

**PERINATAL EXPERIENCE ALTERS BRAIN DEVELOPMENT AND
FUNCTIONAL RECOVERY AFTER CEREBRAL INJURY IN RATS**

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DEDICATION

For Bill

I would never have done it without your encouragement.

THESIS ABSTRACT

Brain damage in the first week of life is behaviorally and anatomically devastating for a rat. I investigated the use of pre- and/or postnatal experience as interventions that might improve outcome in rats with postnatal day 4 (P4) frontal cortex lesions. Prenatal maternal tactile stimulation or maternal complex housing facilitated recovery in P4 lesion animals and produced changes in brain organization. Post-lesion tactile stimulation also was found to be beneficial possibly via experience dependent changes in FGF-2 expression. Levels of FGF-2 were increased in both skin and brain after tactile stimulation and correlated with behavioral and anatomical changes. Direct post-lesion administration of FGF-2 had similar effects. These results are the first demonstration that prenatal experience can be prophylactic for postnatal brain injury and that behavioral experience can act on brain organization via enhanced trophic factor expression originating in skin.

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LIST OF ABBREVIATIONS

AchE	Acetylcholinesterase
ANOVA	Analysis of Variance
Bcl-2	Anti-apoptotic protein
BDNF	Brain Derived Growth Factor
bFGF	Fibroblast Growth Factor-2
C	Centigrade
Cagemom	Offspring born to untreated mothers
Cg	Cingulate
cm	Centimeter
Condomom	Offspring born to complex housed mothers
CORT	Corticosterone
E	Embryonic
FGF-2	Fibroblast Growth Factor-2
FGFR ₁	Fibroblast Growth Factor Receptor 1
Flg	Fibroblast Growth Factor Receptor 1
Fr	Frontal
GFAP	Glial Fibrillary Acidic Protein
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenal
hr	Hour
HRP	Horse Radish Peroxidase
IGF	Insulin Like Growth Factor
kda	Kilodaltons
kg	Kilograms
ml	Milliliter
NIH	National Institute of Health
NMDA	N-methyl-d-aspartate
P	Postnatal
PBS	Phosphate Buffered Saline

PCNA	Proliferating Cell Nuclear Antigen
Petmom	Offspring born to tactile stimulation mothers
PL	Prelimibic
PLSD	Probability of Least Significant Difference
pmol	Picomole
PPC	Posterior Parietal Cortex
PVDF	Polyvinylidene Difluoride
REM	Rapid Eye Movement
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis
sec	Seconds
μg	Micrograms
μl	Microliters

CHAPTER ONE
GENERAL INTRODUCTION

Injury to the human brain is associated with a wide range of behavioral sequelae that may severely compromise the cognitive and motor capacities of the individual, leading to a significant decrement in quality of life. Brain injury can occur at anytime during the lifespan but some stages of life pose a greater risk for damage than others. Particularly vulnerable times to experience brain injury are during certain periods of brain development, and especially during gestation and early infancy. For example, low birth weight (under 2500 grams) is associated with perinatal illness and a higher rate of long-term cognitive problems such as cerebral palsy and learning difficulties. The sensitivity of the brain to perinatal injury therefore provides an important reason for studying factors that might influence outcomes from damage in the infant mammalian nervous system. Furthermore, by documenting the functional deficits and resulting morphology following brain injury, we can gain insight into the nature of the normal ontogenetic processes that occur within the brain and how they support behavior. The general goal of the current thesis is to investigate perinatal factors that might influence both normal brain development and recovery from cortical injury in rats.

1.1. CORTICAL PLASTICITY

Historically, it was thought the size, structure, and functional connectivity of the brain unfolded according to a genetic blueprint and that once formed, the nervous system was relatively stable in its structure. A more contemporary view acknowledges the contribution of experience to the appropriate formation of connections within the brain and subsequent pruning of inappropriate or redundant connections. Although it is still controversial, there is evidence to suggest that experience also can regulate the mitosis of

cells, their migration, and potentially their end-fate determination. If appropriate experiential treatments could be made available to the developing brain at appropriate times, it is feasible that the negative consequences of early brain injury could be ameliorated.

It is easy to suppose that some parts of the brain are likely to possess a greater potential for change, which I shall refer to as “plasticity”, than others. In particular, the mammalian cortex has many attributes that make it an interesting place to look for a correlation between anatomical flexibility and alterations in behavior. Regional variation in cortical morphology specifies the functional organization of cerebral cortex. Areal-specific differences in architecture include: variation in cell size and density, distribution of neurotransmitters and their receptors, and differences in afferent and efferent connections. The functional specificity of the cerebral cortex thus makes it possible to investigate the consequences of experience. For example, visual stimulation should have anatomical consequences in occipital cortex that may not generalize to cortical areas that are not involved directly in visual function. Another feature of the architecture of the cortex that makes it ideal for studying mechanisms controlling plasticity is the intrinsic cortical circuit.

The intrinsic cortical circuit is a local connection formed by neighboring pyramidal neurons and likely represents the type of connection that is most easily altered. Neurons make extensive arborizations (both dendritic and axonal) to connect with nearby cells and these connections account for approximately 70% of the excitatory input on a layer II/III pyramidal cell (Nicoll and Blakemore, 1993). It appears that these local connections represent most of the output of pyramidal neurons and provide an ideal

substrate for mechanisms of plasticity. By gaining an understanding of how to initiate effective alterations in connectivity through experience or therapeutic treatments, it may be possible to develop strategies that will improve functional recovery after brain damage.

1.1.1. The emergence of ideas regarding the nature of cortical plasticity

The notion that the structure of the nervous system can be modified by sensory experience is not new. Santiago Ramon y Cajal (1894) proposed that learning could produce long lasting modifications in neuronal structure. In addition, Ramon y Cajal was the first to propose that dendritic spines were the major sites of neural connections (DeFilipe and Jones, 1988).

In 1948, Jerzy Konorski introduced the idea that activity could provide a powerful stimulus for changes in neural organization. Donald Hebb, in 1949, furthered these ideas with the hypothesis that activity-dependent changes in neuronal architecture would be expressed primarily at the level of the synapse. Although much research in the past 50 years has provided evidence to support these proposals (e.g., Diamond, Lindner, and Raymond, 1967; Greenough, Black and Wallace, 1987; Rosenzweig, 1971), it has become increasingly clear that environmental stimulation can have a more widespread influence on cerebral architecture than was originally believed. In particular, experience can produce changes in neuronal “environment” as well as changes within neurons. Hence, glial structure and proliferation, vascularization, neurotransmitter levels, and growth factor availability all have been shown to undergo changes as a result of various environmental manipulations (e.g., Sirevaag and Greenough, 1987; 1988). It is therefore

clear that environmental experience can have effects that go much beyond mere changes in neuronal structure. Of course, it is likely that changes in vascularization, glia, or growth factors modulate neuronal structure and function and such non-neuronal changes might also be proposed as the mechanism whereby neurons can be altered by experience. Studies of brain plasticity and behavior therefore have begun to expand their scope and to look at a wider variety of cerebral changes than was even contemplated 25 years ago. One factor that has also become intensely studied is developmental age.

1.1.2. Age-related changes in cortical plasticity

The facility with which cortical plasticity is induced and the degree of its expression changes during the lifetime of an individual. Although it is thought that the infant brain is more mutable than the adult brain, there appear to be stages of development that possess greater potential for plasticity than others. As noted above, many elements contribute to cortical plasticity including the generation of neurons, glia, and blood vessels, and the formation of synaptic space through the addition of axon collaterals, dendrites, and dendritic spines. The production of these elements peaks at different developmental stages and thus we can reasonably predict that the details of cortical plasticity are likely to vary with precise developmental age.

Neuronal birth in the rat cerebral cortex begins on about embryonic (E) day 12 (E12), and continues until about E21, which is roughly the day before full term (E22) (Uylings, Van Eden, Parnavelas, et al., 1990). Neuronal migration continues for at least a week after birth and by the second postnatal week there is intense dendritic differentiation, glial (astrocyte) genesis, and the beginnings of synaptogenesis.

Synaptogenesis continues at a high rate for at least another week. Lesion studies suggest that cortical plasticity is relatively low during the first postnatal week as migration progresses (Kolb, 1995). In contrast, there appears to be a rapid reversal in plastic response to injury during the second postnatal week, which is during the period of gliogenesis and rapid dendritic and synaptic growth (Figure 1.1). Little is known about the relative plasticity of the cerebral neurons over the next few weeks although given that it is a period of intense synaptogenesis and rapid development of behavior, it seems likely that there is considerable plastic capacity in at least synapse number. Once animals reach adulthood it is presumed that there is a gradual reduction in neuronal plasticity with a more dramatic decline during senescence. In view of the age-related changes in cerebral plasticity it seems likely that experiences may have age-related changes in their effects on cerebral organization, both in the intact and injured brain.

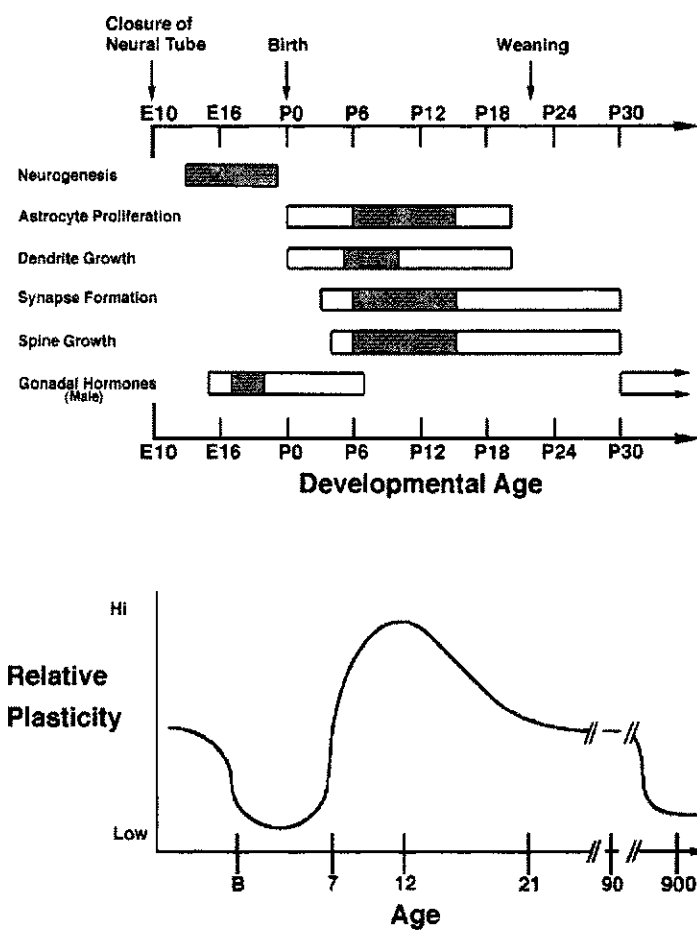


Figure 1.1. Top. Main cellular events related to cortical plasticity. Bars mark the approximate beginning and ending of different processes. The intensity of the shading reflects the intensity of the phenomenon. **Bottom.** Timeline showing variations in cerebral-injury related cortical plasticity during the lifespan of a rat.

1.1.3. Brain injury and cortical plasticity

Systematic study of the effect of early brain damage was begun by Margaret Kennard in the 1930's when she compared unilateral motor cortex lesions in infant and adult monkeys (Kennard, 1938). The impairments in the infant monkeys were milder than those in the adults, which led Kennard to hypothesize that there had been a

change in the cortical organization of the infants. This cortical reorganization was presumed to support the behavioral recovery. In particular, she hypothesized that if some synapses were removed as a consequence of brain injury, “others would be formed in less usual combinations” and that “it is possible that factors which facilitate cortical organization in the normal young are the same by which reorganization is accomplished in the imperfect cortex after injury” (Kennard, 1942, p.239). Although Kennard had much to say regarding the limitations of functional recovery after early brain injury (for a review see Finger and Almlı, 1988), it was her demonstration that the consequences of motor cortex lesions in infancy were less severe than similar injury in adulthood that is usually associated with her name, and is commonly referred to as the “Kennard Principle.” The Kennard Principle holds that recovery is more complete after early brain damage than after damage later in life. This does not explain, however, the contrary observations that some kinds of brain damage are worse if they are experienced during development. In the course of studying children with frontal lobe injuries early in life, Donald Hebb noticed that many children had far more severe functional loss than would be expected from a similar injury in an adult (Hebb, 1947). Hebb concluded that early injuries prevent the development of certain intellectual capacities that are critical to normal cognitive development (e.g., Hebb, 1947; 1949).

Over the 50 years since Kennard and Hebb’s observations, there have been extensive studies on the effects of cortical injury in a variety of laboratory species, especially rats, cats, and monkeys (for a review see Finger and Almlı, 1984). Taken together, these studies support the idea that it is the precise developmental age at which

the injury occurs that predicts the functional outcome (e.g., Kolb, 1995; Villablanca, Hovda, Jackson, and Infante, 1993).

While undertaking the study of the effects of frontal cortex damage in developing rats, Kolb and others (e.g., Kolb and Nonneman, 1976, 1978; Kolb, Sutherland, and Whishaw, 1983; Kolb and Gibb, 1993) noticed that rats with lesions at about seven days of age (a developmental age coincidental with high cortical plasticity) showed remarkable behavioral recovery when tested as adults. Rats given lesions earlier, up to postnatal day five (a developmental age coincidental with low cortical plasticity), showed a much poorer functional outcome than rats with lesions at postnatal days seven through ten or rats with similar removals in adulthood. Lesions performed before birth (E18) allowed complete restoration of behavioral function in adulthood (Kolb, Cioe, and Muirhead, 1998). Thus, the age at which an animal sustains cortical damage is predictive of functional outcome and dependent on the degree of plasticity available to the developing brain at the time (Table 1.1).

Table 1.1. Summary of the effects of frontal cortical injury at different ages

Age at Injury	Result	Reference
E18	Cortex regrows with odd structure Functional recovery	Kolb et al. 1998a
P 1- P 6	Small brain, dendritic atrophy Dismal functional outcome	Kolb and Gibb 1990
P 7-P 12	Dendrite and spine growth Cortical regrowth Functional recovery	Kolb and Gibb 1990 Kolb et al. 1998
P 120	Dendritic atrophy, then growth Partial return of function	Kolb 1995

Abbreviations: E 18, embryonic day 18; P, postnatal day, number refers to age in days

Support for these conclusions is seen in studies using other species, if developmental age is considered as a relevant factor. A similar pattern of results can be seen in parallel studies of the effects of cortical lesions in kittens by Villablanca and his colleagues (e.g. Villablanca, Carlson-Kuhta, Schmanke, et al., 1998). If the kitten cortex is damaged between days E55 (but not before) and P60, there is an excellent functional outcome. Injury prior to 55 embryonic days leads to a poor functional outcome and damage after 60 days leads to a progressive worsening of the lesion effects. Most studies done on monkeys are done much later in development than the rat and kitten studies because monkeys are born relatively mature, even in comparison to humans. The one monkey study by Goldman and Galkin (1978) that did show recovery after a prenatal frontal injury was, in fact, making lesions roughly equivalent to the age at which rats and kittens also show recovery. A key point here is that birth date is irrelevant. It is the developmental stage of the brain at injury that is critical. Thus, because rats and kittens are born at an embryologically younger age than primates, including humans, the time scale for functional outcome must be adjusted to match the neural events that are underway at the time of injury.

1.1.3.1 Functional and anatomical sequelae of early brain damage in rats

Although most of the work done on recovery from early cortical injury in the rat has focused on recovery processes after frontal cortex lesions, there have also been studies on other cortical regions such as motor cortex, posterior parietal cortex (PPC), temporal cortex, and occipital cortex. There are differences in the degree of functional outcome of animals sustaining frontal cortex versus more posterior lesions but for the

most part the general principle holds: focal lesions (unilateral or bilateral) virtually anywhere in the rat cortex around day 7-10 produce much better functional outcomes than similar injuries from days 1-5. This includes injuries to medial frontal, orbital frontal, motor, posterior parietal, posterior cingulate, occipital, and temporal cortex (e.g., Gonzalez, Wishaw, and Kolb, 2003; Kolb and Cioe, 1998; 2003; Kolb, Cioe and Wishaw, 2000; Kolb, Petrie, and Cioe, 1996; Kolb and Wishaw, 1981).

It was the consistency of these findings that led us to conclude that there is something special about the brain's response to injury when it is damaged in the second, versus first, week of life. As we began to look for the anatomical correlates of recovery at different ages, it became apparent that more than one mechanism was at play. Our first hypothesis was that good recovery was related to the generation of new cortical circuits and that the presence of these circuits could be inferred from hypertrophy of dendritic arborization and increased spine density (Kolb and Gibb, 1991). Subsequent studies showed that such changes were correlated with functional recovery after injury to the motor, posterior cingulate, temporal, and occipital cortex during the second (but not the first) week of life and led to a general hypothesis that recovery from early injury is supported by synaptic changes in the remaining cerebral mantle (Kolb and Gibb, 2001).

Further studies showed that there was more to the story, however. In particular, it became clear that some of the behavioral improvement observed was dependent on spontaneous neurogenesis and filling-in of the lesion cavity in the medial frontal area (Kolb, Gibb, Gorny et al., 1998). This novel neurogenic response did not occur after injury across the cortex however, but was found only with midline lesions (e.g., Gonzalez, Wishaw and Kolb, 2003). Furthermore, the variation in the extent of

functional recovery after injury during the second week of life was related to the likelihood of both neurogenic and dendritic changes. Rats with PPC lesions, for example, do not show the same degree of behavioral recovery after lesions around postnatal day 10 and the lesion cavity shows no evidence of filling-in. Given that damage to the midline frontal regions lead to the most plastic response to cerebral injury, I decided to focus on the effects of these lesions in the current study. The general idea was that it might be possible to shift the period of intense plastic change from the second to the first week of life by manipulating different types of experience. Future studies will explore how injuries to other regions, such as the posterior parietal cortex, might respond to the treatments.

1.1.4. Sex hormones and cortical plasticity

Work by Juraska (1990) has shown that the cerebral cortex of male and female rats is structurally different (males have thicker, longer, and wider cortices than do females; sexually dimorphic variation occurs in the thickness of cortical layers of particular brain regions) and respond to experience in different ways. By examining visual cortex, Juraska (1984) showed that males demonstrate greater dendritic proliferation in response to enriched housing than did females. Later studies have shown that the sex-related differences in plasticity are areal-dependent. Thus, Kolb, Gibb and Gorny (2003) replicated Juraska's findings for the visual cortex but also showed that the sensorimotor cortex was equally sensitive to environmental stimulation in males and females.

Other studies of brain-injured animals have also suggested that there are significant sex differences in cortical plasticity. Kolb and Stewart (1995) showed that males with day 7 frontal lesions showed better functional recovery than females and that the dendritic changes associated with the recovery were also sexually dimorphic. In a related set of experiments the same authors also showed that there is a hormone-dependent difference in the morphology of cortical neurons in the prefrontal cortex of rats.

In sum, plastic changes in the cortex appear to be sexually dimorphic and studies of environmental influences on cortical morphology and behavior will require a study of both males and females.

1.1.5. Stress hormones and cortical plasticity

Virtually all animals experience stress at some time during their lives. Chronic stress mediates effects on the neuroendocrine system, which ultimately cause changes in neuronal cell morphology (Stewart and Kolb, 1988). Although much of the stress research conducted to date has focused on the hippocampal formation (e.g., Meaney, Aitken, and Sapolsky, 1987; Gould, Tanapat, Galea, et al., 1997), there is evidence to support the idea that neocortical neurons are also susceptible to stress. Indeed, the receptors for corticosterone (glucocorticoid-receptors -Type II) are located throughout the cerebrum and are particularly dense in frontal cortex. Whether or not stress interacts with environmental stimulation to alter brain morphology is not yet clear but it does seem likely (Kolb and Wishaw, 1998).

1.1.6. Growth factors and Cortical Plasticity

Several studies have reported that brain levels of some growth factors (i.e. insulin-like growth factor (IGLF), brain derived neurotrophic factor (BDNF), fibroblast growth factor-2) are changed after certain experiences. For example BDNF and FGF-2 levels are reported to rise after complex housing (Ickes, Pham, Sanders, et al., 2000; Kolb et al., 1998) whereas IGLF and BDNF expression are increased after exercise (Carro et al., 2000; Garza, Ha, Garcia, et al., 2004). The focus of the current studies was FGF-2, in large part because there is an extensive literature showing a role of FGF-2 in neuronal differentiation and in recovery from cerebral injury in adulthood (e.g., Gregg and Weiss, 2003; Rowntree and Kolb, 1997).

FGF-2 is one of nine members in the fibroblast growth factor family. FGF's are important during development and wound healing, and also have a role in angiogenesis. FGF-2 is a pluripotent growth factor that has been implicated in prenatal, postnatal and adult neurogenesis and it plays a role in healing and repair of both skin and brain. FGFR1 or flg is one of four high affinity receptors for FGF's that has been demonstrated to play an important role in FGF-2 activity in the brain (Logan, Frautschy, Gonzalez et al., 1992).

1.2. ENVIRONMENTAL STIMULATION AND BRAIN PLASTICITY

In the early 1960's Rosenzweig and others (Rosenzweig, 1971) conducted experiments on complex rearing of postweaning animals and showed that by altering an animal's environment the morphological characteristics of its brain were altered. Resulting changes in brain structure were, however, dependent on how the animal

interacted with this novel environment. If interaction was minimal, little change was observed in the animal's behavior and subsequent change noted in brain structure was also limited. If the animal engaged in a new repertoire of behaviors while interacting with the novel environment, many alterations in brain morphology resulted including increases in brain weight, cortical thickness, glial number, neuron size, dendritic branching, and number of synapses per neuron (for a review, see Greenough & Chang, 1989).

1.2.1. Environmental stimulation after brain injury

The notion that environmental enrichment might work as a preventative therapy following cerebral cortex injury was based on the hypothesis that stimulating plastic changes in the injured brain ought to be beneficial in repairing damaged cerebral circuits (for a review, see Will and Kelche, 1992). In fact, there is now considerable evidence that complex housing can improve functional outcome after focal stroke and other types of cerebral injuries in adult rats (for a review, see Schallert, Leasure and Kolb, 2000).

There is considerably less evidence for beneficial effects of complex housing on animals with neonatal lesions but it seems reasonable to predict that the developing brain might be more sensitive to environmental stimulation than the older brain and this does appear to be the case (e.g., Bland and Cooper, 1969; Kolb and Elliott, 1987; Gibb, Gorny, and Kolb, 2004). For example, when I placed rats with large frontal lesions on day 4 into complex environments (see Figure 1.2) at weaning there were significant behavioral improvements in animals when they were examined as adults (Gibb, 2001). In contrast, when animals were placed into the same environments in adulthood, there were no

significant behavioral benefits (Gibb, 2001). The importance of age-at-experience in those studies suggested that the greatest benefit of postinjury environmental treatments would be earlier rather than later. The challenge was to develop a treatment that could be initiated immediately after the injury.

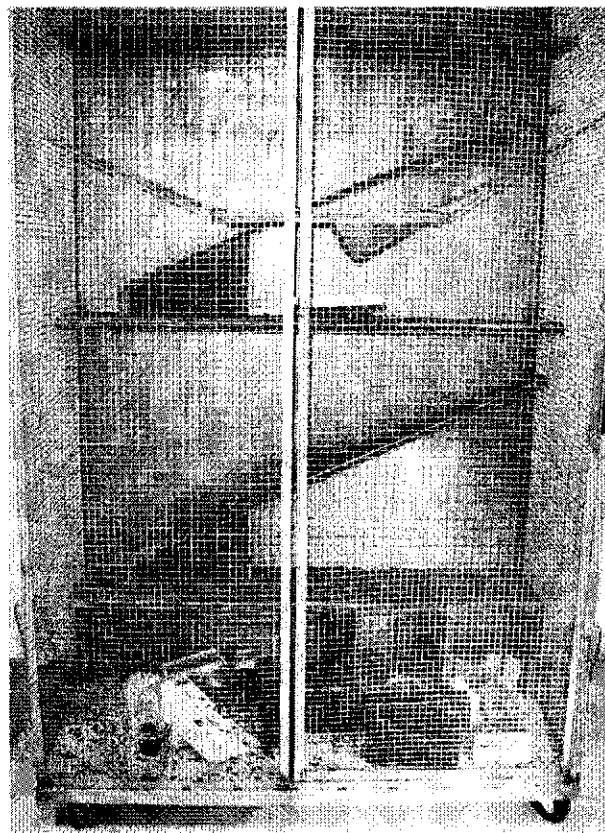


Figure 1.2. Complex housing for rats (Condominiums). The hardware cloth construction and a series of runways and swings allow exploration of the condominiums in all dimensions. PVC pipes provide dark shelter for inhabitants. A variety of hard plastic toys are placed on the condo floor and are changes weekly to provide additional stimulation.

1.2.2. Environmental stimulation in brain-damaged infants

Morphological changes found in the brain following complex housing vary depending on the age of the animal at the time of enrichment (Kolb, Gibb, and Gorny, 2003). Animals with perinatal cortical injury that were placed in complex housing at weaning showed greater behavioral recovery than did lesion animals placed in complex housing as adults. With this in mind I explored the possibilities for providing enriching experience even earlier, specifically in the preweaning or prenatal period. Although enrichment therapies such as rearing animals in complex environments provide a lot of opportunity for animals to interact with novel stimuli in the environment, such therapies cannot be used for preweaning animals. In order to determine if environmental interventions were more effective when the treatment was introduced immediately following the lesion, a different method of stimulation had to be employed. Young rats are unable to see and hear until approximately two weeks of age and so olfaction and somatosensation comprise the majority of all sensory processing until then. Because tactile stimulation has been shown to stimulate growth in premature infants (Field, Schanberg, Scafaldi, et al., 1986) and newborn rats (Schanberg and Field, 1987), we decided to try tactile stimulation as a treatment for early cortical damage in perinatal rats (Figure 1.3). For the rat pups, stimulation of this sort would be a similar experience to the licking and grooming they receive from their mother.

The results of these studies were dramatic. Animals that were given tactile stimulation after medial frontal injuries on day 4 showed significant recovery on the performance of both cognitive and motor tasks (Gibb, 2001). But many questions remained unanswered. Specifically, what was the mechanism that led to the enhanced

functional recovery? Would other sorts of treatments prove to be beneficial too? Did the treatments have to be after the injury or would prenatal stimulation also prove to be beneficial? It was these questions that formed the basis of the current thesis.

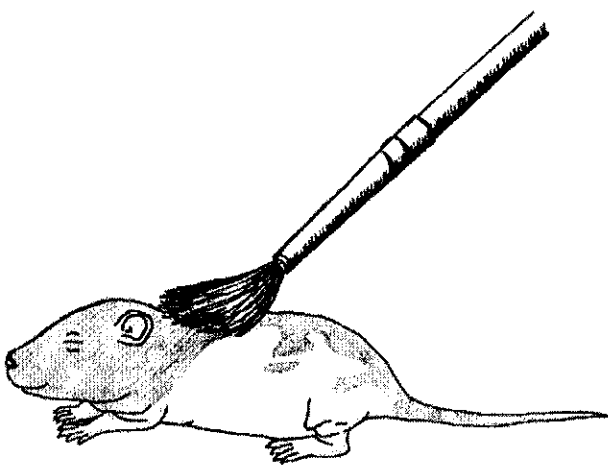


Figure 1.3. Tactile stimulation of an infant rat with a camelhair brush.

The promising results from the postnatal tactile stimulation experiment led to the idea that prenatal stimulation may also be useful as a therapeutic intervention for early brain damage. In Experiment 3, adult female rats were brushed and handled one week before a male was introduced to their home cage. Tactile stimulation proceeded through the entire pregnancy and ceased upon the day of parturition. For Experiment 4 we returned to the use of the rat condominiums to explore the effectiveness of prenatal maternal complex housing as a form of experiential stimulation.

1.3. ORGANIZATION AND RATIONALE OF THE THESIS STUDIES

The thesis began by looking for an answer to the question of what might be the mechanism underlying the tactile stimulation results. Experiment 1 examined the

behavioral and anatomical consequences of tactile stimulation after early injury. I also examined skin samples obtained from the stimulated animals and compared the expression of proteins in the skin to levels found in unstimulated animals. I discovered that the protein FGF-2 was upregulated in the stimulated animals, and thus tried to mimic the effects of the tactile stimulation with administration of subcutaneous FGF-2 during the week following surgery. During tactile stimulation and to a lesser degree during the FGF-2 administration, animals were subjected to short periods of maternal separation. It has been well-documented that maternal separation or “handling” during the early preweaning period can alter the physiology and emotionality of the offspring. I wanted to determine if some degree of the behavioral recovery I observed was attributable to the handling process. With this in mind, I designed Experiment 2. In this experiment, the rat pups were removed from the nest for 15 minutes, 3 times per day without any additional stimulation. This treatment mimicked the separation period the tactile stimulation litters experienced. The results showed that although handling altered the brain of the animals, by itself it did not influence functional recovery. Experiments 3 and 4 assessed the potential for prenatal experience to alter cortical plasticity and attenuate behavioral deficits associated with early cortical lesions. In Experiment 3, maternal tactile stimulation was the therapeutic intervention whereas Experiment 4 employed the use of maternal complex housing to enrich offspring during fetal development. Both treatments altered brain organization and stimulated functional recovery.

Taken together, these experiments provide an overview of how brain structure and function after perinatal cortical injury are affected by pre-and post-natal experience.

There is a chapter appended to this work that reviews of the effects of environment on the behavior and physiology of the laboratory rat. Although this work does not fit into the storyline of this thesis, it provide additional information on the power of experience in shaping the behavior and brain anatomy of the rat.

Before describing the experiments in detail, I first briefly present the general behavioral and anatomical methods used in the studies and the rationale for their choice.

1.4. BEHAVIORAL AND ANATOMICAL ASSESSMENTS

1.4.1. Behavioral Tasks

1.4.1.1. Morris water task. The Morris water task was developed by Richard Morris (1981) and has been used extensively as a test of learning ability in both normal and brain damaged rats. In one version of this task, rats locate a hidden platform in a large tank of opaque water by learning the location of static extra-maze cues (Figure 1.4). Rats are good swimmers and learn to locate the platform quickly. Performance on this task can be assessed by latency to find the platform, heading angle accuracy, and swim path length. Animals with specific cortical lesions show performance deficits on this task and some are entirely unable to learn how to solve it. The observed deficits are not due to motor problems with swimming or standing on the platform but seem due to problems with learning the location of the platform and/or how to navigate efficiently to it.

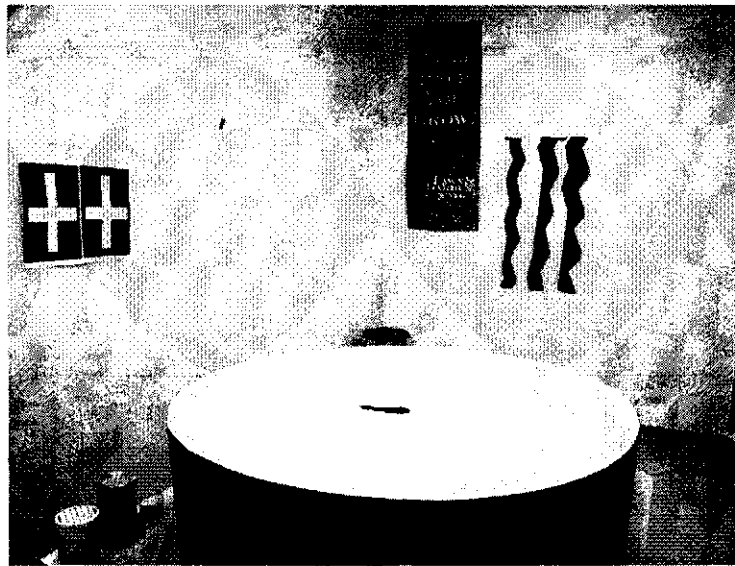


Figure 1.4. The Morris Water Task

1.4.1.2. Whishaw reaching task. Ian Whishaw and his colleagues (e.g., Whishaw, Gorny, Pellis, et al., 1991; Whishaw, O'Connor, Dunnett, 1986; Whishaw, Haun, and Kolb, 1999) have developed a procedure to assess the ability of rats to use their forepaws to retrieve food. A rat is trained to reach through metal bars to retrieve chicken feed from a tray at the front of the cage (Figure 1.5). This test is specific for motor skills and performance is measured by the success of the animal to retrieve and ultimately consume food.



Figure 1.5. The Whishaw Tray Reaching box.

1.4.1.3. Circadian activity. Circadian rhythms are the day-night rhythms found in most animals. In mammals, sleep-waking behavior and general activity are thought to be controlled by a circadian pacemaker within the suprachiasmatic nucleus of the hypothalamus. Rats are nocturnal animals and as such show a higher level of activity in the dark (Kolb and Whishaw, 2001). Using a computer monitoring system (Figure 1.6) it is possible to assess circadian activity and determine if there is a shift from the normal species-typical behavior following cortical lesions and/or environmental stimulation.

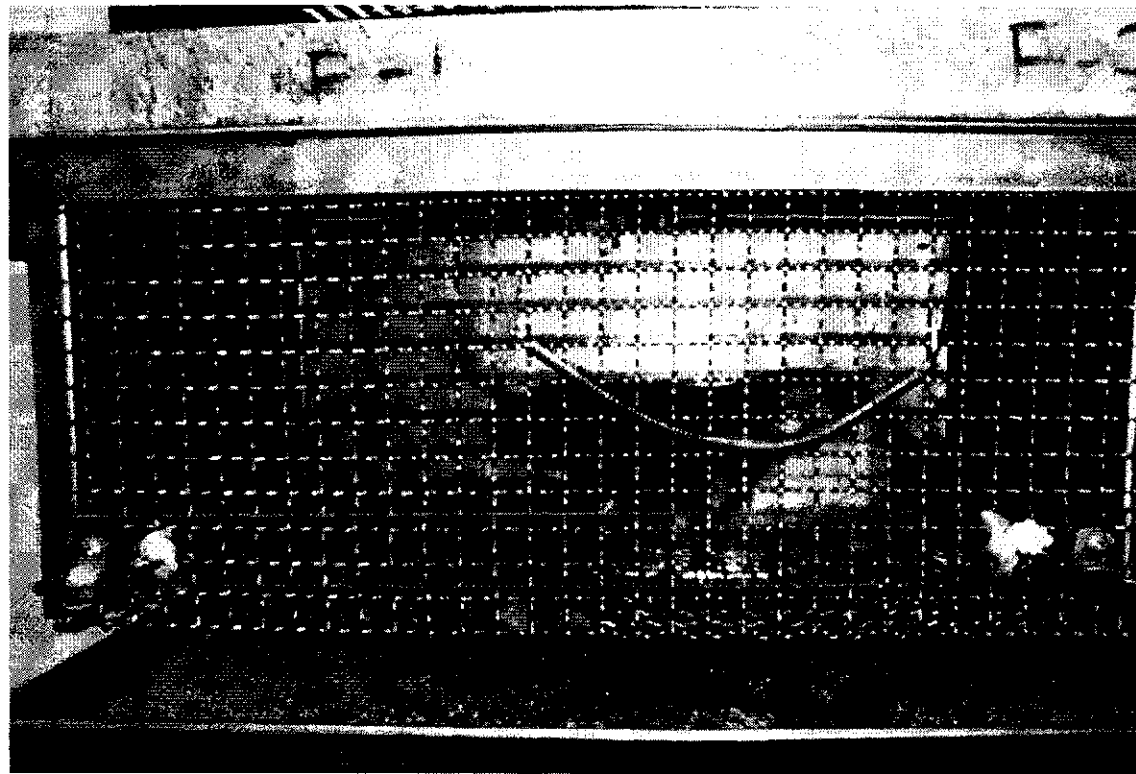


Figure 1.6. Circadian Activity Monitoring System

1.4.2. Physiological and Anatomical Assessments

1.4.2.1. Urine corticosterone. Activation of the hypothalamic-pituitary-adrenal (HPA) axis is an adaptive response to stress and is characterized by increased glucocorticoid secretion. There is evidence to suggest that stress during prenatal and postnatal periods of life can alter the adaptive capacities of adult subjects to stress (Levine, Haltmeyer, Karras et al., 1967; Liu, Dioro, Tannebaum et al., 1997). In a study of the effects of postnatal handling on age-related impairments associated with hippocampal function, Meaney and colleagues (Meaney, Aitken, van Berkel et al., 1988) showed that early postnatal handling of rat pups causes a lower basal level of corticosterone that persists throughout the lifetime of the animal. Assessment of urine corticosterone in adulthood

can thus provide evidence for the re-organization of the HPA system following prenatal or postnatal interventions.

1.4.2.2. Golgi-Cox method. The Golgi technique was developed in 1873 by Camillo Golgi and was used extensively by early investigators (notably Ramon y Cajal) to define structural features of brain architecture (DeFelipe and Jones, 1988). A major advantage of the Golgi technique is that a small percentage of neurons (1-5%) are randomly stained and these neurons are stained completely (Figure 1.7). As a result it is possible to draw individual neurons and to quantify the amount of dendritic space available, as well as the location and density of dendritic spines. A comparison of neurons from “enriched” animals with neurons from appropriate control animals would thus enable characterization of changes induced by environmental stimulation and in doing so provide a means of correlating behavioral outcome with brain anatomy.



Figure 1.7. Neuronal morphology as revealed with Golgi-Cox staining

1.4.2.3. Acetylcholinesterase histochemistry. Acetylcholinesterase (AChE) is a catabolic enzyme that is bound to extracellular matrix material found in the synaptic cleft of cholinergic neurons (neurons that secrete acetylcholine). Traditionally, AChE histochemistry has provided a method to study the cholinergic transmitter system in the brain (Figure 1.8). One can assess the normal distribution of AChE and then look for altered distribution or density of staining following experimental intervention such as cortical lesions or environmental stimulation. Although the distribution of AChE is not necessarily correlated with the presence of cholinergic axons, in some systems such as the basal forebrain projection into cortex, it is thought to be a reasonably good marker (Johnston, 1988). It is clear that the maturation pattern of AChE staining in the cortex occurs at an earlier time than the pattern seen for choline acetyltransferase (an acetylcholine synthesizing enzyme) so the ontogenetic pattern of AChE staining may not mimic the development of cholinergic synapses (Appleyard, 1992). Recent studies examining the function of AChE have revealed that it may play an important role as a neuromodulator with effects on neurite extension and the structural regulation of post-synaptic differentiation (Bravo, Henley, and Rodriguez-Ithurralde, 2000). This makes an assessment of AChE staining even more valuable as a tool for studying synaptic plasticity as AChE may play an important role in this process.

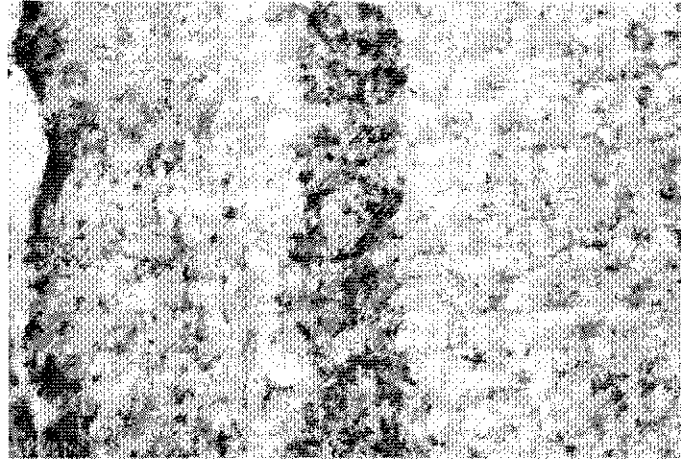
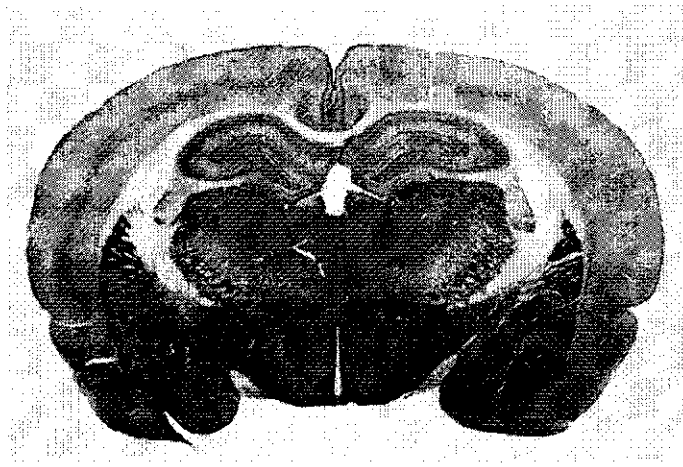


Figure 1.8. Top. Acetylcholinesterase staining of a whole brain section

Bottom. Magnified view of staining in Cingulate Cortex.

1.4.2.4. Western blot. Western blot is a technique that allows detection of a protein of interest from a sample of mixed proteins. A mixture of proteins is run through a gel that has an electrical charge applied to it. The proteins separate out in the gel according to their weight (smaller proteins run through the gel faster than larger proteins). The proteins are transferred to a membrane that is incubated with an antibody against the

protein of interest. Higher expression of protein binds more antibody. The antibody is linked to a molecule that enables its visualization and the amount of protein is thus determined (Fig. 1.9). In this regard, Western blot is a useful technique to identify protein changes that occur after lesion or after various types of experience. We limited our examination of proteins to a few that we considered might be important for plasticity:

- Fibroblast growth factor-2 (FGF-2), and one of its receptors, FGF receptor1 (FGFR1 or flg)

- Glucocorticoid receptor (GR) a receptor for corticosterone. Changes in its expression can reflect alterations in reactivity of the HPA axis in response to stress

- Proliferating cell nuclear antigen (PCNA), a protein expressed in the G₁ and S phase of a cell undergoing mitosis,

- Glial fibrillary acidic protein (GFAP) an intermediate filament protein that is found exclusively in glial cells (increased expression of GFAP implies proliferation of glial cells or increased reactivity of glia already present)

- Bcl-2 an anti-apoptotic protein, increased expression implies a reduction in apoptotic processes.

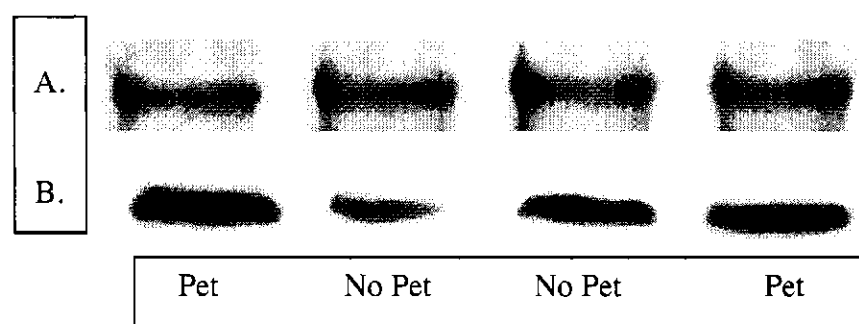


Fig. 1.9. **A.** Coomassie stained band of unidentified protein. **B.** Protein band of FGF-2 (18Kda). Relative amount of protein of interest (FGF-2) is determined by obtaining optical density measures of FGF-2 and expressing it as a ratio of the optical density of the unidentified protein in the same column. This procedure compensates for pipetting errors.

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CHAPTER 2
EXPERIMENT 1

**TACTILE STIMULATION OF FUNCTIONAL RECOVERY AFTER
PERINATAL CORTICAL DAMAGE IS MEDIATED BY FGF-2**

2.1. ABSTRACT

The behavioral consequences of perinatal brain injury can be devastating and there are few effective treatments to reduce the behavioral loss. Here we examine whether tactile stimulation or Fibroblast Growth Factor-2 (FGF-2) might facilitate recovery. Some infant rats received frontal cortex removals on postnatal day 4 (P4) and the remainder underwent a sham surgery. On the day following surgery and until the time of weaning, tactile stimulation (petted) animals received thrice daily 15-minute bouts of stimulation with a camel hair paintbrush (Experiment 1). Other treated animals received subcutaneous injections of FGF-2 once daily for one week post-surgery beginning the day after surgery. On P21 a subset of animals from all groups were decapitated for Western Blot analysis. Behavioral testing of the remaining animals began on postnatal day 60. Following testing on the Morris water task, subjects were trained on a reaching task. At the conclusion of behavioral testing, brains were removed, weighed and later processed for Golgi analysis. Tactile stimulation of infant rats with perinatal cortical injury stimulated functional recovery and reversed injury-related changes in neuronal morphology in the cerebral cortex. The tactile-stimulated induction of recovery and morphological change was associated with changes in expression of FGF-2 in both skin and brain. Direct administration of FGF-2 also was effective in facilitating recovery, although less so than tactile stimulation. These results suggest that early behavioral intervention after perinatal cortical injury can stimulate plastic neuronal changes that can underlie functional recovery and that these changes are mediated, in part, through an upregulation of FGF-2.

2.2. INTRODUCTION

Perinatal cortical injury has severe behavioral and anatomical sequelae in both laboratory animals and human infants. For example, rats with cortical lesions on the first days of life have more severe behavioral deficits than animals with similar injuries in adulthood. Furthermore, this poor behavioral outcome is associated with a thin cortical mantle and a general atrophy of dendritic fields in remaining cortical pyramidal cells (Kolb, 1995). Similarly, human children with prenatal and perinatal cerebral injuries are at high risk for chronic behavioral disturbances (Banich et al., 1990; Vargha-Khadem et al., 1985). We therefore asked if there might be environmental treatments that could attenuate the devastating functional consequences of early brain injuries. Because it had been shown that tactile stimulation is effective in stimulating growth in premature infants (Field et al. 1986) and newborn rats (Schanberg and Field, 1987) we decided to evaluate the effect of tactile stimulation on recovery from cortical injury in newborn rats (Experiment 1). This treatment has the advantage of being simple, noninvasive, and it is possible to begin administration soon after the injury.

Fibroblast growth factor-2 (FGF-2, bFGF) is a growth factor that stimulates mitosis and synaptogenesis in brain of both infant and adult animals and promotes survival of neurons following brain damage. A study by Wagner et al. (1999) demonstrated that subcutaneous injection of FGF-2 could stimulate neurogenesis in developing and adult rats. The potential curative property of subcutaneous administration of FGF-2 was investigated in Experiment 2. Interestingly, FGF-2 is also expressed in skin where it is important for proliferation and differentiation of dermal melanocytes, and wound healing. A study by Buntrock et al. (1982) demonstrated that an extract prepared

from brain tissue of cattle containing FGF-2 was useful in promoting healing of skin wounds. Knock-out mice lacking FGF-2 protein have been produced and are characterized as having abnormalities in the cytoarchitecture of the cerebral cortex, and delayed wound healing (Ortega et al., 1998). These studies led us to wonder if there might be a connection between the reported benefits of tactile stimulation and FGF-2 expression in the skin. Increased availability of growth factors produced by skin could conceivably affect brain tissue in a similar manner to subcutaneous injection of exogenous FGF-2. With this focus we chose to decapitate a subset of the petted and non-petted animals from Experiment 1 to test for relative expression of FGF-2 and its receptor FGFR₁ (flg) in frontal cortex and skin.

2.3. SUBJECTS AND TREATMENT PROTOCOLS

2.3.1. Experiment 1. Tactile Stimulation

The tactile stimulation study was done with 110 rats from 14 litters of animals. Rat pups sustained a frontal lesion or sham surgery on postnatal day four (P4). Half of the litters then received tactile stimulation for two weeks beginning on the day following surgery. This yielded 24 non-stimulated controls, 28 stimulated controls, 25 non-stimulated frontals, and 33 stimulated frontals. There were approximately equal numbers of males and females in each group. The tactile stimulation consisted of sequentially stroking individual pups with a soft camel hair paintbrush for 15 minutes 3 X per day. The pups in the stimulated groups were removed from their mother and placed in a Plexiglas cage that had a 1 cm deep layer of "bed of cobs" on the bottom. The pups were transported to an adjacent room and were given gentle tactile stimulation with a .5 cm diameter camel's

hair histology brush for 15 min three times daily (9 AM; 1 PM; 4 PM). They were then returned to their mother, having been away from her for no more than 20 min. The stimulation procedure continued until weaning at postnatal day 21. During the first week of stimulation the animals typically went into REM sleep, as characterized by twitching. By the time the animals reached about 14 days old they had become quite active and the experimenter had to follow the animals with the brush to provide the stimulation. At the time of weaning a subset of stimulated animals was decapitated and their brains were quickly dissected and frozen for Western blot analysis. A section of skin removed from the rostral portion of the back was also harvested for protein analysis.

2.3.2. Experiment 2. FGF-2 Treatment

The FGF-2 study was done with 110 rats from 9 litters of animals. Rat pups sustained frontal or sham surgery on P4. Approximately half of the animals in each litter then received subcutaneous injections of 10 μ g/kg FGF-2 for 7 days beginning the day after surgery. This yielded 10 sham-operated females, 16 P4 females, 16 sham-operated males, and 19 P4 males that were given FGF-2 treatment. There were 8 sham-operated and 18 P4 females; 10 sham-operated and 13 P4 males in the group that received injections of vehicle.

Human recombinant FGF-2 (R & D Systems, Minneapolis MN: # 233-FB) was dissolved in a phosphate buffer solution containing 1mg/ml of bovine serum albumin. The FGF-2 was administered so each rat received 10 μ g FGF-2/kg body weight. The subcutaneous injections of FGF-2 began the day after surgery and continued for one week. Animals not receiving FGF-2 received placebo injections of the diluent.

2.4. SURGERY

On postnatal day 4 (P4) the pups were removed from the nest and cooled in a Thermanon® cooling chamber until their core temperature reached approximately 20°C. The lesion animals had their scalp opened then the frontal bone carefully removed after it was incised with iris scissors. The medial frontal cortex was then removed bilaterally with gentle aspiration. The tissue targeted for removal was the medial subfield of the prefrontal cortex including Zilles (1985) regions Cg1, Cg3, and PL as well as the medial portion of Fr2 of the motor cortex. After aspiration of the cortical tissue, the animals' scalp was sutured with silk thread drawn by a very fine needle. The remaining control animals underwent a sham surgical procedure in which the scalp was opened and then sutured closed but the skull was not removed. These animals were identified by removal of the tip of the outer toe on their right rear foot. Beginning on the following day, they were given tactile stimulation three times daily for the next two weeks or subcutaneous injections of FGF-2 for one week.

2.5. BEHAVIORAL METHODS

2.5.1. Morris Water Task

Beginning at P60 animals were trained on the Morris Water Task using a similar procedure to that described by Sutherland et al. (1983) based on the original task described by Morris (1981). The maze consisted of a circular pool (1.5 m diameter X 0.5 m deep) with smooth white walls. The pool was filled with approximately 25 °C water mixed with 500 ml of skim milk powder, used to render the water opaque. A clear plexiglas platform (11 X 12 cm) was placed in a constant position inside the pool

approximately 30 cm from the pool wall. The water level was adjusted so that the platform was invisible to a viewer outside the pool and to a rat swimming in the water. A trial consisted of placing a rat into the water facing the pool edge at one of four compass locations (north, south, east, or west) around the pool's perimeter. Within a block of four trials each rat started at the four locations in random sequence, and each rat was tested for four trials a day over five consecutive days. If on a particular trial a rat found the platform, it was permitted to remain on it for 10 seconds. A trial was terminated if the rat failed to find the platform after 90 seconds. Each rat was returned to its holding cage for approximately five minutes before the next trial commenced. The swim path for each rat on every trial was recorded using a Poly Track video tracking system (San Diego Instruments) which tracks the swim path and records the latency, distance and dwell time within each quadrant.

2.5.2. Whishaw Tray Reaching

Following water task training, animals were trained in a skilled reaching task developed by Whishaw et al. (1991). In this task rats were trained to retrieve chicken feed through metal bars at the front of the Plexiglas training cage (28 cm deep x 20 cm wide x 25 cm high). The front of each cage was constructed with 2 mm bars separated from each other by 1 cm, edge to edge and the floor was constructed of wire mesh. A tray (5 cm deep x 2 cm wide x 1 cm high) containing chicken feed pellets was mounted in the front of each cage. To obtain food, the rats had to extend their forelimbs through the bars, grasp, and retract the food pellet. The food tray was mounted on runners to adjust the distance of the food from the bars. Distance adjustment ensured that each rat could not simply rake

the food into the cage. Any pellets that the rat dropped inside the cage were irretrievably lost through the mesh on the floor and the animal would have to reach again. During the first few days the rats were trained in pairs in the reaching cages for a period of one half hour per day. Once reach training commenced, the animals were provided with 15 grams of rat chow daily following the training period. The rats were subsequently trained individually for one half hour per day and then at the end of a two- week training period their performance was videotaped for a five- minute interval. Each time the rat reached through the bars whether or not food was obtained was scored as a “reach” and each time food was successfully returned to the cage and consumed was scored as a “hit”. The percentage of hits to total reaches was then calculated for each animal’s taped performance.

2.6. ANATOMICAL METHODS

2.6.1. Western Blot

Brain tissue was removed from decapitated animals and placed on ice. Following rapid dissection of frontal cortex, brain samples were placed in microcentrifuge tubes cooled on dry ice. Brain samples were sonicated with 800 μ l of 1% SDS then aliquoted. All samples were held at -75°C until analysis. Samples were diluted 1/20 to determine protein concentration (Bradford Assay) before resolving the protein of interest on 8- 12% acrylamide gels (5 μ g of protein per well) using SDS-PAGE gel electrophoresis. Frozen skin samples were sonicated with 5mls of 1% SDS. The tissue was then removed and cut into .5 cm squares and resonicated in 5 mls of fresh 1% SDS. After the second sonication the sample was pooled and 1ml aliquots were made and stored frozen at -75C . Gels were

blotted on polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for non-specific binding for 1 hr. with 5% non-fat dry milk in Tris-buffered saline and .1% Tween 20. FGF-2 primary antibody (Santa Cruz #sc-7911) was diluted 1:1000 (flg antibody [Santa Cruz #sc-121; 1:1000], in the same solution as was used for blocking. Membranes were incubated in primary antibody for 2 hours followed by 5 washes in PBS (5 mins.) and 1 hour incubation in secondary antibody (HRP- linked donkey anti-rabbit; Amersham #NA934V; 1:5000). Following 5 more PBS washes, FGF-2 protein was revealed with an ECL+ detection kit from Amersham (#RPN2132) and the resulting image was captured on Hyperfilm ECL (Amersham #RPN1647K). Exposed film was imaged with a Kodak digital camera and the blot density was then analyzed using NIH Image software. The membrane was then stained with .1% Commassie Blue (OmniPure EM Science) to reveal all proteins in order to ensure pipetting consistency. A random protein band was selected for each blot and the amount of target protein was calculated as the density of the target / density of random sample protein in the same well. This method allowed compensation for any pipetting errors that may have occurred.

2.6.2. Histological Procedures

At the conclusion of behavioral testing the remaining animals were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline. The trimmed brains were weighed and then immersed whole in 20 mls of Golgi-Cox solution. The brains were then stored (in the dark) in the Golgi-Cox fixative for 14 days before being transferred to a solution of 30% sucrose for seven days. The tissue was

cut at 200 μm on a Vibratome™ then developed using a method described by Gibb and Kolb (1998).

2.6.3. Golgi-Cox Analysis

Layer III pyramidal cells in Zilles' area Par 1 were traced using a camera lucida at 250X. In order to be included in the data analysis, the dendritic trees of pyramidal cells had to fulfill the following criteria: (a) the cell had to be well impregnated and not obscured with blood vessels, astrocytes, or heavy clusters of dendrites from other cells; (b) the apical and basilar arborizations had to appear to be largely intact and visible in the plane of section. The cells were drawn and analyzed using a Sholl analysis for estimation of dendritic length (Sholl, 1956) was performed. For this analysis a transparent overlay of concentric circles spaced 20 μm apart was placed over the neuron drawing by centering the innermost ring in the middle of the cell body. The number of dendrite-ring intersections was counted for each ring and the total number used to estimate total dendritic length in μm (number of intersections X 20). Five cells were drawn in each hemisphere of each rat. The statistical analyses were done by taking the mean of the measurements on the five cells for each hemisphere of each subject.

Spine density was measured from one apical dendritic branch in the terminal tuft and one basilar terminal branch on five different cells per hemisphere. Spine density measures were made from a segment greater than 10 μm in length, and usually about 50 μm . The dendrite was traced (1000X) using a camera lucida and the exact length of the dendritic segment calculated by placing a thread along the drawing and then measuring the thread length. Spine density was expressed as the number of spines per 10 μm . No

attempt was made to correct for spines hidden beneath or above the dendritic segment so the spine density values are likely to underestimate the actual density of the dendritic spines.

2.7. STATISTICAL ANALYSES

All statistical analyses were ANOVA's performed on Statsview 5®. If an ANOVA did not show a significant effect of sex, the data were collapsed across this variable to increase the number of subjects per group and to simplify the analysis.

2.8. BEHAVIORAL RESULTS

2.8.1. Experiment 1: Morris Water Task

2.8.1.1. Latency. Animals with P4 frontal lesions took longer to locate the hidden platform in the water task than did sham operates. Post-operative tactile stimulation attenuated this impairment. A two-way ANOVA with lesion and treatment as factors showed a main effect of lesion ($F(1,66)=88.5, p<0.0001$), treatment ($F(1,66)=9.17, p=0.0035$), and the interaction ($F(1,66)=6.34, p=0.014$). The stroking treatment had a greater effect on the lesion animals than on the sham-operates giving rise to the interaction (Fig.2.1).

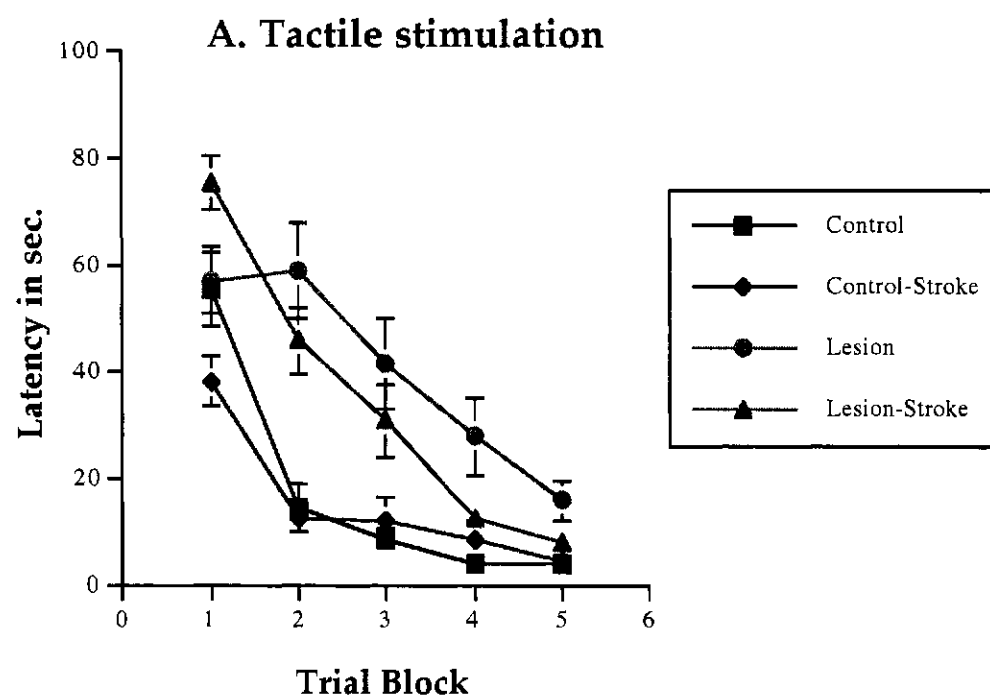


Fig. 2.1. Acquisition curve (latency to find the platform in seconds) for Experiment 1 over 5 test days.

2.8.1.2. Distance. Animals with P4 frontal lesions also swam further to find the platform than sham operates. Once again, tactile stimulation improved the performance of P4 lesion animals in this task (Fig. 2.2). A two-way ANOVA with lesion and treatment as factors showed a main effect of lesion on total swim distance ($F(1,42)=9.78, p<.005$), and a marginal effect of treatment ($F(1,42)=3.8, p<.06$). In addition, the Lesion by Treatment interaction was significant ($F(1,42)=7.5, p<.01$), reflecting the greater effect of the treatment on the performance of the lesion animals.

A. Tactile Stimulation

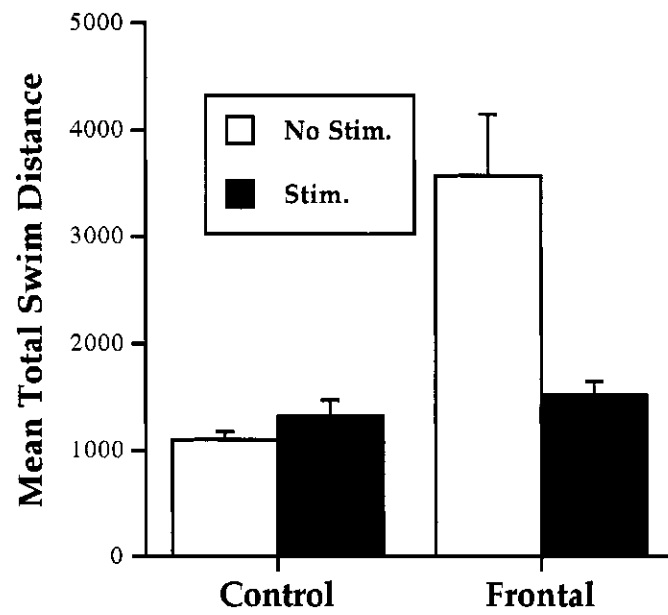


Fig.2.2. Experiment 1: Total swim distance over 5 test days.

Units are arbitrary computer units.

2.8.2. Experiment 2: Morris Water Task

2.8.2.1. Latency. Administration of FGF-2 improved spatial learning in the P4 operates.

A two-way ANOVA on escape latency with lesion and treatment as factors showed a marginal effect of lesion ($F(1,61)=3.47$, $p<.07$) but no main effect of treatment ($F(1,61)=2.25$, $p=.14$). The Lesion by Treatment interaction was significant ($F(1,61)=4.92$, $p<.05$) reflecting the improved performance of lesion animals following FGF-2 administration (Fig 2.3).

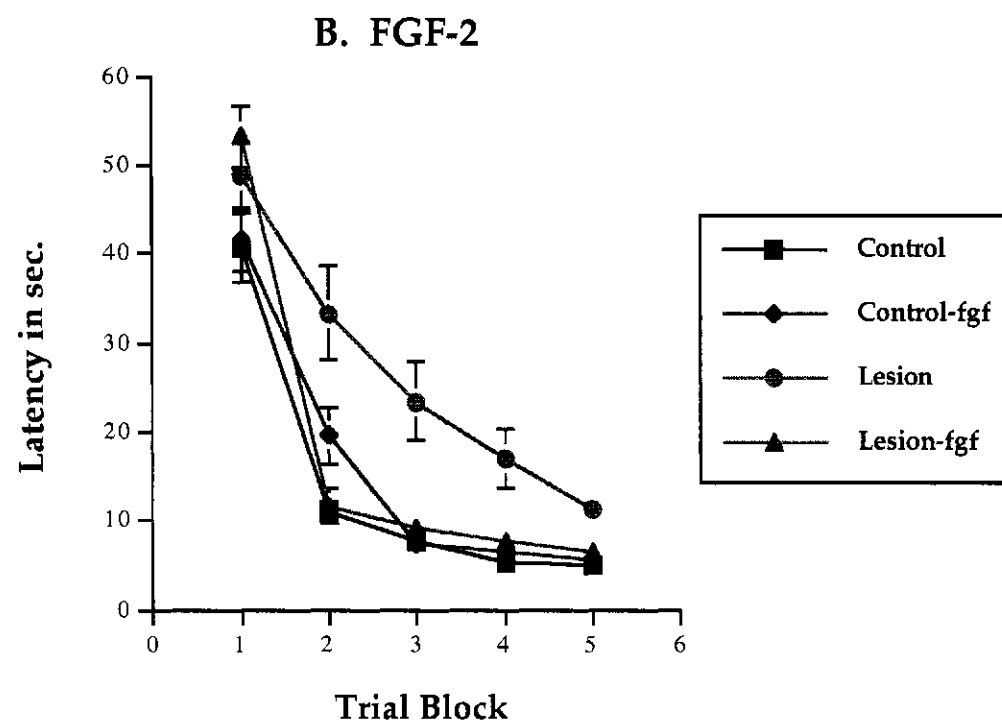


Fig. 2.3. Acquisition curve (latency in seconds) for Experiment 2 over 5 days of testing.

2.8.2.2. Distance. A two-way ANOVA on swim distance with lesion and treatment as factors showed a main effect of lesion ($F(1,50)=5.12, p=0.028$) but no main effect of treatment ($F(1,50)=2.58, p=0.115$) and a marginal interaction ($F(1,50)=3.52, p=0.066$). Once again the interaction reflected the improvement of the lesion animals that received FGF-2 administration (Fig. 2.4).

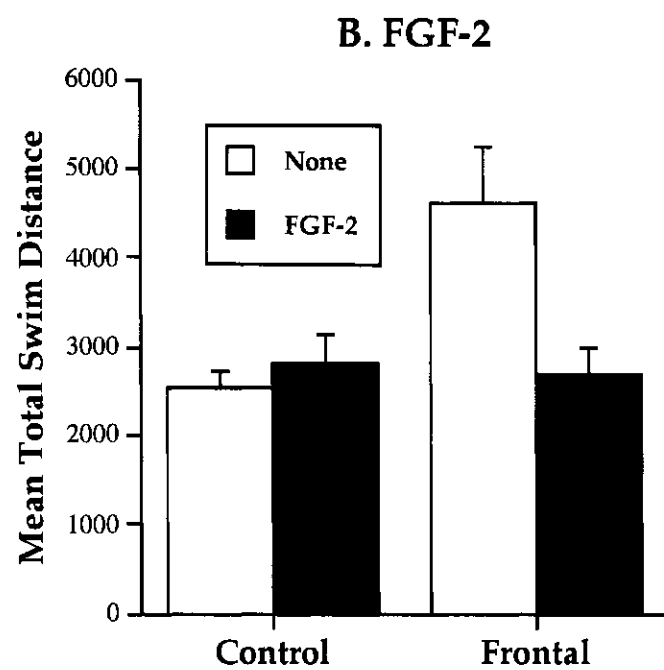


Fig. 2.4. Total swim distance for Experiment 2 in arbitrary computer units.

2.8.3. Experiment 1: Whishaw Tray Reaching

Lesion animals showed impairments in successfully retrieving pellets for consumption when compared to littermate controls. This impairment was reduced by tactile stimulation (Fig. 2.5). A three-way ANOVA performed with lesion, treatment, and sex as factors showed a significant main effect of lesion ($F(1,68)=89.5, p<.0001$), treatment ($F(1,68)=8.0, p<.0002$), and sex ($F(1,68)=5.9, p<.02$). None of the interactions were significant ($p's>.48$). Posthoc tests showed that both the lesion and control animals benefited significantly from the early experience and that overall, females were better at this test than males.

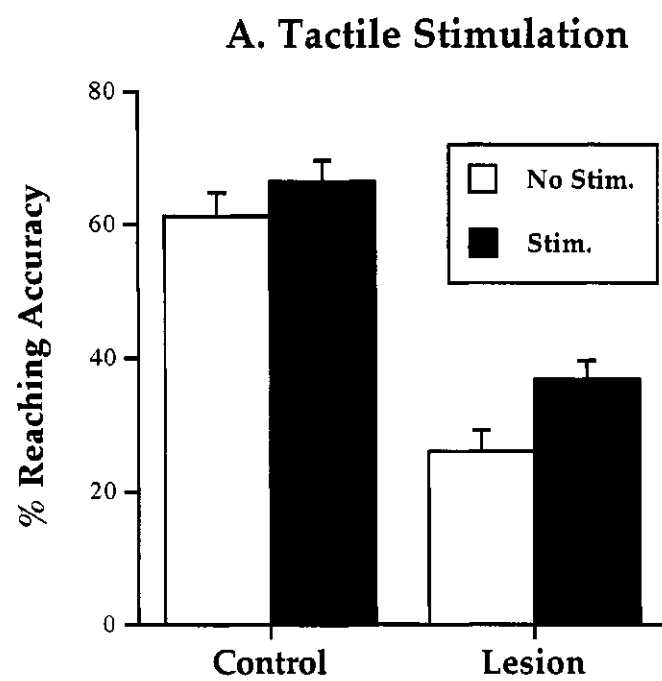


Fig. 2.5. Reaching for Experiment 1. Bars represent total number of successful reaches /total number of reaches.

2.8.4. Experiment 2: Whishaw Tray Reaching

Animals in the lesion group showed deficits in reaching performance compared to the sham-operates and treatment with FGF-2 did not reduce these deficits. Females performed better on the task than did males across all groups (Fig.2.6). A three-way ANOVA with lesion, treatment, and sex as factors showed a main effect for lesion ($F(1,95)=61.16, p<0.0001$) and sex ($F(1,95)=10.77, p=0.0014$), but not treatment ($F(1,95)=0.297, p=0.59$). None of the interactions were significant ($p's>0.21$).

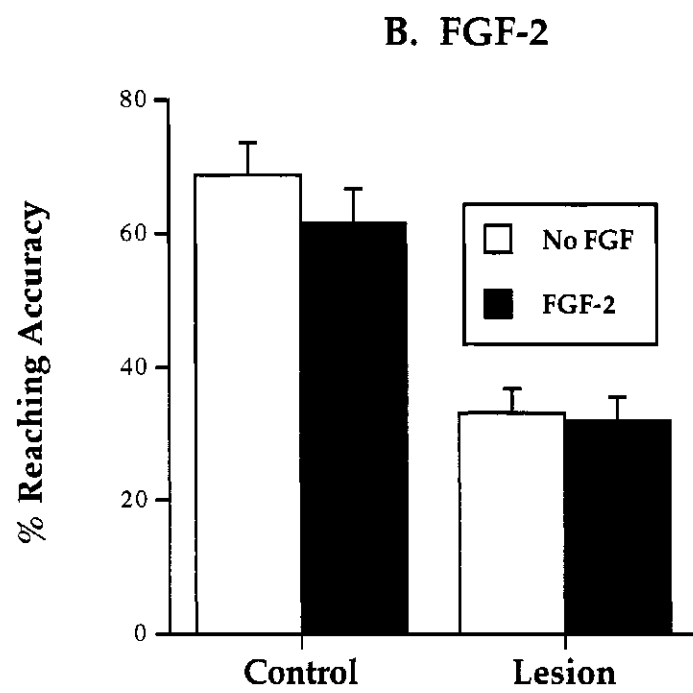


Fig. 2.6. Reaching for Experiment 2. Bars represent number of successful reaches/ total number of reaches.

2.9. ANATOMICAL RESULTS

The frontal lesions were similar in both experiments (Fig 2.7). The lesions removed the anterior midline cortical tissue including Zilles' areas Fr2, Cg1, and Cg3. The infralimbic cortex was spared as were the medial and ventral orbital regions. There was no direct damage to the striatum although the anterior striatum was visibly smaller than normal, likely because of the loss of the fibers of passage that would normally course through from the tissue that was removed. The lesions extended posterior to about the level of the septum but did not include the septum or fimbrial regions. For clarity, the anatomical results of Experiments 1 and 2 are described separately.



Fig. 2.7. Representative lesion for Experiment 1 and 2.

2.10. EXPERIMENT 1

2.10.1. Brain weight

Tactile stimulation increased brain weight in both sham and lesion animals (Table 2.1). A three-way ANOVA on brain weight with lesion, treatment, and sex as factors revealed significant main effects for each factor ($F(1,96)=116.2, p<.0001$; $F(1,96)=3.92, p=.05$; $F(1,96)=33.9, p<.0001$). None of the interactions were significant ($p's>.30$)

Table 2.1. Experiment 1: Summary of brain weight

<u>Group</u>	<u>Brain weight (Males)</u>	<u>Brain weight (Females)</u>
Sham	2.055±.03	1.883±.04
Sham + stimulation	2.080±.03	1.919±.03
Frontal	1.750±.03	1.637±.03
Frontal + stimulation	1.812±.04*	1.699±.02

Numbers represent means ± SEs. Brain weight (in gms) shown

*Differs significantly from same sex frontal group ($p < .05$)

2.10.2. Golgi Analysis

2.10.2.1. Dendritic length. Dendritic length was reduced in the apical tree by the lesion and in the basilar tree by the treatment (Table 2.2). A two-way ANOVA on apical length with lesion and treatment as factors found a significant main effect of lesion ($F(1,48)=6.3$, $p < .02$) but not treatment ($F(1,48)=0.74$, $p=0.4$). A two-way ANOVA on basilar length found a significant main effect of treatment ($F(1,48)=3.99$, $p=0.05$), but not lesion ($F(1,48)=0.507$, $p=0.48$). No interactions were significant on either tree ($p's > .4$).

Table 2.2. Experiment 1: Summary of dendritic measures

<u>Group</u>	<u>Apical Length</u>	<u>Basilar Length</u>
Sham	1658±34	1740±52
Sham + stimulation	1608±34	1632±44*
Frontal	1550±44	1700±52
Frontal + stimulation	1536±32	1598±56*

*Differs significantly from untreated sham or lesion group, respectively ($p < .05$).

2.10.2.2. Spine Density. The tactile stimulation treatment altered spine density in a qualitatively different manner in the sham and lesion animals: it was decreased in the sham operated and increased in the lesion animals. A two-way ANOVA (Lesion X Treatment) on spine density in the apical field of layer III pyramidal cells in parietal cortex revealed main effects of lesion ($F(1,184)=37.4$, $p<.0001$), stimulation ($F(1,184)=6.5$, $p<.01$), and the interaction ($F(1,184)=113.7$, $p<.0001$) (Table 2.3). Thus, in unstimulated animals the lesion reduced spine density whereas in the stimulated lesion animals there was an increase in spine density relative to control animals.

ANOVA (Lesion X Treatment) on spine density in the basilar field of layer III pyramidal cells in parietal cortex revealed no main effects of lesion or stimulation ($p's>.17$), but the interaction of Lesion by Treatment was significant ($F(1,184)=37.2$, $p<.0001$), again reflecting the differential effect of the treatment on the lesion animals.

Table 2.3. Experiment 1: Summary of Spine density

<u>Group</u>	<u>Apical Spines</u>	<u>Basilar Spines</u>
Sham	6.18±0.06	6.59±.05
Sham + stimulation	5.47±0.19*	5.90±.05*
Frontal	4.92±0.09	6.45±.09
Frontal + stimulation	6.13±0.02*	6.96±.03*

Numbers are spines per 10 μ m of dendritic length \pm SE

*Differs significantly from untreated sham or lesion group, respectively ($p<.05$).

2.10.3. Western Blot

Frontal cortex samples were harvested from control and lesion animals. In the control animals the sample included all cortical tissue anterior to +3.0 mm ahead of the

Bregma. In the lesion animals the sample was taken from approximately the same location but only included the remaining lateral cortex including Frontal area 3 (Fr3) and Agranular insular cortex (AID) according to the nomenclature used by Zilles (1985). The olfactory bulb region was discarded in all samples. Skin samples were taken from just below the neck to mid-torso along the back. Excess hair was quickly removed before the sample was frozen on dry ice. Both brain and skin samples were run to detect the expression of FGF-2 and its receptor FGFR1 (flg) in petted and non-petted animals (Table 2.4).

An ANOVA looking at the effects of treatment on protein expression of FGF-2 in skin samples of petted and non-petted animals revealed a significant main effect of treatment ($F(1,11)=5.37$, $p=0.041$). The petted animals had significantly more FGF-2 in their skin than did the untreated animals. An ANOVA examining flg levels in the skin showed no differences in the levels of this protein in petted or non-petted animals.

An ANOVA on FGF-2 levels in remaining frontal cortex of lesion animals showed a significant main effect of treatment ($F(1,6)=6.4$, $p=0.045$). FGF-2 levels were elevated in the petted animals. ANOVA on flg expression showed no differences in petted and non-petted rats ($F(1,12)=0.53$, $p=0.48$).

In parietal cortex there were no differences observed in FGF-2 expression. ANOVA showed no effect of treatment ($F(1,12)=0.024$, $p=0.88$). There was an increase in expression of the flg protein, however ($F(1,12)=4.99$, $p=0.047$).

Table 2.4. Summary of the findings from the Western Blot experiments.

Protein	Frontal Cortex	Parietal Cortex	Skin
FGF-2	↑	=	↑
FGFR1 (flg)	=	↑	=

Note: ↑ reflects a significant increase relative to non-stimulated controls, $p < .05$ or better
 = no significant change

2.11. EXPERIMENT 2

2.11.1. Brain Weight

In contrast to the effects of tactile stimulation, there was no significant effect of FGF-2 administration on brain weight (Table 2.5). A three-way ANOVA on brain weight with lesion, sex, and treatment as factors revealed significant main effects for lesion and sex but not treatment ($F(1,89)=110.2, p < .0001$; $F(1,89)=12.8, p = .0006$; $F(1,89)=.132, p = .72$). The Lesion X Sex X Treatment interaction was significant ($F(1,89)=5.3, p = .02$) and reflected a decrease in brain weight in the female control group following FGF administration.

Table 2.5. Experiment 2: Summary of brain weight

Group	Brain weight (Males)	Brain weight (Females)
Sham	1.929±.02	1.929±.03
Sham + FGF-2	1.972±.02	1.809±.02
Frontal	1.709±.05	1.596±.03
Frontal + FGF-2	1.704±.03	1.643±.03

Numbers represent means ± SEs. Brain weight (in gms) shown.

2.11.2 Golgi Analysis

2.11.2.1. Dendritic length. The P4 lesion reduced dendritic length in the apical tree as did the FGF-2. ANOVA on apical length with lesion and treatment as factors showed a significant effect of lesion ($F(1,80)=12.7$, $p=0.0006$) and treatment ($F(1,80)=15.9$, $p=0.0002$) but not the interaction (Table 2.6). Similar effects were seen in the basilar tree. A two-way ANOVA revealed a significant main effect of lesion ($F(1,80)=8.74$, $p=0.04$) and treatment ($F(1,80)=32.8$, $p<0.0001$) and a trend in the interaction ($F(1,80)=3.62$, $p=0.06$). The interaction reflected a reduction in dendritic length following P4 lesion in the untreated animals as compared to the increase in dendritic length observed in the FGF-2 treated animals.

Table 2.6. Experiment 2: Summary of dendritic measures

<u>Group</u>	<u>Apical Length</u>	<u>Basilar Length</u>
Sham	1325±76	1390±78
Sham + FGF-2	1252±67*	1357±88
Frontal	1388±56	1396±36
Frontal + FGF-2	1235±58*	1439±42

*Differs significantly from untreated sham or lesion group, respectively ($p<0.05$).

2.11.2.2. Spine Density. Treatment with FGF increased spine density (Table 2.7). A two-way ANOVA (lesion, treatment) on the apical tree showed no main effect for lesion ($F(1,80)=1.81$, $p=0.18$), or the interaction ($F(1,80)=1.16$, $p=0.28$) but there was a main effect of treatment ($F(1,80)=4.69$, $p=0.033$). Posthoc tests (Fisher's PLSD, $p<0.05$) showed a significant treatment effect in the lesion but not the control animals. In the

basilar tree ANOVA revealed no main effect of group ($F(1,88)=0.15$, $p=0.70$) or the interaction treatment ($F(1,80)=0.01$, $p=0.92$, but there was a main effect of treatment ($F(1,80)=4.62$, $p=0.035$). Posthoc tests showed that neither control nor lesion group differed significantly from their respective FGF-treated groups ($p>.10$).

Table 2.7. Experiment 2: Summary of Spine density

Group	Apical Spines	Basilar Spines
Sham	5.95±0.20	6.24±.15
Sham + FGF-2	6.17±0.20	6.65±.15
Frontal	6.00±0.29	6.10±.16
Frontal + FGF-2	6.68±0.16*	6.47±.18

Number of spines per 10µm dendritic length ± SE

*Differs significantly from untreated frontal group.

2.12. DISCUSSION

There were three novel findings of these studies. First, both the tactile stimulation and FGF-2 significantly reduced the behavioral impairments after cortical injury. Injury to the frontal region produced a marked deficit in behavioral performance on both tasks relative to sham-control animals and this deficit was significantly reduced by the tactile stimulation and by administration of FGF-2. In fact, the brain-injured rats showed such an improvement in performance that they were able to perform almost as well as control animals in the spatial navigation task. Skilled reaching was improved by tactile stimulation but not by FGF-2. This latter result may have been a problem of effective dose of FGF-2. In a recent study (Waite and Kolb, 2003) FGF-2 (10µg/kg) solution was

made fresh daily and a significant behavioral recovery was observed on skilled reaching. In the current study the FGF-2 was made fresh at the beginning of the treatment and then stored at 4° C. We must note, however, that although the tactile stimulation was beneficial, the lesion animals still showed significant impairments.

Second, tactile stimulation altered brain morphology and did so differently in sham and cortically injured animals. The overall findings were that: 1) tactile stimulation reduced dendritic length in the basilar field; and 2) frontal cortical lesions significantly reduced dendritic length in the apical field. The stimulation-related decline in dendritic length was unexpected but the lesion-related decline replicates our previous findings (Kolb and Gibb, 1990). FGF-2 administration also decreased dendritic length in both the apical and basilar fields. The frontal lesion also caused a reduction but in the basilar tree the reduction was less dramatic in the FGF-2 treated animals than in the untreated operates.

The analysis of dendritic spines led to an unexpected result: Sham-operated animals that received tactile stimulation had a significant decline in spine density in both the apical and basilar fields whereas frontal-operates with similar stimulation had a significant increase in spine density. Thus, a similar experience differentially affects the intact and injured brain. The reduction in spine density with early experience has precedents (Kolb et al., 2003) but the opposite effect of experience on the injured brain is a novel finding. A similar finding was noted in the FGF-2 experiment. Although FGF-2 did not affect spine density in the apical tree, in the basilar field there was trend for FGF-2 treatment to reduce spine density in controls and increase spine density in the frontal cortex operates.

Third, tactile stimulation increased the expression of FGF-2 in both the skin and brain. We hypothesized that the change in cortical morphology was related to an increase in one or more neurotrophic factors and in particular to FGF-2. FGF-2 is known to pass the blood-brain barrier and is made not only in the brain but also in the skin in response to injury or stimulation. We therefore used Western blotting to investigate the effects of tactile stimulation on FGF-2 expression in skin and cortex, and found an increase in both places in the stimulated groups (Fig. 2.3). The presence of an increase in FGF-2 expression in both skin and cortex of tactilely-stimulated rats, combined with the functional improvement and associated anatomical changes suggests that the behavioral treatment acted at least in part, through its actions on FGF-2 expression. The fact that direct administration of FGF-2 was also able to enhance functional outcome, although somewhat less effectively, is consistent with this hypothesis. It is well established that behavioral treatments can influence the production of neurotrophic factors (Carro et al., 2000; Berchtold et al., 2002; Vaynman et al., 2003). What has not previously been recognized, however, is that the same behavioral treatment can differentially affect the intact and injured brain. It is not immediately obvious why a neurotrophic factor treatment would have differential effects on the normal and injured brain but the injured brain presumably has altered regulation of many factors, including gene expression that could be affected by neurotrophic factors.

There is a rich behavioral literature showing that early experiences in infancy can permanently affect the brain and behavior of adult rats (Denenberg and Whimbey, 1963; Denenberg et al., 1967; Levine, 1967; Levine et al., 1967, Meaney et al., 1987; Meaney et al. 1988). The current study suggests that early intervention after cerebral injury is

especially powerful in influencing brain and behavioral development. The importance of early intervention after cortical injury cannot be underestimated. We have shown elsewhere that complex-environment versus cage housing can stimulate recovery after early frontal lesions but only if it begins at weaning (Kolb et al., 2003). In fact, even four months of complex rearing has a minimal affect upon recovery from early frontal lesions if it does not begin until adulthood. Furthermore, the magnitude of the effects observed in the current study after just two weeks of tactile stimulation are greater than those we have found after four months of complex rearing. We should note, however, that the addition of FGF-2 to complex housing experience has greater effects on functional recovery after cortical injury in adulthood (B. Kolb and A. Witt-La Jeunesse, unpublished observations). Perhaps the failure of behavioral therapies later in life to facilitate functional recovery is that they are not effective in sufficiently raising FGF-2 levels. Or, it seems equally likely that the tactile stimulation affected more than just FGF-2 and whatever other factors that are altered by the tactile stimulation may also be necessary to facilitate functional recovery.

Finally, we would be remiss if we did not address the question of whether our tactile stimulation treatment is related to its effects on stress. There is a large literature showing that exposure to stressful experiences, such as brief, repeated removal from the mother during development, can affect cognitive processes in adulthood and senescence (Meaney et al., 1987; 1988). We have shown, however, that such procedures have no beneficial effect on functional recovery from early injury and, in addition, may actually retard recovery (Gibb and Kolb, 2004b). Furthermore, such procedures also do not reverse the injury-related drop in spine density (Gibb and Kolb, 2004b). The tactile-

stimulation procedure in the current study may actually have reduced stress insofar as we noted that during the first week or so of tactile stimulation the animals typically entered into a sleep pattern that was punctuated by twitching, suggesting that the animals were in rapid eye movement sleep. It thus seems plausible to hypothesize that whereas brief periods of neonatal stress may be beneficial in the adult animal, it may not be advantageous to the animal suffering from perinatal brain injury.

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CHAPTER 3
EXPERIMENT 2

**NEONATAL HANDLING ALTERS BRAIN ORGANIZATION BUT DOES NOT
INFLUENCE RECOVERY FROM PERINATAL CORTICAL INJURY**

3.1. ABSTRACT

Rats given frontal cortex lesions in the first week of life show poor motor and cognitive recovery when tested as adults. These behavioral deficits are associated with decreased dendritic arbor and reduced spine density in remaining cortical pyramidal neurons. Postinjury tactile stimulation reverses both the behavioral and anatomical effects of the early lesions. Here we test the possibility that simply handling the animals postinjury might reduce the behavioral deficits and reverse the dendritic atrophy. Rats received medial frontal cortex removals on postnatal day 4 (P4) or sham surgeries. Beginning the day after surgery, rat pups were removed from the nest for 15 minutes 3 times per day until weaning. This procedure has been termed “handling” and postnatal handling has been shown to reduce cognitive decline in aging adult rats. At 60 days of age, animals were tested in the Morris Water Task and on skilled reaching. The “handled” animals showed no improvement in performance on either task. Following behavioral testing, brains were weighed and prepared for Golgi analysis. P4 handled male operates showed a decline in brain weight compared to untreated animals but there were no other differences observed. A subset of animals was perfused with Lana’s fix to prepare sections for Cresyl violet and acetylcholinesterase (AChE) stain. Cortical thickness, and thalamic cross-sectional area were measured on Cresyl violet stained sections. Cortical thickness and thalamic area was increased in sham-operates following handling. P4 operates showed no changes in cortical thickness and a reduction in thalamic area following the handling treatment. Relative density was measured on AChE stained sections but no differences were observed between treatment groups. Pyramidal cells were drawn from layer 3 in parietal cortex. Postnatal handling led to a decrease in

dendritic length in the both sham and operated animals but there were no changes in spine density. Handling did increase cortical thickness and thalamic size, however, in sham operates. The absence of handling-related effects on functional recovery after perinatal injury suggests that the beneficial effects of tactile stimulation do not result from handling alone.

3.2. INTRODUCTION

The procedure known as “handling” wherein newborn rat pups are separated from their mothers for brief periods of time was first described in the 1950’s (Denenberg and Karras, 1959; Levine and Lewis, 1959). This experimental paradigm showed that early separation of pups and mother had lifelong consequences in the emotional reactivity of the pups and altered the physiology of the hypothalamic-adrenal-pituitary axis (HPA) and its function (Denenberg et al., 1967). Rats given handling treatment were reported to have superior avoidance learning (Levine and Lewis, 1959), higher body weights (Denenberg and Karas, 1959), and superior cognitive performance (Lehmann et al., 2002) in adulthood, as well as a lack of cognitive decline in senescence (Meaney et al, 1988). Handling causes a variety of physiological changes including: changes in plasma levels of norepinephrine and epinephrine (McCarty et al., 1981), increased hippocampal glucocorticoid (GR) expression (Meaney et al., 1989), increased hippocampal nerve growth factor (NGF) expression (Pham et al., 1997), reduced cell number and volume in the locus coeruleus (Lucion et al, 2003), and increased expression of immature gamma-aminobutyric acid type A receptors in hippocampal neurons (Hsu et al., 2000).

The handling procedure has been described as therapeutic in reversing behavioral abnormalities induced by prenatal stress (Wakshlak and Weinstock, 1989) and prenatal exposure to alcohol (Lee and Rabe, 1998). I wondered if the handling procedure might also be therapeutic in reversing the behavioral and anatomical deficits observed in rats following perinatal cortical injury. One previous study (Chou et al., 2001) showed that handling was of some benefit to rats with unilateral hypoxia/ischemia at 7 days of age, which led us to suspect that the same treatment might benefit rats with frontal cortical injuries on day 4. Rats were given postnatal day 4 (P4) medial frontal cortex removals and were then removed from the maternal cage for 15-minute periods, three times per day from postoperative day 1 until weaning. The animals were allowed to mature to adulthood before commencement of behavioral testing on the Morris water task, skilled reaching, and circadian activity. After behavioral testing, the brains were processed for Golgi-Cox analysis.

3.3. SUBJECTS AND HOUSING PROCEDURES

Four litters of Long-Evans rats were handled (10 male frontal, 11 male control, 12 female frontal, 14 female control) and three litters were without treatment (10 male frontal, 10 male control, 6 female frontal, 7 female control). Animals in the handled group were removed from the nest and placed in a plexiglas cage in a behavioral testing room away from the main animal colony, for 15 minutes three times per day. Mothers of the handled animals were offered food treats when the pups were removed from the nest and again when the pups were returned. The pups received no further stimulation after weaning. Mothers of the untreated animals were offered food treats at similar intervals as

the mothers of the handled animals. The animals were housed in accordance with the regulations of the Canadian Council of Animal Care.

3.4. SURGERY

On postnatal day 4 (P4) the pups were removed from the nest and cooled in a Thermanon® cooling chamber until their core temperature reached approximately 20°C. The lesion animals had their scalp opened then the frontal bone carefully removed after it was incised with iris scissors. The medial frontal cortex was then removed bilaterally with gentle aspiration. The tissue targeted for removal was the medial subfield of the prefrontal cortex including Zilles (1985) regions Cg1, Cg3, and PL as well as the medial portion of Fr2 of the motor cortex. After aspiration of the cortical tissue, the animals' scalp was sutured with silk thread drawn by a very fine needle. The remaining control animals underwent a sham surgical procedure in which the scalp was opened and then sutured closed but the skull was not removed. These animals were identified by removal of the tip of the outer toe on their right rear foot.

3.5. BEHAVIORAL METHODS

3.5.1. Morris Water Task

Beginning at P60 animals were trained on the Morris Water Task using a similar procedure to that described by Sutherland et al. (1983) based on the original task described by Morris (1981). The maze consisted of a circular pool (1.5 m diameter X 0.5 m deep) with smooth white walls. The pool was filled with approximately 25 °C water mixed with 500 ml of skim milk powder, used to render the water opaque. A clear

plexiglas platform (11 X 12 cm) was placed in a constant position inside the pool approximately 30 cm from the pool wall. The water level was adjusted so that the platform was invisible to a viewer outside the pool and to a rat swimming in the water. A trial consisted of placing a rat into the water facing the pool edge at one of four compass locations (north, south, east, or west) around the pool's perimeter. Within a block of four trials each rat started at the four locations in random sequence, and each rat was tested for four trials a day over five consecutive days. If on a particular trial a rat found the platform, it was permitted to remain on it for 10 seconds. A trial was terminated if the rat failed to find the platform after 90 seconds. Each rat was returned to its holding cage for approximately five minutes before the next trial commenced. The swim path for each rat on every trial was recorded using a Poly Track video tracking system (San Diego Instruments) which tracks the swim path and records the latency, distance and dwell time within each quadrant.

3.5.2. Whishaw Tray Reaching

Following water maze training, animals were trained in a skilled reaching task developed by Whishaw et al. (1991). In this task rats were trained to retrieve chicken feed through metal bars at the front of the plexiglas training cage (28 cm deep x 20 cm wide x 25 cm high). The front of each cage was constructed with 2 mm bars separated from each other by 1 cm, edge to edge and the floor was constructed of wire mesh. A tray (5 cm deep x 2 cm wide x 1 cm high) containing chicken feed pellets was mounted in the front of each cage. To obtain food, the rats had to extend their forelimbs through the bars, grasp, and retract the food pellet. The food tray was mounted on runners to adjust

the distance of the food from the bars. Distance adjustment ensured that each rat could not simply rake the food into the cage. Any pellets that the rat dropped inside the cage were irretrievably lost through the mesh on the floor and the animal would have to reach again. During the first few days the rats were trained in pairs in the reaching cages for a period of one half hour per day. Once reach training commenced, the animals were provided with 15 grams of rat chow daily following the training period. The rats were subsequently trained individually for one half hour per day and then at the end of a two-week training period their performance was videotaped for a five-minute interval. Each time the rat reached through the bars whether or not food was obtained was scored as a "reach" and each time food was successfully returned to the cage and consumed was scored as a "hit". The percentage of hits to total reaches was then calculated for each animal's taped performance.

3.5.3. Circadian Activity

Following reach training the animals were placed in computer monitored circadian activity cages. These cages were designed to assess activity over a 24-hour period by monitoring motion in cages fitted with infrared light beams and detectors. Each time the animal disrupted the light beam the computer recorded the side of the cage at which the activity occurred and a combined activity (for activity occurring across both the left and right sides of the cage) was also computed. The animals occupied the activity cages for two consecutive 24-hour periods and their combined activity for the second day was analyzed. During activity monitoring the animals had ad lib access to both food and

water and were maintained on a 12:12 hr light/dark cycle. The time the animals were placed in the cages was noted and the subsequent analyses were matched for time of day.

3.6. ANATOMICAL METHODS

3.6.1. Histological Procedures

At approximately 30 days of age a subset of animals representing all the groups were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline in 0.1 M phosphate buffer (pH 7.2) followed by a solution of 4% paraformaldehyde and 11% picric acid in 0.1 M phosphate buffer (Lana's Fixative). The brains were then removed from the skull and trimmed by cutting the olfactory bulbs approximately 5 mm ahead of the frontal edge of the cortex and the optic nerves 2 mm ahead of the optic chiasm. The pineal body and paraflocculi were removed and the spinal cord cut even with the posterior edge of the cerebellum. The trimmed brains were weighed and then postfixed in the Lana's solution at 4° C for 24 hours before cutting at 50 μ m on a Vibratome™. Five consecutive sets of tissue were saved and one was mounted immediately for acetylcholinesterase histochemistry. Three sets were saved for immunohistochemical staining and the final set was mounted and processed for Cresyl violet staining.

At the conclusion of behavioral testing the remaining animals were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline. The trimmed brains were weighed and then immersed whole in 20 mls of Golgi-Cox solution. The brains were then stored (in the dark) in the Golgi-Cox fixative for 14 days before being transferred to a solution of 30% sucrose for seven days. The tissue was

cut at 200 μm on a Vibratome™ then developed using a method described by Gibb and Kolb (1998).

3.7. ANATOMICAL ANALYSES

3.7.1. Cortical thickness measurements

Cortical thickness measurements were obtained from Cresyl violet stained coronal sections projected on a Zeiss-Jena MF2 projector at a magnification of 20X (following the method described by Stewart and Kolb, 1988). Briefly, three cortical measures were made at points medial, central and lateral on five sections of tissue identified by the following landmarks; Plane 1: first caudate-putamen visible, Plane 2: anterior commissure, Plane 3: first hippocampal section, Plane 4: posterior commissure, Plane 5: last hippocampal section. A plastic metric ruler was used to measure from the edge of the cortex to the edge of the white matter. An average for each plane and for each animal was calculated and used for statistical comparison.

3.7.2. Assessment of Thalamic Size

Thalamic cross-sectional area was measured from two coronal sections stained with Cresyl violet using a Kodak digital camera to capture the image and the Scion Image program to measure thalamic area. One measure was taken of the anterior thalamus (approximately -1.80mm from the Bregma). The second measure was made in posterior thalamus at approximately -4.30mm from the Bregma (as described in a study by Kolb and Whishaw (1981)).

3.7.3. Acetylcholinesterase Quantification

Acetylcholinesterase (AChE) staining was assessed using the Scion Image densitometry program. The tissue was placed on a Zeiss microscope using a 20X objective and the image captured on computer with a video camera. The lighting levels for photography were held constant through the entire sampling period to minimize any variations in density measures. The same planes as were used for cortical thickness measurements were analyzed for AChE density. An average density was then computed for each plane for each animal and used for statistical comparison.

3.7.4. Golgi-Cox Analysis

Layer III pyramidal cells in Zilles' area Par 1 were traced using a camera lucida at 250X. In order to be included in the data analysis, the dendritic trees of pyramidal cells had to fulfill the following criteria: (a) the cell had to be well impregnated and not obscured with blood vessels, astrocytes, or heavy clusters of dendrites from other cells; (b) the apical and basilar arborizations had to appear to be largely intact and visible in the plane of section. The cells were drawn and analyzed using two different procedures. In the first, each branch segment was counted and summarized by branch order using the procedure of Coleman and Riesen (1969). Branch order was determined for the basilar dendrites such that branches originating at the cell body were first order; after one bifurcation, second order; and so on. Branch order was determined for the apical dendrites such that branches originating from the primary apical dendrite were first order and so on. In the second, a Sholl analysis for estimation of dendritic length (Sholl, 1956) was performed. For this analysis a transparent overlay of concentric circles spaced 20

μm apart was placed over the neuron drawing by centering the innermost ring in the middle of the cell body. The number of dendrite-ring intersections was counted for each ring and the total number used to estimate total dendritic length in μm (number of intersections X 20). Five cells were drawn in each hemisphere of each rat. The statistical analyses were done by taking the mean of the measurements on the five cells for each hemisphere of each subject.

Spine density was measured from one apical dendritic branch in the terminal tuft and one basilar terminal branch. Spine density measures were made from a segment greater than 10 μm in length, and usually about 50 μm . The dendrite was traced (1000X) using a camera lucida and the exact length of the dendritic segment calculated by placing a thread along the drawing and then measuring the thread length. Spine density was expressed as the number of spines per 10 μm . No attempt was made to correct for spines hidden beneath or above the dendritic segment so the spine density values are likely to underestimate the actual density of the dendritic spines.

3.8. STATISTICAL ANALYSES

All statistical analyses were ANOVA's performed on Statsview 5®. If an ANOVA did not show a significant effect of sex, the data were collapsed across this variable to increase the number of subjects per group and to simplify the analysis.

3.9. BEHAVIORAL RESULTS

3.9.1. Morris Water Task

3.9.1.1. Latency. Animals with P4 frontal lesions showed significant impairments in their latency to find the platform in the water maze. Animals with lesion and handling showed no behavioral recovery on the performance of this task (Fig. 3.1). A two-way ANOVA with lesion and treatment as factors showed a significant main effect of lesion ($F(1, 41)=16.8, p=0.0002$), but not treatment ($F(1,41)=0.16, p=0.69$), or the interaction ($F(1, 41)=0.19, p=0.67$).

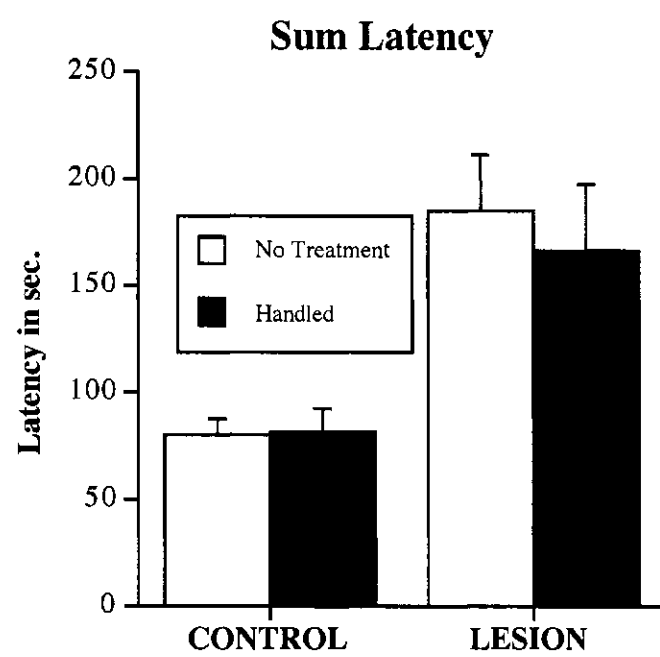


Fig. 3.1. Sum average latency (in seconds) for the untreated and handled animals.

3.9.1.2. Distance. Distance swum to find the platform was higher in P4 lesion animals than in sham-operates and again, handling was without benefit (Fig 3.2). A two-way ANOVA with lesion and treatment as factors showed no main effect of group ($F(1,$

39)=14.6, $p=0.0005$), treatment ($F(1,39)=1.19$, $p=0.28$), or the interaction ($F(1,39)=1.41$, $p=0.24$). We note, however, that as seen in Figure 2, there was a trend for the handled lesion animals to benefit from the treatment. A post hoc comparison of those two groups was marginally significant ($p=0.058$).

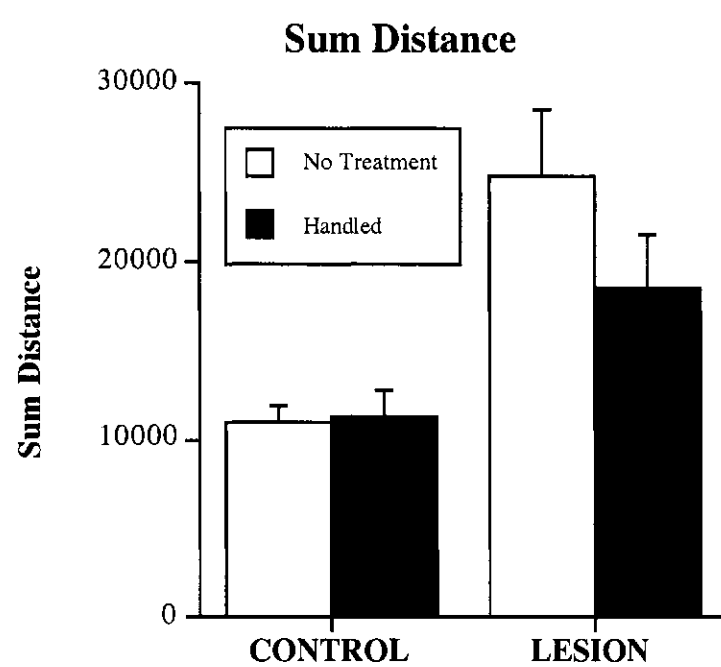


Fig. 3.2. Swim distance summed over 5 test days
(Arbitrary computer units)

3.9.2. Whishaw Tray Reaching

Animals with P4 frontal cortex removals were impaired in their performance on the skilled reaching task and handling did not attenuate these deficits in lesion animals although handled female controls performed better than untreated female controls (Fig. 3.3). A three-way ANOVA with lesion, sex, and treatment as factors showed a main effect of group ($F(1,33)=64.4$, $p<0.0001$), and sex ($F(1,33)=4.6$, $p=0.40$), but not treatment ($F(1, 33)=0.07$, $p=0.79$). None of the interactions reached significance

(F 's<2.2, p 's>0.15). Post hoc analysis using Fisher's PLSD revealed a significant difference between handled and untreated female control animals ($p=0.003$).

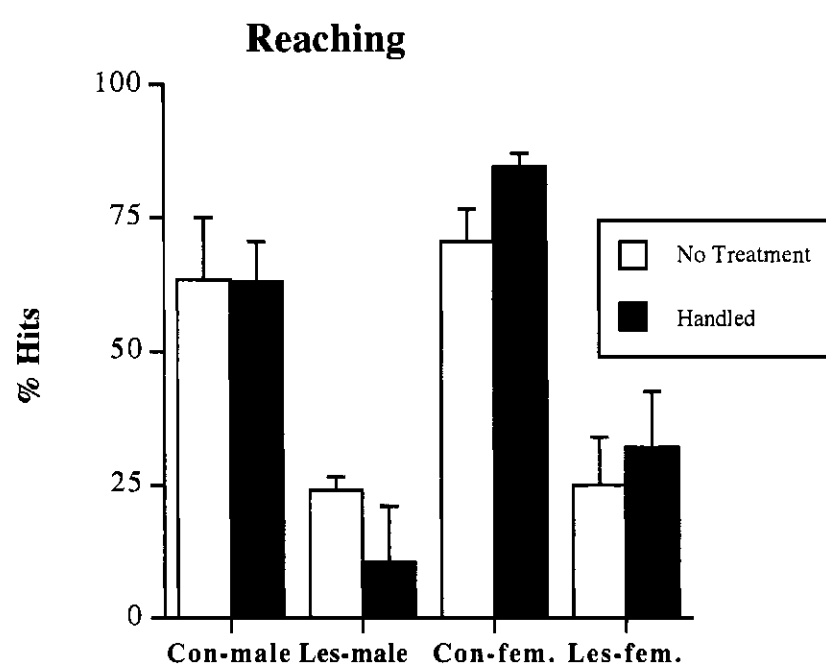


Fig. 3.3. Reaching performance of male and female handled animals.

% Hits represents total successful reaches/ total attempts.

3.9.3. Circadian Activity

An assessment of activity over the 24-hour light/dark cycle showed that lesion animals were generally less active than control animals except for untreated lesion females. This group had the highest overall level of activity. Handled animals were less active than untreated animals, especially in the lesion groups (Fig. 3.4). A three-way ANOVA with lesion, sex and treatment as factors revealed a main effect of lesion ($F(1, 34)=4.98, p=0.03$) and treatment ($F(1, 34)=4.44, p=0.04$), but not sex ($F(1,34)=2.43, p=0.13$). The Lesion by Sex interaction was significant ($F(1,34)=8.00, p=0.008$) and

reflected the tendency for control males to be more active than control females, and lesion females to be more active than lesion males.

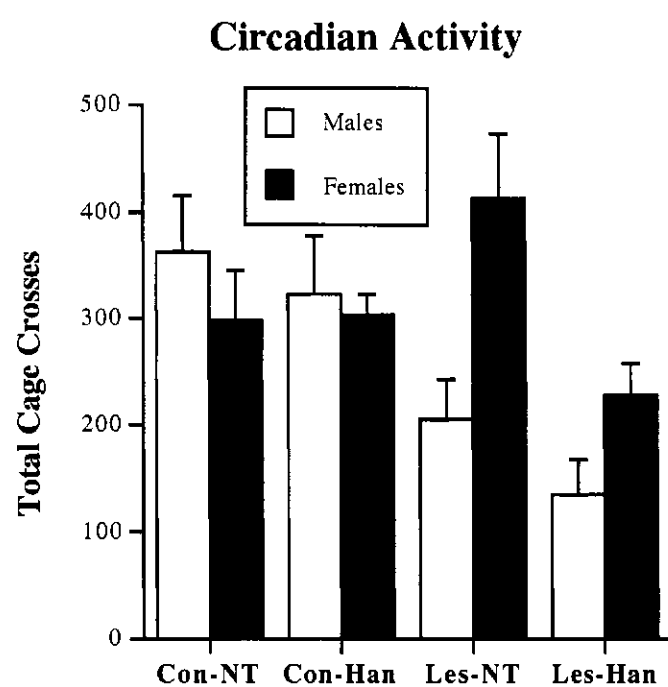


Fig. 3.4. Circadian Activity of male and female handled animals.

Bars represent total number of cage crosses in a 24-hour period.

3.10. ANATOMICAL RESULTS

3.10.1. Body Weight

Lesion animals have reduced body weight compared to sham-operates and females are smaller than males. There was no significant effect observed of handling on body weight although the handled controls showed a trend for increased body weight compared to the non-handled controls (Table 3.1). A three-way ANOVA with lesion, sex, and treatment as factors showed a main effect of lesion ($F(1, 37)=5.11, p=0.03$) and sex ($F(1, 37)=68.52, p<0.0001$), but not treatment ($F(1, 37)=0.92, p=0.34$). None of the interactions were significant ($p's >0.1$).

Table 3.1. Summary of body weights

Group	Experience	
	Handle	No Treatment
Con. Males	400.4±12.1	362.6±7.34
P4 Males	335.7±6.4	357.8±22.4
Con. Females	279.5±19.3	274.6±18.0
P4 Females	271.4±11.4	247.4±11.4

Numbers refer to means ±standard errors in grams

3.10.2. Brain Weight

Frontal lesion animals had lighter brains than control animals but sex and treatment had no effect on brain weight (Table 3.2). A three-way ANOVA with lesion, sex, and treatment as factors showed a main effect of lesion ($F(1,37)=53.5, p<0.0001$), but not sex ($F(1,37)=0.71, p=0.41$) or treatment ($F(1,37)=0.19, p=0.66$). None of the interactions were significant (p 's >0.1).

Table 3.2. Summary of brain weight

Group	Experience	
	Handle	No Treatment
Con. Males	2.009±0.027	1.958±0.007
P4 Males	1.577±0.066*	1.743±0.054
Con. Females	1.951±0.012	1.900±0.064
P4 Females	1.647±0.069	1.652±0.053

Numbers refer to means ±standard errors in grams

*differs significantly from No Treatment values in same lesion group

3.10.3. Cortical Thickness

To determine regional effects of lesion and treatment the analysis of cortical thickness was divided into 2 groups of cortical planes. The first group included anterior planes 1 and 2, and the second group included posterior planes 3-5. Handling increased cortical thickness in the anterior planes whereas lesion decreased it (Table 3.3). A two-way ANOVA with lesion and treatment as factors showed a significant main effect for both ($F(1,80)=12.96, p=0.0005$) ($F(1,80)=132.6, p<0.0001$). The interaction was not significant ($F(1,80)=2.14, p=0.15$). Similar effects were observed for the posterior planes. A two-way ANOVA revealed a significant main effect of lesion ($F(1,80)=8.45, p=0.005$) and treatment ($F(1,80)=52.7, p<0.0001$), but not the interaction ($F(1,80)=0.23, p=0.63$).

Table 3.3 Summary of Cortical Thickness at Anterior and Posterior Planes

Planes	Group	Experience	
		Handle	No Treat.
1-2	Con	42.5±0.4*	41.3±0.4
	P4	37.0±0.8	34.2±0.5
3-5	Con	35.4±0.2*	34.1±0.4
	P4	32.4±0.6	31.4±0.4

Numbers refer means± standard errors in mm.

*differs significantly from No Treatment values in same lesion group

3.10.4 Acetylcholinesterase Quantification

There was no effect of lesion on acetylcholinesterase (AChE) expression within the handled group ($F(1,20)=3.3$, $p=0.080$), and no difference between handled and untreated sham-operates in AChE expression (Table 3.4). A repeated measures ANOVA across all planes with treatment as a factor showed no main effect of treatment ($F(1,182)=0.12$, $p=0.91$) and no interaction with plane of measure ($F(14,182)=0.83$, $p=0.63$).

Table 3.4. Summary of AchE Density Measures

Plane	Percent Untreated Control
1	100.7
2	100.3
3	99.9
4	99.5
5	100.7

Numbers refer to % untreated control values obtained at the same plane

3.10.5. Thalamic Measures

The P4 lesion caused a reduction in thalamic area at both anterior and posterior planes. The handling treatment increased thalamic area at both planes in sham-operates only. This treatment was without effect in the lesion animals (Table 3.5). A two-way ANOVA with lesion and treatment as factors revealed a significant main effect of lesion ($F(1, 35)=39.4, p<0.0001$) but not treatment ($F(1,35)=1.65, p=0.21$) at the anterior thalamus. The Lesion by Treatment interaction was significant ($F(1,35)=9.33, p=0.004$) and reflected the observation that thalamic area was enlarged in handled controls but remained unchanged in handled lesion animals. Post hoc analysis showed the handling effect to be significant ($p=0.003$). At the posterior thalamic plane, a two-way ANOVA revealed a significant effect of lesion ($F(1,35)=43.7, p<0.0001$), treatment ($F(1,35)=17.5, p=0.0002$), and the interaction ($F(1,35)=30.4, p<0.0001$). Again, the lesion reduced thalamic area and handling increased it in the control group only.

Table 3.5. Summary of Thalamic Area in Anterior (A) and Posterior (P) Planes

Plane	Group	Experience	
		Handle	No Treatment
A	Control	115.9*	100.0
A	P4 lesion	81.7*	88.2*
P	Control	134.0*	100.0
P	P4 lesion	91.7	94.1

Numbers refer to percent of No Treatment control values

*differs significantly from No Treatment control value

3.10.6. Golgi Analyses

3.10.6.1. Branch Order

3.10.6.1.1. Apical Dendrites. In order to gain a better understanding of where changes in neuronal morphology might be occurring, the branch order analysis was divided into two areas: branching near the soma (branches 1-3) and branching near the terminal fields of the dendrites (branches 4-6+). In the proximal branches in the apical tree, both lesion and treatment decreased the number of branches (Table 3.6). A two-way ANOVA with lesion and treatment as factors showed a main effect of lesion ($F(1,70)=15.7, p=0.0002$) and treatment ($F(1,70)=22.6, p<0.0001$). The interaction was not significant ($F(1,70)=0.81, p=0.37$).

In the distal branches on the apical tree a two-way ANOVA showed that there was no effect of group ($F(1,70)=0.6$, $p=0.45$), but there was a main effect of treatment ($F(1,70)=6.5$, $p=0.01$) and the interaction ($F(1,70)=9.7$, $p=0.003$). The interaction reflected the tendency for the branch number to be reduced in the handled controls but increased in the handled lesion animals.

Table 3.6. Summary of Branch Order in the Apical Tree

Branches	Group	Experience	
		Handle	No Treat.
1-3	Con	19.7±0.7*	22.8±1.1
	P4	15.9±0.6	20.4±0.7
4-6+	Con	2.1±0.4*	2.3±0.4
	P4	3.3±0.3*	1.6±0.2

Numbers refer number of branches±standard errors

*differs significantly from No Treatment values in same lesion group

3.10.6.1.2. Basilar Dendrites. In the basilar tree the handling caused a reduction in the number of branches near the soma (1-3) and the lesion produced no significant effects (Table 3.7). A two-way ANOVA revealed no effect of lesion ($F(1,70)=0.8$, $p=0.4$), but a main effect of treatment ($F(1,70)=5.4$, $p=0.023$). The interaction was not significant ($F(1,70)=0.35$, $p=0.55$).

In the distal branches of the apical tree, the lesion caused a decrease in the number of branches and the treatment did as well. A two-way ANOVA with lesion and treatment as factors showed a main effect of lesion ($F(1,70)=9.8, p=0.003$) and treatment ($F(1,70)=37.6, p<0.0001$). The interaction was not significant ($F(1,70)=2.3, p=0.13$).

Table 3.7. Summary of Branch Order in the Basilar Tree

Branches	Group	Experience	
		Handle	No Treat.
1-3	Con	21.3±0.5	22.6±1.4
	P4	21.5±0.4*	23.7±0.7
4-6+	Con	9.3±0.7*	13.8±1.8
	P4	4.6±0.5*	12.3±1.2

Numbers refer number of branches± standard errors

*differs significantly from No Treatment values in same lesion group

3.10.6.2. Dendritic Length

3.10.6.2.1. Apical Dendrites. The Sholl analysis of dendritic length was divided into 3 ranges. The first one covered rings 1-6 (dendrite crossings near the soma). The second covered rings 7-11 or ring crossings that occurred in the central portion of the apical tree and the third covered the last rings (12-16+). The third range allowed analysis in the terminal portions of the dendritic tree. Handling and lesion reduced dendritic length near the soma. In the proximal portion of the apical tree both lesion and handling reduced the

branch length. A two-way ANOVA (rings 1-6) with lesion and treatment as factors showed a main effect of lesion ($F(1,76)=25.9$, $p<0.0001$) and treatment ($F(1,76)=19.2$, $p<0.0001$), but not the interaction ($F(1,76)=1.15$, $p=0.29$).

There were no effects of either lesion or treatment on the branch length in the middle portion of the apical tree. A two-way ANOVA (rings 7-11) showed no significance of lesion ($F(1,76)=1.8$, $p=0.18$), treatment ($F(1,76)=0.17$, $p=0.68$), or the interaction ($F(1,76)=0.06$, $p=0.81$).

In the terminal portion of the apical tree, handling reduced branch length but there was no longer an effect of lesion (Table 3.8). A two-way ANOVA showed no main effect of lesion ($F(1,76)=0.24$, $p=0.6$). The treatment effect was significant ($F(1,76)=7.5$, $p=0.008$) and the interaction was also significant ($F(1,76)=3.9$, $p=0.05$). The interaction reflected the effect of handling to reduce the length in terminal branches in sham-operates but not in the lesion animals.

Table 3.8. Summary of Sholl Analysis in the Apical Tree

Rings	Experience		
	Group	Handle	No Treat.
1-6	Con	27.1±0.7*	30.1±0.9
	P4	21.5±1.0*	26.5±1.2
7-11	Con	15.6±0.6	15.5±0.7
	P4	14.9±0.6	14.5±0.6
11-16+	Con	28.1±1.2*	35.8±2.2
	P4	30.5±1.3	31.8±1.7

Numbers refer number of ring crossings± standard errors

*differs significantly from No Treatment control values

3.10.6.2.2. Basilar Dendrites. In the basilar field the Sholl analysis was performed for rings 1-6 and rings 7-11 as the basilar dendrites were not as long as were the apical dendrites. On the proximal portion of the basilar tree (rings 1-6) both handling and lesion reduced dendritic length (Table 3.9). A two-way ANOVA with lesion and treatment as factors showed a significant main effect of lesion ($F(1,76)=14.5, p=0.0003$) and treatment ($F(1,76)=60.1, p<0.0001$), but not the interaction ($F(1,76)=0.7, p=0.40$).

Table 3.9. Summary of Sholl Analysis in the Basilar Tree

Rings	Group	Experience	
		Handle	No Treat.
1-4	Con	47.0±0.8*	63.1±3.1
	P4	41.5±4.0*	54.5±2.4
5-12	Con	26.6±1.5*	35.6±2.8
	P4	24.5±1.3	27.5±2.2

Numbers refer to number of ring crossings±standard errors

*differs significantly from No Treatment control values

3.10.6.3. Spines

3.10.6.3.1. Apical Dendrites. There was no effect of lesion or treatment on spine density in the apical field (Table 3.10). A two-way ANOVA with lesion and treatment as factors showed no main effect of sex ($F(1,74)=0.47$, $p=0.50$) or treatment ($F(1,74)=1.506$, $p=0.22$). The interaction was also not significant ($F(1,74)=1.0$, $p=0.32$).

Table 3.10. Summary of Spine Density on the Apical Terminal

Group	Experience	
	Handle	No Treatment
Control	5.98±0.24	6.03±0.21
P4 lesion	5.91±0.21	6.42±0.24

Numbers refer to number of spines/10µm dendritic branch length

*differs significantly from No Treatment control values

3.10.6.3.2. Basilar Dendrites. There was no effect of lesion or handling on spine density in the basilar tree (Table 9). A two-way ANOVA showed no main effect of lesion ($F(1,66)=0.75$, $p=0.39$) or treatment ($F(1,66)=1.75$, $p=0.19$) and the interaction was also non-significant ($F(1,66)=0.03$, $p=0.86$).

Table 3.11 Summary of Spine Density on the Basilar

Group	Terminal Experience	
	Handle	No Treatment
Control	6.54±0.22	6.41±0.18
P4 lesion	6.33±0.21	6.10±0.18

Numbers refer to number of spines/10µm dendritic branch length

*differs significantly from No Treatment control values

3.10. DISCUSSION

There were 4 principal findings in this experiment. First, there was no significant reduction in behavioral deficits on spatial cognition or motor skill in P4 lesion animals that underwent a handling procedure. Second, circadian activity levels were influenced by handling. Third, handled sham-operates showed significant increases in cortical thickness and thalamic cross-sectional area. Fourth, neuronal morphology of cells in layer 3 Par1 cortex showed significant alterations in both lesion and sham-operates following neonatal handling. Each result will be considered in turn.

Although handling has been reported to ameliorate deficits associated with prenatal stress (Wakshlak and Weinstock, 1989), prenatal ethanol exposure (Lee and Rabe, 1998), and hypoxia/ischemia there was no therapeutic benefit observed after perinatal frontal cortex injury. Reaching ability was enhanced in the handled control females compared to the untreated female controls. In the study by Wakshlak and Weinstock, the behavioral measures studied included open field, plus maze and amphetamine induced rotation. All of these measures are reportedly related to anxiety and fearfulness and prenatally stressed rats showed significant behavioral recovery on these tests. The handling was beneficial in reducing anxiety and emotionality in rats that had been prenatally stressed. Enhanced performance on the skilled reaching task has been reported after ethanol consumption in female rats (Metz et al., 2003). The authors attribute the beneficial effects of ethanol consumption to stress reduction in the test subjects. The increased accuracy of handled female controls in this study may be a result of altered stress responses of these individuals to the testing procedure. The Lee and Rabe (1998) study reported enhance performance of handled animals on reversal learning

in the T-maze following prenatal ethanol exposure. In this study, ethanol exposed animals showed no acquisition deficits in position discrimination, but showed deficits in reversal learning. Handling was effective in ameliorating the reversal deficits observed in this task. The authors reported that the handling procedure they employed did involve some sensory stimulation of the individual rat pups during the maternal separation period (lifting and placement on a soft mat 3-4 times during each period of separation). This additional stimulation may have contributed to the effectiveness of handling in reversing prenatal alcohol induced impairments.

Neonatal handling reduced the activity of adult rats over a 24-hour period of testing compared to untreated animals. The reduction in activity occurred in both lesion and control animals. Handling has been shown to reduce HPA axis reactivity to stressors and as such the handled animals may have experienced a reduced stress response after placement in the novel environment of the activity cages compared to untreated animals. This could account for the hypo-activity observed in the handled animals.

The increased cortical thickness and thalamic cross-sectional area found in handled sham-operates suggests that neonatal handling interacts with the normal processes of cerebral organization. Increased cortical thickness has been observed in rats following environmental enrichment and is associated with superior cognitive abilities (Rosenzweig and Bennett, 1978). The enhanced performance of aged rats after neonatal handling on tasks of spatial cognition (Lehmann et al., 2002; Meaney et al., 1989) has been attributed to increased survival of hippocampal neurons but may also be related to increased cortical thickness and thalamic cross-sectional area. These measures have not been reported previously.

The dramatic alterations of neuronal morphology observed after neonatal handling were surprising. The reduction of dendritic branch length and number after early environmental intervention is not without precedent. Gibb and Kolb (2004) and Gibb et al., (2004) have shown similar declines in dendritic length after prenatal experiential treatments but studies of tactile stimulation in the preweaning period have shown increases in dendritic length of layer 3 Par1 neurons (Gibb and Kolb, 2004). The paradoxical decline in branch length and cell complexity accompanied by enhanced cognitive performance is puzzling. The paradoxical anatomical and behavioral results lead us to speculate that processes of apoptosis or neurogenesis may also be affected by handling and could lead to increased neuronal survival and cell density. This possibility has not yet been addressed. The observation that lesion further reduced dendritic length and complexity may account for the lack of behavioral recovery observed in the P4 operates. Dendritic spine density was not affected by handling or lesion in either the apical or basilar fields and thus did not contribute to enhancing the behavioral performance of sham-operates or the lack of recovery observed in lesion animals. We do note that all of our other pre- and postnatal manipulations that have proven beneficial for behavioral recovery after early cortical injury have produced increased spine density in the lesion animals. The absence of spine changes in the current study is thus different and may be related to the absence of behavioral benefit of the handling treatment.

In sum, handling was not beneficial for animals with early cortical injuries. Handling thus contrasts with the effects of postinjury tactile stimulation, which does provide a functional benefit. It appears that the effects of tactile stimulation cannot be attributed simply to the effects of removing pups from the mother. We have shown

elsewhere that tactile stimulation produces significant changes in the expression of various proteins in skin and brain, the most interesting one being Fibroblast Growth Factor-2 (Gibb and Kolb, 2004). Although we did not measure protein expression in the current study, we can speculate that a major difference between the effects of handling and tactile stimulation on recovery from early brain injury may be related to the changes in factors such as protein expression.

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CHAPTER 4

EXPERIMENT 3

**PRENATAL ENRICHMENT AND RECOVERY FROM PERINATAL
CORTICAL DAMAGE: EFFECTS OF MATERNAL TACTILE
STIMULATION**

4.1. ABSTRACT

Post lesion tactile stimulation is effective in improving behavioral recovery following perinatal brain injury in rats. Here we wondered if prenatal tactile stimulation of the pregnant dam might also provide some prophylaxis for later perinatal cortical injury. Pregnant dams were stroked with a baby hairbrush, three times per day for 15-minute bouts beginning one week prior to mating. On postnatal day 4 (P4) animals received medial frontal cortex removals or sham surgeries. Untreated litters received the same surgical procedures but no stimulation. Animals were tested on the Morris Water Task, a skilled reaching task, and circadian activity beginning at P60. At P90 urine samples were collected and tested for corticosterone (CORT). The brains were then processed for Golgi-Cox analysis. P4 lesion animals born to petted mothers showed behavioral recovery on the Morris water task. P4 lesion male offspring of petted mothers showed improvement on skilled reaching but females did not. Similarly, prenatal tactile stimulation reduced CORT levels in male offspring, but elevated CORT levels in female offspring as compared to untreated animals. Although brain weight did not differ between treatment groups, cortical thickness and thalamic cross-sectional area were both increased in animals born to petted mothers. Animals that received prenatal tactile stimulation (petmom animals) showed an elevated expression of AchE compared to untreated (cagemom) animals. Dramatic changes were observed in the analysis of neuronal morphology. Branch length and number were decreased after prenatal tactile stimulation whereas spine density was increased. These results indicate that prenatal tactile stimulation alters cerebral organization and improves functional recovery in P4 frontal lesion operates.

4.2. INTRODUCTION

The beneficial effects of experiential treatments such as complex housing (Hebb, 1947; Greenough and Volkmar, 1973; Rosenzweig et al., 1962; Rosenzweig, 1971; Rosenzweig and Bennett, 1978) or exercise (Berchtold et al., 2002; Carro et al., 2000) on cognition and cerebral architecture have been well documented. Tactile stimulation has also been reported to be of benefit in both human and rat infants (Field et al., 1986; Schanberg and Field, 1987) for stimulating growth and accelerating maturity. We have recently demonstrated that the beneficial effects of tactile stimulation are at least in part mediated by fibroblast growth factor-2 (FGF-2) protein produced by the skin (Gibb and Kolb, 2004). Animals given perinatal cortical injury and postinjury tactile stimulation until weaning show dramatic behavioral recovery on spatial cognition and motor performance. This functional recovery is correlated with increased production of FGF-2 in the skin of tactilely-stimulated animals and increased expression of FGFR1 (FGF receptor 1) in the brains of these animals. In a parallel set of studies, we also have found that housing pregnant dams in complex environments while they are pregnant alters the brain of the unborn pups and, in addition, improves the outcome of frontal lesions performed on postnatal day 4, which is after the environmental manipulation of the dam was completed. The fact that prenatal experience can alter the brain's response to later injury led us to wonder whether tactile stimulation of the pregnant dam might have similar consequences. Adult females were accustomed to the petting protocol for one week prior to conception. Animals then were petted for 15 minutes, three times per day throughout their pregnancy. At parturition no further stimulation of the dam or her pups was offered. At postnatal day 60 behavioral testing began and at the conclusion of testing

the brains of the subjects were processed for Golgi-Cox analysis. Results show prenatal tactile stimulation is effective in mediating recovery and brain reorganization after perinatal cortical injury.

4.3. SUBJECTS AND HOUSING PROCEDURES

Three litters of rat pups were born to Long-Evans mothers that received tactile stimulation one week prior to impregnation and throughout the entire pregnancy term (petmom animals: 7 male frontal, 6 male control, 12 female frontal, and 12 female control). The stimulation consisted of three-15 minutes bouts of petting with a baby hairbrush at 9:00 a.m., 1:00 p.m., and 4:00 p.m. Three litters of pups were born to mothers that received no stimulation during their pregnancy (cagemom animals: 10 male frontal, 10 male control, 6 female frontal, and 7 female control). After the pups were born no further stimulation was offered. The animals were not handled again until behavioral testing commenced at postnatal day 60 (P60). All animals were housed in accordance with guidelines of the Canadian Council on Animal Care.

4.4. SURGERY

On postnatal day 4 (P4) the pups were removed from the nest and cooled in a Thermanon® cooling chamber until their core temperature reached approximately 20°C. The lesion animals had their scalp opened then the frontal bone carefully removed after it was incised with iris scissors. The medial frontal cortex was then removed bilaterally with gentle aspiration. The tissue targeted for removal was the medial subfield of the prefrontal cortex including Zilles' (1985) regions Cg1, Cg3, and PL as well as the medial

portion of Fr2 of the motor cortex. After aspiration of the cortical tissue, the animals' scalp was sutured with silk thread drawn by a very fine needle. The remaining control animals underwent a sham surgical procedure in which the scalp was opened and then sutured closed but the skull was not removed. These animals were identified by removal of the tip of the outer toe on their right rear foot.

4.5. BEHAVIORAL METHODS

4.5.1. Morris Water Task

Beginning at P60 animals were trained on the Morris Water Task using a similar procedure to that described by Sutherland et al. (1983) based on the original task described by Morris (1981). The maze consisted of a circular pool (1.5 m diameter X 0.5 m deep) with smooth white walls. The pool was filled with approximately 25 °C water mixed with 500 ml of skim milk powder, used to render the water opaque. A clear plexiglas platform (11 X 12 cm) was placed in a constant position inside the pool approximately 30 cm from the pool wall. The water level was adjusted so that the platform was invisible to a viewer outside the pool and to a rat swimming in the water. A trial consisted of placing a rat into the water facing the pool edge at one of four compass locations (north, south, east, or west) around the pool's perimeter. Within a block of four trials each rat started at the four locations in random sequence, and each rat was tested for four trials a day over five consecutive days. If on a particular trial a rat found the platform, it was permitted to remain on it for 10 seconds. A trial was terminated if the rat failed to find the platform after 90 seconds. Each rat was returned to its holding cage for approximately five minutes before the next trial commenced. The swim path for each rat

on every trial was recorded using a Poly Track video tracking system (San Diego Instruments) which tracks the swim path and records the latency, distance and dwell time within each quadrant.

4.5.2. Whishaw Tray Reaching

Following water maze training, animals were trained in a skilled reaching task developed by Whishaw et al. (1991). In this task rats were trained to retrieve chicken feed through metal bars at the front of the Plexiglas training cage (28 cm deep x 20 cm wide x 25 cm high). The front of each cage was constructed with 2 mm bars separated from each other by 1 cm, edge to edge and the floor was constructed of wire mesh. A tray (5 cm deep x 2 cm wide x 1 cm high) containing chicken feed pellets was mounted in the front of each cage. To obtain food, the rats had to extend their forelimbs through the bars, grasp, and retract the food pellet. The food tray was mounted on runners to adjust the distance of the food from the bars. Distance adjustment ensured that each rat could not simply rake the food into the cage. Any pellets that the rat dropped inside the cage were irretrievably lost through the mesh on the floor and the animal would have to reach again. During the first few days the rats were trained in pairs in the reaching cages for a period of one half hour per day. Once reach training commenced, the animals were provided with 15 grams of rat chow daily following the training period. The rats were subsequently trained individually for one half hour per day and then at the end of a two-week training period their performance was videotaped for a five- minute interval. Each time the rat reached through the bars whether or not food was obtained was scored as a “reach” and each time food was successfully returned to the cage and consumed was

scored as a “hit”. The percentage of hits to total reaches was then calculated for each animal’s taped performance.

4.5.3. Circadian Activity

Following reach training the animals were placed in computer monitored circadian activity cages. These cages were designed to assess activity over a 24-hour period by monitoring motion in cages fitted with infrared light beams and detectors. Each time the animal disrupted the light beam the computer recorded the side of the cage at which the activity occurred and a combined activity (for activity occurring across both the left and right sides of the cage) was also computed. The animals occupied the activity cages for two consecutive 24-hour periods and their combined activity for the second day was analyzed. During activity monitoring the animals had ad lib access to both food and water and were maintained on a 12:12 hr light/dark cycle. The time the animals were placed in the cages was noted and the subsequent analyses were matched for time of day. All animals had urine samples collected within four hours of their initial placement in the activity cages. The urine samples then underwent radioimmunoassay for corticosterone levels.

4.6. ANATOMICAL METHODS

4.6.1. Histological Procedures

At approximately 30 days of age a subset of animals representing all the groups were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline in 0.1 M phosphate buffer (pH 7.2) followed by a solution of 4%

paraformaldehyde and 11% picric acid in 0.1 M phosphate buffer (Lana's Fixative). The brains were then removed from the skull and trimmed by cutting the olfactory bulbs approximately 5 mm ahead of the frontal edge of the cortex and the optic nerves 2 mm ahead of the optic chiasm. The pineal body and paraflocculi were removed and the spinal cord cut even with the posterior edge of the cerebellum. The trimmed brains were weighed and then postfixed in the Lana's solution at 4° C for 24 hours before cutting at 50 μ m on a Vibratome™. Five consecutive sets of tissue were saved and one was mounted immediately for acetylcholinesterase histochemistry. Three sets were saved for immunohistochemical staining and the final set was mounted and processed for Cresyl violet staining.

At the conclusion of behavioral testing the remaining animals were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline. The trimmed brains were weighed and then immersed whole in 20 mls of Golgi-Cox solution. The brains were then stored (in the dark) in the Golgi-Cox fixative for 14 days before being transferred to a solution of 30% sucrose for seven days. The tissue was cut at 200 μ m on a Vibratome™ then developed using a method described by Gibb and Kolb (1998).

4.6.2. Western Blot

Brain tissue was removed from decapitated animals and placed on ice. Following rapid dissection of frontal cortex, brain samples were placed in microcentrifuge tubes cooled on dry ice. Brain samples were sonicated with 800 μ l of 1% SDS then aliquoted. All samples were held at -75 °C until analysis. Samples were diluted 1/20 to determine

protein concentration (Bradford Assay) before resolving the protein of interest on 8-12% acrylamide gels (5 μ g of protein per well) using SDS-PAGE gel electrophoresis. Gels were blotted on polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for non-specific binding for 1 hr. with 5% non-fat dry milk in Tris-buffered saline and .1% Tween 20. FGF-2 primary antibody (Santa Cruz, [Santa Cruz, CA] #sc -7911) was diluted 1:1000 in the same solution as was used for blocking. Membranes were incubated in primary antibody for 2 hours followed by 5 washes in PBS (5 mins.) and 1 hour incubation in secondary antibody (HRP- linked donkey anti-rabbit; Amersham [Piscataway, NJ] #NA934V; 1:5000). Following 5 more PBS washes, FGF-2 protein was revealed with an ECL+ detection kit from Amersham (#RPN2132) and the resulting image was captured on Hyperfilm ECL (Amersham #RPN1647K). Other antibodies used were Flg (Santa Cruz #sc-121; 1:1000), GR(Santa Cruz #sc-1004; 1:5000), PCNA (Santa Cruz #sc-56; 1:1000), bcl-2 (Santa Cruz #sc-783; 1:1000), and GFAP (Sigma [St. Louis, MO] #G3893; 1:10,000). The PCNA and GFAP antibodies were raised in mouse and thus HRP-linked sheep anti-mouse secondary antibody (Amersham # NA931) was used. Exposed film was imaged with a Kodak digital camera and the blot density was then analyzed using NIH Image software. The membrane was then stained with .1% Comassie Blue (OmniPure EM Science) to reveal all proteins in order to ensure pipeting consistency. A random protein band was selected for each blot and the amount of target protein was calculated as the density of the target / density of random sample protein in the same well. This method allowed compensation for any pipetting errors that may have occurred.

4.7. ANATOMICAL ANALYSES

4.7.1. Cortical Thickness Measurements

Cortical thickness measurements were obtained from Cresyl violet stained coronal sections projected on a Zeiss-Jena MF2 projector at a magnification of 20X (following the method described by Stewart and Kolb, 1988). Briefly, three cortical measures were made at points medial, central and lateral on five sections of tissue identified by the following landmarks; Plane 1: first caudate-putamen visible, Plane 2: anterior commissure, Plane 3: first hippocampal section, Plane 4: posterior commissure, Plane 5: last hippocampal section. A plastic metric ruler was used to measure from the edge of the cortex to the edge of the white matter. An average for each plane and for each animal was calculated and used for statistical comparison.

4.7.2. Assessment of Thalamic Size

Thalamic cross-sectional area was measured from two coronal sections stained with Cresyl violet using a Kodak digital camera to capture the image and the Scion Image program to measure thalamic area. One measure was taken of the anterior thalamus (approximately -1.80mm from the Bregma). The second measure was made in posterior thalamus at approximately -4.30mm from the Bregma (as described in a study by Kolb and Whishaw (1981)).

4.7.3. Acetylcholinesterase Quantification

Acetylcholinesterase (AChE) staining was assessed using the Scion Image densitometry program. The tissue was placed on a Zeiss microscope using a 20X

objective and the image captured on computer with a video camera. The lighting levels for photography were held constant through the entire sampling period to minimize any variations in density measures. The same planes as were used for cortical thickness measurements were analyzed for AchE density. An average density was then computed for each plane for each animal and used for statistical comparison.

4.7.4. Golgi-Cox Analyses

Layer III pyramidal cells in Zilles' area Par 1 were traced using a camera lucida at 250X. In order to be included in the data analysis, the dendritic trees of pyramidal cells had to fulfill the following criteria: (a) the cell had to be well impregnated and not obscured with blood vessels, astrocytes, or heavy clusters of dendrites from other cells; (b) the apical and basilar arborizations had to appear to be largely intact and visible in the plane of section. The cells were drawn and analyzed using two different procedures. In the first, each branch segment was counted and summarized by branch order using the procedure of Coleman and Riesen (1968). Branch order was determined for the basilar dendrites such that branches originating at the cell body were first order; after one bifurcation, second order; and so on. Branch order was determined for the apical dendrites such that branches originating from the primary apical dendrite were first order and so on. In the second, a Sholl analysis for estimation of dendritic length (Sholl, 1956) was performed. For this analysis a transparent overlay of concentric circles spaced 20 μm apart was placed over the neuron drawing by centering the innermost ring in the middle of the cell body. The number of dendrite-ring intersections was counted for each ring and the total number used to estimate total dendritic length in μm (number of

intersections X 20). Five cells were drawn in each hemisphere of each rat. The statistical analyses were done by taking the mean of the measurements on the five cells for each hemisphere of each subject.

Spine density was measured from one apical dendritic branch in the terminal tuft and one basilar terminal branch. Spine density measures were made from a segment greater than 10 μm in length, and usually about 50 μm . The dendrite was traced (1000X) using a camera lucida and the exact length of the dendritic segment calculated by placing a thread along the drawing and then measuring the thread length. Spine density was expressed as the number of spines per 10 μm . No attempt was made to correct for spines hidden beneath or above the dendritic segment so the spine density values are likely to underestimate the actual density of the dendritic spines.

4.8. STATISTICAL ANALYSES

All statistical analyses were ANOVA's performed on Statsview 5®. If an ANOVA did not show a significant effect of sex, the data were collapsed across this variable to increase the number of subjects per group and to simplify the analysis.

4.9. BEHAVIORAL RESULTS

4.9.1. Morris Water Task

4.9.1.1. Latency. Latency to find the hidden platform has been shown in our previous studies to be an effective measure of cognitive impairment in P4 frontal cortex lesion animals (e.g., Kolb, 1987). Frontal operates in the current study required more time to find the hidden platform than did their littermate controls and this deficit was attenuated

in the petmom animals (Fig. 4.1). A repeated measures ANOVA of water maze performance over the five days of testing, with lesion and treatment as factors showed a main effect of lesion ($F(1,144)=15.9, p=0.0003$), trial block ($F(4,144)=55.04, p<0.0001$) but not treatment ($F(1,144)=2.9, p=0.16$). The interactions of Trial Block by Lesion ($F(4,144)=3.03, p=0.02$) and Trial Block by Lesion by Treatment ($F(4,144)=7.1, p<0.0001$) were significant. The Trial Block by Lesion interaction demonstrated that the sham-operates learned the task more quickly than the P4 lesion animals. The Trial Block by Lesion by Treatment interaction indicated that the petmom P4 operates learned the location of the platform more quickly than did the cagemom P4 operates. The Treatment by Trial Block interaction was not significant ($F(4,144)=0.89, p=0.47$).

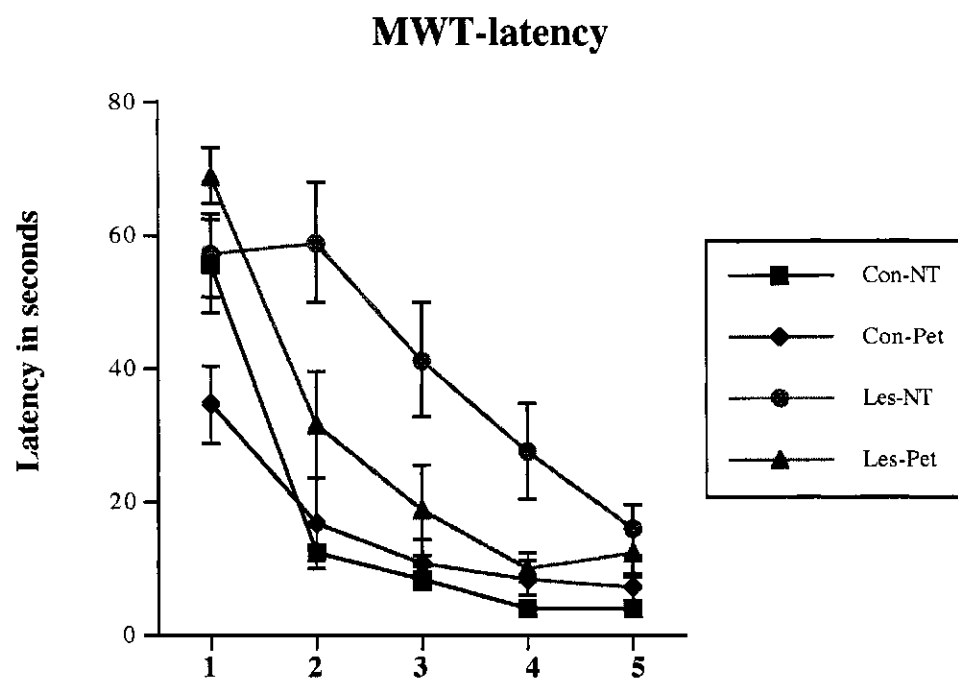


Fig. 4.1. Acquisition curve for Petmom vs. Cagemom animals.

(Latency in seconds)

4.9.1.2. Distance. Analysis of swim distance found results similar to those for swim latency (Fig. 4.2). A repeated measures ANOVA over five trial blocks with lesion and treatment as factors showed a significant main effect of lesion ($F(1,124)=15.1, p=0.0005$) and trial block ($F(4,124)=72.7, p<0.0001$), but not treatment ($F(1,124)=0.99, p=0.33$). The interactions of Trial Block by Lesion ($F(3,124)=6.9, p<0.0001$) and Trial Block by Lesion by Treatment ($F(4,124)=4.8, p=0.012$) were significant. The Trial Block by Lesion interaction indicated that over the five days of testing the controls found the platform after swimming shorter distances than did the P4 lesion animals. The Trial Block by Lesion by Treatment interaction reflected the observation that the petmom lesion animals were more accurate at swimming to the platform than were the cagemom lesion animals.

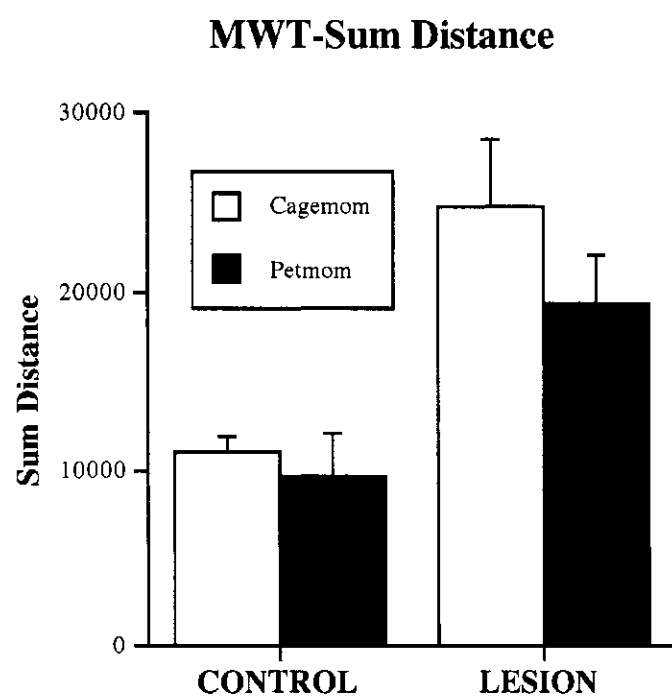


Fig. 4.2. Sum distance in the water maze for petmom vs. cagemom animals. Units are arbitrary computer units.

4.9.2. Whishaw Tray Reaching

Lesion animals showed impairments in successfully retrieving pellets for consumption when compared to littermate controls. The prenatal stroking treatment did not ameliorate these deficits in female operates although visual inspection of the data suggested improvement in the male operates (Fig. 4.3). Sex was therefore considered as a factor in the ANOVA. A three-way ANOVA with lesion, sex, and treatment as factors showed a main effect of lesion ($F(1, 31)=43.9, p<0.0001$) but not sex ($F(1,31)=1.18, p=0.29$) or treatment ($F(1,31)=0.019, p=0.66$). None of the interactions reached significance. Post hoc analysis of the data showed the male petmom P4 operates were marginally different from male cagemom operates ($p=0.066$) and not different from the cagemom male controls ($p=0.11$)

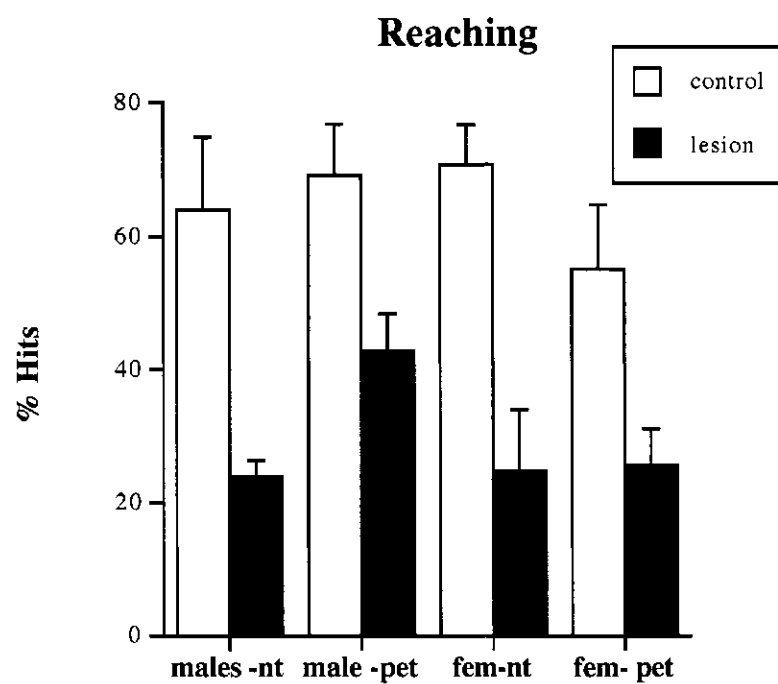


Fig. 2. Reaching performance of the petmom males and females.

Bars represent number of successful reaches/ total number of reaches.

4.9.3. Circadian Activity

Circadian activity patterns were monitored over a 24-hour period to determine if a shift in species typical activity may have resulted from the lesion or the environmental treatment. In this test, females were more active than males, but there was no main effect of lesion or treatment (Table 4.1). A three-way ANOVA with lesion, sex, and treatment as factors showed a main effect of sex ($F(1,32)=9.2, p=0.005$), but not treatment ($F(1,32)=1.5, p=0.23$) or lesion ($F(1,32)=0.89, p=0.35$). The only significant interaction was the Lesion by Sex interaction ($F(1,32)=4.5, p=0.042$). This interaction reflected the finding that the cagemom male controls showed high activity levels whereas the P4 lesion males showed low levels of activity.

Table 4.1. Summary of Circadian Activity

Group	Experience	
	Petmom	Cagemom
Control-male	155.7±44.9*	364.0±51.0
P4 lesion-male	220.8±18.8	206.1±36.7
Control-female	293.6±56.6	298.0±46.7
P4 lesion-female	418.8±62.0	413.3±61.5

Numbers refer to cage crosses ±standard errors

*differs significantly from Cagemom control

4.9.4. Corticosterone Assay

There was no main effect of lesion or treatment on basal corticosterone levels. There was a significant main effect of sex: females had higher basal levels of corticosterone (CORT) expression than did males. There was also a significant interaction of treatment and sex. This interaction reflected the finding that the prenatally stroked males had decreased basal levels of CORT, whereas prenatally stroked females had elevated basal levels of CORT (Fig. 4.4). A three-way ANOVA with lesion, treatment, and sex showed a main effect of sex ($F(1,27)=6.14, p=0.02$) but not treatment ($F(1,27)=0.59, p=0.45$) or lesion ($F(1,27)=1.3, p=0.26$). The only interaction to reach significance was the Treatment X Sex interaction ($F(1,27)=4.8, p=0.037$).

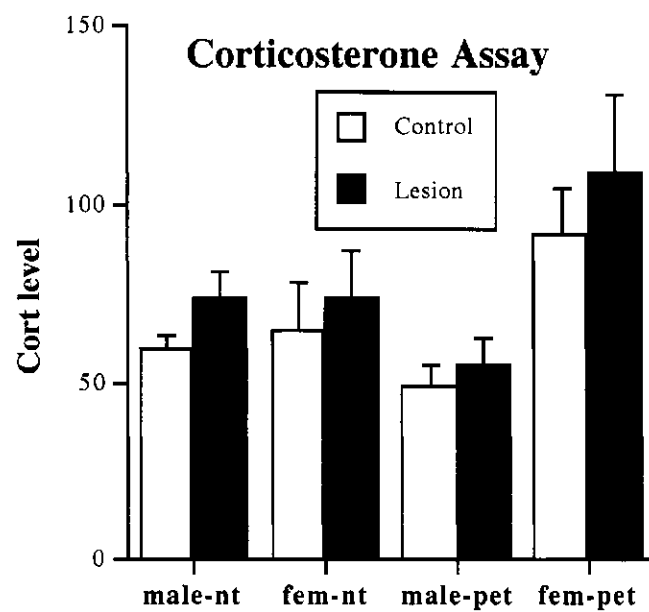


Fig. 4.4. Basal corticosterone levels for petmom males and females.

Values are expressed as pmols per mg. creatinine.

4.10. ANATOMICAL RESULTS

4.10.1. Body Weight

The normal pattern of sexual dimorphism in body weight was observed in this experiment: males were heavier than females. In addition, the petmom animals were heavier than the cagemom animals across all groups. Although there was a tendency for the lesion animals to weigh less than the sham-operates, this effect did not reach statistical significance (Table 4.2). A three-way ANOVA (Lesion by Sex by Treatment) showed a significant main effect of sex ($F(1,36)=79.2, p<0.0001$) and treatment ($F(1,36)=5.55, p=0.024$), but not lesion ($F(1,36)=2.31, p=0.14$). None of the interactions reached significance (p 's >0.26).

Table 4.2. Summary of body weights

Group	Experience	
	Petmom	Cagemom
Con. Males	423.8±34.4	365.6±8.7
P4 Males	390.0±17.6	357.8±22.4
Con. Females	281.9±15.0	274.6±18.0
P4 Females	271.9±11.4	247.4±11.4

Numbers refer to means ±standard errors in grams

4.10.2. Brain Weight

Female animals had smaller brains than males, and lesion animals had smaller brains than sham-operates. There was no effect of treatment on brain weight (Table 4.3).

A three-way ANOVA with lesion, sex, and treatment as factors showed a significant main effect of lesion ($F(1,36)=54.5$, $p<0.0001$) and sex ($F(1,36)=7.05$, $p=0.012$), but not treatment ($F(1,36)=0.91$, $p=0.35$). None of the interactions were significant (p 's >0.2).

Table 4.3. Summary of brain weight

Group	Experience	
	Petmom	Cagemom
Con. Males	2.03±0.03	1.96±0.01
P4 Males	1.74±0.04	1.74±0.05
Con. Females	1.88±0.02	1.90±0.06
P4 Females	1.72±0.03	1.70±0.05

Numbers refer to means ±standard errors in grams

4.10.3. Cortical Thickness

The P4 lesion caused a reduction in cortical thickness in the anterior and posterior planes. The petmom treatment reduced cortical thickness in the sham-operates but increased cortical thickness in the lesion animals in the anterior planes (Table 4.4). A two-way ANOVA with lesion and treatment as factors showed a main effect of lesion ($F(1,74)=214.1$, $p<0.0001$). There was no main effect of treatment ($F(1,74)=0.25$, $p=0.62$) but the Lesion by Treatment interaction was significant ($F(1,74)=5.1$, $p=0.027$)

In the posterior plane males had thicker cortices than did females. Lesion again reduced cortical thickness. A three-way ANOVA on cortical thickness in the posterior planes showed a main effect of lesion ($F(1,70)=43.6$ $p<0.0001$) and sex ($F(1,70)=4.38$,

p=0.40), but not treatment ($F(1,70)=0.002$, $p=0.97$). None of the interactions were significant (F 's <0.7 , p 's >0.4).

Table 4.4 Summary of Cortical Thickness at Anterior and Posterior Planes

Planes	Group	Experience	
		Petmom	Cagemom
1-2	Con	40.6±0.3	41.3±0.4
	P4	35.4±0.3*	34.2±0.5*
3-5	Con	33.9±0.2	34.1±0.4
	P4	31.7±0.3	31.4±0.4

Numbers refer means± standard errors in mm.

*differs significantly from Cagemom control

4.10.4 Acetylcholinesterase Quantification

Prenatal stroking increased acetylcholinesterase (AChE) expression in control animals. The lesion petmom animals showed an overall drop in AChE expression relative to their sham-operated cohorts whereas the lesion cagemom animals showed an increase (Table 4.5). A two-way ANOVA with lesion and treatment as factors revealed a main effect of treatment ($F(1,12)=28.3$, $p=0.0002$), but not lesion ($F(1,12)=1.3$, $p=0.28$). The interaction of Lesion by Treatment was significant ($F(1,12)=80.3$, $p=0.0001$) and

reflected the increase in AchE expression in cagemom operates and the decrease in AchE expression in petmom operates.

Table 4.5. Summary of AchE Density Measures: Treatment Effects

Group	Petmom	Cagemom
Control	121.5	100
P4 Lesion	109.8	115.3

Numbers refer to % untreated control values obtained at the same plane

An evaluation of AchE staining within petmom litters revealed that male sham-operates had a higher expression of AchE than females, but the no differences were observed in lesion animals (Table 4.6). A two-way ANOVA with sex and lesion as factors revealed a main effect of lesion ($F(1,22)=32.1, p=0.0001$) but not treatment ($F(1,22)=2, p=0.17$). The interaction of Sex by Lesion neared significance ($F(1,22)=28.4, p=0.06$). A post hoc analysis of the data showed that male controls showed significantly higher expression of AchE than female controls ($p=0.05$).

Table 4.6. Summary of AchE Density Measures: Sex differences

Petmom	Males	Females
Control	126.6 ±0.2*	122.7±1.1
P4 Lesion	117.9±1.0	118.5±1.1

Numbers refer to arbitrary density units ± S.E.

*differs significantly from females

4.10.5. Thalamic Measures

In the anterior plane of measure, males had a larger cross-sectional area. The lesion reduced cross-sectional thalamic measure and the prenatal stroking increased it (Table 7). A three-way ANOVA with lesion and treatment as factors revealed a significant main effect of lesion ($F(1,28)=30.12, p<0.0001$), sex ($F(1,28)=9.0, p=0.005$), and treatment ($F(1,28)=14.8, p=0.0006$). The interactions were not significant (F 's < 1.5, p 's > 0.24).

Table 4.7. Summary of Thalamic Area in the Anterior plane

Group	Experience	
	Petmom	Cagemom
Control-male	117.0*	100.0
P4 lesion-male	94.2	85.9*
Control-female	109.4	100
P4 lesion-female	96.9	87.1*

Numbers refer to percent of No Treatment control values

*differs significantly from Cagemom control

In the posterior plane, the lesion reduced thalamic area whereas the prenatal stroking increased it (Table 4.8). There was no sex effect for the posterior plane. A three-way ANOVA showed a main effect of lesion ($F(1,28)=5.2$ $p=0.03$) and treatment ($F(1,28)=$ but not sex. The only significant interaction was Lesion X Sex X Treatment ($F(1,28)=6.66$, $p=0.015$) and this reflected the finding that petmom control males and petmom lesion females had the largest thalamic cross-sectional areas.

Table 4.8. Summary of Thalamic Area in the Posterior plane

Group	Experience	
	Petmom	Cagemom
Control-male	112.1	100.0
P4 lesion-male	97.9	96.5
Control-female	99.3	100
P4 lesion-female	104.1	89.5*

Numbers refer to percent of No Treatment control values

*differs significantly from Cagemom control

4.10.6. Golgi-Cox Analyses

4.10.6.1. Branch Order

4.10.6.1.1. *Apical dendrites.* The novel overall finding was that prenatal treatment altered the morphology of cortical neurons in adulthood by reducing the amount of dendritic material (Fig. 4.5). In order to gain a better understanding of where changes in neuronal morphology might be occurring, the branch order analysis was divided into two areas: branching near the soma (branches 1-3) and branching near the terminal fields of the dendrites (branches 4-6+). In the apical tree near the soma there was no effect of lesion, and the treatment reduced the number of branches. A two-way ANOVA with lesion and treatment as factors showed a main effect of treatment ($F(1,50)=24.6, p<0.0001$) but not lesion ($F(1,50)=1.6, p=0.2$). The Lesion by Treatment interaction was also not significant ($F(1,50)=2.89, p=0.099$).

The distal branches on the apical tree were reduced in number by lesion, but treatment had no effect (Table 4.9). A two-way ANOVA showed a main effect of lesion ($F(1,50)=6.66$, $p=0.13$), but not treatment ($F(1,50)=1.2$, $p=0.28$), nor the interaction ($F(1,50)=0.36$, $p=0.85$).

Table 4.9. Summary of Branch Order in the Apical Tree

Branches	Group	Experience	
		Petmom	Cagemom
1-3	Con	17.3±0.5*	22.8±1.1
	P4	17.6±0.9*	20.4±0.7
4-6+	Con	1.9±0.2	2.3±0.4
	P4	1.3±0.2	1.6±0.2

Numbers refer number of branches±standard errors

*differs significantly from Cagemom control

4.10.6.1.2. Basilar Dendrites. In the basilar tree near the soma (branches 1-3) there was no effect of lesion but the treatment caused a reduction in number of branches (Table 4.10). A two-way ANOVA with lesion and treatment as factors showed no main effect of lesion ($F(1,50)=1.4$, $p=0.24$), but a significant effect of treatment ($F(1,50)=3.9$, $p=0.05$). The interaction was not significant ($F(1,50)=0.0004$, $p=0.98$). In the distal branches of the basilar tree there was no effect of lesion but the prenatal stroking caused a dramatic reduction in branch number. A two-way ANOVA showed a main effect of

treatment ($F(1,50)=72.0, p<0.0001$), but not lesion ($F(1, 50)=0.39, p=0.54$) nor the interaction ($F(1,50)=0.66, p=0.42$).

Table 4.10. Summary of Branch Order in the Basilar Tree

Branches	Group	Experience	
		Petmom	Cagemom
1-3	Con	20.8±1.0	22.6±1.4
	P4	21.9±0.7	23.7±0.7
4-6+	Con	3.4±0.4*	13.9±1.8
	P4	3.7±0.6*	12.3±1.3

Numbers refer number of branches± standard errors

*differs significantly from Cagemom control

4.10.6.2. Dendritic Length

4.10.6.2.1. Apical Dendrites. The Sholl analysis of dendritic length was divided into 3 ranges. The first one covered rings 1-6 (dendrite crossings near the soma). The second covered rings 7-11 or ring crossings that occurred in the central portion of the apical tree and the third covered the last rings (12- 16+). The third range allowed analysis in the terminal portions of the dendritic tree. Prenatal stroking reduced branch length across all the ranges examined whereas lesion reduced branch length only in the portion of the apical tree nearest the soma (rings 1-6). Sex was only a significant factor in the distal portion of the apical tree. Petmom males showed branch lengths that were virtually

identical to cagemom males whereas petmom females showed a dramatic drop in branch length compared to cagemom females (Table 4.11).

A three-way ANOVA (rings 1-6) with lesion, sex, and treatment as factors revealed a main effect of lesion ($F(1,74)=6.3$, $p=0.014$) and treatment ($F(1,74)=47.7$, $p<0.0001$), but not sex ($F(1,74)=2.46$, $p=0.12$). None of the interactions were significant (F 's <2.2 , p 's >0.14).

On the medial portion of the apical tree (rings 7-11) a three-way ANOVA revealed no main effect of lesion ($F(1,74)=2.6$, $p=0.11$) or sex ($F(1,74)=1.3$, $p=0.26$), but the effect of treatment was significant ($F(1,74)=13.6$, $p=0.0004$). The only significant interaction was the Lesion by Treatment by Sex interaction ($F(1,74)=5.7$, $p=0.019$). This interaction reflected a decrease in branch length in lesion petmom females and an increase in branch length in lesion cagemom females. The petmom males showed no further decline in branch length following lesion.

On the distal portion of the apical tree (rings 12-16+) a three-way ANOVA revealed a significant main effect of treatment ($F(1,74)=10.0$, $p=0.002$) and sex ($F(1,74)=4.9$, $p=0.03$), but not lesion ($F(1,78)=1.2$, $p=0.28$). The only interaction to reach significance was the Sex X Treatment interaction ($F(1,74)=9.7$, $p=0.003$). This interaction reflected the observation that treatment reduced branch length in females but not in males.

Table 4.11. Summary of Sholl Analysis in the Apical Tree

Rings	Group	Experience	
		Petmom	Cagemom
1-6	Con	21.5±1.1*	30.0±1.1
(male)	P4	20.9±0.9*	25.4±1.6*
1-6	Con	23.4±0.9*	30.4±1.5
(female)	P4	22.1±1.2*	27.6±1.7

7-11	Con	13.3±0.9*	16.2±1.1
(male)	P4	13.7±0.7*	13.8±0.8*
7-11	Con	13.3±0.7	14.7±0.7
(female)	P4	11.1±0.6*	15.2±0.9

12-16+	Con	33.7±2.0	33.6±1.7
(male)	P4	32.4±1.7	32.8±2.0
12-16+	Con	24.4±1.7*	38.4±4.4
(female)	P4	25.1±1.5*	30.8±2.9*

Numbers refer number of ring crossings± standard errors

*differs significantly from Cagemom control

4.10.6.2.2. Basilar Dendrites. In the basilar field the Sholl analysis was performed for rings 1-4 and rings 5-12 as the basilar dendrites were not as long as were the apical dendrites. Prenatal stroking and lesion reduced branch length at both levels measured (Table 4.12).

On rings 1-4, a two-way ANOVA revealed a significant main effect of lesion ($F(1,78)=4.6, p=0.35$) and treatment ($F(1,78)=42.6, p<0.0001$). The Lesion by Treatment interaction was also significant ($F(1,78)=5.4, p=0.023$) and reflected the finding that while lesion reduced branch length in cagemom animals it did not further reduce branch length in petmom animals. On rings 5-12, a two-way ANOVA revealed a significant effect of lesion ($F(1,78)=6.3, p=0.14$) and treatment ($F(1,78)=16.7, p=0.0001$). The interaction was not significant ($F(1,78)=0.8, p=0.38$).

Table 4.12. Summary of Sholl Analysis in the Basilar Tree

Rings	Group	Experience	
		Petmom	Cagemom
1-4	Con	46.0±1.5*	63.1±3.1
	P4	46.3±0.9*	54.5±2.4*
5-12	Con	25.5±1.8*	35.6±2.8
	P4	22.2±1.5*	28.7±2.1*

Numbers refer number of ring crossings±standard errors

*differs significantly from Cagemom control

4.10.6.3. Spines

4.10.6.3.1. Apical Dendrites. Prenatal stroking increased spine density in the apical tree (Table 4.13). A two-way ANOVA with lesion and treatment as factors showed a main effect of treatment ($F(1,72)=6.6$, $p=0.12$), but not lesion ($F(1,72)=0.07$, $p=0.80$). The Lesion by Treatment was significant ($F(1,72)=4.0$, $p=0.05$) and reflected the finding that lesion increased spine density in cagemom animals but decreased spine density in petmom animals.

Table 4.13. Summary of Spine Density on the Apical Terminal

Group	Experience	
	Petmom	Cagemom
Control	7.05±0.24*	6.03±0.20
P4 lesion	6.55±0.20*	6.44±0.20

Numbers refer to number of spines/10 μ m dendritic branch length

*differs significantly from Cagemom control

4.10.6.3.2. Basilar Dendrites. On the basilar terminal the petmom animals had a significant increase in spine density relative to the cagemom animals (Table 4.14; Fig.4.5). There was no effect of group or sex. A three-way ANOVA with lesion, sex, and treatment as factors revealed a main effect of treatment ($F(1,60)=17.7$, $p<0.0001$), but not lesion ($F(1,60)=0.19$, $p=0.66$) or sex ($F(1,60)=0.05$, $P=0.82$). The only interaction to reach significance was the Treatment by Sex interaction ($F(1,60)=8.2$, $p=0.006$) and this reflected the observation that the females had higher spine densities in

the cagemom animals whereas the males had higher spine densities in the petmom animals.

Table 4.14. Summary of Spine Density on the Basilar Terminal

Group	Experience	
	Petmom	Cagemom
Control-male	7.50±0.32*	5.77±0.19
Control-fem.	7.08±0.11*	6.62±0.12*
P4 lesion-male	7.45±0.36*	5.94±0.16
P4 lesion-fem.	6.66±0.41*	6.50±0.49*

Numbers refer to number of spines/10µm dendritic branch length

*differs significantly from same sex Cagemom control

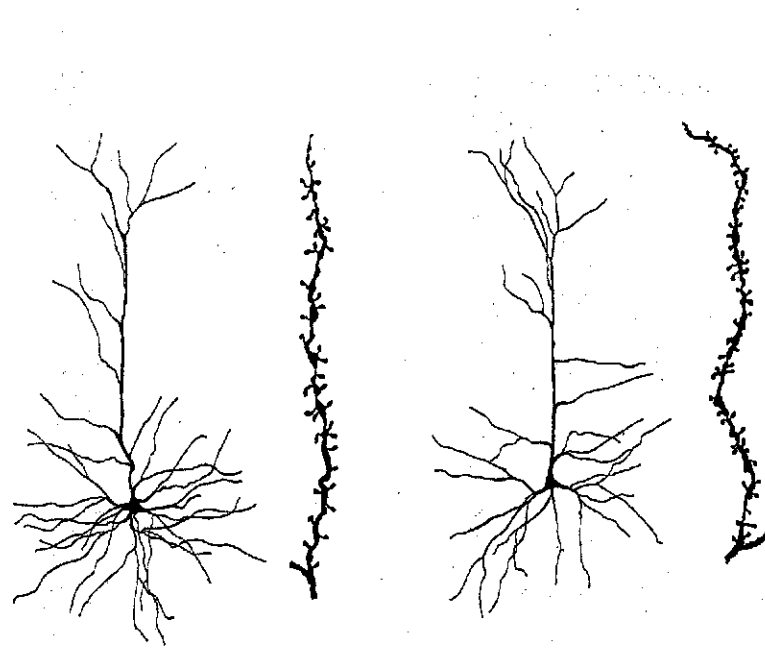


Fig. 4.5. Representative cells and spines drawn from cagemom (left) and petmom (right) animals.

4.10.7. Western Blot

Frontal cortex samples were harvested from control and lesion animals. In the control animals the sample included all cortical tissue anterior to +3.0 mm ahead of the Bregma. In the lesion animals the sample was taken from approximately the same location but only included the remaining lateral cortex including Frontal area 3 (Fr3) and Agranular insular cortex (AID) (Zilles, 1985). The olfactory bulb and nucleus accumbens regions were discarded in all samples.

Blots were run on frontal cortex samples to analyze the effects of treatment on control animals (petmom and cagemom sham-operate samples run on same blot); treatment effects on lesion animals (petmom and cagemom P4 lesion samples run on same blot); lesion effects within petmom litters (control and P4 lesion samples from

petmom litters run on same blot). Westerns were run for the following proteins: Fibroblast growth factor-2 (FGF-2), a growth factor that stimulates mitosis and synaptogenesis in brain of both infant and adult animals and promotes survival of neurons following brain damage, FGF receptor1 (FGFR1 or Flg) a receptor for FGF-2, Glucocorticoid receptor (GR) a receptor for corticosterone and changes in its expression can reflect alterations in reactivity of the HPA axis in response to stress, proliferating cell nuclear antigen (PCNA) a protein expressed in the G₁ and S phase of a cell undergoing mitosis, glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is found exclusively in glial cells (increased expression of GFAP implies proliferation of glial cells or increased reactivity of glia already present), bcl-2 is a anti-apoptotic protein and increased expression implies a reduction in apoptotic processes.

On the blot designed to compare petmom controls with cagemom controls, only two of the proteins tested showed changes in their expression (Table 4.15). Both GR and PCNA expression were decreased in the petmom animals. A simple ANOVA on GR expression showed a main effect of treatment ($F(1,11)=11.7, p=0.006$). ANOVA on PCNA expression also showed a main effect of treatment ($F(1,11)=21.9, p=0.0007$). ANOVA's for all other proteins showed no differences between petmom and cagemom controls ($p's >0.08$)

The blot designed to assess differences in protein expression between P4 lesion petmom and cagemom animals showed that PCNA expression was down and GFAP expression was up in the petmom animals. ANOVA showed a main effect of treatment on PCNA ($F(1,9)=9.8, p=0.012$) and GFAP ($F(1,11)=7.3, p=0.21$) expression.

The comparison within the petmom litter of control and lesion animals demonstrated elevations in protein expression of both FGF-2 and GFAP in the P4 operates. A two-way ANOVA (lesion and sex as factors) on FGF-2 expression showed a main effect of lesion ($F(1,9)=5.9, p=0.04$) but not sex ($F(1,9)=1.88, p=0.20$). The interaction of Lesion by Sex was significant ($F(1,9)=4.8, p=0.056$) and reflected the observation that FGF-2 expression was elevated in male but not female operates. The ANOVA on GFAP expression showed a significant main effect for lesion ($F(1,8)=12.6, p=0.008$), but not sex ($F(1,8)=0.16, p=0.7$) or the interaction ($F(1,8)=0.05, p=0.82$).

Table 4.15. Relative expression of protein in blot comparisons

<u>Analysis</u>	Petmom vs NT Control	Petmom vs. NT P4 Lesion	Petmom P4 vs. Petmom Control
<u>FGF-2</u>	=	=	↑
<u>Flg</u>	=	=	=
<u>GR</u>	↓	=	=
<u>PCNA</u>	↓	↓	=
<u>GFAP</u>	=	↑	↑

4.11. DISCUSSION

The overall finding of the experiment was that prenatal experience significantly altered both brain and behavior of the rats and that the effects were often different in the lesion and sham-operated littermates. The general findings can be separated into 8 different sets of results. First, prenatal stroking afforded significant recovery to P4 lesion animals on the MWT. In addition, sex-dependent recovery was seen on the skilled

reaching task and favored male operates. Second, urine CORT levels were altered by prenatal stroking and the effect was sex-dependent: there was an increase in basal levels in females and a decrease in males. Third, petmom animals were significantly heavier than the untreated animals. Fourth, cortical thickness was altered by prenatal stroking in a lesion-dependent manner. Sham-operates had *decreased* cortical thickness whereas P4 lesion animals had *increased* cortical thickness. Fifth, AchE levels were changed in petmom animals and the observed effects were both sex- and lesion- dependent: the lesion petmom animals showed a drop in AchE expression relative to sham-operated cohorts whereas the lesion cagemom animals show an increase. Sixth, thalamic cross-sectional area was increased by prenatal stroking at both anterior and posterior planes. Seventh, neuronal morphology was extensively reorganized in petmom animals in layer 3 neurons from Par1 cortex. Eighth, protein expression was altered after prenatal stroking in a sex- and lesion- dependent manner. Some of the key results will be considered in more detail.

Behavioral recovery was observed for the prenatally stroked animals on the Morris water task. Animals born to petted mom found the platform more quickly and with greater accuracy (shorter swim distances) than did the cagemom offspring. On the Whishaw skilled reaching task, prenatal stroking reduced the performance of females relative to males. This result was surprising because in our experience females generally outperform males on this task. Motor recovery was observed in the P4 lesion male offspring of petted mothers but P4 lesion females showed no improvement. Basal corticosterone (CORT) levels were reduced in the male petmom animals and increased in the female petmom animals independent of lesion. Animals with higher basal CORT

levels display reduced sensitivity to glucocorticoid receptor regulated negative feedback and more anxious behavior (Liu et al., 2000). Changes in CORT levels may alter motor performance as it has been demonstrated, for example, that low doses of alcohol improved performance of female rats on tests of skilled reaching (Metz et al., 2003). The authors proposed that the anxiolytic properties of the ethanol served to enhance motor performance. The lack of recovery on skilled reaching in P4 female petmom animals thus may have resulted from increased emotional tone and related anxious behavior.

Two gross measures of cerebral organization were altered by prenatal stroking, namely cortical thickness and thalamic area. Both of these are indirect measures of cerebral organization but they do provide some insight into how behavior of the P4 lesion animals may have benefited from the prenatal experience. First, prenatal stroking interacted with lesion to influence cortical thickness. Sham-operated petmom animals had *reduced* cortical thickness and lesion petmom animals *increased* cortical thickness compared to the cagemom animals. It is reasonable to suppose that the increase in cortical thickness in lesion animals may have contributed to their observed behavioral recovery. The decrease in cortical thickness in the sham operates was unexpected but there is a precedent for this finding as we found a reduction in cortical thickness after postnatal tactile stimulation in sham operates (Gibb and Kolb, 2004a). Second, prenatal stroking increased thalamic cross-sectional area in sham-operates at both the anterior and posterior planes whereas the P4 frontal lesion caused reductions at these sites. Prenatal stroking did not prevent the reduction in thalamic area caused by the lesion except at the posterior plane for the P4 lesion females. This sex-related difference is difficult to

interpret but is at least consistent with the other findings that the prenatal treatments differentially affected the two sexes.

The Golgi-Cox analysis of the petmom animals revealed dramatic changes in neuronal morphology. Overall, neurons drawn from the petmom animals had smaller, less complex, dendritic profiles than those drawn from cagemom animals. In contrast, there was a general increase in spine density on the same dendrites. We generally associate loss of dendritic complexity with reduced behavioral capacity but this was not the case in this experiment. Nonetheless, it appears that the intrinsic organization of the cortex must have been altered by the prenatal experience and this appears to have been beneficial for the animals with P4 lesions. We have found previously that increased spine density is associated with functional improvement in P4 frontal operates after a variety of treatments including complex housing and postnatal tactile stimulation (e.g., Gibb and Kolb, 2004a; Kolb, 1995) so there is some precedent for the relationship between increased spine density and functional recovery. The failure to see improved cognitive or motor functioning in the unlesioned animals with prenatal petting may simply reflect the relative insensitivity of the behavioral measures to enhanced behavioral capacity in intact rats. The observed changes gross cerebral morphology obviously must be related to changes at a more molecular level. In the current study our only hints must come from the results from the AchE and protein analyses. We will consider each separately.

Petmom animals showed increased expression of AchE compared to the cagemom animals. Postnatal tactile stimulation had similar effects on AchE expression (Kolb, 1995). Complex-housing of adults rats has also been reported to have similar effects on AchE expression (Rosenzweig, 1971). This could indicate that upregulation of

acetylcholine (ACh) expression in the brain. Increases in cortical ACh are associated with spontaneous arousal, sensory stimulation, and heightened attention and have been implicated in supporting learning and memory (e.g., Acquas et al., 1998). AchE also has been shown to influence membrane conductance, enhance excitatory amino acid transmission, and to hydrolyze peptides (Appleyard, 1992), changes that can be presumed to alter cortical functioning. The differential changes in AchE expression in the petmom males and females was unexpected but we can postulate that it may have contributed to the enhanced motor recovery observed in the male animals. It is feasible that increased expression of AchE in prenatally stroked animals reflects an increased potential for plastic changes in the cortex.

It is difficult to directly relate changes in gene expression to altered cerebral organization and behavior, and especially after cerebral injury, but a couple of the observed changes may provide some hints as to how the prenatal experience affected both brain and behavior. For example, the increased GFAP expression in the petmom animals is likely associated with increased astrocytic activity, which in turn could be associated with increased production of neurotrophic factors, such as FGF-2. We previously have shown that increased expression of FGF-2 is associated with behavioral improvement after P4 lesions and it also was increased in the current experiment (Gibb and Kolb, 2004). Furthermore, the FGF-2 increase in the current study was greater in males than females, as was the functional recovery. The other observed changes in protein expression are less easily correlated with our brain and behavioral measures but we recognize that we measured only a small subset of possible proteins related to cerebral organization. Further, we note that the proteins were measured 20 days after the injury

and measurements at other times may very well have found a different pattern or magnitude of changes. Nonetheless, the key point is that prenatal experience alters later protein expression, cerebral organization, and functional outcome after perinatal brain injury. These findings are novel and will provide the grist for considerable followup study.

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CHAPTER 5
EXPERIMENT 4

**PRENATAL ENRICHMENT AND RECOVERY FROM PERINATAL
CORTICAL DAMAGE: EFFECTS OF MATERNAL COMPLEX HOUSING**

5.1. ABSTRACT

Rats with perinatal frontal cortex removals (postnatal days 1-5) show a dismal behavioral outcome as adults. To test the effect of prenatal experience on functional outcome, 7 pregnant dams and 1 male rat were placed in complex housing for 12 hours per day (in the light cycle) for the duration of the dams' pregnancy. At parturition the dams were left in their home cages with their pups. At postnatal day 4 (P4) some of infants received frontal cortex removals and the remainder underwent a sham surgery. On P21, 36 animals were decapitated for Western Blot and on P40 a subset of animals were perfused for acetylcholinesterase analysis. Behavioral testing of the remaining animals began on postnatal day 60. Following testing on the Morris water task subjects were trained on a reaching task. Brains were processed for Golgi-Cox analysis. Frontal-lesion animals born to complex-housed mothers showed significant behavioral improvement on the water task and skilled reaching relative to the offspring of standard-housed mothers. Prenatal complex housing did not alter brain weight but caused an increase in cortical thickness at anterior planes and thalamic area at both anterior and posterior regions. Golgi analysis revealed that: 1) PN3 frontal lesions caused a reduction in dendritic length in layer III pyramidal neurons in area Par1; 2) prenatal enrichment caused a further decrease in dendritic branch length in all animals; 3) prenatal complex housing increased apical terminal branching; 4) prenatal complex housing increased spine density. Prenatal experience has a powerful effect on both behavioral recovery and anatomical organization of the brain.

5.2. INTRODUCTION

Environmental effects on behavior and cerebral architecture in normal (undamaged) animals have been well documented (Hebb, 1947; Rosenzweig, et al., 1962; Rosenzweig, 1971; Greenough and Volkmar, 1973). These studies demonstrated that complex housing improves behavioral performance and increases such anatomical features as brain weight, cortical thickness, and number of synapses. Although experiments designed to assess the effects of complex housing on recovery after brain damage in adult animals have met with limited success (e.g. Johansson, 2002; Kolb and Gibb, 1991; Will and Kelche, 1992) work with environmental treatments for brain-damaged infants has been more effective. For example, complex housing of weanling rats has been shown to reverse some of the behavioral deficits caused by early cortical lesion and early environmental rehabilitation following perinatal brain injury has a greater impact on functional recovery than later treatment (Kolb, et al., 2003). In a review of the effects of pre- and postnatal stimulation on emotion and cognition in rats (Chapillon et al., 2002), prenatal stress was shown to cause impairments on some cognitive tasks that could be reversed by later environmental enrichment. A study by Koehl et al. (1999) demonstrated that prenatal stress altered the function of the hypothalamic-pituitary-adrenal (HPA) axis and caused changes in the circadian rhythm secretion of corticosterone. Although we are unaware of studies examining the effect of prenatal experience on recovery from cerebral injury, a study by Kiyono et al. (1985) demonstrated that offspring born to pregnant complex-housed females were advantaged when tested as adults in a Hebb-Williams maze. The effect of prenatal enrichment on

learning was also examined by Koo et al. (2001). This study reported that spatial learning and memory, and the expression of neuronal cell adhesion molecules (used as a marker of synaptic plasticity) were enhanced. These experiments led us to wonder if prenatal complex housing might provide some measure of protection against later perinatal brain injury.

In the current study nulliparous females were introduced to a complex environment one week before a male was placed in the same housing. These females were exposed to the complex environment for the duration of their pregnancy and at the time of parturition, returned to standard housing. At postnatal day 4 the offspring of the complex-housed mothers underwent surgery to remove medial frontal cortex bilaterally. After weaning they were placed in groups in standard cages where they were allowed to mature to adulthood before commencement of behavioral testing. After behavioral testing, their brains were processed for Golgi-Cox analysis. Animals born to mothers housed in complex environments showed attenuation of behavioral deficits following perinatal brain injury and altered brain morphology as compared to their untreated cohorts.

5.3. SUBJECTS AND HOUSING PROCEDURES

Seven adult female Long-Evans rats (90 days old) were placed in complex housing for one week prior to the introduction of an adult male rat. The complex housing enclosure (condominium) was a large pen measuring 63 X 148 X 187 cm. Three walls were constructed of sturdy wire mesh and the fourth wall consisted of plywood overlaid with blue arborite®. Within these enclosures were swings, plastic pipes, ramps and

runways and a variety of 'toys' that were moved and/or changed weekly when the pen was cleaned (Fig. 5.1). The females remained in the complex housing for 24 hours per day (12 hours light/12 hours dark) for the first 10 days and then were removed from the condominiums and placed in standard plastic breeding cages for 12 hours each day during the dark cycle. This procedure was introduced to accustom the mothers to the cages they would be occupying once their pups were born. They were returned to the condominiums each morning and this pattern of housing changes continued up to the day of parturition. The male remained in the condominium until all dams were delivered of their pups. In total 93 pups were born to the 7 females and within these litters there were 50 female and 43 male pups (condomom animals). Within the female group, 27 received postnatal day 4 (P4) frontal removals and 23 were sham-operates and in the male group, 24 received the frontal surgery and 19 were sham-operates. The dams remained with their pups until postnatal day 21 (P21) when the pups were weaned and placed in standard plastic cages (3-6 animals per cage). No further 'enrichment' was experienced by the offspring animals and they were subsequently handled only when their cages were cleaned. Four litters of animals born to mothers in standard breeding cages (cagemom animals) served as the control group for this experiment. There were a total of 47 animals in these litters (19 female, 28 male). Within the female group there were 11 frontal operates and 8 shams and in the male group, 16 frontals and 12 shams. At P21, 36 animals were decapitated for Western Blot analysis (4 female control, 4 female P4, 3 male control, 4 male P4 from condomom litters and 2 female control, 2 female P4, 4 male control, and 3 male P4 from untreated litters).

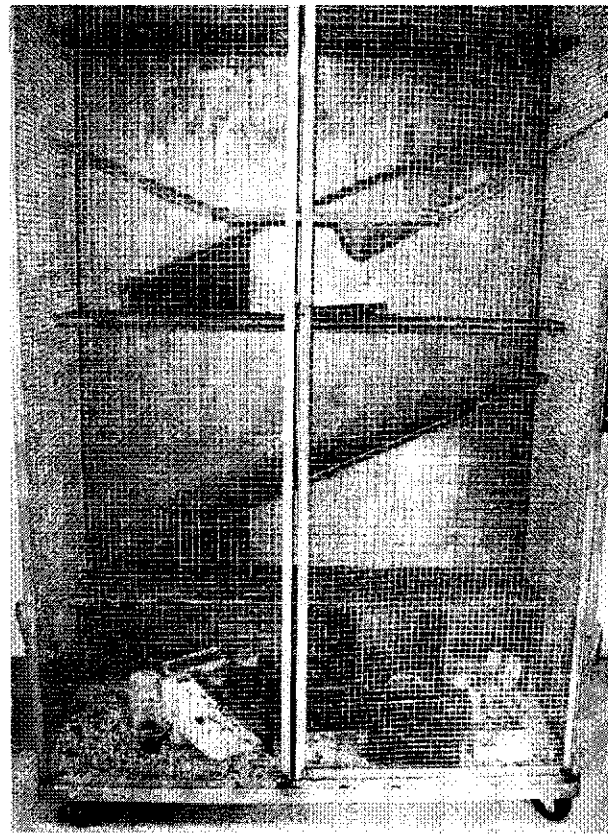


Fig. 5.1. Complex housing for rats (Condominiums).

5.4. SURGERY

On postnatal day 4 (P4) the pups were removed from the nest and cooled in a Thermanon® cooling chamber until their core temperature reached approximately 20°C. The lesion animals had their scalp opened then the frontal bone carefully removed after it was incised with iris scissors. The medial frontal cortex was then removed bilaterally with gentle aspiration. The tissue targeted for removal was the medial subfield of the prefrontal cortex including Zilles (1985) regions Cg1, Cg3, and PL as well as the medial portion of Fr2 of the motor cortex. After aspiration of the cortical tissue, the animals' scalp was sutured with silk thread drawn by a very fine needle. The remaining control

animals underwent a sham surgical procedure in which the scalp was opened and then sutured closed but the skull was not removed. These animals were identified by removal of the tip of the outer toe on their right rear foot.

5.5. BEHAVIORAL METHODS

5.5.1. Morris Water Task

Beginning at P60 animals were trained on the Morris Water Task using a similar procedure to that described by Sutherland et al. (1983) based on the original task described by Morris (1981). The maze consisted of a circular pool (1.5 m diameter X 0.5 m deep) with smooth white walls. The pool was filled with approximately 25 °C water mixed with 500 ml of skim milk powder, used to render the water opaque. A clear plexiglas platform (11 X 12 cm) was placed in a constant position inside the pool approximately 30 cm from the pool wall. The water level was adjusted so that the platform was invisible to a viewer outside the pool and to a rat swimming in the water. A trial consisted of placing a rat into the water facing the pool edge at one of four compass locations (north, south, east, or west) around the pool's perimeter. Within a block of four trials each rat started at the four locations in random sequence, and each rat was tested for four trials a day over five consecutive days. If on a particular trial a rat found the platform, it was permitted to remain on it for 10 seconds. A trial was terminated if the rat failed to find the platform after 90 seconds. Each rat was returned to its holding cage for approximately five minutes before the next trial commenced. The swim path for each rat on every trial was recorded using a Poly Track video tracking system (San Diego

Instruments) which tracks the swim path and records the latency, distance and dwell time within each quadrant.

5.5.2. Whishaw Tray Reaching

Following water maze training, animals were trained in a skilled reaching task developed by Whishaw et al. (1991). In this task rats were trained to retrieve chicken feed through metal bars at the front of the Plexiglas training cage (28 cm deep x 20 cm wide x 25 cm high). The front of each cage was constructed with 2 mm bars separated from each other by 1 cm, edge to edge and the floor was constructed of wire mesh. A tray (5 cm deep x 2 cm wide x 1 cm high) containing chicken feed pellets was mounted in the front of each cage. To obtain food, the rats had to extend their forelimbs through the bars, grasp, and retract the food pellet. The food tray was mounted on runners to adjust the distance of the food from the bars. Distance adjustment ensured that each rat could not simply rake the food into the cage. Any pellets that the rat dropped inside the cage were irretrievably lost through the mesh on the floor and the animal would have to reach again. During the first few days the rats were trained in pairs in the reaching cages for a period of one half hour per day. Once reach training commenced, the animals were provided with 15 grams of rat chow daily following the training period. The rats were subsequently trained individually for one half hour per day and then at the end of a two-week training period their performance was videotaped for a five- minute interval. Each time the rat reached through the bars whether or not food was obtained was scored as a “reach” and each time food was successfully returned to the cage and consumed was

scored as a “hit”. The percentage of hits to total reaches was then calculated for each animal’s taped performance.

5.5.3. Circadian Activity

Following reach training the animals were placed in computer monitored circadian activity cages. These cages were designed to assess activity over a 24-hour period by monitoring motion in cages fitted with infrared light beams and detectors. Each time the animal disrupted the light beam the computer recorded the side of the cage at which the activity occurred and a combined activity (for activity occurring across both the left and right sides of the cage) was also computed. The animals occupied the activity cages for two consecutive 24-hour periods and their combined activity for the second day was analyzed. During activity monitoring the animals had ad lib access to both food and water and were maintained on a 12:12 hr light/dark cycle. The time the animals were placed in the cages was noted and the subsequent analyses were matched for time of day.

5.6. ANATOMICAL METHODS

5.6.1. Histological Procedures

At approximately 30 days of age a subset of animals (n=65) representing all the groups were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline in 0.1 M phosphate buffer (pH 7.2) followed by a solution of 4% paraformaldehyde and 11% picric acid in 0.1 M phosphate buffer (Lana’s Fixative). The brains were then removed from the skull and trimmed by cutting the olfactory bulbs approximately 5 mm ahead of the frontal edge of the cortex and the optic nerves 2 mm

ahead of the optic chiasm. The pineal body and paraflocculi were removed and the spinal cord cut even with the posterior edge of the cerebellum. The trimmed brains were weighed and then postfixed in the Lana's solution at 4° C for 24 hours before cutting at 50 μm on a Vibratome™. Five consecutive sets of tissue were saved and one was mounted immediately for acetylcholinesterase histochemistry. Three sets were saved for immunohistochemical staining and the final set was mounted and processed for Cresyl violet staining.

At the conclusion of behavioral testing the remaining animals were given an overdose of sodium pentobarbital and intracardially perfused with a solution of 0.9% saline. The trimmed brains were weighed and then immersed whole in 20 mls of Golgi-Cox solution. The brains were then stored (in the dark) in the Golgi-Cox fixative for 14 days before being transferred to a solution of 30% sucrose for seven days. The tissue was cut at 200 μm on a Vibratome™ then developed using a method described by Gibb and Kolb (1998).

4.6.2. Western Blot

Brain tissue was removed from decapitated animals and placed on ice. Following rapid dissection of frontal cortex, brain samples were placed in microcentrifuge tubes cooled on dry ice. Brain samples were sonicated with 800 μl of 1% SDS then aliquoted. All samples were held at -75 °C until analysis. Samples were diluted 1/20 to determine protein concentration (Bradford Assay) before resolving the protein of interest on 8- 12% acrylamide gels (5 μg of protein per well) using SDS-PAGE gel electrophoresis. Gels were blotted on polyvinylidene difluoride (PVDF) membranes. Membranes were blocked

for non-specific binding for 1 hr. with 5% non-fat dry milk in Tris-buffered saline and .1% Tween 20. FGF-2 primary antibody (Santa Cruz, [Santa Cruz, CA] #sc-7911) was diluted 1:1000 in the same solution as was used for blocking. Membranes were incubated in primary antibody for 2 hours followed by 5 washes in PBS (5 mins.) and 1 hour incubation in secondary antibody (HRP- linked donkey anti-rabbit; Amersham [Piscatawy, NJ] #NA934V; 1:5000). Following 5 more PBS washes, FGF-2 protein was revealed with an ECL+ detection kit from Amersham (#RPN2132) and the resulting image was captured on Hyperfilm ECL (Amersham #RPN1647K). Other antibodies used were Flg (Santa Cruz #sc-121; 1:1000), GR(Santa Cruz #sc-1004; 1:5000), PCNA (Santa Cruz #sc-56; 1:1000), bcl-2 (Santa Cruz #sc-783; 1:1000), and GFAP (Sigma [St. Louis, MO] #G3893; 1:10,000). The PCNA and GFAP antibodies were raised in mouse and thus HRP-linked sheep anti-mouse secondary antibody (Amersham # NA931) was used. Exposed film was imaged with a Kodak digital camera and the blot density was then analyzed using NIH Image software. The membrane was then stained with .1% Commassie Blue (OmniPure EM Science) to reveal all proteins in order to ensure pipeting consistency. A random protein band was selected for each blot and the amount of target protein was calculated as the density of the target / density of random sample protein in the same well. This method allowed compensation for any pipetting errors that may have occurred.

5.7. ANATOMICAL ANALYSES

5.7.1. Cortical thickness Measurements

Cortical thickness measurements were obtained from Cresyl violet stained coronal sections projected on a Zeiss-Jena MF2 projector at a magnification of 20X (following the method described by Stewart and Kolb, 1988). Briefly, three cortical measures were made at points medial, central and lateral on five sections of tissue identified by the following landmarks; Plane 1: first caudate-putamen visible, Plane 2: anterior commissure, Plane 3: first hippocampal section, Plane 4: posterior commissure, Plane 5: last hippocampal section. A plastic metric ruler was used to measure from the edge of the cortex to the edge of the white matter. An average for each plane and for each animal was calculated and used for statistical comparison.

5.7.2. Assessment of Lesion Size

Lesion size was estimated for all animals in lesion groups from whole brain pictures taken with a digital camera. The digital images were opened in the Scion Image program then the lesion traced around its perimeter and the area analyzed. The same procedure was repeated for the whole brain (including the lesion area but excluding the cerebellum) and then the ratio of lesion/brain was calculated for an estimation of lesion size.

5.7.3. Assessment of Thalamic Size

Thalamic cross-sectional area was measured from two coronal sections stained with Cresyl violet using a Kodak digital camera to capture the image and the Scion Image program to measure thalamic area. One measure was taken of the anterior thalamus

(approximately -1.80mm from the Bregma). The second measure was made in posterior thalamus at approximately -4.30mm from the Bregma (as described in a study by Kolb and Whishaw (1981)).

5.7.4. Acetylcholinesterase Quantification

Acetylcholinesterase (AChE) staining was assessed using the Scion Image densitometry program. The tissue was placed on a Zeiss microscope using a 20X objective and the image captured on computer with a video camera. The lighting levels for photography were held constant through the entire sampling period to minimize any variations in density measures. The same planes as were used for cortical thickness measurements were analyzed for AChE density. An average density was then computed for each plane for each animal and used for statistical comparison.

5.7.5. Golgi-Cox Analyses

Layer III pyramidal cells in Zilles' area Par 1 were traced using a camera lucida at 250X. In order to be included in the data analysis, the dendritic trees of pyramidal cells had to fulfill the following criteria: (a) the cell had to be well impregnated and not obscured with blood vessels, astrocytes, or heavy clusters of dendrites from other cells; (b) the apical and basilar arborizations had to appear to be largely intact and visible in the plane of section. The cells were drawn and analyzed using two different procedures. In the first, each branch segment was counted and summarized by branch order using the procedure of Coleman and Riesen (1968). Branch order was determined for the basilar dendrites such that branches originating at the cell body were first order; after one

bifurcation, second order; and so on. Branch order was determined for the apical dendrites such that branches originating from the primary apical dendrite were first order and so on. In the second, a Sholl analysis for estimation of dendritic length (Sholl, 1956) was performed. For this analysis a transparent overlay of concentric circles spaced 20 μm apart was placed over the neuron drawing by centering the innermost ring in the middle of the cell body. The number of dendrite-ring intersections was counted for each ring and the total number used to estimate total dendritic length in μm (number of intersections X 20). Five cells were drawn in each hemisphere of each rat. The statistical analyses were done by taking the mean of the measurements on the five cells for each hemisphere of each subject.

Spine density was measured from one apical dendritic branch in the terminal tuft and one basilar terminal branch. Spine density measures were made from a segment greater than 10 μm in length, and usually about 50 μm . The dendrite was traced (1000X) using a camera lucida and the exact length of the dendritic segment calculated by placing a thread along the drawing and then measuring the thread length. Spine density was expressed as the number of spines per 10 μm . No attempt was made to correct for spines hidden beneath or above the dendritic segment so the spine density values are likely to underestimate the actual density of the dendritic spines.

5.8. STATISTICAL ANALYSES

All statistical analyses were ANOVA's performed on Statsview 5®. If an ANOVA did not show a significant effect of sex, the data were collapsed across this variable to increase the number of subjects per group and to simplify the analysis.

5.9. BEHAVIOURAL RESULTS

5.9.1. Morris Water Task

5.9.1.1. Latency. With the Morris Water Task, latency to find the hidden platform has been shown to be an effective measure of cognitive impairment in P4 frontal cortex lesion animals. Animals with lesions of this type required more time to find the hidden platform than did their littermate controls. Behavioral performance was enhanced in P4 lesion animals that had prenatal complex housing experience (see Figs. 5.2 and 5.3). A two-way ANOVA revealed a main effect of lesion ($F(1,57) = 18.45, p < 0.0001$) and treatment ($F(1,57) = 7.564, p = 0.008$) whereas the interaction of Lesion X Group was not significant. This result reflected the impairment shown by the P4 lesion animals at finding the hidden platform in the water maze and the effectiveness of prenatal complex housing in reversing that impairment. Further, a Fisher's PLSD was performed and this analysis revealed that although the condomom P4 lesion animals were still relatively impaired when compared to their control (sham-operates) cohorts ($p = 0.03$), they showed no impairment relative to the cagemom control group ($p = 0.33$) and were significantly better than the cagemom lesion animals ($p = 0.0009$).

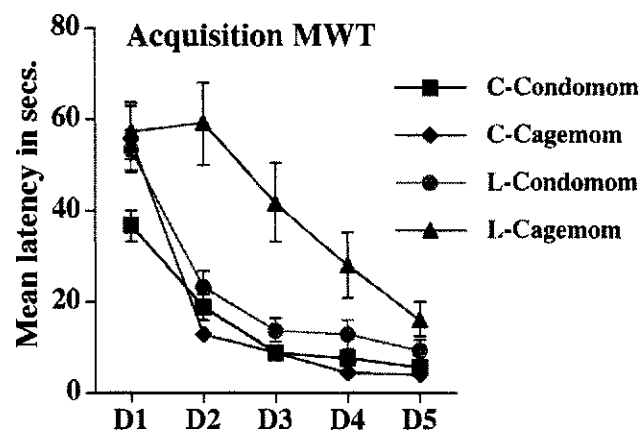


Fig. 5.2. Acquisition of learning the location of a hidden platform in the Morris Water Task. Latency is reported in seconds.

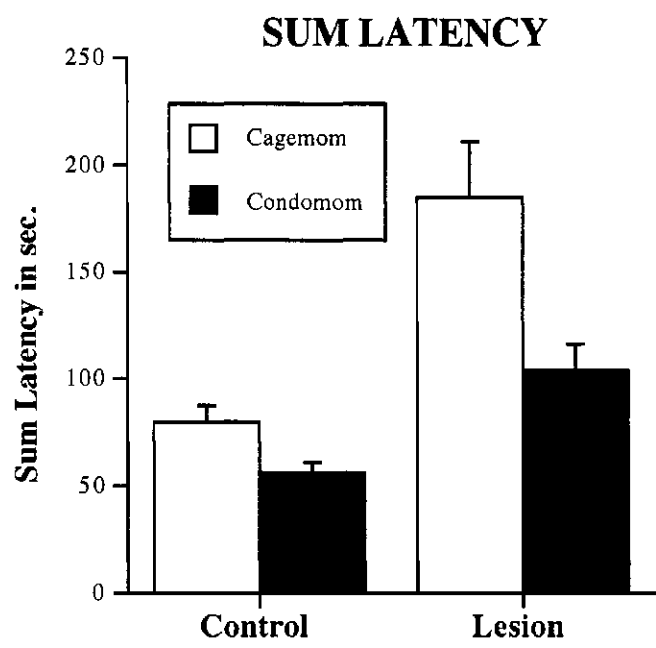


Fig. 5.3. Latency in seconds to find a hidden platform averaged and summed over 5 days of testing.

5.9.1.2. Distance. The distance the rats swam was also recorded and a similar trend was noted in the distance measures as was observed for the latency measures. The untreated P4 lesion animals had the longest swim distances; the P4 condomom animals swam much less; and the control animals swam the least. A two-way ANOVA revealed a significant effect of lesion ($F(1,55)=22.5$, $p<0.0001$) and treatment ($F(1,55)=13.3$, $p=0.0008$) as well as the Lesion X Treatment interaction ($F(1,55)=5.8$, $p=0.02$). The interaction reflected the observation that prenatal complex housing improved the performance of the lesion animals much more than the effect seen on the control animals (Fig. 5.4). Post hoc analysis of the data revealed that the distance the P4 condomom animals swam was significantly less than the distance swum by the cagemom P4 animals ($p<0.0001$) and the P4 condomom animals did not differ from the cagemom controls ($p=0.46$).

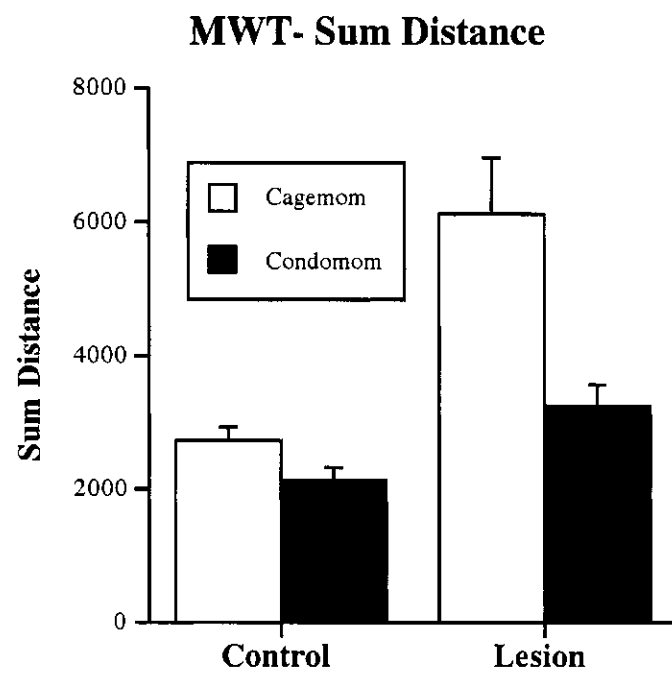


Figure 5.4. Total distance swam (in arbitrary computer units) searching for a hidden platform in the Morris water task.

5.9.2. Whishaw Tray Reaching

Lesion animals showed impairments in successfully retrieving pellets for consumption when compared to littermate controls. A two-way ANOVA with lesion X treatment as factors, showed that there was a main effect of lesion ($F(1,55) = 39.8$, $p < 0.0001$). No main effect was seen for treatment ($F(1,55) = 1.81$, $p = 0.18$), but the interaction of Lesion X Treatment was significant ($F(1,55) = 5.04$, $p = 0.01$). The Lesion by Treatment interaction reflected the observation that although prenatal complex housing did not improve the performance of control subjects, P4 lesion animals did benefit from the treatment (Fig. 5.5). A post hoc Fisher's PLSD indicated that prenatal complex housing significantly improved performance of lesion animals ($p = 0.016$).

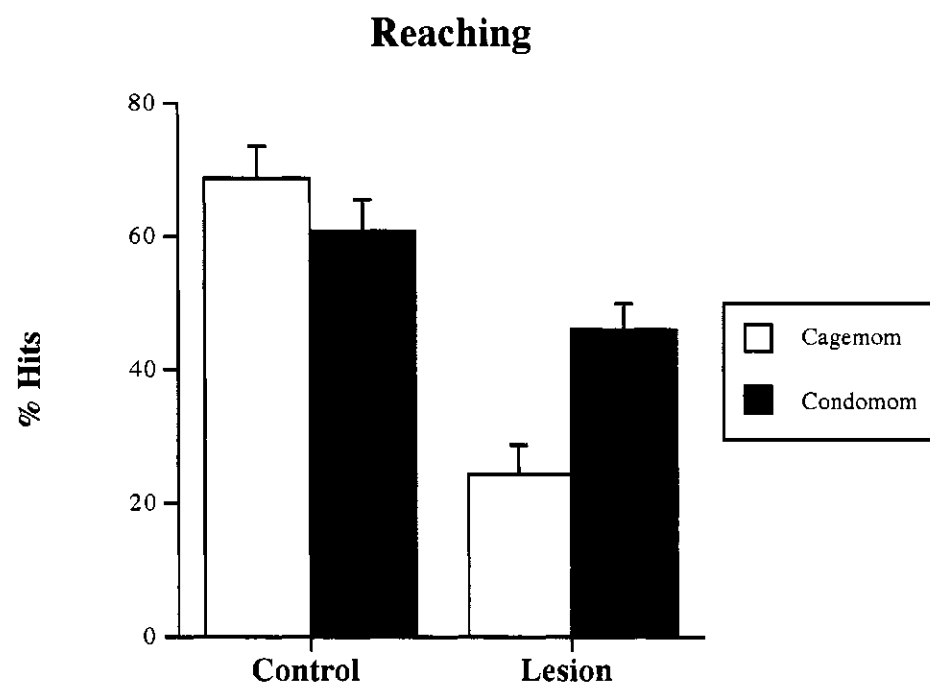


Fig. 5.5. Percent successful reaches versus total number of attempts.

5.9.3. Circadian Activity

Circadian activity patterns were monitored over a 24-hour period to determine if a shift in species-typical activity may have resulted from the lesion or the environmental treatment. During the activity monitoring sessions a number of cages failed to record data so the results presented are based on the data gathered from animals placed in functional activity monitoring cages. Males were not as active as females and the condomom animals were more active than their cagemom cohorts. A three-way analysis of variance (lesion X sex X treatment) performed on the total number of cage crosses in a 24 hour period revealed that the lesion effect was not significant ($F(1,31) = 0.65, p=0.43$) but there was a significant main effect of sex ($F(1, 31) = 4.3, p=0.046$) and treatment ($F(1,31) = 5.32, p=0.028$) on activity. A repeated-measures ANOVA performed on the

same data showed that there was an interaction of treatment with activity over the 24 hour period ($F(23, 713) = 3.92, p < 0.0001$). Closer inspection of the data showed both male and female condomom animals to be more active in the second half of the 24 hour period. A three-way analysis of variance (lesion X sex X treatment) on activity during the first twelve hours showed no main effects for any of the factors (F 's < 2.8 and p 's > 0.1). There was, however, a significant Lesion X Sex interaction ($F(1,31) = 6.6, p = 0.015$). The lesion males were less active than the control males but the lesion females were not.

A preliminary three-way ANOVA for the second twelve hour period (corresponding with 10:00 p.m. to 10:00 a.m.) showed once again that there was no main effect of lesion ($F(1,31) = 0.013, p = 0.9$) or any interactions so the data were collapsed across lesion. The effects of sex ($F(1, 35) = 7.2, p = 0.011$) and treatment ($F(1,35) = 18.8, p = 0.0001$) were robust. The females overall showed higher activity levels than did the males and the condomom animals showed higher activity levels than the cagemom animals across all groups (Fig. 5.6). None of the interactions reached statistical significance.

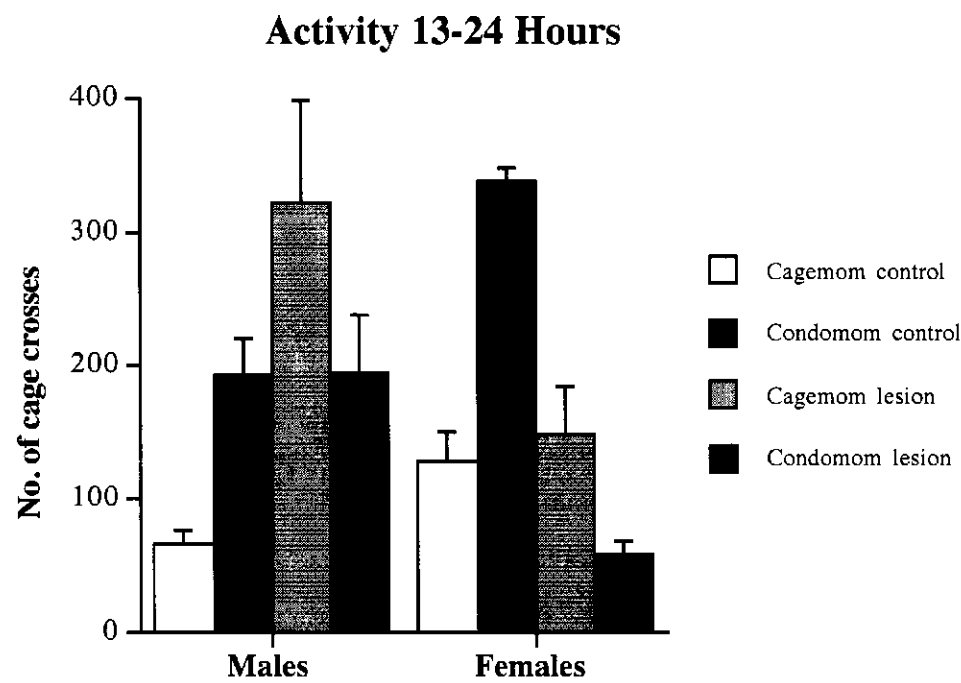


Fig. 5.6. Total number of cage crosses in during the second 12- hour period of testing (corresponding from 10:00 p.m. to 10:00 a.m.).

5.10. ANATOMICAL RESULTS

5.10.1. Body Weight

Statistical analysis of adult body weight using a three-way ANOVA (lesion X sex X treatment) revealed that sex was the factor that had the greatest influence on body weight ($F(1, 58) = 49.6, p < 0.0001$) (Table 5.1). The lesion animals showed reduced body weight ($F(1, 58) = 4.24, p = 0.044$) when compared to controls but treatment showed no main effect on body weight ($F(1, 58) = 1.67, p = 0.20$). The only interaction in this analysis that was significant was the Lesion by Sex by Treatment interaction ($F(1, 58) = 3.93, p = 0.05$). This result reflected the trend for the condomom control males to be heavier than the cagemom control males and the condomom lesion female animals to

show a similar increase in body weight. In both cases the difference was in the order of 40-50 grams.

Table 5.1. Summary of body weights

Group	Experience	
	Prenatal Condo	No Treatment
Con. Males	414.2±21.1	365.3±8.7
P4 Males	332.4±33.5	357.8±22.4
Con. Females	281.4±8.6	274.6±18.0
P4 Females	287.0±4.6	247.4±11.4

Numbers refer to means ±standard errors in grams

5.10.2. Brain Weight

Exposure to complex housing during the prenatal period of development had no effect on brain weight in adulthood. A three way ANOVA with lesion, sex, and treatment as factors revealed a main effect of lesion ($F(1,58) = 84.4, p < 0.0001$) wherein the lesion animals had a reduced brain weight compared to controls and a main effect of sex ($F(1,58) = 8.26, p = 0.0056$) as male rats had larger brains than their female counterparts (Table 5.2). This result reflects the sexual dimorphism normally seen in brain weight. There was no main effect of treatment ($F(1,58) = 1.79, p = .187$), however, nor were any of the interactions significant (p 's > 0.3).

Table 5.2. Summary of brain weight

Group	Experience	
	Prenatal Condo	No Treatment
Con. Males	2.009±0.027	1.958±0.007
P4 Males	1.752±0.034	1.743±0.054
Con. Females	1.909±0.025	1.900±0.064
P4 Females	1.716±0.017	1.652±0.053

Numbers refer to means ±standard errors in grams

5.10.3. Cortical Thickness

To determine regional effects of lesion and treatment the analysis of cortical thickness was divided into 2 groups. The first group included anterior planes 1 and 2, and the second group included posterior planes 3-5. The P4 frontal lesion reduced cortical thickness across all planes measured whereas cortical thickness was increased in the anterior sections by condomom treatment.

A two-way ANOVA on the anterior planes revealed a significant main effect of lesion ($F(1,74)=110.0, p<0.0001$) and treatment ($F(1,74)=18.7, p<0.0001$). The interaction of Lesion X Treatment was also significant ($F(1,74)=7.4, p=0.008$). The interaction reflected the finding that the lesion caused less reduction in cortical thickness in the condomom animals than in the cagemom animals (Table 5.3).

A two-way ANOVA on the posterior planes revealed a significant effect of lesion ($F(1,81)=64.2, p<0.0001$) but not treatment ($F(1,81)=0.06, p=0.81$) nor the interaction ($F(1,81)=0.095, p=0.76$).

Table 5.3. Summary of Cortical Thickness at Anterior and Posterior Planes

Planes	Group	Experience	
		Prenatal Condo	No Treat.
1-2	Con	42.2±0.5	41.3±0.4
	P4	38.0±0.6*	34.2±0.5
3-5	Con	34.1±0.3	34.1±0.4
	P4	31.5±0.2	31.4±0.4

Numbers refer means± standard errors in mm.

*differs significantly from No Treatment values in same lesion group

5.10.4. Lesion Size

A one-way ANOVA revealed that the prenatal complex housed P4 operates had significantly smaller lesions than did the untreated P4 operates ($F(1,35)=6.72$, $p=0.014$). The condomom animals had lesions that averaged 8.0% of the cerebral hemispheres whereas the cagemom animals had lesions that averaged 17.8% of the cerebral hemispheres. This difference in lesion size is not likely due to variations in the size of lesion made at the time of surgery. The surgeon (B.K.) has more than 30 years experience in making this type of lesion and was blind to the treatment conditions of the animals at the time of the surgery.

5.10.5. Acetylcholinesterase Quantification

Acetylcholinesterase histochemistry was performed on a subset of animals that included only sham-operates from the two treatment groups. A repeated-measures ANOVA on the sham-operated animals revealed that there was no main effect of treatment on acetylcholinesterase expression ($F(1,64) = 0.194, p=0.66$) but there was a significant interaction of treatment and plane ($F(4,64) = 3.857, p=0.0071$). This result reflected the decrease in AchE expression in the condomom animals across all planes except plane 2 (Table 5.4).

Table 5.4. Summary of AchE Density Measures

Plane	Percent Untreated Control
1	99.2
2	103.8
3	98.5
4	98.7
5	96.1

Numbers refer to % untreated control values obtained at the same plane

5.10.6. Thalamic Measures

The P4 lesion caused a reduction in thalamic area in the cagemom animals. The prenatal condo-housed animals showed a larger thalamic area in both control and lesion groups than did the untreated animals (Table 5.5). At the anterior plane of measure, a two way ANOVA showed a significant main effect of lesion ($F(1,47)=5.6, p=0.022$) and

treatment ($F(1,47)=22.1$, $p<0.0001$) but no interaction ($F(1,37)=3.05$, $p=0.087$). Although the interaction was not significant there was an obvious trend for the P4 lesion to reduce thalamic area less in the condomom group than in the cagemom group.

Table 5.5. Summary of Thalamic Area in the Anterior plane

Plane	Experience	
	Prenatal Condo	No Treatment
Control	108.9	100.0
P4 lesion	107.1	87.6*

Numbers refer to percent of No Treatment control values

*differs significantly from No Treatment control values

A two-way ANOVA on the thalamic measures derived from the posterior plane showed no significant effect of lesion ($F(1,47)=2.07$, $p=0.16$) but the effect of treatment was highly significant ($F(1,47)=23.0$, $p<0.0001$). The interaction of these factors was not significant ($F(1,37)=0.33$, $p=0.57$). As was observed in the anterior plane, the prenatally complex-housed animal showed larger thalamic areas than did the untreated animals (Table 5.6).

Table 5.6. Summary of Thalamic Area in the Posterior plane

Plane	Experience	
	Prenatal Condo	No Treatment
Control	116.2*	100.0
P4 lesion	112.8*	92.4

Numbers refer to percent of No Treatment control values

*differs significantly from No Treatment control values

5.10.7. Golgi-Cox Analysis

5.10.7.1. Branch Order

5.10.7.1.1. *Apical Dendrites.* In order to gain a better understanding of where changes in neuronal morphology might be occurring, the branch order analysis was divided into two areas: branching near the soma (branches 1-3) and branching near the terminal fields of the dendrites (branches 4-6+). A two-way ANOVA with lesion and treatment as factors, on branches near the soma (1-3) revealed a significant main effect of lesion ($F(1,72)=7.2$, $p=0.009$) but not treatment ($F(1,72)=0.69$, $p=0.41$). The interaction of Lesion X Treatment was also non-significant ($F(1,72)=0.39$, $p=0.53$). The lesion effect was a reduction in branches as compared to the sham-operates.

A two-way ANOVA on branches near the terminal field (4-6+) revealed no effect of lesion ($F(1,72)=1.44$, $p=0.23$) but a main effect of treatment ($F(1,72)=39.5$, $p<0.0001$). The Lesion X Treatment interaction was not significant ($F(1,72)=0.325$, $p=0.57$). The

treatment effect reflected a dramatic increase in terminal branching in the prenatal complex housed animals as compared to the untreated animals (Table 5.7).

Table 5.7. Summary of Branch Order in the Apical Tree

Branches	Group	Experience	
		Prenatal Condo	No Treat.
1-3	Con	22.3±1.0	22.5±1.3
	P4	18.9±0.8	20.3±0.7
4-6+	Con	4.3±0.4*	2.2±0.4
	P4	4.1±0.3*	1.6±0.2

Numbers refer number of branches± standard errors

*differs significantly from No Treatment control values

5.10.7.1.2. Basilar Dendrites. A two-way ANOVA on branches 1-3 showed no main effect of lesion ($F(1,76)=0.175$, $p=0.68$) or treatment ($F(1,76)=0.614$, $p=0.436$). The interaction was also non-significant ($F(1,76)=0.711$, $p=0.402$).

Analysis of the terminal branches using a two-way ANOVA revealed a main effect of lesion ($F(1,76)=4.67$, $p=0.033$) but not treatment ($F(1,76)=2.83$, $p=0.097$) and the interaction was not significant ($F(1,76)=0.025$, $p=0.874$). The P4 lesion caused a reduction in branching compared to the sham-operates (Table 5.8).

Table 5.8. Summary of Branch Order in the Basilar Tree

Branches	Group	Experience	
		Prenatal Condo	No Treat.
1-3	Con	22.6±0.6	22.6±1.4
	P4	22.3±0.4	23.5±0.7
4-6+	Con	12.3±1.2	14.7±1.7
	P4	9.7±1.0*	11.7±1.3

Numbers refer number of branches±standard errors

*differs significantly from No Treatment control values

5.10.7.2. Dendritic Length

5.10.7.2.1. Apical Dendrites. The Sholl analysis of dendritic length was divided into 3 ranges. The first one covered rings 1-6 (dendrite crossings near the soma). The second covered rings 7-11 or ring crossings that occurred in the central portion of the apical tree and the third covered the last rings (12-16+). The third range allowed analysis in the terminal portions of the dendritic tree. A three-way ANOVA (Lesion X Sex X Treatment) on rings 1-6 showed a significant effect of lesion ($F(1,76)=16.65, p=0.001$) and treatment ($F(1,76)=7.124, p=0.0093$) but not sex ($F(1,76)=0.002, p=0.96$) on ring crossings. The P4 lesion animals showed a reduction in the number of ring crossings as compared to the sham-operates. Likewise, the condomom animals showed fewer ring crosses than the cagemom animals except the male control group. The Lesion X Sex interaction was also significant ($F(1,76)=6.02, p=0.016$), which reflected the tendency for the male operates to show a larger reduction in ring crossings than their female cohorts.

A three-way ANOVA performed on rings 7-11 revealed a main effect of lesion ($F(1,76)=18.83, p<0.0001$) but no main effects of either treatment ($F(1,76)=0.84, p=0.36$) or sex ($F(1,76)=1.692, p=0.20$). Again the lesion animals showed a reduction in ring crosses relative to the sham-operates. The Lesion X Sex interaction ($F(1,76)=4.15, p=0.045$) and Lesion X Treatment interaction ($F(1,76)=8.95, p=0.0037$) were significant. The Lesion X Sex interaction once again reflected the tendency for the male lesion animals to show a greater reduction in ring crossings than did the female lesion animals. The Lesion X Treatment interaction indicated that the condomom animals showed a more dramatic drop in ring crossings following lesion than did the cagemom animals.

Using a three-way ANOVA to analyze the rings 12-16+ revealed a main effect of lesion ($F(1,76)=11.87, p=0.0009$) but not sex ($F(1,76)=1.3, p=0.26$) nor treatment ($F(1,76)=0.527, p=0.47$). The Lesion X Sex X Treatment interaction was significant and reflected the finding that the condomom control males had the highest number of ring crosses followed by the cagemom control females. The condomom control females and cagemom control males had approximately the same number of ring crossings and the lesion animals in all groups had fewer ring crossings (Table 5.9).

Table 5.9. Summary of Sholl Analysis in the Apical Tree

Rings	Group	Experience	
		Prenatal Condo	No Treat.
1-6	Con	30.8±1.2	30.0±1.1
(male)	P4	21.7±1.4	25.4±1.6
1-6	Con	25.1±1.5*	30.3±1.5
(female)	P4	24.5±1.4*	25.6±1.7

7-11	Con	19.7±1.4	16.2±1.1
(male)	P4	13.2±0.9*	13.7±0.8*
7-11	Con	16.6±1.0	14.7±0.7
(female)	P4	12.8±0.6	15.2±0.9

12-16+	Con	43.5±2.5	33.6±1.7
(male)	P4	32.1±2.3	32.8±2.0
12-16+	Con	34.2±1.9	38.5±4.4
(female)	P4	30.8±1.5*	30.8±2.9*

Numbers refer number of ring crossings± standard errors

*differs significantly from No Treatment control values

5.10.7.2.2. Basilar Dendrites. In the basilar field the Sholl analysis was performed for rings 1-4 and rings 5-12 as the basilar dendrites were not as long as were the apical

dendrites. A similar pattern of results was observed for both areas of the basilar tree. A two-way ANOVA on rings 1-4 showed a significant main effect of both lesion ($F(1,84)=9.16, p=0.003$) and treatment ($F(1,84)=10.4, p=0.002$) but the interaction was not significant. The P4 lesion animals had shorter dendritic length than did the sham - operates and the condomom animals had shorter branches than the untreated animals (Table 5.10).

A two-way ANOVA on rings 5-12 showed a significant main effect for lesion ($F(1,84)=4.26, p=0.04$) and treatment ($F(1,84)=5.51, p=0.02$) but not the interaction ($F(1,84)=0.41, p=0.52$). Once again the lesion reduced the length of branches and so did the prenatal complex housing.

Table 5.10. Summary of Sholl Analysis in the Basilar Tree

Rings	Group	Experience	
		Prenatal Condo	No Treat.
1-4	Con	54.1±1.9*	61.9±2.9
	P4	48.9±1.1*	54.5±2.4*
5-12	Con	28.1±2.0*	34.1±2.7
	P4	25.2±1.2*	28.7±2.1*

Numbers refer number of ring crossings±standard errors

*differs significantly from No Treatment control values

5.10.7.3. Spines

5.10.7.3.1. Apical Dendrites. Analysis of spine density on the apical tree using a two-way ANOVA with lesion and treatment as factors revealed a significant effect of lesion ($F(1,58) = 4.26, p = 0.043$) and treatment ($F(1,58) = 6.69, p = 0.012$). The interaction of Lesion X Treatment was non-significant ($F(1,58) = 0.95, p = 0.33$). The P4 lesion caused an increase in spine density in the operate animals (Table 5.11). The effect of treatment was to increase spine density as well, so the animals that were prenatally condo-housed had the largest increase in spine density.

Table 5.11. Summary of Spine Density on the Apical

Group	Terminal Experience	
	Prenatal Condo	No Treatment
Control	6.32±0.24	5.95±0.20
P4 lesion	7.02±0.17*	6.20±0.30

Numbers refer to number of spines/10µm dendritic branch length

*differs significantly from No Treatment control values

5.10.7.3.2. Basilar Dendrites. A three-way ANOVA with lesion, treatment, and sex as factors revealed that there was no lesion effect ($F(1,54) = 0.035, p = 0.853$) on spine density on basilar dendrites but there was a main effect of sex ($F(1, 54) = 6.954$) and of treatment ($F(1,54) = 40.69, p < 0.0001$). None of the interactions reached the level of significance. Overall, females had a higher spine density than males and both males and females

exposed to prenatal complex housing had more spines than did untreated animals (Table 5.12).

Table 5.12. Summary of Spine Density on the Basilar Terminal

Group	Experience	
	Prenatal Condo	No Treatment
Control-male	7.31±0.24*	5.77±0.19
Control-fem.	7.58±0.21*	6.62±0.12
P4 lesion-male	7.29±0.41*	5.94±0.16
P4 lesion-fem.	7.69±0.45*	6.50±0.49

Numbers refer to number of spines/10µm dendritic branch length

*differs significantly from No Treatment control values of the same sex

5.10.8. Western Blot

Frontal cortex samples were harvested from control and lesion animals. In the control animals the sample included all cortical tissue anterior to +3.0 mm ahead of the Bregma. In the lesion animals the sample was taken from approximately the same location but only included the remaining lateral cortex including Frontal area 3 (Fr3) and Agranular insular cortex (AID) (Zilles, 1985). The olfactory bulb and nucleus accumbens regions were discarded in all samples.

Blots were run on frontal cortex samples to analyze the effects of treatment on control animals (condomom and cagemom control samples run on same blots); treatment

effects on lesion animals (condomom and cagemom P4 lesion samples run on same blots); lesion effects within condomom- housed litters (control and P4 lesion samples from condomom litters run on same blot). Westerns were run for the following proteins: Fibroblast growth factor-2 (FGF-2), a growth factor that stimulates mitosis and synaptogenesis in brain of both infant and adult animals and promotes survival of neurons following brain damage, FGF receptor1 (FGFR1 or Flg) a receptor for FGF-2, Glucocorticoid receptor (GR) a receptor for corticosterone and changes in its expression can reflect alterations in reactivity of the HPA axis in response to stress, Proliferating cell nuclear antigen (PCNA) a protein expressed in the G₁ and S phase of a cell undergoing mitosis, Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is found exclusively in glial cells (increased expression of GFAP implies proliferation of glial cells or increased reactivity of glia already present), Bcl-2 is a anti-apoptotic protein and increased expression implies a reduction in apoptotic processes. Treatment effects on the expression of bcl-2 protein were also analyzed on the blots run on lesion samples.

The ANOVA for FGF-2 showed no significant effect of treatment ($F(1, 10)=0.014, p=0.91$). The one-way ANOVA's performed for all the rest of the proteins run were significant: Flg ($F(1,10)=66.6, p<0.0001$), GR ($F(1,10)=207.8, p<0.0001$), PCNA ($F(1,10)=69.2, p<0.0001$), GFAP ($F(1,10)=10.7, p=0.0083$). In all cases except GFAP, protein expression was reduced in the prenatal complex- housed animals. For GFAP, the reverse pattern was observed (Table 5.13).

Once again FGF-2 did not differ in expression in condomom lesion animals and cagemom lesion animals ($F(1,10)=0.59, p=0.46$). Bcl-2 ($F(1,10)=1.07, p=0.33$) and

GFAP expression was not different between treatment groups ($F(1,10)=1.61$, $p=0.23$).

All other proteins examined showed significantly reduced expression in condomom treated animals as compared to controls: Flg ($F(1,10)=479.7$, $p<0.0001$), GR ($F(1,10)=36.8$, $p=0.0001$), PCNA ($F(1,10)=57.4$, $p<0.0001$).

In the analysis of lesion within the condomom-treated animals, sex proved to be a significant factor for only the GR blots. A two-way ANOVA on these data showed a main effect of lesion ($F(1,9)=10.3$, $p=0.01$) and a trend for sex differences ($F(1,9)=4.23$, $p=0.07$). The lesion reduced the expression of GR and females had a lower expression than did males. A post hoc analysis using Fisher's PLSD showed the effect of sex to be significant ($p=0.048$). The other proteins measured were analyzed for lesion only. GFAP expression was significantly different between the control and lesion animals ($F(1,11)=6.1$, $p=0.03$) with the P4 lesion animals showing the elevated expression of GFAP that normally accompanies damage to frontal cortex. There were no changes in expression of the other proteins measured as a result of the lesion: FGF-2 ($F(1,11)=0.93$, $p=0.35$), Flg ($F(1,11)=0.38$, $p=0.55$), PCNA ($F(1,11)=0.49$, $p=0.50$).

Table 5.13. Relative expression of protein in blot comparisons

Analysis	Condo vs. NT Control	Condo vs. NT P4 Lesion	Condo P4 Lesion vs. Condo Control
EGF-2	=	=	=
Flg	↓	↓	=
GR	↓	↓	↓
PCNA	↓	↓	=
GFAP	↑	=	↑
Bcl-2	n/a	=	n/a

5.11. DISCUSSION

There were 8 principal findings in this experiment. First, prenatal complex housing attenuated the deficits normally observed in spatial cognition and skilled reaching following P4 frontal cortex removals. Second, circadian activity was altered in the offspring of complex-housed mothers. Third, cortical thickness was increased at anterior planes of measure particularly in P4 lesion condomom animals. Fourth, lesion size was markedly reduced in adult condomom operates as compared to the adult cagemom operates. Fifth, there was a reduction in expression of acetylcholinesterase in condomom animals. Sixth, there was an increase in thalamic cross-sectional area at both anterior and posterior planes in the prenatal complex housed animals. Seventh, neuronal morphology in area Par1 layer 3 in condomom animals showed a number of changes including: an overall decrease in dendritic length in both the apical and basilar trees; an increase in branching in the terminal field of the apical dendrites; and an increase in spine density in both apical and basilar fields. Eighth, the expression of a variety of proteins was significantly altered in the condomom animals. Each result will be considered in turn.

Prenatal complex housing significantly improved the behavioral performance of P4 frontal cortex lesion animals on tasks of both cognitive (MWT) and motor (Whishaw tray reaching) abilities. Such improvement in performance implies the complex housing of the mothers acted as a prophylactic for the offspring that received frontal cortex lesions shortly after birth. What is not known is whether the enhanced behavioral performance of the animals resulted from a reduced lesion effect or a potentiated recovery.

Prenatal complex housing altered circadian activity by increasing activity levels from 10:00 p.m. to 10:00 a.m. Alterations in the circadian activity of animals born to complex-

housed mothers is a puzzle. Activity is a species-typical behavior and increases in activity are sometimes associated with brain damage or altered stress levels. Control condomom animals as well as their P4 lesion cohorts showed increased activity to the same degree, which demonstrates that brain damage was not a factor in this case. The changes in circadian activity observed in the condomom offspring may reflect alterations in corticosterone secretion controlled by circadian rhythm similar to effects observed following prenatal stress (Koehl et al., 1999). Further study to determine corticosterone levels in these animals would be instructive.

The increased cortical thickness observed in control and P4 lesion condomom animals on the anterior planes suggests that prenatal complex housing has changed the anatomy of the anterior portions of the cerebral cortex and has made this part of the brain less susceptible to the damaging effects of early frontal cortex lesion. Although there was some thinning of the anterior cortex in the condomom lesion animals the loss of neuropil particularly in areas near the lesion was not as dramatic as that observed in the cagemom animals.

The finding that lesion size was considerably smaller in prenatal complex housed animals suggests that there may have been a reduction in apoptotic cell death near the lesion site. Alternatively, the prenatal complex housing may have changed the expression of growth factors in the brain and as a result stimulated postnatal neuronal proliferation to partly compensate for the tissue loss. We have shown previously that rats with medial frontal lesions around days 7-10 do show spontaneous neurogenesis (e.g. Kolb et al., 1998) so it is possible that the prenatal treatment has somehow made the brain

respond more like a brain with a lesion in the second week of life, but this remains conjecture at this point.

AchE expression was reduced at every plane measured except plane 2 in condomom animals. Environmental enrichment has been associated with increases in acetylcholine expression in weanling and adult animals thus housed. The finding that AchE expression drops after maternal complex housing seems counterintuitive. However, prenatal dietary choline supplementation also induces a paradoxical drop in AchE expression for up to four weeks postnatally (Yang et al., 2000; Cermak et al. 1998; Halliwell, et al., 2004). This decrease in AchE expression may allow higher acetylcholine availability at the synapse and longer acetylcholine-receptor interaction. Both of these events could have a positive effect on synapse strength and efficacy.

Thalamic area increased at both anterior and posterior planes in condomom animals. In a study by Higashi et al. (2002) thalamocortical synaptic connections were reported to be functional by E19 in rats. The authors suggest that these prenatal thalamocortical connections can influence cortical circuitry before birth. We are unaware of previous studies showing increases in thalamic volume or cross-sectional area in response to behavioral treatments. Increased thalamic area might be partly responsible for the thicker cortex and could be related to the smaller lesion cavities in the lesion animals. The increased thalamic area could also be related to the behavioral advantages seen in the condomom offspring.

The Golgi results suggest that the intrinsic cortical organization may be fundamentally different in the animals born to prenatally complex-housed mothers. Thus, both the general topology and dendritic length was altered by the treatment.

Specifically, branch order in the terminal apical branches was increased in both control and lesion control animals whereas the dendritic length was reduced. Furthermore, spine density was increased on both apical and basilar branches of the control animals.

Finally, the Western blot results suggest that the prenatal experience can have a profound effect on the production of a wide variety of proteins. It is difficult to relate such changes directly to any behavioral or anatomical changes but it is a reasonable assumption that both behavioral and anatomical changes will ultimately be related to changes in gene expression and protein synthesis. The drop in expression of some protein markers that we normally associated with improved functional outcome (e.g., bcl-2, GR, PCNA, and flg) after prenatal control experience was a surprise. One possible difficulty with the Western blot results is that we chose to measure changes at 21 days of age. One could imagine that there might be a different pattern of protein changes in the early days after the injury. This could be especially important for measures such as PCNA and bcl-2 that are thought to reflect cell generation and apoptosis, respectively.

Taken together the behavioral and anatomical results suggest that prenatal experiences can produce a fundamentally different brain. This brain can be presumed to have a different synaptic organization and thus it is not surprising that there may be a different response to cortical injury in animals with different prenatal experiences. Although we have previously emphasized the relationship between dendritic morphology and enhanced recovery from perinatal lesions (e.g., Kolb & Gibb, 1993), such a simple hypothesis cannot account for the current results. There could be changes in cell generation (both neurons and glia), apoptosis, growth factor production, stress responses

and so on, all of which may play a role in the observed behavioral and anatomical changes.

The key question that now must be addressed is what it might be about the experience that alters the brain and what the mechanisms underlying the changes might be. An understanding of these changes should provide a strategy to understanding basic brain-behavior relations as well as developing new treatments to modulate recovery from brain injury in both the young and adult-injured brain.

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CHAPTER 6
GENERAL DISCUSSION

The primary goal of the current work was to determine how pre- and postnatal environmental interventions might be used to improve behavioral recovery after early brain damage. A second goal was to determine what the mechanisms underlying the beneficial (or detrimental) effect of the environmental interventions might be. The studies took advantage of a model of early brain injury in which there is limited spontaneous behavioral recovery, namely cortical injury in the first few postnatal days in the rat. The model provided an opportunity to examine a range of treatments, including both pre- and postnatal experiences. Behavioral recovery and its morphological correlates were then assessed in adulthood.

Perhaps the most impressive finding that resulted from these studies is that prenatal experiences can be prophylactic for perinatal brain injury. The experience-induced protection seems to arise from changes in the anatomical organization of the brain but the relationship between the alterations in anatomy and the resultant behavior is not yet clear. A great deal is known about the negative effects of prenatal stress on brain development (Salm, Pavelko, Krouse et al., 2004) and subsequent behavior (Weinstock, 2001). Similarly, maternal diet during pregnancy is also known to affect the organization of the brain and its response to later brain injury. For example, prenatal choline supplementation through maternal diet protects against later neural insult (Guo-Ross, Clark, Montoya et al., 2002; Halliwell, Tees, & Kolb, 2004; Yang, Liu, Cermak, et al., 2000). In contrast, Experiments 3 and 4 provide the first demonstration that prenatal *experience* can protect the brain from later brain injury.

The experiments described in this thesis also detail several other important findings. First, the skin can act as an important interface between sensory experience and

brain repair. Second, perinatal experiential therapies change brain organization and behavior. Third, there are sex differences in response to experiential stimulation. Fourth, damaged brains sometimes respond differently than normal brains to environmental treatment. Fifth, early environmental intervention alters HPA responsiveness and may ultimately have an impact on functional recovery. Sixth, some behavioral deficits are more resistant to remediation than are others. I shall consider each of the major findings separately before considering the mechanisms underlying the experience-dependent effects and then concluding with a vision of what directions my future research might take.

6.1. NOVEL FINDINGS

6.1.1. The skin acts as an interface for sensory experience and produces proteins that may be useful in stimulating recovery after brain injury.

Postnatal tactile stimulation is effective in stimulating cortical plasticity and functional recovery and this effect is derived, at least in part, from the production of FGF-2 in the skin. This conclusion is supported by the finding that animals that received postlesion tactile stimulation showed increased expression of FGF-2 in remaining frontal cortex, increased expression of flg protein in parietal cortex, and enhanced behavioral outcome. These results imply that production of brain-responsive proteins by the skin can influence their expression in the CNS. Interestingly, epidermal growth factor (EGF) is another protein produced by skin that plays a role in cell mitosis in the CNS. Although we have not characterized changes in skin levels of EGF expression after tactile stimulation, it is feasible that EGF may undergo changes in expression similar to that

observed for FGF-2. If so, we can hypothesize that the tactile stimulation may have led to neuro- or gliogenesis, and that the generation of new cells played a role in the observed functional recovery.

6.1.2. Experiential therapies change behavior and brain organization

Postnatal tactile stimulation has a tremendous impact on the functional recovery of animals with perinatal cortical injury as well as the behavior of sham-operates. For example, tactilely-stimulated animals with early lesions of frontal cortex show behavioral improvements on the water task and increased accuracy on the reaching task following tactile stimulation. Similarly, stimulated-sham operates show improved accuracy on skilled reaching relative to unstimulated controls. Anatomical changes also result from this early stimulation. Brain weight is increased following tactile stimulation whereas the effect of tactile stimulation on spine density is lesion dependent. Lesion animals showed increased spine density following postnatal tactile stimulation whereas spine density is reduced in both the apical and basilar neuronal fields of sham-operates. Normally, reduced spine density is associated with functional loss but this is not the evident with the sham-operated animals. Although loss in spine density usually indicates synaptic loss, this may not always hold true. There is a possibility that the early stimulation is preventing some of the normal neuronal loss caused by apoptosis in the developing brain. For instance, in a study done by Young, Lawlor, Leone, Dragunow, and During (1999), the effect of environmental enrichment on apoptosis in the hippocampus was studied. Juvenile rats placed in complex housing showed a 45% decrease in spontaneous apoptosis in the granule cell layer of the hippocampus after only three weeks of exposure.

This result shows that experience can modify cell death and supports the notion that environmental enrichment may reduce spontaneous apoptosis in neocortical areas as well. If more neurons were available to make contacts with other cells the number of spines per cell could be reduced but total synapse number might actually be increased. Further investigation of the possibility of experience-dependent changes in neuron number would be needed to address this possibility, however. Subcutaneous administration of FGF-2 increased behavioral recovery on cognitive tasks and causes changes in neuronal organization (Table 6.1). Although the observed effects of FGF-2 on behavioral recovery were not as dramatic as those of tactile stimulation, it might be the case that the FGF effects are dose-dependent and the tactile stimulation induced more FGF-2 than was injected. The effects of FGF-2 on neuronal morphology were similar to those seen with tactile stimulation, with the exception of the changes in spine density. Thus, tactile stimulation caused a *decrease* in spine density in sham-operates whereas FGF-2 caused an *increase* in sham operates. It is difficult to reconcile the qualitative differences in the spine density changes, although they could be related to the putative effects of tactile stimulation on either neurogenesis or apoptosis. Postnatal handling did not offer the same therapeutic benefit as did postnatal tactile stimulation for animals with early cortical injury, but it was not without effect (Table 6.1). Normal females showed significantly improved accuracy on the skilled reaching task whereas lesion males had a diminished performance as compared to untreated lesion males. Water maze acquisition was somewhat impaired in the postnatally handled lesion animals and their latency scores remained higher than untreated animals in the last few days of testing. Both male and female lesion animals showed significantly reduced overall circadian activity following

the postnatal handling treatment. Postnatal handling is known to reduce basal levels of corticosterone (Denenberg, Brumaghin, Haltmeyer, et al., 1967; Meaney, Mitchell, Aitken, et al., 1991) and, as a result, produces animals that show superior adaptive responses to stress. It is thought that task-induced stress may interfere with performance on some tests of skilled reaching, particularly in females (Metz, Gonzalez, Piecharka et al., 2003) and it could be that the improved performance seen in the control female group is a result of the enhanced responsiveness of these animals to stressful situations. It is likely that the reduced circadian activity seen by postnatally-handled lesion animals is also a reflection of the altered responses to stress.

Anatomically, postnatal handling had less effect upon brain development than the tactile stimulation or FGF-2 treatment. The only reliable change was a reduction in neuronal branch length that occurred independently of changes in spine density. The decline in dendritic length, coupled with the unchanged spine density, suggests that the handling had a different effect on brain morphology than the tactile stimulation. The simplest explanation is that tactile stimulation may have altered neurogenesis or apoptosis whereas the handling did not. This remains to be shown, however.

Table 6.1. A comparison of the behavioral and anatomical effects of postnatal experiential therapies.

Treatment	MWT		Reaching		Dendrit. Length		Spine Density	
	Sham	P4	Sham	P4	Sham	P4	Sham	P4
Tactile stim.	=	↑	↑	↑	↓	↓	↓	↑
FGF-2	=	↑	=	=	↓	↓	↑	↑
Handling	=	=	↑	=	↓	↓	=	=

Prenatal tactile stimulation and complex housing also had a potent effect on the functional recovery of animals that sustained an early brain injury but there were differences in the behavioral and anatomical effects of these (Table 6.2). Although both treatments proved effective in improving functional outcome after the P4 lesions, the condom treatment had a greater impact on stimulating behavioral and anatomical recovery than the petmom treatment. Nonetheless, the prophylaxis for behavioral outcome following perinatal cortical injury demonstrated by both treatments was impressive and larger than the postnatal treatments. It may be that the fetal animal is at the developmental stage that possesses the most potential for cortical plasticity and, under certain conditions, this potential can be expressed. The idea that the prenatal period is especially plastic is supported by the finding that brain damage at E18 results in virtually normal behavior despite persistent abnormal brain morphology (Kolb, Cioe, & Muirhead, 1998). One reason for this plasticity may be that because the cortex is still generating cortical neurons until about E22, it is relatively easy for the brain to replace lost neurons, and for the neurons to migrate to the appropriate locations, when the brain is damaged prenatally. Similarly, it is possible that neuronal generation can be influenced by other

prenatal experiences. I cannot rule the possibility that there is some experience-dependent change in gene expression as well, however, and that such a change leads to later effects on brain development or in the brain's response to experience.

Table 6.2. A comparison of the behavioral and anatomical effects of prenatal experiential therapies.

<u>Treatment</u>	<u>MWT</u>		<u>Reaching</u>		<u>Ctx Thick</u>		<u>ThalX-sec</u>		<u>Den.Leng</u>		<u>SpineDen.</u>	
	Sham P4		Sham P4		Sham P4		Sham P4		Sham P4		Sham P4	
Petmom	=	↑	=	↑	=	=	↑	=	↓	↓	↑	↑
Condomom	↑	↑	=	↑	=	↑	↑	↑	↓	↓	↑	↑

6.1.3. There are sex differences in response to experiential stimulation

The effect of gonadal hormones on the organization of the brain begins before birth and continues throughout life. According to McEwen (1999) “testosterone secretion during embryonic, neonatal, peripubertal and adult life masculinizes and defeminizes the brain. Estrogen actions in the female brain activate functions that have been allowed to develop in the absence of testosterone... experiences during the lifespan interact with the hormone actions ...”. Thus, experiential treatment may have different consequences on both behavior and brain morphology of males and females. In Experiment 2, handling improved the reaching performance of control females and had no effect on reaching in males. Prenatal tactile stimulation (Experiment 3) produced the most sexually divergent results of all the reported experiments. Whereas both male and female lesion offspring showed a functional benefit of prenatal tactile stimulation on the

water task, males alone show enhanced performance on skilled reaching. Control males also showed reduced circadian activity following prenatal stimulation. Although prenatal stimulation had no effect on brain weight, both control and lesion males showed increased body weight as adults. Acetylcholinesterase expression also increased in animals exposed to prenatal stimulation and the expression was sex-dependent. Male animals showed denser AchE staining following prenatal stimulation than did females. One of the most dramatic sex differences noted in this experiment was the divergent glucocorticoid response to prenatal tactile stimulation. Whereas prenatally-stimulated males showed a similar reduced response in basal glucocorticoid levels to that seen in postnatally stroked (Gibb, 2001) and postnatally handled animals (Gibb and Kolb, 2004b), prenatally-stroked females showed a markedly *elevated* response. A response in this direction could have negative implications for these females as hippocampal neurons are susceptible to long-term elevation of glucocorticoids (Meaney, Aitken, van Berkel, et al. 1988). Thus, during the aging process, continued glucocorticoid hypersecretion causes negative feedback insensitivity and loss of hippocampal neurons. These deficits form a self-perpetuating cascade that results in accelerated hippocampal damage and spatial memory impairments in senescence.

6.1.4. Damaged brains respond differently than normal brains to environmental treatment

Environmental stimulation interacts with brain injury and as a result brain morphology in lesion animals is sometimes different than that observed in normal animals following treatment. This result is not surprising when one considers the potency

of early injury in defining resultant brain architecture. Although altered connectivity may help sustain functional improvement after injury by replacing lost connections with new or altered contacts, changes in connectivity may not be beneficial. For example, Kolb, Gibb & van der Kooy (1994) found that rats with P4 frontal lesions show extensive aberrant connectivity while having significant behavioral abnormalities (Kolb et al., 1994). Postlesion experience may thus have an altered template to act upon relative to animals with intact brains. We can hypothesize that one effect of the postinjury experience is to modify the abnormal circuits, possibly by enhancing pruning of aberrant connections. Indeed, one of the abnormalities in neuronal connectivity in the P4 lesion animals appears related to a failure of the normal processes of pruning to eliminate exuberant connections (Kolb et al., 1994).

A similar logic may account for the effects of prenatal experience on the injured brain. If brain development is altered by the prenatal experience, the injury is acting to change a different brain than the brain without the experience. Just how this might prevent, or alter, the development of aberrant connections remains a matter of conjecture but like the postnatal experience, it may somehow influence the process of eliminating abnormal exuberant connections.

6.1.5. Early environmental intervention alters HPA responsiveness and may ultimately have an impact on functional recovery

Early experience affects resulting emotionality in rats. As early as 1957, work by W. Thompson showed that prenatal maternal anxiety could influence the emotionality of her offspring. Denenberg and Whimbey (1963) did a follow-up study that showed these

emotional modifications were mediated through both mother-fetus interactions and postnatal mother-young interactions. Denenberg and colleagues continued to research the effect of early postnatal experience on emotionality and stress responses and found that by simply removing animals for 3 minutes per day from their nest (handling), there was a dramatic effect on basal corticosterone levels and responsiveness to novel stimuli (Denenberg et al., 1967). Parallel studies established that the pituitary adrenocortical response to stress was reduced in handled animals (Levine, Haltmeyer, Karas, et al., 1967) and work by Meaney and colleagues showed that basal glucocorticoid levels and glucocorticoid receptor levels in hippocampus and frontal cortex were altered by the handling experience (Meaney et al., 1988; Diorio, Viau, & Meaney, 1993).

In Experiment 3, it was shown that basal glucocorticoid levels were not altered by early frontal cortex damage. This result is surprising in light of the discovery that frontal cortex is a major site of glucocorticoid receptor II (Diorio, Viau, & Meaney, 1993) and this receptor plays a role in the negative feedback loop of glucocorticoid production (Sullivan & Gratton, 1999). In contrast, however, the basal glucocorticoid levels were to both prenatal and postnatal tactile stimulation (Gibb and Kolb, 2004c; Gibb, 2001). Prenatal alteration of HPA organization might occur by the transfer of stress hormones from the dam through the placenta into the developing fetus. The prenatal tactile stimulation may serve as a low-level stressor of the pregnant dam and thus her emotional tone could influence development of her offspring. Postnatal tactile-stimulation required removal of the pups from the maternal nest for 15-minute periods thrice daily. Similarly, handled rats were removed from the nest for similar time periods but with no subsequent additional tactile stimulation. Both treatments lowered basal

glucocorticoid levels, confirming previous studies of handled animals (Denenberg et al., 1967; Meaney 1988). It may be the case that lower basal glucocorticoid levels in the postnatal tactile stimulation animals results primarily from removal from the maternal nest and develops in a manner similar to that seen in handled animals. Taken together, these experiments demonstrate that stress hormones play a primary role in brain organization during both the prenatal and postnatal periods of life and the resulting morphology has behavioral and anatomical implications that extend through adulthood into senescence.

6.1.6. Some behavioral deficits that result from early brain damage are more resistant to remediation than are others.

The degree of recovery observed following therapeutic intervention is task-dependent. Whereas cognitive tasks such as spatial navigation seem to benefit dramatically from environmental stimulation, other tasks such as skilled reaching are more resistant to improvement. This is likely due to the differing nature of the systems underlying the behavior. Cortico-cortical connections are largely responsible for spatial performance and are more easily reorganized than are the cortico-spinal connections that primarily support motor ability. Although behavioral recovery is sometimes seen on skilled reaching it is possible that spared motor cortex is subserving the observed restoration of function. Even when there is evidence of restored motor capacities it may be that the animal is using compensatory strategies rather than exhibiting specific recovery of the affected behavior. The reaching task employed to assess recovery of skilled limb use relies on an endpoint measure of success. In instances where skilled

forelimb use is improved by environmental treatments it is impossible to say the lesion animals “recovered” skilled forelimb use as the tactics they rely on to successfully retrieve food in this task may be entirely different from those used by normal animals. Perhaps a more detailed analysis of limb use after brain injury and subsequent treatment would reveal which aspects of motor behavior are benefited by therapy.

6.2. PROPOSED MECHANISMS OF EXPERIENTIAL TREATMENT AS A THERAPY FOR EARLY BRAIN DAMAGE

Experience can be a powerful modulator of brain organization and can (under appropriate conditions) provide an effective treatment for perinatal brain damage. The following proposals detail the mechanisms that may underlie the observed changes in functional recovery and brain morphology after prenatal complex housing or pre- or postnatal tactile stimulation.

6.2.1. Mechanisms that may mediate the *postnatal* tactile stimulation treatment effect

1. *Stimulation induced increased availability of FGF-2.* The role of FGF-2 in nervous system development and differentiation has been established (Raballo, Rhee, Lyn-Cook, et al., 2000) but the developmental significance and trophic role of FGF-2 is not limited to the nervous system. Many other organ systems rely on FGF-2 for proliferation and differentiation, and among these is the skin. Dermal fibroblasts synthesize FGF-2 and expression of FGF-2 is increased following a wound. We have shown that tactile stimulation also upregulates the expression of FGF-2 in the skin. This

increased availability may result in higher serum FGF-2 levels and does result in higher cortical levels of FGF-2. A study by Wagner, Black and DiCicco-Bloom (1999) demonstrated that systemic levels of FGF-2 regulated neurogenesis in the brains of newborn and adult rats by crossing the blood brain barrier to mediate its effects. We have demonstrated that subcutaneous administration of FGF-2 improves behavioral recovery and cortical organization in P4 operates. It remains to be seen if these effects are in part mediated by FGF-2 stimulation of neurogenesis. FGF-2 expression may also be linked with the expression of other physiological markers associated with tactile stimulation. Further study is required to determine the relative contribution of FGF-2 to changes in expression of ornithine decarboxylase, acetylcholinesterase, and serum lactate (see below).

2. *Increased tissue ornithine decarboxylase.* Ornithine decarboxylase (ODC) is an enzyme that is involved in the control of nervous system development. Each area within the brain has a characteristic pattern of expression during ontogenesis and areas with high levels of ODC expression are undergoing rapid growth (Slotkin and Bartolome, 1986). Maternal deprivation is associated with decreased levels of ODC (Wang, Bartolome, & Schanberg, 1996) whereas tactile stimulation induces increased expression of this enzyme (Evoniuk, Kuhn, & Schanberg, 1979). Thus, tactile stimulation may influence the growth and development of brain through a mechanism related to ODC activity.

3. *Increased acetylcholinesterase.* Tactile stimulation induces upregulation of AchE and may increase cortical plasticity through mechanisms related to AchE or acetylcholine. Increased expression of AchE may reflect increased acetylcholine

expression and acetylcholine is thought to play an important role in mediating cortical plasticity (Rasmusson, 2000). AchE alone has been implicated in alterations of synaptic efficacy, and growth and differentiation of neurons (Appleyard, 1992; Bravo et al., 2000).

4. *Increased serum lactate.* Serum lactate is reported to be a preferred metabolic substrate for neonatal rat brain tissue (Dombrowski, Swiatek, & Chao, 1989) and tactile stimulation during the first week of life has been shown to increase circulating levels of serum lactate (Aslami, Pickens, & Hoath, 1997). Reorganization of the brain is likely very energy demanding and tactile stimulation provides a way of increasing metabolic substrate availability.

5. *Increased neural activity.* A study of the effects of early experience on cortical dendrites (Schapiro & Vucovich, 1970) showed that exposure to noise, flashing light, tactile stimulation (mechanical and thermal), and electric shock resulted in increased spine density on dendrites of neurons located in visual and auditory cortex. Tactile stimulation probably mimics the effects of maternal licking and grooming. Liu, Diorio, Day, Francis, & Meaney (2000) showed that mothers that engage in a higher frequency of licking and grooming of their pups, and an arched back nursing posture, reared offspring that showed enhanced performance on tests of spatial learning and memory. The enhanced performance was correlated with increased expression of NMDA receptors and mRNA for BDNF. These chemical effects were hypothesized to be mediated, in part, by neural activity in somatosensory cortex.

6. *Transmission of maternal glucocorticoids through nursing.* Levine and co-workers have determined that maternal stress during nursing can lead to altered neuroendocrine maturation and behavior in her pups (Levine, 1967), an effect that

presumably acts through transmission of stress hormones in milk. In studies by Hansson, Cintra, Belluardo, et al. (2000) and Mocchetti, Spiga, Hayes, Isackson, & Colangelo (1996), glucocorticoids were found to regulate the gene expression of bFGF, Brain derived neurotrophic factor (BDNF), NGF, and neurotrophic factor- 3 (NT-3) in rat dorsal hippocampus and neocortex.

7. *Alteration in maternal care and mother-pup interactions.* A study done by Vilecas, Bell, Wright, and Kufner (1976) examined the effect of “handling” on maternal behavior following the return of the pups to the nest. The mother spent more time with the pups and engaged in more licking and grooming after a short absence from the nest. The authors noted that “ultrasonic signaling by the infants and careful assessment of the mother’s hormonal and behavioral state during the separation from her pups is necessary to gain a better interpretation of maternal responsiveness and how it may influence later differences in pup outcome measures”. Thus, maternal behavior can have a major impact on resultant offspring behavior and brain morphology and should be considered as a possible factor in stimulating functional recovery from early injury.

6.2.2. Mechanisms that may mediate the *prenatal* tactile stimulation treatment effect

1. *Increased availability of basic Fibroblast Growth Factor (bFGF).* We have demonstrated that postnatal tactile stimulation increases expression of FGF-2 in the skin of preweanling animals. If this were also the case in pregnant dams, one might suppose that additional bFGF may become available to fetal brain tissue by transfer through the placenta.

2. *Maternal expression of low levels of glucocorticoids.* Tactile stimulation of a rat may mediate an increased expression of glucocorticoid in response to the novelty of human handling and stroking. Glucocorticoids may (via placental transfer) become available to developing fetal brain where they could play a role in resulting expression of genes for neurotrophic factors.

3. *Increased neural activity.* Neural activity is required in the visual system for pattern formation and synaptic plasticity (Katz & Shatz, 1996) and it is thought that NMDA receptors mediate this developmental cortical plasticity (Catalano, Chang, & Shatz, 1997). It may be that prenatal tactile stimulation alters the developmental regulation of NMDA receptors in somatosensory cortex resulting in increased cortical plasticity in this area.

4. *Alteration in maternal care and mother-pup interactions.* As noted earlier, removing pups from the mother alters mother-pup interactions, and that in turn is hypothesized to influence brain and neuroendocrine development. Although we have no evidence that prenatal treatments might alter later maternal care, this would seem to be a plausible hypothesis to examine.

5. *Increased expression of acetylcholinesterase.* Prenatal tactile stimulation causes increased expression of AchE in the brains of offspring and males show higher levels of expression of this enzyme than do females. This may indicate that males derive more benefit from prenatal tactile stimulation than do females. Given the putative role of acetylcholine in brain plasticity, the sex difference in AchE expression may account for some of the observed sex differences in the effects of the experiential treatment.

6.2.3. Mechanisms that may mediate the *prenatal complex housing* treatment effect

1. *Alterations in AchE expression.* AchE expression in offspring brains was reduced by maternal complex housing. This effect is opposite to that observed after postweaning or adult environmental enrichment. Similar decreases of AchE expression have been documented after prenatal choline supplementation. It is reported that reduced AchE levels could have a positive effect on synapse strength and efficacy by allowing longer availability of acetylcholine in the synapse (Yang, Liu, Cermak, et al., 2000, Cermak, Holler, Jackson, et al., 1998).

2. *Alterations in growth factor expression.* Many studies have shown that complex housing changes the expression of neurotrophic factors in the brains of adult animals (Ickes, Pham, Sanders, et al., 2000; Vanyman, Ying, Gomez-Pinilla, 2003; Farmer, Zhao, Van Praag, et al., 2004)). If alterations in brain protein expression are mediated through changes in serum protein levels (Gold, Schulz, Hartmann, et al., 2003), it is reasonable to suppose that the developing fetus could be exposed to changes in growth factor availability. Increased availability of growth factors in the blood could mediate changes in neurogenesis, brain circuitry, and cell survival in the fetus.

3. *Stimulation of neurogenesis.* Enriched housing can stimulate neurogenesis and increase neuron survival in the dentate gyrus of adult mice (Kempermann, Kuhn, & Gage, 1997). It may be that neocortical neurogenesis is also stimulated by prenatal exposure to enriched housing.

4. *Decreased apoptosis.* Environmental enrichment has been shown to inhibit spontaneous apoptosis in the hippocampus (Young et al., 1999) and although a similar

effect has not been demonstrated in the neocortex, it is not unreasonable to propose that it may exist.

5. *Altered stress reactivity.* Mohammed, Henriksson, Soderstrom et al. (1993) demonstrated that enriched housing caused changes in expression of glucocorticoid receptors in hippocampus similar to that seen after neonatal “handling” of rats. Upregulation of these receptors increases the sensitivity of the negative feedback loop in the stress response and causes lowered basal levels of glucocorticoid expression. This altered response in HPA reactivity could have a beneficial effect on resulting functional recovery.

6.3. CAVEATS

The behavioral tasks used in these experiments were selected for their utility in examining cognitive recovery (Morris Water Task), motor recovery (Whishaw Tray reaching task) and any changes in species-typical behavior (Circadian activity) after perinatal cortical injury (e.g., Kolb, 1995). Other tasks could have been used to make these assessments (radial arm maze, single pellet reaching, nest building etc.) and might provide additional insight as to the nature of the behavioral recovery observed after experiential treatment. I note, however, previous studies using a more extensive behavioral batteries to examine experiential treatments have found these tests to be predictive of performance on the broader battery (Kolb and Elliot, 1987).

It should be noted that all behavioral and anatomical measures reported were endpoint measures. As such there was no consideration of the changes in behavior and anatomy that was occurring between the time of therapeutical intervention and

assessment. The rationale for conducting the thesis studies using endpoint measures was simply to establish that there is an effect of experience on functional and anatomical outcome. Having established this, it follows that multiple outcome assessments should now be considered to detail the qualitative nature of the anatomical and behavioral effects. In particular, studying the behavior of the developing animals using a test battery designed to examine onset of reflexes and other emerging behaviors (e.g. Kolb, Zaborowski and Whishaw, 1989) would be useful to determine if the behavioral outcome reflects sparing or recovery of function. In addition, careful examination of the mechanics of motor behavior such as reaching would reveal whether observed improvements result from normal reaching movements or if behavioral recovery is associated with new adaptive movements (Whishaw, Pellis, Gorny et al., 1991).

The anatomical measurements used for assessment were also endpoint measures. The Western Blot technique may have revealed more dramatic changes if the animals had been staged killed at time points closer to the time of lesion and up to the time of weaning. Similarly, Golgi-Cox analysis done on stage-killed animals may have revealed how the neuronal anatomy changes over time after experience.

In sum, although the measures reported in this thesis were all endpoint in nature, they were useful in establishing that early experience plays an important role in brain development and behavioral recovery. Future experiments should include multiple outcome measurement analyses to provide greater detail as to the nature and development of the observed changes.

6.4. CONCLUSION

The results of the current studies show that experiential treatments can provide effective therapy for early brain damage. Postnatal tactile stimulation provides excellent restorative therapy for early brain injury by effecting changes in expression of FGF-2 in skin that in turn mediates a host of anatomical changes within the central nervous system. Remarkably, prenatal stimulation works extremely well as a *prophylaxis* for later perinatal brain injury. Although the relationship of the anatomical changes in the brain to the mechanisms underlying the behavioral effects of these treatments is currently unknown, further investigation will undoubtedly yield many interesting insights. Recently, a paper by Rampon, Jiang, Dong, et al. (2001) detailed the effect of environmental enrichment on the genome in the adult rat. Using the oligonucleotide microarray technique to analyze gene expression in the brain, the authors determined that no less than 100 genes were affected by complex housing. The time course of expression levels of the genes varied over the period of exposure to complex housing with initial increased expression of genes coding proteins involved in macromolecule synthesis and processing and enzymes involved in DNA, RNA and protein processing. Genes coding for proteins involved in apoptosis were downregulated whereas genes coding for proteins involved in the formation of new synapses and reorganization or strengthening of existing synapses were upregulated. Late changes in the genome affected transcripts for proteins involved in neuronal transmission and structural changes. In view of these results, one might suppose that the effect of the environment on the genome of the developing rat might have an even greater impact with more diverse consequences.

6.5. FUTURE DIRECTIONS

The benefit of environmental therapies in promoting behavioral recovery from early brain damage having been clearly established (Gibb and Kolb, 2004a; 2004b; 2004c; Gibb, Gonzalez, and Kolb, 2004), it is appropriate to investigate, in detail, the anatomical mechanisms that support the improved behavioral outcome. Some additional studies will be possible from tissue saved from brains in the current studies. For example, tissue from animals that have undergone pre- and postnatal tactile stimulation, prenatal complex housing, and postnatal handling has been prepared for immunohistochemistry. Immunohistochemistry has the advantage over Western blotting in that it can localize changes in expression of these proteins. (Of course, it has the disadvantage that it does not allow easy quantification of the expression of the proteins that is possible with Western blots.)

I noted above that there are changes in expression of genes in response to complex housing. In view of the likelihood that similar changes might be observed in rats with perinatal experiential treatments, it is reasonable to explore genetic changes in animals raised as in the current studies. The equipment and supplies to perform *in situ* hybridization using radiolabeled riboprobes for FGF-2, Tyrosine hydroxylase (an enzyme involved in the production of noradrenaline and dopamine), GR and mineralcorticoid receptors (MR; also involved in HPA axis function), and ARC (an immediate early gene) have recently been obtained. This technique will allow the localization of the *genetic* message (mRNA) for production of proteins so I will be able to determine how the expression of mRNA is affected by perinatal experience both in normal and injured brains.

The possibility that these prenatal and postnatal treatments may stimulate neurogenesis in brain-damaged animals will be investigated by using bromodeoxyuridine (a mitotic marker) to label newly- formed cells and then using appropriate antibodies to determine whether the new cells are neurons or glia. Similarly, environmental treatment may alter the course of apoptosis during development. Thus, by using antibodies to proteins involved in apoptotic processes we could determine how these processes are influenced by experiential therapy.

As serotonin is implicated in the restructuring of glucocorticoid receptor expression in the first week of life (Meaney, Mitchell, Aitken, et al., 1991), administration of a serotonergic agonist to early brain damaged animals may be an effective means of promoting behavioral recovery in adulthood. A preliminary study using fluoxetine (Prozac) which acts on 5HT1A receptors and inhibits the reuptake of serotonin, showed prenatal use of this drug can have devastating consequences on the offspring (Day, Gibb, and Kolb, 2003). Animals born to Prozac-treated mothers were small and showed deficits in cognition and motor skills. They also had smaller brains and showed reduced emotionality in stressful situations. Additional work aimed at determining how Prozac interacts with brain organization to affect behavior is in progress.

The significant functional improvement seen in early lesion animals administered FGF-2 subcutaneously raises the question of whether or not administration of other exogenous neurotrophic compounds might also influence functional recovery after perinatal brain injury. Expression of both brain derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) is increased following complex housing or exercise in

adult rats (Torasdotter et al., 1998; Ickes et al., 2000). Postlesion administration of neurotrophic factors such as these may also promote behavioral recovery in animals that sustain perinatal brain injury by inducing cortical plasticity.

Other pharmacological compounds that may promote behavioral recovery in animals with early brain injury are psychomotor stimulants. Animals that sustain brain injury as adults show improved functional performance after exposure to moderate doses of nicotine (Brown, Gonzalez, and Kolb, 2000). Prenatal administration of nicotine may also influence behavioral recovery in developing rats. A preliminary study examining the behavioral and anatomical consequences of low doses of nicotine administered prenatally showed that animals with perinatal damage do show behavioral recovery but sham-operates are adversely affected (McKenna, Gibb, Brown, and Kolb, 1999).

In a preliminary experiment designed to look at the relative effectiveness of prelesion versus postlesion tactile stimulation in animals with P10 frontal cortex lesions, we noted that prelesion stimulation seemed to have negative effects on resulting functional performance (R. Gibb and B. Kolb, unpublished observations, 1998). If there are circumstances that contraindicate therapeutic intervention, it is important to know what these circumstances are. Further studies employing an animal model that allows good spontaneous recovery (i.e., frontal cortex damage at P7-P10 in rats) should provide the means to elucidate when environmental and pharmacological interventions are not appropriate or at least less beneficial.

In sum, my proposal is to continue to investigate the biochemical and anatomical changes that underlie the behavioral recovery seen in animals that have been exposed to early environmental stimulation to gain a better understanding of the mechanisms that

allow this recovery to occur. If an insight can be gained into how experience can alter brain organization to allow plasticity following perinatal cortical injury, we could propose alternate therapies that work on the same substrates to produce similar or even more dramatic recovery.

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APPENDIX I

Environmental Effects on the Physiology and Behaviour of the Laboratory Rat

Introduction

Desirable qualities in rats have been selected for by captive breeding. These include tameness, curiosity, and reduced fear and aggression in response to handling (Barnett, 1975). Genetic selection may have made laboratory rats well suited to the laboratory. But the question that arises is what exactly are laboratory conditions? For example, within a colony, standard housing may range from singles in Plexiglas shoeboxes to group-housing. Across laboratories, variation may be greater. I shall discuss how environmental parameters influence the physiology and behaviour of rats and thus experimental outcome. The review will consider lighting, humidity, airflow, noise, cage construction, diet, and social opportunity. Maternal influences, age at weaning, animal care, exercise regimes, and enrichment are also discussed. These factors can induce changes in brain weight, cerebral vascularization, adrenal size, and body weight.

Housing Considerations

The environment of the rat can be considered at the macroenvironment and microenvironment levels. Macroenvironment refers to the ambient conditions in the animal colony. These include lighting, temperature, humidity, airflow, and noise. Microenvironment refers to the conditions within the cage. The material used to construct the cage, the design, size, type of lid, bedding, access to food and social opportunity are variables that affect microenvironment. Cage design will also influence the ventilation, lighting, temperature and noise level that its occupants will perceive. For example, hanging wire cages are well ventilated and reduce animal contact with their excreta but do not allow the animal to modify its microenvironment. Macroenvironmental conditions will have a greater impact on animals housed in this type of cage. Shoebox cages with bedding are less well ventilated but allow the animal to make alterations in its environment. In addition, bedding affords the animal opportunity to dig, a natural behaviour that laboratory rats' share with their wild predecessors (Canada). A study (Krohn et al., 2003) used telemetry to monitor heart rate, body temperature, and blood pressure of rats kept on three types of flooring: grid-floor, plastic floor or bedding. Grid flooring caused elevations in blood pressure and heart rate and was thus rated most undesirable. Bedding was most acceptable. Anzaldo and colleagues (Anzaldo et al., 1994) allowed rats to inhabit cages that were equipped with L-shaped partitions (high-perimeter housing), cages that allowed increased floor space with a 3-dimensional design or standard cages. The 3-dimensional cage was least preferred whereas the high perimeter cage was most preferred. The rats' preference for the "high perimeter" caging may have reflected their thigmotactic (edge-using) tendencies. As the population density within the cages increased the preference for the high-perimeter caging over standard caging was reduced. This study showed that rats preferred social interaction and security over increased floor space and that population density within a cage can alter the choice of spatial design.

Lighting

Three characteristics of light that affect housing conditions are intensity, quality (wavelength), and photoperiod (Canada, 1993). Monitoring the intensity of light in the animal colony will ensure adequate illumination for providing animal care without causing blindness in the rats. Lighting that is considered normal for humans can cause retinal damage in rats, especially albinos (Bellhorn, 1980). A recent study (Wasowicz et al., 2002) showed that retinas of pigmented animals such as Long-Evans rats are affected by prolonged exposure to a moderate light source. Light intensity varies on cages near the light source and those closer to the floor. In some animal rooms as much as an 80-fold difference in illumination can occur in the vertical dimension (Schofield and Brown, 2003). Animals housed in the uppermost levels of racks are more susceptible to blindness than those housed lower down. Eye problems could interfere with experiments that depend on the animal detecting visual cues.

Few studies have been conducted on the effects of light quality on rats. Spalding et al. (Spalding et al., 1969) showed the wavelength of ambient light influenced wheel-running in mice, and the degree of the effect was dependent on the strain of mouse. A study of the effects of different types of fluorescent lighting (full spectrum, cool white, black, etc.) on organ and body weights in mice showed that the type of lighting affected both organ and body weight in males but not females (Saltarelli and Coppola, 1979). The Canadian Council on Animal Care (1993) recommends that light used in animal housing be as close to natural sunlight as possible.

Light synchronizes circadian rhythm with environmental time through photo-transduction by retinal ganglion cells (Berson et al., 2002). Circadian rhythms control an animal's sleep/wake cycle and can influence its performance in an experiment, especially in older animals (Poulos and Borlongan, 2000)(Winocur and Hasher, 1999). Most animal housing revolves on a 12 hour light-dark cycle. If lights are switched on during the dark cycle or left on for a 24-hour period, retinal ganglion cells will respond by altering the circadian cycle. Breeding cycles of rats can be affected by circadian timing. Hoffman (1973) reported that 12 hour light/ 12 hour dark cycle produces a 4 day estrous period in Sprague-Dawley rats whereas 16 hour light/ 8 hour dark cycle increases the estrous period to 5 or more days. Circadian rhythms influence the physiology of rats. Changes in body temperature, corticosterone levels, neurotransmitter receptor binding, drug sensitivity, size of experimentally induced cortical infarct, and motor activity have been associated with the circadian cycle (Benstaali et al., 2001); (Ixart et al., 1977), (Ixart et al., 1977); Robuelto et al., 2002; (Vinall et al., 2000)). As such, schedules for behavioural testing and surgical procedures should be consistent to reduce variation in experimental outcome.

Temperature

Although rats have fur coats they are sensitive to fluctuations in ambient temperature. The normal temperature for a rat room is in the range of 20°-24°C. This range allows optimal growth of rats and seems most compatible with their behavioural preferences (Allmann-Iselin, 2000). Temperatures outside this range induce activity and metabolic changes that can affect experimental design. Dose-response curves for drugs can be shifted by changes in ambient temperature. A 4°C variation in temperature can cause a

ten-fold variation in drug toxicity (Clough, 1987)). Shifts in ambient temperature also affect the amount of food and water an animal consumes. Changes in ingestive behaviors can alter the effective dose of an administered drug.

The number of inhabitants in the cage will influence the temperature of the microenvironment. Body temperature can be influenced by changes in animal care personnel, stormy weather, and handling (Clough, 1987).

Humidity

Relative humidity in an animal facility is recommended to be approximately 50%, although a range from 40-70% can be tolerated (CCAC, 1993). Airborne microorganisms are less viable at a relative humidity of 50%. Low humidity can cause health problems such as dry skin and ringtail whereas high humidity can increase ammonia production from the cages (Clough, 1987) thereby increasing the incidence of respiratory distress.

Ventilation

Ventilation within the animal room will influence temperature, humidity, and air quality. Cage design and placement will also affect the airflow at the microenvironment level. Draft-free ventilation that allows 15 to 20 air exchanges per hour is recommended. Rats housed in shoebox cages with filter tops require monitoring to ensure that ammonia from soiled bedding does not reach toxic levels. High levels of ammonia in the environment are associated with respiratory distress or disease (Broderson et al., 1976). The human threshold level for detection of ammonia (8 ppm) is above the concentration capable of inducing pathology (Schofield, 2003; CCAC Guide, 1993). Bedding should also be free from aromatic carcinogens and pesticides. Both contaminants are associated with sawdust bedding (Clough, 1987).

Noise

Whether or not a sound will have damaging effects depends on its loudness, frequency, and duration. Sounds of 160 decibels will cause damage to hearing in rats and in humans. It is recommended that animal room noise does not exceed 85 decibels, although auditory damage in rats has been found after intermittent exposure to sounds at 83 decibels (CCAC, 1993). Rats hear sounds that range in frequency from 1000 Hz to 100,000 Hz, depending on the strain (Gamble, 1982). Thus, they are insensitive to lower frequency tones that fall within the human auditory range but their upper threshold is well beyond the human range. This makes monitoring noise in animal facilities more difficult. Sounds that we are incapable of detecting in addition to those that we hear may cause changes in plasma corticosterone levels, immune system function, reproductive fitness, and body weight in rats (Clough, 1987). Certain sudden, loud sounds can induce a startle response or audiogenic seizure in rats and mice. Nursing dams have been known to cannibalize their young following exposure to sudden, loud noise. Sounds can also induce aggression or changes in tolerance to electric shock (Gamble, 1982).

Rodents produce ultrasonic vocalizations to communicate during mating, aggressive behaviours and maternal care (Harding and McGinnis, 2003; Von Frijtag et al., 2002, (Smotherman et al., 1974). Excessive noise can reduce the effectiveness of this means of communication.

Noise in the environment can influence the development of audition in young rodents. Chang and Merzenich (Chang and Merzenich, 2003) demonstrated that rats reared in the presence of continuous moderate noise showed delayed auditory cortical maturation. This effect was reversed by returning the animals to a normal acoustic environment. Thus animal holding facilities should be well away from sources of mechanical noise, as constant exposure to sounds can alter the timing of normal auditory development in young rats.

Dietary Considerations

The nutritional requirements of an animal can be influenced by many factors. Genetic strain, sex, age, physiological status, and environment contribute to the nutrient requirements of the rat. Rats of different genetic strains grow at variable rates and thus have specific nutritive requirements. Because male rats grow faster than females and have a higher proportion of body protein than do females, they require a higher proportion of protein in their diet. Similarly, growing, lactating, and postoperative animals require a higher percentage of dietary protein than do adult animals that are simply maintaining their body weight. Rats living in cooler conditions will increase food intake to maintain a constant body temperature, whereas rats living in warmer conditions reduce food intake and may require higher nutritional density in their food.

Dietary Restriction or Optimization

The negative consequences of *ad libitum* feeding has now been established in every outbred, inbred and hybrid cross strain of rat examined (Keenan et al., 2000). Rat diet formulations are based on the nutritional requirements of weanling rats and lactating dams and contain between 18% and 23% protein. Animals in the growing or nursing phases of life require approximately 15% protein in their diet whereas adult animals in a maintenance phase require 5% to 12% protein (Keenan, et al., 2000). Animals recovering from surgical procedures also require more protein in their diet to ensure rapid healing. Because it is simpler to give all rats in a colony the same food, most research facilities over-nourish their adult inhabitants. Unrestricted access to food is “unnatural” and compromises the health of the animal. Amongst laboratory animals only rodents are commonly given *ad libitum* access to food. Other species have their food intake restricted in accordance with good scientific and veterinary practices (Keenan, 2002).

Dietary restriction has a positive impact on the health of rats. Feeding rats *ad libitum* highly nutritious rat chow causes obesity, diabetes, tumors, shortens the life span and tends to reduce cognitive performance particularly as the animal ages (Means et al., 1993) Means, Higgins and Fernandez, 1993). Formation of free radicals and/or glycation reactions of sugars with proteins may be responsible for the aging effects associated with *ad libitum* feeding. Dietary restriction is associated with increased production of proteins known to enhance neuroplasticity and confer resistance to metabolic insult such as brain-derived neurotrophic factor (Mattson et al., 2002); (Mattson et al., 2003). Anson and colleagues (Anson et al., 2003) have shown that the pattern of feeding dietary restricted animals affects the degree of benefit derived from the procedure. Mice fed every other day ate the same amount as unrestricted animals and maintained their body weight but showed an increased resistance of neurons in the brain to the effects of excitotoxic stress (Anson et al., 2003). The number of dendritic spines found on neurons in the rat

neocortex decline with aging but 24 month old rats that were restricted to every other day feeding had the same number of dendritic spines as 6 month old *ad lib* fed rats (Moroi-Fetters et al., 1989).

A study by Markoska and Slovenko (2002) showed that effectiveness of dietary restriction varies with the genetic strain of the rat. For example, Fischer-344 rats failed to show significant benefit from dietary restriction but offspring of a Fischer-344 and Brown-Norway cross showed improvement on tests of both cognitive and sensorimotor behaviours.

Control of Environment

Joffe and colleagues (1973) raised rats in an environment in which they were able control food, light and water availability by bar pressing. These rats, when compared to animals raised in standard housing under identical food, light, and water availability conditions, were more exploratory, less emotional and more confident in open-field testing. This study supports the notion that animals prefer to exert control on their environment and having such control reduces their affective response to stress.

Social Opportunity

Rats are social creatures and benefit from opportunities for social interaction. Social isolation is known to cause changes in behaviour (i.e. alcohol consumption) and temperament as well as physiology including changes in size of the adrenals and thyroid glands (Baker, Lindsey, and Weisbroth, 1979) in adult rats. A study by Hurst and colleagues (Hurst et al., 1997) examined the effects of housing male rats as singles or in groups of three in two joined but divided cages. The type of barrier that was used to divide the cages varied the degree of social contact between the two cages. Rats housed in isolation engaged more frequently in behaviours related to escape or seeking social information. Singly-housed male rats were more aggressive if not exposed to neighbours or other cage-mates, yet they showed reduced corticosterone concentration and organ pathology compared to group-housed rats. Although single housing may reduce social stress, animals thus housed are motivated to seek social interaction. Sharp and colleagues (Sharp et al., 2003) examined the effect of group housing on stress responses to witnessing common experimental procedures and husbandry. They found that group housing reduced the stress response to witnessing tail injections, restraint, cages changes, and decapitation. Group housing is thus preferable to solitary housing and if housing rats singly is part of the experimental design it should be justified to and approved by the local Animal Care Committee (CCAC, 1993). Short-term social isolation of rats (4-7 days) has been shown to increase the frequency of social interaction when the opportunity arises (Niesink and van Ree, 1982). This finding suggests that rats find social interaction rewarding. However, overcrowding leads to stress and increased aggression.

Maternal Influences

Natural variations in maternal care can influence the cognitive development of offspring. Meaney and his group have shown that mothers that spend more time in an arched-back nursing posture and licking and grooming their pups have offspring that show enhanced

spatial learning in adulthood (Liu et al., 2000). These animals have elevated levels of glutamate receptors and growth factors in the hippocampus. Levine (Levine, 1967) demonstrated that removing the pups from the nest for brief time intervals during the early postnatal period resulted in reduced response to stress in adulthood. This procedure was called "handling". It is now known that "handling" reduces basal levels of corticosterone (Levine, 1967; (Beane et al., 2002) and alters expression of glucocorticoid receptors in the hippocampus and frontal cortex (Bodnoff et al., 1995); Liu et al., 2000; (Diorio et al., 1993). It has been shown that mothers who experienced "handling" in infancy had offspring that showed reduced plasma steroids in response to novel stimuli (Denenberg and Whimbey, 1963); Levine, 1967). This finding indicates that early experiences of a mother can affect stress responses in subsequent generations. We have shown that complex housing of a pregnant dam throughout the duration of her pregnancy ameliorates the behavioural devastation normally associated with perinatal cortical lesion. Both normal and frontal lesion offspring showed enhanced spatial cognition following prenatal condo experience (SFN 2001). Similar results were found with prenatal tactile stimulation. Pregnant dams were "petted" with a soft hairbrush 3 times a day for 15 minutes throughout the duration of their pregnancy. Offspring that were given postnatal day 4 lesions of frontal cortex showed marked improvement on behavioral tests (Thesis). Both prenatal complex housing and tactile stimulation altered neuronal morphology in sham and lesion animals. Thus, experiences of the mother rat can have an impact on behaviour and physiology of her offspring. Social isolation of rats during the pre-weaning period of life (3 to 6 hours per day for 5 days) alters the behaviour of both the pups and the mother rat (Zimmerberg et al., 2003) by increasing their activity and the number of mother-pup interactions. Taken together, these studies show that although there is some natural variation in maternal care that can influence the behavior of offspring, it is prudent to attempt to control the early experiences of rat pups in order to prevent further confounding effects.

Age at weaning

Pre-weanling rats are sensitive to a maternal pheromone present in the mother's feces and respond by eating maternal feces to promote development of myelin in the brain. Rats respond to this pheromone until 27 days of age but rats deficient in myelin continue to respond to the pheromone beyond this age (Schumacher and Moltz, 1985). Weaning rat pups too early has been shown to have serious physiological and behavioural consequences. Increased susceptibility to gastric pathology, and delayed maturation of responses to restraint stress were noted in pups weaned at 15 days rather than 22 days of age (Ackerman, 1975; LaBarba and White, 1971; Milkovic et al., 1975)

Husbandry Variables

Many environmental factors can act as uncontrolled variables in an experiment: music in the colony, strong smells, different care conditions, animal transportation, and even frequency of cage cleaning.

Using music in the colony as a controlled source of sound is thought to be helpful in reducing the disruptiveness of uncontrolled noise. Sounds associated with normal husbandry procedures will have a greater impact on the magnitude of the subsequent stress response in animals accustomed to silence than those used to constant sounds.

Music can also provide a form of enriching experience for rats. Rauscher, Robinson, and Jens (Rauscher et al., 1998) showed that rats exposed *in utero* and 60 days post-partum to Mozart compositions were able to complete a maze more quickly and with fewer errors than rats exposed to white noise or silence.

Rats possess a highly specialized sense of smell. Just as much of our behaviour is guided by sight, rats use their keen sense of smell to familiarize themselves with their environment. Pheromones are smells that help rats identify the presence of neighbours and can give signals that affect development, reproductive fitness and some behaviours of other nearby rats. Strong cleaning odors, ammonia build-up and the use of perfumes by laboratory personnel can all interfere with the acquisition of odor information by rats.

Another important variable to consider is the handling of the animal provided by both experimenter and animal care personnel. Some animal healthcare technicians treat the animals they care for like pets and handle them a great deal whereas others treat them as though they are wild and handle them with reticence. Likewise, some technicians talk to and handle animals in the cage they are cleaning whereas others avoid contact. These disparate methods of handling can cause the animals to mount varying degrees of a stress response that could affect their performance when subjected to testing procedures.

Transportation of animals via plane or truck affects corticosterone levels and immune function (CCAC, 1993). It is recommended that a minimum period of adjustment of two days be allowed to ensure stabilization of physiological parameters. Timed-pregnant females that are subjected to transportation stress may have offspring that are very different behaviourally and neuroanatomically than offspring from mothers that do not experience this stress during pregnancy (Stewart and Kolb, 1988).

Frequency of cage cleaning affects the fitness and number of useable rats at weaning. Litters that had their cages changed twice per week had more healthy survivors than litters exposed to once a week bedding changes (Cisar and Jayson, 1967). This may have resulted from increased exposure to ammonia in the once a week litters, or from increased handling in the twice a week litters but the frequency of cage cleaning may impact experimental outcome.

Exercise

A rat's natural inclination is to explore its environment for food and mating opportunities. Access to a running wheel provides laboratory animals with a means for exploration beyond the limits of their caged environment. Although exercise is not normal behaviour of animals in the wild, there is mounting evidence that exercise is beneficial to the health of rats. Exercise has been shown to increase the production of neurotrophic factors in the CNS (Gomez-Pinilla et al., 2001) and neurons in the hippocampus and motor cortex (van Praag et al., 1999), Galvez et al., 2002). There is also evidence that exercise reduces an animal's response to stress (Greenwood et al., 2002). In addition, exercise has been shown to be therapeutic but not prophylactic for rats that have sustained cortical injury (Gentile et al., 1987).

Enrichment

In the 1940's Donald Hebb raised a group of laboratory rats in his home. When he tested these animals in a maze (as adults), he found that they were faster and made fewer errors than did animals raised under standard laboratory conditions. This was the first

demonstration that an enriching environment can influence the behavioural performance of rats. Rosenzweig and his colleagues extended this finding by showing that the brains of “enriched” animals were heavier and showed an increase in cortical thickness, acetylcholinesterase activity, synaptic contacts and dendritic arborization (Rosenzweig et al., 1959; Rosenzweig, 1971). Rats housed in a complex environment also undergo brain changes that include increases in glial density and vasculature (Black et al., 1987). It is interesting to note that environmental enrichment has a greater effect on open-field behaviour and body weight of wild rats than on their domesticated counterparts (Huck and Price, 1975). This finding suggests that genetic changes that accompanied the domestication process have made laboratory rats more resistant to the influences of experience.

Greenough and Black (1986, 1992) proposed that environment can influence brain morphology in one of two ways: experience dependant and experience expectant changes in the brain. Experience expectant changes occur during development and require proper input for a system like the visual system to develop normally. This involves stabilizing useful synapses and deleting redundant ones. Experience dependent changes are those that allow experiences to alter the animal throughout its lifespan. This type of learning includes maze learning, and motor learning and is influenced by housing conditions.

We have determined that the impact of complex housing on neuroanatomical changes in the cerebral cortex varies with age and sex in rats (Kolb et al., 2003). Animals placed in enriched environments as adults showed an increase in spine density whereas animals enriched at weaning showed a decrease in spine density. Male rats at all ages showed increases in dendritic length yet only adult females showed similar increases.

Enriching experience is not only derived from complex housing. Sensory stimulation and behavioural testing can also be considered a form of enrichment. Brief periods of tactile or olfactory stimulation following brain injury in rats can improve their behavioural outcome (Gibb et al., Gonzalez et al.) not only during development but also in adulthood. Participation in an experiment exposes an animal to a variety of experiences that animals in the colony are not subjected to. These experiences have the potential to alter the subsequent behaviour and neuroanatomy of test subjects. Kolb and colleagues (1996) conducted an experiment to determine the relative effects of solving the place version of the Morris water task (learning condition), swimming in the pool without a platform (yoked condition: animals were allowed to swim for the same length of time that it took the animals in the learning condition to find the platform), and no behavioural testing on spine density and dendritic arbor in the occipital cortex. Rats that solved the problem had the greatest dendritic arbor and spine density, yet rats that swam for an equal length of time showed a significant elevation of these measures above the baseline found for non-tested animals. This result shows that simple participation in the experiment was enough to change cortical circuitry.

Conclusion

Enrichment can take many forms: access to running wheels, handling, sensory stimulation, group housing, or complex housing. Currently, there is a debate as to whether or not standard housing for rats should include some form of enrichment. Proponents of this view believe that standard housing produces “impoverished” animals

that have underdeveloped brains and a limited behavioural repertoire. But despite the minimal stimulation provided by standard housing, very little pathological behaviour has been ascribed to rats raised in these conditions. Although environmental stimulation produces a smarter rat, the value of “intelligence” to animals that do not need to compete for food, housing or mating opportunities is difficult to assess. The use of laboratory rats that have been reared in standard housing as a baseline group to study the effects of the environment on brain development and function have yielded many valuable insights over the past 60 years. Although it is important to consider optimal housing conditions for the experimental subjects, one should be mindful of the impact of adding enriching devices or protocols to standard laboratory rat care. Minimal changes in environmental conditions can have huge effects on the behaviour and physiology of lab animals.

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