast any postnatal time before or after this time period, poor functional recovery is found especially between postnatal day 1-6. Medial frontal cortical lesions at this age result in poor functional recovery on cognitive tasks such as the water task and on fine motor skills. Anatomical correlates include atrophy of dendritic branching in the parietal cortex and an extremely small brain.

Earlier studies (Kolb & Whishaw, 1985) of the orbital prefrontal cortex found that lesions at postnatal day 7 were not as detrimental as similar lesions in adulthood. In our study, we found that lesions of the orbital frontal cortex on postnatal day 3 lead to functional sparing on the water task. As first hypothesized by Goldman (1976), the sparing of function may be attributed to the orbital frontal's proximal location to and connectivity with the medial frontal cortex. During development, the orbital region is

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developmentally advanced from the medial cortex by approximately one day. It is possible that injury to the orbital frontal cortex at a young age will trigger the less mature medial frontal cortex to reorganize its circuitry and compensate for the lost tissue. As a consequence, sparing of spatial navigation occurs.

6. 5. SUMMARY AND FUTURE DIRECTIONS

The overall findings of these studies demonstrate that both environmental and pharmacological treatments are beneficial after perinatal prefrontal cortex injury. It is also apparent that the developmental stage of the brain is a crucial factor to consider when deciding on what treatment would have the greatest benefit, as in some instances it is possible to worsen the behavioral outcome with therapeutic intervention. In particular, the impact of behavioral therapies should not be overlooked as it appears that pharmacological treatments may produce a more favorable outcome in functional recovery if paired with behavioral therapy.

Basic fibroblast growth factor has proven to be a useful tool in promoting functional recovery following brain injury in both animal and clinical studies. Because most studies focus on the role of bFGF in the adult brain, future research may include gaining a better understanding of the role of bFGF in the developing brain as it possibly has a different function than in the adult brain. Studies currently in process through the western blot technique are finding differences in proteins and the corresponding receptors following the administration of bFGF in brain-injured infants.

In addition, as demonstrated by experiments 1 and 2, bFGF appears to work in a dose-dependent manner. Therefore, it would be beneficial to give subjects escalating

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doses of bFGF to determine the dose that provides optimal recovery of function. On the other hand, as high levels of bFGF are found in the diseased brain such as in Alzheimer's, it would also be anticipated that in addition to an optimal dose there must also be a lethal or toxic level of bFGF in the developing brain.

Because environmental factors play an important role in functional recovery, studies that continue to investigate how to optimize the benefits associated with behavioral treatments should continue. For example, our study demonstrated that complex-housing after bFGF administration in the injured brain increased the number of subjects with new tissue in the lesion cavity. The filling in was not complete, however, and given that animals killed at 22 days of age show complete filling of the lesion cavity (R. Diaz-Heijtz & R. Gibb, unpublished observations, 2002), it seems likely that the new tissue must have died over time. As suggested by Kelche and colleagues (1995), survival of tissue may be more complete if specific training targeted at exercising the injured site is enforced. Therefore one possible experiment would be to administer bFGF after perinatal medial frontal cortex injury, followed by training on tasks that target medial prefrontal cortex functioning such as spatial navigation tasks. It would be hypothesized that this combination may be more effective than general sensory stimulation provided by complex-housing.

Finally, the functioning and response to experience (e.g., injury, pharmacological manipulations and an enriched environment) of the medial prefrontal cortex is understood to a greater extent than similar changes produced in the orbital prefrontal cortex. Future studies may therefore investigate the effects of experience on the orbital prefrontal cortex and how this region differs from the medial region.

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8. APPENDIX I: Water Task Pilot Study: The Effect of 20 minute Intervals

8. 1. INTRODUCTION

The water task data found in experiment 1 were unique from previous data collected in this lab. Usually, subjects were run for only 5 days (4 or 8 trials per day) and control subjects are known to asymptote at around 5 seconds by the last day. In our study, however, control subjects did not reach this criterion. Instead, by day 5 control subjects had an average latency of 30 seconds and continued to search the pool in a random pattern for the platform, and therefore testing continued for two more days. By the end of day 7, the average latency was 15 seconds, but the control group still had an extremely high variance in performance. To help reduce the variance, seven additional control subjects were added, but it remained unclear why the animals performed so poorly on the task. There are several aspects of this task that are different in this experiment than from the others that were run in the University Hall labs.

One explanation thus is the "building effect", as this experiment was carried out in a different building, different room, and with different equipment than previous studies. The current room used is much larger with cues farther away and fewer in numbers than the previous testing room, and thus may have made the water task more challenging. Other explanations include the use of a round platform as opposed to a square one, thus making it less likely in the initial learning phase that the animal would bump into the platform. Second, although the location of the platform was randomly chosen in this experiment to be in the north-east quadrant, farthest away from the experimenter, previous studies always had the platform located in a southern quadrant,

which is nearer the experimenter. Third, subjects from experiment 1 were tested at precisely 20 minute intervals. The intertrial interval in previous studies became progressively shorter as the animals learned the task (The intertrial interval was the time it took the other animals being tested in the same grouping to locate the platform). Because these differences in methodology may have influenced performance on this task, a study was conducted to analyze systematically the differences between the past studies and experiment 1.

8. 2. MATERIALS AND METHODS

8. 2. 1. Subjects

A total of 16 Long Evans hooded rats were divided into 4 groups as follows: 20 minute interval between trials, as performed in experiment 1 (20 min.); 5 min interval between trials (5 min.); 5 minute interval between trials with the presence of a square platform as opposed to the round platform initially used (square plat.); 5 minute interval between trials with a square platform located in a southern quadrant close to the experimenter (plat. location). Each group was comprised of 2 males and 2 females.

8. 2. 2. Procedure

The setup of the water task was similar to that described in experiment 1 methods. Each subject was ran four trials per day for seven days, according to the group to which they belonged. The effect of procedure was analyzed by comparing the final performance of all groups, thus comparing the mean latency achieved by day 7 of the task.

8. 3. RESULTS

As found in experiment 1, subjects in the 20 minute interval group had a very high variance in latency. As demonstrated in Figure 8. 1, although all three groups that were tested at five minute intervals demonstrated a clear learning curve, this was not found in the 20 minute group. Instead, the mean latency varied from day to day, and unlike the other groups did not asymptote by seven days. By day 7, the average latency was 29.40 ± 5.05 seconds. This is extremely high in comparison to the other groups. In all other cases, although latencies were not at 5 seconds as found in studies conducted in another building and testing center, there was a profound reduction in latency (Figure 8. 2), primarily due to imposing 5 minute intervals between trials.

Owing to the high variance in the 20 min group (thus violating homogeneity of variance), this group was not included for a univariate comparing all other groups. This analysis, did not find a statistical difference between any of the groups $(F(2,9) = 1.27, p = .33)$. Therefore, the shape of the platform and the location of the platform did not make a difference when subjects were run in 5 minute intervals. Non-parametric tests using Mann-Whitney U, determined that the 20 minute interval group was significantly different from all other groups (20 min vs. 5 min: $U = 1.00, p < .05$; 20 min vs. square plat.: $U = .000, p < .05$; 20 min vs. plat. location: $U = .000, p < .05$).
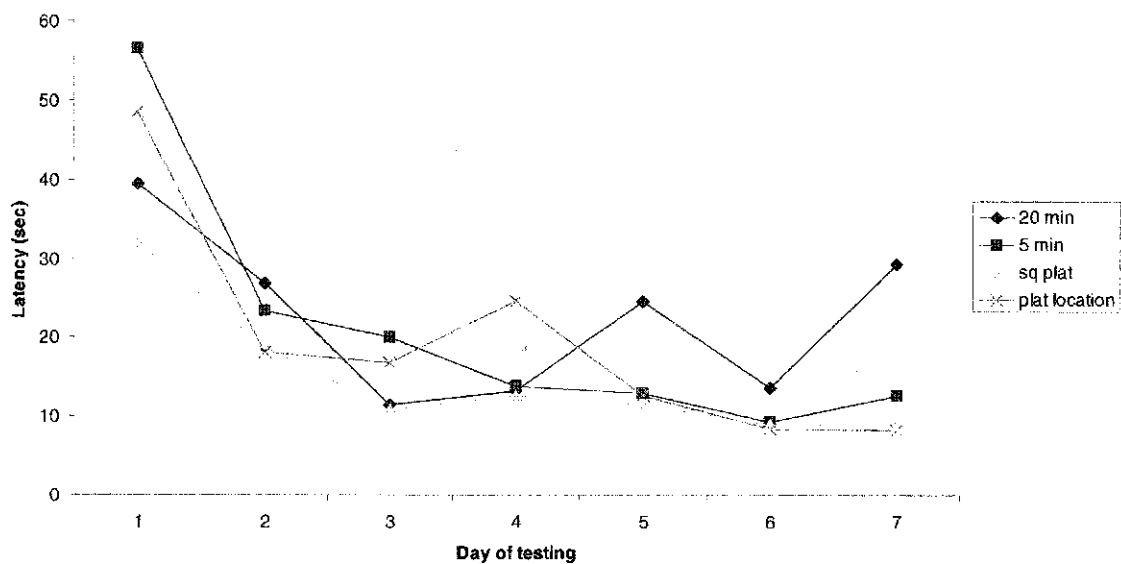
Figure 8. 1. Mean water task latency day by day. Unlike the other groups, the 20 minute group did not asymptote in latency by day 7.
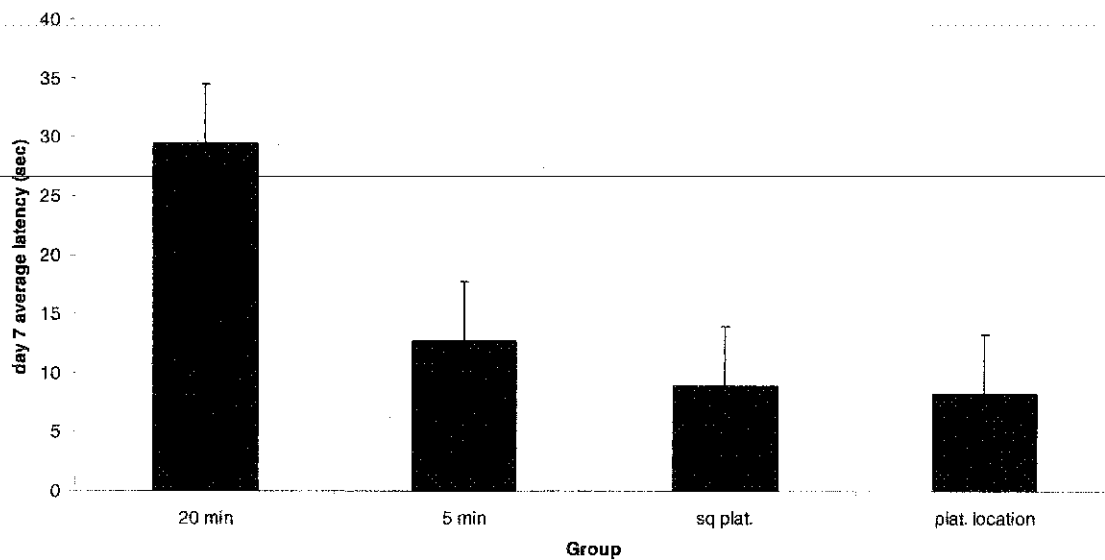

Figure 8. 2. Water task performance after seven days of testing. Twenty minute intervals between trials increased latency on this task. Platform shape and platform location did not affect water maze performance.

8. 4. DISCUSSION

The results indicate that the location of the hidden platform and the shape of the platform did not affect water task performance, but the waiting period between trials did influence performance. By subjects waiting 20 minutes between trials as opposed to the usual 5 minutes used in our lab, the task became more difficult as the subjects had to remember the platform location over a much longer waiting period. Because there was not that immediate reinforcement of finding the platform location every five minutes, this made it more difficult for the subjects to remember the exact location of the platform and thus lead to an improved search strategy as opposed to forming a precise cognitive map of the platform location.

Note that although there was not a significant difference between the other three groups tested the group that had the lowest latency was the group that had five minute intervals between trials, with a square platform located in the southern quadrant of the pool, closest to the holding tubs. Even with a latency of less than 10 seconds, it is important to realize that with all of these variables controlled, subjects still did not asymptote to 5 seconds even by this seventh day. Therefore, there must be other variables that are making this water task more difficult than the conditions in the previous testing center. As described above, the room size, different room cues, and possibly auditory cues are different in this testing room, thus making direct statistical comparisons between data collected from the two different environments impossible.