2005

Basic fibroblast growth factor improves physiological, anatomical, and functional outcome from bilateral lesions to motor cortex at postnatal day 10 in the rat

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Basic fibroblast growth factor improves physiological, anatomical, and functional outcome from bilateral lesions to motor cortex at postnatal day 10 in the rat

by

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A THESIS

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF NEUROSCIENCE

LETHBRIDGE, ALBERTA

NOVEMBER 2005

ABSTRACT

Basic fibroblast growth factor (FGF-2) is a trophic molecule involved in a number of functions within the central nervous system (CNS), including a prominent role in the regulation of CNS responses to injury. Prior studies suggest that rats recover differently from injury inflicted to different regions and at different ages throughout development, and that FGF-2 might underlie this phenomenon. This thesis examined whether the functional and structural outcome following bilateral injury to the motor cortex inflicted at postnatal day (P10) could be ameliorated by exogenous administration of a growth factor (FGF-2). Four complimentary studies were conducted that each assessed the role of FGF-2 in mediating recovery from bilateral motor cortex injury inflicted at P10. We found that FGF-2 improves physiological, anatomical, and functional outcome from bilateral lesions to motor cortex at P10.

ACKNOWLEDGEMENTS

From the formative stages of this thesis to its final version, I owe an immense debt of gratitude to my supervisor Bryan Kolb. Bryan, it has been a great experience to work with you. No length of time spent in your company could ever allow me to learn everything I'd like from you. It has been a pleasure to learn as much as I could. Thank you for letting me conduct so many experiments in your lab, and for introducing me to different ways of seeing the world! To my co-supervisor, Jeff, thank you for the many opportunities you've afforded me. It was a pleasure to work with you.

Thank you to Rob Sutherland, Gerlinde Metz and Cam Goater for accepting to serve on my thesis committee. Rob, thank you for allowing me to conduct work in your lab.

To Dr. Stewart Rood, thank you for accepting to be the Chair of my thesis examination. To Dr. Jane Stewart, thank you so much for agreeing to be my external examiner. It was a pleasure to get to know you better and to have you on the committee.

To all the members of the Kolb and Kleim labs, past and present, thank you for sharing your knowledge with me. I am particularly indebted to Grayznita and Dawn for their excellent technical assistance, as well as Wendy and Greg for helping me settle into the lab when I first arrived. To Cathy Carroll, thank you for your constant attempts to keep us all organized! I would like to thank Penny and Nagheme for being such great office mates, and David (Leloup) for his support and friendship.

To Cam Teskey, thank you for continuing to be such a great mentor. I look forward to many years of collaborations with your lab. To Flynn-flynn, thank you for accepting to collaborate with me. I trust the experience is rewarding for both of us! To Karim Fouad, it's really been a pleasure to collaborate with you and the members of your lab!

A number of key people have undoubtedly enhanced my stay at the CCBN. To Sergio, thank you for entertaining my thoughts on a number of research ideas and for collaborating with me. It has been great to learn from you. To Dr. Whishaw, thank you for spending time discussing with me and for critiquing my work. To Neal Melvin, thank you for sharing your knowledge.

To Afra and Evelyn, thank you for your friendship and support. It's always a pleasure to discuss research idea with you both. To Matt and Martina, thank you for your kindness and generosity. You have been such great friends. To Nazia, thank you for being such a great friend. To Ira, thank you for collaborating with me on so many projects. You have been a great friend, you are an outstanding scientist, and I feel privileged to work with you.

A ma famille, merci à vous tous pour votre support!

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LIST OF ABBREVIATIONS

µm	micrometer
A/D	analog to digital
ANOVA	analysis of variance
BDA	biotin dextran amine
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C	Celsius
CA1	Cornus Ammons 1
CC	corpus callosum
CFA	caudal forelimb area
CNS	central nervous system
DCX	doublecortin
E13	embryonic day 13
EM	electron microscopy
EMG	electromyographic
FGF-2	basic fibroblast growth factor
FGFR1	fibroblast growth factor receptor 1
g	grams
GA	glutaraldehyde
GFAP	Glial fibrillary acidic protein

HMW	high molecular weight
HPC	hippocampus
Hz	hertz
i.p.	intraperitoneal
ICMS	intracortical microstimulation
IG	indusium griseum
kD	kilodalton
kg	kilograms
kHz	kilohertz
M	molar
mA	microamps
MCx	motor cortex
mg	miligrams
ml	mililiters
mL	microliters
mm	millimeters
mPFC	medial prefrontal cortex
NaOH	sodium hydroxide
ng	nanograms
NIH	national institute of health
P10	postnatal day 10
PBS	phosphate buffered saline

PFA	paraformaldehyde
PVE	pseudoventricular epithelium
RFA	rostral forelimb area
RMS	rostral migratory stream
RNA	ribonucleic acid
Shh	sonic hedgehog
SVZ	suvbventricular zone
TBS	tris buffered saline
TT	tenia tecta
VZ	ventricular zone

Basic fibroblast growth factor improves physiological, anatomical, and functional outcome from bilateral lesions to motor cortex at postnatal day 10

1. General Introduction

Overview

The specific focus of the present thesis was to examine brain plasticity at postnatal day 10 (P10) in response to two different types of experience: exposure to FGF-2 and/or bilateral lesions of the motor cortex. More specifically, the thesis examined whether the functional and structural outcome following bilateral injury to the motor cortex inflicted at P10 could be ameliorated by exogenous administration of a growth factor (FGF-2). In the introduction, the crucial steps involved in neocortical development, and the role of FGF-2 during development and following brain damage, are reviewed. Then, the specific objectives and hypotheses on which this thesis rests are outlined.

Development of neocortex in the rat

In vertebrates, the entire central nervous system (CNS) is derived from a group of epithelial cells contained within the neural tube of the developing embryo. The neural tube forms when the neural plate along the dorsal surface of the embryo rolls up and closes off from a strip of overlying ectoderm. The cells from this region form the pool of stem cells from which the entire brain and spinal cord originate. Early during development, most cells are undergoing active proliferation. This proliferative phase continues throughout embryogenesis, but is restricted to the cells from ventricular and

subventricular zones. As neural development continues, many of the cells exit the proliferative region, migrate out, and differentiate into neuron or glia (Ford-Perriss et al., 2001). The mammalian CNS develops through a sequence of complex cellular processes. The classical account of brain development was predominantly based on the use of titriated thymidine to label different phases of development – neurogenesis, migration, synaptogenesis. More recent work looks at CNS development within the framework of an embryology program defined through distinct molecular expression patterns (Hatten, 1999). Within this framework, genes that are expressed during neural induction initiate the program of CNS development (Harland, 1997), the first step of which involves the establishment of anterior/posterior axis (Rubenstein, 1998). As different transcription factors express themselves within different regions of the emerging CNS, different pools of cells acquire dorsal or ventral identity on the axis. The cells' position on the axis dictates whether they will become cortical or subcortical. This gene expression program is in progress at early embryonic periods (E8.5- E13) that coincide with a period of neurogenesis. Then, different cues guide the migration of neurons along radial glial cells to their appropriate targets. Early in development, proliferating precursor cells in the neuroepithelium move from one rhombomere to the other (Jessell & Lumsden, 1997), but eventually, they settle into a specific position, and from there, many will begin radial migration along glial fibers.

Radial glial migration

Much evidence supports the theory that radial glia provide a scaffold for directed

migration in the brain (Rakic 1971, 1972, 1978; Hatten, 1983; Anton et al., 1996). Early in development, glial cells extend processes and create the primary route for migration. As the first populations of neurons migrate away from the ventricular zone, a region of axons is created and forms the intermediate zone (IG). Around E8-E9.5, the dorsal/ventral axis is established by the expression of bone morphogenic proteins (BMPs) and Sonic Hedgehog (Shh). Migration occurs after regionalization specifies a plan for cell fate specification. This plan orchestrates the blue print particular to different brain regions. Neurons eventually stop their migration, thanks to cues provided by target axons, as well as de-adhesion signals. The majority, but not all, of cells migrate through the radial glial scaffold. A subpopulation of cells migrate tangentially (O'Rourke et al., 1997).

Within the developing neocortex, another important area develops above the ventricular zone- the subventricular zone (SVZ). The SVZ, first described by Altman (1969) maintains a proliferative pool of stem cells throughout life (Garcia et al., 1998). The SVZ is one of three areas that remain proliferative into adulthood, the other two being the olfactory bulbs and the hippocampus (Seaberg & van der Kooy, 2002).

Differential developmental plasticity- P3≠ P10≠ P90…

Rats that receive neocortical injury display a broad range of behavioral and anatomical outcomes that vary depending on the age at the time of lesion, whether the injury is unilateral or bilateral, the site and extent of the damage inflicted, and the precise

behavioral tests employed (e.g., Kolb, 1995). For example, bilateral injury of the medial prefrontal cortex (mPFC) inflicted around postnatal day 10 (P10) leads to remarkable functional and anatomical recovery including a filling of the lesion cavity with newly generated neurons and glia, and increased dendritic arborization in pyramidal cells throughout the remaining neocortex (e.g., Kolb, 1995; Kolb et al., 1998). In contrast, injury inflicted earlier (postnatal days 1-6) in development or in adulthood leads to severe behavioral deficits and no filling of the lesion cavity or dendritic hypertrophy. These results *a priori* suggest that there may be something particular about P10 that allows recovery of function and its underlying anatomical changes. Curiously, the filling of the lesion cavity is not observed after P10 lesions in other cortical areas, including both anterior and posterior regions (e.g., Gonzalez, Whishaw & Kolb, 2003). Interestingly, despite the fact that P10 lesions in other cortical regions do not show a spontaneous filling of the lesion cavity, those rats that receive lesions at P10 recover better than their P1 or P90 counterparts, but in no case do they show recovery that is as extensive as in animals with mPFC lesions that show generation of new tissue. It thus appears that the recovery seen after mPFC lesions at P10 may be related both to synaptic changes in the remaining brain as well as to the generation of new neurons and glia. Different 'things' are happening throughout development. It should not come as a surprise, therefore, that the brain reacts differently to injury depending on the time at which it is affected. We are, in essence, damaging a 'different brain' when inflicting injury at various time-points throughout development. For example, at P3 the rat brain is still undergoing neurogenesis. At P10, however, neurogenesis is complete (with the exception of two

focused areas), and the brain is undergoing the peak of synapse formation. Knowing this, we can capitalize on the fact that the brain is different throughout development. Doing so provides us with the opportunity not only to understand the developing brain better, but understand the different principles that function to hinder or facilitate recovery of structure and function in response to injury. Once we understand these principles better, we may be in a position to apply the derived mechanisms to different situations (i.e., different regions or developmental timepoints) to enhance recovery of function.

Different signaling molecules play important roles during brain development, and contribute to the differential recovery seen at different developmental ages. Members of the FGF family, for example, have multiple critical functions during the formation of the CNS as well as following injury, and several members are differentially regulated and expressed throughout development. Of particular interest to the present thesis, is basic fibroblast growth factor (FGF-2).

Basic fibroblast growth factor (FGF-2)

FGF-2 was discovered in 1984 (Thomas et al., 1984). Along with FGF-1, it was found to be a potent modulator of proliferation in the developing CNS. FGF-2 is expressed early in development, and persists into adulthood. It is also expressed in a wider range of tissue than FGF-1 (Vaccarino et al., 1999). Whereas FGF-2 is expressed in both neuronal and non-neuronal cells, FGF-1 is predominantly neuronal (Eckenstein, 1994). FGF-2 exists as 4 different molecular forms, including 3 high molecular weight (HMW; 21.5,

22, and 24 kD), and one 18 kD forms. The HMW FGF-2 are usually expressed in the nucleus, whereas the 18 kD form is cytoplasmic. Extracellularly, FGF-2 exerts its effects by interacting with specific cell surface receptors. Four major receptors have been identified for FGF-2: FGFR1, FGFR2, FGFR3, and FGFR4.

The 18 kD FGF-2 is found in the cytoplasm, but it is also released from the cell. This process involves 2 components: (1) release of 18 kD FGF-2 from the inside of the extracellular compartment; and, (2) release of cell surface, or matrix-bound FGF-2 into the extracellular environment.

FGF-2 and development

Several FGFRs and their ligands (including FGF-2) are expressed in the developing CNS. FGF-2 is a potent proliferative factor for telencephalic progenitor cells in vitro and in vivo (Vaccarino et al., 1999). For example, Murphy, Drago and Bartlett (1990) found that in E10 mice, 50% of neuroepithelial cells divide in the presence of FGF-2. In the absence of FGF-2, those cells die in culture within 6 days. It appears that FGF-2 might stimulate proliferation of precursor cells for the entire CNS- Ray and Gage (1994) found that neural precursor cells from embryonic spinal cord also proliferate in the presence of FGF-2. FGF-2 null mutants are viable, but display cerebral cortex defects. They show, for example, a reduction in parvalbumin positive neurons in cortical layers and an overall reduction in neurons and glia. This is most pronounced in frontal and sensory areas. Neuronal deficiencies are also found in the spinal cord of these mice. Cell density in

many other areas, however, appears normal. The knock-out mice show a reduced density of neurons in sensory and motor areas of the cortex, but not in the striatum or cerebellum (Vaccarino et al., 1999), and Raballo et al. (2000) have suggested that the major effects of FGF-2 loss are localized to the dorsal regions of the brain (i.e., cortex). Raballo et al. (2000) have shown that FGF-2 is necessary for cell proliferation and neurogenesis in the developing neocortex by demonstrating that: (1) FGF-2 is expressed in the pseudostratified ventricular epithelium (PVE) in a dorsoventral gradient; (2) FGF-2 and its receptors are downregulated during the later stages of neurogenesis; and, (3) in FGF-2 knock-outs, the dorsal PVE shows a 50% decrease in founder cells as well as a reduced expansion of the progenitor pool throughout neurogenesis such that by the end of neurogenesis, cortical number is reduced, and the mice lack large neurons in the deep layers of the cortex, but the number of neurons in the basal ganglia remains unaffected. Bromodeoxyuridine labeling in FGF-2 knock-out mice has demonstrated that FGF-2 increased the population of dividing and proliferating cells cells without affecting the length of the cell cycle (Vaccarino et al., 1999; Raballo et al., 2000). The degree of apoptosis within the VZ was not changed in FGF-2 knock-outs, suggesting that FGF-2 does not act as a survival molecule *in vivo* (Raballo et al., 2000). FGF-2 and its receptors are highly expressed during early stages of embryogenesis (E10-12 in rodents), and the deepest layers of the cortex are dividing and differentiating. This result correlates with the finding that the neurons that are lost in FGF-2-deficient mice are the large neurons in deep cortical layers (Raballo et al., 2000). Raballo et al. (2000) conclude that FGF-2 is likely necessary for a specific class of cortical neurons arising from the PVE. FGF-2 has

a spatially- and temporally-defined role, which is to initiate or maintain cell division of neural precursors in the dorsal regions of the telencephalon during the early stages of embryogenesis. Vaccarino et al (1999) have shown that cortical volume and neuron number were increased by FGF-2 microinjection at E15.5. Similar injections at E20.5 resulted in an increase in glia (but not neurons). In their study, they counted the number of neurons and glial cells at P21. It appears that FGF-2 might serve to amplify 'what is already there'. A different study has shown that the regulation of progenitor maturation within the developing cerebral cortex by Wnt depends on Shh or FGF-2. As previously alluded to, the cerebral cortex develops from two germinal layers- the ventricular zone (VZ), a pseudostratified epithelium that lines the lateral ventricles, and the subventricular zone (SVZ), which begins to develop at approximately E13 in mice. Viti et al. (2000) found that BMPs and FGF-2 act antagonistically to regulate the development of a subset of SVZ progenitors that normally express a high level of EGF receptors and divide in response to EGF. In addition, Wnt 7a, Wnt 7b, and Shh together, or FGF-2 alone, promote progenitor maturation in culture. Wnt 7a and Wnt 7b also stimulate the proliferation of neurogenic progenitors and increase the number of cells that can generate neurospheres. Viti et al. (2000) investigated whether Wnts, FGF-2 and Shh act independently or in a common pathway, by inhibiting each factor individually in cortical explant, and found that all three normally contribute to progenitor maturation. Furthermore, Wnt 7a depends on FGF-2 or Shh to promote maturation (but not proliferation). Maturation induced by bone morphogenic proteins (BMPs) is also dependent on FGF-2. FGF-2, however, promotes maturation by a Shh-independent

mechanism. In addition to its role in development, FGF-2 has also been shown to play a role following injury.

FGF-2 and injury

Reilly and Kumari (1996) have shown that FGFRs are expressed by astrocytes, and that they (FGFRs) are upregulated in response to injury. They have also shown that FGF-2 itself is also expressed in the nucleus of astrocytes that stain for FGFRs. The authors also demonstrate that there is a time course for astrocyte expression of FGFRs that precedes and parallels astrocyte hypertrophy in response to injury. Reilly and Kumari's study (1996) suggests that an autocrine action of endogenous FGF-2, acting directly on FGFR-expressing astrocytes contributes to astrogliosis in response to injury. Other studies report a similar mechanism. Baird and Walicke (1989) as well as Logan (1990) have independently shown that FGF-2 acting through FGFR1 plays a role in the regulation of CNS injury processes, including transformation of reactive astrocytes, angiogenesis, scar formation, and promotion of neurotrophic factor release. FGF-2 is found on astrocytes (Reilly & Kumari, 1996) as well as neurons (Gonzalez et al., 1995). Evidence suggests that FGF-2 may translocate to the nucleus after binding to FGFR1 in the nuclear periphery (Stachowiak, 1997). Providing further evidence to that effect, Clarke et al. (2001) have demonstrated upregulation and trafficking of FGF-2 and FGFR1 to the nucleus of reactive astrocytes *in vivo*. Using a different model of injury- hypoxia- Ganat et al. (2002) have shown that levels of FGF-1 and FGF-2 increase following 2 weeks of hypoxia. According to Ganat et al.'s results, in undamaged brains, FGF-2 is expressed in

the nucleus of GFAP-expressing astrocytes. After hypoxia, however, FGF-2-containing cells seem to revert to a more immature state as now they express vimentin, but no longer express GFAP. Hypoxia evidently promotes the appearance of radial glia throughout the subventricular and subependymal zones. A subset of these cells express FGFR1 and are in close proximity with FGF-2 positive cells in the SVZ. It is thought that FGFR signaling in radial glia might act to regulate cell genesis following injury (Ganat et al., 2002).

Why study bilateral motor cortex lesions inflicted at P10?

The roles that FGF-2 play both throughout development as well as following injury suggest that it may prove to be different depending on the age at injury, and the area damaged. In the present thesis, we have chosen to focus on one specific timepoint, P10, and one particular area, motor cortex. We chose to study animals with bilateral P10 motor cortex lesions for two reasons. First, although rats with P10 motor cortex lesions show a better outcome than animals with day 1 or adult lesions, they are still profoundly impaired at skilled motor tasks (e.g., Kolb, Cioe & Whishaw, 2000). Second, we made bilateral rather than unilateral lesions because we wished to ensure that any enhanced motor function was not due to changes in the normal hemisphere of unilaterally-injured animals. Importantly, both the damage itself, but also the changes that occurred as a result of the damage, will have an effect on behavioral outcome. For that reason, we believe that using a combination of complimentary techniques is most effective in assessing outcome following injury.

Objectives and hypotheses

The main objective of the present thesis was to evaluate whether exogenous administration of FGF-2 could improve functional and structural outcome following P10 bilateral injury to the motor cortex. Based on prior work conducted in the Kolb lab (Gibb et al., 2004) we hypothesized that it would. If so, we were interested in isolating what the mechanism(s) underlying the functional and structural recovery might be. We set out to examine theses questions with four complimentary studies that each assessed the role of FGF-2 in mediating recovery from bilateral motor cortex injury inflicted at P10. In order to best describe the changes induced as a result of bilateral lesion to the motor cortex and exogenous administration of FGF-2, data were collected from multiple levels of analysis (behavior, cortical mapping, EEG activity, multi-unit activity, EMG activity, dendritic morphology, electron microscopy, neuroanatomical tracing, and immunohistochemistry).

The logic and sequence of experiments were as follows: Study 1 aimed to thoroughly describe the spatial and phenotypic distribution of FGF-2 throughout development. The goal was to evaluate whether a differential temporal and spatial expression of FGF-2 throughout development might be partially responsible for the differential recovery observed from cortical lesions inflicted at different time points after birth.

We then aimed to examine the effects of exogenously applying FGF-2 on the functional recovery following a bilateral motor cortex lesion inflicted at P10. We show that FGF-2

is associated with functional recovery, as well as a filling of the lesion cavity with some tissue (Study 2). The goal of Study 2 was to assess the functionality of the tissue filling the cavity, tissue surrounding it, and tissue from the motor cortex (in control rats) using intracortical microstimulation to evoke movement, and to derive and analyze the caudal forelimb area map in each rat. Following mapping, the rats were perfused and processed for Golgi-Cox staining. Medium spiny neurons from the striatum were drawn and analyzed to evaluate whether the functional recovery observed might be, at least in part, due to a change in dendritic morphology in the striatum.

In Study 3, we evaluated the electrophysiological and anatomical integrity of corticospinal projections in motor cortex lesioned rats that receive FGF-2. Rats received bilateral MCx lesions on P10. Beginning with day 11, they received subcutaneous injections of either vehicle or FGF-2 for 7 days. In adulthood, the physiology of corticospinal projections was evaluated using intracortical microstimulation and recording electromyographic (EMG) activity in wrist extensors. Corticospinal projections were also anterogradely traced using biotin dextran amine (BDA).

Finally, in Study 4 we examined the structural and anatomical integrity of this filled region in 5 different experiments. In the first experiment, rats were sacrificed at P14 and P21 and their brains processed for Ki67 and doublecortin immunohistochemistry to identify migrating neuroblasts and proliferating cells. In the second experiment, the synaptic organization of the tissue from the filled the cavity, area Cg3, and MCx (in

control rats) was assessed in adulthood (P90), using light and electron microscopy techniques. Unbiaised stereology was used to derive a measure of synaptic density, neuron density, and synapses per neuron from each area. In the third experiment, EEG and multi-unit recordings were performed in P90 rats from MCx (in controls) and from the filled region (in lesion, FGF-2 treated rats). Experiment 4 aimed to examine whether preventing the filling of the lesion cavity with FGF-2 administration would prevent the functional recovery. Pregnant dams were injected with Bromodeoxyuridine (BrdU) on embryonic day 13 (E13). BrdU treatment at E13 is thought to put a halt to the pool of stem cells from the subventricular zone that would be destined to migrate to the deep layers of cortex- and that might be responsible for filling the lesion cavity following FGF-2 injections. The progeny received bilateral P10 motor cortex (or sham) lesions, as well as injections of FGF-2 or saline. When they reached P45 the 4 groups of BrdU-exposed rats, as well as groups of matched controls, were trained and assessed on the Whishaw tray reaching task. In Experiment 5, control rats and lesion rats that received FGF-2 were re-lesioned in adulthood. They received a unilateral lesion of motor cortex (or filled region) contralateral to their reaching forelimb. Their reaching ability and forelimb asymmetry were assessed before and after the re-lesioning.

Chapter 2: Differential expression of FGF-2 in the developing rat brain: a potential mechanism underlying recovery from injury

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Submitted to Neuroscience, September 12, 2005.

Abstract

Basic fibroblast growth factor (FGF-2) is a trophic molecule involved in a number of functions within the central nervous system (CNS), including a prominent role in the regulation of CNS responses to injury. Prior studies suggest that rats recover differently from injury inflicted to different regions and at different ages throughout development, and that FGF-2 might be underlie this phenomenon. In the present study, we describe the distribution of FGF-2 at P0, P2, P6, P10, P12, P14, P18, P21 and P30 in the indusium griseum, the external capsule, the hippocampus, the medial prefrontal cortex, the motor cortex, the rostral migratory stream, and the subventricular zone. Our results suggest a differential temporal and spatial expression of FGF-2 throughout development, which may explain the differential recovery observed from cortical lesions inflicted at different time points after birth.

Key words: Vimentin, FGF-2, GFAP, subventricular zone, development.

Basic fibroblast growth factor (FGF-2) is a trophic molecule involved in a number of functions within the central nervous system (CNS) (Bikfalvi et al.,1997; Ford-Perriss, 2001). Four different molecular forms of FGF-2 are found in the rat brain (18, 21, 21.5, and 22K). The 18K FGF-2 is predominantly cytoplasmic or excreted and stored in the extracellular matrix, whereas the higher molecular mass FGF-2 isoforms preferentially localize to nuclear and ribosomal fractions (Prats et al., 1989). FGF-2 is expressed in both neurons and astrocytes (Emoto et al., 1989; Endoh et al., 1994), and is known to stimulate astrocytic differentiation (Reilly & Kumari, 1996), induce neurogenesis (Wagner et al., 1999), enhance neuronal survival and outgrowth both *in vivo* and *in vitro* (Ramirez et al., 1989; Walicke, 1988), and to play a prominent role in the regulation of central nervous system injury responses (Baird and Walicke, 1989; Logan, 1990), including scar formation (Logan, 1990), and promotion of neurotrophic factor release. Recently, FGF-2 infusion was found to improve sensorimotor deficits and to reduce infarct size following cerebral ischemia in adult rats (Li & Stephenson, 2002). Furthermore, application of FGF-2 leads to anatomical, physiological, and behavioural recovery following motor cortex lesions inflicted at postnatal day 10 (Monfils et al., 2005), and neutralizing antibodies to FGF-2 blocks recovery from motor cortex lesions in adult rats (Rowntree & Kolb, 1997).

Prior studies suggest that rats recover differently from injury inflicted to different regions and at different ages throughout development (Kolb et al., 2000a; Kolb et al., 2000b; Kolb et al., 1996). Specifically, rats show poor recovery after injury in the first

week of life whereas they show dramatic recovery in the second week (Kolb et al., 2000a). One explanation for this difference in recovery is that there is a time-dependent expression of trophic factors such as FGF-2 in recovery (e.g., Gibb & Kolb, 2005). Yet, despite evidence for a plausible role of FGF-2 in recovery from early injury, little is known regarding its protein expression throughout the brain throughout postnatal development.

We suggest that a different spatial distribution of FGF-2 at various time points throughout development could in part underlie the differential recovery observed following cortical lesions inflicted at different time points after birth. Thus, in the present study, we examined the distribution and phenotypic characteristics of FGF-2-expressing cells at P0, P2, P6, P10, P12, P14, P17, P21 and P30 in the indusium griseum (IG), corpus callosum (CC), hippocampus (HPC), medial prefrontal cortex (mPFC), motor cortex (MCx), rostral migratory stream (RMS), and subventricular zone (SVZ).

Materials and Methods

All experiments were conducted in accordance with the guidelines set forth by the Canadian Council for Animal Care. Male Long-Evans hooded rats were intracardially perfused with phosphate buffered saline (PBS) (0.1 M, 0.9 %) followed by phosphate buffered paraformaldehyde (PFA) (4%) at postnatal day 0 (P0), 2, 6, P10, P12, P14, P18, P21 and P30 (n=3 for each age group). Following perfusion, the brains were stored in phosphate buffered sucrose (30%), then microtome-sectioned coronally at 30 μm, and

stored in PBS until processing.

Three series of sections were obtained from each brain. One series was processed for FGF-2 immunochemistry with DAB reaction. The DAB processing steps were as follows. Free-floating sections were first placed in a 0.3% peroxide solution and washed in 1X PBS, then incubated overnight in a 1:400 dilution of FGF-2 rabbit anti-FGF-2 (Santa Cruz Biotechnologies). The next day, the sections were developed using the ABC method (Vector-Elite). A subportion of the brains (P6, P10, P12, P18, P21) were also processed for FGF-2 as well as either doublecortin, NeuN, or GFAP and vimentin to establish the phenotype of the cells that expressed FGF-2. Free-floating sections were incubated overnight in a dilution of primary antibodies (rabbit anti-FGF-2, 1:250; Mouse anti-NeuN, 1:500; chicken anti-vimentin, 1:1000; Mouse anti-GFAP, 1:1000). The next day, the sections were processed for fluorescence using secondary antibodies specific to the primary antibodies (Cy3 anti-rabbit, biotinylated anti-mouse and Streptavidin 488, Cy5 anti-chicken).

Results

Indusium griseum

Cells from the indusium griseum expressed FGF-2 at P0 (see Fig, 2-1). The reactivity was faint, but was observed consistently in this region across all the P0 brains that were processed (n=3). There was no qualitative difference between P0 and P2. By P6, however, FGF-2 expression in the IG increased in intensity. It peaked at P21, and

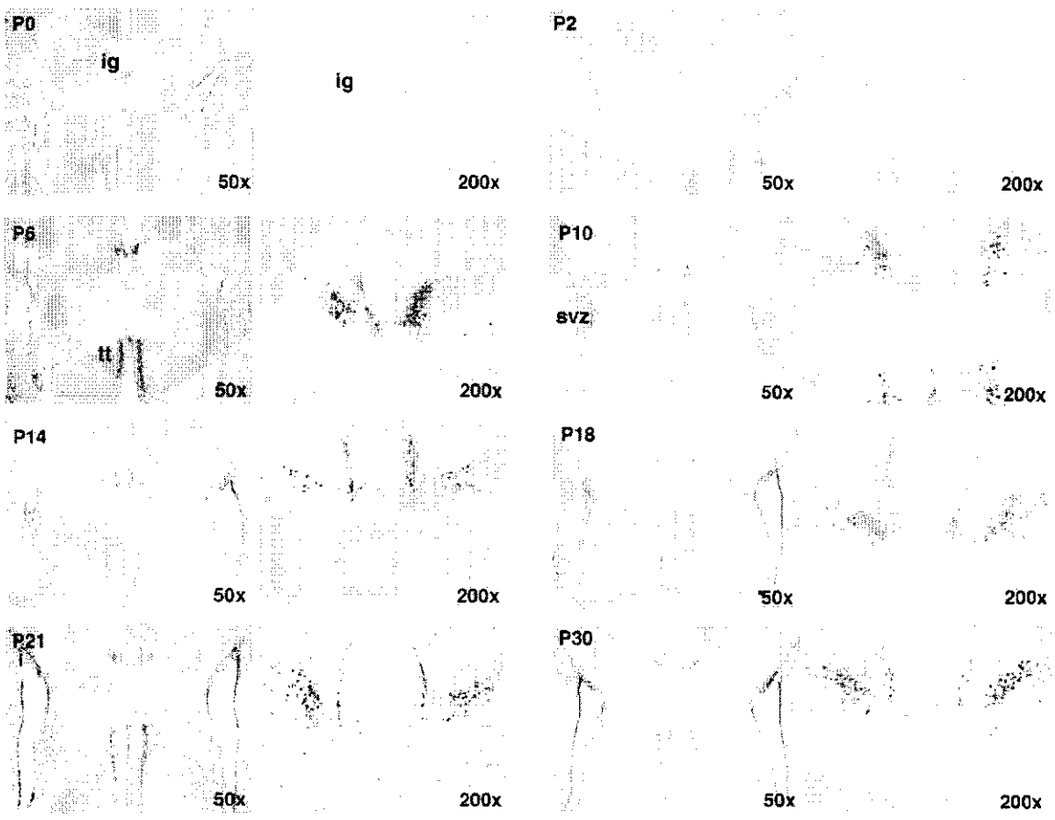

Figure 2- 1 Expression of FGF-2 in the indusium griseum, tenia tecta, and subventricuar lining throughout development. FGF-2 first appeared in the indusium griseum (IG) at P0. It increased in intensity at P6 and the level of expression was maintained thereafter. In the tenia tecta (tt), FGF-2 was first expressed at P6 and peaked at P21. In the subventricular lining (svz), FGF-2 was first expressed at P10, and was consistently expressed thereafter.

seemed to return to a slightly lower intensity by P30. As can be seen in Fig. 2-2, the IG cells that expressed FGF-2 were double-labelled with NeuN and thus were neurons.

Septum

Cells from the septum expressed FGF-2 at P6 (see Fig. 2-1). FGF-2 expression was qualitatively consistent across later ages, and peaked at P21 just like in the IG. As can be observed in Fig. 2-2, the septal cells that expressed FGF-2 were neurons.

Hippocampus

In the hippocampus (HPC), FGF-2 was expressed at P0 in the anterior portion of CA1 (see Fig. 2-3). In caudal sections of the HPC, it was expressed in the most lateral and medial portions of CA1 (see Fig. 2-5). Beginning with P10, an interesting pattern of FGF-2 distribution developed in the anterior portion of the HPC. Specifically, as shown in Fig. 2-3, from P10 and older there appears to be a gap between two robust bands of FGF-2 expression. The gap seems to be located in a portion of CA1, and the 2 areas expressing FGF-2 appear to comprise the medial and lateral edge of CA1, as well as CA2. Interestingly, there are still some FGF-2 positive cells within the gap described here, but the cells are far less dense in that region and they display a phenotype that is clearly distinct from what is seen in the FGF-2-dense regions. Fig. 2-4 shows that, in those FGF-2-dense regions, FGF-2 is expressed in neurons.

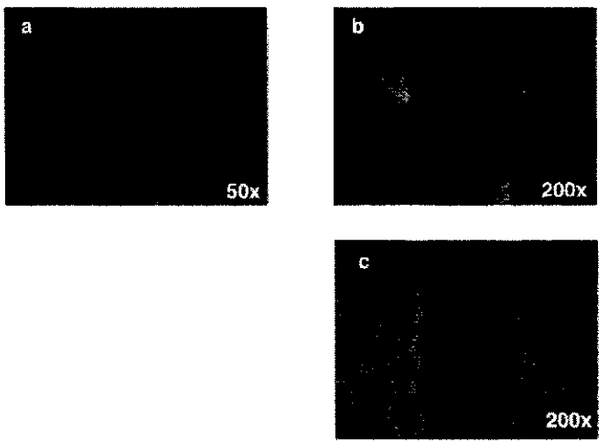

Figure 2- 2. Co-localization of FGF2 and NeuN in the indusium griseum and tenia tecta. In a, co-localization of NeuN (green) and FGF-2 (red) in the indusium griseum and tenia tecta at 50x magnification. In b and c, higher magnification (200x) picture showing that FGF-2 and NeuN are both expressed in cell nuclei from the indusium griseum (b) and tenia tecta (c). The example shown was obtained from a P10 rat. However, this expression pattern was consistent across all ages examined (P6, P10, P12, P14, P18, P30).

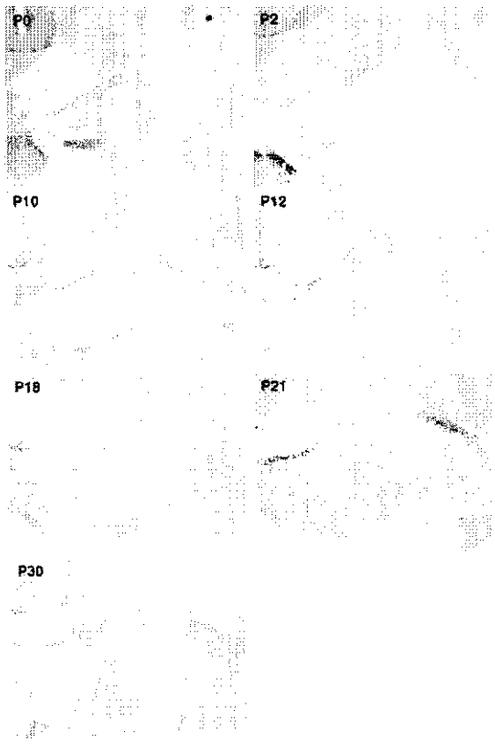

Figure 2- 3 FGF-2 expression in rostral hippocampus throughout development. From P0 to P10, FGF-2 was only expressed in the most medial region of the hippocampus. Beginning with day 10, and persisting thereafter, an interesting expression pattern developed: there appears to be a gap between two robust bands of densely expressed FGF-2 positive cells. The gap seems to be located in a portion of CA1, and the 2 areas expressing FGF-2 appear to comprise the medial and lateral edge of CA1, as well as CA2. Interestingly, there are still some FGF-2 positive cells within the gap described here, but the cells are far less dense in that region and they display a phenotype that is clearly distinct from what is seen in the FGF-2-dense regions.

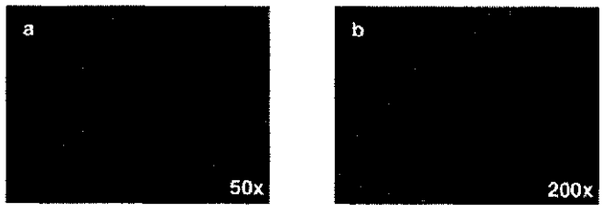

Figure 2- 4 Co-expression of FGF-2 and NeuN in distinct regions of the rostral hippocampus. As shown in a, FGF-2 and NeuN were co-expressed in CA2, and in the medial portion of CA1. In b, higher magnification (200x) showing that the nuclei of cells nuclei in CA2 co-expressed FGF-2 and NeuN.

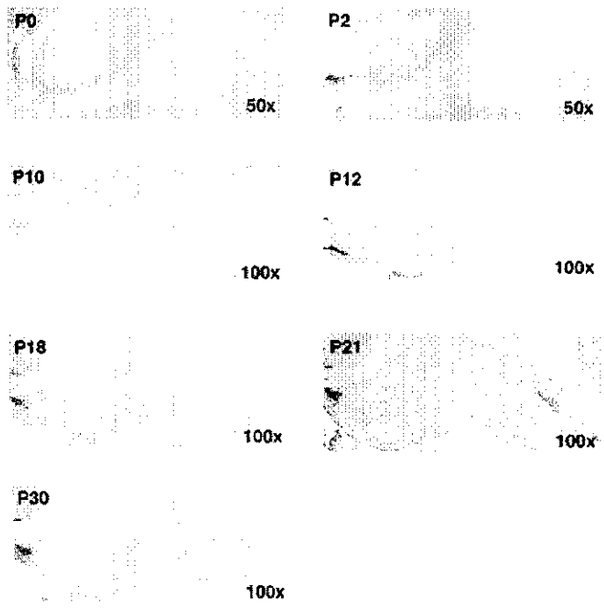

Figure 2- 5 Expression of FGF-2 in the caudal hippocampus throughout development. In caudal sections of the hippocampus, FGF-2 was expressed in dense clusters in the most medial portions of CA1 from P0 onward. From P10 on, FGF-2 was expressed ubiquitously throughout the hippocampus, yet, densely packed clusters of FGF-2 positive cells were still prominent in the most medial and lateral portions of CA1.

External capsule, motor cortex and medial prefrontal cortex

FGF-2 was differentially expressed within different cortical regions. Within the medial prefrontal cortex (mPFC), FGF-2 first appeared at P14 (data not shown), was consistently expressed thereafter, and peaked at P21, similarly to the IG and septum. In motor cortex (MCx), FGF-2 was first expressed at P10, and was consistently expressed thereafter (see Fig. 2-6). The expression of FGF-2 in the external capsule paralleled that seen in the MCx. Thus, as shown in Fig. 2-6, FGF-2 in the CC was first expressed at P10, and was consistently expressed thereafter. In the mPFC, the MCx, as well as the CC, FGF-2 was expressed in mature astrocytes (data not shown).

Rostral migratory stream and subventricular zone

FGF-2 was not expressed in either the rostral migratory stream (RMS) or the ventricular lining until P10 (see Fig. 2-6). FGF-2 expression peaked at P14, and remained at that level across all subsequent ages. As can be seen in Fig. 2-7, the cells that expressed FGF-2 in the ventricles were not neurons. They did not express NeuN, and they can also be recognized as distinct from neurons based on their smaller cell bodies. Interestingly, a number of SVZ FGF-2 positive cells were colocalized with vimentin only between P10 and P14 (See figure 2-8). By P18, however, the majority of FGF-2 positive cells expressed both vimentin and GFAP (See figure 2-9). By P30, the vimentin positive cells

in the SVZ no longer expressed FGF-2. Furthermore, FGF-2 expression was predominantly restricted to the nucleus across all ages, with the exception of P10-P14, at which point it was expressed both in the nucleus (in a portion of cells), and in the cytoplasm (in other cells).

Discussion

The results of our study suggest that there is a differential distribution of FGF-2 throughout development that is region-specific and age-dependent. To our knowledge, this is the first study to thoroughly describe the developmental distribution and phenotypic characterization of FGF-2 protein expression within the rat brain. Other studies (e.g., Gomez Pinilla et al., 1995) have reported protein expression of FGF-2 in the developing brain with an emphasis on certain structures, but here we attempted to globally describe FGF-2 expression. Our results suggest that the differential temporal and spatial expression of FGF-2 throughout development may play a role in the differential recovery observed from cortical lesions inflicted at different time points after birth. We discuss this possibility within the framework of past research conducted on early cortical lesions in the rat.

First, in a number of regions described here, FGF-2 expression peaks at P21. This finding is in accordance with a previous study, which reported that FGF-2 messenger ribonucleic acid (mRNA) peaked at P21 (Riva & Mocchetti, 1991). Second, FGF-2 is expressed in neurons, as well as glial

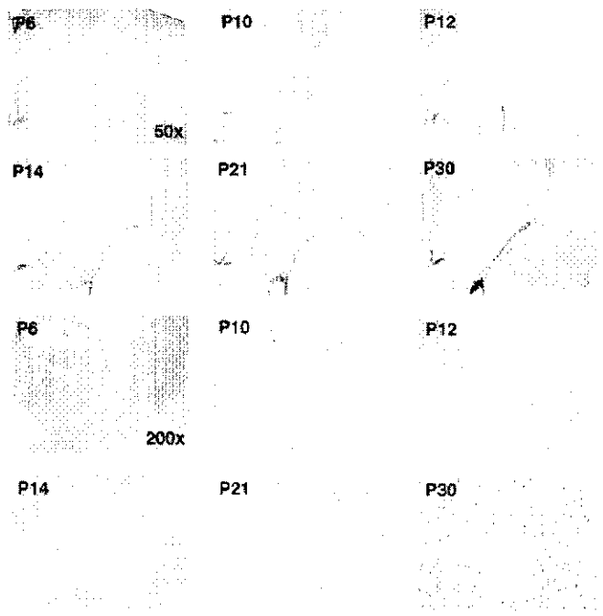

Figure 2- 6. Distribution of FGF-2 in the subventricular zone, motor cortex, and external capsule. FGF-2 was first expressed in the subventricular zone, the motor cortex and the external capsule at P10. Higher magnification (200x) shows an originally faint expressed of FGF-2 in the motor cortex at P10. FGF-2 expression in the motor cortex was robustly expressed from P21 onward.

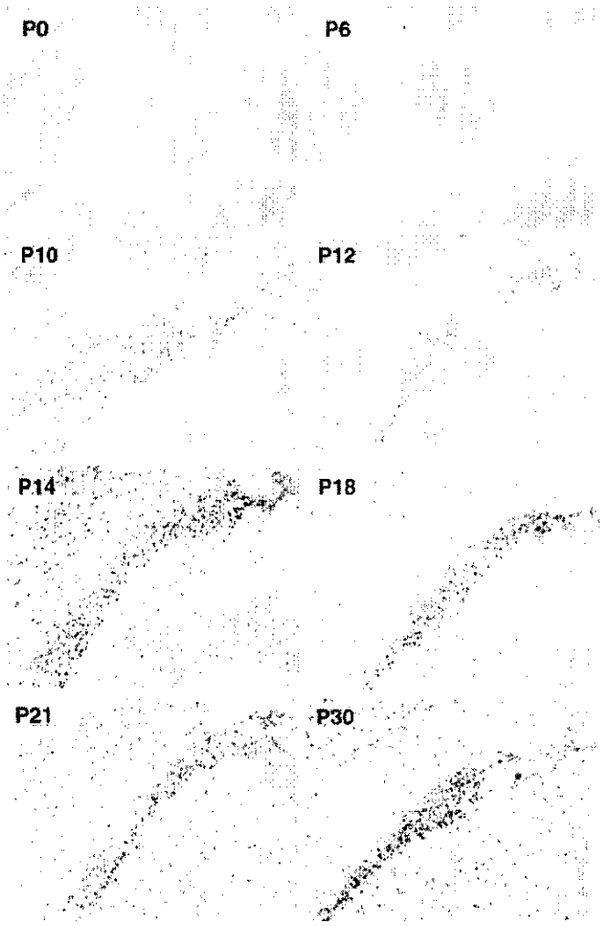

Figure 2- 7. Distribution of FGF-2 in the lateral portion of the subventricular zone (SVZ). FGF-2 was first expressed in the subventricular zone at P10. Higher magnification (200x) shows an originally faint expressed of FGF-2 in the SVZ at P10. It peaked at P14, and was robustly expressed from then onward.

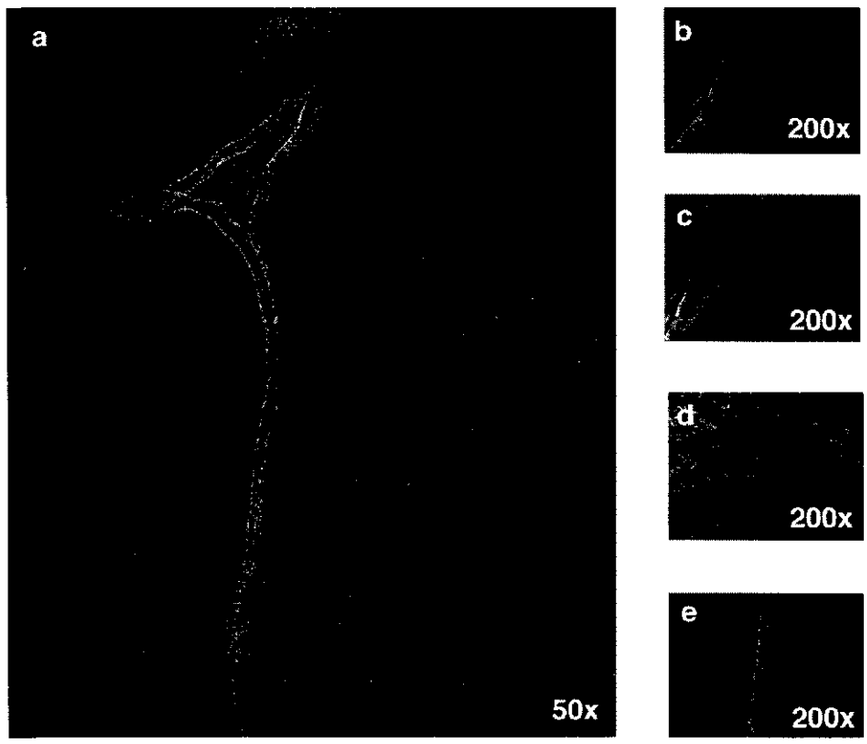

Figure 2- 8. Glial phenotype of FGF-2 expressing cells in the subventricular zone and rostral migratory stream at postnatal day 12. At P12, FGF-2 positive cells were found at the lateral edge of the subventricular wall. Higher magnification (b, c, and d) shows that FGF-2 positive cells co-expressed the immature astrocytic marker vimentin (shown in green). A portion of FGF-2 positive cells were co-localized with GFAP (b). In the rostral migratory stream (e) both FGF-2 and vimentin positive cells were expressed, but were not co-localized.

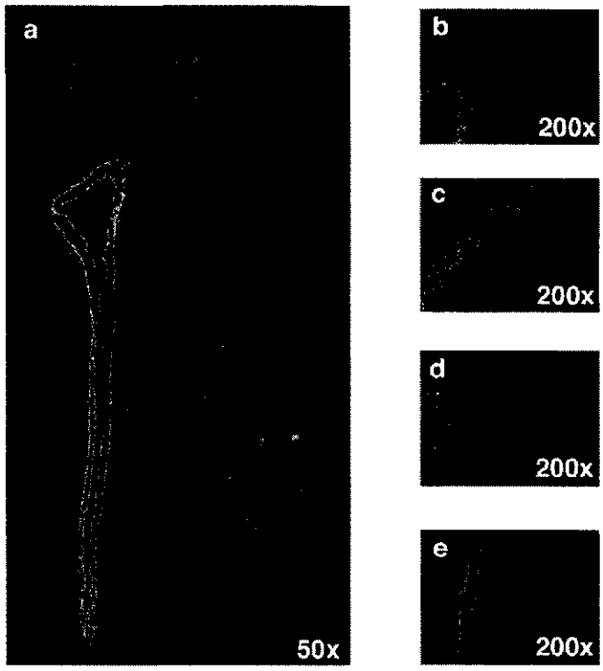

Figure 2- 9. Glial phenotype of FGF-2 expressing cells in the subventricular zone and rostral migratory stream at postnatal day 30. At P30, FGF-2 positive cells were found at the lateral edge of the subventricular wall. Higher magnification (b, c, and d) shows that most FGF-2 positive cells did not co-expressed the immature astrocytic marker vimentin (shown in green). Vimentin positive but not FGF-2 positive cells were expressed in the In the rostral migratory stream (e).

cells. The spatial distribution of FGF-2 expression by the two different cell phenotypes appears to be consistent throughout development. This finding confirms the conclusions of prior studies that reported that FGF-2 was expressed by both neurons and glial cells in different brain regions (Gomez-Pinilla et al., 1994). Our study extends these findings to distinct regions throughout the developing brain and suggests that the phenotypic distribution within structures remains constant throughout postnatal development. Third, FGF-2 expression appears to be differentially expressed across subregions of the hippocampus. This finding was previously reported to occur in the adult (Kuzis et al., 1995), and developing rat brain (Gomez-Pinilla et al., 1994; Kuzis et al., 1995). Although our results differ slightly from those reported by Gomez-Pinilla et al. (1994), there are methodological differences in the protocols and the precise antibodies that may account for the differences. Their FGF-2 antibody recognized the molecular conformation related to the biological activity of FGF-2. Because the brains were perfused in PFA (which cross links proteins) prior to immunoprocessing, it is possible that the biological activity of FGF-2 was altered and affected its expression, and thus could account for the discrepancy between our and their findings. The significance of the differential distribution of FGF-2 throughout the hippocampus is currently unknown, but suggests a differential role of FGF-2 for distinct subpopulations within this region. Interestingly, however, all the brain regions in which FGF-2 shows a neuronal expression are strongly interconnected (i.e., the IG, HPC and septal area) and originate from the same embryonic subregion.

The results we observed in the subventricular zone are particularly interesting because they show that (1) there are no FGF-2 expressing cells in this region until P10, and (2) the co-localization of FGF-2 expressing cells with glial cells is differentially regulated across development. Thus, between P10 and P18, a portion of cells expressed FGF-2 and vimentin only, whereas others co-expressed FGF-2, vimentin, and GFAP. At P30, the vimentin positive/GFAP negative cell population no longer expressed FGF-2.

The differential expression of FGF-2-reactive glial cells in the SVZ may be important for understanding the recovery observed after perinatal cortical lesions. Rats with lesions anywhere in the cortical mantle from postnatal day 1-5 show a dismal functional outcome whereas similar lesions from day 7-15 show a much better outcome, with the best outcome roughly around day 10 (e.g., Kolb, 1995). The enhanced production of glial cells in the second week could result in the increased production of FGF-2 and we thus have proposed that FGF-2 may play an important role in mediating the observed recovery (Gibb & Kolb, 2005). Indeed, we have recently shown that exogenous application of FGF-2 after medial frontal or posterior parietal lesions on day 3 (Comeau et al., 2005) or motor cortex lesions on day 10 (Monfils et al., 2005) facilitates recovery. We have also shown that postinjury tactile stimulation enhances functional recovery from day 3 lesions to medial frontal cortex, motor cortex, or posterior parietal cortex (e.g., Gibb & Kolb, 2005) and that this is correlated with an enhanced expression of FGF-2 and FGF-2 receptors in both skin and brain. It thus seems reasonable to

propose that the good endogenous recovery from injury around postnatal day 10 is related to the expression of FGF-2 by the glial cells that begin to appear after the injury.

The fact that there are no FGF-2 positive cells in the SVZ prior to P10 (which coincides with the time at which the most potential for recovery is observed) provides support for the idea that it might play a role in recovery. Subventricular zone astrocytes have received much attention in recent years (for a review, see Doetsch et al., 2003a; Doetsch et al., 2003b). They have been shown to act as stem cells *in vitro* and have been characterized as the primary *in vivo* precursors for new neurons in the adult brain (Doetsch et al., 1999). Doetsch et al.'s work (Doetsch et al., 1999, 2003a, 2003b) offers that the adult SVZ is populated by migrating neuroblasts (type A cells), precursor cells (type C cells), SVZ astrocytes (type B cells) and ependymal cells, and that SVZ astrocytes are pluripotent. Effectively, after elimination of type A and C cells, the SVZ repopulates and a few days later type A, B and C cells can be found in the SVZ.

The results of the present study, in light of prior work reported from our laboratory, does not preclude the possibility that at least a portion of FGF-2 expressing cells within the SVZ might be pluripotent (between P10 and P18) (Kolb et al., 1996; Monfils et al., 2005). They are not A cells (as evidence by the fact that they were not labeled with doublecortin), and they express some mature and immature astrocytic markers (vimentin and/or GFAP). Interestingly, Doetsch et al.'s experiment has shown that after elimination of type A and C cells with Ara-C treatment, the type B cells show

staining for vimentin, which is indicative of immature astrocytes (1999). The fact that we see a different expression of FGF-2 in seemingly different types of SVZ astrocytes (vimentin positive/GFAP negative, vimentin positive/GFAP positive, vimentin negative/GFAP positive) throughout different postnatal days may provide support for a role of FGF-2 in the differential recovery observed across those days. Our study did not directly test this possibility.

Recently, Zheng et al. (2004) showed that in the absence of FGF-2 gene product, the dividing progenitor population of the SVZ is reduced by 50%. Furthermore, there is a precedent in the literature suggesting that vimentin positive FGF-2 cells might show pluripotential. According to Ganat et al. (2002), FGF-2-containing astrocytes within the normal adult HPC and SVZ are non-dividing. Ganat et al. (2002) have shown, however, that under chronic hypoxic conditions, the majority of FGF-2 containing cells do not show any detectable levels of GFAP staining (but are vimentin positive) suggesting that hypoxia might induce those cells to revert to an immature state. In the adult brain, FGF-2 has been shown to play a role in the response of astrocytes to injury (Reilly & Kumari, 1996). FGF-2 expression is enhanced following injury (Clarke et al., 2001; Ganat et al., 2002), exogenous FGF-2 injection post injury increases astrocytic hypertrophy, and the expression FGF receptors 1 and 2 (FGFR1 and FGFR2) is also upregulated in response to cortical ischemia (Reilly & Kumari, 1996). We therefore propose that the mechanisms leading to astrocytic hypertrophy and FGF-2 upregulation in adulthood might recapitulate the permissive environment observed around P10 in the rat. In line with this idea, we

would predict that (1) an injury inflicted prior to P10 would *not* lead to recovery and would *not* induce astrocytic hypertrophy nor FGF-2 upregulation; and (2) that certain types of injury, provided they lead to an upregulation of FGF-2, might be beneficial to recovery following a second insult. There is evidence supporting both of these predictions. Prior to P10, hypoxia fails to induce an upregulation of FGF-2 (Ganat et al., 2002). Furthermore, cortical lesions inflicted between P1 and P5 lead to a dismal recovery (Kolb, 1995). Additionally, Matsushima et al. (1998) found that ischemic preconditioning, which is known to be neuroprotective during a subsequent ischemic incident, induced an increase in FGF-2 levels.

There is a well-documented effect of exogenously applied FGF-2 on cell proliferation and recovery from injury, as well as a role of FGF-2 in cortical development (Comeau et al., 2005; Monfils et al., 2005; Reilly & Kumari, 1996; Ford-Perriss et al., 2001). The findings presented here suggest that the differential distribution of FGF-2 during development may account for the differential recovery observed following lesions inflicted at different time points. Nevertheless, the question remains as to what mechanism(s) might be at play to initiate such processes. Recent work (Olsnes et al., 2003) suggests that translocation of FGFR1 to the nucleus by FGF-2 stimulation is induced by the 18 K molecular form. Endogenous FGF-2 can thus act on FGFR-expressing astrocytes to modulate their response to injury. It is conceivable that, under permissive conditions, this mechanism might translate into a mobilization of cells to the site of injury, and that in turn these cells promote recovery. Evidence suggests that

subpopulations of SVZ astrocytes (namely, the ones expressing vimentin and FGF-2) might operate through such a mechanism. A previous study has shown that the different isoforms of FGF-2 may serve to differentially modulate proliferation and migration (Bikfalvi et al., 1997). Namely, the high molecular forms, located predominantly in the nucleus, control proliferation through a receptor-independent mechanism, whereas the low molecular form (18 K), which is found in the cytoplasm, would stimulate migration by autocrine receptor activation (Bikfalvi et al., 1997).

Many studies suggest a role of FGF-2 and its receptors in brain development as well as in recovery from injury (e.g., Reilly & Kumari, 1996). The present results show that there is a differential distribution if FGF-2 expression throughout development that varies as a function of the brain region under investigation. We propose that this serve to explain the differential recovery observed following postnatal neocortical lesions inflicted at different time points after birth (Kolb et al., 2000a; Kolb et al., 2000b; Kolb et al., 1996). Future studies will be geared towards harnessing these principles to facilitate recovery from injury throughout the life span, as well as to establish whether administering FGF-2 exogenously might prove to be beneficial following lesions to a brain area that is typically associated with persistent deficits (e.g., motor cortex).

Chapter 3: FGF-2 stimulates functional recovery after neonatal lesions of motor cortex in rats

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Published in Neuroscience, 2005.

ABBREVIATIONS

mPFC	-	medial prefrontal cortex
P10	-	postnatal day 10
P1	-	postnatal day 1
P90	-	postnatal day 90
FGF-2	-	basic fibroblast growth factor
BSA	-	Bovine serum Albumin
i.p.	-	intraperitoneally
CFA	-	Caudal forelimb area
RFA	-	Rostral forelimb area
ANOVA	-	Analysis of Variance
ICMS	-	Intracortical microstimulation

Abstract

Rats were given bilateral lesions of the motor cortex on the tenth day of life (P10), and then received a daily subcutaneous injection of either basic fibroblast growth factor (FGF-2) or vehicle for seven consecutive days. In adulthood, they were trained and assessed on a skilled forelimb reaching task. Although all lesion groups were impaired at skilled reaching, the P10 lesion group that received FGF-2 was less impaired than the lesion group that received the vehicle. Furthermore, the lesion rats that received FGF-2 showed a filling of the lesion cavity with tissue, whereas the lesioned vehicle-treated rats still had a prominent lesion cavity. The functionality of the tissue filling the cavity, tissue surrounding it, and tissue from the motor cortex (in control rats) was assessed using intracortical microstimulation, and showed that stimulation of some sites from the filled cavity could evoke movement. The rats were perfused and processed for Golgi-Cox staining. Medium spiny neurons from the striatum were drawn and analyzed, and the results suggest that P10 lesions of the motor cortex induced an increase in the length and complexity of these cells compared to those of no lesion rats. Our results suggest that FGF-2 may play an important role in recovery from early brain damage.

Key words: caudal forelimb area, dendritic morphology, golgi-cox, ICMS, reorganization

Rats that receive neocortical injury display a broad range of behavioural and anatomical outcomes that vary depending on the age at the time of lesion, whether the injury is unilateral or bilateral, the site and extent of the damage inflicted, and the precise behavioral tests employed (e.g., Kolb, 1995). For example, bilateral injury of the medial prefrontal cortex (mPFC) inflicted around postnatal day 10 (P10) leads to remarkable functional and anatomical recovery including a filling of the lesion cavity with newly generated neurons and glia, and increased dendritic arborization in pyramidal cells throughout the remaining neocortex (e.g., Kolb, 1995; Kolb et al., 1998). In contrast, injury inflicted earlier (postnatal days 1-6) in development or in adulthood leads to severe behavioral deficits and no filling of the lesion cavity or dendritic hypertrophy. These results *a priori* suggest that there may be something particular about P10 that allows recovery of function and its underlying anatomical changes. Curiously, the filling of the lesion cavity is not observed after P10 lesions in other cortical areas, including both anterior and posterior regions (e.g., Gonzalez, Whishaw & Kolb, 2003). Interestingly, despite the fact that P10 lesions in other cortical regions do not show a spontaneous filling of the lesion cavity, those rats that receive lesions at P10 recover better than their P1 or P90 counterparts, but in no case do they show recovery that is as extensive as in animals with mPFC lesions that show generation of new tissue. It thus appears that the recovery seen after mPFC lesions at P10 may be related both to synaptic changes in the remaining brain as well as in the generation of new neurons and glia.

The goal of the current study was to determine if exogenous stimulation of cell generation might further improve functional outcome after P10 lesions. We chose to

study animals with bilateral P10 motor cortex lesions for two reasons. First, although rats with P10 motor cortex lesions show a better outcome than animals with day 1 or adult lesions, they are still profoundly impaired at skilled motor tasks (e.g., Kolb, Cioe & Whishaw, 2000). Second, we made bilateral rather than unilateral lesions because we wished to ensure that any enhanced motor function was not due to changes in the normal hemisphere of unilaterally-injured animals. Furthermore, there is good evidence in humans that whereas unilateral injuries in infancy allow significant functional recovery, recovery from bilateral injuries, even when the injury in one hemisphere is limited, is surprisingly poor. For example, an infant with an injury to language zones in the left hemisphere and a small injury outside the homologous zones in the right hemisphere will show a dismal outcome, including permanent aphasia (e.g., Vargha-Khadem, Watters, & O'Gorman, 1985). It is thus of clinical interest to see if there might be a treatment that can be effective in stimulating recovery from bilateral injuries in the infant brain.

Basic fibroblast growth factor (FGF-2) was recently proposed to account for the functional and anatomical recovery seen following P10 damage to the mPFC (Gibb & Kolb, 2005). FGF-2 is also known to induce neurogenesis (Wagner et al., 1999), enhance neuronal survival and outgrowth both *in vivo* and *in vitro* (Ramirez et al., 1989; Walicke, 1988), and to play a prominent role in the regulation of central nervous system injury responses (Baird and Walicke, 1989; Logan, 1990); including scar formation (Logan, 1990), and promotion of neurotrophic factors. Recently, FGF-2 was found to improve sensorimotor deficits and to reduce infarct size following cerebral ischemia in adult rats

(Li & Stephenson, 2002), and neutralizing antibodies to FGF-2 blocks recovery from motor cortex lesions (Rowntree & Kolb, 1997).

The present study sought to investigate whether peripheral administration of FGF-2 could ameliorate the functional and anatomical outcome following P10 bilateral injury to the motor cortex. Functional outcome was evaluated by testing the rats' performance on a skilled reaching task, as well as by assessing the integrity of motor cortex by using intracortical microstimulation to evoke movements (at P100). Furthermore, because prior work reported some changes to occur in the dorsolateral striatum in response to adult motor cortical injury (Gonzalez & Kolb, 2003), we also measured the effects of motor cortex lesions and FGF-2 administration on dendritic morphology in this structure.

Experimental procedures

Subjects

The present study used 20 male rats, derived from the Charles River Long-Evans hooded strain, which were divided into groups that received either bilateral motor cortex lesions (N=10) or sham operations (N=10) on postnatal day 10. The animals were group-housed, and then placed with same sex littermates in clear plastic cages in a colony room maintained on a 12h on/12h off light cycle. All experimentation was conducted during the light phase. Rats were maintained on Lab Diet no. 5001 (PMI feeds Inc., St-Louis, MO) and water *ad libitum*, except during the Whishaw tray reaching task, and were handled and cared for according to the Canadian Council for Animal Care guidelines.

Materials

FGF-2 injections

Basic fibroblast growth factor (FGF-2) was diluted in Bovine serum Albumin (BSA) (1 μg/ml), and administered at 0.1 ml/10g of body weight (for a final concentration of 10 ng/g). This concentration was determined based on the work of Wagner et al. (1999), which had shown this concentration to be sufficient to enhance neurogenesis in P7-P28 rats. BSA was prepared into 0.1 M PO_4 as a concentration of 1 mg/ml.

Treatment groups

The lesion and sham-operated rats were divided into groups that received 7 daily sessions of either FGF-2 (10) or BSA (10) administered subcutaneously. In a previous study conducted in our lab, this injection protocol was found to be effective in inducing partial behavioral recovery following medial prefrontal cortex aspiration lesions at P3 (Comeau, Hastings & Kolb, 2005). Following injections, the rats were allowed to age until P100, at which time they were trained on the Whishaw tray reaching task for 14 days. Then, the hemisphere contralateral to their preferred forelimb was mapped using intracortical microstimulation, and the rats were intracardially perfused and their brains processed for Golgi-Cox staining.

Lesion procedure

The neonatal rats were anesthetized by cooling in a Thermatron cooling chamber until rectal temperature was in the range of 18-20°C. The bone over the motor neocortex area was removed with iris scissors and motor decortication was achieved by aspiration using procedures previously described by Kolb (1987). Kolb (1987) previously showed that, on postnatal day 1, the frontal cortex lies more anterior relative to bregma than it does in adulthood, but that the relation between the bregmoidal junction and the underlying cortex is constant after about postnatal day 5. Thus, the lesion was performed from +2 to –2, relative to the bregmoidal junction. The scalp wound was sutured with 5-0 Vicryl thread as soon as the surgery was complete. The control rats were anesthetized, the skin incised, and then sutured with 5-0 Vicryl thread.

Skilled Reaching task

Forelimb use was measured with a procedure that was adapted from the method devised by Whishaw et al. (1991). Each animal was food deprived to 85 % free feeding level throughout training and testing. The rats were placed in the test cages (10X18X10 cm high) with floors and front constructed of 2 mm bars, 9 mm apart edge to edge. A 4 cm wide and 5 cm deep tray, containing chicken feed pellets, was mounted in front of each box. The rats were required to extend a forelimb through the gap in the bars, grasp and obtain the food. The tray was mounted on runners and was retracted 0.5 cm from the cage so that the rats could not scrape the food into the cage. If the animal attempted to

rake the pellet out of the tray, the pellet would inevitably fall through the gap. An attempt was scored only if the rat reached into the tray and touched the food. If it reached into the tray without touching the pellet, no attempt was scored. A successful reach was defined as grasping a pellet, and bringing it to the mouth. The rats were trained for 20 minutes per day for 14 consecutive days. Once trained, the rats received a 10 minute reaching test during which time they were videotaped for later scoring. All rats showed a forelimb preference, and only the reaches performed by the preferred limb were scored and analyzed.

Intracortical Microstimulation

Two weeks following the last reach training session (to rule out immediate effects of training and food deprivation on the maps), intracortical microstimulation (ICMS) was used to generate detailed maps of the motor cortex forelimb regions (Kleim et al., 1998) by experimenters that were unaware of the rats' treatment condition. Rats were anesthetized with ketamine hydrochloride (70 mg/kg, injected intraperitoneally [i.p.]), and xylazine (5 mg/kg, i.p.), and ketamine (20 mg/kg, i.p.) as supplementary injections when needed. A craniotomy was performed over motor cortex, and the dura was carefully removed. The exposed cortex was then covered with warm (37.4C) silicone oil. A small puncture was made in the cisterna magnum to reduce cortical edema. A glass microelectrode (between 0.2 and 0.7 MOhms) controlled by a hydraulic microdrive was used to make penetrations to a depth of ~ 1550 μm (corresponding to cortical layer V), with an interpenetration distance of 375 μm. Stimulation consisted of thirteen, 200 μs

cathodal pulses delivered at 350 Hz from an electrically isolated stimulation circuit. Animals were maintained in a prone position, with the limb contralateral to the side being supported in a consistent position. At each penetration site the minimal threshold required to elicit a movement was recorded. Sites where no movement was detected at ≤ 100 μA were recorded as unresponsive. The level of anesthesia was assessed by monitoring the breathing rate and by revisiting positive-response sites to check for changes in movement thresholds as mapping progressed. In the control rats, mapping was restricted to CFA and RFA, as well as the necessary boundaries defining those regions. In the lesioned rats, mapping was extended to include most of the hemisphere in order to assess whether motor cortex had simply relocated.

Movement representation analysis

An image analysis program (CANVAS v. 8) was used to calculate the aerial extent of the caudal forelimb area (CFA). In control rats, the CFA is separated from the rostral forelimb area (RFA) by a band of neck/whisker representations (Kleim et al., 1998) and was chosen for analysis because previous work demonstrated its capacity to undergo reorganization following behavioural manipulations (Kleim et al., 1998, Remple et al, 2001) or stimulation (Nudo, et al., 1990; Teskey, et al., 2002; Monfils et al., 2004). The RFA has proven, thus far, to be resistant to modifications.

Perfusion and histology

Following mapping, the rats were intracardially perfused with 0.9% saline, and the brains were removed and weighed. The mapped hemisphere was then immersed in 40 ml of Golgi-Cox solution (Glaser and van der Loos, 1981). The solution was changed after two days and the brains remained immersed for an additional 14 days before being placed in a 30% sucrose solution for 2 days, cut on a vibratome at 200 μm, and developed using a procedure outlined by Gibb and Kolb (1998).

Analysis of Dendritic Branching, Length and Spine Density

Medium spiny neurons from the dorsolateral striatum were traced using a camera lucida at 260x (Occular setting- 10x, camera lucida- 1.3x, and microscope objective- 20x). While there were possible distance distortions generated in the peripheral portions of the field, tissue from all animals was treated in exactly the same manner, under blind conditions to eliminate any systematic bias. Dendritic trees had to maintain the following criteria to be included in the data analysis: (1) the cells had to be well impregnated and in full view, unblocked by blood vessels, astrocytes, or clustering of dendrites from other cells; (2) the arborizations had to appear intact and visible in the plane of section. Cells were chosen by locating the dorsolateral striatum (Zilles, 1985) and then drawing each cell in the section that maintained the above criteria. Following drawing of the cells, each branch segment was counted and summarized by branch order according to the methods of Coleman and Riesen (1968). As such, dendrites were determined to be first order if the branch originated from the cell body increasing in order with every

bifurcation. Five cells were drawn for each rat. The mean of the measurements on five cells per rat was used for statistical analyses.

A Sholl analysis of ring intersections was used to estimate dendritic length (Sholl, 1956). The number of intersections of dendrites with a series of concentric spheres at 20 μm intervals from the center of the cell body was counted for each cell. Total dendritic length (in μm) was estimated by multiplying the number of intersections by 20. Spine density measures were made from a segment between 10-50 μm in length. The dendrite was traced (at 1300x: Occular setting- 10x, camera lucida- 1.3x, and microscope objective- 100x) using a camera lucida drawing tube and the exact length of the dendritic segment was 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, therefore the spine density values are likely to underestimate the actual density of the dendritic spines.

Statistical Analyses

Factorial Analyses of Variance (ANOVAs) were performed and compared the four experimental groups on 6 dependent measures: brain weight, skilled reaching performance, CFA size, as well as striatal dendritic length, dendritic morphology, and spine density. Significant interactions were followed-up with Fisher's least significant difference tests. All statistical analyses were performed using StatView5.

Results

Gross anatomy

As in our previous studies, the untreated lesion rats all showed some degree of lesion cavitation in adulthood. In contrast, there was filling of the lesion cavity in the lesion rats that received FGF-2. Figure 3-1 illustrates that although the lesion cavity of the P10 FGF-2 treated rats was filled with cells, the organization was haphazard with little evidence of lamination. Inspection of the Golgi-stained sections revealed that in contrast to normal brains in which the apical dendrites of pyramidal neurons project perpendicular to the cortical surface, many pyramidal cells in the in-filled cavity project at other orientations (see Figure 3-1D).

Brain weight

As expected, the brains of the lesion rats weighed less than the no lesion animals. There was, however, an effect of FGF-2 as the FGF-2-treated lesioned rats had significantly heavier brains than their untreated counterparts. A factorial (2X2) Analysis of Variance (ANOVA) yielded a significant main effect of Lesion ($F(1,15)=13.024$, $p=0.0026$), and a Lesion X FGF-2 interaction, ($F(1,15)= 5.28$, $p= 0.0363$) in brain weight. Follow-up tests revealed that whereas the untreated lesion rat brains weighed less than controls, the FGF-2 treated lesion rat brain weights did not differ from their control counterparts (see Figure 3-2, $p< .05$), thus leading to the significant interaction.

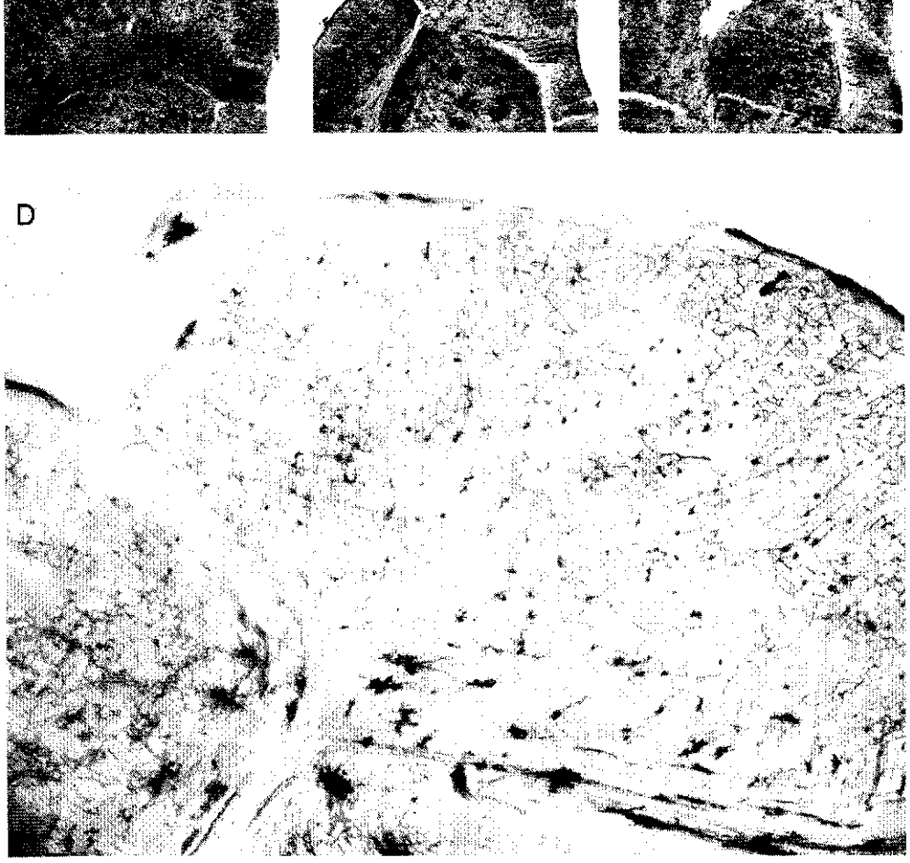

Figure 3- 1. Distribution of cells within the filled cavity of rats that received motor cortex aspiration lesions at postnatal day 10 and FGF-2 treatment. The cells from the filled cavity did not show clear laminar distribution (B and D) compared to no lesion rats (A). The lesion rats that did not receive FGF-2 all showed a lesion cavity in adulthood (C). Pictures A, B, and C were taken at a magnification of 2.5x, and D was taken at a magnification of 10x.

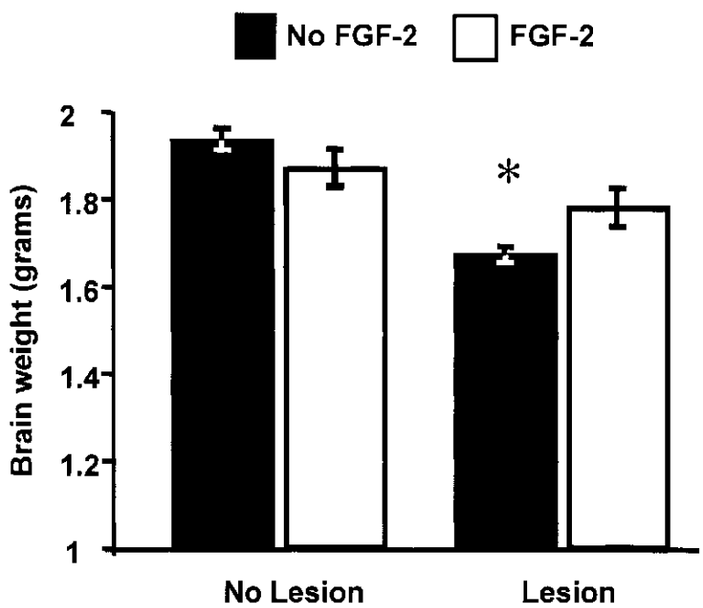

Figure 3- 2. Effect of postnatal day 10 motor cortex lesion and/or FGF-2 administration on the brain weights of adult rats. There was an effect of lesion, which indicated that the lesion rats had lighter brains than no lesion rats. There was also a Lesion X FGF-2 treatment interaction whereby there was no differential effect of FGF-2 treatment for the no lesion rats, but the FGF-2 treated lesion rats' brains were, on average, heavier than those of the lesion rats that did not receive FGF-2.

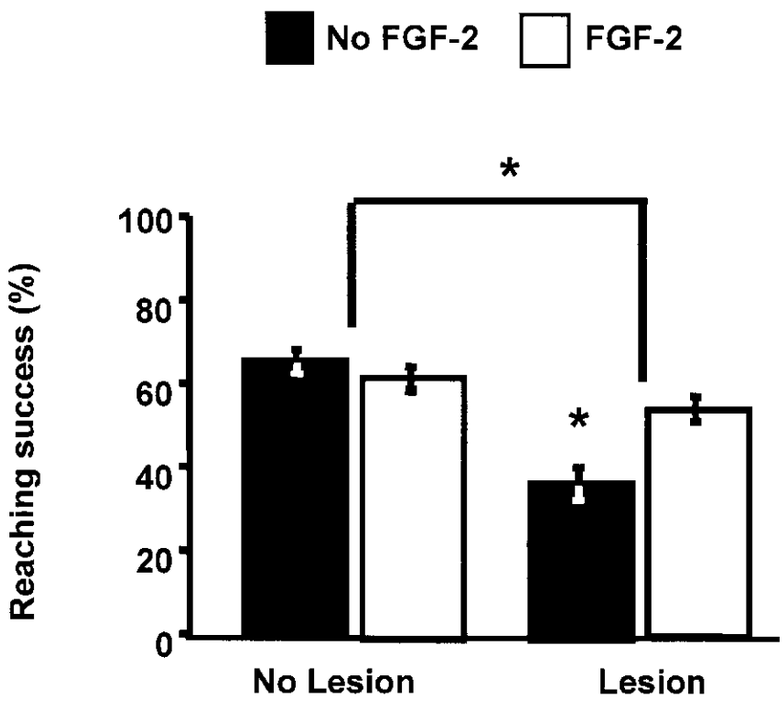

Figure 3- 3. Effect of postnatal day 10 motor cortex lesion and/or FGF-2 administration on the performance of adult rats in the Whishaw tray reaching task. The no lesion groups are shown in white, and the lesion groups shown in gray. There was an overall effect of lesion on the rats' success in the skilled reaching task. There was also a Lesion X FGF-2 treatment interaction whereby the magnitude of the difference between the lesion and no lesion rats was reduced in the rats that received FGF-2. Importantly, the lesion rats that received FGF-2 outperformed the lesion-untreated rats.

Skilled reaching task

There was an overall effect of lesion on the rats' success in the skilled reaching task. More importantly, however, Figure 3-3 shows that FGF-2 enhanced motor performance in the lesion rats relative to the untreated lesion rats. A 2X2 factorial ANOVA revealed a main effect of lesion, $F(1,15)= 70.157$, $p< 0.0001$, as well as a significant Lesion X FGF-2 interaction, $F(1,15)= 19.203$, $p<0.0009$, on rats' reaching success. Follow-up tests revealed that there was a significant difference between the control and lesion rats on the reaching task for both the FGF-2 and the non-FGF-2 treated groups, $p< .05$, but the FGF-2 rats performed significantly better (Figure 3-3).

Mapping of the motor cortex and filled lesion cavity

Whereas all control rats showed clear forelimb representations, none of the lesion untreated rats showed any forelimb representations (Figure 3-4). In contrast, some movements could be elicited in all of the FGF-2 treated lesion animals. There was, however, a lot of variability in the type of movement that could be elicited. Two examples of mapping variability can be found in Figure 3-5. It is clear in Figure 5C and 5D that, in some of the FGF-2 treated lesion rats, forelimb movements could be elicited, whereas in others, the movements that could be elicited were limited to other types of movement (shown here, predominantly vibrissae sites). Such variability in the types of movements that could be elicited from the filled cavity may be due to lesion variability.

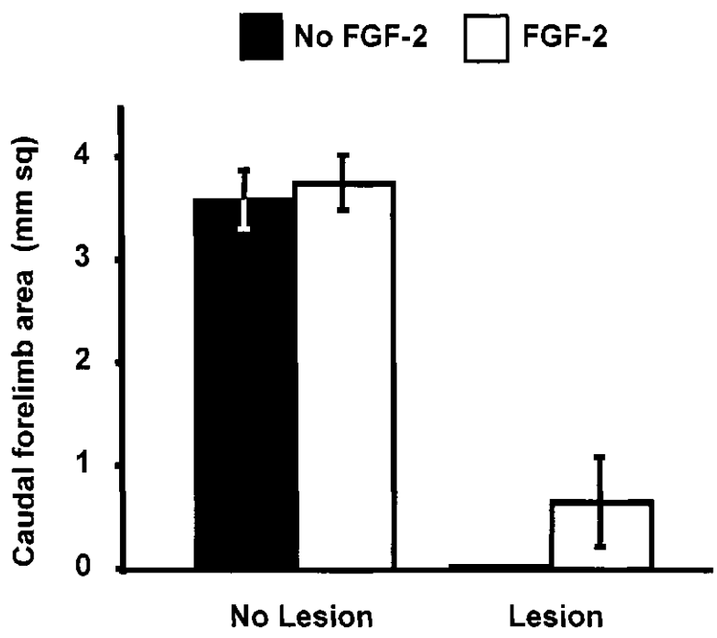

Figure 3- 4. Quantitative representation of the effect of postnatal day 10 motor cortex lesion and/or FGF-2 administration on the mean areal size of the caudal forelimb area in adult rats. The no lesion groups are shown in white, and the lesion groups shown in gray. No statistical tests were performed for these comparisons, because the lesion group that did not receive FGF-2 did not have any variability (due to a floor effect). However, it is obvious that there was an overall effect of lesion. Note: in all cases, electrode penetrations were performed around the lesion site, as well as in and around the filled lesion cavity to attempt to elicit movement from these regions.

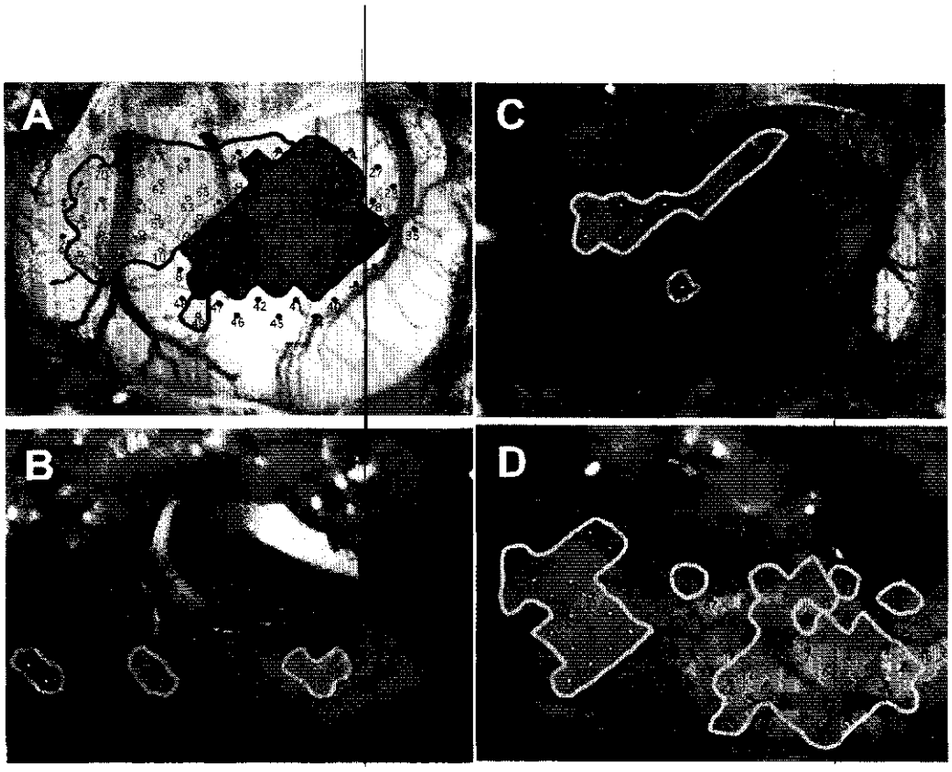

Figure 3- 5. Qualitative mapping data of representative rats from (A) a no lesion rat, (B) a lesion rat that did not receive FGF-2, (C) a lesion rat that received FGF-2 from which forelimb movements could be elicited, and (D) a lesion rat that received FGF-2 from which forelimb movements were not elicited (but that showed other responsive sites- predominantly vibrissae). Forelimb movements are shown in dark grey, and other movements are shown in light grey. For each animal, a black line is also shown, representing bregma. There was a lot of variability in the lesioned-FGF-2 treated rats. In all cases, however, some type of movements could be elicited from the tissue that filled the lesion cavity.

Dendritic morphology of striatal medium spiny neurons

Our analyses revealed that, overall, striatal cells from the lesion rats had longer and more complex dendritic branches than the no lesion animals. Those groups did not differ, however, in terms of spine density, and we did not find any FGF-2 effects or differential FGF-2 by lesion treatment interactions. ANOVAs performed to test Lesion X FGF-2 effects on dendritic length, branch complexity, and spine density revealed a main effect of lesion on dendritic length and branch complexity, $F(1,15)= 6.58$, $p =.023$, and $F(1,15)= 13.54$, $p= 0.0028$ (see Figure 3-6). There was no effect on spine density, and for either comparison, there was no significant interaction ($p>.05$).

Discussion

The main findings of the current study were that administration of FGF-2, following bilateral motor cortex aspiration lesions at postnatal day 10, leads to: 1) a filling of the lesion cavity; 2) a partial recovery of function on a skilled reaching task; and, (3) partial physiological recovery of the filled region. Importantly, neither the cavity filling nor the recovery of function were observed in the untreated lesion rats.

Our finding of enhanced functional recovery in the FGF-2 treated animals is significant in the larger view of the effects of neonatal cortical lesions. We have shown in an extensive series of studies that neonatal cortical injury virtually anywhere in the cerebral cortex will produce persistent deficits in skilled reaching tasks (e.g., Kolb, Cioe, & Whishaw, 2002; Kolb et al., 2000), even if the lesions are produced at the optimal time

for recovery, which is around 10 days of age (Kolb, 1995). In contrast, lesions to most cortical regions around 10 days of age allow significant recovery on cognitive tasks. Thus, it appears that motor deficits are more persistent, and as such, less affected by age-at-injury than cognitive functions. Indeed, even in the case of unilateral neonatal motor cortex injury, which does allow more extensive recovery than bilateral injury, there are still significant motor deficits (e.g., Castro, 1990; Kartje-Tillotson, Neafsey, & Castro, 1985; Whishaw & Kolb, 1988). Thus, our finding that FGF-2 increased performance of lesioned rats on the skilled reaching task is very encouraging.

A number of factors could explain the FGF-2 effect. For example, it is possible that FGF-2 acts on the cascade of events that already occurs following damage. Wei et al. (2000) found an increase in FGF-2 expression in the frontal cortex and striatum following middle cerebral artery occlusion in rats. They suggested that this increased expression of FGF-2 likely served to protect neurons following focal ischemia. Previous work from our lab has shown that the medial frontal lesioned P10 rats display an enhanced behavioural recovery, as well a filling of the lesion cavity (Kolb et al., 1998). Recently, FGF-2 was proposed to account for this recovery (Gibb & Kolb, 2005). It thus is possible that peripheral administration serves to 'amplify' the FGF-2 cascade that occurs following injury and provides support to the remaining cells. It also could be the case that FGF-2 repairs the lesioned area by mobilizing cells from a distant location such as the subventricular zone to the site of damage, much as in the spontaneous regeneration after mPFC lesions on P10. The newly generated cells could either participate directly in

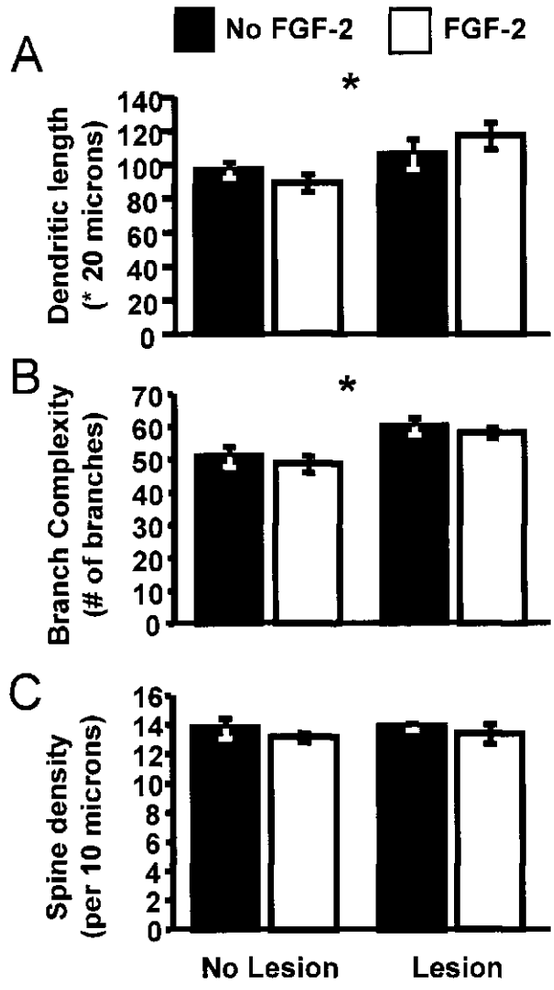

Figure 3- 6. Effect of postnatal day 10 motor cortex lesion and/or FGF-2 administration on the dendritic length, cell complexity, and spine density of medium spiny neurons of the striatum. Overall, striatal cells from the lesioned (gray) rats had longer (A) and more complex branches (B) than the non-lesioned rats (white). Those groups did not differ with respect to spine density (C). There were no FGF-2 effects or Lesion X FGF-2 interactions.

functional recovery by forming connections with the rest of the brain, or indirectly by providing some type of trophic support that facilitates function in the nearby intact brain. There is some evidence in the literature suggesting that incremental administration of FGF-2 following injury can lead to an increase in cell proliferation (Matsuoka et al., 2003). It is also possible that FGF-2 simply acts on completely different brain regions that provide compensation, such as the anterior cingulate corticospinal projections. A combination of these scenarios likely accounts for the changes observed.

Mapping of the filled lesion cavity suggests that stimulation could elicit movements from a number of sites, although forelimb movements were sparse at the stimulation intensities used here. The fact that any movements at all could be elicited (in the present case, mostly vibrissae movements), however, is of some interest, and at the very least suggests that there are cells in a brain region that would otherwise be largely absent (without the administration of FGF-2), and that such cells are functional. The fact that most of the elicited movements were from the vibrissae is puzzling and may represent some fundamental difference in the organization of vibrissal and forelimb movements or it may be related to the stimulation parameters. Certainly, the corticospinal projections related to forelimb movements are much longer, and may be more difficult to develop after neonatal injury. Thus, it may require a recruitment of more fibers, possibly with more intense stimulation, to observe more ICMS-related forelimb movements. One difficulty with the ICMS experiment is that this technique is performed by lowering a micro-electrode into layer V of the motor cortex, then sending a

small amount of current that activates horizontal networks of corticospinal neurons, which in turn elicits movements (Jankowska, 1975). In the present case, it is possible that although we thought we were stimulating layer V neurons, we were in fact not adequately targeting the appropriate networks necessary to elicit forelimb movements. We chose to limit deviations to the ICMS protocol to a minimum, because the technique itself can induce plastic changes within motor cortex (Nudo et al., 1998; Monfils, Plautz & Kleim, 2005). Furthermore, we wanted to ensure that our stimulation did not spread beyond the boundary of the regrown tissue and into the intact brain, when stimulating the filled region. Thus, it is possible that different stimulation parameters, or stimulation through different depths may have yielded more forelimb responses. It is also possible that a higher intensity of stimulation would have been more effective in eliciting forelimb responses. We chose not to use intense stimulation parameters, however, because it would have been difficult to compare the maps in control and lesion rats. Had we increased the intensity in the controls we would have activated a broader pool of neurons, which would have produced larger maps in the control rats. Thus, we chose to keep the intensity constant to allow for a more appropriate comparison. Finally, motor cortex mapping using ICMS relies on the subjective perception of movements. It is possible that such a method was not sensitive enough to detect subtle activation of the muscles upon stimulation of the filled cavity.

The absence of the rostral forelimb area in all animals with motor cortex lesions is curious given that the lesions would likely not have removed this region. It is possible

that one effect of the lesion was to produce a large-scale reorganization of the motor map. We did map well beyond the motor area and in some animals much of the hemisphere was stimulated but no anomalous map was seen. It thus seems unlikely that rostral forelimb areas simply relocated to some intact cortical region.

Our results do not conclusively demonstrate exactly how the FGF-2 treatment leads to better functional outcome. We have shown previously that neutralizing antibodies to FGF-2 block recovery in rats with motor cortex lesions in adulthood, and this was correlated with dendritic atrophy in the perilesional cortex (Rowntree & Kolb, 1997). Thus, it is possible that the FGF-2 treatment had the opposite effect here and acted on the neural networks in perilesional cortex. The absence of ICMS-positive sites in perilesional cortex does not support this idea. A second possibility is that FGF-2 increased neuro- or gliogenesis both in the region of the lesion cavity and elsewhere in the brain. We have obvious evidence of new cells in the lesion cavity but have no measure of cell generation elsewhere. Nonetheless, we believe that taken together, our behavioral and ICMS results support the conclusion that at least some of the cells that filled the lesion cavity contributed to motor function and are responsible, at least in part, for the functional improvements after FGF-2 treatment. This can be tested directly by either removing the new tissue or by preventing the generation of the new tissue. We have previously shown, in parallel studies in rats with medial prefrontal lesions on day 10, that removal of the spontaneously generated neurons that fill the lesion area reverses the behavioral benefits (Dallison & Kolb, 2003) and that prevention of the regeneration

by prenatal administration of bromodeoxyuridine blocks the regeneration and the accompanying functional recovery (Kolb et al., 1998). Such studies in motor cortex are currently in progress.

Another question that arises from our results relates to the source of the new cells, which would most likely be from the subventricular zone, and whether the cells form connections with the intact brain. The simplest way to answer these questions is to use the mitotic marker bromdeoxyuridine to label subventricular cells the day before surgery and then to do both retrograde and anterograde tracing studies in the adult brain. It should be possible to double label cells in the regrown tissue and thus support the putative role of these cells in recovery. These studies remain to be performed.

Because we thought it possible for the striatum to subserve some of the recovery of function seen in our study, we also investigated the effects of lesion and/or FGF-2 administration on the dendritic morphology of medium spiny neurons from the striatum. The fact that there was an effect of lesion, but no interaction or main effect of FGF-2 on any of the measures of dendritic morphology, suggests that changes within the striatum alone are likely not responsible for the recovery of function observed in the skilled reaching task. The fact that neonatal lesions to the motor cortex led to dendritic hypertrophy in the striatum is interesting. Gonzalez & Kolb (2003) reported differential changes within the striatum following unilateral injury to the motor cortex inflicted in adulthood. Effectively, whereas middle cerebral artery occlusion led to an increase in

dendritic arbor in the striatum, aspiration lesion led to a decrease in the same measures. This latter result contrasts with the current findings and suggests that changes in striatal neurons vary not only with lesion etiology but also with age at injury.

In sum, our work provides evidence that FGF-2 may play a significant role in recovery from early cortical injury. Our findings are in agreement with previous reports showing that administration of FGF-2 after cortical injuries on postnatal day 3, as well as the administration of treatments that stimulate the endogenous production of FGF-2, can positively affect recovery on cognitive tasks (Gibb & Kolb, 2005; Comeau, Hastings & Kolb, 2005). In these past studies, however, FGF-2 did not stimulate the filling of the lesion cavity. Indeed, to our knowledge, the present study provides the first evidence of a treatment that can stimulate functional and physiological recovery following *bilateral* motor cortex damage at any age. We have also found that FGF-2 is ineffective in stimulating recovery in adult rats with motor cortex injury (B Kolb, unpublished studies). The key difference here is that the injury and subsequent FGF treatments began on day 10, rather than earlier or later. An important question for future research is to determine what is special about day 10 and how we might use this knowledge to stimulate recovery at other ages. Additionally, in future studies we should aim to establish whether the tissue filling the cavity plays a significant role in mediating the functional improvement observed in the lesioned-FGF-2-treated rats.

Chapter 4. FGF-2-induced functional improvement from neonatal motor cortex injury via corticospinal projections

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Submitted to the Journal of Neuroscience, November 8, 2005.

Abstract

We have recently shown that the application of basic fibroblast growth factor (FGF-2) in postnatal 10 (P10) rats with motor cortex (MCx) lesions results in functional improvements accompanied with a filling of the lesion cavity with tissue. In contrast, lesion rats that do not receive FGF-2 do not show a filling of the lesion cavity and are impaired relative to their treated counterpart. The present experiment sought to evaluate the electrophysiological and anatomical connectivity of the projections from the tissue filling the cortex in lesioned neonatal rats that received FGF-2. To that effect, rats received bilateral MCx lesions on P10. Beginning with P11, they received subcutaneous injections of either vehicle or FGF-2 for 7 days. In adulthood, the physiology of presumptive corticospinal projections was evaluated using intracortical microstimulation, then recording the evoked electromyographic (EMG) activity in wrist extensors, and comparing the delay to elicit EMG activity to that of control rats. The regrown corticospinal projections were anterogradely traced using biotin dextran amine (BDA). Results revealed that in 5 out of 7 FGF-2 treated lesioned rats, activity could be induced in wrist extensors following stimulation of the filled cavity. Furthermore, in the rats in which EMG activity could be elicited, long descending axons could be labeled with projections into the spinal cord, which showed delays comparable to undamaged 'cortico'spinal fibers. Together our results suggest that the FGF-2-induced recovery from early MCx injury is supported by projections from the filled region.

Key words: EMG, corticospinal, FGF-2, reaching, motor cortex.

Basic fibroblast growth factor (FGF-2) is involved in a number of physiological processes including a potential role in regulating developmental processes (e.g., Li & DiCicco-Bloom, 2004; Raballo et al., 2000) as well as mediating recovery from injury in adult and neonatal animal models of focal brain injury (e.g., Ganat et al., 2002; Rowntree & Kolb, 1997; Gibb & Kolb, 2004; Monfils et al., 2005a). There is evidence suggesting that the different spatio-temporal developmental expression of FGF-2 might play a role in modulating recovery from cortical damage in neonatal rats (Ganat et al., 2002; Monfils et al., 2005a; Gibb & Kolb, 2004). In particular, Kolb et al. (1998) have reported spontaneous anatomical and behavioral recovery from medial prefrontal cortex aspiration lesions inflicted at postnatal day 10 in rats, and such recovery does not occur if the injury occurs earlier in development. Notably, the better outcome coincides with elevated FGF-2 expression in the cortex (Gibb & Kolb, 2004). Rowntree and Kolb (1997) have also shown that blocking FGF-2 following motor cortex lesions in adulthood worsens the behavioral outcome and was associated with dendritic atrophy in layer V pyramidal cells contralateral to the lesion. FGF-2 blockade also reduces FGF-2 reactivity following a unilateral aspiration lesion while preserving normal GFAP reactivity. Together, these studies suggest that endogenous FGF-2 plays a critical role in improving outcome following injury in neonatal and adult rats. Recently, we investigated whether exogenous administration of FGF-2 could be beneficial following aspiration lesions of cortex in neonates (see Comeau et al., 2005; Monfils et al., 2005b). We found that FGF-2 application leads to functional improvement in a reaching task following medial prefrontal cortex lesions inflicted at P3 (Comeau et al., 2005). Furthermore, FGF-2

induces partial recovery of function on a skilled motor task following bilateral motor cortex lesions inflicted at P10, accompanied by a filling of the lesion cavity with tissue (Monfils et al., 2005b). This result is especially important, considering that motor impairments following cortical injuries are quite persistent, regardless of age at injury or area damaged (Kolb et al., 2000). Our earlier findings emphasize the therapeutic potential of FGF-2 following cortical injuries, yet, the mechanism by which functional recovery might be promoted is currently unknown. For example it is uncertain whether the tissue filling the cavity directly contributes to functional improvements. Understanding the role and functionality of the regrown tissue may provide important insight on how to repair the injured central nervous system. A first step in this direction is to assess the integrity of connections from this region. We thus sought to evaluate whether the filled region where the formerly forelimb area of the motor cortex was located, contains neurons capable of activating forelimb muscles as seen in uninjured rats. In the present study, we examined the electrophysiological and anatomical integrity of regrown cortico spinal projections in motor cortex lesion rats that received FGF-2 to assess whether they form functional connections and possibly contribute to the functional recovery observed following FGF-2 administration.

Following bilateral aspiration lesions to the motor cortex at P10, rats received exogenous administration of either FGF-2 or BSA (vehicle). In adulthood, functional outcome was evaluated by testing the rats' performance on a skilled reaching task. Then, the connectivity of the corticospinal projections from the filled cavity was evaluated

using microstimulation and recording the electromyographic activity in wrist extensors. The anatomy of the regrown projections was assessed by anterograde tracing using biotin dextran amine.

Experimental procedures

Subjects

The present study used 24 male rats, derived from the Charles River Long-Evans hooded strain, which were divided into groups that received either bilateral motor cortex lesions (N=14) or sham operations (N=10) on postnatal day 10. The animals were group-housed, and then placed with same sex littermates in clear plastic cages in a colony room maintained on a 12 h on/12 h off light cycle. All experimentation was conducted during the light phase. Rats were maintained on Lab Diet no. 5001 (PMI feeds Inc., St-Louis, MO) and water *ad libitum*, except during the Whishaw tray reaching task, and were handled and cared for according to the Canadian Council for Animal Care guidelines.

Experimental Design

Lesion and sham-operated rats were divided into groups that received 7 daily injections (s.c.) of either FGF-2 (N=12) or bovine serum albumin (BSA) (N=12). In a previous study conducted in our laboratory, this injection protocol was found to be effective in promoting behavioral recovery following medial prefrontal cortex aspiration lesions at P3 (Comeau, et al., 2005), as well as functional improvements accompanied by a filling of

the lesion cavity following MCx lesions inflicted at P10 (Monfils et al., 2005b). Following injections, the rats were allowed to age until P100, at which time they were trained and tested in the Whishaw tray reaching task for 14 days (Whishaw, 1991). Then, the filled region contralateral to their preferred forelimb was electrophysiologically assessed using intra'cortical' microstimulation and EMG recordings, and traced using biotin dextran amine (BDA, Vector Laboratories, Burlington, ON, Canada). Two weeks later the rats were intracardially perfused, and their brains sectioned. One series was processed for cresyl violet staining, and another was processed to visualize BDA.

Materials

FGF-2 injections

Basic fibroblast growth factor (FGF-2, R & D systems, Minneapolis, MN #233-FB) was diluted in BSA (1 μg/ml), and administered at 0.1 ml/10 g of body weight per day (for a final concentration of 10 ng/g). This was based on the work of Wagner et al. (1999), which had shown this concentration to be sufficient to enhance neurogenesis in P7-P28 rats. BSA (Sigma-Aldrich) was prepared into 0.1 M PO_4 as a concentration of 1 mg/ml.

Lesion Procedure

P10 rats were anesthetized by cooling in a Thermatron cooling chamber until rectal temperature was in the range of 18-20°C. The bone over the motor neocortex area was removed with iris scissors and motor decortication was achieved by aspiration using

procedures described by Kolb (1987). Kolb (1987) previously showed that, on postnatal day 1, the frontal cortex lies more anterior relative to bregma than it does in adulthood, but that the relation between the bregmoidal junction and the underlying cortex is constant after about postnatal day 5. Thus, to completely ablate the forelimb area of the motor cortex, the lesion was performed from +2 to –2, relative to the bregmoidal junction. The control rats were anesthetized, the skin incised, and then sutured with 5-0 Vicryl thread.

Skilled Reaching Task

Forelimb use was measured with a procedure adapted from the method devised by Whishaw et al. (1991). Each animal was food deprived to 85 % free feeding level throughout training and testing. The rats were placed in the test cages (10x18x10 cm high) with floors and front constructed of 2 mm bars, 9 mm apart edge to edge. A 4 cm wide and 5 cm deep tray, containing chicken feed pellets, was mounted in front of each box. The rats were required to extend a forelimb through the gap in the bars, grasp and obtain the food. The tray was mounted on runners and was retracted 0.5 cm from the cage so that the rats could not scrape the food into the cage. If the animal attempted to rake the pellet out of the tray, the pellet would inevitably fall through the gap. An attempt was scored only if the rat reached into the tray and touched the food. If it reached into the tray without touching the pellet, no attempt was scored. A successful reach was defined as grasping a pellet, and bringing it to the mouth. The rats were trained for 20 minutes per day for 14 consecutive days. Once trained, the rats received a

10 minute reaching test during which time they were videotaped for later scoring. All rats showed a forelimb preference, and only the reaches performed by the preferred limb were scored and analyzed. The success score was expressed as a percentage, and was computed by dividing the number of successful reaches by the total number of reaches, and then multiplying the ratio by 100.

Intracortical Microstimulation and Electromyographic Recordings

Two weeks following the last reach training session (to rule out potential effects of training and food deprivation [Smith & Metz, 2005]), intra'cortical' microstimulation (ICMS) was used and evoked potentials in wrist extensors were recorded. Rats were anesthetized with ketamine hydrochloride (70 mg/kg, injected intraperitoneally [i.p.]), and xylazine (5 mg/kg, i.p.). Supplementary injections of ketamine (20 mg/kg, i.p.) were administered as needed. Teflon coated stainless-steel (Cooner wire Inc Chatsworth, Ca) bipolar electrodes with exposed tips of approximately 2 mm were acutely implanted in wrist extensors (Raineteau et al., 2001). A craniotomy was performed over the filled region (or motor cortex, in controls), and the dura was carefully removed. A tungsten-wire electrode (approximately 3.5 MOhms) controlled by a manual microdrive was used to make approximately 3-5 penetrations to a depth of ~ 1550 μm (corresponding to cortical layer V), or to the coordinates corresponding to the dorsolateral striatum (Paxinos & Watson, 1992). Stimulation consisted of twenty cathodal pulses, 0.2 ms in duration, delivered at 330 Hz from an electrically isolated stimulation circuit to up to 100 μA. The EMG signal was amplified (cyberamp, Axon instruments), filtered (20-300 Hz) and

digitized at a sampling rate of 5 kHz using the Axoscope program (Axon instruments). In the control rats, cortical stimulation was restricted to the caudal forelimb area (CFA). In the lesion rats, stimulation was extended to include most of the hemisphere in order to assess whether shifts in the motor maps had occurred. EMG recordings were performed in 7 lesion and FGF-2-treated animals, 7 lesion and control BSA-treated animals, 5 no lesion animals that received FGF-2, and 5 no lesion rats that did not receive FGF-2.

Analysis of EMG recordings

The occurrence and the delay of EMG responses was assessed using Axoscope. The delay was defined as the time from the first stimulus pulse to the onset of EMG activity. A minimum of 10 sweeps of EMG activity was analyzed and averaged for each stimulation site.

Anterograde Tracing (BDA)

Following EMG recordings, the presumptive caudal forelimb area of motor cortex (in controls) (Neafsey et al., 1986; Kleim et al., 1998) and the filled region (Monfils et al., 2005b) was traced using pressure injection of biotin dextran amine (BDA; 10,000 molecular weight, Molecular Probes, Eugene, OR). A single stereological injection of 0.5 μl of a 10% BDA solution in 0.1 phosphate buffer, pH 7.2 was made approximately 0.5 mm rostral, and 2.0 mm lateral to Bregma at a depth of 1.5 mm, using a 10 μL Hamilton syringe fitted with a glass micropipette with an opening of approximately 50

μm in diameter (Z'Graggen et al., 1998). In the lesion rats that received FGF-2, BDA was injected in the filled cavity in a region from which EMG activity could be stimulated.

Perfusion and histology

Fourteen days following BDA injection, the rats were intracardially perfused with 0.9% phosphate buffered saline followed by 4% PB PFA, and the brains and spinal cords were removed and placed in 4% PFA for 24 hours. They were then transferred to 30% buffered sucrose for cryoprotection. The brain, brain stem and spinal cord were embedded separately in Tissue Tek and immediately frozen by immersion in –40 degrees Celsius isopentane. The brain and brain stem were cut saggitally, a portion of the spinal cord was cut horizontally and the cervical (C1) spinal cord was cut coronally. All sections were cut at 30 μm, and stored in two separate series. The brain and spinal cord were cut in different planes to maximize the likelihood of finding labeling in each region, as well as to facilitate following the labeled fibers from cortex (or filled cavity) to spinal cord.

BDA Immunohistochemistry

One series of the sections from all tissue sections was processed for BDA staining. Staining for BDA was performed according to earlier reports (Fouad et al 2001). The slides were washed 3 × 30 minutes in a 50 mM Tris buffered saline, pH 8.0, containing 0.5% Triton X-100 (TBS) on a magnetic stirrer. Afterwards the slides were incubated

over night with an avidin-biotin-peroxidase complex in TBST (ABC elite, Vector Labs, Burlingame, CA) according to the instructions of the manufacture. Subsequently the DAB reaction was performed using the Vector DAB kit (SK4100, Vector Labs). The reaction was monitored and stopped by extensive washing in TBS. The slides were dried for two days and dehydrated through alcohol and xylene and coverslipped in Permount (Fischer Scientific). The second series of sagittal brain sections was Cresyl violet stained to allow us to examine the laminar distribution of cells within the filled region. Following staining, the slides were dehydrated in increasing series of alcohol and coverslipped.

Analysis of Anterograde Tracings

The diffusion of BDA was measured by taking a picture at 50x using a camera mounted on a Zeiss microscope of the site across all sections it spread through. The volume of BDA diffusion was then calculated using NIH image. The saggital brain and brain stem sections, the horizontal spinal cord sections and the spinal cord cross sections were examined for the presence of anterogradely labeled fibers, and the cross-sectioned fibers from C1 were counted.

Statistical Analyses

Factorial Analyses of Variance (ANOVAs) were performed and compared the four experimental groups on 4 dependent measures: standardized brain size, skilled reaching

performance, EMG delay from cortical stimulation, and EMG delay from striatal stimulation. A regression was derived to evaluate the relationship between the number of fibers counted in the spinal cord and the success rate on the skilled reaching task. Significant interactions were followed-up with Fisher's least significant difference tests. All statistical analyses were performed using SPSS 11.

Results

Gross anatomy of the lesion site

As in our previous studies, the untreated lesioned rats all showed some degree of cavitation at the lesion site in adulthood. In contrast, there was evidence of lesion size reduction induced by a partial filling of the lesion cavity in the lesioned rats that received FGF-2 (Figure 4-1A and 4-1C). Inspection of the cresyl violet-stained sections revealed that in contrast to normal brains in which neurons showed an organized laminar distribution, the organization of the filled cavity appeared haphazard.

Skilled Reaching Task

The Whishaw tray reaching task is a sensitive measure that evaluates forelimb motor function. Here we show an overall effect of lesion on the rats' success in the skilled reaching task. As previously reported, FGF-2-treated lesioned rats outperformed their untreated no lesion counterparts on reaching success (Figure 4-2). A 2x2 factorial ANOVA revealed a main effect of lesion, $F(1,15)= 70.157$, $p< 0.0001$, as well as a

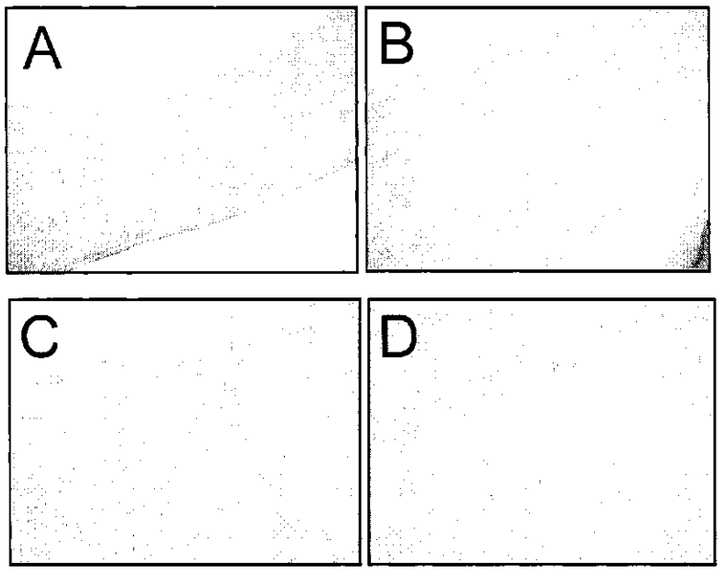

Figure 4- 1. Distribution of cells within the filled cavity of rats that received motor cortex aspiration lesions at postnatal day 10 and FGF-2 treatment. Lesion rats that did not receive FGF-2 show a prominent lesion cavity (not shown here), whereas those that were lesioned and received FGF-2 show a filling of the cavity with cells (1A, 1C). Compared to that of a normal rat (1B, 1D), the filled region in the lesioned-FGF-2-treated rats was disorganized (1A, 1C).

significant Lesion x FGF-2 interaction, $F(1,15)= 19.203$, $p<0.0009$, on rats' reaching success. Statistical follow-up tests revealed no significant difference between the control rats that received FGF-2 and those that did not receive FGF-2, $p>.05$. However, the FGF-2-treated no lesion rats performed significantly better than the no lesion animals that did not receive FGF-2, $p<.05$ (Figure 4-2).

EMG responses from stimulating the motor cortex or filled lesion cavity

The elicitation of wrist extensor EMG activity following stimulation of the motor cortex (controls) or filled cavity (lesioned-FGF-2-treated rats) was assessed to evaluate the electrophysiological integrity of projections from the filled region. We found that whereas 8 out of 9 control rats showed EMG activity from the wrist extensors in response to cortical stimulation, none of the lesion untreated rats showed any EMG activity in response to stimulation of areas adjacent to the lesion site (Figure 4-3A). In contrast, EMG activity could be evoked in 5 out of 7 of the FGF-2 treated lesion animals following stimulation of the tissue filling the cavity. All controls, 2/5 lesion untreated, and 5/7 lesioned-FGF-2-treated rats showed EMG responses following striatal stimulation (see Figure 4-4). Interestingly there was no difference between groups with respect to the delay of EMG onset following stimulation of either the cortex (or filled region) (Figure 4-3B) or the striatum (Figure 4-4B) suggesting a monosynaptic connection between neurons filling the lesion cavity, and wrist extensor motoneurons (Figure 4-3B, D, and E).

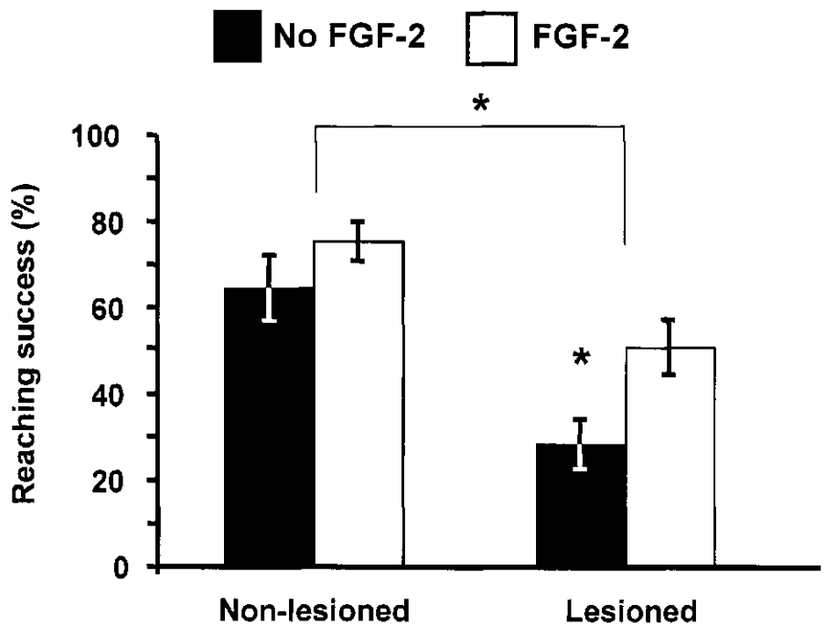

Figure 4- 2. Effect of postnatal day 10 motor cortex lesion and/or FGF-2 administration on the skilled reaching performance. Overall, the no lesion outperformed the lesion rats on the skilled reaching task. There was also a lesion X FGF-2 interaction which revealed that the lesion rats that received FGF-2 performed better than the lesion rats that did not receive FGF-2.

BDA tracings

BDA was injected in the filled region (or motor cortex) to assess the anatomical projections of fibers from that area. Analysis of BDA diffusion suggests that in the lesion rats that received FGF-2, the injections were limited to the filled cavity (i.e., BDA did not diffuse to non lesioned cortical areas). Anterogradely labeled fibers were found in all control rats that were injected with BDA, and 6 out of 7 rats that were lesioned and received FGF-2 (Figure 4-5). A regression analysis to evaluate the strength of the relationship between the number of labeled fibers counted in the spinal cord (C1), and the reaching success on the Whishaw tray reaching task, revealed a significant relationship of moderate strength, $R(16)= .64$, $p< .05$ (Figure 4-6). In addition, a regression for the lesioned-FGF-2 treated rats only, revealed a strong significant relationship between the variables, $R(7)= .80$, $p<.05$, suggesting that number of fibers labeled in the spinal cord accounted for 64% of the variability in reaching success.

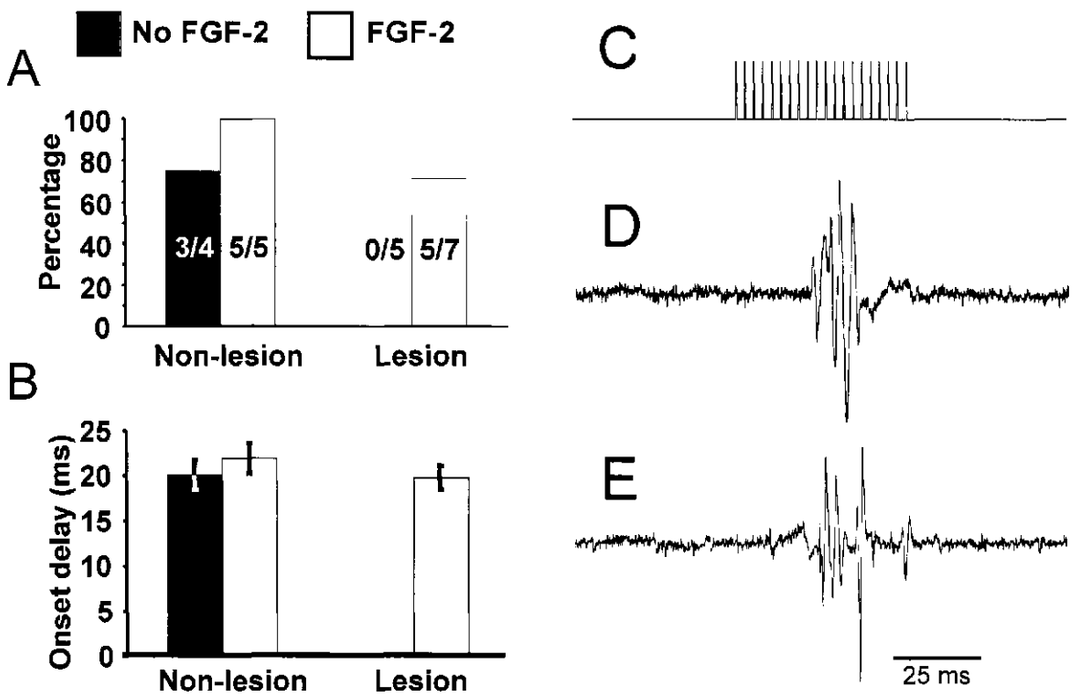

Figure 4- 3. EMG recordings from wrist extensors following cortical stimulation in P10 motor cortex lesioned rats that received FGF-2 injections. 3A summarizes the number of rats in each group from which EMG activity could be recorded in the wrist extensors. EMG activity was elicited from cortical stimulation in 8/9 of the control rats and 5 out of 7 lesion-FGF-2-treated rats. The delay of onset following cortical stimulation was compared, and revealed that there were no differences between the groups of rats from which EMGs were recorded (3B). In 3D and 3E, representative EMG recordings from a control rat (3D) and a lesion FGF-2-treated rat (3E) in response to cortical stimulation (3C).

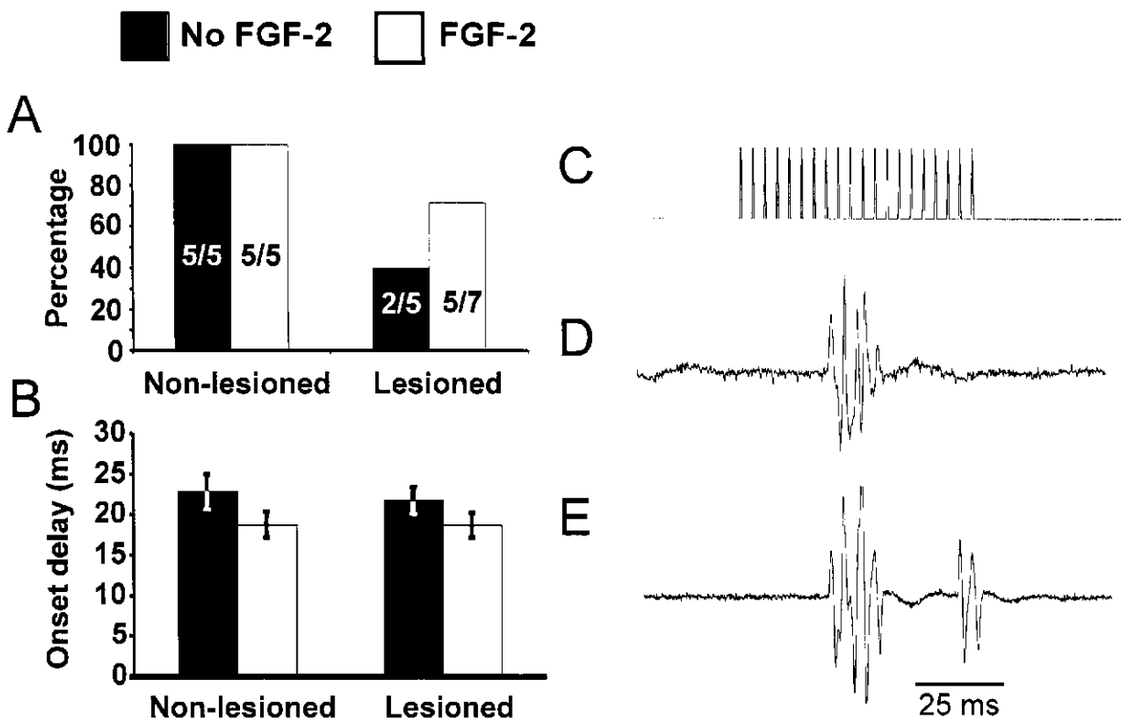

Figure 4- 4. EMG recordings from wrist extensors following striatal stimulation in P10 motor cortex lesioned rats that received FGF-2 injections. 4A summarizes the number of rats in each group from which EMG activity could be recorded in the wrist extensors in response to striatal stimulation. EMG activity was elicited from striatal stimulation in 9/9 of the control rats, 5 out of 7 lesioned-FGF-2-treated rats., and 2 out of 5 lesioned rats that did not receive FGF-2. The delay of onset following cortical stimulation was compared, and revealed that there were no differences between the groups of rats from which EMGs were recorded (4B). In 4D and 4E, representative EMG recordings from a control rat (3D) and a lesioned FGF-2-treated rat (3E) in response to striatal stimulation (3C).

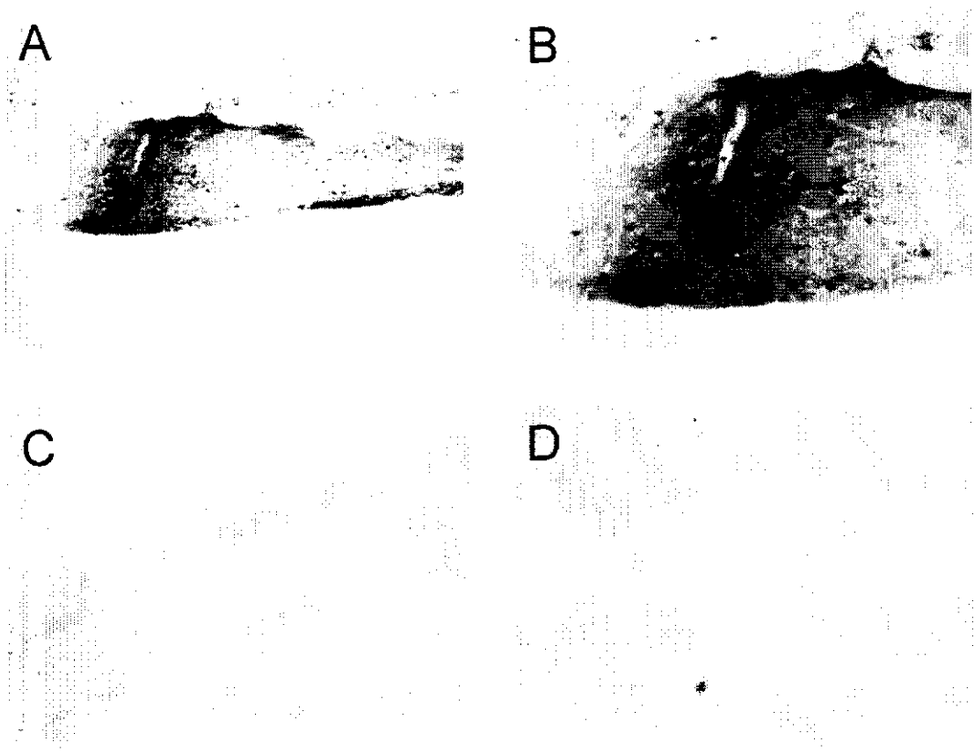

Figure 4- 5. Anterogradely traced corticospinal fibers from a lesioned rat that received FGF-2 injections. Fibers were found in C1 in all injected controls, in 6 out of 7 lesioned-FGF-2-treated, and in 1 out of 7 lesioned non-treated rats. In 4-5A, BDA labeling at the injection site. In 4-5B, labeled cells at the injection site at higher magnification (200x). In 4-5C, anterogradely traced fibers in the brain stem, and 4-5D, fibers in a horizontal section of the spinal cord.

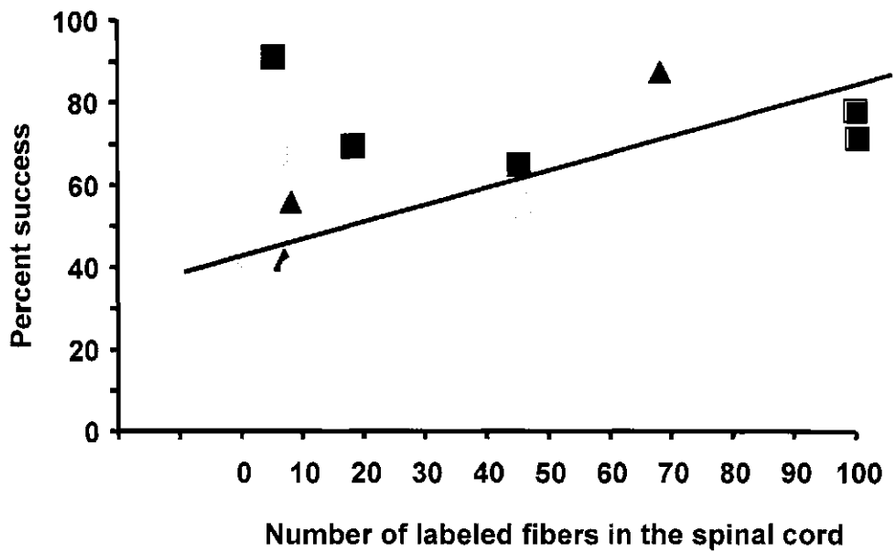

Figure 4- 6. Scatterplot and regression line representing the relationship between the number of fibers counted in the spinal cord and skilled reaching ability in control and lesion rats that received FGF-2 or vehicle. The black triangles represent control rats that received FGF-2. The black squares represent control rats that did not receive FGF-2. The grey circles represent lesion rats that received FGF-2. A simple regression revealed a significant relationship between the number of fibers labeled in the spinal cord and reaching performance ($R(16)= .64$, $p<.05$). The number of fibers in the spinal cord accounts for 43% of the variance in reaching success.

Discussion

The current study builds on our earlier findings that following bilateral injury to the motor cortex inflicted at P10, FGF-2 leads to filling of the lesion cavity with tissue in parallel with improvement in a skilled reaching task. Here, we show that the filled region contains neurons that project into the spinal cord. Importantly, these neurons are functional as their activation results in the activation of neurons in the cervical spinal cord. Furthermore, neither cavity filling, recovery of function, spinal cord projections nor evoked wrist extensor EMG activity are present in untreated lesion rats. To our knowledge this is the first study to demonstrate corticospinal tract restoration in motor cortex lesion rats. Consequently, our results support the idea that the filled region is involved in mediating the improvement seen in the FGF-2 treated rats (Monfils et al., 2005b).

We recently reported that chronic administration of FGF-2 following bilateral lesions to the motor cortex inflicted at P10 led to a partial recovery accompanied by a filling of the lesion cavity with some cells (Monfils et al., 2005b). These results raised obvious questions: (1) Is the filled region involved in mediating recovery? (2) If the filled region is involved, what are the mechanisms by which it subserves the behavioral recovery? and (3) where do the cells that fill the cavity come from? A number of factors could explain the effect of FGF-2 effect, including that FGF-2 might act on different brain regions that provide compensation, such as the striatum or the sensory cortex. Our earlier results suggested that the striatum alone was likely not responsible for the

functional recovery observed, because hypertrophy of medium spiny neurons in that structure occurred in response to motor cortex lesions, but no lesion x FGF-2 interaction was observed (Monfils et al., 2005b). The sensory cortex alone (or any other cortical regions) is also unlikely to account for the behavioural improvement, because layer V microstimulation of cortical areas around the lesion site did not elicit any movements or evoked potentials (Monfils et al., 2005b and current experiments respectively).
In the present study, we demonstrate that the filled region could play a key role in promoting FGF-2 induced recovery, as functional neuronal projections from that region to the spinal cord and onto motoneurons exist.

Importantly, in the present study, the same lesioned-FGF-2 treated rats from which EMG activity could be elicited also showed anterogradely labeled fibers in the spinal cord (with the addition of a rat from which EMG activity could not be evoked, as well as improved behavioral performance. Another indication that these reestablished cortical projections are involved in the recovery is the finding that the number of fibers counted in the spinal cord correlates significantly with the reaching success. Although statistically significant the strength of the relationship between those two variables was moderate, and this may seem deceiving at first glance: i.e., if the filled region was solely responsible for the behavioral recovery, we might expect to see a tighter relationship between the two variables. A few reasons can explain this discrepancy: (1) it is reasonable to anticipate that other brain regions might at least in part be responsible for the functional outcome. Indeed, bilateral motor cortex lesions inflicted at P3, P10, or P90

all lead to impairments on skilled reaching ability in adulthood. Compared to P3 and P90 lesioned rats, the P10 group shows some degree of functional recovery, yet, all three groups show a prominent lesion cavity (Kolb et al., 2000), suggesting that the partial recovery seen in the P10 lesioned rats is due to compensation from other brain areas. The FGF-2 treatment might thus be incremental to the other changes that occur as a result of injury at P10, and thus results in a greater degree of recovery. (2) We only injected a small amount of BDA to ensure that the injection did not spread beyond the filled region. As a result, we likely only labeled a fraction of the total number of fibers. Since we can not assume that the cells are evenly distributed with in the regrown tissue, the traced cells do not necessarily represent the density of fibers in other regrown areas.

The mechanisms by which FGF-2 leads to the formation of neurons that extend within the filled region with projections into the spinal cord are not clear. A few necessary steps are likely involved, a) the cells must migrate to the site of injury, and differentiate into neurons, and b) these neurons must grow, extend viable projections and make appropriate connections with motor neurons in the spinal cord.

Past studies have shown that exogenous administration of FGF-2 in adult rats induces cell proliferation in the subventricular zone (Tao et al., 1996; Kuhn et al., 1997; Wagner et al., 1999), and promote neuronal differentiation (Kuhn et al., 1997). In addition, increases in endogenous levels of FGF-2 in response to injury (e.g., hypoxia) have been found to be associated with the presence of FGF-2-reactive radial glial cells in

the subependymal zone, which are thought to be important in improving anatomical outcome following brain injury (Ganat et al., 2002). FGF-2 mRNA was also found to be increased following ischemic preconditioning, and to protect the brain from a subsequent insult (Matsushima et al., 1998). Future studies are underway to elucidate the mechanisms that promote cell mobilization to the site of injury in our model.

Our study suggests the presence of anatomical and electrophysiological connectivity from the filled region to the cervical spinal cord. It is somewhat surprising that the regrown fibers project in a similar way to the cortico spinal tract in uninjured rats. How this is achieved is not fully understood, however, a number of studies have implicated FGF-2 in promoting axonal growth. Kawamata et al. (1997) reported an increase in growth-associated protein 43 (GAP-43) immunoreactivity, a molecular marker of axonal sprouting, in the intact sensorimotor cortex contralateral to cerebral infarcts (induced by middle cerebral artery occlusion) following FGF-2 treatment, suggesting that FGF-2 enhances functional improvement after stroke via stimulation of neuronal sprouting in the intact brain. FGF-2 was also found to significantly increase sprouting by the cholinergic septodentate pathway following entorhinal cortex injury (Ramirez et al., 1999). In addition, FGF-2 has been shown to increase astrocyte-associated fibronectin (Mahler et al., 1997), an axonal growth-promoting molecule (Price and Hynes, 1985; Liesi et al., 1986; Matthiessen et al., 1989). In addition to axonal growth, relevant cues must be present in order for projecting neurons to extend their projections to the appropriate target, i.e., cervical motor neurons, and the mechanism by which this might occur in the

current preparation is unknown. Of relevance here, however, FGF-2 signaling is known to play a role in axonal guidance to the appropriate target in Xenopus retinal ganglion cells *in vivo* (McFarlane et al., 1995).

In conclusion, we demonstrate for the first time that following cortical lesions in neonatal rats that received FGF-2 the lesioned region fills with cells that contain neurons, which project to the spinal cord and make contacts with motoneurons. Thus, the presented findings provide a mechanism explaining our earlier findings (Monfils et al., 2005b) and supports further research in the therapeutic potential of FGF-2. Additional experiments are needed to elucidate the source of cells filling the cavity and to derive suitable treatments for brain injury inflicted in different regions or developmental time points.

Chapter 5. FGF-2-induced cell proliferation is associated with anatomical, neurophysiological, and functional improvement from neonatal motor cortex injury

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Abstract

We have recently shown that postnatal day 10 (P10) motor cortex (MCx) lesioned rats that receive basic fibroblast growth factor (FGF-2) following the lesions show partial recovery of function accompanied by filling of the lesion cavity with new tissue. The primary objectives of the current study were to identify the mechanism by which the lesion cavity fills with tissue in response to FGF-2, to evaluate the anatomical and neurophysiological integrity of this filled region, and to establish whether preventing the filling of the cavity with new tissue would prevent functional recovery. The results show that: 1) FGF-2 stimulates neuroblasts from the subventricular region to migrate to the site of injury; 2) the cells that fill the lesion cavity show normal-appearing spontaneous electrical activity; 3) the cells that fill the cavity form synapses; 4) blockade of neuroblast migration by embryonic day 13 injections of bromodeoxyuridine prevents the later FGF-2 induced filling of the lesion cavity and inhibits functional recovery; and, 5) removal of newly generated tissue reversed the functional gains. Taken together, the results suggest that after day 10 motor cortex injury exogenous FGF-2 stimulates the brain to produce new cells that migrate to the of injury, form synapses and neuronal networks, and contribute to motor function.

Key words: FGF-2, reaching, motor cortex, electron microscopy, ki67, doublecortin.

Basic fibroblast growth factor (FGF-2) is involved in regulating developmental processes (e.g., Li & DiCicco-Bloom, 2004; Raballo et al., 2000) as well as mediating outcome in response to various types of injury (e.g., Ganat et al., 2002; Rowntree & Kolb, 1997). There is much evidence suggesting that the developmental expression of FGF-2 might play a role in modulating plasticity following cortical damage (Ganat et al., 2002; Monfils et al., 2005a). Notably, we have shown in two independent studies that after receiving motor cortex (MCx) lesions on postnatal day 10 (P10) Mcx-lesion rats that receive FGF-2 perform better on a skilled motor task in adulthood (Monfils et al., 2005b; Monfils et al., 2005c). In addition, the lesion-FGF-2-treated rats show a filling of the lesion cavity with both neurons and glia.

Indirect evidence from our prior studies suggests that the filled cavity might be involved in the functional improvement observed in the lesion FGF-2-treated rats. Specifically, we have shown that stimulation of the filled region leads to EMG activity in the forelimb extensors and to the induction of some movements and that cells in the filled region have corticospinal connections (Chapter 4). It is unknown, however, whether the FGF-2-induced filling of the cavity is necessary for the behavioral improvement. Furthermore, the mechanism by which the lesion cavity fills with cells in response to FGF-2 treatment is currently unknown.

The primary objective of the current study was to evaluate the anatomical and neurophysiological integrity of the filled region, and to establish whether preventing the

filling of the cavity with tissue would prevent functional recovery. A complimentary objective was to identify the mechanism by which the lesion cavity fills with tissue in response to FGF-2. Four experiments were conducted and were specifically designed to (1) identify the types of cells that fill the lesion site, (2) establish whether the cells that fill the lesion site show spontaneous electrical activity and to compare the firing pattern to that of undamaged rats, (3) estimate the number of synapses from the filled region or motor cortex, as well as area Cg3, (4) replicate our prior findings that FGF-2 induces functional recovery, and (5) evaluate whether preventing (or removing) the filling of the lesion cavity would prevent the functional recovery associated with FGF-2 injections.

Materials and Methods

General Experimental Design

All experiments were performed on rats that received motor cortex or sham lesions at postnatal day 10, followed by chronic injections (up to 7 days) of either BSA (vehicle) or FGF-2. Experiment 1 investigated the effects of P10 motor cortex lesions followed by FGF-2 administration on Ki67 and doublecortin expression in the filled region and striatum at P14, and P21. Experiment 2 looked at the effects of P10 motor cortex lesions followed by FGF-2 administration on the firing pattern of cells from the adult motor cortex (in control rats) or the filled region (in lesion rats that received FGF-2). Experiment 3 was conducted on tissue from rats that were used in a prior study (Monfils et al., 2005b). Tissue from the motor cortex (controls), the filled region (lesion+FGF-2 rats), and area Cg3 (all rats) obtained from one hemisphere was processed

for light and electron microscopy, and the number of neurons as well as the number of synapses was estimated using unbiaised stereology techniques. In Experiment 4, we attempted to prevent the filling of the cavity by injecting rats with BrdU on embryonic day 13 (E13). We have shown previously that such treatment blocked both functional recovery and the spontaneous generation of cells in rats with medial frontal lesions on P10 (Kolb et al., 1998). On P10, these rats received either motor cortex or sham lesions, then either BSA (vehicle) of FGF-2 chronic injections (7 days). In early adulthood (P45), all rats were trained and assessed on a reaching task. In Experiment 5, 5 control rats and 5 lesion rats that received FGF-2 (from experiment 4) were assessed in the skilled reaching task and the cylinder asymmetry test before and after receiving a unilateral lesion to the motor cortex (or filled region) contralateral to their preferred forelimb.

The rats were group-housed, and then placed with same sex littermates in clear plastic cages in a colony room maintained on a 12h on/12h off light cycle. For each experiment, testing was conducted during the light phase. Rats were maintained on Lab Diet no. 5001 (PMI feeds Inc., St-Louis, MO) and water *ad libitum*, except during the Whishaw tray reaching task, and were handled and cared for according to the Canadian Council for Animal Care guidelines.

Experiment 1

Animals

In Experiment 1, 36 male Long-Evans hooded rats were used and were divided into groups that received either bilateral motor cortex lesions (N=12) or sham operations (N=12) on postnatal day 10 using a split litter design.

General Procedures

The lesioned and sham-operated rats were divided into groups that received up to 7 daily injections of either FGF-2 (n=12) or BSA (n=12) administered subcutaneously. Rats were perfused and processed for immunohistochemistry at either P14 (n=12), or P21 (n=12).

Surgery

The neonatal rats were anesthetized by cooling in a Thermatron cooling chamber until rectal temperature was in the range of 18-20°C. The bone over the motor neocortex area was removed with iris scissors and motor decortication was achieved by aspiration using procedures previously described by Kolb (1987). The lesion was performed from +2 to –2, relative to the bregmoidal junction. The scalp wound was sutured with 5-0 Vicryl thread as soon as the surgery was complete. The control rats were anesthetized, the skin incised, and then sutured with 5-0 Vicryl thread.

FGF-2

Basic fibroblast growth factor (FGF-2) was diluted in Bovine serum Albumin (BSA) (1 μg/ml), and administered at 0.1 ml/10g of body weight (for a final concentration of 10 ng/g). This concentration was determined based on the work of Wagner et al. (1999), which had shown this concentration to be sufficient to enhance neurogenesis in P7-P28 rats. BSA was prepared into 0.1 M PO_4 as a concentration of 1 mg/ml.

Treatment groups

The lesioned (n=12) and sham-operated (n=12) rats were divided into groups that received 7 daily sessions of either FGF-2 (n=12) or BSA (n=12) administered subcutaneously. In a previous study conducted in our lab, this injection protocol was found to be effective in inducing behavioral improvement following medial prefrontal cortex aspiration lesions at P3 (Comeau, Hastings & Kolb, 2005), and motor cortex lesions at P10 (Monfils et al., 2005b).

Histological Methods and Tissue Preparation

All rats were sacrificed by overdose of sodium pentobarbitol (100 mg/kg i.p.) and perfused transcardially with phosphate buffered saline (PBS; 0.9% saline in 0.1 M phosphate buffer) followed by 4% buffered paraformaldehyde. The brains were extracted, stored overnight in the perfusate solution, and then transferred to 30% phosphate buffered

sucrose. Hemispheres were stereotaxically blocked in the coronal plane, and serial coronal sections were cut on a freezing microtome at 40 μm.

Doublecortin (DCX) and Ki67

Sections were incubated overnight (~20 hrs) at room temperature with goat anti-Doublecortin (DCX, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-Ki67 (1:250; Novacastra, UK) in 0.3% Triton X in PBS. The sections were then rinsed 3X in PBS, and placed in a secondary Alexa Fluor 488 anti-goat IgG (1:250) and Cy3 Conjugated Affinipure Donkey Anti-rabbit IgG (1:250; Jackson Laboratories; West Baltimore Pike, PA) for 2 hrs at room temperature.

Following the labeling procedures, all sections were rinsed in 0.1 M PBS, mounted on gelatin/chrome alum-coated slides, air-dried, and cover slipped. DCX and Ki67-labeled cells were viewed on a Zeiss Axioskop Z microscope under fluorescence using 5, 10 and 20X objectives.

Stereology

The number of ki67 positive cells were counted within an 82 x 82 pixel grid placed onto photomicrographs of the dorsolateral striatum and the dorsomedial striatum. Each grid was placed at equidistant locations from the subventricular zone and the callosal white matter, which served as landmarks. The exact thickness of each section from all the

striatal sections was measured using a programmed z-sectioning routine, and the number of ki67 positive cells was expressed per volume (mm^3).

Experiment 2

Animals

The present experiment used 13 male rats, derived from the Charles River Long-Evans hooded strain, which were divided into groups that received either bilateral motor cortex lesions (N=6) or sham operations (N=7) on postnatal day 10. The animals were group-housed, and then placed with same sex littermates in clear plastic cages in a colony room maintained on a 12h on/12h off light cycle. All experimentation was conducted during the light phase. Rats were maintained on Lab Diet no. 5001 (PMI feeds Inc., St-Louis, MO) and water *ad libitum*, except during the Whishaw tray reaching task, and were handled and cared for according to the Canadian Council for Animal Care guidelines.

General Procedures

The lesion rats received 7 daily injections FGF-2 (n=7), and the sham rats received 7 daily injections of BSA administered subcutaneously. Following injections, the rats were allowed to age until P100. Then, EEG and multi-unit activity were recorded from the motor cortex (in controls) or the filled cavity (in lesion rats that received FGF-2).

Surgery

Bilateral motor cortex lesions were performed at P10, using the protocol outlined in Experiment 1.

FGF-2

Basic fibroblast growth factor (FGF-2) was diluted in Bovine serum Albumin (BSA) (1 μg/ml), and administered at 0.1 ml/10g of body weight (for a final concentration of 10 ng/g). This concentration was determined based on the work of Wagner et al. (1999), which had shown this concentration to be sufficient to enhance neurogenesis in P7-P28 rats. BSA was prepared into 0.1 M PO_4 as a concentration of 1 mg/ml.

Field and Single Unit Recordings

All rats were initially anaesthetized with a combination of isoflurane and oxygen (4% isofluorane). A rubber silastic tube cannula was then inserted into the jugular vein dorsal to the thoracic cavity, and secured with silk suture thread. The isoflourane/oxygen was then discontinued and deep anaesthesia was continued with urethane (0.8 mg/ml) administered i.v. through the jugular cannula. Urethane was administered as needed throughout the remainder of the surgical procedure. Rats were then placed in a standard stereotaxic apparatus. A hole was drilled in the skull above the medial prefrontal cortex (A/P: +4.0 mm, M/L: +0.5 mm). Electrodes consisted of insulated and sharpened tungsten wire (~1 μm tip). One electrode was lowered 0.5 – 2 mm into medial prefrontal

cortex and was used as a reference. The other electrode (0.5 – 0.8 MΩ) was lowered into the tissue that filled in the previously aspirated cortex (A/P: +1.0 mm, M/L: 3.0 mm).

Recorded electrical activity was amplified (1000X) with a Grass QPS11 high impedance amplifier/filter (West Warwick RI, USA). The signal from the electrode located in filled in tissue was split and filtered in order to record both field and multiunit activity. Field activity was filtered at half amplitude below 0.3Hz and above 100 Hz, and multiunit activity was filtered at half amplitude below 100 Hz and above 1 KHz. Both signals required the addition of a notch filter at 60 Hz to remove background electrical noise. The multiunit activity was displayed on a digital storage oscilloscope (Hitachi VC-6023), and also amplified and played through a speaker to aid in the isolation of single units. The electrode located in the filled in tissue was moved ventrally in small increments, and the visual (oscilloscope) and auditory (audio amplifier and speaker) signals were used to aid in the isolation of single unit activity. A cell was considered sufficiently isolated when amplitude of the single cell spike was at least twice that of the background noise. Once a cell was isolated, 10 minutes of field activity and multiunit activity were recorded as separate signals on a Vetter signal recorder/player for subsequent off-line analysis. If the signal to noise ratio of the isolated cell was reduced below 2:1 during the 10 minutes of recording, the record was discontinued, and isolation was attempted again. Following the successful recording of at least 10 minutes of a well-isolated cell, the reference and cell electrodes were switched. Single cells were then

isolated in medial prefrontal cortex so serve as a control example of activity in unmanipulated cortex.

Analysis

The recorded signals were played back with the Vetter recorder/player into and digitized with a Datawave 256 bit analog to digital (A/D) board. The digital signal was analyzed using the SciWorks Data Acquisition/Analysis package (Datawave, Denver, CO). The field activity was sampled at a rate of 66Hz, and was analyzed for differences in the peak frequency. Fourier transforms were performed on several 5-second segments of stable EEG collected from both filled in tissue and medial prefrontal cortex. The multiunit activity was sampled at a rate of 20 points/ms (20 KHz). Single units were isolated based on the peak amplitude of the spike, and comparisons were made for mean firing rate and the total firing sum. An inter-event interval histogram was also created in order to investigate any gross differences in stationarity or bursting of the isolated cells.

Histological Methods and Tissue Preparation

All rats were sacrificed rats by overdose of sodium pentobarbitol (100 mg/kg i.p.) and perfused transcardially with phosphate buffered saline (PBS; 0.9% saline in 0.1 M phosphate buffer) followed by 4% paraformaldehyde. The brains were extracted, stored in the perfusate solution overnight, and then transferred to a phosphate buffered 30%

sucrose solution. The brains were blocked in the coronal plane, and serial coronal sections were cut on a freezing microtome at 50 μm.

Experiment 3

Tissue Samples

Perfusion and initial fixation

The tissue samples used in this experiment were obtained from rats that were used in a previous study (Monfils et al., 2005b). The brains were processed as follows. All rats were deeply anesthetized with sodium pentobarbital (120 mg/kg) prior to perfusion. Rats were transcardially perfused with 0.9% saline only. Each brain was cut in front of the cerebellum, and mid-saggitally. One hemisphere was immersed in Golgi-Cox solution (data previously reported, Monfils et al., 2005d). The other hemisphere was processed using a fixation by immersion procedure (Monfils et al., 2005b). The hemisphere was immersed in a 37 °C fixative solution containing 0.1 M cacodylate buffer, 2.5% GA, 4% PFA and 2.5% dimethysulfoxide (DMSO) for 5 minutes, after which it is transferred to the same fixative solution at a temperature of 4° C. This hemisphere was stored at 4° C for seven days and then dissected and processed for EM as described below.

Electron Microscopy Preparation

The brains were coronally sectioned (300 μm) on a vibratome, and the sections viewed under a dissecting microscope at low power (2X). Samples of tissue (approximately 500

μm wide) extending from pia to white matter were dissected from the caudal forelimb area of motor cortex using a sharp razor blade. In rats that were lesioned and received FGF-2, the 'geographically relevant' area corresponding to motor cortex was dissected. For all the groups, area Cg3 was also dissected. The tissue blocks were then processed according to the methodology previously reported (Kleim et al., 1996) Briefly, the blocks were washed in 0.1M cacodylate buffer for 2 hours, postfixed in 2% osmium tetroxide/1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 2 hours and en bloc stained with 2% uranyl acetate for 45 min. The samples were then dehydrated through an ascending series of ethanol concentrations before being transferred into propylene oxide followed by gradual embedding in Eponate resin.

A few (approximately 10) 1 μm sections were cut from a block of embedded tissue from each rat. Sections were stained with toluidine blue, and cortical layer V was identified by the presence of large pyramidal cells and the absence of layer IV granule cells using the light microscope. Next, most of the block was trimmed away in a pyramidal shape, leaving a small strip containing layer V of the motor cortex. From this strip, silver/gray serial sections (approximately 70 nm thick) were cut using a diamond knife. These sections were collected on Formvar-coated, slotted, copper grids, and stained sequentially with uranyl acetate followed by lead citrate.

Stereology

Thin sections were surveyed with the electron microscope and an area of the neuropile that was essentially free of nuclei was selected in one of the serial sections. Within this area, a composite of four micrographs were taken at X 6,000 such that each micrograph represented one quadrant of a square. Next, the same area in a non-consecutive section (i.e. the intervening section was skipped) was photographed at the same magnification. A minimum of 16 micrographs were prepared for each area dissected for each rat. The resulting negatives were then printed at a final magnification of X 23,800 and subsequently analyzed for synaptic density using the physical disector method (Sterio, 1984). In this method, an unbiased counting frame of known area (105 μm2) is placed over each micrograph of the first composite (reference sections) and the number of synapses counted. The same frame is then placed over each micrograph from the second composite (lookup sections) and synapses not visible in the reference section are counted. In this way, synapses are counted only once. In the present study, synapses were identified by the presence of synaptic membrane specializations and only those with at least three vesicles were included in the counts. From these counts, the synaptic density per volume (μm3) could be determined.

Experiment 4

Animals

Seven pregnant rats, derived from Charles River Long–Evans strains, were randomly chosen to receive injections of BrdU at embryonic day 13 (4 rats) or to receive no

injection (3 rats). Two injections (spaced 6 hours apart) of 60 mg/kg of BrdU in 0.007N NaOH, or vehicle, were given intraperitoneally. All rats were then left to continue their pregnancy naturally. Postnatally, approximately half the rat pups from each litter were given bilateral motor cortex lesions at ten days of age. In a prior study, we found that animals with P10 frontal lesions after BrdU injections on E13 showed no filling of the lesion cavity whereas non-BrdU injected rats show a spontaneous filling of the cavity (Kolb et al., 1998).

After weaning, the rats were housed in pairs in shoebox cages where they were maintained on a 12:12 h light/dark schedule. Food and water were provided *ad libitum* except during the reaching task where they were food deprived to no less than 85% their original body weight. The body weights of all animals were recorded daily for the first 3 weeks postnatally and then weekly until behavioral testing was completed.

General Procedures

The BrdU- (N=17) and non-BrdU-exposed (N=25) lesioned and sham-operated rats were divided into groups that received 7 daily injections of either FGF-2 (n=22) or BSA (n=19) administered subcutaneously. Following injections, the rats were allowed to age until P65. Then, all rats were trained and assessed on the Whishaw tray reaching task. The BrdU-exposed rats were then perfused. The non-BrdU-exposed rats were further tested in Experiment 5.

Surgery

Bilateral motor cortex lesions were performed at P10, using the protocol outlined in Experiment 1.

FGF-2

FGF-2 was prepared and administered using the same protocol as that outlined in Experiment 1.

Whishaw tray reaching task

Forelimb use was measured with a procedure that was adapted from the method devised by Whishaw et al. (1991). Each animal was food deprived to 85 % free feeding level throughout training and testing. The rats were placed in the test cages (10X18X10 cm high) with floors and front constructed of 2 mm bars, 9 mm apart edge to edge. A 4 cm wide and 5 cm deep tray, containing chicken feed pellets, was mounted in front of each box. The rats were required to extend a forelimb through the gap in the bars, grasp and obtain the food. The tray was mounted on runners and was retracted 0.5 cm from the cage so that the rats could not scrape the food into the cage. If the animal attempted to rake the pellet out of the tray, the pellet would inevitably fall through the gap. An attempt was scored only if the rat reached into the tray and touched the food. If it reached into the tray without touching the pellet, no attempt was scored. A successful reach was defined as grasping a pellet, and bringing it to the mouth. The rats were trained for 20 minutes per day for 14 consecutive days. Once trained, the rats received a 10 minute reaching test during which time they were videotaped for later scoring. All

rats showed a forelimb preference, and only the reaches performed by the preferred limb were scored and analyzed.

Experiment 5

Animals

From the non-BrdU-exposed rats of Experiment 4, the control rats, as well as lesion rats that received FGF-2 were used for Experiment 5.

Experimental Design

At P90, an additional baseline measure of the reaching success on the Whishaw tray reaching task was obtained on control rats, as well as those that received a lesion+ FGF-2. In addition, these rats were tested in the cylinder apparatus, to derive a measure of asymmetry. Then, all rats received a unilateral motor cortex lesion contralateral to their preferred limb. In the lesion rats that received FGF-2, we aimed to remove the filled region. Two days following the lesion, all rats were retested in the Whishaw tray reaching task and the cylinder apparatus.

Whishaw tray reaching task

The rats were assessed on tray reaching task during a single 10 minute-session before the lesion, and a single 10 minute-session 2 days following the lesion. The reaching task was described in the methods for Experiment 4.

Cylinder

The cylinder (30 cm high, 16 cm dia) of 3-mm-thick Plexiglas was mounted on a glass table, beneath which was positioned an inclined mirror. The test consisted of placing a rat into the cylinder and leaving it to explore for 5 min during which its behavior was filmed. Each contact of a forepaw with the cylinder wall during rearing was measured upon replay of the videotape. In addition, the duration of the first 10 contacts for each forelimb was timed. An asymmetry score was derived by calculating the number of contact from the reaching forelimb, divided by the total number of contacts made by both limbs multiplied by 100.

Results

Experiment 1

Doublecortin and Ki67

In the lesion rats that received FGF-2, the lesion cavity shows a number of migrating neuroblasts (doublecortin positive- DCx +) as well as cycling (ki67+) cells at the site of injury at P14 as well as P21. In comparison, the lesion rats that did not receive FGF-2 do not show cells migrating to the site of injury (see figure 5-1). Analyses were performed to quantify and compare the number of cells in the dorsolateral and dorsomedial striatum in lesion rats that received FGF-2, lesion rats that did not receive FGF-2, as well as the non-lesion matched controls. Two 2x2x2 ANOVAs with age, FGF-2 treatment, and

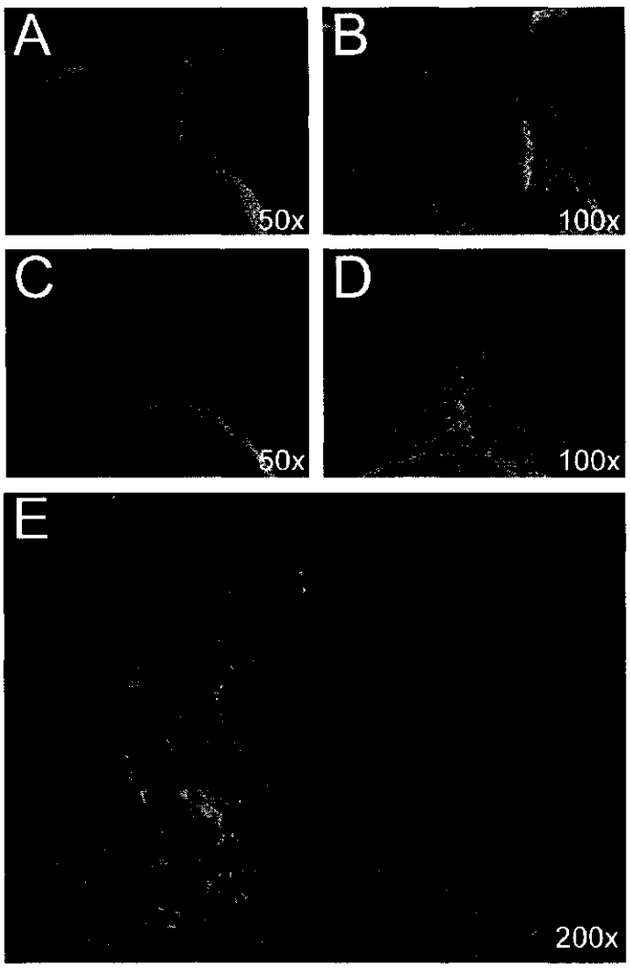

Figure 5- 1. The lesion rats that did not receive FGF-2 (A and B) do not show cells migrating to the site of injury. In comparison, in the lesion rats that received FGF-2 (C, D, and E), the lesion cavity shows a number of migrating neuroblasts (doublecortin positive- DCx +, in green) as well as cycling (ki67+, in red) cells at the site of injury at P14 as well as P21.

lesion condition as between subject factors were conducted to compare the groups on the number of ki67 cells in dorsolateral striatum, dorsomedial striatum. Our analyses revealed a main effect of age on both measures, $F(1,28)= 11.72$, $p<.005$, $F(1,28)=19.921$, $p<.005$, for the dorsolateral and the dorsomedial reigons, respectively. This suggested a decrease in the number of ki67 positive cells in P21 rats compared to P14 rats. There was also a main effect of lesion, $F(1, 28)= 9.858$, $p<.005$, $F(1,28)= 11.634$, $p<.05$, and an age by lesion interaction on both measures, $F(1,28)= 4.235$, $p<.05$, $F(1,28)= 4.036$, $p=.05$. Simple main effects were then conducted for each of the age groups, and revealed an effect of lesion on number of ki67 positive cells in both areas investigated, $p<.05$, suggesting an increase in the number of proliferating cells in the dorsolateral striatum and dorsomedial striatum of lesion rats. In addition to those regions, a number of ki67 positive cells were found in the filled cavity at both P14 and P21. Due to a rather large number of doublecortin cells, and the difficulty this created in dissociating individual cells, we only discuss these results in qualitative terms. A large number of doublecortin positive cells was found in the subventricular zone of all rats at both P14 and P21. There was a qualitatively larger amount in the lesion rats, and no apparent differential effect of FGF-2.

Experiment 2

EEG and multi unit recordings

We were able to acquire multi unit activity and EEG recordings from the filled region in 6 out of 7 rats that were lesioned and received FGF-2, and in all control rats. In 1 out of

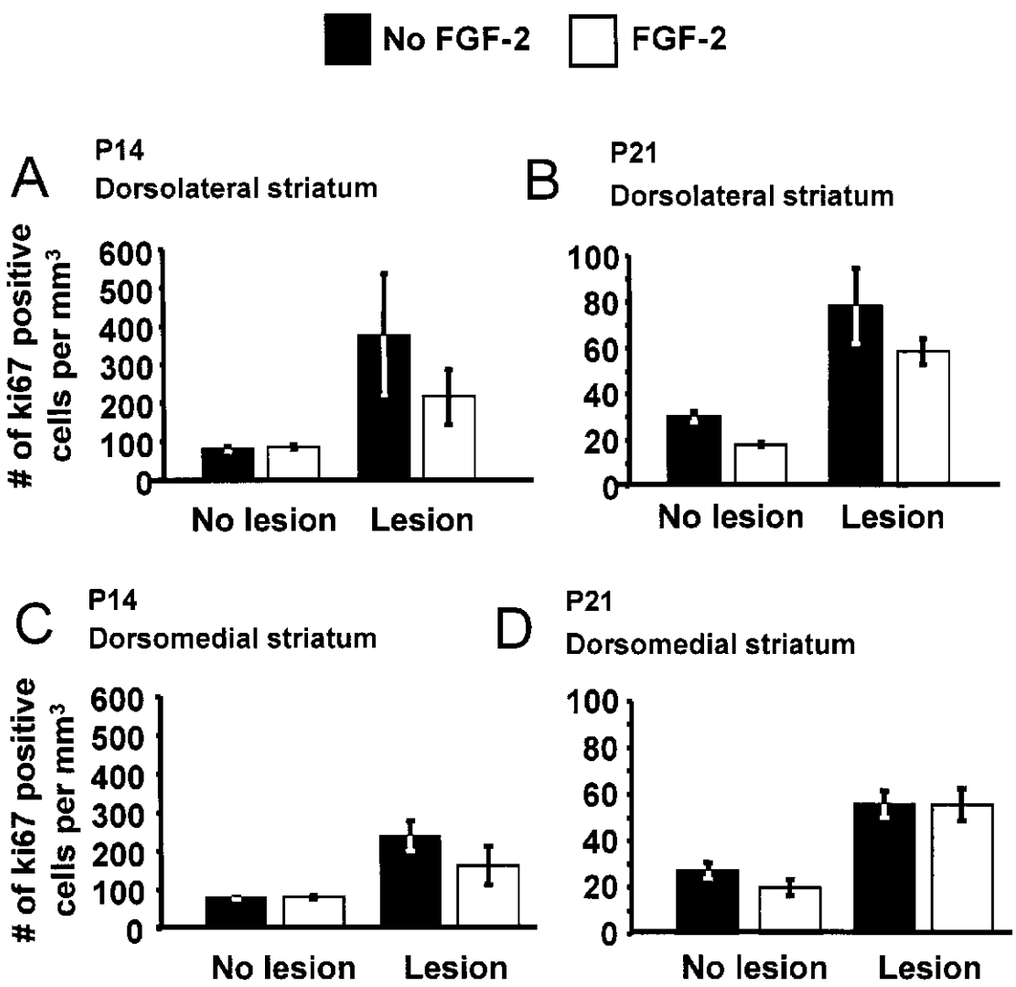

Figure 5- 2 Effects of FGF-2 and bilateral motor cortex lesions at postnatal day 10 on the number of ki67-positive cells in the dorsolateral and dorsomedial striatum at P14 and P21. There was a main effect of lesion for both P14 and P21 rats, suggesting the P10 motor cortex lesion induced an increase in the number of proliferating cells.

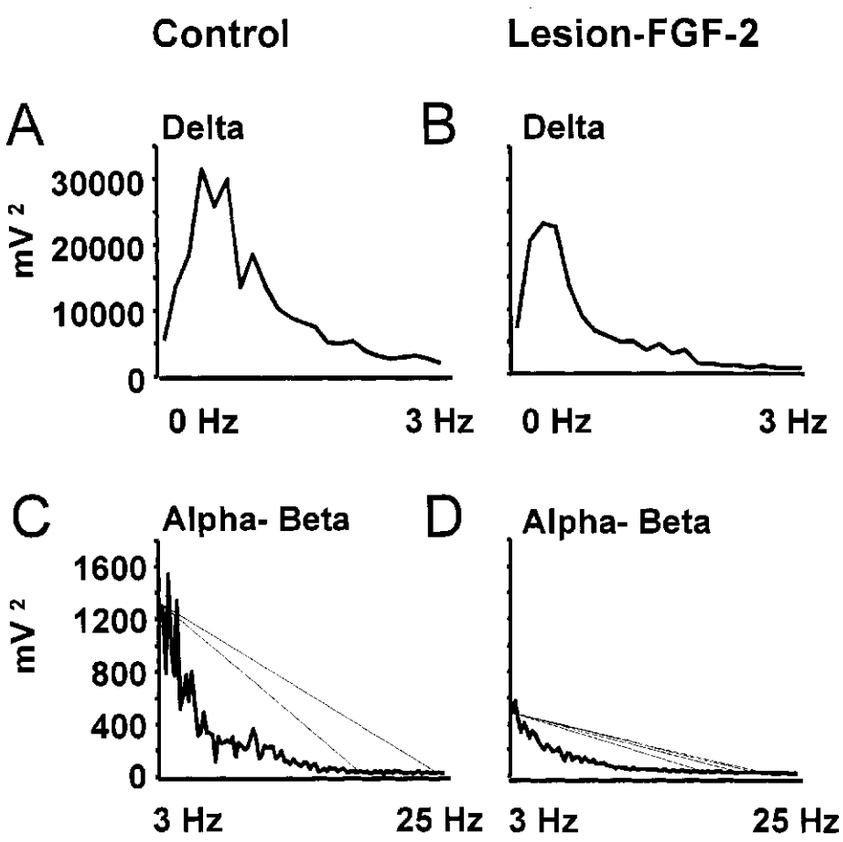

Figure 5- 3 Fourier Transform analyses from field EEG recorded from controls and lesion rats that received FGF-2. For all rats, there was attenuation of higher frequencies. Represented here are data from delta range (A and B) as well as theta, and alpha (C and D) for the lesioned rats that received FGF-2 (B and D) and control rats (A and C). Overall, the analyses revealed a higher power from the control rats than their lesioned counterpart. Importantly, however, the frequency distribution was qualitatively similar.

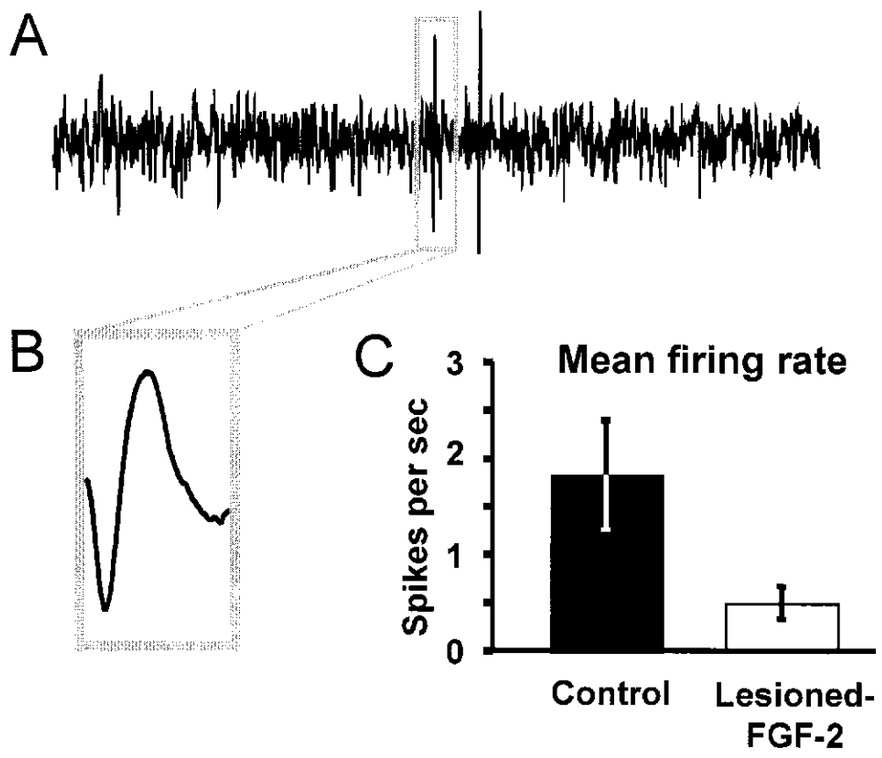

Figure 5- 4 In A, Example of multi-unit activity recorded from a lesion rat that received FGF-2. In B, example of one spike from a cell. Multiunit activity was recorded in 6 out of 7 lesioned-FGF-2 treated rats and in all controls (6 rats). Analyses revealed that the mean firing rate was greater in the cells from motor cortex (controls) than in the cells from the filled cavity (lesion rats that received FGF-2) (C).

7 lesion-FGF-2 rats, we lowered a recording electrode though dura in the region where we expected the filled cavity to be, and were unable to record any unit activity. When this rat was perfused, we found a prominent cavity at the lesion site. Fourier Transform analysis was performed on the EEG signal acquired from all the rats. A t-test was performed to compare the mean firing rate of cells from a control rat and those from the region filling the lesion cavity, and revealed a lower spontaneous firing rate of the cells from the filled cavity, $t(27)= 2.57$, $p=.016$. A qualitative comparison of the Fourier Transform performed on EEG from control rats and from lesion rats that received FGF-2 showed that the pattern of EEG, which was mostly low-voltage fast activity, was similar across both groups. Upon closer scrutiny, however, the power associated with peak frequencies in the delta, alpha, and beta range was lower in the EEG recorded form the filled region compared to that recorded from the control rats, $p<.05$. EEG and multiunit activity were also obtained from a control region (medial prefrontal cortex) in both the control and the lesion rats that received FGF-2. Our analyses showed that there was no difference between the groups on either the mean firing rate (data not shown), $p>.05$, or the Fourier Transforms for the gamma or the alpha and beta frequency range, $p> .05$ in both cases (data not shown).

Experiment 3

Number of synapses per neuron in the motor cortex or filled region

The filled-in region is not nicely laminated as in normal cortex (Monfils et al., 2005b), a finding that led us to wonder if the synaptic density was abnormal as well. A one-way

ANOVA was performed to compare the control groups and the lesion rats that received FGF-2 on synaptic density, neuron density, and number of synapses per neuron in the filled region. Our analyses revealed that the lesioned rats that received FGF-2, the no lesion rats that received BSA and the no lesion rats that received FGF-2 did not differ on either of these measures, $p>.05$ (see Figure 5-5).

Number of synapses per neuron in area Cg3

Although the major corticospinal projection in the rat is from the motor cortex, there is also a projection from anterior cingulate cortex, Zilles' area Cg3. Because this region could potentially support recovery of motor function after motor cortex injury, we asked whether there were changes in synaptic density in Cg3. A 2X2 factorial ANOVA was performed to compare the groups on synaptic density, neuron density, and the number of synapses per neuron in area Cg3 between our lesion and FGF-2 groups, and revealed no significant lesion X FGF-2 interaction, main effect of FGF-2, or main effect of lesion on synaptic density or neuron density, $p>.05$ (see Figure 5-6). In addition, there was no interaction or main effect of FGF-2 on the number of synapses per neuron, $p>.05$. There was, however, a main effect of lesion on the number of synapses per neuron, $F(1,17)=8.192$, $p=.019$, suggesting that the lesion induced a decrease in the number of synapses per neuron in area Cg3.

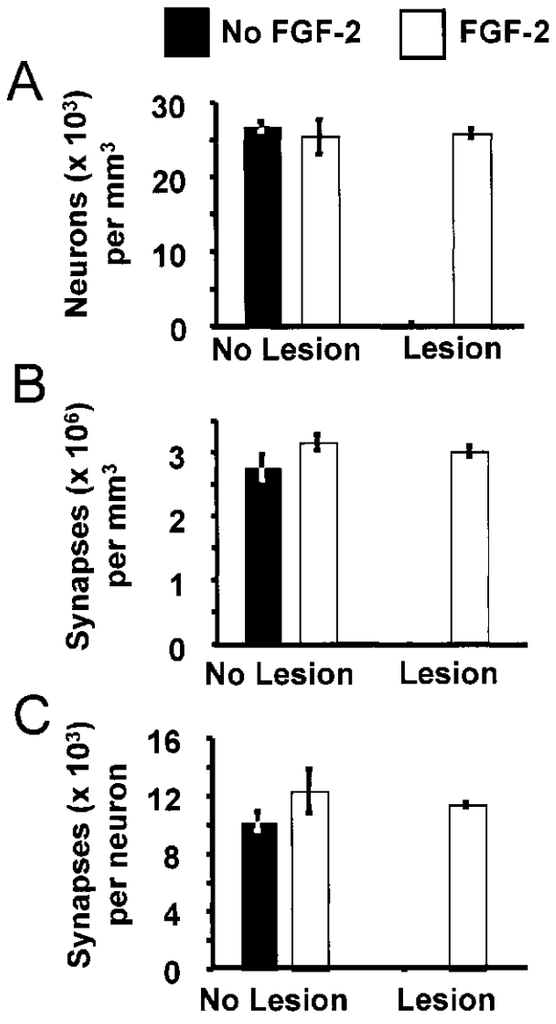

Figure 5- 5. Comparison between controls, and lesion rats that received FGF-2 on neuron density, synapse density and number if synapses per neuron in motor cortex or filled region. There was no difference between the controls or the lesion rats that received FGF-2 in either Neuron density (A), Synapse density (B), or number of synapses per neuron (C) in the motor cortex or filled region. There was a main effect of lesion on the number of synapses per neuron in area Cg3 (F), $p<.05$.

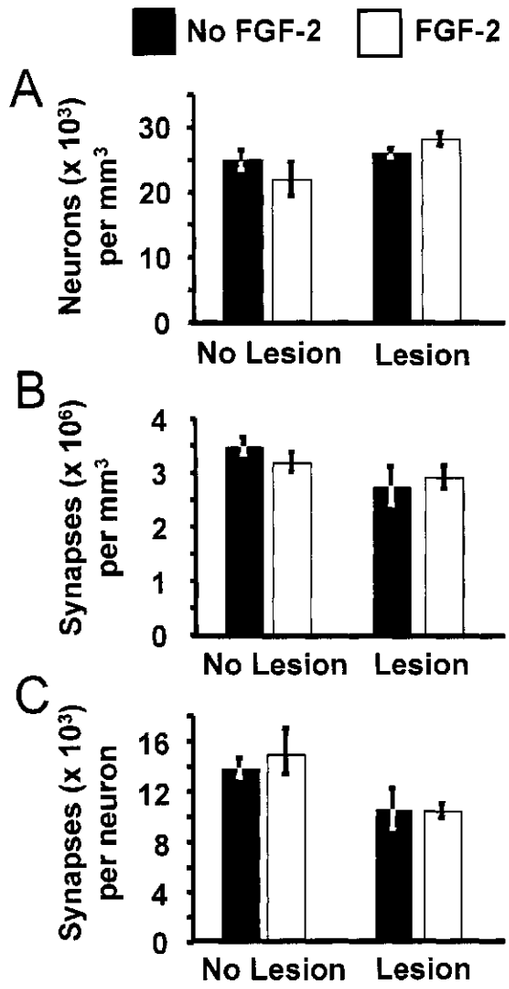

Figure 5- 6. Comparison between controls, and lesioned rats that did not receive FGF-2 and those that received FGF-2 on neuron density, synpase density and number of synapses per neuron in area Cg3. There was no difference between the controls or the lesioned rats that received FGF-2 in either Neuron density (A) or Synapse density (B). There was a main effect of lesion on the number of synapses per neuron in area Cg3 (C), $p<.05$.

Figure 5- 7. Effects FGF-2 on the skilled reaching performance of E13 BrdU (or saline)-exposed rats that received a bilateral motor cortex-lesion on postnatal day 10. In rats exposed to Saline on E13, FGF-2 administered following a bilateral lesion to the motor cortex is associated with an improved outcome on the skilled reaching task (A). In the lesion rats that were exposed to BrdU on E13, a prominent lesion cavity was present even in the FGF-2 treated group. In rats exposed to BrdU on E13 lesion rats are significantly impaired on the skilled reaching task, whether they receive FGF-2 or not (B).

Experiment 4

Skilled Reaching

As in our previous studies, FGF-2 treatment enhanced performance on a skilled reaching task but this improvement was blocked by prenatal treatment with BrdU. A 2x2x2 factorial ANOVAs was computed to compare the percent success on the skilled reaching task and revealed a 3-Way interaction (BrdU-treatment X lesion X FGF-2 treatment), $F(1,35)= 6.062$, $p=.019$ (see Figure 5-7). Follow-up tests revealed that in the rats that were not exposed to BrdU, whereas there was no difference between the BSA and FGF-2-treated rats that did not receive a lesion, the lesion rats that received FGF-2 outperformed their non-FGF-2-treated lesion counterpart, $F(1,20)= 4.063$, $p<.05$. In contrast, there was no effect of FGF-2 in the BrdU-treated rats, regardless of whether or not they were lesioned, $p>.05$. Finally, as in our previous studies, there was a main effect of BrdU, suggesting there was a deleterious effect of BrdU on performance on the skilled reaching test, $F(1,35)= 33.836$, $p<.001$. In addition, there was a main effect of lesion, suggesting that overall the lesion rats did not perform as well as their no lesion counterparts, 47.709, $p<.001$. The BrdU-exposed rats that were lesioned did not show a filling of the cavity, regardless of whether or not they had received FGF-2.

Experiment 5

Skilled reaching task

Rats were tested in the skilled reaching task before and after a unilateral lesion to the motor cortex (or filled region) at P90. The basic finding was that the FGF-2-mediated improvement in skilled reaching was reversed by removal of the filled-in tissue and that the performance level of such rats approximated that of untreated control animals that received similar-sized motor cortex removals in adulthood. A 2x2 ANOVA revealed a main effect of re-lesion, suggesting that all the rats performed more poorly on the reaching task following the lesion, $F(1,16)=40\ 9$, $p<.001$. There was no re-lesion x treatment group interaction, or main effect of group, $p>.05$.

Cylinder task

Rats were tested in the cylinder task before and after a unilateral lesion to the motor cortex (or filled region) to assess forelimb asymmetry. Our results show that whereas the rats did not show an asymmetry before they received a unilateral lesion, both the controls and the lesion rats that received FGF-2 made more contacts with the ipsilateral forelimb following the unilateral lesion. A 2x2 ANOVA revealed a main effect of unilateral lesion on forelimb asymmetry, $F(1,16)= 28.57$, $p<.001$.

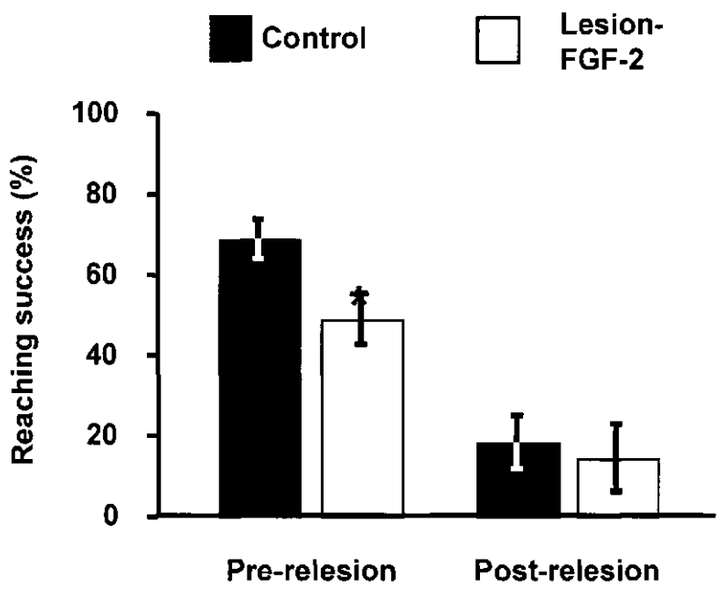

Figure 5- 8. Effect of unilaterally lesioning (re-lesioning) motor cortex contralateral to the preferred forelimb on the reaching success in the Whishaw tray reaching task in control rats and rats that received FGF-2 treatment following motor cortex lesions at P10. The relesion was associated with a skilled reaching deficit in both the controls and the lesion rats that received FGF-2, $p<.05$. In addition, whereas the groups different on skilled reaching performance before the re-lesion, they did not differ after the re-lesion, $p<.05$.

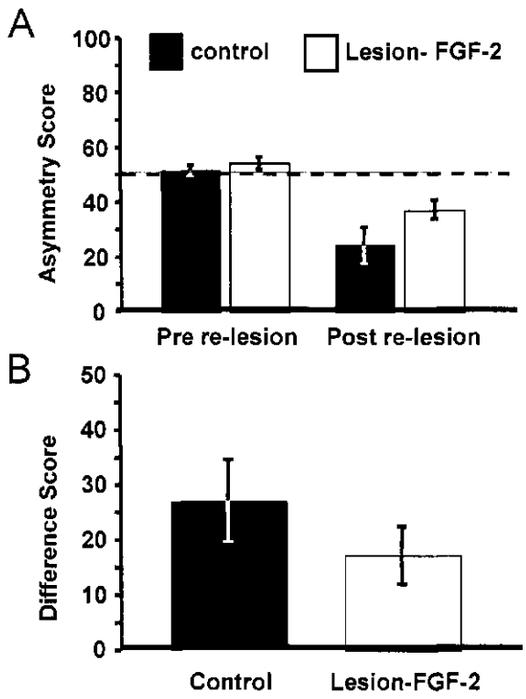

Figure 5- 9. Effect of lesioning (relesioning) on forelimb asymmetry in controls and lesion-FGF-2-treated rats. In A, asymmetry score pre and post re-lesion for the control and the lesion-FGF-2-treated rats. Following the re-lesion, both groups showed a reduced asymmetry score for the forelimb contralateral to the re-lesion., $p<.05$ In B, difference score (pre – post asymmetry score) for the control and the lesion-FGF-2-treated rats. There were no differences between the 2 groups on the difference score.

Discussion

We have shown previously that FGF-2 administration following bilateral motor cortex lesions inflicted at postnatal day 10 results in a filling of the lesion cavity with tissue and behavioral improvement on a skilled motor task. In the current study, we show evidence that: (1) FGF-2 stimulates neuroblasts from the subventricular region to migrate to the site of injury; 2) the cells that fill the lesion cavity spontaneously fire and show electrophysiological characteristics qualitatively similar to those from an undamaged motor cortex; and, 3) the cells that fill the cavity form synapses. In addition, we demonstrate that the FGF-2-induced motor cortex regrowth is necessary for the behavioral improvement by demonstrating that preventing the 'regrowth' by blocking cortical neurogenesis prevents the functional improvement; and, re-lesioning the regrown region contralateral to the preferred forelimb in adulthood reverses the functional improvement to the same degree as that of a unilaterally lesioned control. This study is the first to provide a detailed analysis of the behavioral, anatomical, and electrophysiological characteristics of newly-generated tissue that fills the lesion cavity after FGF-2 administration.

Neurogenesis

A number of studies have implicated FGF-2 in mediating neurogenesis, proliferation, and survival *in vivo* (Jin et al., 2003; Mehler et al., 2002; Yoshimura et al., 2001; Cheng et al., 2001). Endogenous FGF-2 has also been shown to be upregulated in response to cortical injury in a number of preparations (e.g., Ganat et al., 2002; Gibb & Kolb, 2005). Here,

we show an increase in proliferating (cycling-ki67-positive) cells in the SVZ, as well as the striatum, in response to the lesion. This is in agreement with previous reports that have shown an increase in neurogenesis in the subventricular zone as a result of a cortical lesion (e.g., Faiz et al., 2005; Gotts & Chesselet, 2005; Gotts & Chesselet, 2005b). Other studies have also shown increased neurogenesis (e.g., Gotts & Chesselet, 2005b), or dendritic hypertrophy (Monfils et al., 2005b) to occur in the striatum as a result of cortical injury. It is important to note that in the present study the increased proliferation of cells in the striatum occurred for both lesion groups, with no differential effect of FGF-2. In addition, we show that in lesion rats, FGF-2 leads to the migration of neuroblasts (doublecortin-positive) to the lesion cavity. To our knowledge, this is the first report of neuroblasts migrating to the site of injury and permitting a replacement of missing tissue with viable cells. Our results suggest that whereas the lesion itself leads to the generation of new cells in the subventricular zone, without administration of FGF-2, a number of them migrate to the striatum, but never leave to rostral migratory stream to be mobilized to the injury site. Future endeavors should establish the mechanism by which FGF-2 administration leads the newly-born cells to migrate to the injury site. Gott & Chesselet (2005) have recently observed that the number of blood vessels in the SVZ of animals with lesions was highly predictive of the number of cells in the SVZ, and suggested that these two processes may be mechanistically linked. FGF-2 is known to exert potent effects on endothelial vascular cells (Gospodarowicz et al., 1979; Logan et al., 1992), and thus a similar mechanism may be at play in the present case.

Firing characteristics

In adulthood, the cells filling the lesion cavity spontaneously fire. We show that there are quantitative differences in the firing pattern of cells from the filled region compared to that of a normal control: the mean firing rate was reduced in the lesion rats that received FGF-2. In addition, Fourier Transforms from field EEG suggest quantitative differences between the EEG of the lesion rats that received FGF-2 and control rats. Importantly, however, the qualitative pattern of multi-unit cell firing as well as the Fourier Transform analyses revealed a similar qualitative pattern from field EEG as well as cells isolated from the filled region of lesioned FGF-2-treated rats. The mean firing rate of isolated cells and Fourier Transforms from field EEG recorded in a control region (the medial prefrontal cortex) did not differ between the lesion-FGF-2-treated and control rats. Prior studies have reported a reduction in multi-unit activity and changes in field EEG in response to cortical injury (e.g., endothelin induced middle cerebral artery occlusion; Moyanova et al., 2003), as well as a decrease in the mean firing rate of cortical cells in response to hippocampal injury (O'Donnell et al., 2002). Our results are in general agreement with these findings, in that we show an overall decreased firing rate in the cells in the newly generated region. What is not known, however, is what the reduced firing rate might mean to the functioning of the cells.

Synapses in motor cortex, filled region, and Cg3

Our results suggest that there was no difference between the number of synapses per neuron in the filled region vs. motor cortex. Furthermore, there was an overall effect of

lesion, independent of FGF-2 treatment, which led to a decreased number of synapses per neuron counted in area Cg3. A number of studies have examined the effect of unilateral motor cortex lesions (in adulthood) on synapse numbers or dendritic changes in the contralateral cortex (Allred & Jones, 2004; Luke et al., 2004; Hsu & Jones, 2005). Recently, Hsu & Jones (2005) reported an increase in the number of efficacious synapses on the side contralateral to the lesion that was associated with an enhanced performance on a skilled reaching task with the limb ipsilateral to the injury (Hsu & Jones, 2005). Unilateral motor cortex lesions induced earlier in development (P1-P10) lead to dendritic hypertrophy in areas adjacent to the lesion, as well as the homotopic regions on the contralateral side (Kolb et al., 2000). The present study differs from previous ones, however, in that we inflicted bilateral damage. We have reported bilateral motor cortex lesion-induced changes in dendritic morphology in the striatum (Monfils et al., 2005b), and here we show lesion-induced changes in the number of synapses in area Cg3. The fact that those changes are not differentially affected by FGF-2 administration suggests that they do not support the additional recovery observed in the lesion rats that received FGF-2. Instead, we propose that the cells filling the lesion cavity support the functional improvement. Our laboratory previously reported that after day 10 medial prefrontal lesions the tissue spontaneously partially regrows and that in adulthood, the cells have dendritic fields that are similar to those of normal tissue (De Brabander & Kolb, 1997). We are unable to do a similar analysis of dendritic organization in the FGF-2-stimulated motor cortex growth because the lamination is abnormal and it would be impossible to identify comparable cells in normal brains and the filled-in region. Nonetheless, the

frontal tissue, the FGF-2-generated motor tissue has relatively normal neuronal connectivity.

The newly-generated tissue supports functional improvement

We demonstrate with two different methodologies that the filled region is responsible for the behavioral improvement reported in the lesion rats that received FGF-2. In the fourth experiment, we prevented the filling of the lesion cavity with proliferating cells by exposing the rats to BrdU on embryonic day 13 (Kolb et al., 1998). This procedure interferes with later postnatal mobilization of subventricular neurons both in vivo and in vitro and blocks recovery after day 10 medial prefrontal lesions (e.g., Kolb, Pederson & Gibb, in submission). Here we show that the BrdU-exposed rats that received bilateral motor cortex lesions still showed a prominent lesion cavity, regardless of whether they received FGF-2 or not, and this was associated with a decreased performance relative to their non-lesion counterparts. One caveat on this finding is that because the BrdU treatment has teratogenic effects on the behavior of rats without cerebral injury, the behavioral and anatomical effects on the FGF-treated lesion animals is not without question (for a review of common symptoms associated with E13 BrdU toxicity, refer to Kolb et al., 1998b). We therefore chose to test the hypothesis that the filled region was involved in the functional improvement using a complimentary method. In Experiment 5, we assessed the skilled reaching performance of lesioned-FGF-2-treated rats and control rats before and after a unilateral lesion to the motor cortex (or filled region)

normal synaptic density suggests that like rats with spontaneous regrowth of the midline

contralateral to their preferred forelimb. The results show that the adult lesion was associated with a significant decrease in reaching performance that was equivalent in both the control and the lesioned-FGF-2 rats that received unilateral lesions in adulthood. Together, these two experiments provide evidence for a role of the filled region in supporting the improved functional outcome. Our laboratory has previously shown that bilateral removal of the filled region in adulthood (which spontaneously occurs following a medial prefrontal cortex lesion inflicted at P10, and is associated with an improved functional outcome) induced a functional deficit (Dallison & Kolb, 1998). Thus, we have shown after FGF-2-stimulated motor cortex growth as well as spontaneously generated medial prefrontal growth that the functional recovery associated with the newly-generated tissue can be blocked using two complementary methods. We conclude that taken together, the studies show that the new cells contribute to recovery of motor functions after both types of injuries on postnatal day 10.

Broader context and general considerations

We have previously shown that medial prefrontal neonatal injury inflicted between P1 and P5 results in a dismal functional and anatomical outcome, whereas a similar injury inflicted around 10 days of age is associated with a remarkable functional outcome associated with a spontaneous filling of the lesion cavity with tissue. This remarkable outcome is restricted to injury to midline cortical regions inflicted around P10, and was recently suggested to be mediated by FGF-2 (Gibb & Kolb, 2005). In addition, we recently reported a differential expression of FGF-2 throughout development (Monfils et

al., 2005a) and found that compared to the first postnatal week FGF-2 expression was upregulated in the cortex and the subventricular zone between postnatal day 10 and 18. Those results suggested an important role of endogenous FGF-2 in modulating outcome from cortical injury around day 10.

The current findings emphasize a role for exogenous administration of FGF-2 in promoting a better outcome following injury. This is supported by behavioral, anatomical, and neurophysiological evidence, as well as the fact that the cells filling the cavity support the functional improvement on a skilled motor task. We have previously reported that the tissue filling the cavity lacks an elaborate topographic representation of forelimb movements even though stimulation of the tissue induces EMG activity in the contralateral forelimb (Monfils et al., 2005c). Future work will aim to enhance functional improvement and attempt to stimulate the formation of an integrated network of movement representations by combining focused rehabilitative motor training in conjunction with the FGF-2 treatment.

6. General Discussion

The goal of the present thesis was to examine brain plasticity in response to two different types of experience: exposure to FGF-2 and/or bilateral lesions of the motor cortex at postnatal day 10. We set out to examine these questions with four complimentary studies that assessed the potential role of FGF-2 in mediating functional improvements, and associated structural changes following bilateral motor cortex injury inflicted at P10.

Background

The research presented here was *a priori* driven by two main principles that emerged from decades of research on cortical injury: (1) outcome from brain injury varies as a function of age; and, (2) outcome from brain injury varies as a function of area damaged.

Outcome from brain injury varies as a function of age

Over three decades of research from the Kolb lab (e.g., Kolb, 1995) suggest that regardless of cortical area damaged, a focal aspiration lesion inflicted around postnatal day 10 (P10) is associated with a better functional outcome than a lesion inflicted either before or after that time. The better functional outcome is correlated with anatomical changes, with the details depending upon the precise region injured.

Outcome from brain injury varies as a function of area damaged

Although the functional recovery is overall better at P10, it is clear that the functional outcome differs depending on area damaged. Research suggests that the midline

structures, i.e., cingulate cortex and medial prefrontal cortex, display a remarkable functional and anatomical recovery following a lesion. In contrast, lesions to the more lateral cortical regions, i.e., motor cortex and parietal cortex, do not show as pronounced a functional recovery (e.g., Gonzalez, Whishaw & Kolb, 2003; Kolb, Cioe & Whishaw, 2000).

In an attempt to isolate a shared factor underlying both of these principles, Gibb & Kolb (submitted) conducted experiments in which they attempted to find a molecular 'stamp' common to mPFC and P10 that could potentially explain the improved functional and anatomical outcome relative to other times or regions. They looked at a number of potential proteins, and found that a particular growth factor, basic fibroblast growth factor (FGF-2), was more highly expressed after P10 than P3 injury to the mPFC. They proposed that an increased FGF-2 availability could play an key role in the improved behavioral and anatomical outcome observed in rats with P10 mPFC lesions.

The idea that FGF-2 *per se* might play a role following injury was not new. A number of studies reported a beneficial role of exogenous FGF-2 administration following injury (e.g., Comeau et al., submitted; Monfils et al., 2005a). Others reported an upregulation of FGF-2 in response to an ischemic insult (e.g., Ganat et al., 2004), and others demonstrated that blocking FGF-2 following injury worsens outcome (Rowntree & Kolb, 1997). The fact that endogenous FGF-2 availability at a certain time and place (P10, mPFC) might foster an amazing behavioral recovery, however, was novel, and raised

important questions. In particular, if the presence of FGF-2 is one of the 'conditions' that fosters a better outcome, could it be applied to induce recovery at different times or after injury to different regions?

Comeau et al. (2005) answered the first question in an experiment in which they evaluated whether exogenous administration of FGF-2 could improve the outcome following a bilateral P3 lesions to the mPFC (Comeau et al., 2005). Their results showed that FGF-2 induced a behavioural improvement on cognitive tasks, associated with structural changes in areas adjacent to the lesion. This lent further support for a role of FGF-2 in mediating recovery after brain damage.

In an attempt to further elucidate whether this new found 'condition' could be applied to animals with injury to a different brain region, we sought to establish whether exogenous administration of FGF-2 could improve outcome following bilateral aspiration lesions to the motor cortex. We chose to perform bilateral lesions to motor cortex for two reasons. First, although rats with P10 motor cortex lesions show a better outcome than animals with day 1 or adult lesions, they are still profoundly impaired at skilled motor tasks (e.g., Kolb, Cioe & Whishaw, 2000). Second, we made bilateral rather than unilateral lesions because we wished to ensure that any enhanced motor function was not due to changes in the normal hemisphere of unilaterally-injured animals.

The results of our first study suggested that the distribution and phenotypic expression of FGF-2 during early development provided a possible mechanism by which P10 may benefit from a better outcome following injury (Study 1). In our second study, we found that FGF-2 administration following P10 lesions to the motor cortex (MCx) leads to behavioral improvement associated with a filling of the lesion cavity with some tissue (replicated in Studies 3, and 4). The fact that cells filled the cavity in FGF-treated animals was unanticipated and played a very important role in shaping the remaining studies in this thesis. A strong focus of studies 3 and 4 was to assess the integrity of the filled region using a combination of techniques. We also sought to assess how adjacent areas were modified in response to the MCx lesion and/or FGF-2 compared to control animals, as well as to establish whether the filling of the cavity was necessary for the functional improvement on a skilled reaching task.

We found that stimulation of the filled region elicits a modest number of observable movements (Study 2) as well as EMG activity in the wrist extensors (Study 3). Furthermore, FGF-2 administered following P10 lesions to the MCx leads to the migration of neuroblasts to the site of injury (Study 4), and blocking the filling of the lesion cavity prevents anatomical and functional recovery (Study 4).

In order to understand our results in the broader context of brain plasticity after perinatal injury, we must first revisit what 'typically' underlies functional recovery following injury to the brain. Specifically, if recovery or compensation on a behavioral task is

observed after cerebral injury, the functional change typically is associated with structural changes in the remaining brain. We might expect to see one or a combination of three different types of changes to occur as a result of focal injury, some resulting in a positive outcome and some that do not. First, novel circuitry may be generated and reorganized across broad regions of the remaining cortex. Second, following a lesion, the local circuitry of the remaining cortex may reorganize. Such changes typically would involve reorganization of areas adjacent to the insult, or of other cerebral areas typically involved in supporting the behaviour. Third, there may be generation of new glial cells and/or neurons, with or without mobilization of those cells to the site of injury. These changes in cerebral organization occur following various types of injury, and typically are associated with differential functional outcomes. For example, following P1 lesions to either frontal or parietal cortex, there is extensive reorganization of corticocortical and corticostriatal connections (Kolb, Gibb, and van der Kooy, 1994). The reorganization involves the formation (or maintenance) of aberrant connections, such as projections from the medial geniculate nucleus to the visual cortex, and are associated with a poor functional outcome. In contrast, however, when similar lesions are applied to the frontal or parietal cortex at P10, there is no evidence of extensive aberrant connections, and there is a better functional outcome (Kolb, Gibb and van der Kooy, 1994). A similar phenomenon occurs as a result of bilateral motor cortex injury inflicted at different times throughout development. P1 injury results in the presence of extensive abnormal connections from cortical areas adjacent to the injury and is associated with a poor functional outcome, whereas P10 injury is not associated with the presence of abnormal

connections, and animals with P10 lesions show a better outcome than animals with P1 lesions (Kolb et al., 2000).

P10 mPFC lesions are associated with increased dendritic arbor and spine density in areas both adjacent to the injury as well as more distally. That is, there are modifications to the internal circuitry of the remaining cortex. In contrast, P1 mPFC lesion rats show a decrease on those same measures. Together, these findings suggest that whereas extensive aberrant reorganization of connections hinders function, changes to the intrinsic circuitry of the cortex are far more beneficial.

The last type of change that may occur as a result of injury is neuro- and/or gliogenesis. Kolb and colleagues (e.g., Gonzalez et al., 2003; Kolb et al., 1998) have found in a number of studies that removal of midline structures (olfactory bulbs, mPFC, Cg) at P10 results in a spontaneous filling of the lesion cavity with neurons and glia and that the presence of this tissue is associated with a remarkable functional recovery. The role of cell genesis after medial prefrontal cortex lesions has been most extensively studied, and the results suggest that the tissue filling the cavity comprises newly generated neurons and glial cells. Blocking the regeneration by administering BrdU on E13 inhibits the functional recovery, and removing the tissue filling the cavity in adulthood induces a functional deficit (Dallison & Kolb, 2004), lending support to the idea that the filled region supports functional recovery. Retrograde tracing from the striatum indicates that

the connectivity of the filled region is not abnormal, although it lacks long descending corticospinal projections (Kolb et al., 1998).

The behavioural and anatomical results presented in the present thesis (bilateral motor cortex lesions, and FGF-2) are reminiscent of the changes seen following P10 mPFC lesions. Together, the mPFC and Motor cortex studies constitute the only two instances I am aware of that suggest that new tissue might be responsible for subserving functional recovery after perinatal brain injury.

In both models, the injury is associated with a filling of the cavity with some tissue, this 'regrowth' supports the improved functional outcome, and there is no evidence of aberrant connections between cortical regions. In both cases, there are cells firing from the filled region that show physiological activity and in both cases although there is clear motor recovery, there is still some evidence of skilled motor deficits. In the case of the mPFC lesion, there is partial restitution of projections (to the striatum, for example). There is a lack of long corticospinal axons projecting to the spinal cord, however. In contrast, following motor cortex lesions and FGF-2 injections, there are long projections from the filled region to the spinal cord, and those projections are electrophysiologically functional (Monfils et al., 2005b).

General principles emerging from these findings

The role of FGF-2

Evidence from a number of studies has highlighted a role of FGF-2 in both development and recovery from brain injury. There is also evidence suggesting that the different spatio-temporal developmental expression of FGF-2 might play a role in modulating recovery from cortical damage in neonatal rats (Ganat et al., 2002; Monfils et al., 2005a). It appears, however, that the mere presence of FGF-2 is not sufficient. The FGF must be present at the right time and in a physiologically relevant dose. It is likely that a complex interaction of FGF-2 with its receptors (and in particular FGFR1) mediates the reaction of this ligand in response to injury (see Clarke et al., 2001). Further, endogenous and exogenous FGF-2 seem to be additive in their role following injury throughout early development and combined endogenous and exogenous FGF-2 levels may be deleterious if they are too high (Hastings, 2003).

It is clear that FGF-2 plays a role in the recovery observed in a number of experimental conditions (Gibb & Kolb, submitted; Comeau et al., 2005; Monfils et al., 2005a, 2005b, 2005d, 2005e; Rowntree & Kolb, 1997). What could be the underlying mechanisms by which FGF-2 exerts its beneficial effects? Are there mechanisms common to the different injuries sustained?

Regenerative plasticity, and degenerative plasticity

There appears to be a qualitative difference in the changes that occur in response to various forms of experience. First, in some instances, changes that occur as a result of brain injury are associated with a beneficial functional outcome (e.g., P10 medial prefrontal cortex lesion). In other cases, modifications found following lesions appear to hinder function (e.g., P3 motor cortex lesion). A good example illustrating these principles is the case of bilateral vs. unilateral injury. Following focal bilateral injury (e.g., a motor cortex lesion), a number of changes occur in different motor areas, or in regions adjacent to the lesion. Those changes are considered to be regenerative. It is not exactly known *what* signals or mechanism cause these changes to occur, but we do know that they are associated with a positive functional outcome. In fact, inhibiting those changes also inhibits the improved outcome. Furthermore, when focal motor cortex lesions are inflicted at a time when those changes do not occur (e.g., P3), there is no functional improvement. Following a unilateral motor cortex lesion (at P10 or adulthood), the changes are different; namely, on the side contralateral to the lesion we see changes that are similar to those described for bilaterally-lesioned rats- regenerative processes. However, on the side ipsilateral to the lesion, the changes take a different form- we actually see a decrease on measures of dendritic morphology. Furthermore, tracing studies have shown that the side contralateral to the lesion extends projections to the regions originally subserved by the now-lesioned side (Kolb, Gibb & van der Kooy, 1994). Here again, the precise mechanism at the source of this effect is not fully

understood. It is likely, however, that those plastic changes are derived from 'naturally-occurring' events that just happen to be enhanced following an injury.

Developmental brain plasticity

There must be some key developmental principles involved that modulate the window of plasticity at different ages and different areas. There are several stages to cortical development; namely, cell proliferation, migration, differentiation, dendritic and axonal growth, synaptogenesis, gliogenesis, and synaptic pruning and cell death. Those different steps occur a different rates and over a different length of time in different species, but nevertheless show a consistent pattern. In rats, most cell proliferation is complete at birth, whereas migration continues for approximately one additional week (Bayer & Altman, 1990). Dendritic arborization, synaptogenesis, and gliogenesis reach a peak in the second and third weeks of development. This is important, because various types of experiences (including lesions and/or the presence of growth factors) have different effects on the brain that likely largely depend on the major developmental events that are occurring during that time. For example, during dendritic development and synaptogenesis, an increase in afferent input enhances the formation of additional synapses, whereas a decrease leads to synaptic pruning (e.g., Fifkova, 1970). Importantly, because different animals develop at different rates (and so do their brains), a useful way to make sense of changes that occur in response to various treatments is to relate any changes to the different steps that are known to occur in a number of species. For example, the same developmental events that take place around P12 in the rat occur

around 8 months of age in humans (Kolb et al., in press). Of course, identifying how the brain responds to different treatments inflicted at various time points also furthers our knowledge of these different steps, and refines our ability to capitalize on 'naturally-occurring' mechanisms to promote recovery. We tried to close in on the potential mechanisms that could explain the behavioural improvements seen in the lesioned FGF-2 treated rats, but of course these give rise to many unanswered questions. The bottom line: in the treated rats, there is tissue that would not otherwise be there, and this tissue is functional.

Limitations and future studies

The design employed in the present study was such that we attempted to evaluate whether the filled region was involved in the recovery seen in the lesioned-FGF-2 treated rats. In doing so, we attempted to (1) assess the 'integrity' of the filled region using a variety of converging techniques, and, (2) break the relationship between the filled region and functional improvement. The limitations in assessing the first point were in that whereas we made progress in assessing the physiology, functionality, connectivity, and cellular make up of the filled region, we failed to isolate distinct mechanisms that could explain each of the steps involved in the generation and formation of the new tissue. Future experiments should study each of these phenomena in isolation to evaluate the interactions of FGF-2, P10 and motor cortex injury in modulating the various levels of recovery. For example, what is the role of FGF-2 in neurogenesis? What is the relationship of motor cortex lesion at P10 to cell migration? What is the role of FGF-2

signaling in promoting the extension of long descending axons to an appropriate target in the spinal cord? With respect to our second goal, again, we made progress in ruling out the role of other regions in subserving the functional recovery (indirectly testing the prediction that the filled region was involved), and tried to dissociate the relationship between function and filled region using two different experimental designs. The obvious problem, of course, is that neither design is without flaws: E13 BrdU prevents cortical neurogenesis, but also has an overall effect on the whole brain, which in some respects clouds the role of the filled region in subserving recovery. The re-lesioning design sidesteps the problems with the BrdU experiment but (1) re-lesioning *only* the *new* tissue is impossible, and (2) removal of the tissue via aspiration undoubtedly disturbs the function of areas that were connected to the lost tissue, leading again to a larger functional lesion than intended.

An important point that was not addressed in the current thesis was to attempt to disambiguate between the role of the filled region and the role of other areas in their contributions to the functional recovery. Future studies will aim to address this discrepancy by manipulating FGF-2 levels following P10 lesions to the motor cortex in the opposite direction- i.e., downregulating or blocking FGF-2. If FGF-2 is involved in mediating the changes that support partial recovery of function dissociated from a filling of the cavity in lesioned rats that do not receive FGF-2, then reducing FGF-2 levels with neutralizing antibodies should inhibit *the majority* of functional improvements.

Implications

Some of the principles that emerged (or were emphasized) from our experiments should be applied to different time points, different areas, and different types of injury, in order to determine how our findings generalize to other ages and injuries. It is known already that FGF-2 does not stimulate tissue growth after P3 mPFC lesions (e.g., Comeau et al., 2005) or after P3 motor cortex lesions (Monfils, unpublished observations). But it is not known if FGF-2 given after P10 lesions to more posterior regions might also stimulate cell growth and functional recovery. It is also not known if combinations of FGF-2 and experiential treatments known to influence functional recovery after perinatal injury might be synergistic and further enhance functional recovery after perinatal injury. Finally, it is not known if our results generalize to other species, and especially species with larger brains in which there is a longer distance from the SVZ to the site of injury.

7. References

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APPENDIX 1. A quantitative comparison of synaptic density following perfusion vs. immersion fixation in the rat cerebral cortex.

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Published in Microscopy Research and Technique, June 2005.

Abstract

The main objective of the present study was to perform an unbiased comparison of immersion vs. perfusion techniques to assess whether we could use the former to quantify synapses through electron microscopy. Using the immersion technique include is ideally suited for instances in which the specimen under study could not be perfused under the standard EM protocol. Our results suggest that, despite suboptimal qualitative results, fixation by immersion allows for adequate quantification of synapses.

Key words: Electron microscopy, Golgi-Cox, synaptic density

The main objective of the present study was to perform an unbiased comparison of immersion vs. perfusion techniques to assess whether we could use the former to quantify synapses through electron microscopy (EM). The standard EM protocol involves fixation by perfusion. An advantage of using the immersion technique is that it is ideally suited for instances in which the specimen under study could not be perfused with Glutaraldehyde (GA) and Paraformaldehyde (PFA) (the standard EM protocol). Additionally, fixation by immersion would allow processing of different brain regions with different protocols. A relevant example from studies conducted in our laboratory is the Golgi-Cox method. Effectively, Golgi-Cox staining and EM both enable estimation of the number of excitatory synapses, however the two techniques are qualitatively different. Golgi-Cox impregnation permits the visualization of whole cells, including dendritic arborization and spines, and provides a global overview of treatment-induced changes (Gibb & Kolb, 1998). EM, on the other hand, provides higher resolution imaging of cell ultrastructure including synaptic terminals, but is limited to smaller areas of the brain (Bozzola & Russell, 1991). Quantification following Golgi-Cox staining can provide information about the complexity of the stained neurons, the length of the dendrites, as well as the density of dendritic spines. With EM, unbiased stereology can be performed on electron micrographs, enabling identification of synapse type and quantification of synaptic density per unit volume of tissue.

However, to perform Golgi-Cox and EM on the same specimens presents a challenge. The Golgi-Cox methodology routinely employed in our laboratory requires a saline perfusion (Gibb & Kolb, 1998), and in order to facilitate the penetration of Golgi-Cox through the tissue, aldehydes should not be included in the perfusion solution. Likewise, it is widely believed that, in order to yield properly preserved tissue for EM, brains require perfusion with glutaraldehyde (GA), either alone or in combination with paraformaldehyde (PFA) (Bozzola & Russell, 1991).

The methodology outlined here is an extension of the work by Morin et al. (1997), which presented a technique allowing fixation of resected human tissue by immersion. We compared synapse quantification derived from samples processed using perfusion vs. immersion fixation. The latter methodology could potentially enable both EM and Golgi-Cox procedures to be performed on different hemispheres of the same brains. The practical applications of using both EM and Golgi techniques in two different hemispheres of the same animal are discussed.

MATERIALS AND METHODS

Perfusion and initial fixation

Two groups of four rats each were used in this study. All rats were deeply anesthetized with sodium pentobarbital (120 mg/kg) prior to perfusion. Rats from the first group, which served as the EM control group, were transcardially perfused with phosphate buffered saline (PBS) (0.9% saline in 0.1M PO_4) followed by 2% paraformaldehyde

(PFA)/2.5% glutaraldehyde (GA) in 0.1M PBS. The brains were then stored in 2% PFA/2.5% GA at 4° C until dissection of the motor cortex took place.

Rats from the experimental group were perfused with 0.9% saline only. Each brain was then cut in front of the cerebellum, and mid-saggitally. One hemisphere was immersed in Golgi-Cox solution (see Gibb and Kolb, 1998) while the other was immersed in a 37 °C fixative solution containing 0.1 M cacodylate buffer, 2.5% GA, 4% PFA and 2.5% dimethysulfoxide (DMSO) for 5 minutes, after which it is transferred to the same fixative solution at a temperature of 4° C. This hemisphere was stored at 4° C for seven days and then dissected and processed for EM as described below. The hemisphere immersed in Golgi-Cox solution was processed according to methods previously described (Gibb & Kolb, 1998).

EM preparation

The control brains, as well as the hemispheres for EM from the experimental group, were coronally sectioned (300 μm) on a vibratome, and the sections viewed under a dissecting microscope at low power (2X). Samples of tissue (approximately 500 μm wide) extending from pia to white matter were dissected from the caudal forelimb area of motor cortex using a sharp razor blade. The tissue blocks were then processed according to the methodology previously reported (Kleim et al., 1996) Briefly, the blocks were washed in 0.1M cacodylate buffer for 2 hours, postfixed in 2% osmium tetroxide/1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 2 hours and en bloc stained with 2% uranyl

acetate for 45 min. The samples were then dehydrated through an ascending series of ethanol concentrations before being transferred into propylene oxide followed by gradual embedding in Eponate resin.

A few (approximately 10) 1 μm sections were cut from a block of embedded tissue from each rat. Sections were stained with toluidine blue, and cortical layer V was identified by the presence of large pyramidal cells and the absence of layer IV granule cells using the light microscope. Next, most of the block was trimmed away in a pyramidal shape, leaving a small strip containing layer V of the motor cortex. From this strip, silver/gray serial sections (approximately 70 nm thick) were cut using a diamond knife. These sections were collected on Formvar-coated, slotted, copper grids, and stained sequentially with uranyl acetate followed by lead citrate.

Stereology

Thin sections were surveyed with the electron microscope and an area of the neuropile that was essentially free of nuclei was selected in one of the serial sections. Within this area, a composite of four micrographs were taken at X 6,000 such that each micrograph represented one quadrant of a square. Next, the same area in a non-consecutive section (i.e. the intervening section was skipped) was photographed at the same magnification. A minimum of 16 micrographs were prepared for each rat. The resulting negatives were then printed at a final magnification of X 23,800 and subsequently analyzed for synaptic density using the physical dissector method (Sterio,1984). In this method, an unbiased

counting frame of known area (105 μm^2) is placed over each micrograph of the first composite (reference sections) and the number of synapses counted. The same frame is then placed over each micrograph from the second composite (lookup sections) and synapses not visible in the reference section are counted. In this way, synapses are counted only once. In the present study, synapses were identified by the presence of synaptic membrane specializations and only those with at least three vesicles were included in the counts. From these counts, the synaptic density per volume (μm^3) could be determined.

RESULTS

Golgi-Cox staining

As shown in Figure 1, the Golgi-Cox method yielded staining of whole cells. The extensive staining permits observation, and quantification (not shown here), of dendritic branches and spines.

EM: qualitative observations

Except for a difference in contrast, the appearance of both control and experimental tissue sections was similar at low magnification (Figures 2A and 2B). Increased contrast of the experimental tissue sections was apparent and likely due to the extraction of cytoplasmic proteins during the interval between saline perfusion and infiltration of the fixative. There was other evidence of suboptimal preservation in the experimental tissue sections but this was minimal and appeared to be restricted primarily to the nuclei. As seen in Figure 2B,

vacuoles were apparent in some nuclei (*), however other membranes appeared normal. Myelin sheaths in both control and experimental tissue showed no signs of de-lamination. Membrane discontinuities and other indicators of inadequate fixation (Hayat, 1981), were not seen. The synapses, which were the primary focus of this study, also showed little variation between fixative regimens (Figures 2C and 2D). Post-synaptic vesicles were easily discernable in all tissues, although not as well preserved in the experimental group tissue. All of the synaptic types could be easily identified in both groups.

Synaptic Density

The physical disector method was performed on thin sections of both tissue groups (control and experimental) to determine whether there was a quantitative difference between synaptic densities (Sterio, 1984). The total synaptic volumes analyzed were 250.56 μm^3 and 195.16 μm^3 respectively. The number of synapses per cubic micrometer was, on average, 0.233 for the control group and 0.278 for the experimental group (Figure 3). A between-subjects t-test was performed to compare the synaptic density between both groups, and revealed that there was no significant difference ($p > 0.05$).

DISCUSSION

The primary goal of the present study was to perform an unbiased comparison of immersion vs. perfusion techniques to assess whether we could use the former to quantify synapses. Such a design would afford the opportunity to process different portions of the same brains with different protocols (e.g., Golgi-Cox staining and EM), or to quantify synapses using EM in tissue that was not prepared using the standard perfusion protocol.

The Golgi-Cox methodology is a well-established analytical procedure that has been used extensively in our lab for over two decades. In order to extend our observations to the EM level and at the same time maintain consistency with previous Golgi-Cox studies, it was imperative that only the EM procedure be modified. The two techniques are qualitatively different, and because each technique affords specific information that the other does not, the two are complimentary.

The protocol presented here is a modified version of Morin et al.'s (1997) methodology, which was originally designed to prepare resected human tissue for EM analyses. In their study it was determined that the addition of DMSO to the immersion fixation solution resulted in acceptable preparation of nervous tissue and maintenance of the structural integrity of synaptic elements. DMSO facilitates the penetration through the tissue, likely by weakening the tissue permeability barrier (Morin et al., 1997; Franz & Van Bruggen, 1967). Inclusion of this reagent in the fixative solution is also believed to enhance preservation of membranes (Sandborn et al, 1975). Using this reagent, our results demonstrate that although some fixation artifact was apparent in the experimental group tissue compared to tissue of the control EM group, the synaptic complex retained its integrity. In fact, the increased contrast of the experimental tissue sections enhanced the visibility of synapses making them more easily identifiable in these sections.

Using unbiased stereology techniques, we confirmed that there was no significant difference between synapse counts for the control and the experimental tissue groups,

thus signifying that suboptimal preservation of the experimental group tissue had no effect on the quantitative results. In addition, the numbers obtained in our counts compared favorably with those published for other studies (Kleim et al., 2004; Kleim et al., 2002) using the same stereological procedures on micrographs produced from tissue prepared using standard procedures. The generalizability of our results is limited, however, because we did not determine whether the fixative penetration was adequate to quantify synapses in deeper brain structures.

Despite the fact that both the Golgi-Cox and EM techniques allow for the quantification of synapses (either directly or indirectly), we do not feel it would be appropriate to directly compare quantitative results obtained using each of them. Effectively, results using those two methodologies independently have proven, in the past, to be comparable in relative terms (i.e., treatments found to induce an increase in dendritic morphology as assessed by the Golgi-Cox method were also found to induce increases in synapses using EM [Juraska, 1984; Greenough et al., 1985). The magnitude of effect and the raw numbers however, cannot be appropriately compared because the unit of analysis derived from each methodology is qualitatively different.

Because each method confers specific advantages over the other, we believe it is advantageous to be able to perform both Golgi-Cox and EM procedures on the same animal. EM techniques are laborious and rather costly in comparison to the rapid and inexpensive Golgi-Cox method. We thus suggest that performing the latter technique is

the most cost-efficient way to derive an index of synaptic changes in large areas of the brain. Combining this technique with EM, however, enables quantitative measurements on targeted areas of interest.

ACKNOWLEDGEMENTS

Supported by CIHR grants to BK and JAK, AHFMR and NSERC scholarships to MHM, and NSF and AHFMR scholarships to ID. The authors would like to thank Nagheme Thomas for technical assistance, and G.C. Teskey for providing tissue samples.

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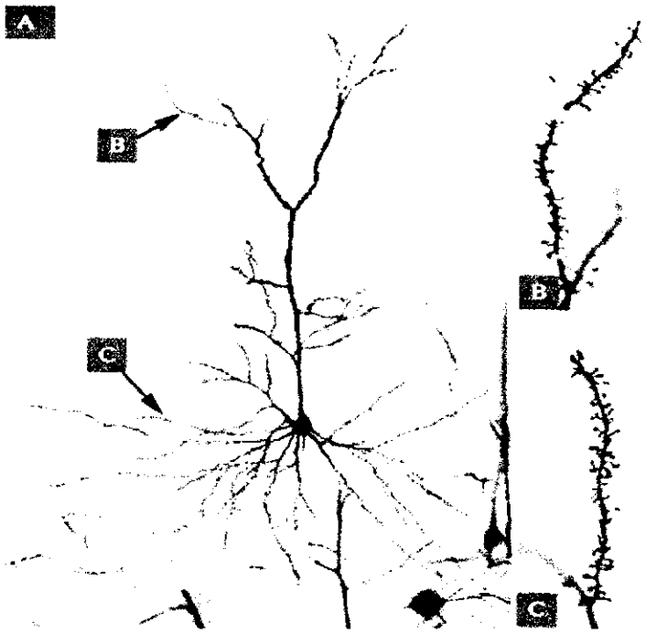

Figure 1. Golgi impregnated pyramidal cell from layer III in area Par1. In A, picture of a golgi impregnated pyramidal cell from layer III in area Par1. In the insets (B and C), higher magnification (1060X) showing complete labeling of dendrites and dendritic spines from an apical (B) dendrite, and a basilar (A) dendrite (after Kolb et al., 2003).

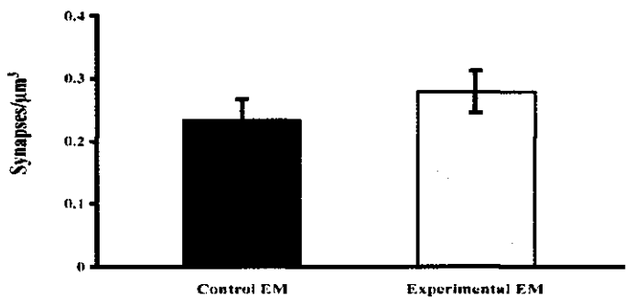

Figure 2. Effect of perfusion (control) vs. immersion (experimental) fixation of tissue on ultrastructure. As can be seen in these representative micrographs, some aberrations were present in the experimental group (A and C), compared to the control EM group (B and D), including pericuclear enlargement (*), intra nuclear artifacts (#), enlarged myelin (^), and extracted ground plasm. Synapses (@) appeared to be unaltered by the experimental methodology.

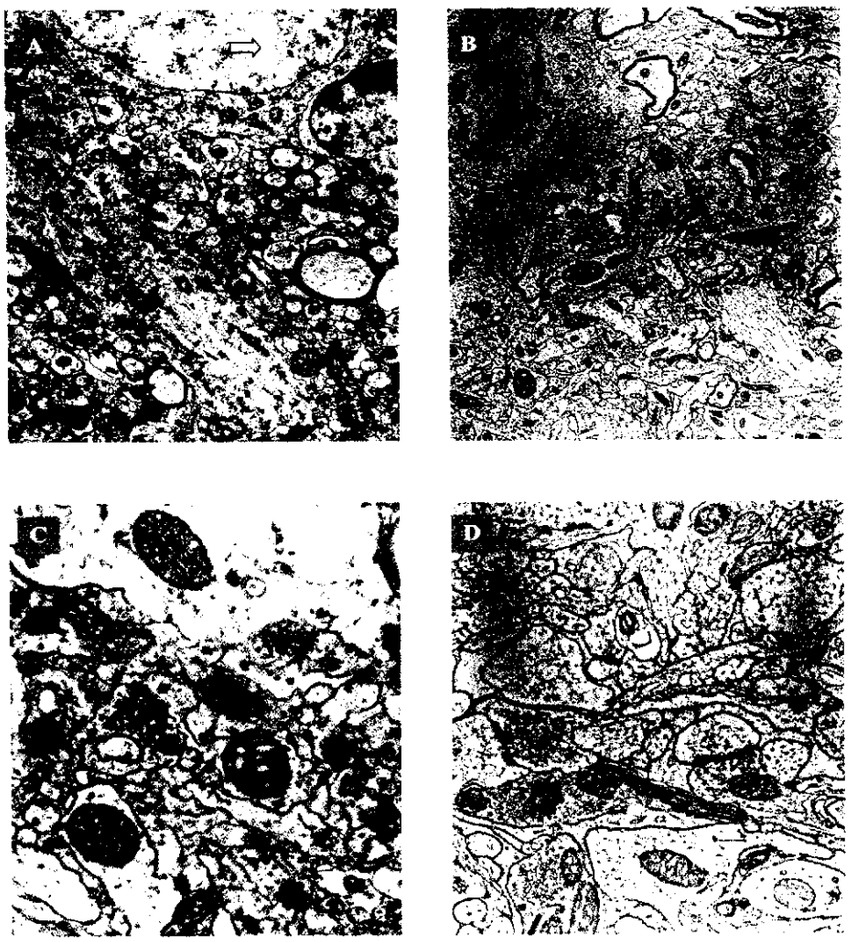

Figure 3. Synaptic density in Layer V of motor cortex for the experimental and control EM groups. There was no difference between the experimental and control EM groups in the number of synapses per μm^3 ($p > .05$).