AN ANALYSIS OF POSTSTROKE MOTOR DYSFUNCTION AND CEREBRAL REORGANIZATION IN RATS

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Dedication

A Laura

Por todas las prosas y versos...

Abstract

This thesis investigates the behavioural and anatomical correlates of recovery from motor cortex damage in rats. The effectiveness of behavioural, pharmacological, and regenerative treatments was investigated using models of focal stroke. Chronic bilateral motor deficits were found after motor cortex damage induced by various methods. These behavioural deficits were similar in severity and duration although they were correlated with different patterns of cortical reorganization seen in Golgi-stained tissue. Animals with motor cortex injury benefited from postinjury olfactory stimulation, chronic administration of nicotine, and infusions of epidermal growth factor followed by erythropoietin. Different mechanisms of plasticity in remaining cortical circuits are discussed as possible candidates responsible for the behavioural improvement. The current thesis expands the current knowledge of the effects of adult cortical damage to areas critical to motor control. It also may stimulate research on therapies and possible mechanisms that might enhance recovery after stroke.

Acknowledgements

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List of Abbreviations

A	Anterior
ANOVA	Analysis of variance
BDNF	Brain derived growth factor
bFGF	Fibroblast growth factor-2
BL	Baseline
BOA	Branch order analysis
BrdU	BromodeoxyUridine
C	Centigrade
Ca	Calcium
Cg	Cingulate
CIMT	Constraint-induced movement therapy
CFA	Caudal forelimb area
cm	Centimeter
EGF	Epidermal growth factor
EPO	Erythropeitin
fMRI	Functional MRI
Fr	Frontal
g	Gram
GPe	External portion of the globus pallidus
GPi	Internal portion of the globus pallidus
h	Hour

HL	Hindlimb
ICMS	Intracortical microstimulation
IL-1β	Interleukin 1β
K	Potassium
Kg	Kilogram
L	Lateral
M	Molar
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
min	Minute
mm	Millimeter
mg	Milligram
NGF	Nerve growth factor
MRI	Magnetic resonance imaging
OS	Olfactory stimulation
PET	Positron emission tomography
PID	Peri-infarct depolarizations
PBS	Phosphate Buffer solution
PMd	Dorsal premotor cortex
RFA	Rostral forelimb area
SA	Sholl analysis
SE	Standard error
SDA	Spine density analysis

SVZ	Subventricular zone
TNFα	Tumor necrosis factor α
t-PA	Tissue plasminogen activator
TMS	Transcranial magnetic stimulation
W	Week
μm	Micrometer
μg	Microgram

Chapter 1

1. Introduction

I was at a Society for Neuroscience meeting in Orlando when I received the call: "Your aunty Tere is in the hospital, she had a stroke." Thinking I knew something about it I asked for her condition, whether she could move, speak, or make sense of herself. She spent 25 days in the hospital connected to a respirator and I.V. feeding. She never moved, spoke, or recovered consciousness again.

My aunty Tere was one of the very unfortunate 20% of people that are hospitalized for stroke and die before leaving the hospital. A brain attack, or stroke, occurs every ten minutes in Canada and is the leading cause of long-term disability. Even if my aunty had lived, she would have needed long-term care. Her life would have been shattered; the stroke damaged the right frontal, parietal and temporal lobes, and her motor and cognitive capacities would have been seriously compromised.

About 50% of all stroke survivors require some form of rehabilitative program. Physiotherapy to regain motor control and speech therapy to regain language skills are among the most common types of therapy. The prevalence, the cost, and the growing aged population constitute important reasons for studying factors that might influence outcomes from damage after stroke. Furthermore, by documenting the functional recovery/compensation and resulting brain morphology following stroke, we can gain insight into the nature of the processes that occur within the brain after it has been damaged, and how can we stimulate some of those processes to enhance recovery. There were two main goals of the present thesis; the first one was to document the behavioral deficits and anatomical sequelae that follow motor cortex damage and the second one

was to test different therapies that might positively influence behavior and enhance brain plasticity after stroke.

Although different models of stroke were used to induce damage to the motor cortex, the experiments that constitute the core of the current thesis deal with recovery of function and not with the neurobiology of stroke. The definition of stroke and its pathophysiology will be briefly summarized followed by a description of the organization of the motor system. Finally, some evidence and current theories of motor recovery after stroke will be reviewed.

1.1 What is stroke?

Stroke is the sudden disruption of the blood flow to the brain. It is the most common cause of brain lesions. There are two main categories of stroke: ischemic (80%) and hemorrhagic (20%). Ischemic stroke results from occlusion of cerebral vessels by thrombosis or embolism. The rupture of a cerebral blood vessel causes a hemorrhagic stroke. When an artery deep within the brain ruptures, the hemorrhage is called intracerebral stroke, if the bleeding occurs on the surface of the brain (between the brain and the skull) is called subarachnoid hemorrhagic stroke. Although stroke most commonly occurs in people over 65, anyone of any age can have a stroke. Hypertension, heart disease, diabetes, smoking, high blood cholesterol, and inactivity are among the most common "controllable" risk factors for stroke.

1.1.1 Pathophysiology of stroke

Most of what is known about the neurobiology of ischemic stroke has come from animal models that have focused on the molecular and cellular processes mediating neuronal death. The primary goal of this research has been at understanding the sequence

of events that follow stroke in order to identify treatments that may stop or reverse this neurotoxic cascade and rescue cell tissue.

The brain has a high consumption of oxygen and glucose and when stroke occurs there is a sudden loss of these substrates. Energy depletion initiates a metabolic sequence of events that evolves over minutes to days and weeks. Although this cascade is a continuous process, for simplicity it can be divided into four different steps (Enders and Dirnagl, 2002): 1) excitotoxicity, 2) peri-infarct depolarization, 3) inflammation, and 4) apoptosis (see Figure 1.1).

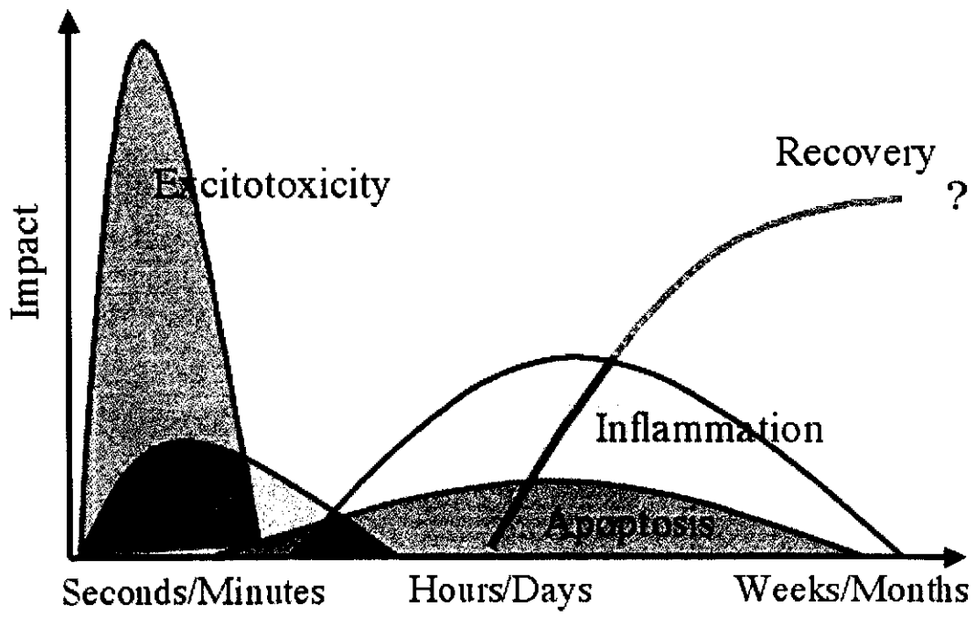

Figure 1.1. The cascade of events that take place after stroke. In the first seconds to minutes, excitotoxicity takes place mainly due to glutamate release. At the same time a cascade of peri-infarct depolarizations (PIDs) continue over hours followed by inflammatory and apoptotic processes which slowly resolves over

days and weeks. Recovery occurs during the few days post stroke and continues over months to years. (Adapted from Dirnagl et al, 1999, and Kolb and Whishaw, 2003).

Excitotoxicity: Within minutes following ischemia the lack of energy disrupts the cell membrane ionic balance. These ionic changes are responsible for a massive release of the excitatory neurotransmitter glutamate into the synaptic cleft, which is triggered by sodium entry into depolarizing ischemic neurons (Scott and Gray, 2000). Glutamate further accumulates in the synaptic cleft and induces activation of calcium channels. The intracellular increase of calcium (Ca^{++}) ions eventually leads to cellular disruption and cell death. Calcium produces not only direct toxic effects but also activates enzymes that degrade the cytoskeleton, the membrane, and structural proteins (Endres and Dirnagl, 2002).

Peri-infarct depolarization: In the core of the ischemic lesion the cells are unable to repolarize and die as a result. In the border-zone ("penumbra") of the lesion, cells are able to re-polarize but at the expense of further energy depletion. The loss of energy and glutamate release produces an electrochemical wave that propagates through neural tissue at 2-5 mm/min, causing prolonged (1-5 min) cellular depolarization (Hartings et al., 2003). This phenomenon was first shown to occur in the context of ischemic injury in 1977 (Branston et al., 1977) and since then has been investigated as a pathogenic mechanism in clinical stroke. These waves of de- and repolarization may be why they are called peri-infarct depolarizations (PID). In the clinic, reduction of PIDs by therapeutic intervention reduces infarct volume and vice versa (Iijima et al., 1992; Hartings et al.,

2003). Lesion volume and cell loss correspond to the number of PIDs (Mies et al., 1993; Hartings et al., 2003).

Inflammation: Brain ischemia is associated with expression of a number of inflammatory mediators. Cytokines, such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) are responsible for the accumulation of inflammatory cells in the injured brain and may affect the survival of damaged neurons. Cytokines are a unique family of growth factors and are secreted primarily from leukocytes. Cytokines stimulate the cellular immune response, as well as the activation of phagocytic cells. During ischemia, cytokines attract leukocytes and stimulate the production of adhesion receptors on leukocytes and endodthelial cells. Leukocytes promote infarction through their toxic byproducts, phagocytic action and by the immune reaction (for review see Han and Yenari, 2003). In experimental settings, it has been shown that administration of exogenous IL-1β exacerbates the ischemic injury and on the contrary IL-1β antagonists attenuate infarct size (Loddick and Rothewell, 1996; Betz et al., 1995).

Apoptosis: Although most cells in the core of the ischemic injury die from necrosis, cells in the penumbra area die mostly from a type of "programmed cell death" or apoptosis (Linnik et al., 1993). During ischemia, the limitation on the availability of oxygen and glucose place a severe restriction on mitochondrial function. Decreases in mitochondrial respiratory capacity leads to a triggering of the genes for the activation of different proteins and enzymes that ultimately destroy neurons. Cytochrome c and Caspase-3 are examples of these chemicals that play a pivotal role in programmed cell death, and are activated during ischemia (Sims and Anderson, 2002).

Excitotoxicity, peri-infarct depolarization, inflammation, and apoptosis are the most relevant mediators of cell death after stroke and a great deal of ongoing research is aimed at targeting each one of these components for early intervention. To date, there is no question that the best neuroprotection is provided by the rapid restitution of oxygen and glucose. Reperfusion of an occluded artery by t-PA (tissue plasminogen activator) is still the most effective early intervention to restore oxygen and glucose. Despite the advances of hyperacute intervention and the emergence of neuroprotectant drugs however, most stroke survivors still have residual functional deficits and require some sort of rehabilitation to help restore lost functions.

1.2. Motor control

Stroke survivors are left with varying degrees and types of neurologic impairments and functional deficits. Motor impairments are the most prevalent of all deficits seen after stroke, usually with involvement of upper and lower extremities. In order to understand some of the consequences that stroke can have on motor functioning, a brief review of the major players in motor control is needed. The following section will discuss the general aspects of the organization of the motor system. (For more detailed discussion, see Afifi and Bergman, 1998; Blumenfeld, 2002; Kandel et al., 2000; Kolb and Whishaw, 2003; Squire et al., 2003).

1.2.1. The motor system

Imagine the simple action of moving your hand toward an object. To do so, a complex series of steps have to take place in the nervous system and many brain structures are involved in executing this action (see Figure 1.2). First, visual information

is sent to the parietal and temporal regions of the cortex, from there is sent to the premotor and supplementary areas of the frontal cortex that are activated in order to organize the sequence of movements, then the information is sent to the motor cortex. At the same time the basal ganglia and the cerebellum process sensory information from the muscle tendons, joints and skin and then determine the pattern of neural activity required to perform the movement. That information is sent via the thalamus to the motor cortex and finally is sent down through the corticospinal tract to the ventral horn of the spinal cord and from there to the muscle fibers that move your hand. The role that the motor cortex, basal ganglia, cerebellum, and descending pathways play in movement is summarized in the following section.

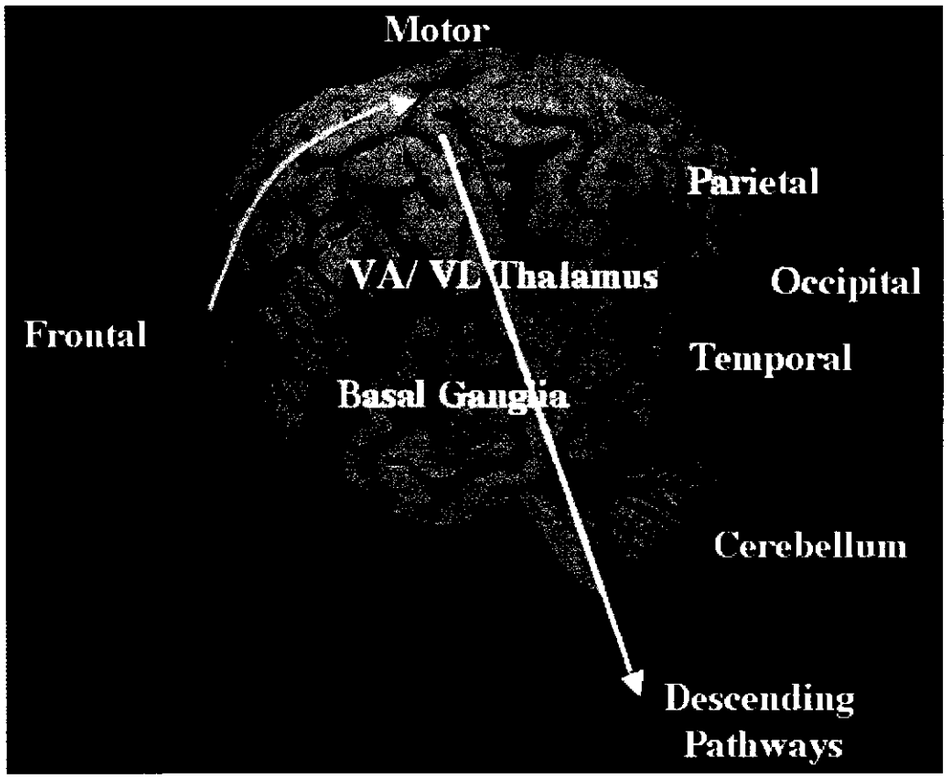

Figure 1.2. Brain structures involved in the simple action of moving your hand toward an object. Visual information of the location and position of the object travels from the occipital cortex to the parietal and temporal cortex, and from there to the frontal cortex which in turn sends projections to the premotor (not shown) and motor cortex. The cerebellum and basal ganglia also are activated and send information to the motor cortex via the thalamus. The motor cortex sends the concluding output through the descending pathways that synapse in the spinal cord (not shown) so you can extend and flex the muscles of your hand and arm.

1.2.1.1.The motor cortex

The motor cortex lies on the precentral gyrus rostral to the central sulcus. The first direct evidence that the neocortex could produce movement came from studies by Fritsch and Hitzing in 1870. Fritsch and Hitzing electrically stimulated the cortex of anesthetized dogs producing movements of different parts on the opposite side of the dog's body. Systematic stimulation of the surface of the cortex in non-human primates revealed somatotopic maps of the body in frontal areas. Early in the 1870s Hughlings Jackson first hypothesized the idea that the motor cortex was systematically organized to control movements of different parts of the body. Jackson observed that in some epileptic patients the convulsive movements systematically progressed from one part of the body to contiguous parts. In the 1950s Penfield electrically stimulated the cortex of conscious humans patients and found that most of the movements elicited by the stimulation where organized in a somatotopic fashion. In the largest area, the legs and feet are represented most medially and the trunk, arm neck and head are represented progressively more laterally. From these studies Penfield constructed a map (the homunculus) of the neural

representation of the body in the motor cortex (Penfield and Rasmussen, 1950). The motor homunculus is disproportionate in the relative size of its body parts compared with their relative sizes in the body itself. In the motor cortex, the fingers, lips, and tongue for example, are very big, and the trunk, arms and legs take much less space. This disproportion of body parts is due to the fact that language (speech) and hand motor control require greater precision and thus a larger cortical processing area than the trunk or legs.

Recently, however, several studies suggest that the motor control of different parts of the body exerted by the motor cortex is not as systematically organized as the homunculus suggests. Although, it remains clear that the head, upper extremity, and lower extremity have sequential and largely separate representations, the representations of smaller body parts are widely distributed within these major regions. Consequently, the territory controlling one body part overlaps extensively with the territory controlling adjacent body parts. For example, the thumb representation in the motor cortex overlaps extensively with the territories controlling other fingers. Furthermore, the representation of different parts of the body in the motor cortex is not fixed. Repetitive stimulation of the thumb area for example, will produce movement of the thumb followed after a while by immobility or movement of the index finger and so on.

The principal cortical input to the motor cortex is the prefrontal cortex, located rostral to it, and the somatosensory cortex found in the postcentral gyrus. The motor cortex also receives fibers from the supplementary motor cortex and the premotor cortex. These two regions receive sensory information from the parietal and temporal lobes, and both send efferent axons to the motor cortex. The motor cortex also receives fibers from

the ventrolateral nucleus of the thalamus that convey information from the basal ganglia and cerebellum. The principal outputs of the motor cortex are the descending pathways to the spinal cord, the basal ganglia, and the brain stem nuclei that project to the cerebellum.

1.2.1.2. Descending motor pathways

Neurons in the motor cortex send axons to the brain stem and spinal cord. These descending motor pathways can be divided into two groups, the lateral group and the ventromedial group, named for their location in the spinal cord (see Table 1). The lateral group includes the corticospinal tract, the corticobulbar tract and the rubrospinal tract. The vestibulospinal, tectospinal, reticulospinal and the ventral corticospinal tracts comprise the ventromedial group. The names of these tracts are given according to the place where they originate and the place where they terminate. For example the corticospinal tract originates in pyramidal cells of layer 5 of the motor cortex and terminate in the spinal cord. Similarly, the rubrospinal tract originates in the red nucleus of the midbrain and descends to the spinal cord. The lateral group controls the movement of the extremities and is in charge in particular for essential rapid dexterous movements. The ventromedial group controls the proximal axial and girdle muscles involved in postural tone, balance, and orienting movements of the head and neck. Most of these tracts (except the ventral corticospinal and the reticulospinal tracts) cross over to the other side en route to the spinal cord and thus controlling muscles of the opposite (contralateral) side of the body with respect to their site of origin. Table 1 shows the descending pathways, their site of origin and termination, as well as their main function.

The corticospinal tract has the highest level of development in primates, especially in humans and is the most clinically important descending motor pathway. It

controls movement of the extremities, and lesions to its origin (the motor cortex) or along its course produce characteristic deficits in skilled movements.

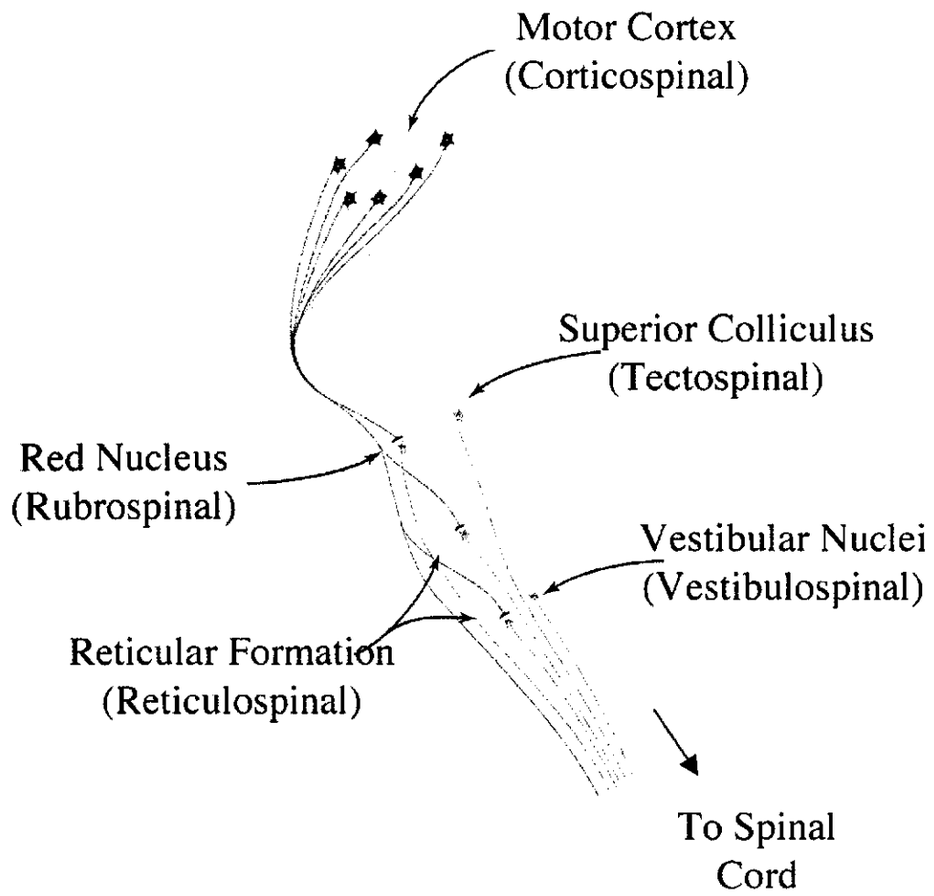

Figure 1.3. Main descending pathways projecting from the cortex or brain stem nuclei to the spinal cord. (Adapted from Squire et al., 2003).

Tract ***Lateral***	Origin	Termination	Function
Corticospinal	Finger, hand, and arm region of the motor cortex	Spinal cord	Movement of fingers, hands, and arms
Corticobulbar	Face region of the motor cortex	Cranial nerves: 5, 7, 9, 10-12	Control of muscles of the face
Rubrospinal	Red Nucleus (Midbrain)	Spinal cord	Hands, lower arms, feet, and lower legs
Ventromedial			
Ventral corticospinal	Trunk and upper leg region of the motor cortex	Spinal cord	Movement of trunk and upper legs
Vestibulospinal	Vestibular nuclei (Medulla)	Spinal cord	Control of postural movements
Tectospinal	Superior colliculus (Midbrain)	Spinal cord	Control of muscles in the neck and trunk
Reticulospinal	Reticular formation of the pons and medulla oblongata	Spinal cord	Flexor muscles of legs (medullar) and extensor muscles of legs (pontine)

Table 1.1 Major motor pathways.

1.2.1.3. The basal ganglia

Perhaps the best evidence of the role of the basal ganglia in motor control comes from clinical observations. Destruction of the basal ganglia by disease or injury produces severe motor impairments. Patients with basal ganglia lesions can have one of the following motor disorders: hyperkinetic (uncontrolled or/and exaggerated involuntary movements) like patients with Huntington's or Tourette's syndromes; or hypokinetic

(rigidity, slowness, and difficulty initiating movements) like those observed in patients with Parkinson's disease. These different kinds of symptoms suggest that a major function of the basal ganglia is to modulate movement. The motor nuclei of the basal ganglia include the caudate nucleus, putamen, globus pallidus, the subthalamic nucleus, and the substantia nigra. The caudate and putamen together are also called the striatum. The basal ganglia receives most of their input from all areas of the neocortex, primarily the motor cortex, and from the substantia nigra. The major outputs of the basal ganglia are to the motor cortex (via the thalamus), the substantia nigra, and the motor nuclei of the brain stem that contribute to the ventromedial pathways. The basal ganglia pathways that contribute to motor control are illustrated in Figure 1.4. There are two main pathways from input to output nuclei through the basal ganglia relevant for motor control. The direct pathway travels from the striatum directly to the internal portion of the globus pallidus (GPi) and is excitatory. The indirect pathway travels from the striatum to the external segment of the globus pallidus (GPe; this pathway is inhibitory), then to the subthalamic nucleus (also inhibitory) and finally reaching the Gpi (excitatory). The major role of the GPi is to project to the thalamus (anterior thalamic nucleus) and the thalamus projects to the motor cortex closing the loop. The ultimate effect of excitatory input from the motor cortex through the direct pathway (net effect is excitatory) will be to inhibit the Gpi, thus the thalamus is free to excite the cortex and thus elicit movements. Conversely, excitation of the indirect pathway will inhibit the thalamus resulting in inhibition of movement through connections back to the cortex.

1.2.1.4. The cerebellum

The cerebellum integrates inputs from many regions of the brain and spinal cord.

This information is used to coordinate ongoing movements, plan motor movement, and acquire and maintain motor skills. When the cerebellum in damaged, people's movements become jerky, erratic, and uncoordinated.

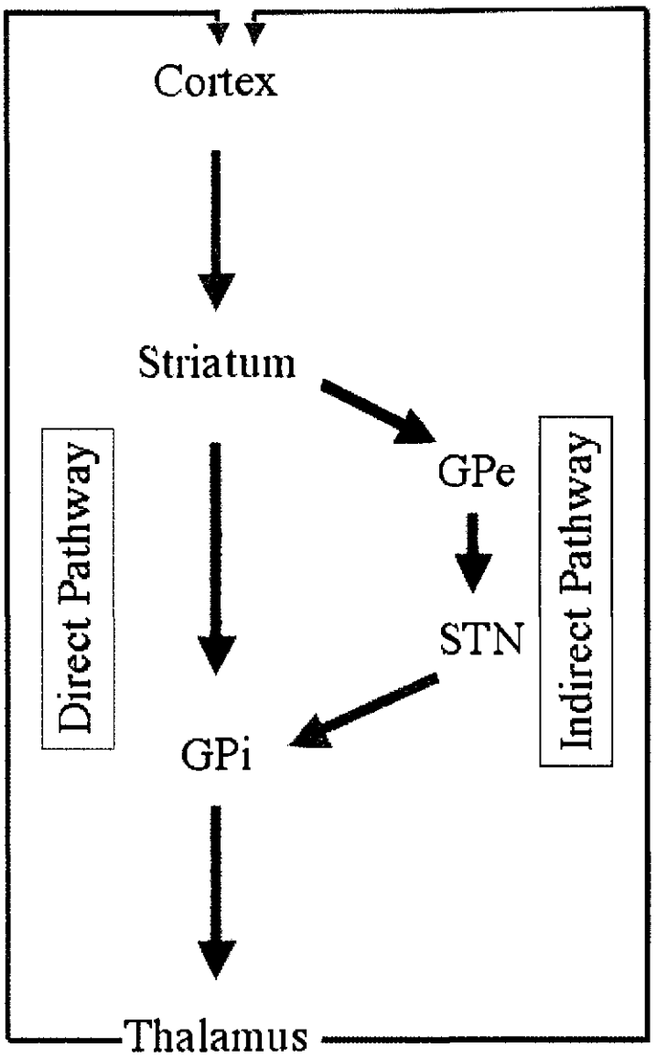

Figure 1.4. Direct and indirect pathways of the basal ganglia. Red arrows represent inhibitory pathways and green arrows represent excitatory pathways.

Although the projections from the thalamus to the cortex are excitatory, the net effect of the direct pathway is to inhibit the cortex and thus movement, and the ultimate effect of the indirect pathway is excitatory. (Adapted from Alexander and Crutcher, 1990).

Four types of neurons are found the cerebellum: granule cells; Purkinje cells; and two types of inhibitory interneurones, the Golgi cells and the stellate/basket cells (for review see Voogd and Glickstein, 1998). The cerebellar cortex can be divided into three layers: the granular layer constitutes the deepest layer, the Purkinje layer that corresponds to the middle layer and the molecular layer that comprise the superficial layer. The granule layer contains the granule and Golgi neurons. The Purkinje layer contains Purkinje cells and the molecular layer consists of stellate and basket cells.

The cerebellar cortex receives extra cerebellar afferents from the mossy fibers, which synapse with terminal axons of many tracts including the pinocerebellar, pontocerebellar and vestibulocerebellar, and the climbing fibers, which contain axons from the olivocerebella and reticulocerebellar neurons.

The cerebellum can be divided into two hemispheres that contain several nuclei, each specializing in a different aspect of motor control. These nuclei can be divided into three functional regions (see Figure 1.5), from medial to lateral, based on their input and output connections (Blumenfeld, 2002): 1) the vermis and flocculonodular lobes control proximal and trunk muscles, and vestibulo-ocular control, respectively; 2) the medial part of the cerebellar hemisphere is involved in the control of distal muscles in the arms and legs; and, 3) the lateral part, the largest cerebellar region, it is involved in planning the

motor program for moving the extremities. The lateral part is devoted to achieving precision in the control of rapid limb movements and in tasks that require fine dexterity.

Based on fiber connectivity, however, the cerebellum can be divided in three functional subdivisions (Afifi and Bergman, 1998): 1) the vestibulocerebellum (corresponding with the flocculonodular lobe) has reciprocal connections with vestibular and reticular nuclei and plays a role in control of body equilibrium and eye movement; 2) the spinocerebellum (corresponds to the anterior lobe) has reciprocal connections with the spinal cord and plays a role in control of muscle tone as well as axial and limb movements; and, 3) the cerebrocerebellum (corresponds to the posterior lobe) has reciprocal connections with the cerebral cortex and plays a role in planning and initiating movement, as well as the regulation of discrete limb movements.

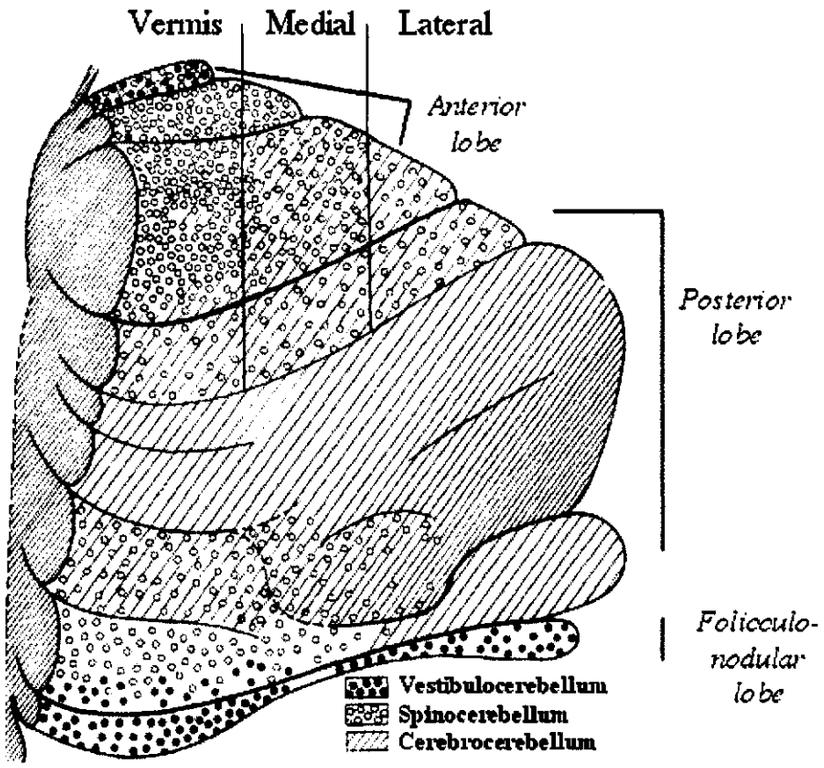

Figure 1.5. Schematic representation of the cerebellum, its major divisions, lobes and functional subdivisions. (Adapted from Squire et al., 2003).

Figure 1.6 illustrates the main pathways from the cerebral cortex and the cerebellum. The cerebral cortex communicates with the cerebellum through the following pathways: 1) corticoolivocerebellar via the red nucleus and inferior olivary nucleus; 2) corticopontocerebellar via the pontine nuclei; and 3) corticoreticulocerebellar via the reticular nuclei of the brain stem.

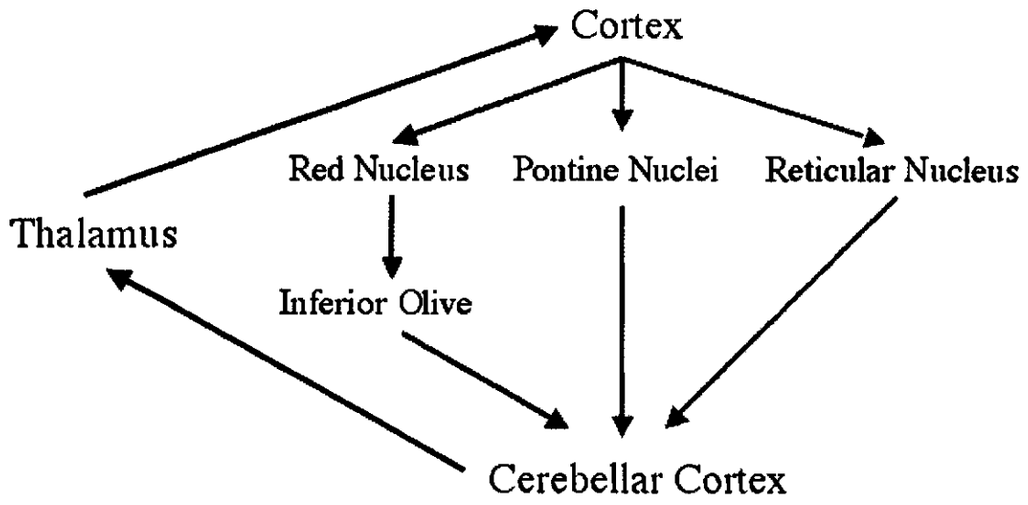

Figure 1.6. Main cerebro-cerebellar pathways. The corticopontocerebellar pathway is the main pathway and is shown in red, the cortticoolivocerebellar pathway is in blue, and the corticoreticulocerebellar is shown in green. (Adapted from Afifi and Bergman, 1998).

Summary

The cortical control of movement could be summarized on Figure 1.7. The motor cortex controls movement in part by direct corticospinal neurons but also by projections to different nuclei in the brain stem that in turn project to the spinal cord. Additionally, the motor cortex projects directly to the basal ganglia and cerebellum, which in turn project back to the cortex via the thalamus. Movement, thus, is a complex, orchestrated interaction of many different structures and parallel pathways.

After a stroke any of these structures and pathways can be damaged and the lesion inevitable will yield to alterations in motor output. Although, after stroke the size and

location of the lesion will constitute the best predictors of the degree of recovery that a patient could reach, different levels of reorganization within these circuits is possible and contribute to functional recovery. The following section will discuss evidence of brain reorganization after stroke.

1.3. Brain plasticity and recovery of function after injury to the motor cortex

The brain has a remarkable ability to reorganize itself throughout the human life span. An illustrative example is the remodeling of the cerebral cortex after it has been injured. It is quite common for stroke patients who have lost the ability to speak or to move a limb, to recover over time and to regain some (in some cases even full) function of the lost capacity. It is this ability of the brain to reorganize after injury or experience that is commonly referred as brain plasticity.

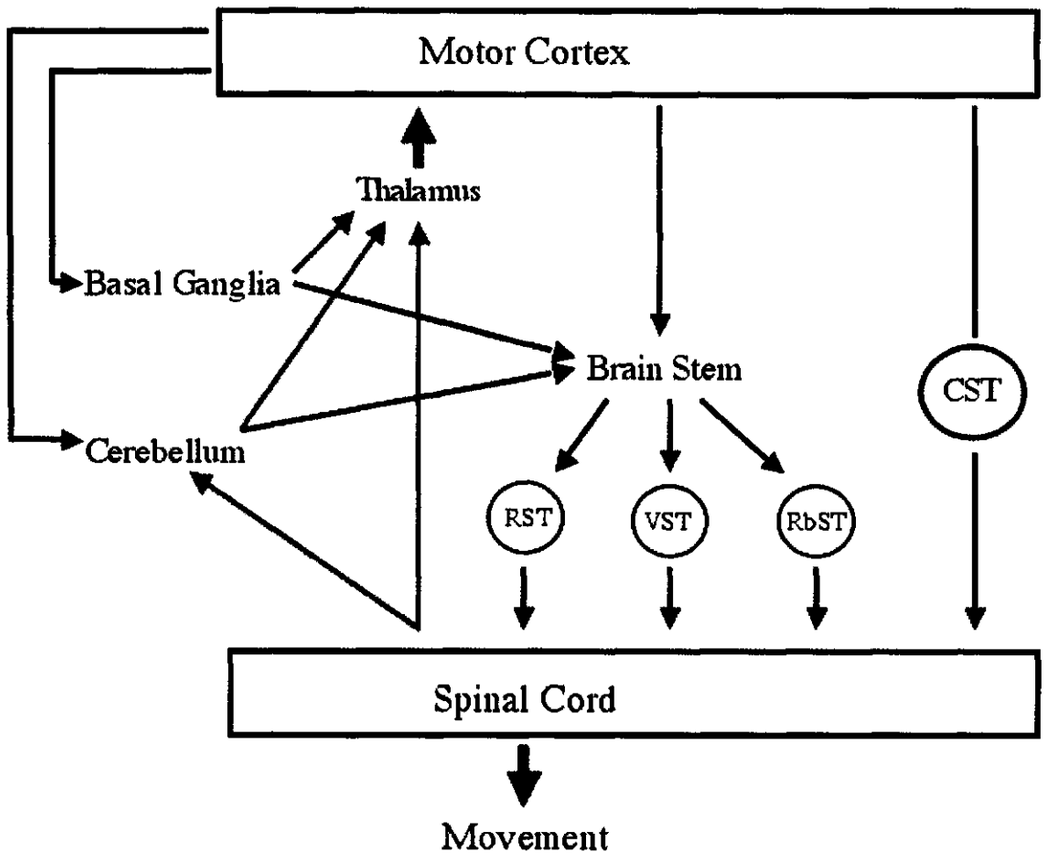

Figure 1.7. Schematic organization of the motor system. The different major components and main pathways are shown. The major descending pathways are shown in black, the feedback inputs to the motor cortex are shown in red. (Adapted from Squire et al., 2003).

1.3.1. *Brevia (from the Latin "brevis" –Short)* on the concept of brain plasticity

The word plasticity comes from the Greek *"plastikos"* that signifies "to mould". It was probably first introduced in the context of psychology by William James in the late 1800's. In 1887 James wrote: "*Plasticity,* then, in the wide sense of the word, means the

possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once...Organic matter, especially nervous tissue, seems endowed with a very extraordinary degree of plasticity of this sort" (James, 1983). In 1906 Ernesto Lugaro introduced the term into the neurosciences. He stated that throughout life the anatomo-functional relations between neurons can change in an adaptive fashion to enable psychic maturation, learning, and even functional recovery after brain damage (Berlucchi, 2002).

Presently, the terms brain plasticity, neural plasticity, neuroplasticity or simply plasticity are usually used to refer to the ability of the nervous system to undergo adaptive functional and morphological modifications in response to internal and/or external environmental changes (Liu and Wang, 2001). It is this context that the word plasticity will be used from here.

1.3.2. Plasticity in the injured brain

There are a limited number of possible ways the brain can recuperate after it has been injured. It can either reorganize existing circuitry, it can create new circuitry, or it can regenerate new tissue to replace some of the lost tissue. Currently, there is evidence that these three mechanisms can take place in the brain after cortical injury. I will briefly describe them with emphasis on recovery of motor function.

1.3.2.1. Reorganization of existing circuits

After the brain is injured by stroke or similar insult, the organization of the cerebral cortex changes because of neuronal loss, and the disruption of networks in undamaged brain areas reciprocally connected to the injured region (Nudo et al., 2000). Motor recovery is possible because much of the motor system will remain intact after the injury and these regions display some plasticity in their organization. Thus, as mentioned

in previous sections, there is great deal of overlap between representation for different body parts within the motor cortex and there are parallel descending pathways from the brain stem. These two features of the motor system allow for some compensation after brain injury. Within the forebrain, at least in principle, reorganization after cortical injury can take place either in intact regions of the damaged hemisphere and/or in the undamaged hemisphere.

Lesions to the frontal cortex in rats produce severe initial deficits in both cognitive and motor tasks. With time however, there is some amelioration of the initial deficits although the recovery is incomplete (for a review see Kolb, 1995). Analyses of dendritic morphology after the lesions reveal initial atrophy on the dendritic field of cortical pyramidal cells in sensorimotor cortex followed by sprouting, such that there is a net gain in dendritic space (Kolb and Gibb, 1991a; Kolb, 1995). These changes are correlated with recovery of performance on cognitive tasks. Animals tested in a spatial navigation task early after the lesions (during the time of atrophy) are devastated in their performance on the task. Animals tested later on (during the time of dendritic expansion) display substantial recovery. Similar results are found with lesions to the motor cortex. After lesions to the motor cortex in rats the extent of functional recovery varies directly with the size of the lesion (Whishaw, 2000). Lesions restricted to the forelimb region of the motor cortex cause animals to show initial impairments in reaching performance but substantial recovery is observed over time. Morphologically, there is initial atrophy of dendritic fields of pyramidal neurons in the remaining motor cortex, followed by expansion of the dendritic fields (Kolb et al., 2000).

Studies on the intact hemisphere after lesions to the sensorimotor cortex have shown similar results. For example, Jones (1992, 1994, 1999) and colleagues have reported dendritic overgrowth in Layer V cells of the forelimb area contralateral to the lesion. These studies have shown that early after the lesion there is a period of dendritic hypertrophy (peaking at day 18) followed by a period of pruning (around day 30 after the lesion) without obvious further changes. At this time point, the volume of dendritic processes is increased with respect to control animals. The dendritic overgrowth is closely related to the time of over-reliance on the unimpaired forelimb whereas the subsequent dendritic pruning is related to a return of more symmetric use of the forelimbs (Jones, 1994). Taken together, there is considerable anatomical evidence of cortical reorganization in both the injured and uninjured hemisphere and functional recovery following lesions to the motor cortex.

At the functional level, evidence supporting the idea that adjacent cortical areas take over some of the functions of the lost tissue comes from both brain stimulation and functional mapping studies. In 1950, Glees and Coles conducted an experiment in monkeys in which they determined the thumb representation of the motor cortex by surface stimulation before producing a lesion to this area. Days later they noted that stimulation to the same area resulted in no thumb response but if the stimulation occurred in an adjacent area (hand area) the thumb would move. This was perhaps one of the first studies to show that other cortical area adjacent from the injury site could take over the function of the damaged one. This phenomenon is often referred as vicariation of function (Xerri et al., 1998) and has gained considerable support from recent studies. Lesions to the primary somatosensory cortex (Xerri et al., 1998) or the primary motor

cortex (Nudo and Milliken, 1996) in monkeys produce severe initial deficits in sensorimotor skills. Over weeks, however, there is substantial recovery and this recovery is accompanied by the re-emergence of the injured representation. This reorganization is highly influenced by experience (see section bellow).

Studies of brain reorganization after stroke have been conducted in humans by making use of neuroimaging techniques like positron emission tomography (PET) and functional MRI (fMRI). For example, during performance of voluntary finger movements by patients recovered from stroke, extensive reorganization of the motor system, such as enhanced bilateral activation of motor pathways and recruitment of additional sensory and motor structures not normally involved directly with motor function (Cramer et al., 1997, 2000; Nelles et al., 1999) has been observed. More specifically, Pineiro et al., (2001) showed posterior shifts (postcentral gyrus) in the location of motor cortical activity after stroke. Similarly, Seitz et al., (1998) showed that after recovery from stroke movement of the previously affected hand did not activate primary motor cortex but rather activated the dorsolateral premotor cortex and somatosensory cortex bilaterally. Finally, other investigators have found increased activation in prefrontal cortex, posterior parietal and cingulate areas in stroke patients during performance of manual tasks with the affected arm (for a review see Rossini and del Forno, 2004).

After recovery from stroke, imaging studies have revealed persistent activation of structures (primarily sensorimotor areas) in the undamaged hemisphere during movement of the recovered hand. Because this activation often occurs in patients that show favorable recovery, it has been hypothesized that recovery/compensation is mediated to some degree by the undamaged hemisphere (Cao et al., 1998; Nelles et al., 1999;

Caramia et al., 2000). In a study of the role of the ipsilateral hemisphere after stroke (Johansen-Berg et al., 2002) transcranial magnetic stimulation (TMS) was used to interfere transiently with processes in the undamaged primary motor or dorsal premotor cortex (PMd) during finger movements of stroke patients. It was found that the stimulation produced different degrees of disruption of the finger movements. Patients with more severe deficits were more affected by the stimulation, suggesting increased activation of the undamaged hemispheres in patients with greater motor impairments.

Finally, a recent report suggests that there is still much to be known about recovery of function and its relationship with brain reorganization because activation of other cortical areas after stroke does not always predicts a positive outcome. Ward and colleagues (Ward et al., 2003) showed that patients with poor recovery were more likely to recruit a number of motor-related brain regions over and above those seen in the control group during the motor task, whereas patients with more complete recovery were more likely to have 'normal' task-related brain activation. This finding demonstrates a negative correlation between outcome and the degree of task-related activation in regions such as the supplementary motor area, cingulate motor areas, premotor cortex, posterior parietal cortex, and cerebellum.

1.3.2.2. Generation of new circuits by intervention

Three different types of therapies have proven beneficial in generating reorganized neural circuits and correlated functional improvement: 1) behavioral therapies, 2) growth factors, and, 3) psychomotor stimulants.

Behavioral Therapies. The relevance of rehabilitative therapies in promoting behavioral recovery after brain damage was addressed as early as 1917. Ogden and Franz

(1917) described almost complete recovery of function after motor cortex lesions in a monkey that was forced to use its affected arm. Since then, there is evidence that administration of behavioral therapies is successful in stimulating cortical reorganization and enhancing behavioral recovery after damage to the cerebral cortex. Taub and his colleagues, for example, furthered the observation of Ogden and Franz and showed that by immobilizing the good arm of patients that had suffered a unilateral stroke and thus increasing the use of the affected limb, significant functional improvement in the impaired arm could be observed (Taub et al., 1993, Liepert et al., 1998, Miltner et al., 1999). The authors hypothesized that this form of therapy (known as constraint-induced movement therapy; CIMT) might work by enhancing cortical plasticity and enlarging the motor representation of the affected limb in remaining cortical tissue. Liepert et al., (1998; 2000) have shown just this: using transcranial magnetic stimulation (TMS) to map the cortical motor output area of the hand of stroke patients before and after CIMT, they found cortical reorganization and functional improvements in stroke patients. There was up to a 50% increase in the map size of the limb representation after 12 days of CIMT. Nudo and his colleagues (1996) have reported similar findings in non-humans primates. They induced an ischemic infarct of the cortical area that controls movement of the hand and then some animals received CIMT of the good limb plus reaching training of the affected limb. Monkeys that did not receive the treatments showed poor outcome (use of the digits) and a reduction of the hand's cortical representation. In contrast, rehabilitation resulted in both improved motor functioning and cortical reorganization.

There is also evidence that rehabilitative training without CIMT can enhance motor recovery and cortical plasticity after stroke in rodents. Placing animals in enriched

environments has been shown to be beneficial in promoting dendritic branching after unilateral or bilateral frontal cortex damage (Kolb and Gibb, 1991b). More recently, Biernaskie and Corbett (2001) and Johansson and Belichenko (2002) have shown similar results after experimental stroke in rodents. In sum, housing animals in complex and stimulating environments after the brain injury, results in functional recovery and enhanced dendritic complexity.

Growth Factor Therapies. Growth factors are proteins that can stimulate cells to grow and/or divide. They can also function to promote synaptogenesis (creation of new synapses). Growth factors such as nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) have been tested in experimental models of stroke. In one of these experiments, Kolb et al., (1997) produced large unilateral lesions to the sensorimotor cortex in rats and then some received intraventricular infusions of NGF. The authors found that rats that received NGF showed reduced behavioral deficits and enhanced dendritic length and spine density. Similarly, Kawamata et al., (1997) showed that intracisternal injections of basic fibroblast growth factor (bFGF) also enhanced functional recovery after experimental stroke in rats. This beneficial effect may have been mediated by increases in the expression of GAP-43 (a protein found in the growth cone of sprouting neurons) that was found in intact cortical areas.

Psychomotor Stimulants. An early study by Feeney and colleagues (1982) found that a single dose of d-amphetamine 24 hours following lesions to the sensorimotor cortex in rats resulted in enhancement of motor functions. Subsequent experiments in rats and cats showed similar beneficial effects of d-amphetamine following lesions to the frontal, motor, and occipital cortex (Hovda and Feeney, 1984; Sutton et al., 1989; Feeney

and Hovda, 1989; Goldstein and Davis, 1990; Dietrich et al., 1990). The issue of using amphetamine as a therapy after stroke in humans, however, remains controversial. Clinical trials in stroke patients have reported contradictory results (for a review see Long and Young, 2003). Successful trials in patients suggest that the rate of recovery is contingent on the administration of amphetamine in combination with rehabilitative therapy.

Although, it has not been tested in animal models of stroke, nicotine is another psychomotor stimulant that has proven beneficial after brain damage. Brown et al., (2000, 2001) has shown that chronic administration of nicotine enhances cognitive performance after lesion to the frontal cortex or hippocampus.

1.3.2.3. Generation of new cells to replace the lost ones

In principle, regeneration of neural tissue can be achieved by transplantation of fetal tissue or stem cells, or by stimulating intrinsic production of stem cells that would migrate and replace cells that have died. Some of the initial experiments looking at grafts of embryonic tissue and its effect on recovery of function go back to the 70's and 80's. In 1979, Perlow showed that dopamine-rich brain grafts reduced motor abnormalities produced by destruction of nigrostriatal dopamine system. Concurrently, Bjorklund and his colleagues showed that transplantation of embryonic dopaminergic cells into the rat's striatum improved functional deficits in a rat model of Parkinson's disease (Bjorklund et al., 1980; Dunnett el al., 1981). Since then, lessons from animal models have led to human transplant trials for patients suffering from Parkinson's and Huntington's disease with moderate success (Freed et al., 2003).

In the treatment of stroke, there have been experimental and clinical tests of several types of donor cells leading to different functional outcomes (for a review see Savitz et al., 2002). Bone marrow stromal cells, umbilical cord blood cells, neural progenitor cells and immortalized cell lines have all been tested on different models of focal and global ischemia. There have been some promising results arising from this research although we still do not know the mechanisms by which the cells might improve function. Furthermore, the location and severity of the injury, the site of implantation, and the age of the patient are likely to influence the effectiveness of the cells.

Finally, multiple efforts have been recently placed on promoting intrinsic mobilization of stem cells to damaged cortical or striatal tissue. In the adult mammalian brain, a population of neural stem cells exists in the lateral ventricle subependyma. These cells migrate to the olfactory bulb and differentiate into new neurons (Reynolds and Weiss 1992; Lois and Alvarez-Buylla, 1994). The idea is to activate these cells after stroke to initiate migration to the site of the lesion, and to induce the cells to differentiate into neurons, that can influence behavior. Although a challenge remains in understanding the mechanism of growth and differentiation of transplanted cells or intrinsic mobilization of stem cells and how they interact with the host tissue, these forms of therapy represent an exciting promise in the study of brain repair.

1.4 Organization and rationale of the thesis studies

The experiments in this thesis were designed with two purposes in mind: 1) to gain general knowledge about the behavioral and anatomical outcomes after cortical lesions of different etiologies, and, 2) to test the effectiveness of behavioral,

pharmacological and regenerative therapeutic approaches to treating stroke. Five experiments were conducted and form the major part of the thesis. A sixth experiment that spun out of these experiments but was only conducted on control animals is included as an appendix.

Experiment 1: *A Comparison of Different Models of Stroke on Behavior and Brain Morphology*. This experiment was designed to address the question of whether or not different methods used to induce motor cortex damage have similar effects on behavioral recovery and brain plasticity. The behavioral and morphological effects of lesions produced by devascularization, aspiration, and two different kinds of MCA occlusions were evaluated. The results suggested that 1) the behavioral deficits observed after large MCA occlusion, devascularization, or aspiration of the motor cortex are remarkably similar; and 2) the effects of the lesions on cortical and striatal morphology are different across the different lesion models.

Experiment 2: *Evidence for Bilateral Control of Skilled Movements: Enduring Ipsilateral Skilled Forelimb Reaching Deficits in Rats Follow Motor Cortex and Lateral Frontal Cortex Lesions*. The purpose of this study was to make a systematic examination of the motor skills of the ipsilateral forelimb after two different kinds of focal stroke. The study found enduring deficits in both quantitative and qualitative measures of skilled reaching. This demonstration that cortical injury can impair ipsilateral-to-lesion limb skilled movements suggested that these movements normally involve some degree of bilateral cortical control.

Experiment 3: *Chronic Low-Dose Administration of Nicotine Facilitates Recovery and Synaptic Change after Focal Ischemia in Rats.* This experiment tested the hypothesis that the plastic changes known to occur after administration of nicotine in normal rats also would take place in injured animals and it would enhance recovery of function. Lesion animals treated with nicotine showed enhanced behavioral recovery in a time dependent manner, and an increase in dendritic length and branching in pyramidal cells of the forelimb area contralateral to the lesion and in cingulate cortex ipsilateral to the lesion.

Experiment 4: *Olfactory Stimulation Promotes Behavioral Recovery From Motor Cortex Injury in Adult Rats.* Different types of behavioral stimulation (e.g. complex housing, motor training) have been successful in promoting behavioral recovery after cortical damage. Here we wondered if other kinds of sensory stimulation would enhance outcome after stroke. Animals received olfactory stimulation for three weeks after the insult and were tested in two motor tasks. The results showed that olfactory stimulation can improve behavioral performance in adult animals after damage to the motor cortex.

Experiment 5: *Growth Factor-Induced Regeneration of Neural Tissue Following Cortical Lesions.* We tested the hypothesis that stimulating proliferation and differentiation of intrinsic forebrain neural stem cells with epidermal growth factor (EGF) and erythropeitin (EPO), respectively, will enhance functional recovery after stroke damage to the cerebral cortex. The results showed that EGF and EPO could stimulate the

production and differentiation of new cells in the cortically-injured brain. Behavioral results demonstrated that combined administration of EGF + EPO enhanced behavioral recovery following focal ischemia and suggested that newly generated neurons that migrate to the lesion site contributed to this functional improvement.

Appendix 1: *Nicotine Stimulates Dendritic Arborization in Motor Cortex And Improves Concurrent Motor Skill But Impairs Subsequent Motor Learning*. This experiment shows that enhancing brain plasticity is not always advantageous with respect to functional outcome. Administration of nicotine in intact animals enhanced the performance of a previously acquired motor task. When rats were given a new motor task however, they were unable to acquire it even after extensive training. The nicotine treated rats failed to show a training-related change in dendritic morphology in motor cortex. They did, however, show large changes in dendritic morphology related to the nicotine treatment. The results suggest that there may be limits to cortical plasticity and that drug-induced changes in cell morphology may interfere with other forms of experience-dependent plasticity.

1.5 Behavioral and anatomical assessments

Several different behavioral tasks sensitive to motor disturbance were given to the animals in the experiments described in this thesis. Not all of them were given for each experiment but a brief description of all of them is summarized in the following section. Measures of brain plasticity are listed in at the end of the behavioral tasks.

1.5.1. Behavioral Tasks

1.5.1.2. Tray reaching task. Whishaw and his colleagues (e.g., Whishaw et al., 1991; Whishaw et al., 1986;) have developed a procedure to assess the ability of rats to use their forepaws to retrieve food. A rat is trained to reach through metal bars to retrieve chicken feed from a tray at the front of the cage (Figure 1.8). This test is specific for motor skills and performance is measured by the success of the animal to retrieve and ultimately consume food.

1.5.1.3. Single Pellet Reaching Task. Whishaw and his colleagues also developed this task. Animals learn to use a forepaw to reach through a slot in a cage for single food pellets located on an external shelf (Whishaw and Pellis, 1990) (Figure 1.9). Following a reach, a short pause precedes the presentation of the next pellet. This procedure encourages animals to move away from the slot after each reach, a procedure that forces them to reposition themselves relative to the reaching slot to prepare for the next reach. This is important because one of the purposes of the task is to study the separate components of forelimb reaching movements.

Figure 1.8. The Tray Reaching Task (Courtesy of I. Whishaw)

This analysis provides additional insight into the organization of skilled movements and into the nature of the deficits (in addition to endpoint measures of reaching success) following perturbation of the motor system.

1.5.1.4. Forepaw Asymmetry. Also called "the cylinder task", this test was developed by Schallert et al., (1997). The task takes advantage of the spontaneous vertical exploration that rats usually display when placed in enclosed environments. For the test, rats are placed into a small circular container (Figure 1.10) and their forepaw contacts on the vertical surface are counted as they rear over a 3–5 min interval. Asymmetries in forepaw contact (forepaw placing) can provide an index of lateralized brain injury. Animals with motor cortex damage for example, use their unimpaired forelimb more when supporting their weight around the cylinder walls.

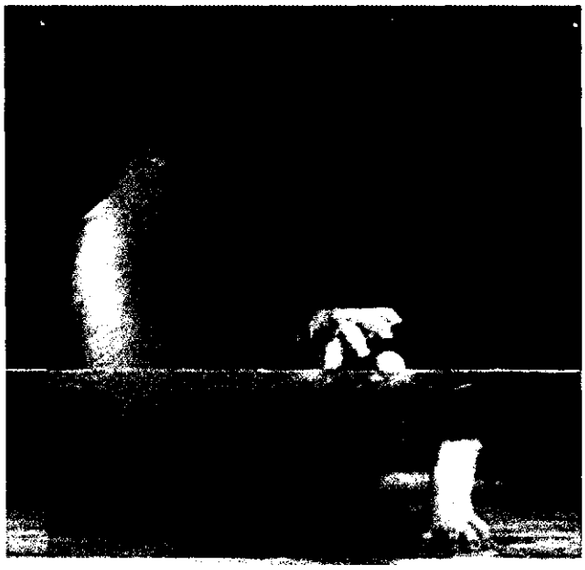

Figure 1.9. The Single Pellet Reaching Task

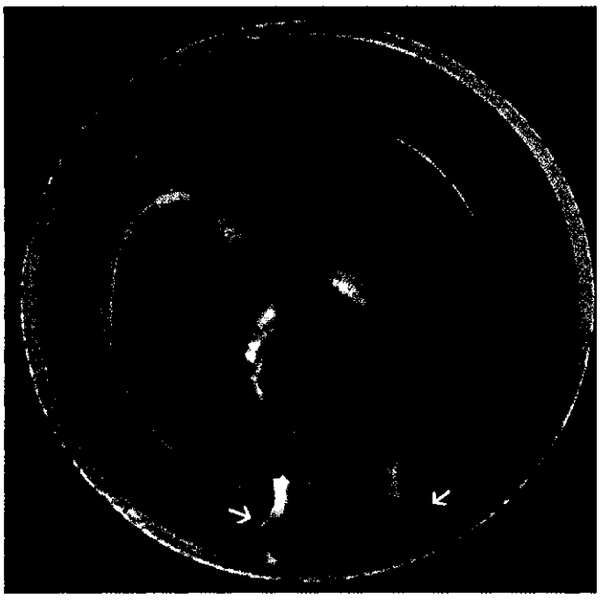

Figure 1.10. The Cylinder Task (Courtesy of O. Gharbawie)

1.5.1.5. Forepaw Inhibition. Kolb and Whishaw (1981) first described that during swimming, rats usually inhibit their forepaws and propel with their hind limbs (Figure 1.11). Schallert later described in detail this task in evaluating animals with cortical damage (Stoltz et al., 1999). Rats are usually placed in a water tank and their job is to swim to a visible platform located at the end of the aquarium. Intact rats normally hold their forelimbs immobile under their chins while swimming. Cortical injury disrupts the normal swim pattern as these animals show a disinhibition of the forepaw contralateral to the lesion thus stroking during swimming.

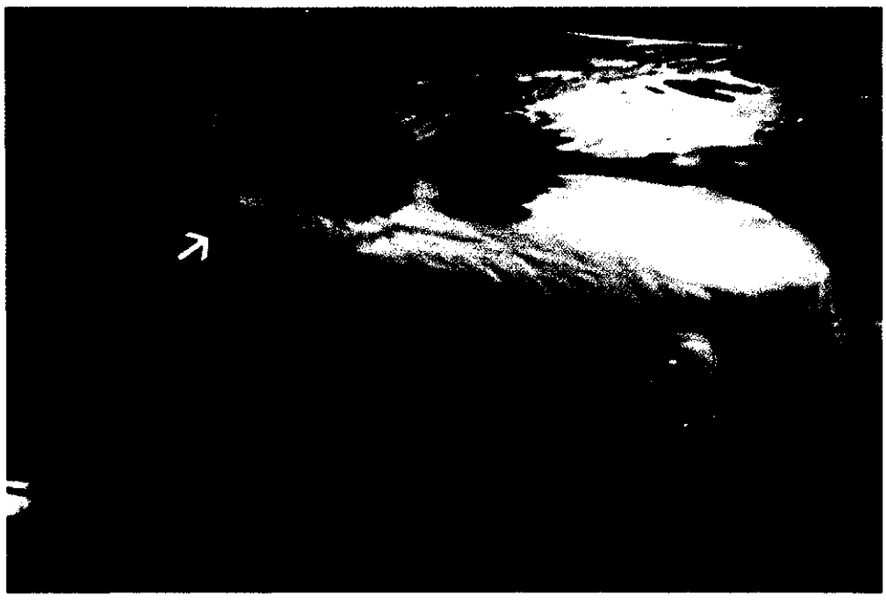

Figure 1.11. The Forepaw Inhibition Task

1.5.1.6. Sunflower seed consumption. The challenge to the rat in this task is to open and successfully consume five sunflower seeds (Figure 1.12). The total amount of time that the animal spent manipulating, opening and consuming the seeds can be recorded as well

as the number of pieces of shell that the animal had to break in order to have access to the seed. The rationale was that animals with cortical lesions would show increased time and increased number of pieces before final consumption of the seed.

Figure 1.12. The Sunflower Seed Task

1.5.1.7. Tongue extension. Motor cortex lesions have been shown to produce deficits in tongue protrusion (Whishaw and Kolb, 1983). The tongue extension test requires the animal to stick out its tongue to lick a palatable food that has been spread onto a ruler (Figure 1.13). Measures of tongue extension are usually made before and after the lesions.

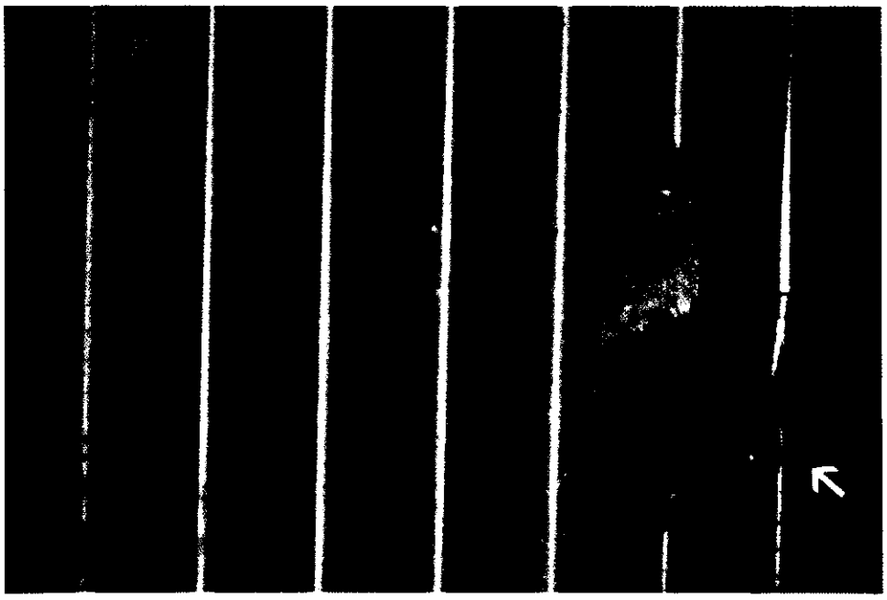

Figure 1.13. The Tongue Extension Test

1.5.2. Physiological and Anatomical Assessments

1.5.2.1. Golgi-Cox method. The Golgi technique was developed in 1873 by Camillo Golgi and was used extensively by early investigators (notably Ramon y Cajal) to define structural features of brain architecture (DeFelipe and Jones, 1988). A major advantage of the Golgi technique is that a small percentage of neurons (1-5%) are randomly stained and these neurons are stained completely (Figure 1.14). As a result it is possible to draw individual neurons and to quantify the amount of dendritic space available, as well as the location and density of dendritic spines.

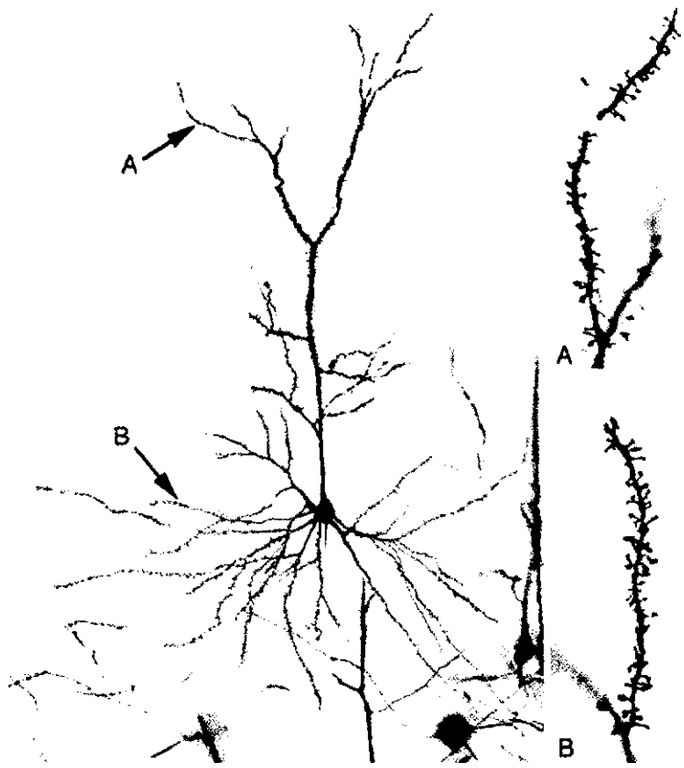

Figure 1.14 The Golgi-Cox Method (Courtesy of G. Gorny)

15.2.2. Intracortical microstimulation (ICMS) In this technique, movement representations are defined as the cortical loci from which movements about individual joints, or specific muscle groups can be evoked. These loci are identified by penetration of a microelectrode into the layer V of the motor cortex where the somata of the corticospinal cells reside. A small amount of electrical current is delivered in a brief train burst producing movements of specific body parts. Because ICMS results in the activation of relatively small groups of corticospinal cells, this approach allows detailed analyses of functional relationship between cortical loci and specific motoneuron pools (Keller, 1999). At each site of the stimulation, the movement evoked at the lowest

possible current level is defined and coded on a digital photograph of the surface vasculature. Sites representing broad categories of movements (e.g finger, wrist. elbow etc) are coded on the map (Nudo, 2003). The result is a mosaic map of the motor cortex with the representations of each muscle group (see Figure 1.15).

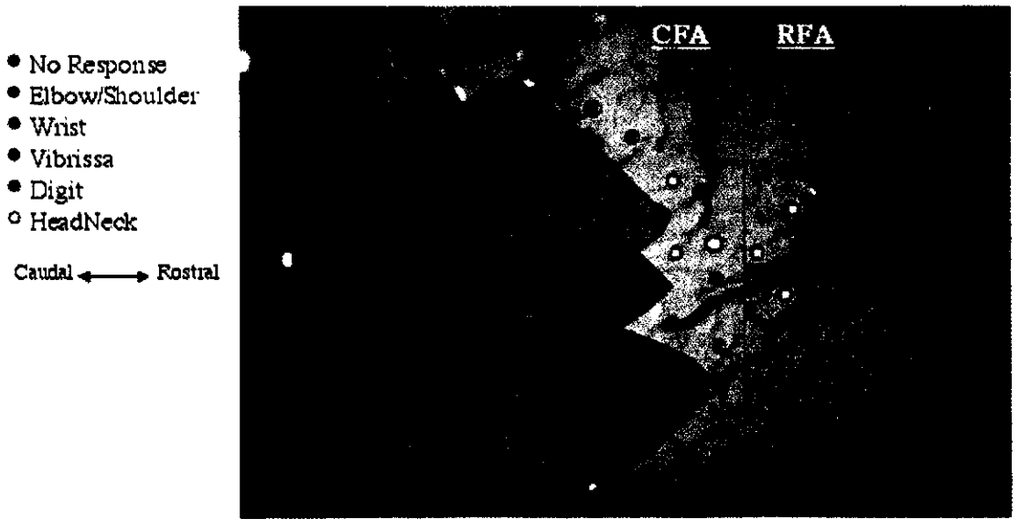

Figure 1.15. The Intracortical Microstimulation Technique (Courtesy of P. Williams)

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A Comparison of Different Models of Stroke on Behavior and Brain Morphology

2.1. Abstract

We compared three models of permanent ischemia plus animals that sustained cortical aspiration on behavior and brain morphology. Rats received a stroke either by devascularization, or by two different procedures of medial cerebral artery occlusion (MCAO; small vs. large). Animals were trained in a reaching task, forepaw asymmetry, forepaw inhibition, sunflower seed task, and tongue extension. Behavior was assessed one week after the lesion and every other week for a total of nine weeks. One week after the surgeries all animals were severely impaired on all tasks and although they improved over time, they only reached pre-operative baselines on tongue extension. Animals with small MCAO's performed better in reaching and sunflower tasks, no other behavioral differences were detected among the groups. Pyramidal cells in forelimb and cingulate areas as well as spiny neurons of the striatum were examined for dendritic branching and spine density using a Golgi-Cox procedure. Each lesion type had a different impact on cell morphology. Overall, different changes (atrophy or hypertrophy) were observed with each kind of lesion and these changes were specific for the region (forelimb, cingulate, striatum), and the condition (intact vs. damaged hemisphere). These results suggest that: 1) different lesions to the motor cortex produce subtle differences in behavior, and 2) the method used to induce the lesion produces striking differences in cortical and subcortical plasticity.

2.2 Introduction

The treatment of chronic behavioral loss following stroke is a major problem in clinical neuroscience. One way to develop new treatments is to use animal models, which have been developed principally in rodents, to mimic the pathology of stroke and to try to understand the basic mechanisms that might underlie functional improvement. There are a wide variety of models of stroke (for a review see Hossmann 1998), however, including models of global and focal ischemia (e.g., transient vessel occlusion), and focal infarction (e.g., permanent vessel occlusion). Behavioral studies of transient ischemia have tended to focus on hippocampal-dependent behaviors (and hippocampal cell loss) whereas studies of focal infarction have tended to be more focused on sensorimotor functions related to the middle cerebral artery's (MCAs) perfusion field. But even within the studies of MCA occlusion, many methods have been used to model the pathology including MCA occlusion by cauterization, MCA occlusion by clip, MCA occlusion by thread, endothelium-induced lesions, and pial stripping (see Hossmann, 1998). Oddly, there have been no studies comparing the behavioral and pathological outcomes across these different models (except Roof et al., 2001). There is reason to believe, however, that different models may not be equivalent in their post injury pathology. For example, ischemic brain damage induced by thermocoagulation of the motor cortex produces very different changes in the striatum than aspiration of equivalent areas. These changes include differences in axonal sprouting as well as the expression of molecules associated with neuronal plasticity such as GAP-43 and bFGF (Szele et al. 1995; Uryu et al. 2001). In another experiment, Voorhies and Jones (2002) compared the effects of electrolytic versus aspiration lesions of the motor cortex and found hypertrophy on pyramidal cells

layer V of the forelimb area opposite to the lesion after electrolytic but not aspiration lesions. Furthermore, interventions after stroke can have different effects depending on the method used to induce the lesion. Zeng et al. (2000), for example, have suggested that the host to graft connections are better when fetal neocortical tissue is transplanted into an infarct cavity than into an aspiration cavity.

In the current study, we elected to compare the effects on behavior of four different procedures that produce sensorimotor loss related to damage to the perfusion field of the MCA. These included groups of animals receiving one of two different MCA cauterization procedures (small and large), a group with devascularization of the sensorimotor cortex, and a group with cortical aspirations. The latter group was included to allow comparisons to a much larger literature looking at the effects of cortical aspirations of motor function and subsequent morphological changes. We hypothesized that animals with small MCA occlusions would generally perform better than animals with large MCA occlusions or animals with aspiration lesions. In addition to the behavioral measures we investigated the possibility that different procedures would have a different impact on cortical plasticity. In order to identify such changes, dendritic arbors of layer III pyramidal cells within anterior cingulate cortex and spiny neurons of the striatum in both the damaged and intact hemispheres were analyzed using a modified Golgi–Cox staining procedure. In addition, layer V pyramidal neurons within the forelimb motor cortex of the undamaged hemisphere were also analyzed.

2.3 Materials and Methods

Subjects

Subjects were 35 male Long-Evans hooded rats, 4 months old and weighing 300-400 g at the beginning of the experiment. Animals were raised in the University of Lethbridge vivarium and were housed in groups of four to five individuals in hanging wire mesh cages. The colony room was maintained on a 12:12h light/dark cycle (08:00-20:00 h) and the temperature regulated at 22 °C. Experiments were conducted according to standards set by the Canadian Council on Animal Care and approved by the University of Lethbridge animal care committee.

Surgery and lesion placement

Animals received lesions of the motor cortex contralateral to the preferred paw for reaching using four different procedures: 1) aspiration (n = 8); 2) devascularization (n = 8); 3) small MCAO (n=10); and large MCAO (n=9). All animals were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (5 mg/kg) and sustained the surgical procedures under a heating blanket to maintain normal body temperature (37.0 ± 0.5 °C). For the aspiration and devascularization groups, subjects were positioned in a stereotaxic operating apparatus and the lesions were produced according to procedures described elsewhere (Sofroniew et al. 1983; Stephens et al. 1985; Kolb et al. 1997). Coordinates for the aspiration and devascularization groups were based upon the extent of cortical injury in preliminary studies of rats with large MCAO lesions (Chen et al. 2002). For the aspiration group, a rectangular hole in the skull was produced at stereotaxic coordinates anterior (A): A= 2.0 to -6.0 mm and lateral (L): L= 1.0 to 5.5 mm using an electric dental drill and the exposed cortex was removed by aspiration. For the devascularization group, a flap of bone and underlying dura mater were removed at coordinates corresponding to the overlapping motor and sensory representation of the

forelimb, areas Fr2, Fr1, Fr3, FL and HL (Zilles, 1985). All vessels and pia mater were rubbed away with sterile, saline-soaked cotton swabs.

For the MCAO groups, procedures described by the Chen et al. (2002) were used. In brief, a vertical incision was made to expose scalp between the right orbit and external auditory canal. The temporal muscle was incised and scraped from the skull. A rectangular hole in the skull (3 mm anterior, 4 mm posterior, and 3 mm lateral to bregma) was made using an electric dental drill whilst avoiding traumatic brain injury. The opening was enlarged with rongeurs by removal of additional temporal bone to expose the medial cerebral artery (MCA) and olfactory tract. At this point the dura mater was carefully removed and either the base of the MCA (large MCAO) or just distal to the inferior cerebral vein (small MCAO) were permanently occluded by bipolar electrocoagulation (Howard Instruments Inc.). Animals were observed for 48 hours and then returned to the colony.

Food restriction

Subjects were maintained on a restricted food regime in which each animal obtained 20 g of food per day (normal daily consumption ranges from 18-25 g) but only an hour after the testing session was completed. Their body weight was maintained at about 95-98% until the completion of the behavioral testing.

Cylinder test (Forepaw Asymmetry)

Forelimb use for weight support during explorative activity was examined by placing the rats in a transparent cylinder 20 cm in diameter and 30 cm high for three minutes (Schallert, 1997). The animals were individually placed in the cylinder during the three minutes of each testing session. A mirror was placed underneath the cylinder at

an angle to allow the experimenter to videotape the animal's activity from a ventral view. The cylindrical shape encouraged vertical exploration of the walls with the forelimbs, but the walls were high enough so that animals could not reach the top. Forelimb use was measured during vertical exploration. Animals were placed and videotaped in the cylinder once before the lesions were made. Each forepaw contact with the cylinder wall was counted. When simultaneous limb contact was observed, a touch was counted for each paw. The asymmetry score of forelimb use in wall exploration was calculated for each group (i.e., affected forelimb/(affected + unaffected)) to obtain a score where 0.5 represents perfect symmetry and any number closer to zero would suggest a decrease in the use of the affected limb.

Reaching boxes and training

Training boxes were made of plexiglass with dimensions 26 cm high, 28 cm deep, and 19 cm wide. The front of the boxes was constructed of 2mm bars separated from each other by a 9 mm gap. Clear plexiglass tops allowed access to the inside of the box. A 4 cm wide and 0.5 cm deep tray was mounted in front of the bars. The tray contained food fragments weighing approximately 30 mg each. Animals had to reach between the bars, grasp the food and retract it where they were able to freely eat. Subjects were trained for a total of 15 days before the lesions to obtain a stable baseline before the surgeries.

Reaching success was calculated as follows: If the rat made a reaching movement (forepaw inserted through the bars, but no food was grasped or the food was dropped), the movement was scored as a "reach", whereas if the rat obtained the food and

consumed it, the movement was scored as a "hit". Success was calculated then as: Success percent = ("hit" / "reach") X 100.

Swimming Task (Forepaw Inhibition)

Rats were trained for two days prelesion to swim to a visible platform located at the end of a rectangular aquarium (120x43x50 cm). The water in the pool was maintained at 25°C at all times. During the training phase, animals were released from the opposite end of the tank and consecutive trials were given until they swam directly to the platform without touching the walls of the aquarium (about 10 trials for each animal). Kolb and Whishaw (1983) described that normal animals hold their forelimbs immobile under their chins while swimming using their hind limbs to propel through the water. By the end of the second day animals were quite familiar with the tank and the task therefore inhibition of the forepaws was observed. On the third day, three trials were videotaped for each animal as they swam directly to the platform.

Disruption to the normal swim pattern (swim score) was quantified by counting the number of strokes made by each forelimb on each trial. The swim score of forelimb inhibition was calculated for each group by subtracting the number of contralateral forelimb strokes minus the ipsilateral number of forelimb strokes (i.e., contralateral forelimb strokes (-) ipsilateral forelimb strokes).

Sunflower Seed Task

Evidence for limb and digit use during sunflower seed consumption among rodents has been reported elsewhere (Whishaw et al. 1998). Rats start by manipulating the seed into a preferred position before shelling. They then chew away a corner of the seed in order to facilitate splitting it longitudinally and finally the seed is split open

(usually in two pieces) with a bite (Whishaw et al. 1998). In the present study the purpose of the task was to challenge the animals to open and successfully consume five sunflower seeds. The total amount of time that the animal spent manipulating, opening and consuming the seeds were recorded as well as the number of pieces of shell that the animal had to breake in order to have access to the seed. Animals were trained for three consecutive days and on the fourth and fifth days the behavior was recorded and videotaped. For training and testing, animals were placed on a clear plexiglass box (50X50X50 cm) and 5 sunflower seeds were placed in one of the corners of the box. A mirror was placed underneath the box at an angle to allow the experimenter to videotape the animal's activity from a ventral view. The experimenter started timing the moment the animal touched the first seed and would stop the timer every time the animal was distracted. If on a given day, the animal spent 5 minutes or longer to retrieve the seeds, 300 sec was recorded and the animal was returned to its home cage. A random maximum of 30 pieces of shell was assigned if the animal shredded the shell in pieces too little to count.

Tongue Extension

Motor cortex lesions have been shown to produce deficits in tongue protrusion (Whishaw and Kolb, 1983). This test requires the animal to stick out its tongue to lick a palatable food that has been spread onto a ruler. Measures of tongue extension were made before the lesions (to have a baseline for each animal) and along with the rest of the tasks. Animals were placed in the boxes used for reaching but the food tray was removed. A slurry of warm water and chocolate chip cookie spread onto a ruler was positioned against the bars of the cages. After a couple of minutes animals engaged in licking and

the ruler was removed when the animal's tongue could not reach the slurry any longer. Measurements of tongue protrusion were made in millimeters.

Anatomical procedures

At the conclusion of the behavioral testing the brains of half of the animals of each group were processed either for Golgi-Cox staining or for Cresyl Violet staining. The brains of four intact controls matched in age, sex, and trained in reaching for three months were included in the Golgi-Cox analyses for comparison purposes.

Golgi-Cox analysis

Four rats from each group were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline. The brains were removed and weighed before being immersed in a 20 ml of Golgi-Cox solution where they remained for 14 days. The brains were then placed in a 30% sucrose solution for 2 days and cut on a vibratome at 200 μm and developed using a procedure described by Gibb and Kolb (1998). Ipsilateral and contralateral apical and basilar dendrites of layer III pyramidal cells in anterior cingulate cortex were traced using a camera lucida at 200X magnification. The basilar tree of layer V pyramidal cells within the forelimb motor cortex of the uninjured hemisphere, and cells on the dorsolateral striatum (both, ipsilateral and contralateral to the lesion) were also drawn. Measures of dendritic length and dendritic branching were obtained from those drawings. To be included in the study, the dendritic trees had to be well impregnated and in full view, unblocked by blood vessels, astrocytes or clustering of dendrites from other cells. They also had to appear intact and visible in the plane of section. Cell bodies of pyramidal neurons had to be located in area Cg3 (Zilles, 1985) or within the sensorimotor cortex (as defined by Zilles and Wree (1995). Spiny neurons in

the striatum were drawn as well from the remaining dorsolateral or mediodorsal striatum. For branch order analysis, each branch segment was counted and summarized according to methods of Coleman and Riesen (1968): branches emerging from either the cell body (basilar) or the primary apical dendrite (apical) were first order. After the first bifurcation, branches were considered second order, etc. Quantification of each branch type using this method provides an indication of dendritic arbor complexity. To obtain an indirect measure of dendritic length, the Sholl analysis (Sholl, 1956) of ring intersections was used. The number of intersections of dendrites with a series of concentric circles at 20 μm intervals from the center of the cell body was counted for each cell. A reflection of total dendritic length (in μm) can be determined by multiplying the number of intersections by 20. The mean of the measurements of five cells per hemisphere per rat was used for statistical analyses.

Spine density was measured on a terminal tip segment on the same brain areas. Spine-density measures were made from a segment 40-50 μm in length. The dendrite was traced at 1,000X 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.

Cresyl Violet staining

Subjects were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde solution. The brains were

removed, weighed and placed in a 30% sucrose-formalin solution for 3 days before being cut frozen at 40 μm. Every seventh section was saved and stained with Cresyl violet.

Infarct Measurements

Images of mounted Cresyl violet-stained and Golgi-Cox impregnated sections at standardized levels (8 different planes, (see Fig. 1) were captured digitally. The cross-sectional area of the neocortex and complete hemisphere were measured on both sides of the brain (NIH IMAGE software, Ver.1.62). Because of the different histological techniques, the data was expressed as percentage of the intact hemisphere.

Statistical analysis

Analyses of variance (ANOVA) were used for all measures and Fisher's LSD (P<0.05) was used for *post hoc* evaluations. In addition, a paired t-test was used for comparisons within groups between the baseline performance and the last week of testing after the lesions.

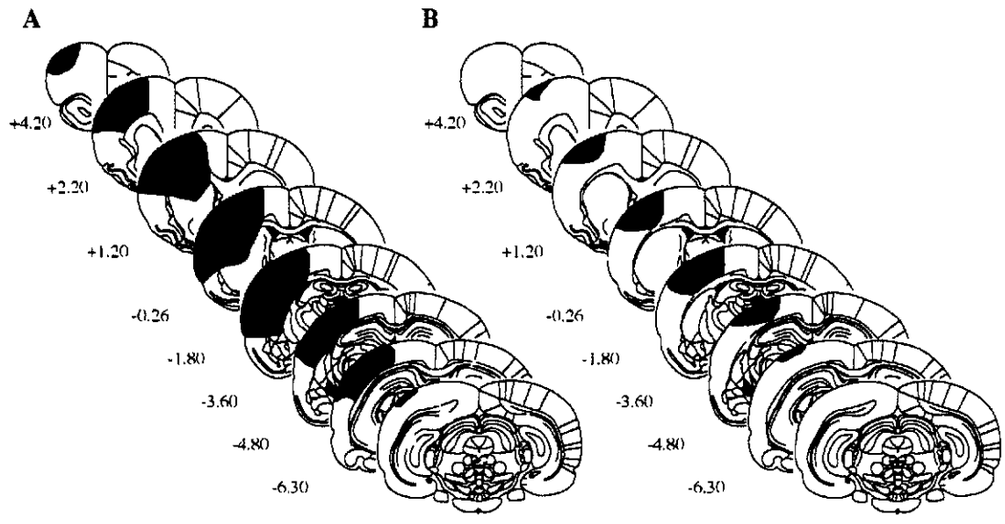

Figure 2.1. : Representative diagrams illustrating the eight different planes where

the measurements for estimating the infarct size were taken. (A) Illustrates a typical infarct after devascularization, aspiration or large MCAO. (B) Illustrates a typical infarct after a small MCAO.

2.4. Results

Behavioral Results

The performance of animals with aspiration, devascularization, small and large MCA occlusions were compared on cylinder test, reaching, swimming task, sunflower seed test and tongue extension using a repeated measures ANOVA. A measure of their performance on these tasks was taken before the lesions and a t-test was used to detect differences before and on week nine after the lesions.

Cylinder Test

All groups actively explored the cylinder and they reared and supported their body against the walls with their forelimbs. The asymmetry score of forelimb use was calculated for each group and it showed that all lesions produced an impairment. A repeated measures ANOVA showed no main effect of lesion group ($F(3,31) = 0.744$, $P = 0.534$), no main effect of test week, ($F(4,124) = 0.88$, $P = 0.478$), nor the interaction ($F(12,124) = 0.411$, $P = 0.957$) (Figure 2). A t-test revealed that by the end of the behavioral testing only the small MCAO group did not differ from preoperative base line: Aspiration ($P = 0.039$); Devasc ($P = 0.031$); MCAO-small ($P = 0.205$); MCAO-large ($P = 0.0062$).

Reaching

All animals quickly learn to reach for food and asymptote at about 65% accuracy before the lesions. On the first week after the surgeries, animals were devastated and their

performance dropped even to 0%. Over the course of the nine weeks post-injury rats acquired an alternate strategy to reach for food: they learned to reach with the non-

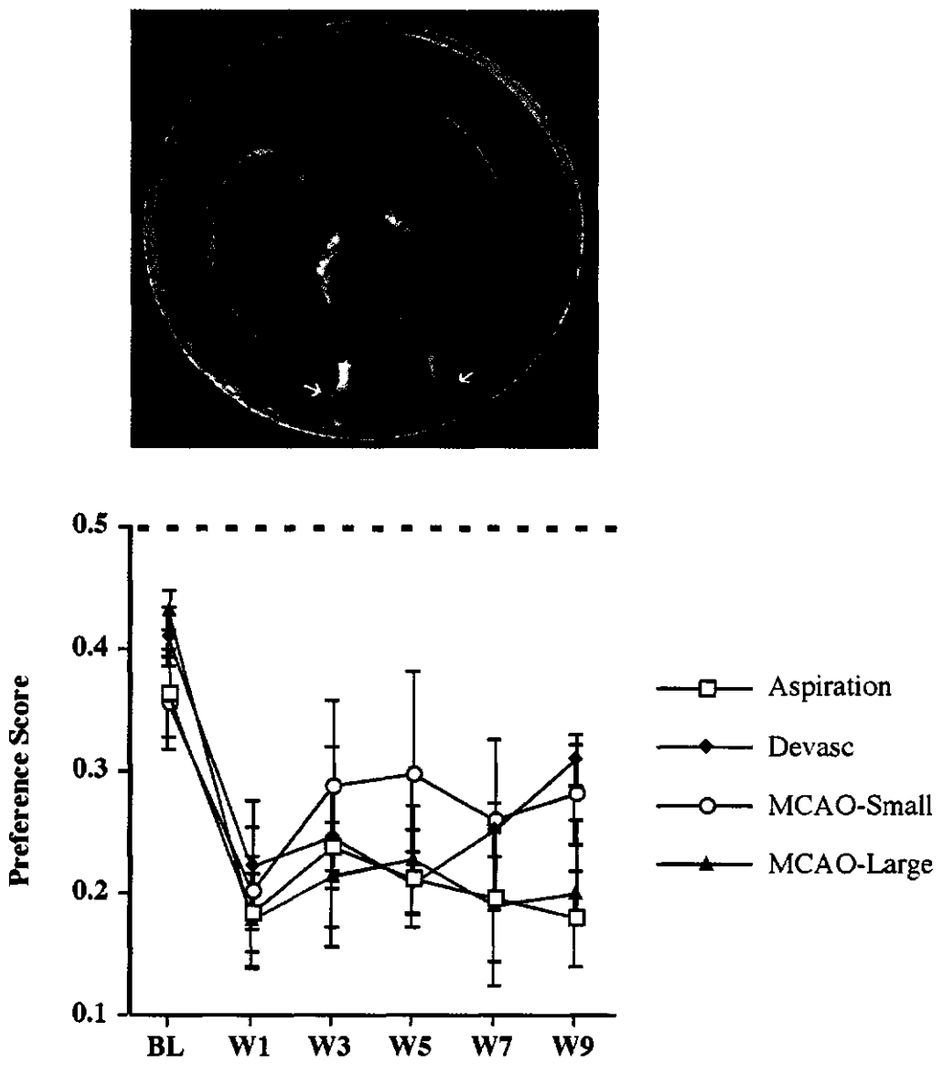

Figure 2.2: Top panel shows a picture of an animal in the cylinder before the surgeries. Arrows show use of both forearms for support during rearing. Bottom panel shows performance on this task before the lesion (BL) and every other week

for a total of nine weeks (W1, W3, W5, W7, W9) post-stroke (±SE). A preference score of 0.5 (dashed line) indicates no preference on the use of left or right forearms. Note than there were no differences among the groups and they were still impaired by the last week of testing with respect to their baseline.

preferred paw. This compensatory mechanism has been observed before in animals that sustained large cortical lesions. Although animals could be forced to use the impaired limb by putting a bracelet on the non-impaired limb (Whishaw, 2000) the purpose of the task in the present study was to detect whether or not any spontaneous recovery would occur. Figure 3 illustrates the animal's performance before and after the lesions while reaching with the preferred paw ("recovery") and reaching with the non-preferred paw ("compensation"). Animals in the small MCAO group showed the least impairment overall when compared to the other three groups. For "recovery", a repeated measures ANOVA comparing all groups showed a significant main effect of group ($F(3,31) = 15.97$, $P < 0.0001$), and week ($F(4,124) = 3.29$, $P < 0.02$), but not the interaction ($P = 0.58$). Follow-up tests (Fisher's LSD) showed that the small MCAO group differed from the other three groups ($P < 0.001$), which in turn did not differ among themselves. A t-test revealed that by the end of the behavioral testing, all groups differed from their preoperative base line performance, ($P < 0.0001$). For "compensation" a repeated measures ANOVA comparing all groups showed a marginal effect of group ($F(3,31) = 2.73$, $P < 0.06$), a significant effect of week ($F(4,124) = 3.45$, $P < 0.02$), but not the interaction ($P = 0.88$). Follow-up tests (Fisher's LSD) showed that the small MCAO used their "non preferred paw" significantly less than the devascularization and large MCAO

groups ($P < 0.05$), and marginally less from the aspiration group ($P = 0.08$). A t-test revealed that by the end of the behavioral testing, all groups differed from their preoperative baseline performance, ($P < 0.0001$).

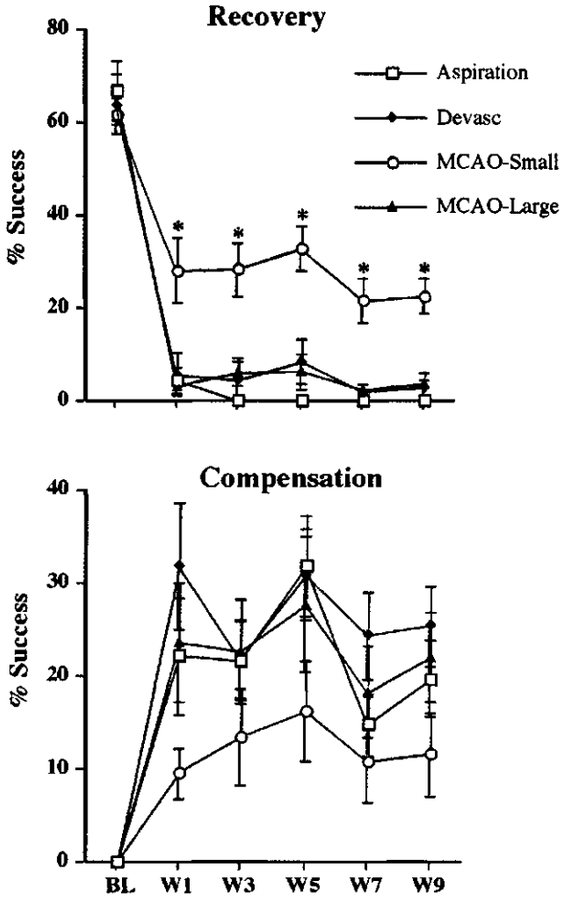

Figure 2.3.: Top panel shows a picture of an animal on the reaching apparatus before the surgeries, arrow points at the animal's grasp for food. Bottom panels show the rat's performance before the lesion (BL) and every other week for a total of nine weeks (W1, W3, W5, W7, W9) post-stroke (±SE). Middle panel shows recovery (±SE, successful reaches with impaired limb/total attempts with impaired limb X 100), and bottom panel shows compensation (±SE, successful reaches with unimpaired limb/total attempts with unimpaired limb X 100) of animals with different kinds of stroke on this task. Note that only animals with small MCAO show recovery whereas the other groups compensate by learning to reach with the unaffected paw. (*p<.05 with respect to the other groups).

Swimming task

Before the lesions, rats in all groups learned to swim directly to the platform and showed almost perfect inhibition (less than one stroke per animal on average) of the forelimbs before climbing on top of the platform. After the surgeries animals showed clear asymmetry in limb inhibition as they stroked with the impaired limb, but not the ipsilateral limb during swimming, (Figure 4). A repeated measures ANOVA showed no main effect of group ($F(3,31) = 2.04$, $P = 0.127$), a significant main effect of test week ($F(4,124) = 5.45$, $P < 0.001$), but not the interaction ($F(12,124) = 1.0$, $P = 0.45$). Follow-up tests (Fisher's LSD) showed, however, that overall the group with aspiration was more impaired than the group with small MCAO ($P = 0.025$). The small MCAO group did not differ from the other two groups. A t-test revealed that by the end of the behavioral

testing all groups still differed from their preoperative base lines: Aspiration (P = 0.004); Devasc (P = 0.041); MCAO-small (P = 0.0044); MCAO-large (P = 0.010).

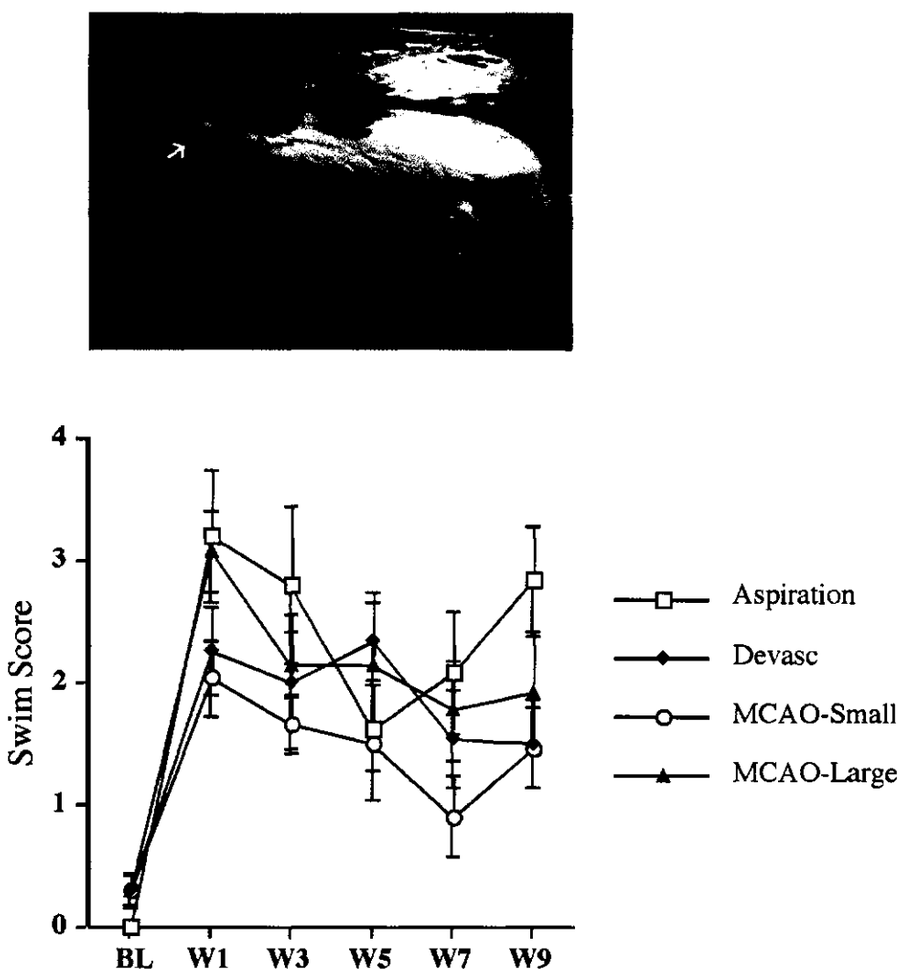

Figure 2.4. : Top panel shows a picture of an animal before the lesions during swimming, arrow shows forepaw inhibited. Bottom panel shows the number of strokes (±SE) made with the affected forelimb before the lesion (BL) and every other week for a total of nine weeks (W1, W3, W5, W7, W9) post-stroke (±SE).

The only statistical difference was found between the small MCAO and the aspiration group in that the small MCAO animals were less impaired.

Sunflower Seed Test

Animals quickly learned how to open and consume the sunflower seeds. By the end of the fifth day pre-surgery, it would take an animal 30 to 35 seconds on average to open and successfully consume the five sunflower seeds. The average number of broken shell pieces after consumption was 11. After the lesions, the time to consume the seeds and the total number of pieces that the animal had to breake in order to consume the seed increased markedly (Figure 5). These effects could be due to paw impairments during the manipulation of the seed, tongue impairments while opening the seed or due to a combination of paw-mouth coordination. Although subjects never reached preoperative baselines animals in the small MCAO group showed greater recovery than the ones in the other three groups. The mean time to consume the seed for groups with aspiration, devascularization, and large MCAO was 78 sec, whereas animals with small MCAO had an average of 52 sec. A repeated measures ANOVA comparing all groups showed a significant main effect of group ($F(3,31) = 4.75$, $P < 0.01$), week $F(4,124) = 19.68$, $P < 0.0001$, and the interaction ($F(12,124) = 2.22$ ($P < 0.02$). Follow-up tests (Fisher's LSD) showed that animals with small MCAO performed better than the other three groups ($P < 0.05$), which in turn did not differ among themselves. A t-test revealed that by the end of the behavioral testing all groups differed from their preoperative base line performance, ($P < 0.002$). A very similar pattern of performance was observed when the pieces of shell were counted: the small MCAO group showed greater recovery than the other three groups. A repeated measures ANOVA comparing all groups showed a significant main

effect of group ($F(3,31) = 7.9$, $P < 0.001$), week $F(4,124) = 16.51$, $P < 0.0001$, but not the interaction ($P = 0.117$). Follow-up tests (Fisher's LSD) showed that the small MCAO differed from the other three groups ($P < 0.05$), which in turn did not differ among themselves. Surprisingly a t test revealed that by the end of the behavioral testing only the group with devascularization differed from its preoperative base line performance ($P < 0.05$).

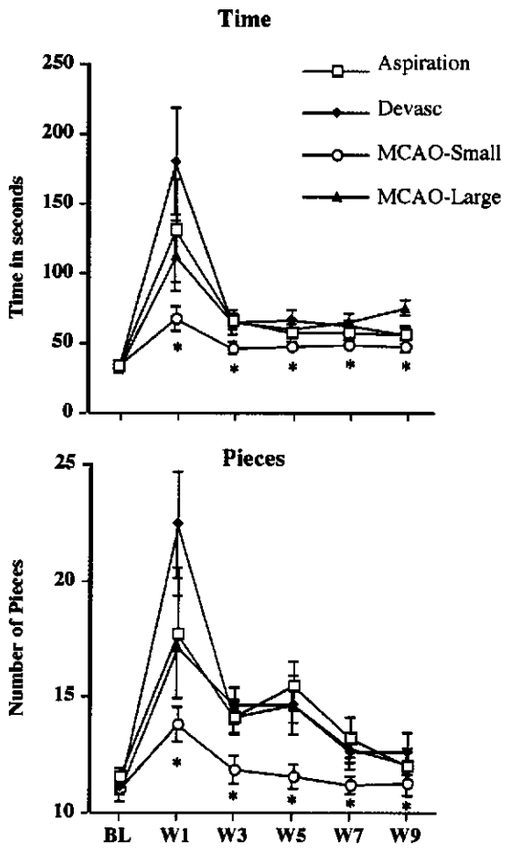

Figure 2.5. : Top panel shows a picture of an animal consuming a sunflower seed before the lesions. Bottom panels show the total amount of time (±SE) and the total number of pieces (±SE) required for the animal to consume the seed before the lesion (BL) and every other week for a total of nine weeks (W1, W3, W5, W7, W9) post-stroke (±SE). Note that animals with small MCAO required less time to open the five sunflower seeds and the number of leftover shell pieces at the end of the consumption was also lower (*p<.05 with respect to the other groups).

Tongue Extension

The mean length that animals could extend their tongues to lick the slurry before the lesions was 11.1 mm. After the surgeries, however, and especially during the first three weeks post-surgery, this mean dropped to 8.1 mm (Figure 6). A repeated measures ANOVA showed no main effect of group ($F(3,31) = 2.12$, $P = 0.116$), a significant main effect of test week ($F4,124) = 32.8$, $P < 0.0001$), but not the interaction ($F(12,124) = 0.73$, $P = 0.71$). Follow-up tests (Fisher's LSD) however, showed that overall the group with devascularization was more impaired than the group with small MCAO ($P = 0.022$). The small MCAO group did not differed from the other two groups. A t test revealed that by the end of the behavioral testing all groups reached their preoperative base lines: Aspiration ($P = 0.40$); Devasc ($P = 0.28$); MCAO-small ($P = 0.85$); MCAO-large ($P = 0.29$).

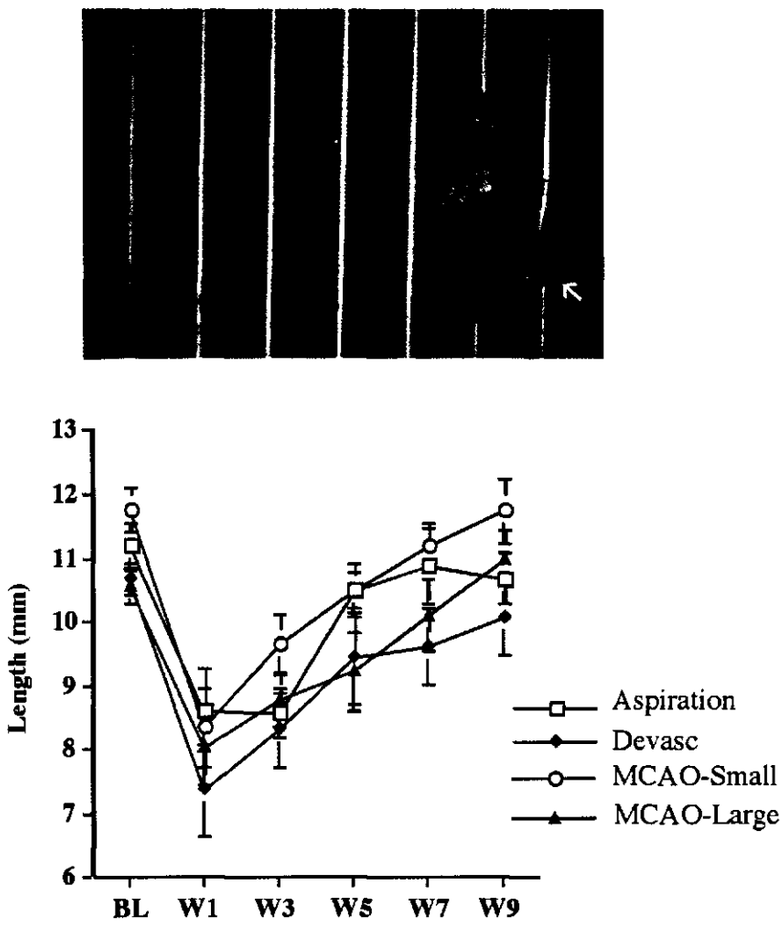

Figure 2.6. : Top panel shows a picture of an animal extending its tongue in order to consume the slurry spread onto the ruler. Bottom panel shows the length in millimeters of the tongue extension before the lesion (BL) and every other week for a total of nine weeks (W1, W3, W5, W7, W9) post-stroke (±SE). Note that animals in the different groups did not differ from each other at any week and this was the only behavioral task in which animals returned to baseline measures by the end of the nine weeks of testing.

Anatomical Results

Infarct Size

Infarct size was determined by measuring areas of non-infarcted tissue in both the damaged and undamaged hemispheres. Measures are represented as percentage of the normal hemisphere and are shown in Table 1.

When the infarct size was calculated as a ratio to the intact hemisphere, a simple ANOVA showed a significant effect of group ($F(3,30) = 6.423$, $P = 0.001$). A follow-up test (Fisher's LSD) showed that this difference was driven by the group with small MCA occlusion as they showed virtually no damage to the striatum, had a smaller cortical

Table 2.1.. Percentage of area of remaining tissue compared with the contralateral hemisphere.

Group	Infarct Size	
	Hemisphere	Cortex
Aspiration	66.93 ± 1.82	45.33 ± 2.42
Devascularization	71.51 ± 4.06	49.70 ± 6.15
MCAO-Small	84.26 ± 2.52*	72.66 ± 3.89*
MCAO-Large	71.06 ± 3.68	47.91 ± 4.10

Data are mean ± SD

*Significantly different from the other three groups ($p < 0.01$).

infarct, ($P < 0.01$), and no damage to the corpus callosum. Partial damage to the dorsolateral striatum was observed in all animals on the other three groups. A simple ANOVA for the cortical measurements showed a significant effect of group ($F(3,30) = 9.446$, $P < 0.0001$). A follow-up test (Fisher's LSD) showed that the group with small MCAO was different than the other three groups ($P < 0.001$). Again, the cortical lesion observed in animals with small MCA occlusions was not as severe as the one produced with the other three methods. Because the lesion extent on groups with aspiration, devascularization and large MCAO were very similar ($F(2,21) = 0.57$, $P = 0.57$ for the % hemisphere, and $F(2,21) = 0.24$, $P = 0.78$ for the % cortex), Figure 1 illustrates a typical infarct after devascularization and small MCA occlusion to show the difference in lesion size. An intact dorsolateral striatum and corpus callosum were observed only in animals with small MCAO.

Morphological analyses

Because training at skilled reaching alters dendritic organization (Biernaskie and Corbett 2001; Withers and Greenough 1989), and because lesions alone alter dendritic branching (Kolb el al. 1997) the brains of four intact control animals trained in tray reaching for a total of three months, were added in order to compare them with the lesion groups. Tables 2, 3, and 4 summarize the effects that the different types of lesion had on cortical and striatal morphology. Figure 7 shows examples of cells drawn from the forelimb area layer V of the intact side of the brain. Figure 8 shows example of cells drawn from the cingulate cortex (Cg 3) layer III of the intact side of the brain.

When pyramidal cells of layer V in the forelimb area contralateral to the lesion were analyzed, a reduction on the number of branches but and increase in spine density

was observed in animals with aspiration lesions. Animals with devascularizations showed and increase in dendritic length. Animals with small MCAO showed a slight increase in dendritic length but a decrease in spine density. Finally, animals with large MCAO showed no changes.

Dendritic Length: A simple ANOVA showed a significant effect of group ($F(4,15) = 7.21$, $P < 0.001$). A follow-up test (Fisher's LSD) showed that the group with devascularizations had longer dendritic trees that differed from controls and from the other three groups ($P < 0.01$; Table 2).

Table 2.2.. Summary of the morphological changes after different kinds of stroke on the forelimb area contralateral to the lesion.

Group	Forelimb Area LV Undamaged Hemisphere		
	SA	BOA	SDA
Control	139.7 ± 4.7	49.3 ± 1.0	7.0 ± 0.13
Aspiration	129.5 ± 6.3	42.7 ± 2.1**	8.2 ± 0.18**
Devascularization	167.4 ± 5.9**	48.6 ± 2.1	6.9 ± 0.08
MCAO-Small	153.3 ± 5.2	47.8 ± 1.2	6.4 ± 0.1*
MCAO-Large	144.3 ± 4.0	48.2 ± 1.2	7.0 ± 0.3

SA (Sholl Analysis), BOA (Branch Order Analysis), SDA (Spine Density Analysis).

*Significantly different from control ($p < 0.05$).

**Significantly different from controls and the other three groups ($p < 0.05$).

Table 2.3. Summary of the morphological changes on the cingulate cortex apical tree after different kinds of stroke.

Group	Cingulate Cortex (Cg3 LIII) Apical Tree Undamaged Hemisphere		
	SA	BOA	SDA
Control	67.1 ± 4.4	22.9 ± 0.5	6.4 ± 0.09
Aspiration	65.4 ± 9.5	22.7 ± 3.1	6.8 ± 0.2
Devascularization	90.3 ± 6.3*	33.5 ± 3.4**	6.5 ± 0.1
MCAO-Small	75.7 ± 5.5	25.6 ± 2.8	6.3 ± 0.1
MCAO-Large	70.1 ± 5.8	22.7 ± 1.9	6.6 ± 0.2

Group	Cingulate Cortex (Cg3 LIII) Apical Tree Damaged Hemisphere		
	SA	BOA	SDA
Control	68.2 ± 4.0	23.8 ± 0.5	6.2 ± 0.04
Aspiration	50.7 ± 4.5**	20.8 ± 2.4	6.9 ± 0.2*
Devascularization	83.8 ± 2.3**	29.1 ± 2.6***	6.0 ± 0.02
MCAO-Small	69.6 ± 3.7	22.2 ± 2.6	6.3 ± 0.2
MCAO-Large	65.0 ± 5.0	21.8 ± 1.5	6.4 ± 0.2

SA (Sholl Analysis), BOA (Branch Order Analysis), SDA (Spine Density Analysis).

*Significantly different from control ($p < 0.05$).

**Significantly different from controls and the other three groups ($p < 0.05$).

***Significantly different form the three other lesion groups ($p < 0.05$).

Table 2.4. Summary of the morphological changes on the cingulate cortex basilar tree after different kinds of stroke.

Group	Cingulate Cortex (Cg3 LIII) Basilar Tree Undamaged Hemisphere		
	SA	BOA	SDA
Control	102.0 ± 1.6	41.3 ± 0.5	7.6 ± 0.2
Aspiration	89.4 ± 4.5	36.0 ± 1.1	9.0 ± 0.1**
Devascularization	101.5 ± 6.7	41.2 ± 2.5	7.7 ± 0.02
MCAO-Small	94.8 ± 3.8	38.6 ± 4.1	7.9 ± 0.2
MCAO-Large	99.1 ± 4.8	36.9 ± 0.3	7.8 ± 0.08

Group	Cingulate Cortex (Cg3 LIII) Basilar Tree Damaged Hemisphere		
	SA	BOA	SDA
Control	111.7 ± 4.2	44.8 ± 1.2	7.6 ± 0.07
Aspiration	80.6 ± 4.7*	37.3 ± 1.9*	8.0 ± 0.07
Devascularization	110.0 ± 5.7	42.4 ± 2.8	7.9 ± 0.2
MCAO-Small	93.6 ± 2.1*	33.9 ± 4.0*	7.8 ± 0.08
MCAO-Large	90.5 ± 2.5*	36.5 ± 1.5 *	7.9 ± 0.2

SA (Sholl Analysis), BOA (Branch Order Analysis), SDA (Spine Density Analysis).

*Significantly different from control ($p < 0.05$).

**Significantly different from controls and the other three groups ($p < 0.05$).

Dendritic Branching: A simple ANOVA showed a marginal effect of group ($F(4,15) = 2.57$, $P = 0.08$. A follow-up test (Fisher's LSD) however, showed that the group with aspiration lesions had fewer branches when compared to controls and to the other three groups ($P < 0.05$; Table 2).

Spine Density: A simple ANOVA produced a significant effect of group ($F(4,15) = 12.95$, $P < 0.0001$). A follow-up test (Fisher's LSD) showed that the group with aspiration lesions had more spines and differed from controls and from the other three groups ($P < 0.001$). Also, the group with small MCA occlusions had fewer spines when compared to control and the large MCAO group, ($P < 0.05$; Table 2).

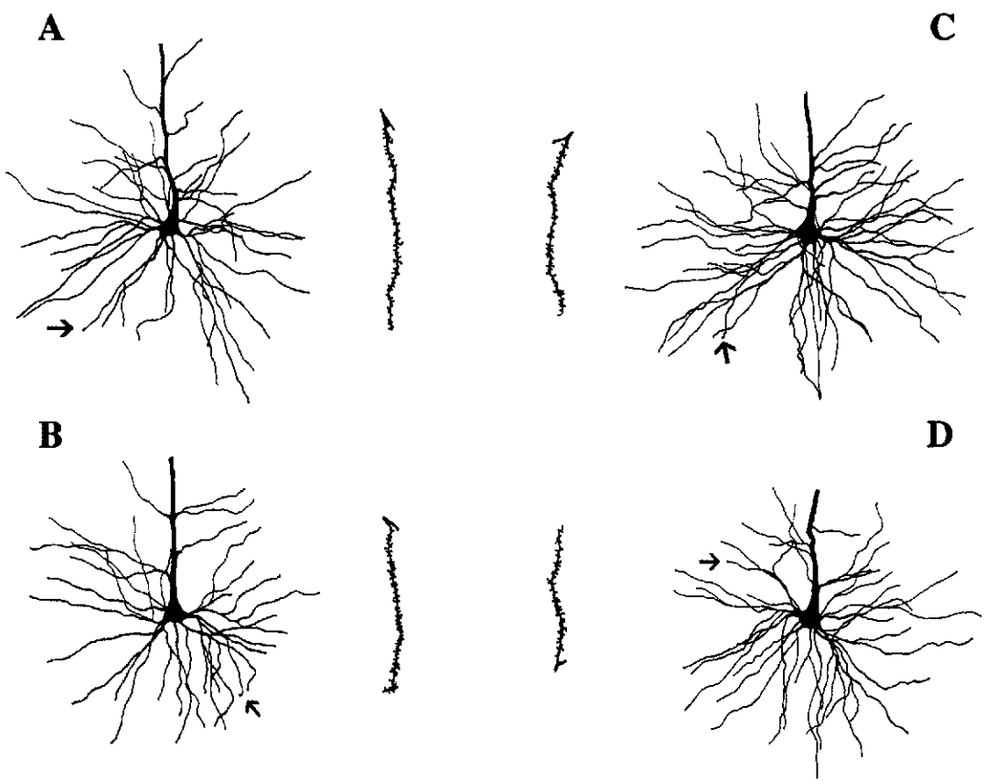

Figure 2.7: Camera lucida drawing of representative layer V pyramidal neurons and spines in Zilles' area FL contralateral to the lesion from (A) control, (B) aspiration, (C) devascularization, and (D) small MCA occlusion groups. Animals with large MCAO were not included because the changes were very similar than in the small MCAO group. Neurons from groups with aspiration lesions had fewer dendrites but more spines on the basilar tree, animals with devascularization lesion show longer dendrites, animals with small MCA occlusion show reduction on the number of spines, and animals with large MCA occlusion showed no changes in any of the three measures (not shown). Arrows indicate the dendritic branch from where the spines were drawn.

The apical tree of pyramidal cells of layer III within the anterior cingulate cortex showed changes depending upon the specific site (ipsilateral vs. contralateral) and type of lesion. Animals with aspiration lesions showed no changes on the contralateral hemisphere but a decrease in dendritic length and an increase in spine density in the ipsilateral hemisphere. Animals with devascularizations showed an increase in dendritic length and dendritic branching in the contralateral hemisphere but no changes in the ipsilateral hemisphere.

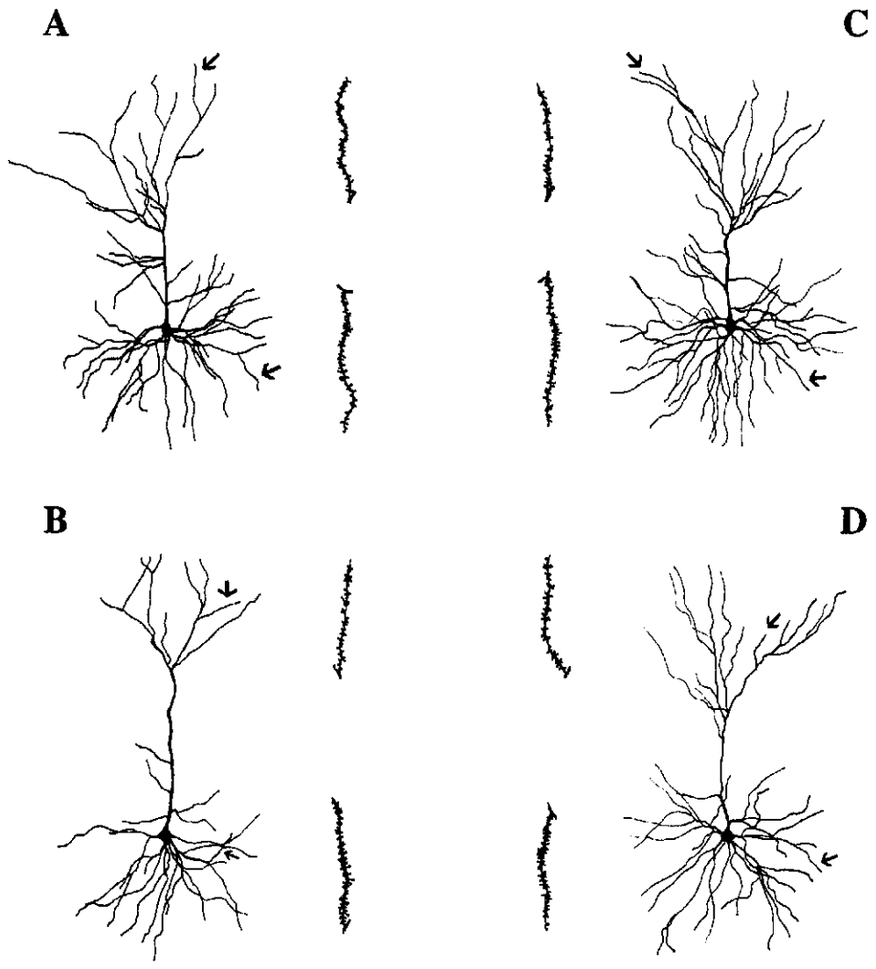

Figure 2.8. : Camera lucida drawing of representative layer III pyramidal neurons and spines in Zilles' area Cg 3 from (A) control, (B) aspiration, (C) devascularization, and (D) small MCA occlusion groups. Animals with large MCAO were not included because the changes were very similar than in the small MCAO group. Overall, neurons from groups with aspiration lesions had fewer dendrites but more spines, animals with devascularization lesion show an increase on the length and number of dendrites, and animals with MCA occlusions show a

slight reduction on the number and length of dendrites. Arrows indicate the dendritic branch from where the spines were drawn.

Animals with small or large MCAO's showed no changes on the ipsilateral nor the contralateral hemispheres. For the basilar trees a different pattern was observed. Animals with aspiration lesions showed an increase in spine density in the contralateral hemisphere but a decrease in dendritic length and arbor on the ipsilateral hemisphere. Animals with devascularization showed no differences, and animals with small and large MCAO's showed a decrease in dendritic length and branching in the ipsilateral hemisphere.

Apical and Basilar trees were drawn from both the intact and lesion hemisphere and the data are presented separately.

Intact Hemisphere

Dendritic Length: When the apical tree was analyzed, a simple ANOVA showed no effect of group ($F(4,15) = 2.33$, $P = 0.10$). A follow-up test (Fisher's LSD) however, showed that the group with devascularizations had longer apical trees that differed from controls and from the aspiration and large MCAO groups ($P < 0.05$; Table 3). When the basilar tree was analyzed, no statistical differences were found among the groups ($F(4,15) = 1.29$, $P = 0.31$; Table 4).

Dendritic Branching: A simple ANOVA showed a significant effect of group when the apical tree analyzed ($F(4,15) = 3.16$, $P < 0.05$. A follow-up test (Fisher's LSD), showed that the group with devascularizations lesions had more branches when compared to

controls and to the other three gourps ($P < 0.05$; Table 3). Analysis of the basilar tree showed no statistical differences among groups ($F(4,15) = 1.15$, $P = 0.37$; Table 4).

Spine Density: A simple ANOVA showed no differences among groups when spines of the apical tree were analyzed ($F(4,15) = 1.06$, $P = 0.40$; Table 3). When spines of the basilar tree were analyzed (Table 4), however, a significant effect of group was observed ($F(4.15) = 9.39$, $P < 0.001$). This difference was driven by the group with aspiration lesions having more spines than control and the other three lesion groups ($P < 0.001$, Fisher's LSD).

Damaged Hemisphere

Dendritic Length: When the apical tree was analyzed, a simple ANOVA showed a significant effect of group ($F(4,15) = 8.53$, $P < 0.001$). A follow-up test (Fisher's LSD) showed, however, that the group with aspirations had a reduction on the length of the apical trees with respect to controls, and the rest of the lesion groups, ($P < 0.05$). On the other hand, animals with devascularizations had longer apical trees and differed from controls and from all the other groups ($P < 0.05$; Table 3). When the basilar tree was analyzed, a simple ANOVA showed a significant effect of group ($F(4,15) = 10.37$, $P < 0.001$). A follow-up test (Fisher's LSD) showed that all groups except animals with devascularization had smaller basilar trees when compared to controls ($P < 0.05$; Table 4).

Dendritic Branching: Analysis of the apical tree using a simple ANOVA showed no statistical differences ($F(4,15) = 2.35$, $P = 0.10$). A follow-up test (Fisher's LSD) however, showed that the group with devascularizations lesions had more branches when compared to animals in all the other experimental groups ($P < 0.05$; Table 3). Analysis of

the basilar tree showed a significant effect of group ($F(4,15) = 3.16$, $P < 0.05$). A follow-up test (Fisher's LSD) showed that only the group with devascularizations lesions did not differ from controls ($P < 0.05$; Table 4).

Spine Density: A simple ANOVA produced a significant effect of group when spines of the apical tree were analyzed ($F(4,15) = 3.129$, $P < 0.05$). A follow-up test (Fisher's LSD) however, showed that the group with aspiration lesions had more spines when compared to controls, devascularized, and small MCAO animals ($P < 0.05$; Table 3). When spines of the basilar tree were analyzed no significant effects were observed ($F(4.15) = 0.63$, $P = 0.64$; Table 4).

Striatum

Spiny neurons of the remaining dorsolateral striatum were analyzed for dendritic length and branching in the contralateral and ipsilateral hemispheres. In two brains the staining observed in the striatum and nucleus accumbens was slightly lighter making the drawing of spines difficult, therefore analysis of spine density was not included. Animals with aspiration lesions showed a decrease in dendritic length and branching. Animals with devascularization showed no changes with respect to controls. Animals with small MCAO showed an increase in dendritic length in both hemispheres and finally animals with large MCAO showed an increase in dendritic length in the contralateral hemisphere and an increase in dendritic branching in the ipsilateral hemisphere.

Intact Hemisphere

Dendritic Length: A simple ANOVA showed a significant effect of group ($F(4,15) = 9.01$, $P < 0.001$). A follow-up test (Fisher's LSD) showed that both groups with MCA occlusions had an increase on the length of the dendrites with respect to controls and the

aspiration group ($P < 0.05$). Also, animals with devascularization lesions showed an increase when compared to aspiration (Table 5).

Table 2.5. : Summary of the morphological changes on the striatum after different kinds of stroke.

Group	Striatum (Remained Dorsolateral)			
	Undamaged Hemisphere		Damaged Hemisphere	
	SA	BOA	SA	BOA
Control	82.0 ± 2.7	42.7 ± 1.0	84.8 ± 1.6	41.9 ± 1.7
Aspiration	73.0 ± 3.4	38.1 ± 1.3	71.6 ± 5.1*	36.1 ± 1.4**
Devascularization	91.2 ± 2.1	44.4 ± 2.0	83.7 ± 4.	42.2 ± 1.0
MCAO-Small	110.4 ± 4.3*	42.6 ± 1.8	121.1 ± 4.9**	43.0 ± 0.07
MCAO-Large	96.52 ± 8.2*	47.9 ± 4.1	96.6 ± 4.5	47.9 ± 2.7*

SA (Sholl Analysis), BOA (Branch Order Analysis).

*Significantly different from control ($p < 0.05$).

**Significantly different from controls and the other three groups ($p < 0.05$).

Dendritic Branching: A simple ANOVA showed no statistical differences among the groups ($F(4,15) = 2.29$, $P = 0.10$). A follow-up test (Fisher's LSD) did reveal, however, that animals with large MCAO had longer branches than animals with aspirations (Table 5).

Lesion Hemisphere

Dendritic Length: A simple ANOVA showed a significant effect of group ($F(4,15) = 18.58$, $P < 0.0001$). A follow-up tests (Fisher's LSD) showed that the group with a small MCA occlusion had a dramatic increase on the length of the dendrites with respect to every other group including the controls ($P < 0.01$). Also, animals with aspiration lesions showed a decrease when compared to controls ($P < 0.05$; Table 5).

Dendritic Branching: A simple ANOVA showed a significant effect of group ($F(4,15) = 6.19$, $P < 0.01$). A follow-up test (Fisher's LSD) revealed that animals with aspirations had fewer branches than animals in any other group ($P < 0.05$). On the other hand, animals with large MCA occlusions showed increased dendritic trees when compared to controls and devascularized animals ($P < 0.05$; Table 5).

2.5. Discussion

There are two major findings from the present study: 1) the behavioral deficits observed after large MCA occlusion, devascularization, or aspiration of the motor cortex were very similar; and, 2) the effects of the lesions on cortical and striatal plasticity differed across the different lesion models.

Behavioral similarities among cortical-injury models

One of the objectives of the study was to investigate whether the behavioral outcome would be equivalent across different models of stroke. We used an extensive behavioral test battery that proved to be effective in detecting behavioral impairments and spontaneous behavioral improvement after injury. Behavioral improvement was limited in animals with large MCA occlusion, devascularization and aspiration, however, and

only animals that received a small MCAO appeared to show significant spontaneous functional improvement. Behavioral impairments in the other three groups were comparable in severity and duration. One explanation for the better behavioral outcome in the small MCAO group is their smaller cortical infarct and the lack of striatal damage. Roof et al. (2001) have shown the same pattern of results when they compared animals with two different MCA occlusions (with striatal damage and without striatal damage) in tests of cylinder, forelimb, and hindlimb placement. Another possibility could be that smaller lesions stimulate more extensive plastic changes in the rest of the brain, although these were not immediately apparent in the current study. Finally, it should be noted that a more challenging task such as the Whishaw single pellet task (Whishaw et al., 1993) and kinematic analyses of the reaching movement, might have picked up behavioral differences across models.

There are few studies of behavioral outcome after different types of cortical injury. In one of such studies, Napieralski et al. (1998) tested animals on four different behavioral tasks after aspiration or thermocoagulation of pial blood vessels. They found no behavioral differences between the groups in two of the tasks (coordinated forelimb placement and vibrissae-stimulated forelimb placing). They found, however, that early after the surgeries, aspiration lesions of the motor cortex produced bigger deficits on tasks of tactile discrimination than comparable lesions produced by thermocoagulation of the vessels. In the same study, the authors found that thermocoagulation lesions produced a slightly more severe deficit than aspiration lesions in the use of the contralateral forelimb during vertical exploration. Voorhies and Jones (2002) found that unilateral electrolytic and aspiration lesions of the motor cortex produced similar behavioral

asymmetries early after the lesions, although these asymmetries were less enduring following electrolytic lesions than following aspiration lesions. In the present study, the behavioral measures failed to reveal any differences in motor asymmetry between animals with aspiration versus devascularization lesions, although we did not include a tactile discrimination task (Napieralsky study) nor did we include detailed analyses of the animals during vertical exploration (Voorhies and Jones study). There were, however, behavioral differences between the small MCAO and aspiration groups in the swimming task and between the small MCAO and the devascularization groups in measures of tongue extension. It is possible that behavioral differences among different lesion models are obvious early after the lesions and we failed to detect differences because we did not assess behavior in the time between week one and week three post-injury. Nonetheless in the present study our results suggest to us that although there is something different about removing the tissue all at once (aspiration) versus allowing it to progressively die (devascularization), this difference seems more apparent in neuronal morphology than in behavior.

Another puzzling result from our study is the fact that animals with devascularizations show increased dendritic length and branching, yet still failed to show behavioral improvement. Several studies from our laboratory (for a review see Kolb et al. 2001), have shown that increases in dendritic arborization and/or spine density after perinatal cortical lesions correlates with functional improvement. Moreover, Bury and Jones (2002) recently showed that a unilateral motor cortex lesion enhances acquisition of a reaching task with the non-impaired limb, and that this improvement correlates with enhanced dendritic plasticity. The authors suggested that the lesion-induced enhancement

of motor cortical neural plasticity is linked to the behavioral improvements that they observed. In contrast, our data showed no correlation between the rate or degree of behavioral improvement and enhanced neural plasticity. Rather, our results suggest that the lesions led to different compensatory mechanisms in the brain, in one case (devascularization group) the lesion increased dendritic arborization whereas in the other (aspiration group) the lesion increased spine density. If we assume that the changes in dendritic arborization and spine density reflect changes in synaptic organization in the brain, then it would appear that different lesion etiologies led to different patterns of synaptic reorganization. What is a bit surprising is that these changes did not produce more detectable behavioral differences. Thus, it appears that there are multiple ways in which the brain can reorganize to produce behavioral compensation.

We should note one additional issue here. In the current study the animals had extensive behavioral training during the postoperative recovery period and it is plausible to suppose that the training influenced the morphological changes that we observed and did so differentially in the different etiologies. This is a difficult problem to control in behavioral studies but should be remembered in the interpretation of the results.

Cortical and Striatal Plasticity after different models of stroke

Evidence is accumulating that partial functional restitution is possible after cortical injury. Studies in both human patients and animal models of stroke suggest that the cerebral cortex undergoes significant functional and structural plasticity that could last up to months following the injury (for reviews see Hallett, 2001; Nudo et al, 2001). These changes could occur in either the intact or injured hemisphere so we consider each separately.

Whishaw and his colleagues have shown that after motor cortex injury, animals rely on the "unaffected" side of the body for postural support and food handling, including reaching and food manipulation (e.g., Whishaw, 2000; Whishaw and Coles, 1996). Anatomical examination of the intact forelimb area after cortical injury has been described in detail by Jones and her colleagues (e.g., 1992; 1994; 1999) who have shown dendritic overgrowth in Layer V cells of the forelimb area contralateral to the lesion in Long-Evans rats. Other studies in the same strain of rats but using different lesion models have failed to find similar results of increased dendritic branching after motor cortex injury; however, (Forgie et al., 1996; Prusky and Whishaw, 1996). This discrepancy suggests differences in morphological changes following cortical injury may vary with lesion etiology and possibly with the intact versus injured hemisphere (e.g., Voorhies and Jones, 2002). In the present study we found lesion-specific morphological changes that are presumably permanent given that animals were sacrificed at least ten weeks after the surgeries. Animals with suction lesions showed reduced branching but increased spine density; animals with devascularization showed increased dendritic length; and, animals with small MCAO showed a marginal increased in length but a decrease in spine density. In Biernaskie and Corbett study (2001) authors showed that after MCA occlusions using endothelin-1, a greater number of dendritic branches were observed in layer V basilar tree in the forelimb area. Although the present study did not find such changes, the results by Biernaskie and Corbett suggest once again that etiology is important in understanding lesion-induced plasticity.

Most of the work of induced cortical plasticity after injury has focused on changes on the intact hemisphere, although some studies have shown anatomical changes in the

area surrounding the lesion. For example, Stroemer et al., (1992) reported increased synaptophysin immunoreactivity in the surrounding tissue suggesting synaptogenesis in the perilesion cortex. The same authors in 1993 showed increased expression of growth associated protein (GAP-43) after focal cortical ischemia suggesting axonal sprouting in areas adjacent to the lesion. In a study looking at the changes in connections after stroke to the somatosensory cortex, Carmichael et al., (2001) have shown that the peri-infarct cortex is structurally abnormal, with new cortical connections and axonal sprouting, but a loss in thalamic connections. In our study, we chose to look for changes in the anterior cingulate cortex because it is adjacent to the site of the lesion, has corticospinal connections, has previously been shown to change after stroke (Kolb et al. 1997), and because it would allow us to examine differences in neuronal plasticity on the ipsilateral versus the contralateral side. We found that whereas aspiration lesions and both MCA occlusions produced a decrease in dendritic length and branching on the basilar tree of the damaged hemisphere, no such changes were observed in animals with devascularizations. Instead, devascularization of the motor cortex produced an increase in length and dendritic branching in the apical tree of both intact and damaged sides. There was also a decrease in length, but an increase in spine density in animals with aspiration lesions in the apical tree of the lesion hemisphere and on the basilar tree of the intact hemisphere. This apparent disorganized pattern of morphological changes following different injuries leaves us wondering which elements may play an important role in cortical reorganization following injury.

Finally, we opted to investigate if the different models of stroke would produce changes in subcortical structures. Work by the Chesselet group (Napieralski et al., 1996;

Uryu et al., 2001) has shown axonal sprouting of contralateral corticostriatal neurons into the denervated striatum after ischemic cortical lesions, but not after aspiration lesions. Thus, it seemed possible to find a similar pattern of results in our study in animals with devascularization versus aspiration lesions. The results of the present study showed that the striatum on the lesion hemisphere was indeed affected with dendritic atrophy in animals with aspiration versus enhanced plasticity in animals with MCA occlusions. No changes were obvious in animals with devascularizations. The fact that small MCA occlusions produced enhanced plasticity in the striatum is intriguing because there was virtually no damage to the striatum but still a robust change in cell morphology.

This complex pattern of behavioral and anatomical results leads us to two general conclusions. First, the behavioral consequences of motor cortex injury are similar across different etiological models. Second, in view of the etiology-related differences in the morphological changes, it seems possible that different pharmacological or behavioral therapies may be appropriate for cerebral injuries of differing etiologies. On the other hand, it is possible that to the extent that therapies act to potentiate the endogenous lesion-induced plasticity, it may be that the same therapeutic intervention may benefit brains with different injuries in different ways.

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Evidence for Bilateral Control of Skilled Movements: Ipsilateral Skilled Forelimb Reaching Deficits and Functional Recovery in Rats Follow Motor Cortex and Lateral Frontal Cortex Lesions

3.1. Abstract

Unilateral damage to cortical areas in the frontal cortex produces sensorimotor deficits on the side contralateral to the lesion. Although there are anecdotal reports of bilateral deficits after stroke in humans or in experimental animals, little is known of the effects of unilateral lesions on the same side of the body. The objective of the present study was to make a systematic examination of the motor skills of the ipsilateral forelimb after frontal cortex lesions to either the motor cortex, by devascularization of the surface blood vessels (pial stroke), or to the lateral cortex, by electrocoagulation of the distal branches of the middle cerebral artery (MCA stroke). In addition, plastic processes in the intact hemisphere were documented using Golgi-Cox dendritic analysis and by intracortical microstimulation (ICMS) analysis. Although tests of reflexive responses in forelimb placing identified a contralateral motor impairment following both cortical lesions, quantitative and qualitative measures of skilled reaching identified a severe ipsilateral impairment from which recovery was substantial but incomplete. Golgi-impregnated pyramidal cells in forelimb area showed an increase in dendritic length and branching. Electrophysiological mapping showed normal size forelimb representations in the lesion rats relative to control animals. The finding of an enduring ipsilateral impairment in skilled movement is consistent with a large but more anecdotal literature in rats, nonhuman primates, and humans and suggests that plastic changes in the intact hemisphere are related to that hemisphere's contribution to skilled movement.

3.2. Introduction

It is well known that damage to the frontal neocortex or to the basal ganglia of one hemisphere results in impairments in movement on the contralateral side of the body [Whishaw et al., 1986; Kolb, 1995; Hesse and Werner, 2003]. In rat models of motor cortex injury [Castro, 1972; Whishaw et al., 1991; Schallert et al., 1997; Napieralski et al., 1998; Gonzalez and Kolb, 2003] or stroke [Hossmann, 1998], contralateral impairments may be displayed in using a paw to reach for food [Whsishaw and Miklyaeva, 1996; Kolb, 1999], in making placing responses [Schallert et al., 2000], in walking over complex surfaces such as an elevated horizontal ladder [Metz and Whishaw, 2002], and in detecting tactile stimulation to the limbs [Schallert et al., 1983; 2000; Schallert and Whishaw, 1984]. Immediately after surgery impairments may be pronounced but with time and/or experience, animals display substantial compensation/recovery [Whishaw 2000; Virley et al., 2000; Reglodi et al., 2003; Gonzalez and Kolb, 2003].

Although contralateral motor deficits follow unilateral frontal cortex damage, there are many reports in laboratory animals and humans [see Table 1] of impairments on the ipsilateral side of the body. For instance, although the arm controlled by the intact side of the brain after middle cerebral artery (MCA) stroke is often referred as being "unaffected", there are reports of patients complaining about loss in strength, speed of movement, and complex action sequences with that arm [Hermsdorfer et al., 1999; Hermsdorfer and Goldenberg, 2002; Jung et al., 2002]. Similarly, in rodent studies in which function of the ipsilateral forelimb is included as a "control" for the contralateral limb, there are also reports of impairments, especially in the immediate postoperative

period [Marston et al., 1995]. To date, there has been little systematic study of the ipsilateral impairments that might follow neocortical injury in the rat, even though such work might be helpful in understanding the nature of movement impairments and in aiding rehabilitation.

The purpose of the present study was to determine whether there are impairments in skilled movements in the forelimb ipsilateral to: a) motor cortex injury produced by devascularization of the surface blood vessels over the forelimb regions of motor cortex [Sofroniew et al., 1983]; or, b) lesions to the lateral sensory cortex, by electrocoagulation of the distal branches of the middle cerebral artery (MCA) with a procedure similar to the one described by Gonzalez and Kolb (2003). These methods have proved to be highly reproducible providing a well-defined infarct. They also produce quantifiable contralateral behavioral impairments while allowing some degree of spontaneous recovery/compensation [Whishaw, 2000; Gonzalez and Kolb 2003]. Before the lesions, the rats were trained in a skilled reaching task in which they had to learn to retrieve food pellets located on a shelf and their behavior was studied postoperatively for a total of four weeks. Impairments were documented by end point measures of success in obtaining food pellets [Whishaw and Pellis, 1990], and the qualitative changes in reaching movements were analyzed frame by frame from video records [Whishaw et al., 1993]. To enhance post-surgical recovery, animals were also given intensive training on a less demanding reaching task [Vergara-Aragon et al., 2003], between the second and fourth post-surgical weeks. At the completion of the behavioral tests, plastic changes in the intact hemisphere were examined using electrical microstimulation mapping of motor

cortex [Kleim et al., 1998], and by Golgi-Cox analyses [Gibb and Kolb, 1998] of pyramidal cells.

Table 3.1. Published studies in rats and humans reporting motor impairments with the ipsilateral limb after unilateral brain damage.

Species	Type of Injury	Reference #
Rat	Cortex	56, 68
	Basal Ganglia	17, 18, 21, 39, 54, 55, 65, 98
	Stroke	7, 12, 25, 37, 52, 57, 75, 90
	Other	34
Human	Stroke	1, 2, 4, 8, 13, 14, 15, 20, 26, 27, 30, 31, 32, 33, 40, 50, 51, 67, 78, 81, 82, 83, 84, 87, 99, 100, 102

3.3 Materials and Methods

Subjects

Subjects were 24 female Long-Evans hooded rats, 4 months old weighing 250-300g at the beginning of the experiment. Animals were raised in the University of Lethbridge vivarium and were housed in groups of two or three individuals in clear plexi-glass cages. The colony room was maintained on a 12/12h light/dark cycle (08:00-20:00 h) and the temperature regulated at 22 °C. Experiments were conducted according to

standards set by the Canadian Council on Animal Care and approved by the University of Lethbridge animal care committee.

Food restriction

Three weeks before the lesions, the rats were changed to a restricted food intake: Each animal received 20 gr. of food per day (normal daily consumption ranges from 18-25gr) an hour after the testing session was completed. Their body weight was maintained at about 95-98% until the completion of the behavioural testing.

Surgery and lesion placement

Subjects were assigned to three different groups, control (n = 8), motor cortex lesion by devascularization of blood vessels (n = 8), and latero-frontal cortex lesion by middle cerebral artery occlusion (n = 8). Animals with motor cortex lesions received an injection of atropine nitrate (0.1 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) to facilitate respiration throughout surgery. Animals were then anesthetized (sodium pentobarbital 45 mg/kg i.p.) and placed in a stereotaxic apparatus. The lesions were produced by unilateral devascularization of the motor cortex (Sofroniew et al., 1983; Kolb et al., 1997). In brief, a flap of bone and underlying dura were removed at coordinates corresponding to the overlapping motor and sensory representation of the forelimb, areas Fr2, Fr1, Fr3, FL and HL (Zilles, 1985). A rectangular hole in the skull was produced at stereotaxic coordinates anterior (A), lateral (L): A= -1.0 to +4.0 mm and L= 1.0 to 4.0 mm using an electric dental drill. All vessels and pia matter were rubbed away with sterile, saline-soaked cotton swabs. The incision was closed and animals were observed for 24 hours before being returned to the colony.

Animals that received MCA occlusions were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine (5 mg/kg). A procedure similar to Tamura et al. (1981) was used. In brief, a rectangular hole lateral to the temporal ridge was made using an electric dental drill whilst avoiding traumatic brain injury. The opening was enlarged with rongeurs by removal of additional temporal bone to expose the MCA. At this point the dura mater was carefully removed and the vessel was permanently occluded by bipolar electrocoagulation (Howard Instruments Inc.) dorsal to the rhinal fissure. The incision was closed and animals were observed for 24 hours and then returned to the colony.

Apparatus and training

Cylinder test: Forelimb use for weight support during explorative activity was examined by placing the rats in a transparent cylinder 20 cm in diameter and 30 cm high (Figure 3A) for three minutes (Schallert et al., 1997). The animals were individually placed in the cylinder during the three minutes of each testing session. A mirror was placed underneath the cylinder at an angle to allow the experimenter to videotape the animal's activity from a ventral view (Pinel et al., 1993). The cylindrical shape encouraged vertical exploration of the walls with the forelimbs, but the walls were high enough so that animals could not reach the top.

Scoring forelimb use asymmetry

Forelimb use was measured during vertical exploration. Each forepaw contact with the cylinder wall was counted. The asymmetry score of forelimb use in wall exploration was calculated as the percent preference for the paw ipsilateral to the lesion,

or for the control rats, the paw that they used for reaching: Preference = ipsilateral paw/(ipsilateral paw + contralateral paw) X 100.

Reaching boxes

Single pellet boxes: They were made of clear Plexiglas, with the dimensions 45 cm deep by 14 cm wide by 35 cm high. In the center of each front wall was a 1 cm-wide slit which extended from 2 cm above the floor to a height of 15 cm. On the outside of the wall, in front of the slit, mounted 3 cm above the floor, was a 2 cm-wide shelf. Two indentations on the floor of the shelf were located 2 cm from the inside of the wall and were centered on the edges of the slit where rats could reach. Food pellets (45 mg Rodent Chow food pellets, Bioserve Inc.) were placed in the indentation contralateral to the limb with which the rat reached (Whishaw, 1990) (Figure 4A). Following each reach, a short pause preceded the presentation of the next pellet. This would encourage animals to return to the back of the box after each reach, which forced them to reposition themselves and prepare for the next reach.

Tray Boxes: They were made of Plexiglass with dimensions 26 cm high, 28 cm deep, and 19 cm wide. The front of the boxes was constructed of 2 mm bars separated from each other by a 9 mm gap. Clear Plexiglass tops allowed access to the inside of the box. A 4 cm wide and 0.5 cm deep tray was mounted in front of the bars. The tray contained food fragments weighing approximately 30 mg each. Animals had to reach between the bars, grasp the food and retract it where they were able to freely eat.

Scoring reaching success

Single pellet: Reaching performance was assessed on two measures: (1) Success on first reach: if a rat obtained the food pellet following the initial limb advance, this

reach was scored as a hit. (2) Total success: if a rat obtained a piece of food either following the first limb advance or after a number of limb advances, the reach was counted as a hit.

Tray reaching: If the rat made a reaching movement (forepaw inserted through the bars, but no food was grasped or the food was dropped), the movement was scored as a "reach", whereas if the rat obtained the food and consumed it, the movement was scored as a "hit". Success was calculated as follows:

Success percent = ("hit" / "reach + hit") X 100

Qualitative reaching analysis

Reaching movements made during the single pellet task were analyzed using a rating scale derived from Eshkol-Wachmann Movement Notation (EWMN: Eshkol and Wachmann 1958; Whishaw, 1993). A reach was subdivided into ten components: (1) Digits to the midline: the limb is lifted from the floor with the upper arm and the digits adducted to the midline of the body. (2) Digits close: as the limb is lifted, the digits are semiflexed and the paw is supinated so that the palm faces the midline of the body. (3) Aim: using the upper arm, the elbow is adducted so that the forearm is aligned along the midline of the body, with the paw located just under the mouth. This movement involves fixation of the distal portion of the limb, so that digits remain aligned with the midline of the body. This is likely produced by a movement around the elbow that reverses the direction of movement of the paw to compensate for the adduction of the elbow. (4) Advance: the head is lifted and the limb is advanced directly forward above and beyond the food pellet. (5) Digits open: as the limb is advanced the digits are extended and opened. (6) Pronation: using a movement of the upper arm, the elbow is abducted,

pronating the paw over the food. Full pronation of the paw onto the food is aided by a movement of the paw around the wrist. (7) Grasp: as the pads of the palm or the digits touch the food, the food is grasped by closure of the digits. This can occur as an independent movement, or the grasp can occur as the paw is withdrawn. (8) Supination I: as the limb is withdrawn, the paw is dorsiflexed and is supinated 90^0 by a movement around the wrist and by adduction of the elbow. These movements can occur as soon as the food is grasped or can occur as the limb is withdrawn. (9) Supination II: as the rat sits back with food held in the paw, the paw is further supinated by 90^0 and ventroflexed to present the food to the mouth. (10) Release: the digits are opened and the food transferred to the mouth.

Five successful reaches were analyzed frame-by-frame from the videotapes. Each movement component was rated on a one-point scale. If the movement appeared normal, it was given a score of "0"; if it appeared slightly abnormal but recognizable it was given a score of "0.5"; and a score of "1" was assigned if the movement was absent or completely unrecognizable.

Procedures and time-line

The first week of training in the single pellet task consisted of daily sessions of ten minutes with unlimited access to the food pellets (45 mg). For the second week and until the end of the experiment, animals were presented with 20 pellets each day. Once the animals consistently achieved daily scores (total success) of at least 50% (successful retrieval and consumption of 10 of 20 pellets) they were video recorded and their performance analyzed. Upon scoring, the animals were divided into three groups such that the mean baseline was similar for the three groups: control (n = 8), devascularization

of motor cortex (n = 8), and MCA occlusions (n = 8). The following testing and training procedures were then given to the three groups following surgery:

1) Single pellet reaching: All rats were tested and video recorded on days 1, 3, 7, 14, and 30 post surgery.
2) Cylinder test: All rats were placed in the cylinder and their activity was video recorded for 3 minutes on days 1, 3, 7 and 14 post surgery.
3) Tray reaching: On day 15 and for the following 8 days, animals were placed in the tray reaching apparatus for one hour in the morning and one hour in the afternoon. On the last day of training (day 22) their performance was video recorded for 10 minutes.

Video recording

Video records were made using a Cannon ZR 30 MC digital video camcorder with a shutter speed of 1000^{th} of a second. Illumination for high shutter speed filming was provided by a two arm Nikon Inc. MII cold light source. Frame-by-frame analysis at 30 frames/s was produced by a Sony digital videocassette recorder DSR-11 or through a computer based frame grabber.

Statistical analysis

Analyses of variance (ANOVA) were used for all measures and Fisher's LSD ($P<0.05$ or better) was used for *post hoc* evaluations.

Inttracortical microstimulation (ICMS)

At the completion of the behavioral testing 4 animals from each group underwent ICMS motor mapping. For this procedure animals were anesthetized with ketamine hydrochloride (70 mg/kg i.p.) and xylazine (5 mg/kg i.p.). A craniotomy was performed

over the cortex contralateral to the preferred reaching paw to create a window for ICMS. To relieve edema, a small puncture wound to the cisterna magna provided a drain outlet for excess cerebrospinal fluid. The dura was deflected without damaging underlying pia mater and the exposed cortex was covered with an inert silicon oil (37 °C). A digital picture was taken over the exposed cortex, and a calibrated high-resolution grid was superimposed onto the picture to serve as a guide for microelectrode penetration sites.

A glass microelectrode, pulled and beveled (15-30 μm tip diameter) was filled with concentrated saline (3.5 M). A tungsten filament was inserted into the glass microelectrode to deliver current from an isolation stimulation unit. Stimulation trains consisted of thirteen 200 μs cathodal pulses delivered at 350 Hz. At each penetration site, current gain was progressively increased from 0 μA to a maximum of 60 μA.

Electrode placement was controlled by a hydraulic microdrive (Kopf) to depths corresponding to layer V of motor cortex (~1550 μm). Rats were maintained in a prone position and the forelimb was elevated from underneath the elbow. At each penetration site, forelimb movements were classified as either proximal (elbow/shoulder) or distal (wrist/digit). In the case of two or more simultaneous forelimb movements, the single movement persisting at the lowest stimulation intensity was recorded and deemed representative of that particular cortical point. Movement thresholds were determined for each site evoking forelimb movements. An image analysis program (Canvas v3.5) was used to calculate the extent of proximal and distal representations in both the caudal forelimb (CFA) and rostral forelimb (RFA) representations (Kleim et al., 1998). Mean thresholds for forelimb representations were calculated for analysis.

After mapping, animals were sacrificed using a lethal dose of sodium pentobarbital. They were intracardially perfused, first with saline in 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS. The brains were removed, post-fixed and cryoprotected in a solution of 30% sucrose in 4% paraformaldehyde solution for three days in 4°C. All brains were then sectioned into 40 μm using a cryostat (2800 Frigocut, Reichert-Jung). Every seventh section from the control and lesion groups was mounted into glass slides and stained for cresyl violet.

Golgi-Cox analysis

At the completion of the behavioural testing the remaining four rats from each group that did not undergo ICMS were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline. The brains were removed and weighed before being immersed in a 20 ml of Golgi-Cox solution where they remained for 14 days. The brains were then placed in a 30% sucrose solution for 2 days and cut on a vibratome at 200 μm and developed using a procedure described by Gibb and Kolb (1998). The basilar trees of layer V pyramidal cells within the forelimb motor cortex of the uninjured hemisphere, were traced using a camera lucida at 200X magnification. Measures of dendritic length and dendritic branching were obtained from those drawings. To be included in the study, the dendritic trees had to be well impregnated and in full view, unblocked by blood vessels, astrocytes or clustering of dendrites from other cells. They also had to appear intact and visible in the plane of section. Cell bodies of pyramidal neurons had to be located within the sensorimotor cortex (as defined by Zilles and Wree, 1995). For branch order analysis, each branch segment was counted and summarized according to methods of Coleman and Riesen (1968): branches emerging from the cell

body (basilar) were first order. After the first bifurcation, branches were considered second order, etc. Quantification of each branch using this method provides an indication of dendritic arbour complexity. To obtain an indirect measure of dendritic length, the Sholl analysis (Sholl, 1956) of ring intersections was used. The number of intersections of dendrites with a series of concentric circles at 20 μm intervals from the centre of the cell body was counted for each cell. A reflection of total dendritic length (in μm) can be determined by multiplying the number of intersections by 20. The mean of the measurements of five cells per hemisphere per rat was used for statistical analyses.

Infarct measurements

Images of mounted cresyl violet-stained or Golgi-Cox sections at standardized levels (6 different planes, figure 2) were captured digitally. The cross-sectional area of each hemisphere was measured using the NIH IMAGE software, Ver.1.62. The data was expressed as percentage of the intact side of the brain.

3.4. Results

Infarct size

Infarct size was determined by measuring areas of non-infracted tissue in both the damaged and the undamaged sides of the brain. Measures are represented as percentage of the normal hemisphere. When the infarct size was calculated as a ratio of the intact hemisphere, a simple ANOVA revealed that overall animals with latero-frontal damage had larger lesions than animals with motor cortex damage ($F(1,14) = 9.19$, $P < 0.05$; Motor = 91.35 ± 1.19 %, Latero-Frontal = 82.42 ± 2.29 %). Figure 1 illustrates an example of an animal with motor cortex damage and an animal with latero-frontal cortex

damage produced by the MCA occlusion.

Cylinder test

All groups actively explored the cylinder and they reared and supported their body against the walls with their forelimbs. The asymmetry score of forelimb use was calculated for each group and it showed that both types of lesions produced an impairment. Control animals placed both forepaws on the cylinder wall during vertical exploration whereas animals with lesions relied on the paw ipsilateral to the lesion having

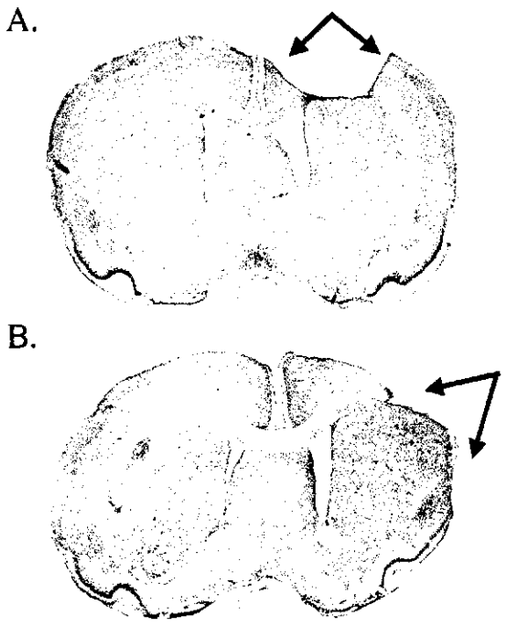

Figure 3.1.: Photomicrographs of a coronal section through the middle of the lesion of (A) a motor cortex lesion and (B) a latero-frontal lesion (cresyl violet). Arrows mark lesion boundary.

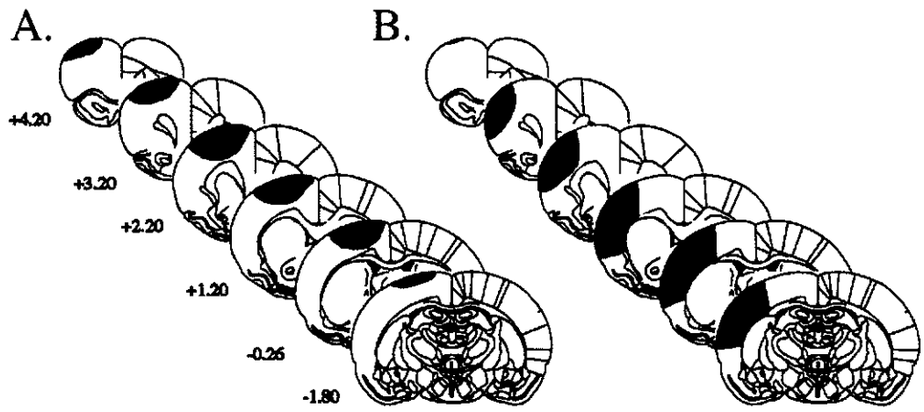

Figure 3.2.: Representative diagrams illustrating the six different planes where the measurements for estimating the infarct size were taken. (A) Illustrates a typical infarct after motor cortex lesion. (B) Illustrates a typical infarct after latero-frontal cortex damage.

fewer contacts with the contralateral paw. A repeated measures ANOVA showed a significant effect of lesion group ($F(2,21) = 22.02$, $P < 0.0001$), no main effect of test day, ($F(3,63) = 1.86$, $P = 0.14$), nor an interaction of group by day ($F(6,63) = 1.12$, $P = 0.36$) (Figure 3B). Follow-up tests (Fisher's LSD) showed that the control group differed form the motor cortex and the latero-frontal cortex group ($P < 0.001$), which in turn did not differ between themselves ($P = 0.61$).

Reaching

Single pellet: Animals learned to reach for the food pellets and before the lesions all animals achieved a baseline of at least 50% success. Animals were tested 1, 3, 7 and 14 days after the lesions and their performance was analyzed. Two kinds of analysis were performed: total success and success on first reach. Both, quantitative and qualitative

analyses showed that the lesions produced significant impairments in reaching success. When total success was analyzed, a repeated measures ANOVA showed a significant effect of group ($F(2,21) = 6.21$, $P < 0.01$), test day, ($F(3,63) = 8.33$, $P < 0.001$), but no significant interaction ($F(6,63) = 2.114$, $P = 0.06$) (Figure 4B). Follow-up tests (Fisher's LSD) showed that the control group differed form the motor and the latero-frontal groups ($P < 0.05$), which in turn did not differ between themselves ($P = 0.14$). When success on first reach was analyzed, a repeated measures ANOVA showed a significant effect of

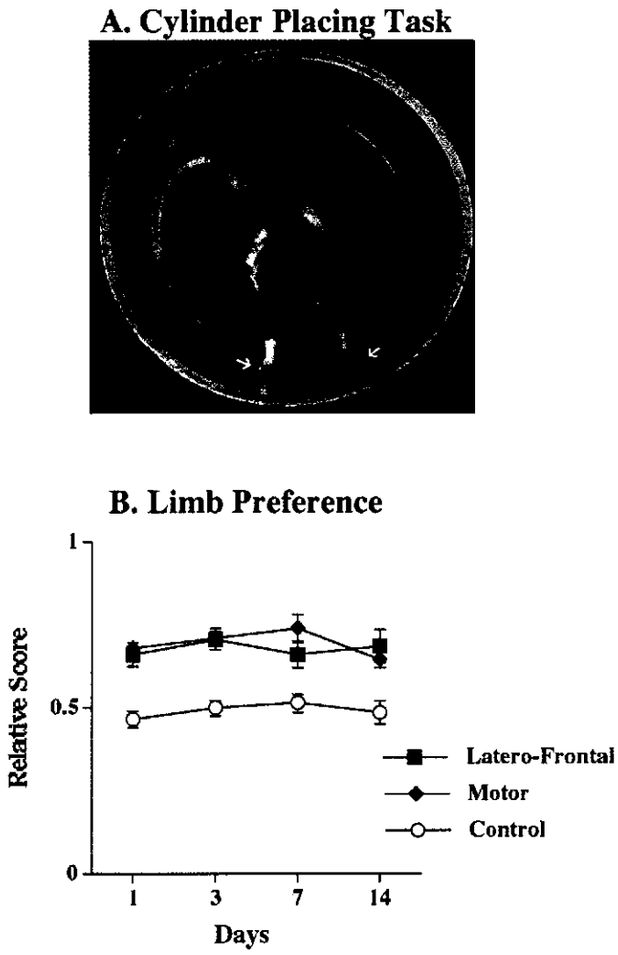

Figure 3.3.: Top panel shows a picture of a control animal in the cylinder. Arrows show use of both forepaws for support during rearing. Bottom panel shows performance on this task before the lesion (BL) and on days 1, 3, 7, and 14 post-stroke (±SE). A preference score of 0.5 indicates no preference on the use of left or right forepaw, a score of 1 indicates complete use of the ipsilateral forelimb. Note that both groups relied more on the ipsilateral forelimb for postural support during vertical exploration.

lesion group ($F(2,21) = 4.71$, $P < 0.05$), test day, ($F(3,63) = 4.25$, $P < 0.01$), but not an interaction ($F(6,63) = 1.85$, $P = 0.10$) (Figure 4C). Follow-up tests (Fisher's LSD) showed that the control group differed form the motor and the latero-frontal groups ($P < 0.05$), which in turn did not differ between themselves ($P = 0.38$). Subsequent to "rehabilitation" in tray reaching no significant differences in total success and first reach success were detected among the groups: ($F(2,21) = 1.64$, $P = 0.21$) and ($F(2,21) = 1.46$, $P = 0.25$) respectively.

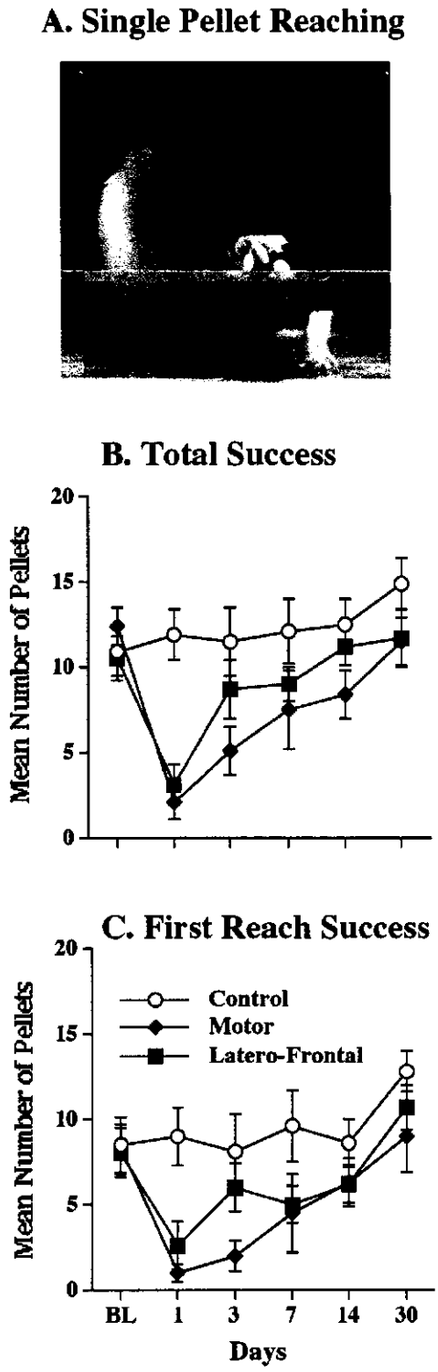

Figure 3.4.: (A) Single pellet-reaching task: a rat reaches through a slot for a single food pellet located on a shelf. (B) Total reaching success and (C) first reach

success in the single pellet task (number of pellets retrieved out of 20 ±SE) for control, motor cortex lesion (motor) and latero-frontal cortex (latero-frontal) groups. Note that before the lesion (BL) all groups were equally successful in obtaining pellets in both measures. Both lesion groups were significantly different than controls on days 1, 3, and 7 post-lesion. On day 30, after "rehabilitation" animals in both lesion groups scored comparable to controls.

Tray Reaching: All animals quickly learned to reach through the bars and successfully retrieve the chicken food pellets. At the end of the seven days of training (day 22) animals were videotaped for 10 minutes and their performance analyzed. Lesion animals performed to similar levels of accuracy when compared to controls (Table 3). A simple ANOVA showed no significant main effect of group ($F(2,21) = 1.46$, $P = 0.25$; Control = 69.5 ± 1.8 %, Motor = 66.3 ± 3.7 %, Latero-Frontal = 62.4 ± 2.8 %).

Qualitative analysis of single pellet

A summary of scores for component movements for motor and latero-frontal cortex groups on days 1 and 30 are illustrated in figures 5A and 5B respectively. A repeated measures ANOVA (all elements X group) showed a significant effect of lesion group ($F(2,21) = 29.52$, $P < 0.001$), test day, ($F(39,780) = 12.28$, $P < 0.001$), and an interaction of group by day ($F(78,780) = 5.12$, $P < 0.001$) (Figure 5C). Follow-up tests (Fisher's LSD) showed that the control group scored better (lower) than animals with either kind of lesion ($P < 0.01$). Animals with latero-frontal lesions scored better (lower) than animals with motor cortex lesions ($P < 0.01$). With recovery time, and after tray training, there were substantial improvements in both the motor cortex and latero-frontal

cortex groups, but residual deficits could still be observed in the animals with motor cortex lesions.

The analysis of day 1 performance indicated that animals with motor cortex lesions were impaired in all elements. By day 30 they showed significant recovery although there were persistent impairments in the lifting, aiming, and advance components of the reach and in supination of the paw upon withdrawal and in releasing the food to the mouth. Some of the movement impairments that contributed to their poor qualitative scores are illustrated in Figure 6. As the animals approached the slot in order to locate the food pellet, rather than pointing the nose at the food pellet, as did the control rats, they sniffed in an opposite direction to the location of the pellet (Figure 6a). The motor cortex lesion rats also failed to use a normal supporting posture of the contralateral-to-reach forelimb and its ipsilateral hind limb. Rather they used the wall of the cage as a crutch in order to support their body weight (Figure 6b,c). The contralateral-to-reach forepaw crossed the midline of the body (and slot; Figure 6d) instead of adopting a normal lateral position. Once the animals grasped a food pellet, they shifted the weight of their forequarters onto the grasping limb. Thus, once they withdrew it from the slot, the forelimb and the paw grasping the food pellet dropped to the floor (Figure 7a). They were also impaired in supinating the paw and oriented their snout to the back of the grasping paw (Figure 7b).

Animals with latero-frontal cortex damage also displayed poor alignment of the nose to the food pellet (Figure 6e) and displayed a wider base of support in the hind limbs, typified by a more lateral placement of the good hind limb (Figure 6f). They were also impaired in retrieving the food pellet from the paw after grasping. Rather than

supinating the paw in order to bring the pellet to the mouth, the rat's mouth came in contact with the dorsal surface of the paw (Figure 7c). On some occasions, consumption occurred because the pellet was dropped and retrieved from the floor. The rats were also impaired in using the contralateral forepaw to assist the good forepaw (Figure 7d).

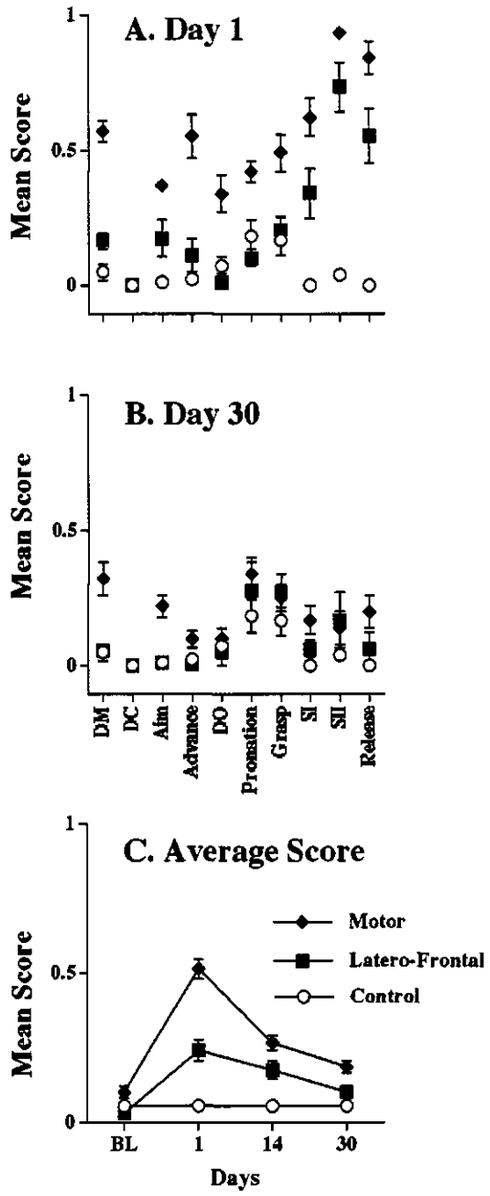

Figure 3.5. Qualitative scores of ten elements comprising a reach on post surgical days 1 (A) and 30 (B). Note that even at 30 days post-lesion animals with motor cortex damage displayed deficits in some of the components of the reach. (C) Qualitative scores of single pellet reaching movements before the lesion (BL) and on days 1, 14 and 30 after the lesions. Digits to the midline (DM); Digits close

(DC); Aim; Advance; Digits open (DO); Pronation; Grasp; Supination I (SI); Supination II (SII); Release.

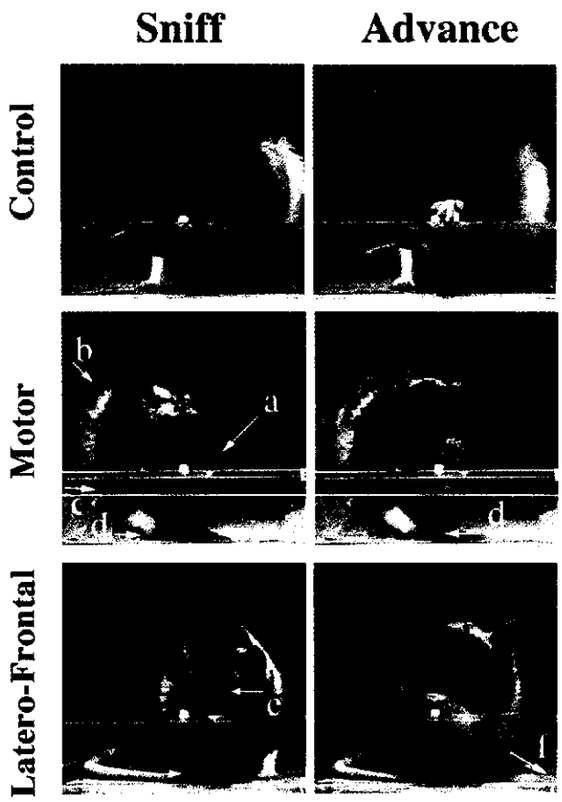

Figure 3.6. Illustration of some of the early components of the reach (Sniff and Advance) by a control rat (top), a motor cortex lesion rat (middle), and latero-frontal cortex lesion rat (bottom). Note (arrows) that in the motor cortex animals the sniff of the pellet is opposite to the food, their body rests against the wall of the box and the forepaw contralateral to the reaching paw is crossed to the midline. Animals in the latero-frontal cortex group also exhibited deficits orienting to the pellet and in supporting their weight with their hind limbs.

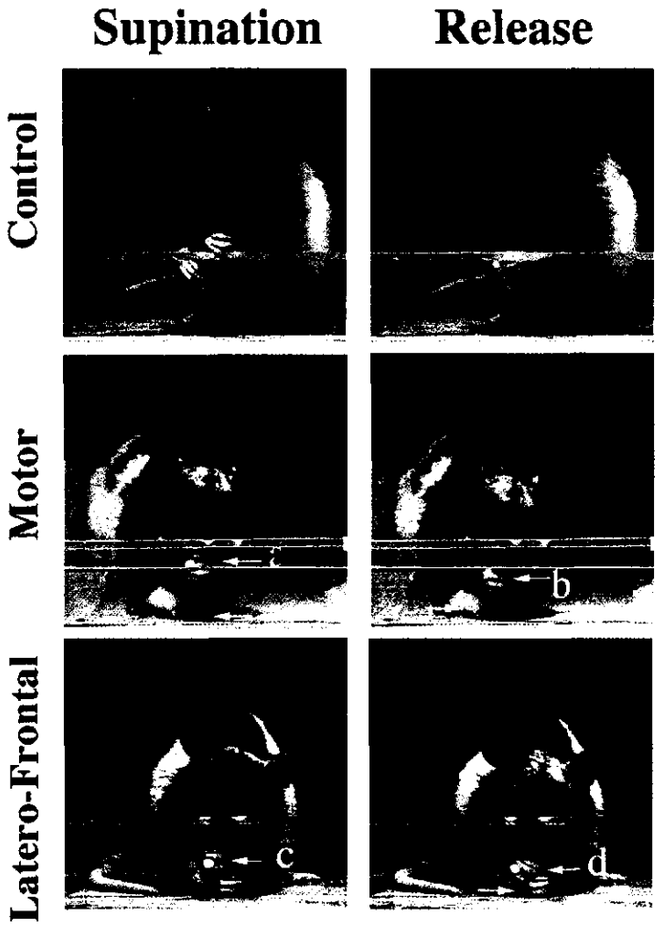

Figure 3.7. : Illustration of the late components of the reach (Supination II and Release) by a control rat (top), a motor cortex lesion rat (middle), and latero-frontal cortex lesion rat (bottom). Note (arrows) that animals in both groups have to drag the paw through shelf to retrieve the pellet and the release is not helped by the contralateral forepaw.

Intracortical microstimulation

Means and standard errors for the area representation of CFA and RFA of the intact hemisphere are shown in Figure 8D. An ANOVA showed no significant differences in the representation of CFA ($F(2,9) = 1.02$, $P = 0.39$), nor of RFA ($F(2,9) = 0.247$, $P = 0.78$) among the injured and control groups. This result suggests that the electrophysiological properties of the forelimb cortical area of the intact motor cortex (the one supporting the skilled movements) remained functional after either lesion. Representative motor maps for control, motor cortex lesion and latero-frontal lesion are shown in figures 8A, 8B, and 8C.

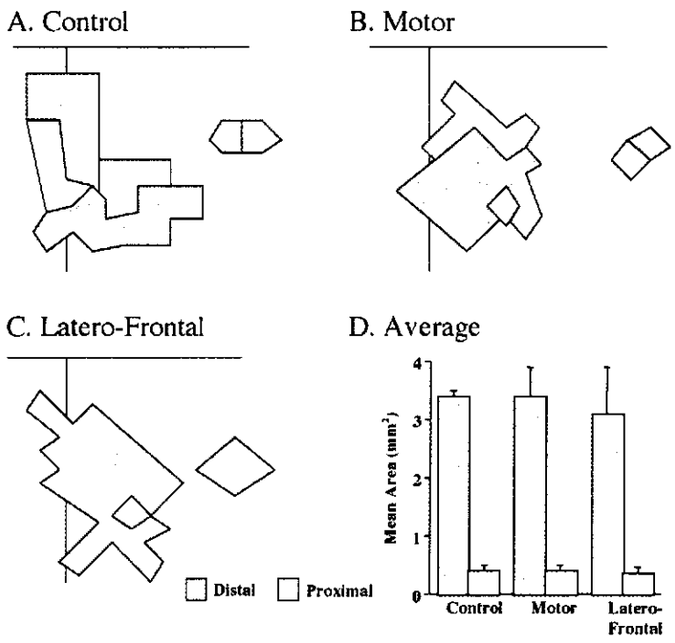

Figure 3.8. : Representative maps of control (A), motor cortex lesion (B), and latero-frontal cortex lesion (C) showing distal (digit and wrist) and proximal (elbow/shoulder) areas. (D) Mean are (mm^2) of movement representations.

Golgi-Cox Analysis

When pyramidal cells of layer V in the forelimb area in the intact hemisphere were analyzed, an increase in dendritic length and in the number of branches in the basilar field was observed in both lesion groups (Figure 9).

Dendritic Length: A simple ANOVA showed a significant effect of group ($F(2,9) = 8.99$, $P < 0.001$). A follow-up test (Fisher's LSD) showed that animals with motor and latero-frontal cortex damage had longer dendritic trees that differed from controls ($P < 0.01$; Table 2).

Dendritic Branching: A simple ANOVA showed a significant effect of group ($F(2,9) = 7.34$, $P < 0.05$). A follow-up test (Fisher's LSD) showed that again both lesion groups had more branches when compared to controls ($P < 0.05$; Table 2).

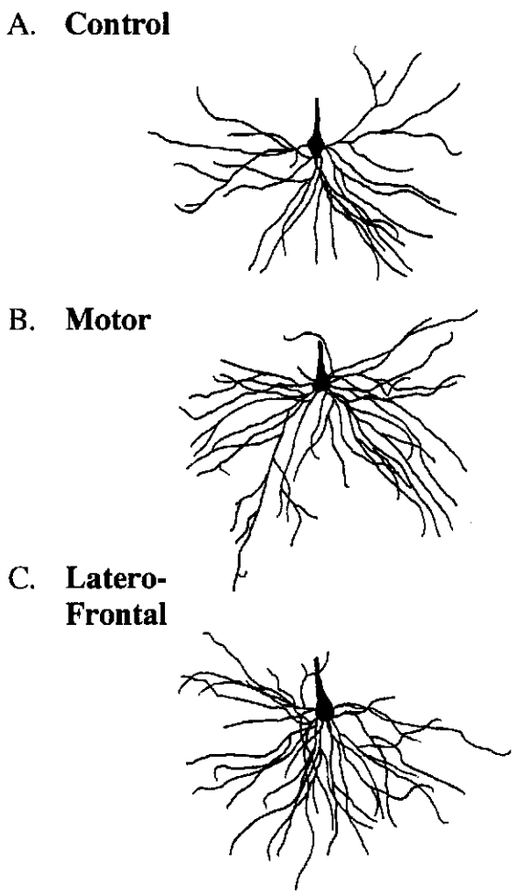

Figure 3.9: Camera lucida drawing of representative layer V pyramidal neurons in Zilles' area FL contralateral to the lesion from (A) control, (B) motor cortex lesion, and (C) latero-frontal cortex lesion groups. Animals with both lesions show longer dendrites and branchier cells.

Table 3.2. Summary of the morphological changes in the basilar fields of the layer V pyramidal neurons of the forelimb area of the intact hemisphere.

Group	SA	BOA

Control	142.76 ± 5.3	39.25 ± 0.8
Forelimb	179.95 ± 5.3*	48.55 ± 1.8*
Latero-Frontal	178.4 ± 9.5*	48.1 ± 2.6*

SA (Sholl Analysis), BOA (Branch Order Analysis)

*Significantly different from control ($p < 0.05$).

3.5. Discussion

It is well known that unilateral motor cortex lesions produce impairments in movements of the contralateral side of the body. Nevertheless, there are anecdotal reports of ipsilateral motor impairments, especially in the performance of skilled movements. The objective of this study was to assess the effects of frontal cortex injury on ipsilateral movements; i.e., movements mainly controlled by the intact hemisphere. Forelimb use was evaluated by spontaneous limb preferences in exploration, as well as in quantitative and qualitative measures of skilled reaching for food and plastic changes in the intact hemisphere as assessed by measures of motor cortex dendrites in Golgi-Cox stained tissue and intracortical microstimulation movement mapping. Although the intact hemisphere displayed plastic changes and normal rostral and caudal forelimb motor maps, there were enduring deficits in both quantitative and qualitative measures of skilled reaching. Similar acute and chronic impairments followed both motor cortex lesions and lateral frontal cortex lesions that spared the motor cortex. This demonstration that cortical injury can impair ipsilateral-to-lesion limb skilled movements suggests that these

movements normally involve some degree of bilateral cortical control not seen in more reflexive limb use. The results also suggest that within hemisphere plastic changes are related to recovery/compensation within that hemisphere instead of (or in addition to) the recovery of contralateral movements.

Some evidence suggests that recovery/compensation may be mediated by intact tissue surrounding the injury [Jenkins and Merzenich 1987; Stroemer et al., 1993; Castro-Alamancos and Borrel, 1995; Nudo et al., 2001 and Nudo 2003]. Other evidence suggests that changes in other brain areas, including the intact hemisphere, mediate compensation/recovery [Jones and Schallert 1992; Schallert et al., 1997; Jones 1999; Biernaskie and Corbett 2001; for reviews see Witte et al., 1997; Nudo 2003]. That the intact hemisphere may be involved in compensation/recovery is supported by findings of time-dependent metabolic and neural changes in that hemisphere [Jones and Schallert 1992, 1994; Szele et al., 1995; Napieralski et al., 1996; Jones 1999; Uryu et al., 2001]. In fact, there is evidence that suggests that plastic processes in the intact hemisphere may be activated to such an extent that the hemisphere becomes functionally supernormal, supporting an increase in movement skill in its contralateral forepaw [Bury and Jones 2002; 2004]. The idea is that these plastic changes in the undamaged hemisphere enhance motor function under the control of that hemisphere. Nevertheless, there is substantial, but perhaps more anecdotal, evidence in animals and humans indicating that bilateral motor deficits can follow unilateral damage [see Table 1]. Such findings suggest that the plastic changes in the intact hemisphere may be compensatory processes that includes that hemisphere's function instead of (or in addition to) functions associated with the lost tissue of the other hemisphere. It was this possibility that was examined in the present

experiments. Using lesions made to the motor cortex and "control" lesions to the lateral frontal cortex, the function of the intact hemisphere was assessed by tests of spontaneous limb use and skilled limb use. To ensure maximum recovery, animals were studied for two weeks following surgery and were then given additional training in a less demanding reaching task [Whishaw et al., 1986; Vergara-Aragon et al., 2003] before being reassessed. To determine if plastic changes occurred in the intact hemisphere, dendritic arbores were quantified in motor cortex layer V cells using Golgi-Cox stained tissue and with intracortical microstimulation mapping of the rostral and caudal regions of the motor cortex.

The surprising finding of the present study was that there were severe and enduring deficits in skilled reaching in the ipsilateral forelimb following both motor cortex and latero-frontal cortex damage. This finding is consistent with multiple reports of patients showing persistent loss in strength, speed, and difficulty in making complex movements with the ipsilateral arm after a unilateral stroke and with similar incidental reports in animals [see Table 1]. The impairment in skilled reaching could stem in part from shock, or postural abnormalities. The persistence of the impairments however, suggests that it is more likely related to the complexity of movements required for skilled reaching. This idea is further supported by the contrasting results obtained in spontaneous limb use in the cylinder task, which seemingly demonstrated normal use of the ipsilateral limb for more reflexive support during vertical exploratory movements. Further evidence for the idea that it is task complexity that involves the ipsilateral limb was the finding that intensive training in the tray-reaching task facilitated compensation/recovery although it did not completely ameliorate the impairment. It is also possible that contralateral

deficits, such as posture aberrations, contribute to the reaching impairments observed on the ipsilateral forelimb. Although the impairments in one side of the body may have an indirect effect on the impairments detected during reaching with the ipsilateral limb, careful kinematic analysis showed an absence of independent multiarticulated wrist and digit movements with the ipsilateral paw. These impairments were obvious in absence of supination of the paw upon withdrawal and in the release of the pellet to the mouth.

The present study confirms multiple reports of increased plasticity in the intact hemisphere after cortical injury [Jones and Schallert, 1992; Witte et al., 1997; Jones, 1999; Gonzalez and Kolb 2003]. Because there was some recovery/compensation from the marked impairments in skilled reaching, the present study suggests that these plastic changes are mediating the recovery of the ipsilateral side of the body instead of (or in addition to) the contralateral side of the body. Substantial evidence suggests that the size and conformation of the motor map reflects plastic processes related to behavior [Nudo et al., 1996; Kleim et al., 1998]. The finding that the motor map of the intact hemisphere was largely unchanged suggests that the recovery/compensation could be mediated by adaptations within brain regions outside the neocortex or through mechanisms that are not manifested as changes in motor maps. Of course, it is possible that because the mapping portion of the experiment was performed at the apex of recovery/compensation, map changes had dissipated [Friel et al., 2000; Nudo and Milliken, 1996]. Although we did not looked for ipsilateral movements induced by the electrical stimulation, bilateral movements elicited by ICMS after aspiration of the motor cortex are known [Kartje-Tillotson and Castro, 1985; Emerick et al., 2003], so although the lesions did not changed

the overall CFA and RFA representations on the contralateral hemisphere, it is possible that the lesions could have changed representations of the ipsilateral hemisphere.

Previous work has shown that skilled reaching performance in the single pellet task improves with training on a simpler reaching task [Vergara-Aragon et al., 2003]. To maximize performance on the single pellet task, here animals received two hours a day of training in the tray task for one week. Although significant improvements were observed when returned to the single pellet task detailed inspection of the movement components revealed persistent deficits. The improvement in the rats with motor cortex lesions was found in posture, in the supination of the forelimb, and in releasing the pellet to the mouth.

One of the strengths of the present study was the use of qualitative measures of skilled limb use in addition to assessments of success. Because skilled reaching for food is a complex act, measures of success can be dissociated from measures of how the limb is used. That is, following an injury it is possible that an animal can recover its ability to grasp food [Whishaw et al., 1991; Whishaw, 2000], while still being unable to recover normal movements. Just such a dissociation and/or differences in lesion parameters may be responsible for reports of normal or improved performance on end point measures that have been used to assess performance [Bury and Jones 2002; 2004]. An additional strength of the present study was the examination of more reflexive limb movements in juxtaposition to skilled limb movements. This examination confirmed that paw placing by the ipsilateral limb during rearing in a small cylinder appeared unaffected by the lesions at the same time that pronounced impairments occurred in skilled reaching.

Thus, together, these findings suggest that assessment of the undamaged hemisphere's contribution to behavior requires a comprehensive movement evaluation.

It is interesting that damage to the latero-frontal cortex was also associated with impairments in use of the ipsilateral limb (although this impairment was not as severe as the one seen with motor cortex lesions). This area of the neocortex represents the face region of sensorimotor cortex [Hall and Lindholm, 1974; Donoghue and Wise, 1982; Neafsey et al., 1986; Remple et al., 2003] and has not previously been implicated in skilled forelimb movements. Nevertheless, the latero-frontal and not the motor cortex per se, is the region of the cortex that is usually damaged following middle cerebral artery (MCA) stroke in rats and this form of stroke is reported to be associated with contralateral limb impairments on a wide variety of tests. Thus, the present findings suggest that latero-frontal cortex can contribute to forelimb control.

In conclusion, an examination of cortical change in the intact hemisphere and an assessment of forelimb function mainly controlled by that hemisphere revealed both plastic changes and functional changes. The results suggest that the plastic changes and the functional changes are related and co-occur because skilled movements involve the participation of both hemispheres. These findings and conclusions in our studies of the rat are consistent with many reports in both human and animal studies indicating that skilled movements are under bilateral control.

3.6. References

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Chronic Low-Dose Administration of Nicotine facilitates Recovery and Synaptic Change After Focal Ischemia in Rats

4.1. Abstract

The current study examines the effects of chronic administration of nicotine on motor behavior after focal stroke in rats. Animals were trained in a tray-reaching task for two weeks and then they were divided into four groups: 1) control, 2) control+ nicotine, 3) stroke, and, 4) stroke+nicotine. A unilateral stroke in the sensorimotor cortex contralateral to the forepaw used for reaching was produced by devascularization of the surface blood vessels. Forty-eight hours after the lesions, and then for a total of twelve days, animals received two daily injections of nicotine (0.3 mg/kg). Animals were tested in a skilled reaching task, and tests of forepaw asymmetry and forepaw inhibition one week after the lesions and every other week for a total of seven weeks. The animals were then trained on a more demanding skilled reaching task for an additional three weeks. Pyramidal cells in forelimb and cingulate areas then were examined for dendritic length and branching using a Golgi-Cox procedure. Behavioral results showed that by the end of behavioral testing lesion animals receiving nicotine showed significant behavioral improvement relative to untreated lesion animals. Lesion animals treated with nicotine showed an increase in dendritic length and branching in pyramidal cells of the forelimb area contralateral to the lesion and in cingulate cortex ipsilateral to the lesion. The results suggest that the behavioral enhancement in the stroke+nicotine group might be attributable to the enhanced dendritic growth in residual cortical motor regions.

4.2. Introduction

Studies on both human patients and animal models of stroke suggest that the cerebral cortex undergoes significant functional and structural plasticity that could last up to months following the insult (for reviews see Hallett 2001; Nudo et al, 2001, 2003). These plastic changes have received much attention in recent years, in part because they may provide a basis for developing better strategies for stimulating functional improvement after stroke. Studies in primates (Nudo and Milliken, 1996; Xerri et al., 1998) and rats (Kleim et al., 2003) have shown that cortical motor representations are modifiable after cortical injury and this property of the cerebral cortex may support improved motor functions. In humans it has also been shown that reorganization of intact adjacent cortical tissue contributes to functional recovery after focal ischemia (Traversa et l., 1997; Cramer et al., 1997, 2000). Given that plastic changes in the cortex may support functional improvement, it is logical to try to enhance the endogenous plastic changes to further behavioral rehabilitation. One way to do this is to give a pharmacological agent capable of enhancing cerebral plasticity and thus reorganization of lost pathways. Nicotine is a psychostimulant known to have beneficial effects on neurological and neurodegenerative conditions. In humans, for example, nicotine has been suggested to have a role as a therapeutic agent and to be beneficial in diseases such as Parkinson's (Fagerstrom et al., 1994; Clemens et al., 1995; but see Lemay et al., 2004). Chronic administration of nicotine has been shown to enhance the execution of cognitive tasks in animals with frontal cortex or hippocampal lesions (Brown et al., 2000, 2001). These favorable effects of nicotine have been explained in terms of increases in trophic factors, increased release of different neurotransmitters, or enhanced brain plasticity.

Increases in dendritic length and spine density for example, have been shown in anterior cingulate cortex and nucleus accumbens after administration of nicotine in otherwise intact rats (Brown and Kolb, 2001).

The purpose of the present study was to examine if chronic administration of nicotine would have any effect on the behavior and brain plasticity of rats that have suffered unilateral focal ischemia that damaged the primary motor representation. The rats were pretrained in a skilled reaching task before being divided into 4 groups: 1) control, 2) control+nicotine, 3) stroke, and 4) stroke+nicotine. Two days after the surgeries and for a total of twelve days animals received two injections a day of 0.3mg/kg of nicotine. The behavior on the tray reaching task, as well as test of forepaw asymmetry and forepaw inhibition was studied postoperatively for a total of seven weeks. After seven weeks animals received an additional three weeks of training in a more demanding skilled reaching task. At the completion of the behavioral tests, dendritic changes in the intact hemisphere were examined using Golgi-Cox analyses of the pyramidal cells of layer V of the forelimb area, or in the injured hemisphere by examining pyramidal cells of layer V of the anterior cingulate region.

4.3. Materials and Methods

Subjects

Subjects were 20 male Long-Evans hooded rats, 4 months old and weighing 300-400 g at the beginning of the experiment. Animals were raised in the University of Lethbridge vivarium and were housed in groups of two individuals in clear plexi-glass cages. The colony room was maintained on a 12:12h light/dark cycle (08:00-20:00 h) and the temperature regulated at 22 °C. Experiments were conducted according to standards

set by the Canadian Council on Animal Care and approved by the University of Lethbridge animal care committee.

Food restriction

Three weeks before the lesions, the rats were changed to a restricted food intake: Each animal received 20 g of food per day (normal daily consumption ranges from 18-25gr) an hour after the testing session was completed. Their body weight was maintained at about 95-98% until the completion of the behavioural testing.

Surgery and lesion placement

Subjects were assigned to four different groups, control (n = 5), control+nicotine (n = 5), stroke (n = 5), and stroke+nicotine (n = 5). Stroke animals received an injection of atropine methyl nitrate (0.1 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) to facilitate respiration throughout surgery. Animals were then anesthetized (sodium pentobarbital 60 mg/kg i.p.) and placed in a stereotaxic apparatus. The lesions were produced by unilateral devascularization of the motor cortex (Sofroniew et al., 1983; Stephens et al., 1985; Kolb et al., 1997). In brief, a flap of bone and underlying dura were removed at coordinates corresponding to areas Fr2, Fr1, Fr3, FL and HL (Zilles, 1985). A rectangular hole in the skull was produced at stereotaxic coordinates anterior (A), lateral (L): A= +2.0 to –3.0 mm and L= 1.0 to 4.0 mm. using an electric dental drill while avoiding traumatic brain injury. All vessels and pia matter were rubbed away with sterile, saline-soaked cotton swabs.

Drug Administration

Two days after the surgeries all animals were taken away from the colony into a separate room for 20 minutes where they received one injection in the morning and one

in the afternoon for a period of 12 days. Rats were injected subcutaneously with saline (control and stroke) or nicotine hydrogen tartrate salt (Sigma, St Louis, MO, USA) 0.3 mg/kg (control+nicotine and stroke+nicotine).

Cylinder test (Forepaw Asymmetry)

Forelimb use for weight support during explorative activity was examined by placing the rats in a transparent cylinder (Figure 4.2) 20 cm in diameter and 30 cm high for three minutes (Schallert et al., 1997). The animals were individually placed in the cylinder during the three minutes of each testing session. A mirror was placed underneath the cylinder at an angle to allow the experimenter to videotape the animal's activity from a ventral view (Pinel et al., 1993). The cylindrical shape encouraged vertical exploration of the walls with the forelimbs, but the walls were high enough so that animals could not reach the top. Forelimb use was measured during vertical exploration. Animals were placed and videotaped in the cylinder once before the lesions were made. Each forepaw contact with the cylinder wall was counted. When simultaneous limb contact was observed, a touch was counted for each paw. The asymmetry score of forelimb use in wall exploration was calculated for each group (i.e., affected forelimb/(affected + unaffected)) to obtain a score where 0.5 represents perfect symmetry and any number closer to zero would suggest a decrease in the use of the affected limb.

Swimming Task (Forepaw Inhibition)

Rats were trained for two days pre-lesion to swim to a visible platform located at the end of a rectangular aquarium (120x43x50 cm), (Figure 4.3). The water in the pool was maintained at 25°C at all times. During the training phase, animals were released from the opposite end of the tank and consecutive trials were given until they swam

directly to the platform without touching the walls of the aquarium (about 10 trials for each animal). Kolb and Whishaw (1983) described that normal animals hold their forelimbs immobile under their chins while swimming using their hind limbs to propel through the water. By the end of the second day animals were quite familiar with the tank and the task therefore inhibition of the forepaws was observed. On the third day, three trials were videotaped for each animal as they swam directly to the platform.

Disruption to the normal swim pattern (swim score) was quantified by counting the number of strokes made by each forelimb on each trial. The swim score of forelimb inhibition was calculated for each group by subtracting the number of contralateral forelimb strokes minus the ipsilateral number of forelimb strokes (i.e., contralateral forelimb strokes (-) ipsilateral forelimb strokes).

Reaching boxes and training

Training boxes (Figure 4.4) were made of plexiglass with dimensions 26 cm high, 28 cm deep, and 19 cm wide. The front of the boxes was constructed of 2mm bars separated from each other by a 9 mm gap. Clear plexiglass tops allowed access to the inside of the box. A 4 cm wide and 0.5 cm deep tray was mounted in front of the bars. The tray contained food fragments weighing approximately 30 mg each. Animals had to reach between the bars, grasp the food and retract it where they were able to freely eat. Subjects were trained for a total of 15 days before the lesions to obtain a stable baseline before the surgeries. The rats were trained individually for one half hour per day and then at the end of a two- week training period their performance was videotaped for a five-minute interval. Each time the rat reached through the bars whether or not food was obtained was scored as a "reach" and each time food was successfully returned to the

cage and consumed was scored as a "hit". The percentage of hits to total reaches was then calculated for each animal's taped performance.

Single Pellet Reaching

Boxes (Figure 4.5) were made of clear Plexiglas, with the dimensions 45 cm deep by 14 cm wide by 35 cm high. In the center of each front wall was a 1 cm-wide slit which extended from 2 cm above the floor to a height of 15 cm. On the outside of the wall, in front of the slit, mounted 3 cm above the floor, was a 2 cm-wide shelf. Two indentations on the floor of the shelf were located 2 cm from the inside of the wall and were centered on the edges of the slit where rats could reach. Food pellets (45 mg Rodent Chow food pellets, Bioserve Inc.) were placed in the indentation contralateral to the limb with which the rat reached (Whishaw and Pellis, 1990). Following each reach, a short pause preceded the presentation of the next pellet, and an additional pellet could be dropped in the back of the box. This would encourage animals to return to the back of the box after each reach, which forced them to reposition themselves and prepare for the next reach. Success scores were computed as follows:

Success percent = (number of successful reaches/total given number of pellets) X 100

Qualitative reaching analysis

Reaching movements made during the single pellet task were analyzed using a rating scale derived from Eshkol-Wachmann Movement Notation (EWMN: Eshkol and Wachmann 1958; Whishaw et al., 1993) analysis of reaching. A reach was subdivided into ten components. (1) Limb lift: the limb is lifted from the floor with the upper arm and the digits adducted to the midline of the body. (2) Digits close: as the limb is lifted, the digits are semiflexed and the paw is supinated so that the palm faces the midline of the body. (3) Aim: using the upper arm, the elbow is adducted so that the forearm is

aligned along the midline of the body, with the paw located just under the mouth. This movement involves fixation of the distal portion of the limb, so that digits remain aligned with the midline of the body. This posture is likely produced by a movement around the elbow that reverses the direction of movement of the paw to compensate for the adduction of the elbow. (4) Advance: the head is lifted and the limb is advanced directly forward above and beyond the food pellet. (5) Digits open: as the limb is advanced the digits are extended and opened. (6) Pronate: using a movement of the upper arm, the elbow is abducted, pronating the paw over the food. Full pronation of the paw onto the food is aided by a movement of the paw around the wrist. (7) Grasp: as the pads of the palm or the digits touch the food, the food is grasped by closure of the digits. This closure can occur as an independent movement, or the grasp can occur as the paw is withdrawn. (8) Supination I: as the limb is withdrawn, the paw is dorsiflexed and is supinated 90^0 by a movement around the wrist and by adduction of the elbow. These movements can occur as soon as the food is grasped or can occur as the limb is withdrawn. (9) Supination II: as the rat sits back with food held in the paw, the paw is further supinated by 90^0 and ventroflexed to present the food to the mouth. (10) Release: the digits are opened and the food transferred to the mouth.

Five successful reaches were analyzed frame-by-frame on the video tapes. Each movement was rated on a one-point scale. If the movement appeared normal, it was given a score of "0"; if it appeared slightly abnormal but recognizable it was given a score of "0.5"; and a score of "1" was assigned if the movement was absent or completely unrecognizable.

Golgi-Cox analysis

Animals were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline. The brains were removed and weighed before being immersed in a 20 ml of Golgi-Cox solution where they remained for 14 days. The brains were then placed in a 30% sucrose solution for 2 days and cut on a vibratome at 200 μm and developed using a procedure described by Gibb and Kolb (1998). The basilar tree of layer V pyramidal cells within the forelimb motor cortex of the uninjured hemisphere, were traced using a camera lucida at 200X magnification. Ipsilateral to the lesion basilar dendrites of layer V pyramidal cells in anterior cingulate cortex (Cg3) were traced using a camera lucida at 200X magnification. Measures of dendritic length and dendritic branching were obtained from those drawings. To be included in the study, the dendritic trees had to be well impregnated and in full view, unblocked by blood vessels, astrocytes or clustering of dendrites from other cells. They also had to appear intact and visible in the plane of section. Cell bodies of pyramidal neurons had to be located in area Cg3 (Zilles, 1985) or within the sensorimotor cortex (as defined by Zilles and Wree (1995). For branch order analysis, each branch segment was counted and summarized according to methods of Coleman and Riesen (1968): branches emerging from the cell body (basilar) were first order. After the first bifurcation, branches were considered second order, etc. Quantification of each branch type using this method provides an indication of dendritic arbor complexity. To obtain an indirect measure of dendritic length, the Sholl analysis (Sholl, 1956) of ring intersections was used. The number of intersections of dendrites with a series of concentric circles at 20 μm intervals from the center of the cell body was counted for each cell. A reflection of total dendritic length (in μm) can be determined by multiplying the number of intersections by 20. The mean of the

measurements of five cells per hemisphere per rat was used for statistical analyses. .

Infarct Measurements

Images of mounted Golgi-Cox impregnated sections at standardized levels (8 different planes, (see Figure 1) were captured digitally. The cross-sectional area of the neocortex and complete hemisphere were measured on both sides of the brain (NIH IMAGE software, Ver.1.62). Because of the different histological techniques, the data was expressed as percentage of the intact hemisphere.

Statistical analysis

Analyses of variance (ANOVA) were used for all measures and Tukey-Kramer was used for *post hoc* evaluations of the behavioral data and Fisher's LSD for the anatomical data.

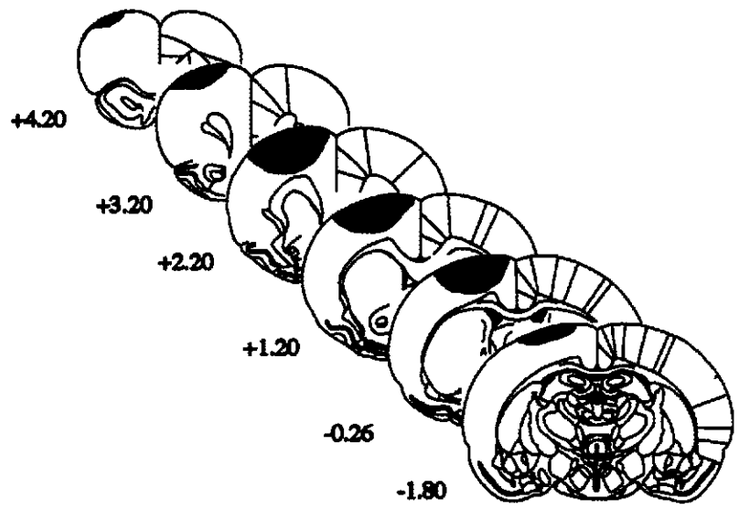

Figure 4.1: Representative diagram illustrating the six different planes where the measurements for estimating the infarct size were taken and a typical infarct after motor cortex lesions.

4.4. Results

Behavioral Results

Cylinder Test

All animals actively explored the cylinder and they reared and supported their body against the walls with their forelimbs on each test session. Overall stroke animals that received nicotine perfomed better than animals without treatment. A repeated measures ANOVA on the 7 weeks of testing showed a significant effect of group ($F(3,16) = 10.94$, $P < 0.001$), a main effect of test week, ($F(3,48) = 5.6$, $P < 0.01$), and a significant interaction ($F(3,48) = 2.21$, $P < 0.05$). Follow-up tests (Tukey-Kramer) showed that the control group differed from the stroke-untreated group and that the control+nicotine group differed from both lesion groups. Because the asymmetry score is calculated by dividing the number or touches with the affected (contralateral) limb over the sum of the affected plus the unaffected (ipsilateral) limb touches, it is possible that the main effect of group resulted from a reduction by the lesion animals in the number of touches with the unaffected limb. When the number of touches with the unaffected limb was calculated over the 7 weeks of testing, repeated measures ANOVA showed a significant effect of group ($F(3,16) = 5.09$, $P < 0.05$), a main effect of test week, ($F(3,48) = 8.14$, $P < 0.001$), and a significant interaction ($F(3,48) = 2.93$, $P < 0.05$). Follow-up tests (Tukey-Kramer) showed that the control+nicotine group used the contralateral paw for vertical support more than the stroke untreated group and significantly differed from it.

In order to ascertain if nicotine had an early or late effect on behavior, statistical analyses were performed for weeks 1 and 3 (early) and weeks 5 and 7 (late). When the asymmetry score of forelimb use was calculated for the early weeks no beneficial effects of nicotine were found. Both groups with lesions showed preferential use of the forepaw ipsilateral to the lesion. When the late weeks were analyzed, however, stroke animals that received nicotine did not differ from control animals (Figure 4.2). A repeated measures ANOVA on the early weeks showed a significant effect of group ($F(3,16) = 15.55$, $P < 0.0001$), no main effect of test week, ($F(1,16) = 0.006$, $P = 0.93$), nor the interaction ($F(3,16) = 0.91$, $P = 0.45$). Follow-up tests (Tukey-Kramer) showed that the control groups differed from the lesion groups. A repeated measures ANOVA on the late weeks showed a significant effect of group ($F(3,16) = 5.0$, $P = 0.012$), no main effect of test week, ($F(1,16) = 0.53$, $P = 0.47$), nor the interaction ($F(3,16) = 1.87$, $P = 0.17$). Follow-up tests (Tukey-Kramer) showed that both control groups differed form the stroke-untreated group. Stroke+nicotine animals did not differ from either control group.

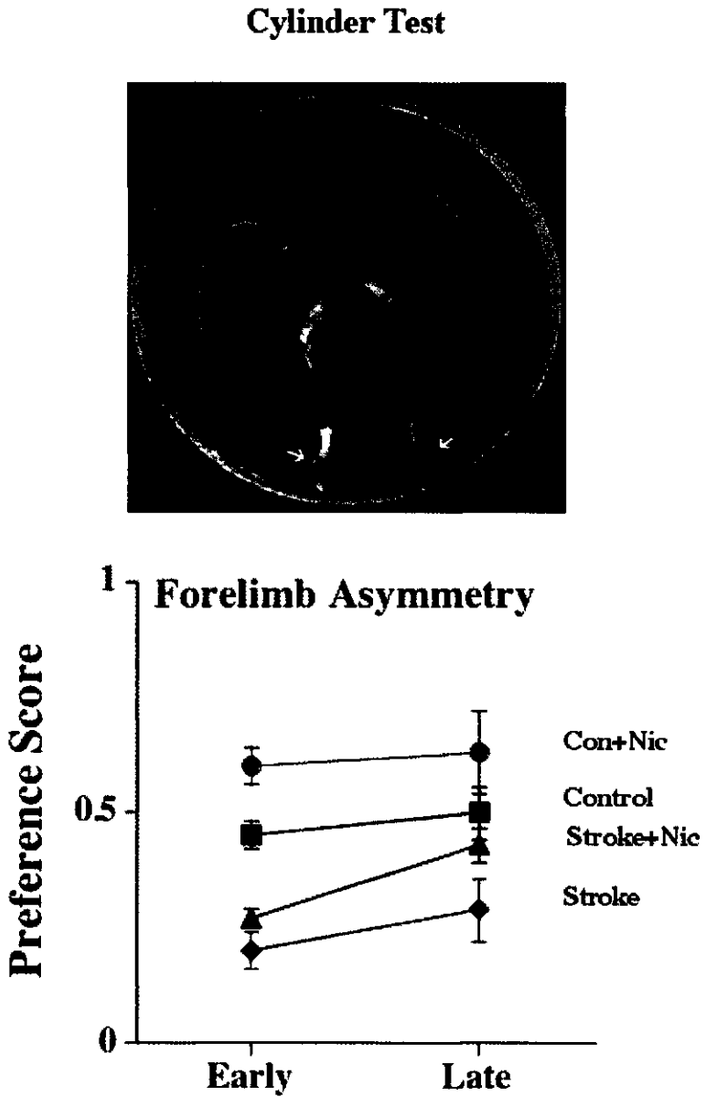

Figure 4.2: Top panel shows a picture of a control animal in the cylinder. Arrows show use of both forepaws for support during rearing. Bottom panel shows performance on this task early after the lesions (weeks 1 and 3) and later (weeks 5 and 7) (±SE). A preference score of 0.5 indicates no preference on the use of left or right forepaw a score of 1 indicates complete use of the ipsilateral forelimb.

Note that stroke animals treated with nicotine performed as well as controls on the later weeks of testing.

Swimming task

Before the lesions, rats in all groups learned to swim directly to the platform in a one day training session. By the end of the session animals showed almost perfect inhibition (less than one stroke per animal on average) of the forelimbs before climbing on top of the platform. After the surgeries, stroke animals in both groups showed asymmetry in limb inhibition as they paddle with the impaired (contralateral) limb, but not with the unimpaired (ipsilateral) limb during swimming. A repeated measures ANOVA over the 7 weeks of testing showed a significant effect of group ($F(3,16) = 14.21$, $P < 0.0001$), no effect of test week, ($F(3,48) = 51.71$, $P = 0.17$), and no significant interaction ($F(3,48) = 0.68$, $P = 0.71$). Follow-up tests (Tukey-Kramer) showed that the control group differed from both stroke groups and that the control+nicotine group differed from only from the stroke untreated group. When the number of paddles with the unaffected limb was calculated over the 7 weeks of testing, repeated measures ANOVA showed a significant effect of group ($F(3,16) = 11.05$, $P < 0.001$), a main effect of test week, ($F(3,48) = 24.18$, $P < 0.001$), but no significant interaction ($F(3,48) = 1.0$, $P = 0.45$). Follow-up tests (Tukey-Kramer) showed that the control+nicotine group paddled significantly more with the contralateral limb than any other group.

During the early weeks (weeks 1-3) after the suregeries stroke animals paddled more with the contralateral limb than the control animals. Nicotine, however, increased the number of paddles that control animals performed so the stroke+nicotine group was not different when compared to the control+nicotine group. On the late weeks (5-7),

nicotine proved to be beneficial to the lesion rats who did not differ from the control groups (Figure 4.3). A repeated measures ANOVA on the early weeks showed a significant effect of group ($F(3,16) = 8.03$, $P < 0.01$), no significant effect of test week ($F(1,16) = 3.78$, $P = 0.069$), and no interaction ($F(3,16) = 1.46$, $P = 0.26$). Follow-up tests (Tukey-Kramer) showed that the control group was different than both lesion groups but that the control+nicotine group was only different than the stroke-untreated group. A repeated measures ANOVA on the late weeks showed a significant effect of group ($F(3,16) = 6.8$, $P < 0.01$), no main effect of test week, ($F(1,16) = 3.20$, $P = 0.092$), nor the interaction ($F(3,16) = 1.25$, $P = 0.40$). Follow-up tests (Tukey-Kramer) showed, that the stroke untreated group significantly differed from both control groups.

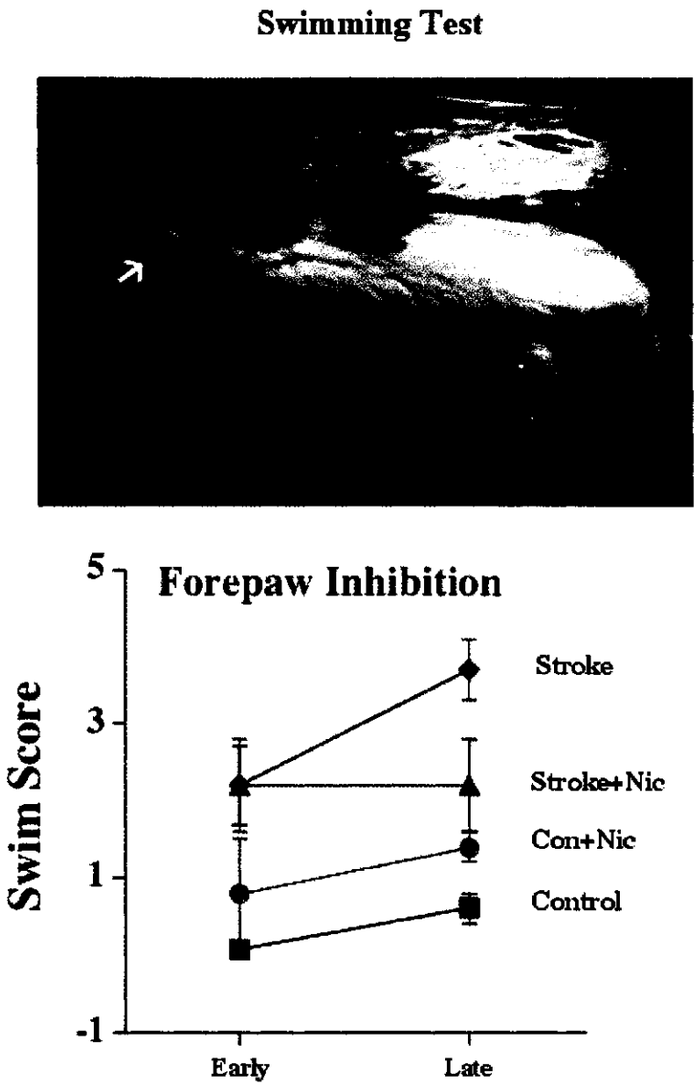

Figure 4.3: Top panel shows a picture of an animal before the lesions during swimming. Arrow shows the inhibition of the forepaws. Bottom panel shows the number of strokes (±SE) made with the affected forelimb early after the lesions (weeks 1 and 3) and later (weeks 5 and 7) (±SE). Animals treated with nicotine (control and stroke) showed similar performance on the last weeks of testing.

Tray Reaching

All animals quickly learned to reach for food and asymptoted at about 68% accuracy before the lesions. When a repeated measures ANOVA was run over the 7 weeks of testing a significant effect of group was found ($F(3,16) = 4.26$, $P < 0.05$), a significant main effect of test week ($F(3,48) = 11.85$, $P < 0.0001$), and a significant interaction ($F(9,48) = 6.46$, $P < 0.0001$). Follow-up tests (Tukey-Kramer) showed that the stroke-untreated group performed worse and differed form the control+nicotine group. No other differences were significant. Early after the lesions however, the stroke untreated group differed from both control groups (Figure 4.4). A repeated measures ANOVA on weeks 1-3 showed a significant effect of group ($F(3,16) = 5.85$, $P < 0.01$), no significant effect of test week ($F(1,16) = 2.4$, $P = 0.14$), but a significant interaction ($F(3,16) = 7.78$, $P < 0.01$). Follow-up tests (Tukey-Kramer) showed, that the stroke group had fewer successful attempts and differed from both control groups. A repeated measures ANOVA on the later weeks (5-7) failed to showed any significant effects of group ($F(3,16) = 2.41$, $P = 0.10$), no main effect of test week, ($F(1,16) = 0.85$, $P = 0.36$), nor the interaction ($F(3,16) = 1.84$, $P = 0.17$).

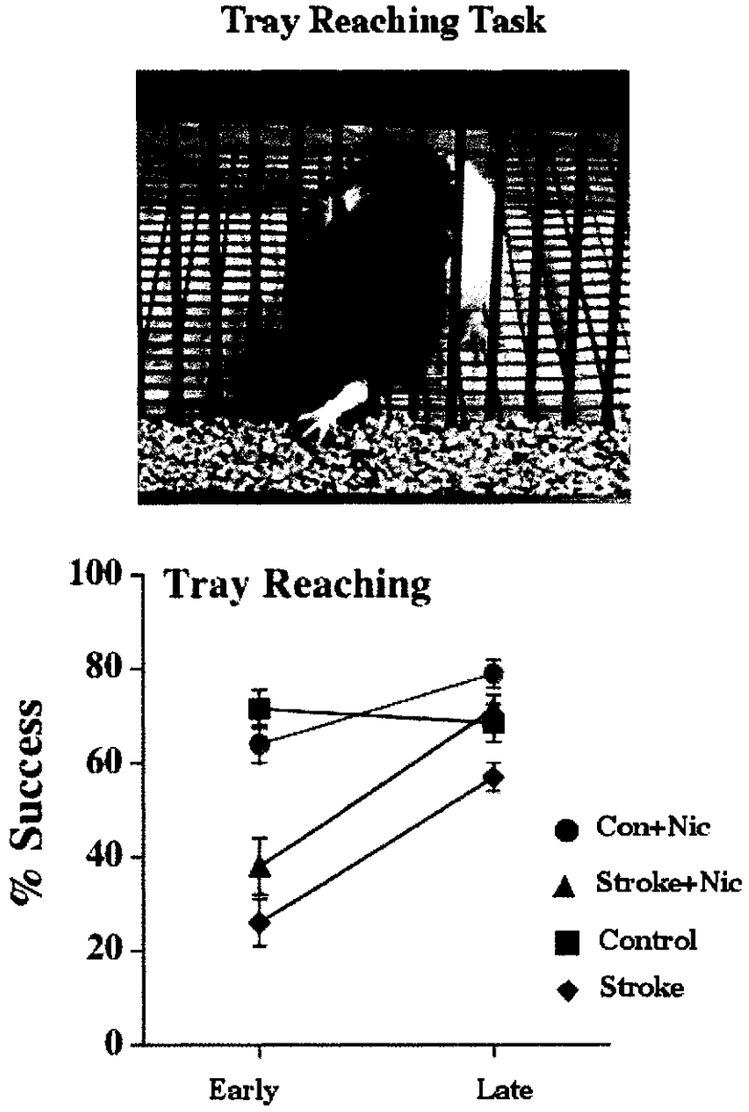

Figure 4.4: Top panel shows a picture of an animal on the reaching apparatus before the surgeries, arrow points at the animal's grasp for food. Bottom panels show the rat's performance early after the lesions (weeks 1 and 3) and later (weeks 5 and 7) (±SE). Note that by the last weeks of testing stroke animals that received nicotine performed as well as control animals.

Single Pellet Reaching

After the seven weeks of testing animals were trained to reach for single food pellets located on a shelf in front of them (Figure 4.5). All the animals learned to retrieve 20 pellets from the shelf within three days and starting on day four performance on a total of 10 days was analyzed. When success was analyzed no differences were found among the groups. A repeated measures ANOVA on the total success showed no effect of lesion group ($F(3,14) = 2.34$, $P = 0.117$), a significant effect of test day, ($F(9,126) = 2.07$, $P < 0.05$), but no significant interaction ($F(27,126) = 1.39$, $P = 0.11$).

Qualitative analysis of single pellet

The ten movement components of five successful reaches for the last day (day 10) were carefully examined frame by frame. Overall, stroke untreated animals showed greater abnormalities than the stroke animals treated with nicotine. An unexpected finding was observed however, the control+nicotine animals showed profound deficits as well. A repeated measures ANOVA showed a significant effect of lesion group ($F(3,14) = 12.18$, $P < 0.001$), movement element, ($F(9,126) = 23.06$, $P < 0.001$), and the interaction between group and element ($F(27,126) = 2.33$, $P < 0.001$) (Figure 4.5). Follow-up tests (Tukey-Kramer) showed that the control group scored better (lower) than the control+nicotine and the stroke-untreated animals. The control+nicotine also scored worse than the stroke+nicotine group. Qualitative analysis of the movements revealed that stroke+nicotine animals benefited from the treatment as they displayed better scores than the stroke-unteated animals in a number of movement elements (Figures 4.6 and 4.7): lift, digits open, grasp, supination I, supination II and release. Interestingly, control animals treated with nicotine showed marked impairments.

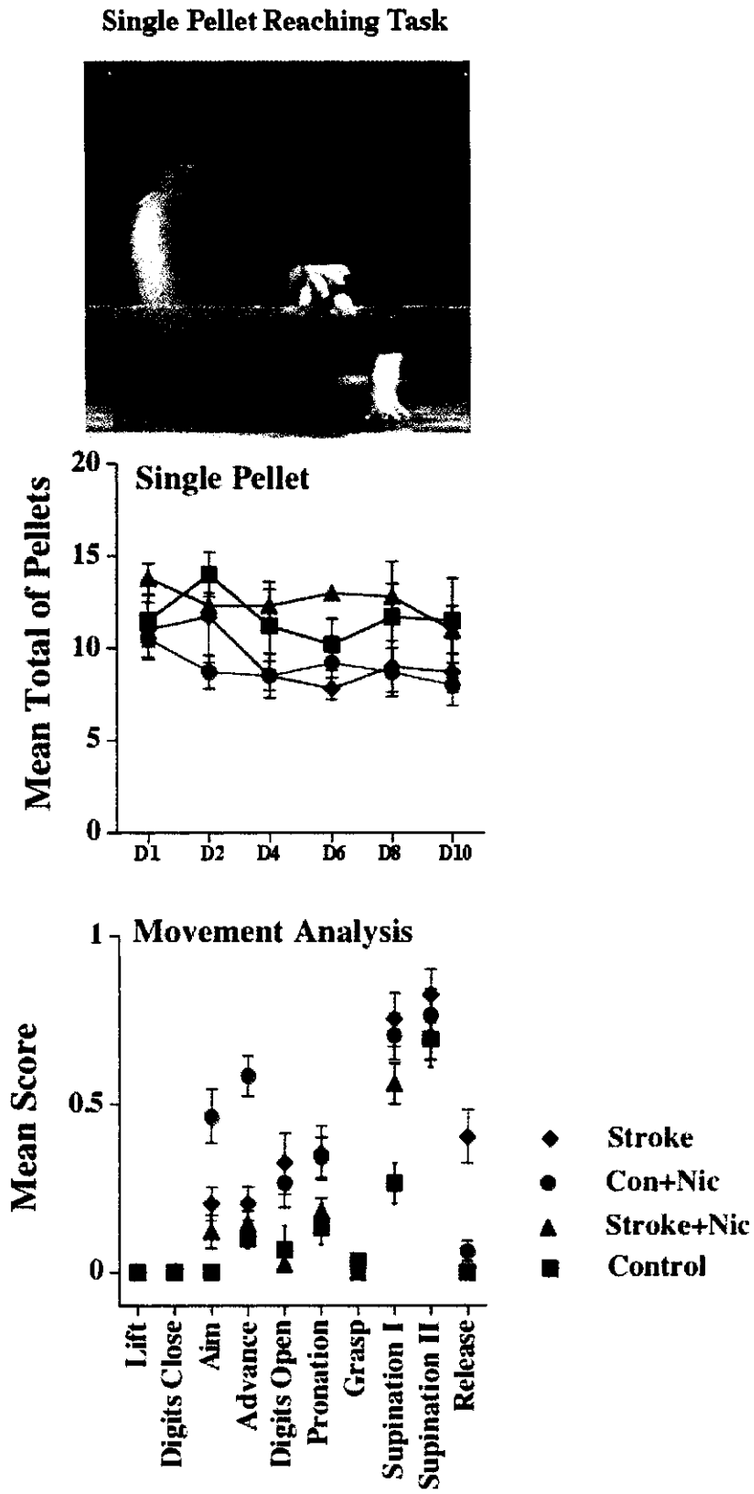
Single Pellet Reaching Task
Single Pellet
Mean Total of Pellets
20
15
10
5
0
D1
D2
D4
D6
D8
D10
Movement Analysis
Mean Score
1
0.5
0
Lift
Digits Close
Aim
Advance
Digits Open
Pronation
Grasp
Supination I
Supination II
Release
Stroke
Con+Nic
Stroke+Nic
Control

Figure 4.5: (Top panel) Single pellet-reaching task: a rat reaches through a slot for a single food pellet located on a shelf. (Middle Panel) Total reaching success displayed by the animals on days (D) 1, 2, 4, 6, 8 and 10 (±SE). Note that stroke animals that received nicotine displayed comparable acquisition rates than control animals. (Bottom Panel) Qualitative scores of ten elements comprising a reach on the last day of testing. Note that nicotine had a beneficial effect on the animals that received a stroke.

The nature of the deficits between the control+nicotine and the stroke-untreated groups however, was very different. Control animals that received nicotine were mainly impaired in the early components of the reach (aim and advance) whereas stroke animals were mostly impaired in the late components of the reach (supinations and release).

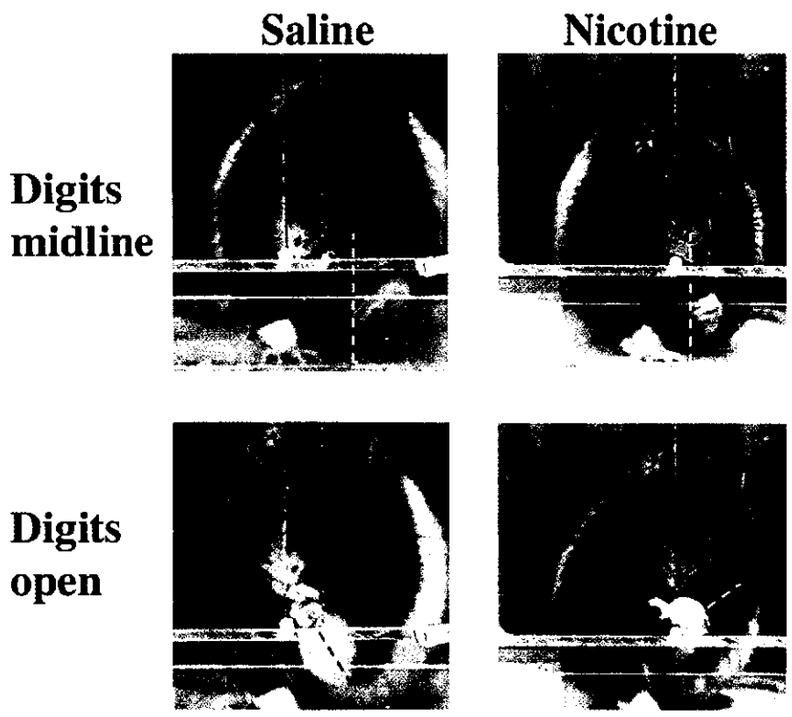

Figure 4.6: Illustration of some of the early components of the reach (Digits to the midline and digits open) by a stroke (saline), and a stroke+nicotine (nicotine) animal. Note (dashed line) that in the saline animal the digits are not aligned with the midline of the body as displayed by the nicotine-treated animal. Also note that lesion animals that received saline were unable to properly open the digits before grasping the pellet.

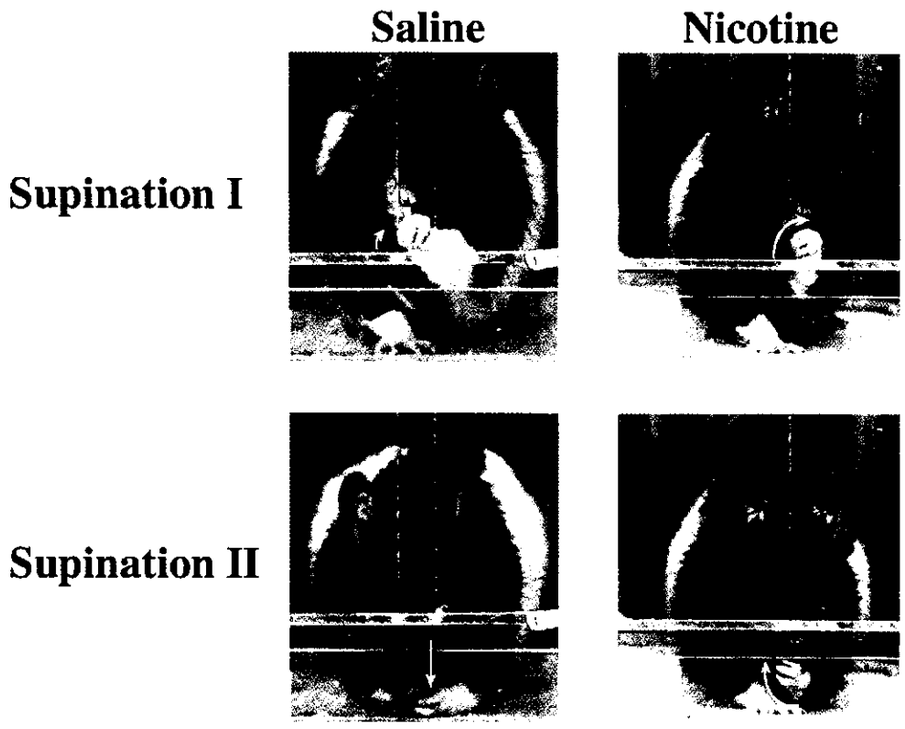

Figure 4.7: Illustration of the late components of the reach (Supination I and II) by a by a stroke (saline), and a stroke+nicotine (nicotine) animal Note (arrows) that stroke animals treated with saline were unable to supinate 90° and instead had to drag the paw through shelf to retrieve the pellet. This animals also displayed severe deficits in supinating the paw another 90° to take the food to the mouth and rather, the head had to find the paw in order to eat the pellet. Animals treated with nicotine displayed a normal supination I and a partial supination II.

Anatomical Result

Infarct Size

Infarct size for the lesion groups was determined by measuring areas of non-

infarcted tissue in both the damaged and undamaged hemispheres (Figure 4.1). Measures are represented as percentage of the normal hemisphere and are shown in Table 4.1. When the infarct size was calculated as a ratio to the intact hemisphere, no significant effect of group was found (P = 0.31 by unpaired Student's t-test). None of the animals exhibited damage to the corpus callosum or striatum. For the control groups measurements of all sections were grouped and no significant effect of treatment was found (P = 0.116 by unpaired Student's t-test).

Table 4.1. Percentage of area of remaining tisue compared with the contralateral hemisphere.

Group	**Infarct Size**
Hemisphere	
Stroke	93.64 ± 0.6
Stroke+Nicotine	91.44 ± 1.9

Data are mean ± SD

Morphological analyses

Tables 2 and 3 summarize the effects that the different types of lesion had on cortical morphology. The principal effect was that nicotine increased dendritic branching and length in both the forelimb and anterior cingulate motor areas.

Forelimb Area Layer V

When pyramidal cells of layer V in the forelimb area contralateral to the lesion were analyzed, increases in length and branching were observed in animals treated with nicotine (Table 2).

Dendritic Length: A simple ANOVA showed no significant effect of group ($F(3,12) = 2.3$, $P = .12$). A follow-up test (Fisher's LSD) however, showed that the control+nicotine group had significantly longer dendritic trees than the control and stroke-untreated groups ($P < 0.05$) but not than the stroke+nicotine group.

Dendritic Branching: A simple ANOVA showed a significant effect of group ($F(3,12) = 4.89$, $P < 0.05$. A follow-up test (Fisher's LSD) showed that the control+nicotine group had more branches when compared to controls and to the stroke-untreated groups ($P < 0.05$). The stroke+nicotine group also had more branches when compared to the control group ($P < 0.05$).

Table 4.2. Summary of the morphological changes in the basilar fields of the layer V pyramidal neurons of the forelimb area of the intact hemisphere.

Group	SA	BOA
Control	137.35 ± 7.9	42.40 ± 2.5
Control+Nicotine	162.67 ± 8.9*	52.60 ± 16*
Stroke	139.30 ± 2.5	43.80 ± 1.2
Stroke+Nicotine	148.20 ± 9.0	50.25 ± 2.9*

SA (Sholl Analysis), BOA (Branch Order Analysis)

*Significantly different from control ($p < 0.05$).

Area Cg3 Layer

The basilar tree of pyramidal cells of layer V within the anterior cingulate cortex ipsilateral to the lesion showed significant changes depending on the group. Stroke+nicotine animals showed an increase in dendritic length when compared to the untreated controls and stroke groups. There was also an increase in dendritic branching in control and stroke+nicotine animals when compared to the stroke-untreated group (Table 3).

Dendritic Length: When the basilar tree was analyzed, a simple ANOVA showed no significant effect of group ($F(3,12) = 3.1$, $P = 0.065$). A follow-up test (Fisher's LSD) however, showed that the stroke+nicotine group had longer basilar trees when compared to untreated controls and untreated stroke ($P < 0.05$).

Dendritic Branching: A simple ANOVA showed a significant effect of group ($F(3,12) = 6.74$, $P < 0.05$). A follow-up test (Fisher's LSD) showed that the control+nicotine and stroke+nicotine groups had more branches than the control and the untreated stroke animals ($P < 0.05$).

Table 4.3. Summary of the morphological changes in the basilar fields of the layer V pyramidal neurons of the anterior cingulate cortex of the lesion hemisphere.

Group	SA	BOA
Control	109.07 ± 6.9	33.55 ± 2.1
Control+Nicotine	116.36 ± 6.5	38.70 ± 1.3*
Stroke	102.45 ± 3.2	30.10 ± 1.6
Stroke+Nicotine	126.17 ± 5.8*	39.16 ± 1.4*

SA (Sholl Analysis), BOA (Branch Order Analysis)
*Significantly different from control ($p < 0.05$).

4.5. Discussion

The objective of the present study was to determine if chronic administration of low doses of nicotine would enhance behavioral recovery/compensation and dendritic arborization after a devascularizing injury to the motor cortex. The principal findings were that: 1) nicotine enhanced behavioral performance in a time-dependent manner after the stroke; and, 2) nicotine stimulated dendritic arborization in residual motor and cingulate areas in the damaged hemisphere.

We employed an extensive battery of behavioral tasks that are sensitive to motor disturbance after devascularizing lesions of the motor cortex (Gonzalez & Kolb, 2003). As in our previous studies, this battery proved to be effective in detecting behavioral impairments and spontaneous behavioral improvement after the stroke in the current

study. More importantly, however, lesion animals that were treated with nicotine showed remarkable improvement over the postoperative recovery period and by the end of the behavioral testing, nicotine-treated lesion animals showed almost complete recovery in all tasks. Furthermore, they acquired the single-pellet reaching task at rates comparable to control animals.

Although there are previous studies looking at the effect of nicotine on recovery from cortical injury (e.g., Decker et al., 1994; Brown et al., 2000; 2001) there is a larger literature demonstrating that amphetamine may be beneficial. An early study by Feeney and colleagues (1982) found that a single dose of d-amphetamine 24 hours following lesions to the sensorimotor cortex in rats resulted in enhancement of motor functions. Subsequent experiments in rats and cats showed similar beneficial effects of d-amphetamine following lesions to the frontal, motor, and occipital cortex (Hovda and Feeney, 1984; Hovda et al., 1989; Sutton et al., 1989; Goldstein, 1990; Dietrich et al., 1990). The issue of using amphetamine as a therapy after stroke in humans, however, remains controversial. Clinical trials in stroke patients have reported contradictory results (for a review see Long and Young, 2003). In the present study, the effect of nicotine in enhancing behavioral recovery after a motor cortex stroke was pronounced and found to be effective across a large battery of behavioral tasks. These results suggest that other psychostimulants (e.g. nicotine) may be beneficial in enhancing functional recovery after stroke.

Little is known about the mechanisms underlying the actions of psychomotor stimulants on the injured brain. It has been suggested that the actions of amphetamine are mediated through its actions on catecholaminergic systems (e.g., Feeney et al., 1982;

Hesse and Werner; 2003; Goldstein, 2003) but this begs the question of how such modulation might facilitate recovery. There is, however, a series of studies showing that in otherwise normal brains, amphetamine acts to stimulate dendritic growth and increased spine density in pyramidal cells in the prefrontal cortex as well as in spiny neurons in the striatum (e.g., Robinson & Kolb, 2004). The effects of amphetamine on recovery from cortical injury could therefore be mediated through its actions on dendritic growth and synaptic reorganization. A similar action is likely for nicotine. It has been shown that nicotine produces changes in prefrontal cortex and striatum that are similar to amphetamine but, in addition, nicotine appears to increase dendritic arborization in motor cortex (Gonzalez et al., 2004). The broader actions of nicotine on dendritic organization as well as its greater effects on functional recovery may be related to its broader effects on the brain. Thus, in the unperturbed nervous system, nicotine stimulates dendritic growth (Brown and Kolb, 2001), upregulates some neurotransmitters such as acetylcholine and dopamine (Narahashi et al., 2000; Clarke, 1990; Buisson and Bertrand, 2001; Risso et al., 2004), and also upregulates nerve growth factor (NGF; Martinez-Rodriguez et al., 2003), basic fibroblast growth factor (bFGF; Blum et al., 1996; Belluardo et al., 1998; Maggio et al., 1998), and brain derived nerve factor (BDNF; Maggio et al., 1998; Kenny et al., 2000). These neurotrophic factors have shown to be effective in promoting functional recovery and morphological changes after a number of nervous system lesions (Stein and Hoffman, 2003). Thus, it is possible that some of the beneficial effects that nicotine had on behavioral recovery were through increasing the availability of these growth factors after the injury.

Because nicotine enhanced behavioral outcome at least one month after the lesions it is unlikely that it had worked through neuroprotection. Studies *in vitro* have suggested that nicotine can neuroprotect against cortical insult through enhancing cell proliferation and preventing apoptosis (Hejmadi et al., 2003; Stevens et al., 2003). In our study this possibility is unlikely, however. Administration of nicotine started 48 hours after the insult and by this time irreparable cell death had taken place. Measures of infracted tissue revealed similar loss between the lesion groups. Finally, improved behavior was only obvious on weeks 5 and 7, if nicotine had acted by neuroprotecting, the behavioral enhancement should have been visible earlier. Rather, the results of later enhanced recovery suggest that "slower", most likely, permanent changes had to take place in order to support the improvement in behavior. Golgi analyses after lesions to the motor cortex have revealed that with functional improvement there is an initial decrease in dendritic arborization in perilesion regions, later followed by an increase that is temporally locked to the functional improvement (Jones and Schallert, 1993; Jones and Schallert, 1994). Because the improved behavioral recovery in the lesion animals treated with nicotine was visible only a month after the lesions and this is the time of increased dendritic growth it is possible that the neuronal changes might be in part responsible for the behavioral improvement.

One unexpected finding in the current study was that intact rats that were treated with nicotine had deficits in later learning of a novel motor task. We have subsequently analyzed this finding further and found that the nicotine appears to interfere with the expected experience-dependent dendritic changes normally associated with motor learning (Gonzalez et al., 2004). Kolb et al (2003) also showed that prior exposure to

amphetamine or cocaine blocks the effects of complex housing on dendritic arborization in nucleus accumbens and parietal cortex, a result that is reminiscent of the current findings of nicotine interfering with motor learning and dendritic change. Furthermore, Hamilton & Kolb (2004) have found essentially the same result with nicotine plus later complex housing. These results are intriguing and may have important implications for using psychomotor stimulants as treatments for stroke. One could imagine that individuals with a prior history of exposure to psychomotor stimulants might not show the same benefits of post stroke stimulant treatment as those individuals who were naïve to the drug. This remains to be shown, however.

In conclusion, the results of the present study suggest that nicotine is capable of enhancing motor recovery after focal stroke and stimulating dendritic growth in remaining cortical regions. These finding add to the literature showing that pharmacological therapies are capable of ameliorating the effects of brain damage by facilitating plastic changes in the brain.

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Chapter 5

Olfactory Stimulation Promotes Behavioral Recovery From Motor Cortex Injury in Adult Rats

5.1 Abstract

The current study examines the effects of olfactory stimulation on motor behavior after focal stroke in rats. Animals were trained on a tray-reaching task for two weeks and then they were placed in one of three groups: 1) control, 2) stroke, and 3) stroke+olfactory stimulation. A unilateral stroke in the sensorimotor cortex contralateral to the forepaw used for reaching was produced by devascularization of the surface blood vessels. Two days after the surgeries and for a total of three weeks animals were exposed to vanilla, coconut, or maple scents for 10 minutes in the morning and afternoon. Rats were tested one week after the lesions and once weekly for a total of six weeks. Results showed that olfactory stimulation enhanced recovery in both behavioral tasks. The results suggest that sensory stimulation can improve behavioral performance in adult animals after damage to the motor cortex. This finding could have implications for therapy in humans following stroke.

5.2 Introduction

It has been known for over 50 years that experience can alter brain structure and behavior. For example, the brains of animals housed in an enriched environment are heavier, have thicker neocortical mantles (for a recent review see Rosenzweig, 2003), and show morphological changes within a number of different brain areas including the visual and the sensorimotor cortex (e.g., Kolb et al., 2003). There is also evidence in experimental models of cortical injury that housing rats in complex environments (for a review see Schallert et al., 2000) or providing some sort of sensory stimulation (e.g. acupuncture) in humans may enhance behavioral outcome after stroke (for review see Ernst and, White, 1996; and Kjendah, 1997). Whether other types of experience might also be beneficial remains to be shown. For example, although the benefits of aromatherapy's 'healing properties' have been hyped in the popular press we are unaware of any studies that have directly assessed the effectiveness of such treatments following brain injury.

Here we examined the effects of olfactory stimulation on recovery in a model of focal stroke in adult animals. For this purpose, rats were pretrained in a skilled reaching task before being assigned to one of three groups: 1) control, 2) stroke, 3) stroke+olfactory stimulation (stroke+OS). Lesions were produced by devascularization of surface blood vessels on top of the sensorimotor cortex contralateral to the preferred paw for reaching. Rats were exposed to one of three different scents twice daily for a total of three weeks post-surgery. Performance on the tray-reaching task, as well as on a test of forepaw asymmetry, was studied postoperatively for a total of six weeks.

5.3. Materials and Methods

Subjects

Subjects were 20 male Long-Evans hooded rats, 4 months old and weighing 300-400 g at the beginning of the experiment. Animals were raised in the University of Lethbridge vivarium and were housed in groups of two in clear plexi-glass cages. The colony room was maintained on a 12:12h light/dark cycle (08:00-20:00 h) and the temperature regulated at 22 °C. Experiments were conducted according to standards set by the Canadian Council on Animal Care and approved by the University of Lethbridge animal welfare committee.

Food restriction

Three weeks before the lesions, the rats were changed to a restricted food intake: Each animal received 20 g. of food per day (normal daily consumption ranges from 18-25 g) one hour after the testing session was completed. Their body weight was maintained at about 95-98% of baseline until the completion of the behavioural testing.

Surgery and lesion placement

Subjects were assigned to three different groups, control (n = 5), stroke (n = 5), stroke+OS (n = 10), (We note that 5 of the rats with olfactory stimulation also received tactile stimulation but because there was no additional benefit of the tactile stimulation the two groups are combined here for simplicity). Stroke animals received an injection of atropine methyl nitrate (0.1 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) to facilitate respiration throughout surgery. Animals were then anesthetized (sodium pentobarbital 60 mg/kg i.p.) and placed in a stereotaxic apparatus. The lesions were produced by unilateral devascularization of the motor cortex (Sofroniew et al., 1983; Kolb et al., 1997). In brief,

a flap of bone and underlying dura were removed at coordinates corresponding to areas Fr2, Fr1, Fr3, FL and HL (Zilles, 1985). A rectangular hole in the skull was produced at stereotaxic coordinates anterior (A), lateral (L): A= -3.0 to +2.0 mm and L= 1.0 to 4.0 mm. using an electric dental drill while avoiding traumatic brain injury. All vessels and pia matter were rubbed away with sterile, saline-soaked cotton swabs.

Olfactory Stimulation

Olfactory experience was provided by moving the animals to a novel cage and placing small glass jars with holes in the lid into the cage (Figure 1). Sealed within the glass jar was 4-6 cotton balls which each had 3-4 drops of one aroma (maple, vanilla, or coconut) placed on them. The aromas were cooking extracts commonly found in the spice and baking section of a grocery store. Animals received olfactory stimulation twice a day for a period of ten minutes (morning and late afternoon) for three weeks post stroke. The olfactory stimulation was conducted in a room separate from the colony throughout the treatment. A different aroma was used each test period by rotating through the three odours in arbitrary fashion.

Cylinder test (Forepaw Asymmetry)

Forelimb use for weight support during explorative activity was examined by placing the rats in a transparent cylinder 20 cm in diameter and 30 cm high for three minutes (Schallert et al., 1997), (Figure 2). The animals were individually placed in the cylinder during the three minutes of each testing session. A mirror was placed underneath the cylinder at an angle to allow the experimenter to videotape the animal's activity from a ventral view (Pinel et al., 1993). The cylindrical shape encouraged vertical exploration of the walls with the forelimbs, but the walls were high enough so that animals could not reach the top. Forelimb use was measured during vertical exploration. Animals were

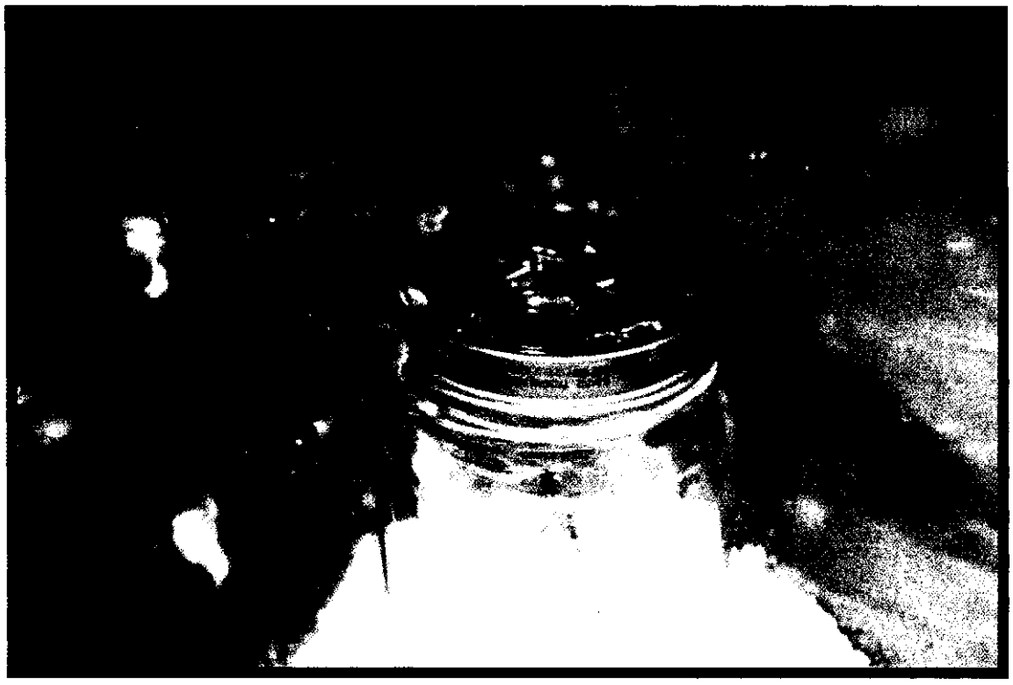

Figure 5.1. An example of animals receiving olfactory stimulation

placed and videotaped in the cylinder once before the lesions were made. Each forepaw contact with the cylinder wall was counted. When simultaneous limb contact was observed, a touch was counted for each paw. The asymmetry score of forelimb use in wall exploration was calculated for each group (i.e., affected forelimb/(affected + unaffected)) to obtain a score where 0.5 represents perfect symmetry and any number closer to zero would suggest a decrease in the use of the affected limb.

Reaching boxes and training

Training boxes were made of plexiglass with dimensions 26 cm high, 28 cm deep, and 19 cm wide. The front of the boxes was constructed of 2mm bars separated from each other by a 9 mm gap. Clear plexiglass flip tops allowed access to the inside of the box. A 4 cm wide and 0.5 cm deep tray was mounted in front of the bars. The tray

contained food fragments weighing approximately 30 mg each. Animals had to reach between the bars, grasp the food and retract it for consumption (Figure 3). Subjects were trained for a total of three weeks before the lesions to obtain a stable baseline. Rats were trained individually for one half hour per day and then at the end of the three weeks pretraining period, their performance was videotaped for a five- minute interval. Each time the rat reached through the bars whether or not food was obtained was scored as a "reach" and each time food was successfully returned to the cage and consumed was scored as a "hit". The percentage of hits to total reaches was then calculated from each animal's videotaped performance.

Histology

At the completion of behavioral testing, the animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% saline. The brains were removed and processed for cresyl violet staining.

Infarct Measurements

The brains of all animals were photographed prior to being sectioned. Lesion size was estimated by tracing the area of the intact neocortex on each side of the brain (NIH IMAGE software, Ver.1.62) and then expressing the data as percentage of the intact hemisphere.

Data Analysis

Behavioural scores were evaluated by analyses of variance (ANOVA) and Fischer's LSD to detect significant differences ($p \leq 0.05$) between treatment and control groups. Student's t-test was used to determine significant anatomical differences between lesion groups ($p \leq 0.05$).

5.4. Results

Cylinder Task

All groups actively explored the cylinder and they reared and supported their body against the walls with their forelimbs. The asymmetry score of forelimb use was calculated for each group and it showed that the lesions produced a reduction in limb use contralateral to the infarct. Control animals placed both forepaws on the cylinder wall during vertical exploration whereas animals with lesions relied on the paw ipsilateral to the lesion and had fewer contacts with the contralateral paw. Animals that received olfactory therapy however, made more contacts with the contralateral paw than animals that did not received therapy (Figure 2). A repeated measures ANOVA over the six weeks of testing showed a significant effect of group ($F(2,17) = 13.49$, $P < 0.001$), no main effect of test week, ($F(4,68) = 0.12$, $P = 0.97$), but a significant interaction of group by week ($F(8,68) = 2.95$, $P < 0.05$). A post-hoc analysis revealed that control animals differed from the other two groups ($P < 0.01$) and that animals treated with olfactory stimulation recovered better and differed from the stroke-no-treated animals, who essentially showed no change over the six postoperative weeks, $P < 0.05$.

Tray Reaching Task

All animals quickly learned to reach for food and asymptoted at about 68% accuracy before the lesions. After the lesions, both stroke groups showed reduced accuracy when compared to the control no treatment group. Animals that received therapy following a lesion however, showed better reaching success with their affected forelimb than animals that did not receive therapy. A summary of the reaching performance of the animals' preferred limb, as measured by baseline percent for each

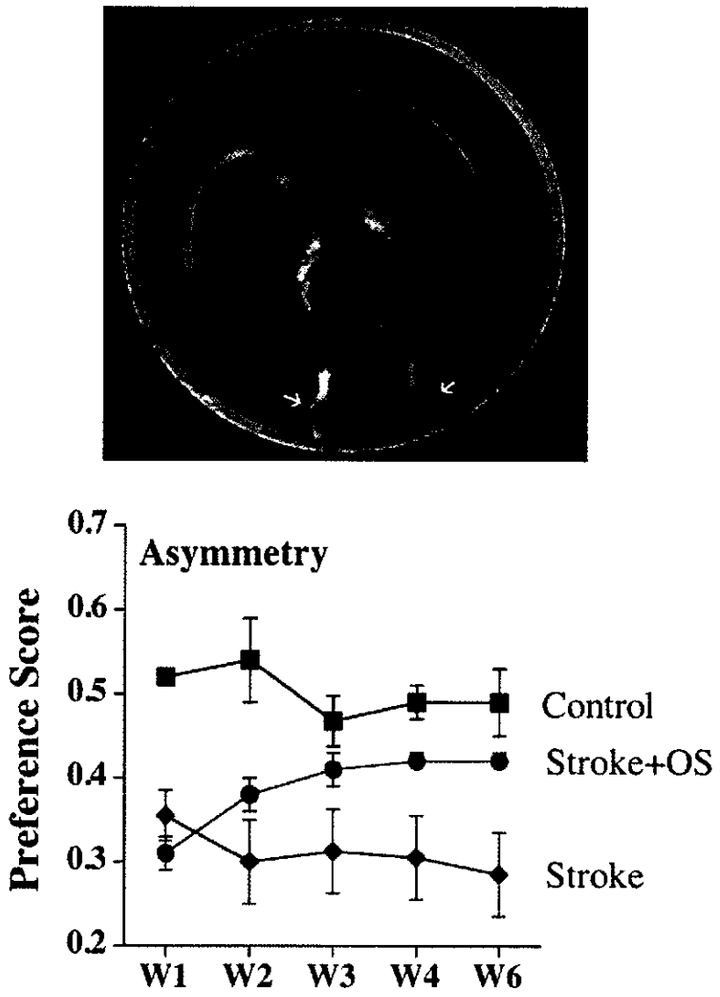

Figure 5.2. Top panel shows a picture of a control animal in the cylinder. Arrows show use of both forepaws for support during rearing. Bottom panel shows performance on this task on weeks (W) 1, 2, 3, 4, and 6 after the surgeries (±SE). A preference score of 0.5 indicates no preference on the use of left or right forepaw a score of 1 indicates complete use of the ipsilateral forelimb. Note that stroke animals exposed to the olfactory stimulation performed much better than the animals with no treatment.

animal, is shown in Figure 3. A repeated measures ANOVA over the six weeks post surgery showed a significant effect of group ($F(2,17) = 6.93$, $P < 0.01$), a significant effect of week ($F(4,68) = 3.27$, $P < 0.05$), but no interaction of group by week ($F(8,68) = 1.33$, $P = 0.24$)). A post-hoc analysis revealed that stroke untreated animals differed from both, the control and the stroke+OS group ($P < 0.5$).

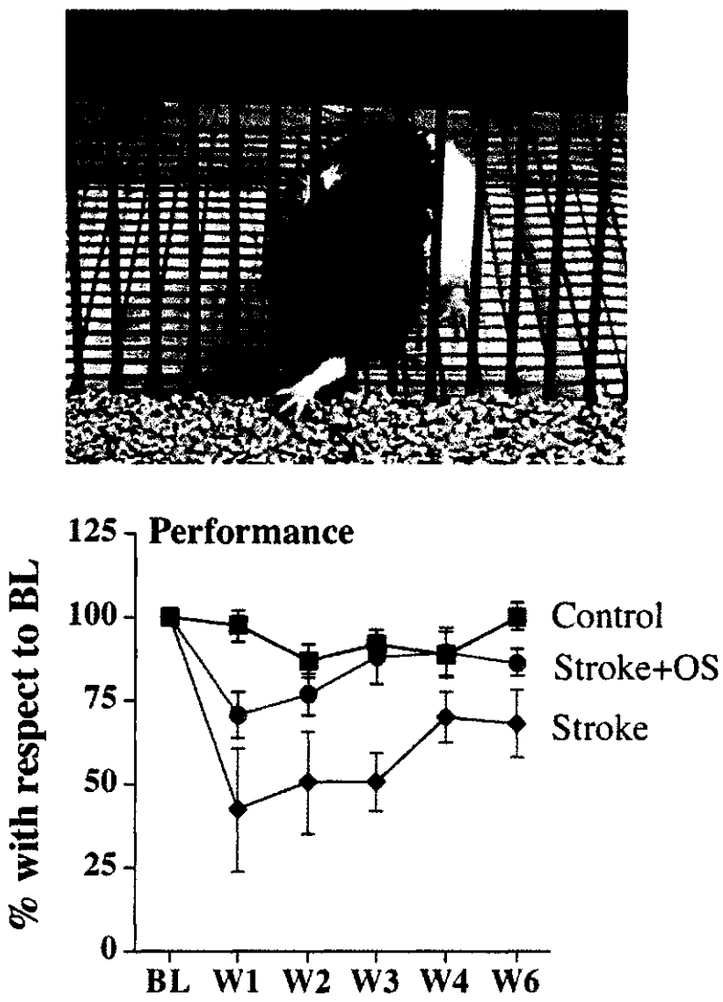

Figure 5.3. Top panel shows a picture of an animal on the reaching apparatus before the surgeries, arrow points at the animal's grasp for food. Bottom panels

show the rat's performance on this task on weeks (W) 1, 2, 3, 4, and 6 after the surgeries (±SE). Note that by the last weeks of testing stroke animals that received olfactory stimulation performed as well as control animals.

Infarct Measurements

Infarct size for the lesion groups was determined by measuring areas of non-infarcted tissue in both the damaged and undamaged hemispheres. Measures are represented as percentage of the normal hemisphere and are shown in Table 1. When the infarct size was calculated as a ratio to the intact hemisphere, no significant effect of treatment was found (P = 0.64 by unpaired Student's t-test).

Table 5.1. Percentage of area of remaining tissue compared with the contralateral hemisphere.

Group	**Infarct Size Hemisphere**
Stroke+OS	82.46 ± 2.9
Stroke	82.15 ± 1.20

Data are mean ± SD

5.5. Discussion

The objective of the present study was to examine whether sensory stimulation would enhance behavioral recovery/compensation after stroke. Animals benefited from olfactory therapy in their performance in both behavioral tests and, surprisingly, the olfactory-treated rats performed nearly as well as control animals by the end of the six weeks of testing.

There is now an extensive literature showing that both global and specific experiences can alter both brain and behavior. The brains of animals housed in complex environments show extensive changes including an increase in brain weight, increased neocortical thickness, increased vascularity, increased astrocyte size, and increased dendritic length in a number of different brain areas including visual, somatosensory, and motor cortex (e.g., Sirevaag & Greenough, 1985). These anatomical changes are correlated with enhanced behavioral capacities on both motor and cognitive tasks in otherwise intact animals. Similarly, more specific experiences such as training in visual mazes or motor tasks produce localized changes in the active regions such as the visual and motor cortices, respectively (e.g., Greenough & Chang, 1988).

Less is known about the effects of global or specific experience in the injured brain although there is a growing body of literature showing that cortical motor representations are modifiable after cortical injury and may support improved motor functions (for review see Nudo, 2001, 2003, Stein and Hoffman, 2003). In humans for example, it has been shown that reorganization of intact adjacent cortical tissue contributes to functional recovery after focal ischemia (Cramer et al., 1997, 2000; Traversa et al., 1997). Given that plastic changes in the cortex may support functional

improvement, it is logical to try to enhance the endogenous plastic changes to further behavioral rehabilitation. One way to do this is to give experiential treatments known to change the normal brain, such as complex housing. Indeed complex housing has been used in a variety of animal models of cerebral injury and has proven beneficial (e.g., Kolb et al., 1998; Schallert et al., 2000; Risedal et al., 2002). Few studies has examined more specific experiences, however, although sensory stimulation mediated by acupuncture (Ernst and White, 1996; and Kjendahl, 1997) and galvanic vestibular stimulation (Magnusson et al., 1994) have been found to be effective in human brain-injured patients and sensory restriction has proven beneficial for recovery from certain neglect syndromes (Crowne et al., 1983; Van Vleet et al., 2003; Burcham and Corwin, 1998).

One novel aspect of the current study is that although there is a considerable literature showing that multisensory experience, such as in complex environments, can influence recovery from cerebral injury (for a review see Schallert et al., 2000), few studies have examined the ability of specific sensory stimulations to facilitate recovery. We are unaware of any previous studies using olfactory stimulation as a treatment for brain injury, although there are anecdotal reports that it may be beneficial for brain-injured children (Gordon Pomares, personal communication; for more information see www.gordonpomarescentre.com). Nonetheless, the surprising magnitude of the treatment effect in the current study suggests that olfactory stimulation may be a powerful treatment for treating brain injury.

One difficult question to answer is what the mechanism of olfactory stimulation's restorative effect might be. One possibility is that olfactory stimulation may stimulate activity in the subventricular zone and rostral migratory stream (Rochefort et al., 2002).

Another is that the olfactory stimulation may increase the production of catecholamines and there is considerable evidence that increases in noradrenaline and perhaps dopamine can facilitate cortical plasticity after injury (for reviews see Goldstein, 2003; Hesse and Werner, 2003). The presence of odorants increased activity in the animals and this increase could certainly have stimulated general neural activity and may have had more specific effects as well. It is obvious that at present the answer to the mechanism question is reduced to speculation but we believe that our results provide the grist for many future studies examining the role of olfactory stimulation in recovery from cerebral injury. Of course, olfaction is likely a more powerful sensory experience for rats than people so there is the question of whether olfactory stimulation would be effective in humans.

Whether these forms of environmental enrichment prove to be beneficial in humans suffering from stroke or not, the effects shown here indicate that sensory stimulation may have therapeutic potential. Future research designed to address the minimum and maximum periods of time capable of providing beneficial behavioral outcome after cortical injury and that help elucidate the possible mechanisms of action of these forms of environmental stimulation are essential.

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Chapter 6

Growth Factor-Induced Regeneration of Neural Tissue Following Cortical Lesions

6.1. Abstract

We tested the hypothesis that stimulating proliferation and differentiation of intrinsic forebrain neural stem cells, with epidermal growth factor (EGF) and erythropoietin (EPO), respectively, will enhance functional recovery after stroke damage to the cerebral cortex. Rats were trained in three behavioral tasks: 1) skilled reaching task (tray reaching), 2) forelimb placing asymmetry (cylinder task), and 3) forelimb inhibition (swimming task). A unilateral stroke in the sensorimotor cortex contralateral to the forelimb used for reaching was produced by devascularization of the blood vessels overlying the targeted cortical area. After the strokes animals received lateral ventricle infusions of: a) EGF followed by CSF, b) EPO followed by CSF, c) EGF followed by EPO, and d) CSF followed by CSF. In subsequent experiments infusions began either immediately or were delayed 3, 7, or 14 days. One week after stroke surgery all animals were severely impaired in all tasks, regardless of treatment condition. For animals receiving infusions on the day of stroke surgery or day 3, animals infused with EGF + EPO began showing clear behavioral improvements. Infusions beginning 7 days after stroke had a slower behavioral improvement whereas infusions beginning 14 days after the stroke had only a small effect on behavioral outcome. After six weeks of testing only animals that received EGF + EPO reached preoperative baselines in tests of cylinder and swimming, and enhanced recovery in skilled reaching. Histological analysis of animals that received EGF+EPO, including doublecortin staining of neuronal progenitors, suggested intrinsic cell mobilization from the subventricular zone to the infarct site and apparent tissue regeneration. Golgi analyses revealed neurons with complex, but abnormal, dendritic fields in the newly-generated tissue. Finally, one group of animals

had the generated tissue plug removed 5 weeks after surgery. The behavioral advantage was lost, suggesting that the tissue plug was playing some role in the behavioral improvement observed. Overall, The results demonstrate that combined administration of EGF + EPO enhances behavioral recovery following focal ischemia and suggest that newly generated neurons that migrate to the lesion site contribute to this functional improvement.

6.2 Introduction

There is minimal spontaneous recovery of function after cerebral injury in any species, including humans (for a review see Kolb, 1995). Most evidence for the capacity for functional recovery comes either from studies of aphasia in humans (Kertez, 1979) or the lack of aphasia in children with perinatal injuries to the language zones (e.g., Lennenberg, 1967). Studies of World War II veterans shows that some recovery of function is possible given enough time (e.g., Teuber, 1975) but the recovery is far from complete and may be associated with loss of other functions (e.g. Corkin, 1989). Laboratory studies have used two general strategies to potentiate recovery. One has been to initiate behavioral "therapies" of various types but this has had mixed results and, in most cases, the recovery is still minimal (e.g., Schulkin, 1989). The second strategy has been to provide pharmacological intervention either with stimulants such as amphetamine (for a review, see McIntosh, 1993) or with growth factors. The initial results with drugs like amphetamine were encouraging, and clinical trials were initiated, but the results have been less impressive than was initially hoped. The treatment with growth factors such as Nerve Growth Factor (e.g. Kolb et al., 1997) may prove to be more useful. In these studies the brain is given infusions of factors such as NGF after a cerebral injury and in some studies there has been substantial recovery, which is correlated with the growth of more dendritic arbor and increased spine density, which implies an increase in synapses. Even in these studies, however, the recovery is incomplete and the subjects still have formidable functional disturbances.

A second approach to stimulating recovery from cerebral injury is to mobilize endogenous stem cells to produce neurons and/or glia that could migrate to the region of

injury and influence functional recovery. It has been known for a long time that fish, amphibians and reptiles are capable of regeneration of neurons (e.g., Harrison, 1947; Nicholas, 1957). More recently, there have been numerous demonstrations of neurogenesis in the bird brain (e.g., Nottebohm & Alvarez-Buylla, 1993). Taken together, the studies of nonmammalian species have shown that neurogenesis is possible in the brains of adult animals. In mammals, however, until recently there have been only hints that neural proliferation might be possible in adults, and for the most part these hints have been treated as curiosities. This was due, in part, to the fact that the number of new neurons in most of these studies is rather small and that these new neurons have not been demonstrated to be functional (see Altman & Bayer, 1993 for a review).

During development most neurons are born in the ventricular and subventricular zones. There is now evidence that there are stem cells in the subventricular zone in both rodents and primates that are capable of producing progenitor cells that, in turn, can produce both neurons and glia (e.g., Kirschenbaum et al., 1994; Weiss et al., 1996). Furthermore, Morshead et al., (1994) showed that intraventricular infusions of epidermal growth factor (EGF) can induce both migration and neuronal differentiation of these cells.

The functional role of neurogenesis in the adult brain is not known but some structures, notably the hippocampus and olfactory bulb, appear to have a higher incidence of new neurons than most other regions (e.g., Bayer et al., 1982). Furthermore, there have been reports that cerebral injury does increase the rate of neuronal division (Altman, 1962) and mitosis has been reported in neurons of the cortex of mice with small cortical lesions (Huang & Lim, 1990). In addition, Szele and Chesselet (1996) induced a

transient increase in cell number in the subventricular zone after small sensorimotor cortex lesions. These is no reported evidence of substantial neuronal regeneration, however, and no evidence that any neural regeneration is associated with functional recovery. There is evidence of neural regeneration being associated with functional recovery in the developing brain, however, (e.g., Dallison & Kolb, 2003; Gonzalez et al., 2003; Kolb et al., 1998a).

The fact that the postmitotic brain, albeit infant, can produce new neurons that will form appropriate connections and support functional recovery shows that it is possible for the mammalian brain to generate new neurons to support recovery. The challenge is to induce the adult brain to do the same. Preliminary experiments showed that infusions of EGF alone or EGF and NGF, FGF-2, or BDNF all stimulated the production of cells that filled the lesion cavity (Kolb et al., 1998b). These cells were poorly differentiated, however, and if the recovery period was prolonged beyond 4-6 weeks, the cells began to die. Given that there was no obvious anatomical difference between EGF alone and the various combinations of growth factors, it was decided to begin the behavioral studies by studying the behavioral effects of EGF alone.

6.3. Experiment 1: Generating new cells

The preliminary studies were solely anatomical studies so Experiment 1 was designed to determine if the newly generated, but poorly differentiated, cells might contribute functionally. Animals were pre-trained in a skilled reaching task (Whishaw et al., 1986) and then given motor cortex lesions contralateral to the preferred forepaw.

6.3.1. Methods and Materials

Subjects. Twenty-one male Long Evans rats (Charles River Breeding Laboratories, 300-350g) were divided into three groups: normal control (n=5), motor cortex (n=8), and motor cortex + EGF (n=8). The animals were group housed and maintained on 12:12 h light/dark schedule and were given free access to food and water, with the exception being the time spent training to reach for food. Animals were cared for under the rules and provisions of the Canadian Council on Animal Care. All efforts were made to minimize animal suffering.

Surgery. Under sodium pentobarbital anesthesia (60 mg/kg), all animals were implanted with permanent stainless steel cannulae (23 gauge) in the lateral ventricle ipsilateral to the preferred paw using skull coordinates -0.8 anterior-posterior from the Bregma, 1.4 lateral from the midline, and 3.5 ventral from the skull surface. The cannulae were connected to filled, pre-tested, s.c. placed osmotic minipumps (Alzet 2001) via coiled flexible polyethylene tubing. These were filled with either vehicle (artificial cerebral spinal fluid plus 0.1% bovine serum albumin) or with the csf and EGF. The EGF alone was made at a concentration of 10 μg/ml. Two weeks following implantation the minipumps and connective tubing were removed under isoflurane anesthesia.

A craniotomy was made in the opposite hemisphere at the time of cannulae implantation by removing the bone from –3.0 to +2.0 mm AP and lateral 1 to 4.0 mm. The dura was retracted and the underlying pia was stripped from the brain using a saline-soaked cotton swab. The wound was sutured closed with 4-0 Vicryl suture.

Behavioral training. Forepaw use was measured using a procedure that was adapted from the method devised by Whishaw et al (1986). Each animal was food-deprived to 95% body weight for the training and testing. The animals were placed in the test cages (10 X 18 X 10 cm high) with floors and fronts 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 the front of each box. The rats were required to extend a forelimb through the gap in the bars, grasp and retract 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 fall irretrievably through the gap. An attempt was scored only when the rat reached into the tray and touched the food. If it reached into the tray without touching a pellet, no attempt was scored. Animals were trained for 20-30 min per day for a minimum of 10 days, by which time their performance had reached asymptote. Once trained, the rats received a 10-min reaching test during which time they were videotaped and then scored later. The animals were retested weekly for four weeks beginning seven days after their surgery.

Anatomical Methods. At the completion of behavioral testing, the animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% saline followed either by 4% paraformaldehyde. The brains were transferred to a 30% sucrose foramlin solution two days later and a week later they were cut on a vibratome at 40 μm. They were stained using immunohistochemical procedures for nestin (an intermediate filament), NeuN (neuron specific), and GFAP (astrocytes).

6.3.2. Results

Anatomical results

There were obvious cavitations in lesion-alone brains, much as seen in previous experiments. In contrast, the EGF-treated animals had largely undifferentiated cells in the lesion cavities (Figure 6.1). There were a few NeuN positive cells but many Nestin positive cells. The origin of the cells was undetermined but the subventricular zone (SVZ) was greatly expanded (Figure 6.1), suggesting that the cells may have originated the subventricular zone (SVZ).

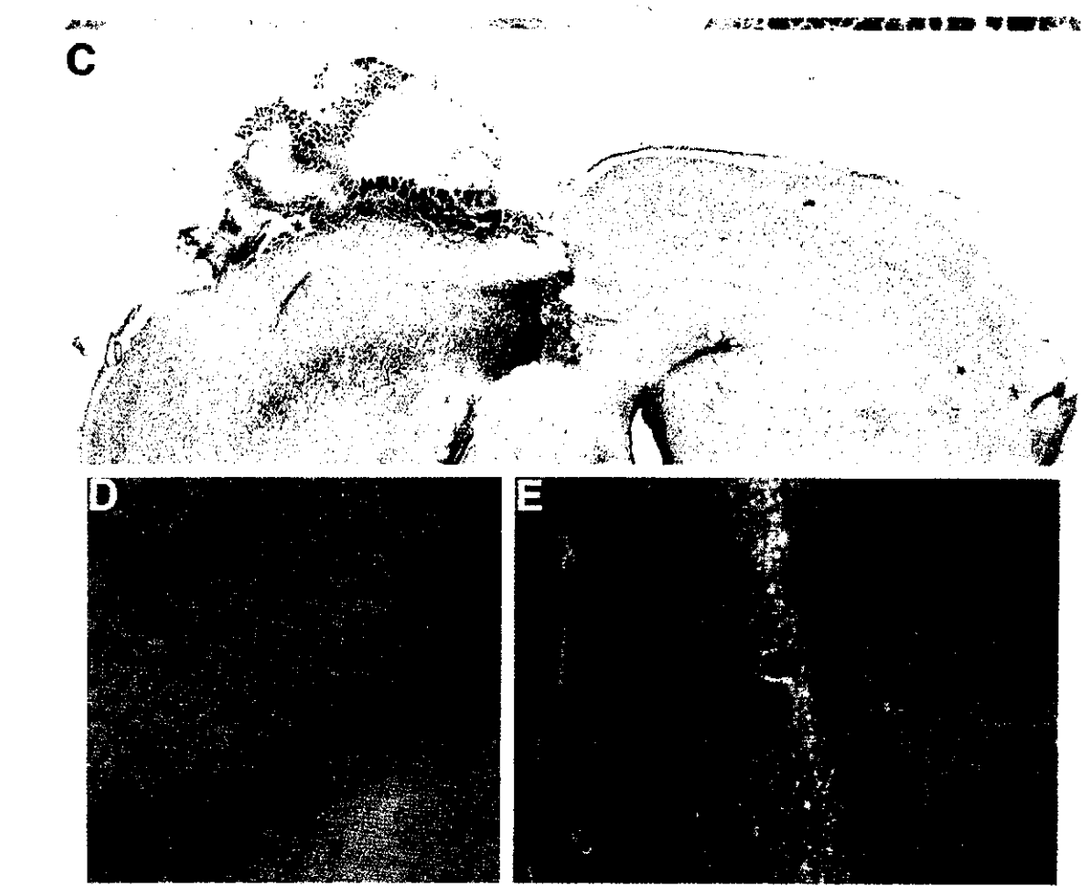

Figure 6.1. Top. A Nissl-stained coronal section through the site of lesion in an animal treated with EGF post-stroke. There are large numbers of poorly differentiated cells both on the surface of the injured brain as well as in the striatum. Bottom left. A section showing Nestin-positive cells in the striatum in a section adjacent to the top section.

Behavioural Results

The lesions produced severe deficits in the skilled reaching task as the lesion animals dropped to about 50% of their preoperative performance level (Figure 6.2). There was some improvement over the test weeks but the EGF treatment was without any benefit, in spite of the generation of cells that filled the cavities. A two-way ANOVA on Groups by Test week showed a main effect of group ($F(2,18)= 4.9$, $P<.02$), Test week ($F(4,72)=22.3$, $P<.0001$), and the interaction ($F(8,72)=3.3$, $P<.003$). The interaction reflected the impairment seen in the lesion groups relative to pre-testing.

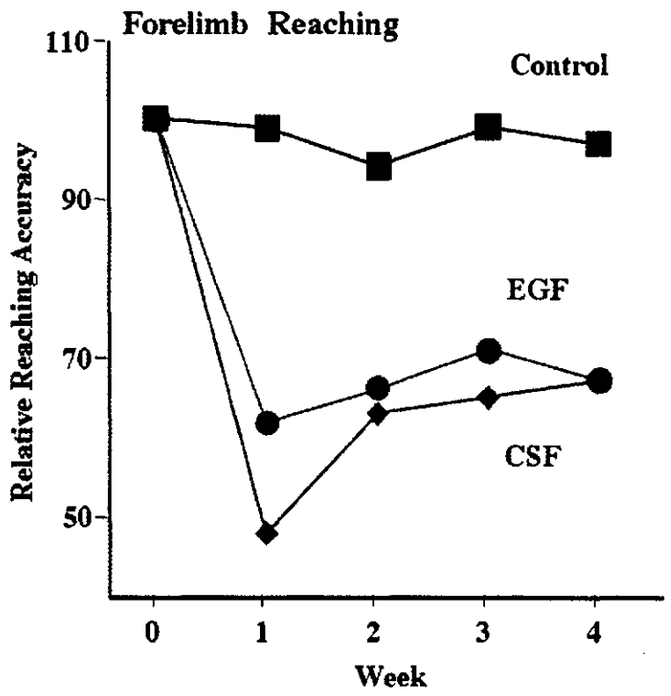

Figure 6.2. Reaching performance of rats given EGF or CSF after a motor cortical injury.

6.3.3. Discussion

The results showed that although it was possible to generate cells and for these cells to migrate to the site of cortical injury, the cells did not benefit functional recovery. One obvious problem was that the cells did not differentiate into neurons or glia but remained undifferentiated cells. The task is to induce the cells to differentiate.

6.4. Experiment 2: Inducing cell differentiation

A major difficulty both with Experiment 1 and with our earlier preliminary studies was that the cells failed to differentiate into neurons or glia. Recently, Weiss and colleagues (Shingo et al., 2001) have shown that erythropoietin (EPO) can stimulate progenitor cells to differentiate into a neuronal phenotype in vitro, leading the possibility that treating EGF-stimulated cells with EPO in vivo might drive the cells towards a neuronal phenotype and thus possibly facilitate some functional restitution. We also considered the possibility that EGF may have provided some functional benefit if a larger test battery had been used so the more extensive test battery used by Gonzalez & Kolb (2003) were also used in the current study.

6.4.1. Methods and Materials

Subjects. Forty-seven male Long Evans rats (Charles River Breeding Laboratories, 300-350g) were divided into five groups : normal control (n=5), motor cortex lesion (n=15), motor cortex lesion+EGF (n=10), motor cortex lesion+EPO (n=6), and motor cortex lesion+EGF+EPO (n=11). Animals were housed and cared for as in Experiment 1.

Surgery. Surgery was generally as in Experiment 1. The one difference is that animals had one osmotic minimpump for 7 days at which time it was replaced by a second pump for an additional 7 days. Control and motor cortex animals received CSF from both their pumps. The animals receiving motor cortex lesions+EGF or motor cortex lesions+EPO received EGF in the first pump and CSF in the second. The animals receiving motor cortex lesions + EGF +EPO received EPO in the second pump

Anatomical methods

At the conclusion of behavioral testing the animals were given an overdose of Ethansol and all but three brains per group were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. The brains were removed, photographed, and postfixed for 48 h. The brains then were placed in 30% surcrose and the region through the lesion was sectioned on a vibratome at 40 μm. Every ninth and tenth sections were saved and stained either with cresyl violet or double cortin. The remaining brains were perfused only with 0.9% saline. They were then processed for Golgi-Cox analysis by placling them in Golgi-Cox solution for 14 days, 30% sucrose for at least 3 days and then cut on a Vibratome at 200 μm, mounted, and stained using procedures described by Gibb & Kolb (1998).

Behavioural methods

Forelimb Asymmetry. Forepaw asymmetry of the animals was determined by filming them from below while in an acrylic cylinder 20 cm in diameter, on an acrylic platform. Preference was determined by separately counting the number of times in 5 minutes that an animal reared and placed the left or right forepaw on the surface of the cylinder in a gesture of postural stabilization. This test allows a measure of asymmetry in forelimb use, a measure that shows a reliable bias to using the limb ipsilateral to the injury. There is typically little change in performance, even with extended postoperative recovery times. Animals were tested preoperatively and then postoperatively on weeks 1, 2, 3, 4, and 6.

Forepaw Inhibition. In normal rats, swimming is accomplished by propulsion from the hind limbs. The forelimbs are normally inhibited from any stroking and are held immobile and together under the animal's neck. Inhibition of the forelimbs was assessed

by filming animals while swimming. Animals are introduced into one end of an aquarium (30 w x 90 l x 43 h cm) filled to a depth of 25 cm with room temperature water (~25oC) and filmed as they swim to a 9.5 cm square visible platform. This platform projects 2 cm above the surface of the water and is positioned at the opposite end of the aquarium. Scoring of inhibition was done by counting the number of strokes, if any, made by each forelimb in three swims along the length of the aquarium. A swim was deemed scorable only if the animal does not touch the sides of the aquarium during the swimming trial. Animals were tested preoperatively and then postoperatively on weeks 1, 2, 3, 4, and 6.

Tray Reaching Task. Tray reaching was conducted as in Experiment 1. Only a subset of animals chosen randomly preoperativelyh was tested in this task (n=5 per group). Animals were tested preoperatively and then postoperatively on weeks 1, 2, 3, 4, and 6.

6.4.2. Results

Anatomical Results

In contrast to the effects of EGF alone in Experiment 1, EGF+EPO led to the partial differentiation of cells that filled the lesion cavity. BrdU/NeuN(+) cells were observed within the tissue plug of all brains when examined at day 18 post-stroke (Figure 6.3). Such cells were not observed in the EGF or EPO alone groups.

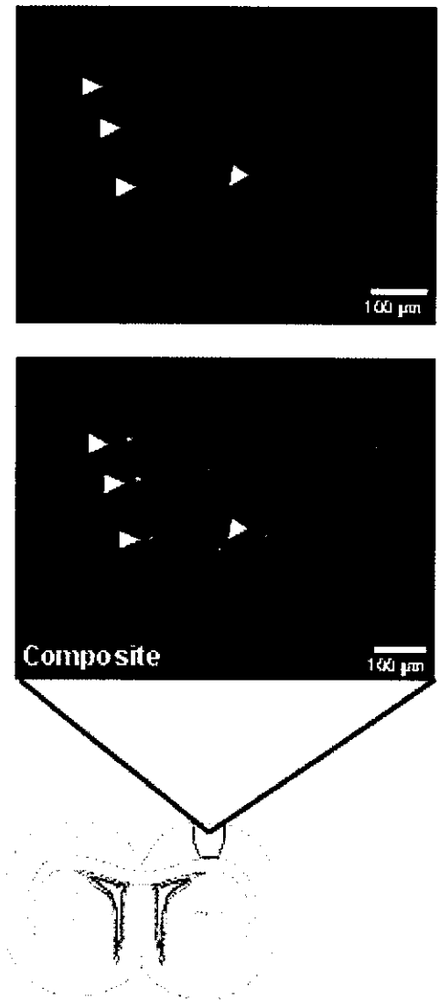

Figure 6.3. BrdU/NeuN(+) cells observed within the tissue plug core at day 18 post-stroke (Group D animal, BrdU injected on day 15). Blue arrows represent cells labelled with either BrdU or NeuN alone.

Examination of the Golgi-stained sections revealed that although there were immature appearing neurons among the cells that filled the lesion cavity (Figure 6.4), no normal looking mature cells were seen. Cells in the regions adjacent to the lesion appeared normal.

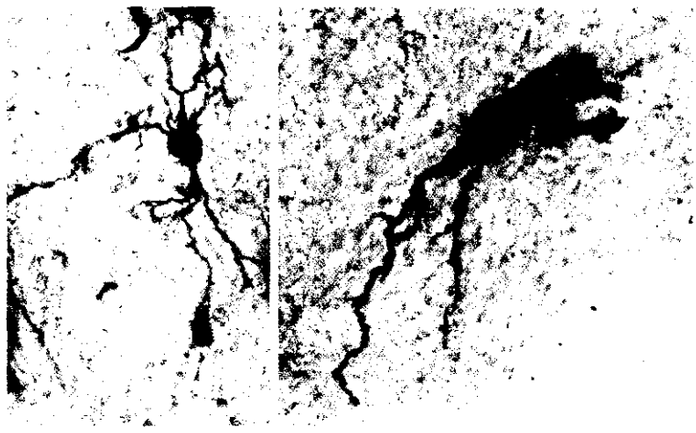

Figure 6.4. Golgi-stained sections illustrating representative cells in the tissue in the lesion cavity. The cells do not have a normal appearance of adult cells although they have similarities to immature neurons.

Behavioural Results

Forelimb Asymmetry. Following the stroke, all lesion rats showed an asymmetry of paw use favouring the ipsilateral paw. The extent of asymmetry decreased over time in all groups but the motor cortex+EGF+EPO group showed a faster, and larger reversal than any of the other groups (see Figure 6.5). A two-way ANOVA (group x week) showed a main effect of Group ($F(4,42)=4.9$, $P=.003$), Week ($F(4,168)=5.5$, $P=.0003$, and the interaction ($F(16,168)=1.9$, $P=.02$. The interaction reflected the better improvement

of the EGF+EPO group, which differed signficantly from from all lesion groups, but not the control group, on weeks four and six.

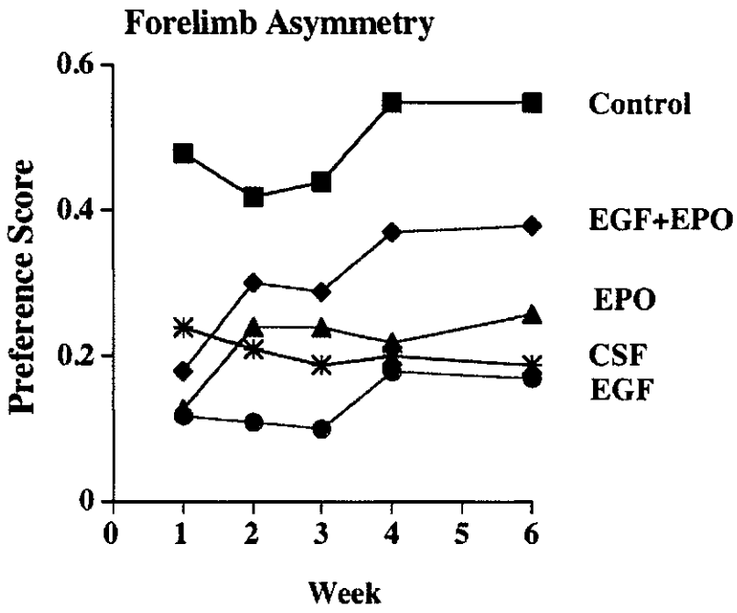

Figure 6.5. Rats in all groups showed an asymmetry favoring the use of the ipsilateral limb. This asymmetry was partially reversed in the animals receiving EGF+EPO.

Forelimb Inhibition. Following the lesion all ischemic groups failed to inhibit the contralateral forepaw as they had preoperatively but all continued to inhibit the ipsilateral forepaw (Figure 6.6). The EGF+EPO lesion group showed a reduction in contralateral forelimb use relative to the other groups, however. A two-way ANOVA (Group X Week) found a main effect of Group ($F(4,42)=6.7$, $P=.0003$), but not of Week ($F(4,168)=1.9$, $P=.11$), nor the interaction ($F(4,168)=0.7$, $P=.79$). Follow up tests showed

that the EGF+EPO group did not differ from the control group on weeks 2-6 whereas the other lesion groups did.

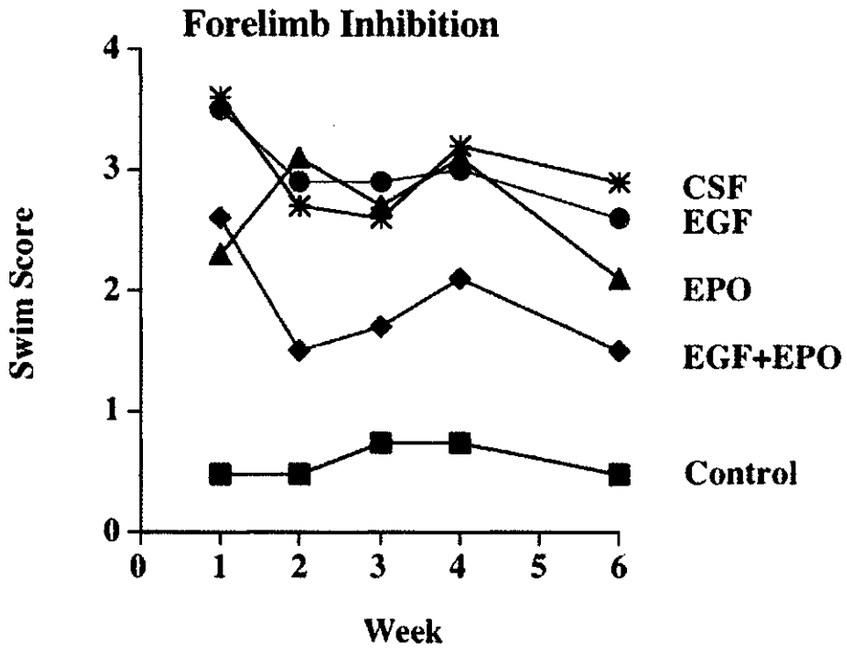

Figure 6.6. Forelimb inhibition in the contralateral limb in the swimming pool. All lesion groups showed a release of forelimb stroking that was partially reversed by infusions of EGF+EPO.

Tray Reaching. All lesion groups were significantly impaired at the task following the lesion (Fgure 6.7). As in the other behavioral tests, the EGF+EPO group showed greater improvement than the other lesion groups. A two-way ANOVA (Group X Week) found a main effect of Group ($F(4,22)=6.4$, $P=.0014$), Week ($F(4,88)=25.9$, $P<.0001$), and the interaction ($F(4,88)=2.8$, $P=.001$). Posthoc tests showed that all of the groups improved over the test weeks but the EGF+EPO group showed the best

performance. Thus, this group did not differ from control from weeks 2-6 and differed from the EGF group on weeks 4 and 6.

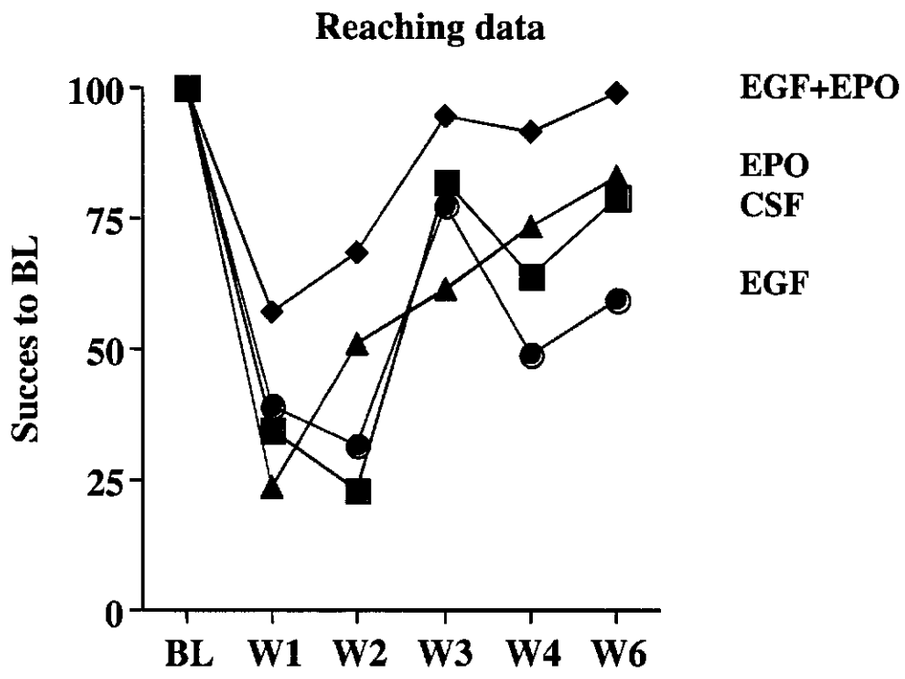

Figure 6.7. Summary of tray reaching performance. Note that animals receiving EGF+EPO displayed the best performance.

6.5. Experiment 3: What is the optimal timing?

The results of Experiment 2 showed that the administration of EGF+EPO provided functional benefit. The question that was not addressed, however, was what the optimal timing of the EGF+EPO administration might be. In order to determine the best time for administration we first examined the time course of cell death after the lesions by studying brains that survived zero, 1, 2, 3, 7, or 14 days after the lesion. Because the

affected tissue was dead by day 3, we then compared the effects of the lesions + factors by administering the factors on postinjury day 0 (as in the previous experiments), day 3, 7, and 14.

6.5.1. Methods and Materials

Subjects. Twelve male Long-Evans rats (Charles River Breeding Laboratories, 300-350g) were used for the staged kill analysis. Thus, all animals recieved surgeries as in Experiment 2 but they were killed either 1, 2, 3, 7 or 14 days later. These animals did not undergo behavioural analysis.

Twenty-five male Long-Evans rats (Charles River Breeding Laboratories, 300-350g) then were divided into five groups (n=5/gp): lesion alone, lesion+EGF+EPO on day 0, lesion+EGF+EPO on day 3, lesion+EGF+EPO on day 7, and lesion+EGF+EPO on day 14. Thus, different groups received EGF on the day of cortical surgery or 3, 7, or 14 days later. The EPO was always administered 7 days after the EGF. The cannulae were implanted in all animals on the day of cortical surgery but they were attached only to a plugged piece of tubing until the appropriate day for connection to the infusion pumps. The details of concentrations and surgical coordinates were as in Experiment 2. In contrast to Experiment 2, however, the lesion alone group did not receive cannulae implantation.

Behavior and anatomy. The behavioural procedures were identical to Experiment 2. At the end of the experiment the animals were given an overdose of Euthansol and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. The brains

were photographed, cryoprotected, and the region through the lesion was cut on a vibratome at 40 um. Every 10th section was stained with Cresyl violet.

6.5.2. Results

Anatomical Results

Staged kills. Within 24h of the pial stripping most cells appeared severely compromised and there were large numbers of microglia in the lesion area. By 48 h postinjury there were no viable neurons visible (Figure 6.8). The cells were slow to be absorbed, however, and even on Day 14 there was still infarcted tissue visible in the lesion area, although it was fragile did survive the histological processing as it disintegrated.

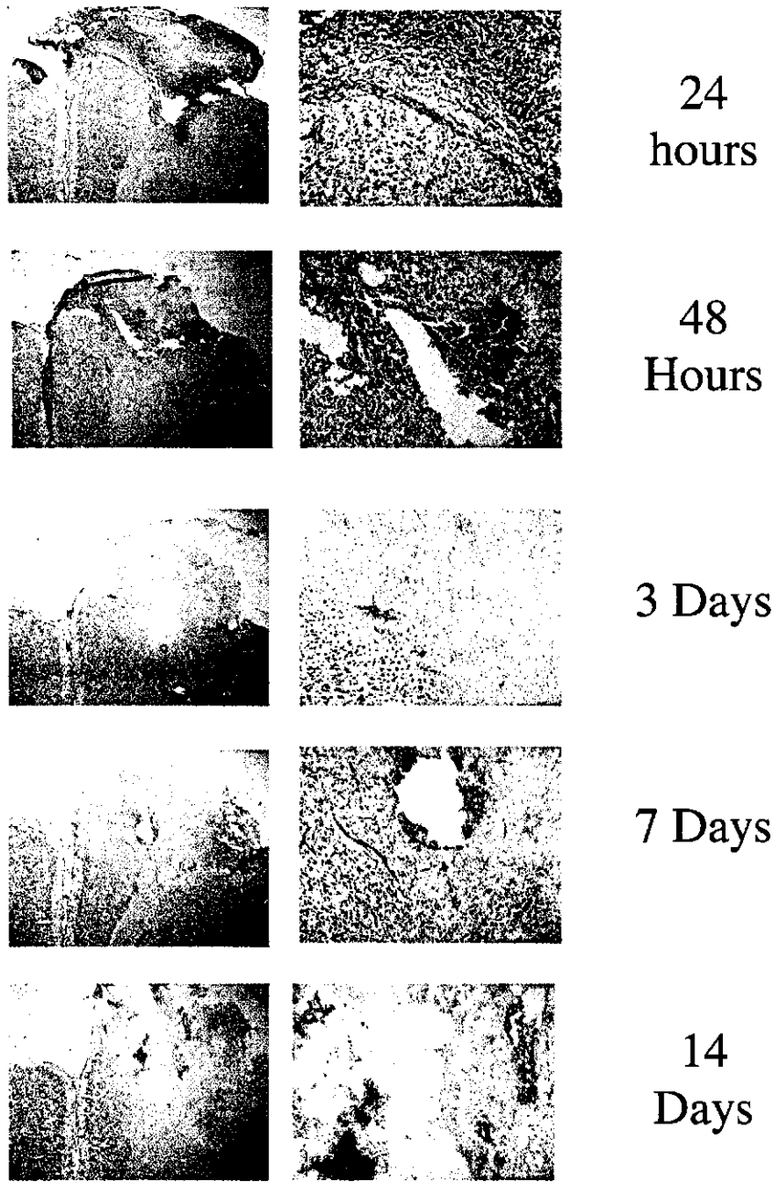

Figure 6.8. Photomicrographs of a coronal section through the pial stripping lesion at different stages (Cresyl violet).

Gross Anatomy. As in Experiment 2, the lesion cavities contained plugs of tissue. There was little difference in the amount of tissue in the plugs in the different groups, and all groups showed considerable variance from virtually no visible cavity to about 50% of the lesion cavity visible (Figure 6.9).

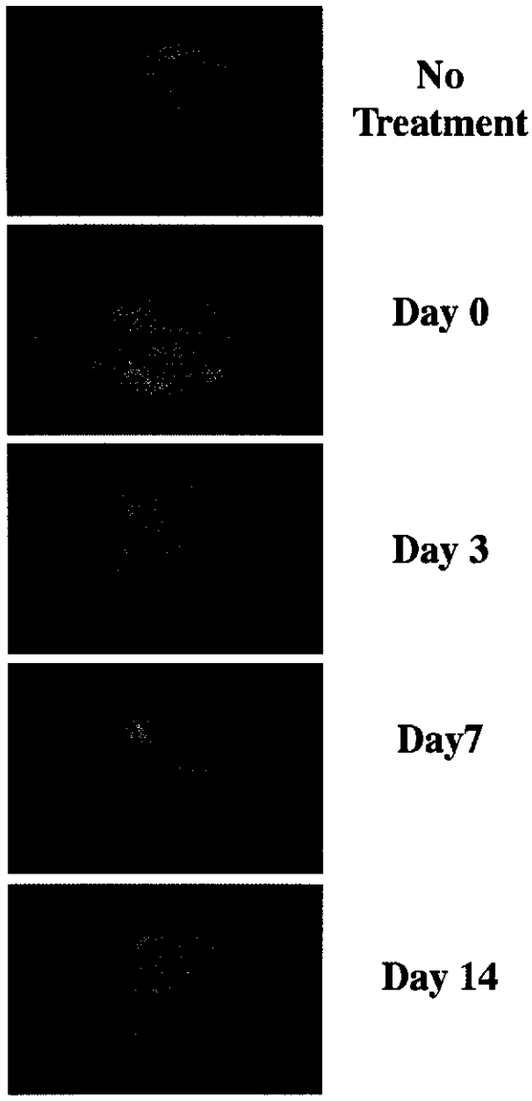

Figure 6.9. Representative brains of animals that sustained lesions to the motor cortex and no treatment, or EGF+EPO treatment starting at different postsurgical times.

Analysis of cresyl violet-stained sections showed that the majority of the cells in the tissue plugs were glia and in many brains there were many patches of white matter. One consistent problem in examining all of the treated brains was the difficulty in determining exactly what was new tissue versus original brain tissue. Regions of normal appearing lamination were presumed to be original brain whereas regions that did not have such lamination was likely new tissue. The areas of new tissue never had a layer I and always had a majority of cells that were small and presumably glia. Comparison of the brains of animals with different pre-infusion periods was difficult but one clear difference was that no neurons were found in any of the day 14 brains. There were patches of what appeared to be white matter, however, within the swirls of presumptive glia forming the tissue plug (Figure 6.10).

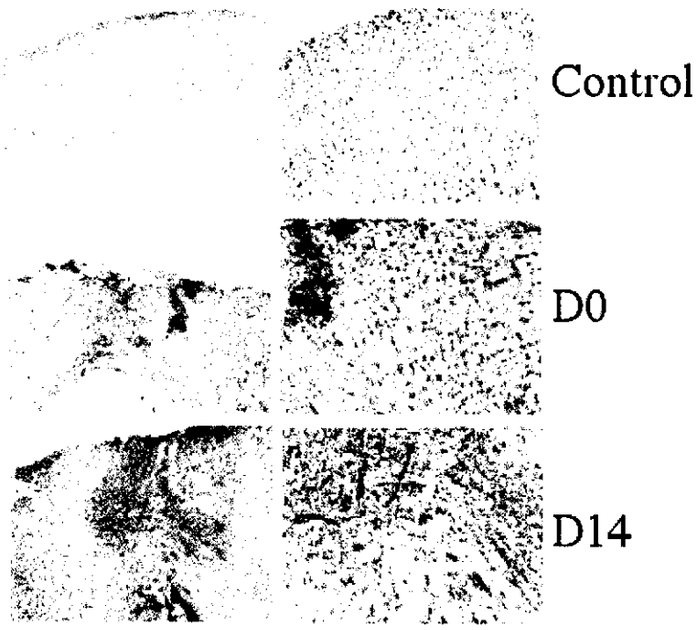

Figure 6.10. Coronal sections through brains of animals that sustained lesions to the motor cortex and EGF+EPO treatment beginning 0 or 14 days after pial stripping. The tissue plugs were largely made of presumptive glia but some neurons were visible in the sections from the day 0 animals.

Behavioral Results

Forelimb Asymmetry. All lesion rats showed an asymmetry of paw use favouring the ipsilateral paw in the first week after the pial stripping. The extent of asymmetry decreased over time in the EGF+EPO groups, but the largest and fastest reduction was seen in the Day 3 and Day 0 group as shown in Figure 6.11).

A two-way ANOVA (group x week) showed a main effect of Group ($F(4,40)=9.9$, $P=.0006$), Week ($F(4,80)=7.0$, $P=.0003$, but no interaction ($F(16,80)=1.6$, $P=.10$).

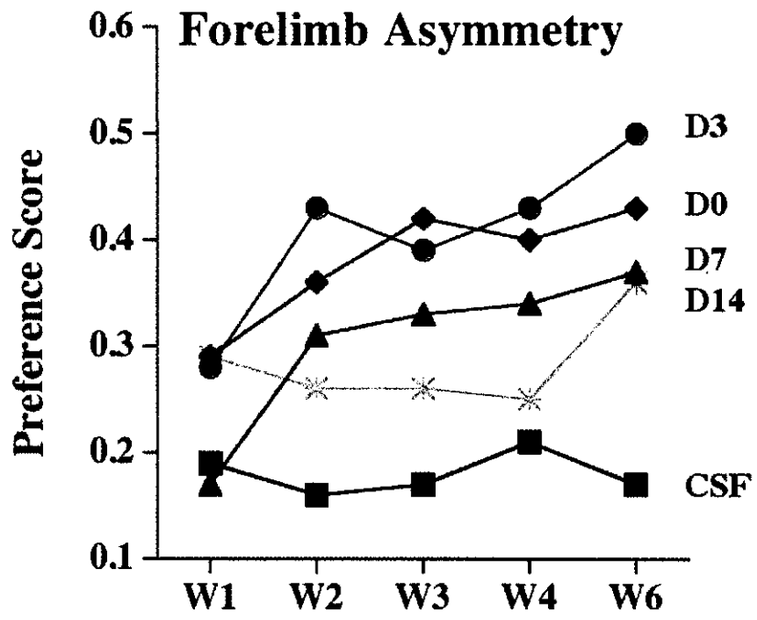

Figure 6.11. Performance on the cylinder task on weeks (W) 1, 2, 3, 4, and 6 after the surgeries (±SE) by animals that received no treatment or EGF+EPO at different postsurgical times. A preference score of 0.5 indicates no preference on the use of left or right forepaw a score of 1 indicates complete use of the ipsilateral forelimb. Note that stroke animals exposed that receive EGF+EPO three days after the stroke performed the best.

Forelimb Inhibition. As in Experiment 2, all ischemic groups failed to inhibit the contralateral forepaw as they had preoperatively and all continued to inhibit the ipsilateral forepaw (Figure 6.12). The Day 0, 3, and 7 groups all showed a decline in their forelimb inhibition deficit whereas the untreated and Day 14 rats showed little change over time.

A two-way ANOVA (Group X Week) found a marginal main effect of Group (F(4,20)=2.7, P=.06), a main effect of Week (F(4,80)=9.5, P=.0001), and an interaction (F(16,80)=2.3, P=.0088). The interaction reflected the differential improvement in the different groups.

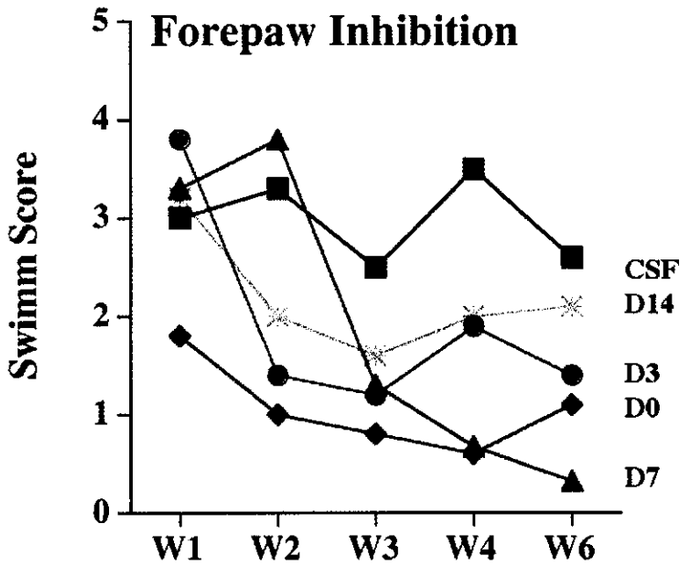

Figure 6.12. Number of strokes (±SE) made with the affected forelimb on weeks (W) 1, 2, 3, 4, and 6 by animals that received no treatment or EGF+EPO at different postsurgical times.

Tray Reaching. All groups were significantly impaired at the task following the lesion and all most groups showed progressive improvement over test weeks (Fgure 6.13). One exception was the Day 14 Group, which failed to improve over the test period. A two-way ANOVA (Group X Week) found no main effect of Group (F(4,20)=1.6, P=.21) but there were significant effects of Week (F(4,80)=10.8, P<.0001),

and the interaction (F(16,80)=2.3, P=.008). The interaction reflected the differential improvement of the groups over the testing weeks.

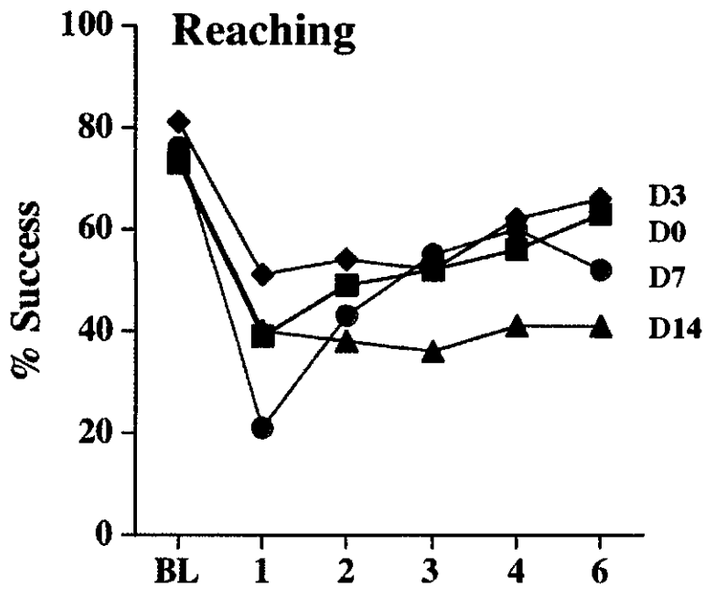

Figure 13. Performance on the tray reaching task on weeks (W) 1, 2, 3, 4, and 6 (±SE) by animals that received no treatment or EGF+EPO at different postsurgical times. Note that by the last weeks of testing stroke animals that received EGF+EPO at days 0 and 3 showed the best performance.

6.5.3. Discussion

The principal results of this experiment were that 1) infusion of the EGF+EPO produced some benefits at all time points tested (Day 0, 3, 7, 14) but that it was most functionally effective when administered beginning on days 3 and 7; and, 2) infusion at

all time points stimulated cell growth and partial filling of the lesion cavities. The fact that the infusions that began after the cells in the infarcted region were dead is consistent with the conclusion that the cells found in the lesion cavity were not simply cells that were rescued from death by the infusions. The fact that all infusion groups showed partial filling of the lesion cavities and some functional benefit is consistent with the hypothesis that the generated cells were somehow participating in the functional benefits observed. The failure to see any advantage of the Day 14 infusions on reaching is contradictory, however, and suggests that something about the timing of the cell generation is important in affecting function.

6.6. Experiment 4: Removing the Tissue

One prediction that comes from Experiments 2 and 3 is that if the generated tissue is contributing to the functional improvement, then removing the tissue should remove the function benefit provided by the tissue. Animals were given EGF+EPO, beginning on Day 3 postinjury. They were then tested repeatedly on two behavioral tests, the cylinder test of forelimb asymmetry and a single pellet-reaching task. After the end of the fifth post-injury week the animals were again anesthetized and half of the animals had the tissue plug removed. The behavior then was reassessed for another four weeks.

6.6.1. Materials and methods

Subjects. Twelve male Long Evans rats (Charles River Breeding Laboratories, 300-350g) were divided into two groups (n's=6): EGF+EPO and EGF+EPO+LX. Animals were housed and cared for as in Experiment 3.

Surgery. Surgery was identical to the EGF+EPO Day 3 group in Experiment 3. At the end of postinjury week 5 all animals were once again anesthetized and for the EGF+EPO+LX group the plug in the lesion cavity was carefully dissected from the "normal" brain and the tissue gently removed by aspiration. There was an obvious color difference in the new and old tissue making the dissection relatively simple. Care was taken not to injure adjacent tissue.

Forelimb asymmetry. The limb asymmetry task was administered as in Experiment 2. Behavior was filmed on postlesion weeks 2, 3, 5, 7, and 9.

Single pellet reaching task. Animals learn to use a forepaw to reach through a slot in a cage for single food pellets located on an external shelf (Whishaw and Pellis, 1990). Reaching boxes were made of clear Plexiglas, with the dimensions 45 cm deep by 14 cm wide by 35 cm high. In the center of each front wall there is a 1 cm-wide slit extending from 2 cm above the floor to a height of 15 cm. On the outside of the wall, in front of the slit, mounted 3 cm above the floor, there was a 2 cm-wide shelf. Two indentations on the floor of the shelf were located 2 cm from the inside of the wall and are centered on the edges of the slit where rats could reach. Food pellets (45 mg Rodent Chow food pellets, Bioserve Inc.) were placed in the indentation contralateral to the limb with which the rat reached. Following a reach, a short pause preceded the presentation of the next pellet. This procedure encouraged animals to move away from the slot after each reach, a procedure that forced them to reposition themselves relative to the reaching slot to prepare for the next reach. The number of successful reaches was scored.

Anatomy. At the end of the experiment the animals were given an overdose of Euthansol and perfused intracardially with 0.9% saline followed by 4%

paraformaldehyde. The brains were photographed, cryoprotected, and the region through the lesion was cut on a vibratome at 40 um. Every 10th section was stained with Cresyl violet.

6.6.2. Results

Anatomical Results

The animals in the EGF+EPO groups had all had at least partial filling of the lesion cavities as in the previous studies. In contrast all of the EGF+EPO+LX groups had the plug removed and the ventricle exposed. All EGF+EPO+LX animals had slight additional damage to the cingulate cortex (Figure 6.14).

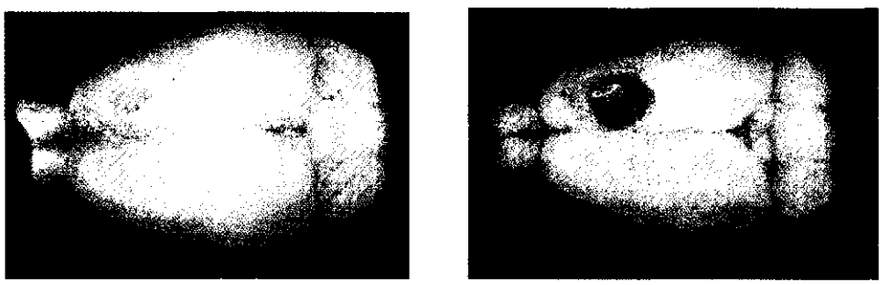

Figure 6.14. Left: The brain of an animal that received EGF+EPO. Right: The brain of an animal that received EGF+EPO+LX. There is tissue visible in the lesion cavity in the top panel but the striatum is visible in the bottom panel.

Behavioral Results

One animal in the EGF+EPO+LX group did not show a behavioral impairment on the reaching test and thus was removed from the behavioral analysis.

Forelimb asymmetry. As in the previous studies, the animals showed a partial return to baseline levels by week 3. After the plug was removed there was a partial loss of the recovered symmetry that continued to decline over the subsequent test weeks (Figure 6.15).

A two-way ANOVA (Group x Week) on the weeks following the plug removal showed a main effect of Group ($F(1,9)=9.9$, $P=.01$) and the interaction ($F(2,18)=3.6$, $P<.05$) but no effect of week ($F(2,18)=2.2$, $P=.14$). The interaction reflected the increasing forelimb asymmetry visible in the EGF+EPO+LX group.

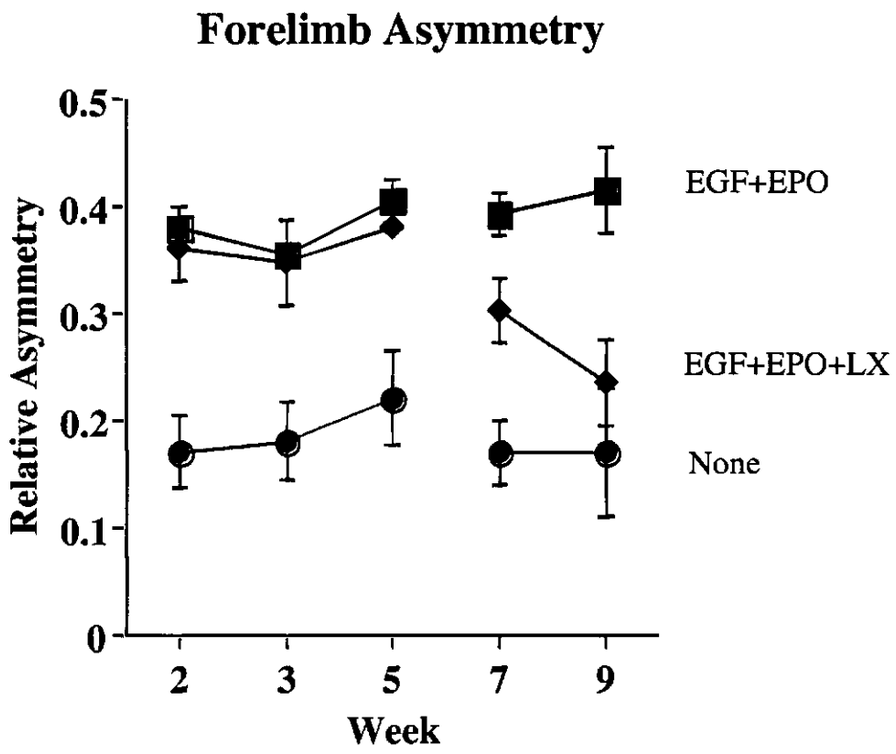

Figure 6.15. Performance on the cylinder task on weeks 2, 3, 5, 7, and 9 after the surgeries (±SE) by animals that received a stroke and no treatment (None), a

stroke and EGF+EPO (EGF+EPO) and animals that on week 6 received a second surgery and the plug was removed by aspiration. A preference score of 0.5 indicates no preference on the use of left or right forepaw a score of 1 indicates complete use of the ipsilateral forelimb. Note that stroke animals that received the second lesion dropped down in their performance and by week 9 they behaved as untreated animals.

Single Pellet Reaching. We had not previously examined performance on this test in animals with the EGF+EPO treatments. All animals (but one) showed a severe initial deficit followed by a gradual improvement over the following four weeks. After the second surgery the EGF+EPO+LX showed an initial loss of function followed by a gradual worsening of behavior over the following two weeks (Figure 6.16).

A two-way ANOVA showed no effect of Group ($F(1,9)=1.8$, $P=.21$) or Week ($F(3,27)=0.5$, $P=.69$), but there was a significant interaction as can be seen in Figure 15 ($F(3,27)=3.6$, $P=.03$).

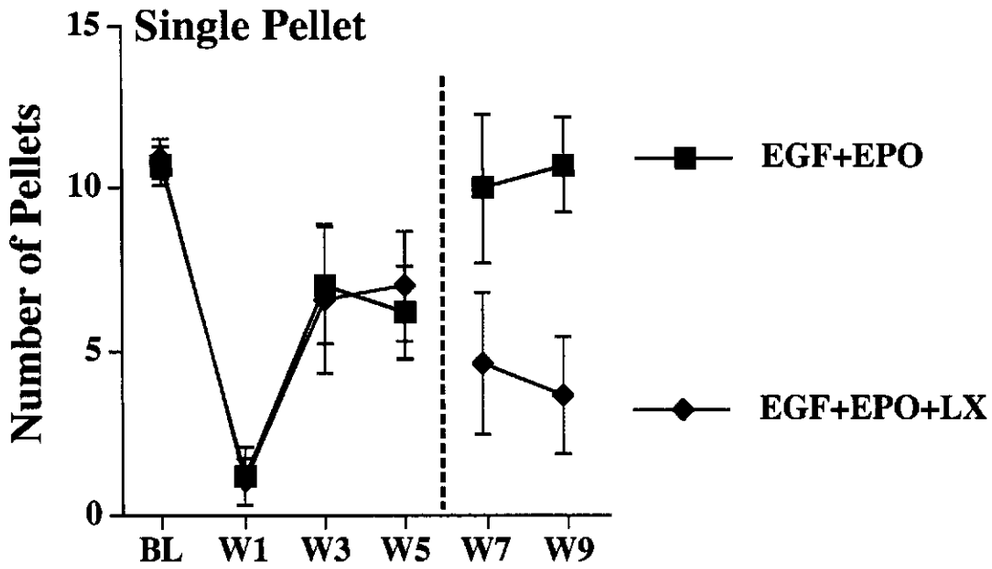

Figure 6.16. Total reaching success displayed by the animals before the lesions (BL) and on weeks (W) 1, 3, 5, 7, and 9 (±SE). Dashed line signifies the time of the second lesion. The lesions produced severe deficits in reaching that slowly recovered over the 5 weeks of testing. Removal of the tissue plug led to a progressive loss of skilled reaching in the EGF+EPO+LX group.

6.6.3. Discussion

The results of Experiment 4 showed once again that infusion of EGF+EPO reduced the behavioral impairments after the cortical injury. The novel finding of the experiment was that removal of the newly generated tissue resulted in a loss of the behavioral savings but, unexpectedly, the loss was not immediately complete and appeared to worsen over time. This latter result suggests that the generated tissue played

a role in the behavioral improvement but it is unclear from this experiment what this role may have been. We return to this issue below.

6.7. General Discussion

The goal of the current studies was to determine whether EGF could sitmulate the production of new cells in the cortically-injured brain and whether such cells would migrate to the lesion area and suppport functional improvement. The results are affirmative but they also show that the EGF-mediated functional recovery is constrained by several factors. We consider several issues arising from these results.

The role of EGF in mediating functional improvement after injury

Infusion of EGF into the cerebral ventricles of animals with vascular lesions of the motor cortex leads to the production of large numbers of newly generated cells. Furthermore, these cells migrate to the site of the lesion and at least partially fill the lesion cavity. The infusion of EGF alone, however, does not facilitate functional restitution and the cells do not show differentiation into a neuronal or glial phenotype. It was only when EPO was infused after the EGF that cells showed a differentiation into a neuronal-like cells that could be identified both by double cortin immunohistochemistry and general cell morphology in the Golgi-stained tissue. The importance of the EPO in stimulating cell differentiation is not surprising given that a similar role for EPO has been identified in vitro (Shingo et al., 2001). The results of Experiment 3 showed that timing of the growth factor administration is important as the best functional outcome was apparent when the EGF was first infused 3-7 days after the injury, which is after the cells in the infarcted region are dead. The effect of the EGF, therefore, is not neuroprotective

but rather must have some other action on the injured brain. Presumably this effect is to stimualate the generation of new neuronal and possibly glial cells.

The fact that EGF+EPO administered 14 days after the stroke still produced thousands of cells that migrated into the lesion cavity but that there was little behavioral benefit was unexpected and suggests that the mere presence of the cells is not sufficient. Rather, it suggests that the cells must be present at a particular critical time postinjury. We note too, however, that there appeared to be a difference in the tissue in the day 14 animals as we did not observe any presumptive neurons in these brains.

The results of Experiment 4 show that these new cells play some role in the improved functional outcome but the precise role of the cells remains uncertain. There are two logical possibilities. First, the fact that the improvement in behavior did not immediately completely disappear suggests that at least part of the effect of the new tissue was to produce some type of factor that influenced the functioning of the remaining brain. In its absence, the brain slowly lost whatever changes had been induced and the behavioral advantage was lost. Second, we have assumed that the new cells were all located in the tissue plug but we have no direct evidence of this. It is possible that removing the tissue plug removed only a portion of the new, and active, cells. Thus, at this point we can only speculate on the mechanism of the behavioral improvement after the EGF+EPO infusions but it seems most likely that the functional effects are related to actions of the new cells on the functioning of adjacent regions.

Where do the new cells originate?

One obvious question that is not easily answered in the current studies relates to the origin of the cells that fill the lesion cavity. Parallel studies by Morshead and

colleagues (e.g., Kim et al., 2003) have shown that if cells in the SVZ are labeled with a retrovirus a few days prior to pial stripping of the motor cortex as in the current studies, there is a proliferation of labelled cells visible in the SVZ and that these cells migrate to the site of injury. Similarly, if animals are injected with the mitotic marker BromodeoxyUridine (BrdU) for the first three days of EGF treatment in rats with lesions as in the current studies, labeled cells can be found in the lesion cavity as well as in the white matter adjacent to the cavity. Taken together with the results of the current studies the Morshead results suggest that the cells originate in the SVZ, migrate to the lesion zone, and then act to alter the synaptic organization of the injured hemisphere. The precise mechanism of this action remains to be determined.

Future Directions

The results of current series of experiments and the Morshead experiments lead to several obvious directions. First, we can identify whether the cells that form the plug in the lesion cavity form any connections with the remaining brain. This can be shown by using anterograde and retrograde tracers. Second, it has been shown in studies by Kolb et al (1998a) that a single injection of BrdU on prenatal day 13 will chronically downregulate the activity of cells in the SVZ. We can speculate that such injections might block cell generation, and functional recovery, in the current model. Third, although we have shown that the effects of the EGF+EPO combination can last at least 9 weeks, we do not know whether the changes may persist indefinitely. We also do not know whether the EGF+EPO combination can be enhanced by behavioral therapies such as complex housing or rehabilitation training. Finally, we do not know if the effects of vascular lesions elsewhere in the cortex will be attenuated by the EGF+EPO treatments,

nor do we know if lesions induced by other means might be affected. These questions will all be the grist for future studies.

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7. General Discussion

Stroke continues to be a leading cause of disability in North America. Most stroke survivors have to deal with chronic functional deficits and few therapies have been developed to deal with the long-term behavioral consequences of stroke. The main goal of this thesis was to characterize the behavioral and anatomical consequences of motor cortex damage. The second goal was to employ different therapeutic approaches to try to enhance behavioral recovery using a model of focal stroke. The studies were conducted in adult rats and several behavioral measures were obtained in each of the experiments. In addition, anatomical, morphological, or electrophysiological measures were used to evaluate changes in several remaining brain areas. The results can be summarized into three principal findings: 1) damage to the motor cortex by different experimental methods produces similar behavioral deficits but different reorganization of remaining cortical structures; 2) there are bilateral behavioral deficits after unilateral motor cortex stroke; and 3) it is possible to enhance recovery/compensation of function after stroke. These findings and other relevant issues arising from these studies will be discussed in the following sections.

7.1. Differences and similarities among stroke models

A major problem that stroke research continues to face is the wide range of animal models used (specifically the methodology used to induce the lesion). Different scientific communities (e.g., basic vs. clinical scientist) constantly debate about which animal model is more relevant for understanding the human condition. Looked at objectively, however, each model has advantages and disadvantages. The size, severity, and location of the infarction can vary from model to model just like it varies from

patient to patient. Thus, depending upon the specific questions that one wishes to address, there is a model of stroke that best fits those needs. For our studies in which we tested different therapeutic agents we chose to use the devascularization of surface blood vessels (pial strip) model. The pial strip model was advantageous for several reasons: First, it was highly reproducible providing a very well-defined infarct. Second, it reliably produced quantifiable behavioral impairments. Third, the model allowed for some degree of spontaneous recovery while at the same time leaving a chronic behavioral syndrome. All these features become relevant when testing drugs or rehabilitation treatments. In order to test the putative role of different therapeutic agents in functional recovery, it is important to have precise control over the area of tissue that is damaged. Lesions produced by other models (e.g. MCA occlusion) generate inconsistent infarcts that can include one or more of the following areas: prefrontal, latero-frontal, parietal and somatosensory cortices as well as subcortical structures like the striatum. Large lesions allow very limited spontaneous recovery and thus the efficacy of the treatment could be minimum or masked due to the severity of the impairments. On the other hand, very small infarcts allow for very good spontaneous recovery (sometimes almost complete) diminishing the effectiveness or importance of the therapeutic agent.

In Experiment 1, we compared behavioral deficits after lesions to the motor cortex induced by aspiration, pial stripping, electrocoagulation of the distal branches of the middle cerebral artery (MCA), and electrocoagulation of the entire MCA. We found severe enduring deficits in all animals regardless of etiology. Nonetheless, the behavioral deficits were not all equivalent and animals with electrocoagulation of the distal branches of the MCA showed the best behavioral recovery. This finding perhaps is not surprising

given that the lesion size in this last group was significantly smaller when compared to the other groups. There are few studies in the literature that address the behavioral outcome after different kinds of lesions to the motor cortex. One recent one, however, by Gilmour et al., (2004) produced lesions to the motor cortex by aspiration, excitotoxicity (quinolinic acid), or vasoconstriction of blood vessels by intracortical infusions of endothelin-1. The authors found identical behavioral deficits and similar rates of recovery among the three lesion groups on a skilled reaching task. Their finding is strikingly similar to the behavioral results obtained in our Experiment 1. Although we did not do single pellet-reaching in Experiment 1, the results of Gilmour et al., and our own observations (unpublished data) suggest that at least with end-point measures no visible differences exist among rats with lesions of different etiologies. It is possible, however, that careful kinematic analysis of reaching behaviors could reveal different compensatory mechanisms in the different lesion groups. Time, cost, and expertise might be reasons why very few comparative studies have been conducted to assess long-term functional outcome after stroke but this issue needs further investigation.

A second finding of Experiment 1 was the contrasting differences in cortical morphology after the different lesion procedures. Animals that received devascualarization lesions showed increases in dendritic arbors in both the lesion and intact hemispheres. Animals that received aspiration lesions showed atrophy in dendritic arbors of areas adjacent to the lesion. In contrast, they displayed increased spine density in the same areas – a result suggestive of different compensatory mechanisms. The observation of these different morphological sequelaes and yet similar behavioral deficits seems somewhat counterintuitive and raises an important question: Is neuronal plasticity

correlated to any degree with recovery of function? This issue will be further discussed in following sections.

7.2. Rules of plasticity and how they relate to recovery

The results of the experiments in this thesis support our initial assumption that there are three ways that the brain can recuperate after injury: reorganization of existing circuits (Experiments 1 and 2), creation of new ones (Experiments 3 and 4), and regeneration of lost ones (Experiment 5). Experiment 1 showed that there is some degree of spontaneous recovery of function after lesions of the sensorimotor cortex and substantial morphological changes in remaining cortical areas. Experiment 2 showed that although there are chronic deficits in reaching with the ipsilateral-to lesion paw after motor and latero-frontal lesions, there is also great deal of spontaneous recovery and reorganization of dendritic fields in the intact motor cortex. In Experiment 3, nicotine enhanced functional recovery in animals with motor cortex lesions, and also enhanced dendritic arborization in neocortical areas of the ipsilateral and contralateral hemispheres suggesting these two phenomena could be correlated. Experiment 4 showed that olfactory stimulation attenuates behavioral deficits after motor cortex lesions, and although no mechanism has been described for the recovery, it is proposed that the treatment promoted neuronal plasticity. Finally, Experiment 5 showed that administration of EGF followed by EPO stimulated the generation and differentiation of neurons. The new cells migrated to the site of the lesion and seemingly replaced the lost ones. Animals treated with EGF and EPO showed enhanced behavioral recovery. Taken together, these results strongly suggest a relationship between recovery of function and neuroplasticity.

If we assume that some form of neuronal plasticity is at least partially responsible for recovery of function after brain damage there area number of caveats that need to be considered. For example, it has traditionally been thought that increased synaptic space (e.g., spines or dendrites) would also increase the potential for recovery. In contrast, we found in Experiment 1 that although there was spontaneous behavioral recovery this recovery did not correlate (to the extent of our investigation) with increased synaptic availability. Three of the four groups of animals showed spontaneous recovery of function at a similar rate for each lesion group. At the same time animals in these three groups showed decreased dendritic arbors and increased spine density (aspiration group), hypertrophy on dendritic fields (pial strip) and marginal increase in dendritic arbors (MCAO). If neuronal plasticity is correlated with recovery of function then it would make sense that animals with suction lesions showed less recovery of function and animals with pial strip lesions showed the best behavioral recovery. There are a few possible explanations that could account for the absence of behavioral differences. First, it could be that the behavioral measures were not sensitive enough to detect behavioral differences among groups. Second, it is possible that there are many different ways that the brain can compensate for injury (e.g., less dendrites but more spines) and for some reason different etiologies stimulate different types of synaptic change. Third, it could be possible that other areas (not studied) like the cerebellum changed to compensate for the functional loss. For example, it has been shown in humans, that after recovery from motor cortex stroke, movement of the recovered hand is associated with increased bilateral activation of the cerebellum (Small et al., 2002; Chollet et al., 1991).

Another caveat to the relationship between brain plasticity and behavior is the puzzling finding of severe deficits in the acquisition of a motor task in intact animals previously treated with nicotine (Experiment 4, Appendix 1). These animals displayed enhanced performance on a reaching task that they acquired before the nicotine administration but their performance on a task acquired later was dreadful. Animals treated with nicotine showed similar increases in dendritic arborization in the motor cortex of both hemispheres and did not display learning-dependant plasticity like control animals did. It is possible then, that enhancing plasticity in the normal brain could actually have a detrimental effect. There is anecdotal evidence (B. Kolb's unpublished studies) suggesting that agents that otherwise produce beneficial effects when administered after a cortical injury such as NGF or choline, can disrupt cognitive and motor performance when given before the injury. It is possible to suppose that these agents influenced plasticity before the injury and therefore attenuated or altered the dendritic reorganization that otherwise supports spontaneous behavioral recovery. A question that remains to be answered is whether or not administration of nicotine during or after the acquisition of the skilled-reaching task would have a different effect on the behavioral outcome. If neurochemical and structural changes in the cortex are required for skilled learning to occur, one would predict that there is a limit in the amount of plasticity that the brain can undergo and thus later administration of nicotine would have little or no effect.

Considering these caveats together it appears that the relationship between neuroplasticity and behavior in the intact or injured brain is not as straightforward as one might have liked!

7.3. Compensation/Recovery

Perhaps one of the most important aspects in developing rehabilitative therapies is the understanding of the precise nature of the deficits that follow brain damage and the relationship between these deficits and remaining functional capacities. It is tempting to think that if behavior improves after injury then there is recovery of the original behavior (Kolb 1995). However, if indeed, there are specific behaviors mediated by local neural areas (i.e., some form of localization of function), evidence of recovery could be challenged on the grounds that the "recovered" behavior is simply a series of clever "tricks" used by the brain to accomplish its ends (Slavin et al., 1988). In this rigorous sense, *recovery* of function is impossible; what is possible is *substitution* of function. This substitution is considered a form of compensation or adaptation to the deficit. Behavioral compensation in the context of the motor system is defined as the ability to achieve a goal by substituting remaining movement abilities for lost movements (Whishaw, 2000). Lesion studies suggest that following injury, mainly compensation and not true recovery mediate improved performance. For example, it has been described that after stroke in humans (Cirstea and Levin, 2000), monkeys (Friel and Nudo, 1998), and rats (Whishaw, 2000) reaching for an object (e.g. piece of food) becomes a task accomplished mainly by compensatory strategies related to postural modifications.

In our studies, although we repetitively allude to the word recovery to indicate that the animals now can do what they could do before the lesions (e.g. reaching for food) or that they can do it better than animals that did not receive a treatment, we are really referring to compensatory mechanisms as revealed by careful kinematic analyses of single-pellet reaching (e.g., Experiment 2). We, like many others, use the term "recovery"

loosely perhaps because if a person can regain the skill of writing or a rat the skill of reaching after a stroke in our minds they succeeded and accomplished the task. This assumption of recovery can always be challenged under the assumption that greater analysis would have revealed an underlying difference form the original behavior (Slavin et al., 1988).

Finally, the concept of compensation of function after brain damage makes more sense than the term "recovery" if we believe that the nervous system is plastic. We have learned that there are multiple examples of studies that support the idea of intact parts of the brain taking over the functions of the lost ones and it is this notion that remains the primary candidate to explain how the brain compensate for functional deficits.

7.4. Treatments that do not work in other models

One obvious question arising from the work of this thesis is whether the treatments that worked in the pial stripping model (e.g., nicotine, olfactory stimulation, EGF+EPO) would also work in other models of stroke. If the nature of progression of different lesion procedures is different, leading to different morphological changes, then the administration of drugs or rehabilitative therapies may have a different effect on recovery of function depending on the technique used to produce the lesion. It may also be reasonable to think that there is a "common" mechanism by which any treatment would work, regardless of the lesion etiology. Although there has not been any direct evidence that supports the "common mechanism" theory, there are multiple examples in the literature of treatments that have worked in one particular setting but not in others. The use of amphetamine as a therapeutic drug after stroke illustrates this point. Administration of amphetamine has been reported to ameliorate motor deficits after

stroke in humans (Crisostomo et al., 1988; Walker-Batson et al., 1995; Sawaki et al., 2002) but it also has been reported to produce no benefit (Sonde et al., 2001; Treig et al., 2003). Variables such as age, sex, lesion size, time after the insult, and method of motor function assessment are other factors that contribute to the difficulty of rating the effectiveness therapeutic agents.

Another interesting example of how the nature of the lesion differentially affects recovery comes from studies of patients with brain tumors versus patients with stroke lesions. Slowly progressing lesions appear to allow time for reorganization within the brain and it has been suggested that this might be the reason why patients with tumors exhibit better outcome than patients with similar lesions caused by stroke. Anderson et al., (1990 in Benton and Tranel, 1988) conducted a systematic comparison of outcome in patients with stroke versus patients with tumors. They reported that stroke patients had more predictable neuropsychological deficits and more severe impairments than tumor patients who, in contrast, had much less impairment than would have been predicted from the location and size of the lesion. Seitz and Freund (1997) reviewed studies showing that slowly progressing lesions often remain asymptomatic for years because the tumors cause a substantial reorganization of function as measured by regional blood flow studies (in Stein and Hoffman, 2003). It is possible to imagine that different treatments could have a different effect on these patients depending not only on the kind of lesion that they have but also if the brain has changed already or not. It would be very interesting to follow up patients with tumors and strokes that have been rehabilitated to find out if they display different rates of recovery.

In addition to the studies described in this thesis, we have also tested the effects of nicotine, olfactory stimulation, sensory deprivation, and administration of EGF plus EPO on a model of motor cortex damage by aspiration. We found that nicotine and sensory deprivation ameliorated behavioral deficits after the lesion but olfactory stimulation and EGF and EPO did not produce any beneficial effects. These results lead us to believe that any given treatment interacts differently with the etiology of the lesion and that possibly there are separate mechanisms of action by which therapeutic agents can or cannot promote recovery of function. This interaction between treatments and the nature of a lesion is obviously a place for considerably more investigation.

7.5. Implications of bilateral deficits

"Thinking, as most neurologists probably do, that in right-handed persons writing is governed solely by the left hemisphere, it came as a surprise to the patient that there were clear-cut changes in his handwriting after the stroke... the changes were observed constantly, and were present even under the most favorable conditions for writing" (Brodal, 1973). The neuroanatomist Alf Brodal (1973) wrote these words after suffering from a stroke. He reported his own performance on different motor tasks after an embolism in a branch of the MCA on his right hemisphere. He noted that although the lesion did not damaged the left hemisphere and the principal deficits were an acute left-sided hemiparesis, his writing (with his right hand) was affected even nine months after the stroke. Numerous studies have shown that after a unilateral stroke the function of the ipsilateral limb is not normal (see Table 3.1). These abnormalities range from reduction in strength and speed of movement, to impairments in accomplishing complex skilled movements (Sunderland et al., 1999, 2000).

Although it has been reported in the human literature repeatedly and several investigators have looked exclusively at the impairments in the ipsilateral limb after stroke, our findings on Experiment 2 are the first to have systematically looked at skilled motor impairments on the ipsilateral paw. We found enduring deficits in the single-pellet reaching task even after extensive training. Curiously, we also observed enhanced dendritic arborization on the intact hemisphere (the one controlling the reaching paw). Despite the fact that there were enduring behavioral deficits there was an increase in dendritic arborization in the contralateral motor cortex. These results can be viewed as further evidence that plasticity may also produce unwanted effects, or (as we proposed in the discussion of Experiment 2) that those plastic changes are responsible for the spontaneous substantial recovery of the ipsilateral paw on skilled reaching.

Studies aimed at examining bilateral deficits after stroke provide evidence that ipsilateral impairments are subtle compared to the gross sensorimotor losses on the contralateral side. Ipsilateral deficits nonetheless can be long lasting and debilitating (Brodal, 1973). We believe that having characterized the deficits with the ipsilateral paw and the course of its recovery provides us (and others) with an excellent tool to study recovery/compensation after pharmacological or rehabilitative treatments. It is reasonable to think that drugs that have broad effects in the brain (e.g. nicotine, amphetamine) and that have shown to be beneficial in ameliorating deficits of the "most affected limb" would have also a beneficial effect on the ipsilateral limb. Having said this, it follows that after stroke rehabilitative treatments like physical therapy should be aimed at working both sides of the body.

7.6. Future Directions and Conclusion

The results of the studies in this thesis showed that motor recovery occurs after stroke and that different pharmacological, experiential, and regenerative treatments can provide effective therapy for brain damage after stroke. The results also showed that after stroke substantial dendritic changes take place in remaining cortical areas. Furthermore, the results of the current studies suggest that enhancing plasticity in the injured brain could be responsible for the improved recovery observed in animals subjected to treatments like nicotine. Although the relationship between the behavioral outcome and the anatomical changes in the brain is correlational at best, it is suggestive and should stimulate further investigation on the factors mediating functional recovery after stroke.

Overall, each one of the experiments described in this thesis could potentially lead to a number of additional studies looking at the anatomical mechanisms that support recovery of function after brain damage. Multiple studies of brain imaging or mapping have shown that after a brain lesion, changes in other brain regions take place (see Nudo 2001, 2003; Cramer, 2003 for reviews). Experiments designed to test the idea that brain reorganization supports functional recovery should be a priority. It is possible to speculate that agents that block plasticity would also block the behavioral recovery often seen after stroke (for a compelling example see Sawaki et al., 2003). It is important, however, to keep in mind that even when dendritic sprouting can be correlated with recovery, sprouting is only one of many regenerative and degenerative processes that co-occur following brain injury. Furthermore, plasticity by itself might not be enough to reestablish function. For example, it has been reported that stroke patients exhibit the emergence of deficits in the ipsilateral hand associated with improvement in the affected

hand (Cramer et al., 1997); so it is possible that gains in one aspect of function might arise at the cost of another.

In an insightful paper on the issues relevant to present and future neurological rehabilitation Bach-Y-Rita (2000) suggested that programs that provide cost-effective, measurable recovery of function will dominate rehabilitation. One exciting result from the experiments in this thesis is the finding that treatments like olfactory stimulation can enhance recovery/compensation after stroke suggesting that inexpensive, simple treatments possess the ability to ameliorate behavioral deficits after brain damage. This result also has implications for human studies because to our knowledge there is no evidence that suggests that "aromatherapy" could have a detrimental effect on the brain or on behavior. The finding that nicotine promotes recovery could have similar implications given that nicotine is a relatively "safe" drug that has been used for centuries and its side effects are far less controversial than the ones produced by amphetamine for example.

Although much research has been devoted to understanding the mechanism by which recovery after brain damage can occur, there are several questions that remain unanswered. Why is there so much variation in recovery among subjects with similar damage? Under what conditions could recovery be maximized? What factors might slow or impede recovery of function? What parts of the brain are responsible for behavioral recovery? These questions might go unanswered for some time to come because even modern imaging techniques may not be capable of directly and unambiguously answering them. With a more complete understanding of the events leading to recovery/compensation after brain injury, we might be able to address some of these

questions. The capacity of the adult brain to change and reorganize is no longer in doubt and it might become possible to design treatments that promote plastic changes that ultimately enhance recovery after brain damage. The results of this thesis have created more questions than answers with respect to the organization of behavior and the nervous system and in particular questions of how brain and behavior influence and modify each other in the presence of neurological demand.

Ultimately, whether the therapeutic treatment is a drug, a behavioral therapy, or any other kind of intervention, a patient suffering from stroke or other type of brain damage only cares (as he/she should) about how life is going to change and how can it be improved. The results of this thesis are promising and suggest that perhaps effective treatment may become available to these patients sooner than later.

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Appendix I

Nicotine Stimulates Dendritic Arborization in Motor Cortex And Improves Concurrent Motor Skill But Impairs Subsequent Motor Learning

A.1. Abstract

The effect of the premature commitment of neurons to exuberant growth by nicotine on concurrent and subsequent learning is unknown and was the focus of the present study. Animals were trained on a tray reaching for food task (where lots of pieces of chicken feed were available) for three weeks before they received two daily injections of nicotine (0.3 mg/kg) or 0.9% saline for twelve days. Measures of tray-reaching performance were obtained before the administration of nicotine and every other week for a total of seven weeks. In addition to the reaching task, motor skill was also monitored in a test of vertical exploration. Starting on week eight, animals were given a novel motor skill problem that required them to learn to use a forepaw to reach through a slot in a cage for single food pellets located on an external shelf. Pyramidal cells in the forelimb area of both hemispheres were then examined for dendritic length and branching using a Golgi-Cox procedure. Animals treated with saline displayed excellent performance in both reaching tasks and increased neuronal branching in Layer V pyramidal cells in the motor cortex contralateral to the reaching paw. Animals treated with nicotine showed bilateral increases in neuronal branching. Behavioral results showed that nicotine improved forelimb use in the concurrently administered tray-reaching task, but severely degraded quantitative and qualitative scores of skilled forelimb use in the subsequently administered single-pellet reaching task. The results suggest that plasticity coincidence with skilled training is essential to skilled motor learning, but this expenditure can impair subsequent learning.

A.2. Introduction

Administration of the psychostimulant nicotine has wide ranging effects on brain and behavior in humans and nonhuman animals alike. Acute administration of nicotine can enhance vigilance (Mancuso et al., 1999; Lee et al., 1997), attention (Young et al., 2004; Hahn et al., 2003; Lawrence et al., 2002), and motor performance on skilled tasks, e.g., hand writing (Tucha and Lange, 2003). The periodic administration of nicotine can result in addiction as well as increases in neuronal dendritic length and synapse number in a number of brain regions, including the nucleus accumbens, and prefrontal cortex (Brown and Kolb 2001). This association between nicotine administration and neuropile changes may underlie addiction (for review see Mathieu-Kia et al., 2002). The ability of nicotine to stimulate neuronal plasticity has suggested that nicotine may be a useful treatment for enhancing recovery from brain injury, especially if behavioral therapy and nicotine administration are coincident (Brown et al., 2000, 2001).

Whereas the close association between nicotine administration and exuberant neuronal change may enhance learning, little is known of the consequences of premature commitment of neurons to plastic changes prior to subsequent motor learning. One line of evidence shows that exposure to an enriched environment can positively alter behavior and the dendritic morphology of cortical pyramidal neurons (Greenough & Chang, 1988) For example, housing animals in complex environments produces a global increase in dendritic length that is correlated with enhanced behavioral capacities on both motor and cognitive tasks. Environmental enrichment, however, can arm animals with a variety of sensory and motor skills that may in themselves enhance subsequent behavioral performance. Recent findings that psychostimulants can enhance dendritic length and

synapse number in the absence of behavior experience (Robinson & Kolb, 1999) raises the question of what effect the prior commitment of neurons to plastic change has on the subsequent ability of an animal to learn. One possibility is that the availability of enhanced neuronal arbor could provide a substrate for enhanced behavioral modification. On the other hand, it is possible that the prior commitment of plastic capacity is disadvantageous. This second possibility is supported by evidence that prior exposure to amphetamine or cocaine blocks experience-dependent changes in dendritic arborization in animals placed in complex environments (Kolb et al., 2003). A more direct answer to the questions related to the effects of the prior commitment of neuronal plasticity requires an explicit examination of how prior drug induced neuronal enhancement affects subsequent novel learning. This was the purpose of the present study.

Here we asked whether exposure to a psychomotor stimulant (nicotine) would alter the concurrent performance of a skilled motor task and affect the learning of a subsequently administered motor task. Furthermore, we asked if nicotine would change the pattern of dendritic changes normally associated with motor learning. Rats were trained for three weeks on a tray-reaching task where many little pieces of chicken feed were available before receiving a schedule of twelve days of saline or low dose nicotine administration. Rats were tested once before the administration of nicotine and every other week after the first day of nicotine for a total of seven weeks. To account for activity changes relevant to forelimb use associated to the nicotine administration, forelimb use for weight support during explorative activity was examined using the "cylinder test" (Schallert et al., 1997). At least two months following the completion of the drug administration schedule, the rats were given a novel motor task in which they

were required to learn to use a forepaw to reach through a slot in a cage to retrieve a single food pellet located on an external shelf. At the completion of the behavioral tests, dendritic changes in both hemispheres were examined using Golgi-Cox analyses of the pyramidal cells of layer V of the forelimb area.

A.3. Materials and Methods

Subjects

Subjects were 14 male Long-Evans hooded rats, 4 months old and weighing 300-400 g at the beginning of the experiment. Detailed kinematic analyses were performed on only 10 rats (5 per group) but the anatomical analyses were done on all rats. Animals were raised in the University of Lethbridge vivarium and were housed in groups of two individuals in clear plexi-glass cages. The colony room was maintained on a 12:12h light/dark cycle (08:00-20:00 h) and the temperature regulated at 22 °C. Experiments were conducted according to standards set by the Canadian Council on Animal Care and approved by the University of Lethbridge animal care committee.

Drug Administration

For the nicotine and saline administration all animals were taken away from the colony into a separate room for 20 minutes where they received one injection in the morning and one in the afternoon for a period of 12 days. Rats were injected subcutaneously with 0.9% saline (control) or nicotine hydrogen tartrate salt (Sigma, St Louis, MO, USA) 0.3 mg/kg (nicotine).

Food restriction

One week before beginning the behavioral testing, the rats were changed to a restricted food intake: Each animal received 20 gr. of food per day (normal daily consumption ranges from 18-25gr) an hour after the testing session was completed. Their body weight was maintained at about 95-98% until the completion of the behavioral testing.

Cylinder test

To account for activity changes relevant to forelimb use associated to the nicotine administration, forelimb use for weight support during explorative activity was examined by placing the rats in a transparent cylinder (Figure 1) 20 cm in diameter and 30 cm high for three minutes starting on the first week of nicotine administration and every other week for a total of seven weeks (Schallert et al., 1997). The animals were individually placed in the cylinder during the three minutes of each testing session. A mirror was placed underneath the cylinder at an angle to allow the experimenter to videotape the animal's activity from a ventral view (Pinel et al., 1992). The cylindrical shape encouraged vertical exploration of the walls with the forelimbs, but the walls were high enough so that animals could not reach the top. Forelimb use was measured during vertical exploration. Each forepaw contact with the cylinder wall was counted. When simultaneous limb contact was observed, a touch was counted for each paw. Two measures were taken: total amount of touches during vertical exploration and an asymmetry score of forelimb use. This score was calculated (i.e., right forelimb/(left + right)) to obtain a score where 0.5 represents perfect symmetry and any number closer to zero would suggest a decrease in the use of the affected limb.

Tray Reaching

Tray boxes were made of plexiglass with dimensions 26 cm high, 28 cm deep, and 19 cm wide (Figure 2). The front of the boxes was constructed of 2 mm bars separated from each other by a 9 mm gap. Clear plexiglass tops allowed access to the inside of the box. A 4 cm wide and 0.5 cm deep tray was mounted in front of the bars. The tray contained food fragments weighing approximately 30 mg each. Animals had to reach between the bars, grasp the food and retract it where they were able to freely eat. If the rat made a reaching movement (forepaw inserted through the bars, but no food was grasped or the food was dropped), the movement was scored as a "reach", whereas if the rat obtained the food and consumed it, the movement was scored as a "hit". Success was calculated as follows:

Success percent = ("hit" / "reach + hit") X 100

Single Pellet Reaching

Behavioral training started eight weeks after the last nicotine administration. Reaching boxes were made of clear Plexiglas, with the dimensions 45 cm deep by 14 cm wide by 35 cm high (Figure 3). In the center of each front wall was a 1 cm-wide slit which extended from 2 cm above the floor to a height of 15 cm. On the outside of the wall, in front of the slit, mounted 3 cm above the floor, was a 2 cm-wide shelf. Two indentations on the floor of the shelf were located 2 cm from the inside of the wall and were centered on the edges of the slit where rats could reach. Food pellets (45 mg Rodent Chow food pellets, Bioserve Inc.) were placed in the indentation contralateral to the limb with which the rat reached (Whishaw, 1990). Following each reach, a short pause preceded the presentation of the next pellet. This would encourage animals to return to the back of the box after each reach, which forced them to reposition themselves

and prepare for the next reach. Reaching performance was assessed on two measures: (1) Success on first reach: if a rat obtained the food pellet following the initial limb advance, this reach was scored as a hit. (2) Total success: if a rat obtained a piece of food either following the first limb advance or after a number of limb advances, the reach was counted as a hit. Success scores were computed as follows:

Success percent = (number of hits/total given number of pellets) X 100

Qualitative reaching analysis

Reaching movements made during the single pellet task were analyzed using a rating scale derived from Eshkol-Wachmann Movement Notation (EWMN: Eshkol and Wachmann 1958; Whishaw, 1993) analysis of reaching. A reach was subdivided into ten components. (1) Limb lift: the limb is lifted from the floor with the upper arm and the digits adducted to the midline of the body. (2) Digits close: as the limb is lifted, the digits are semiflexed and the paw is supinated so that the palm faces the midline of the body. (3) Aim: using the upper arm, the elbow is adducted so that the forearm is aligned along the midline of the body, with the paw located just under the mouth. This movement involves fixation of the distal portion of the limb, so that digits remain aligned with the midline of the body. This posture is likely produced by a movement around the elbow that reverses the direction of movement of the paw to compensate for the adduction of the elbow. (4) Advance: the head is lifted and the limb is advanced directly forward above and beyond the food pellet. (5) Digits open: as the limb is advanced the digits are extended and opened. (6) Pronate: using a movement of the upper arm, the elbow is abducted, pronating the paw over the food. Full pronation of the paw onto the food is aided by a movement of the paw around the wrist. (7) Grasp: as the pads of the palm or

the digits touch the food, the food is grasped by closure of the digits. This closure can occur as an independent movement, or the grasp can occur as the paw is withdrawn. (8) Supination I: as the limb is withdrawn, the paw is dorsiflexed and is supinated 90^0 by a movement around the wrist and by adduction of the elbow. These movements can occur as soon as the food is grasped or can occur as the limb is withdrawn. (9) Supination II: as the rat sits back with food held in the paw, the paw is further supinated by 90^0 and ventroflexed to present the food to the mouth. (10) Release: the digits are opened and the food transferred to the mouth.

Five successful reaches were analyzed frame-by-frame on the video tapes. Each movement was rated on a one-point scale. If the movement appeared normal, it was given a score of "0"; if it appeared slightly abnormal but recognizable it was given a score of "0.5"; and a score of "1" was assigned if the movement was absent or completely unrecognizable.

Golgi-Cox analysis

Animals were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline. The brains were removed and weighed before being immersed in a 20 ml of Golgi-Cox solution where they remained for 14 days. The brains were then placed in a 30% sucrose solution for 2 days and cut on a vibratome at 200 μm and developed using a procedure described by Gibb and Kolb (1998). The basilar tree of layer V pyramidal cells within the forelimb motor cortex of both hemispheres were traced using a camera lucida at 200X magnification. Measures of dendritic length and dendritic branching were obtained from those drawings. To be included in the study, the dendritic trees had to be well impregnated and in full view, unblocked by blood vessels, astrocytes

or clustering of dendrites from other cells. They also had to appear intact and visible in the plane of section. Cell bodies of pyramidal neurons had to be located within the sensorimotor cortex (as defined by Zilles and Wree (1995). For branch order analysis, each branch segment was counted and summarized according to methods of Coleman and Riesen (1968): branches emerging from the cell body (basilar) were first order. After the first bifurcation, branches were considered second order, etc. Quantification of each branch type using this method provides an indication of dendritic arbor complexity. To obtain an indirect measure of dendritic length, the Sholl analysis (Sholl, 1956) of ring intersections was used. The number of intersections of dendrites with a series of concentric circles at 20 μm intervals from the center of the cell body was counted for each cell. A reflection of total dendritic length (in μm) can be determined by multiplying the number of intersections by 20. The mean of the measurements of five cells per hemisphere per rat was used for statistical analyses.

Statistical analysis

Analyses of variance (ANOVA) were used for all measures and Fisher's LSD ($P<0.05$ or better) was used for *post hoc* evaluations.

A.4. Results

Behavioral Results

Cylinder Test

All animals actively explored the cylinder and they reared and supported their body against the walls with their forelimbs. When the total number of touches was calculated for each week, a profound decrease by the nicotine group was observed on

week 3 (Figure 1). This period coincided with the time animals were going through nicotine withdrawal and similar results in general activity have been reported elsewhere

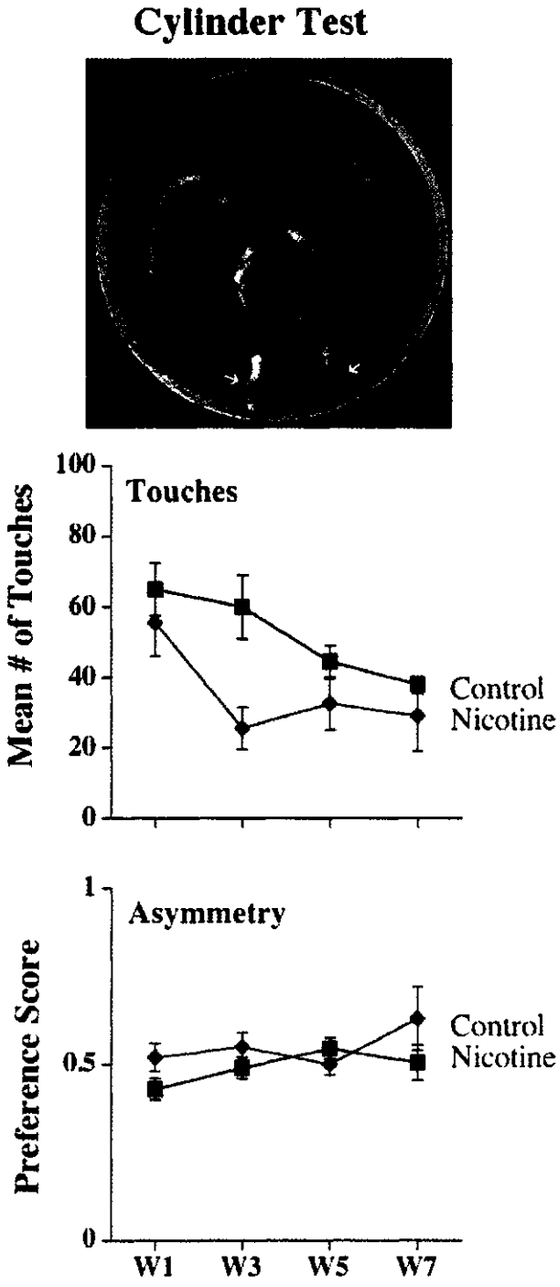

Figure A1: Top panel shows a picture of a rat in the cylinder. Arrows show use of both forearms for support during rearing. Middle panel shows the mean number of touches (±SE) to the walls of the cylinder with both paws during exploration on weeks 1, 3, 5, and 7 for control and nicotine treated animals. Bottom panel shows a preference score in the use of the forelimbs (±SE). A 0.5 indicates no preference on the use of left or right forearm. Note that both groups relayed equally on both forelimb for postural support during vertical exploration.

(Malin et al., 1992; Hildebrand et al., 1997). A repeated measures ANOVA showed a marginal effect of group ($F(1,8) = 4.41$, $P = 0.068$), a significant effect in the number of touches as they decreased in both groups over the seven weeks of testing ($F(1,8) = 7.62$, $P < 0.001$) but no interaction ($F(3,8) = 2.13$, $P = 0.12$). When the asymmetry score of forelimb use was calculated no differences were found between the groups (Figure 1). Both groups showed equal use of both forepaws during vertical exploration. A repeated measures ANOVA showed no effect of group ($F(1,8) = 1.23$, $P = 0.29$), no significant effect in the number of touches ($F(3,24) = 1.45$, $P = 0.25$) and no effect in the interaction ($F(3,24) = 1.58$, $P = 0.21$).

Tray Reaching

All animals quickly learn to reach for food and asymptote at about 60% accuracy before starting the nicotine regimen. Nicotine did not affect the performance of rats in the tray-reaching test. In fact nicotine-treated animals improved by 20% from the time measured before the drug administration to the last week of testing (Figure 2). A repeated measures ANOVA showed no significant effect of group ($F(1,8) = 0.22$, $P = 0.64$), a

significant effect of testing week as nicotine-treated animals steadily improved over the seven weeks whereas the means of control animals randomly fluctuated over this period ($F(4,32) = 6.22$, $P < 0.001$) and a significant interaction ($F(4,32) = 9.16$, $P < 0.001$).

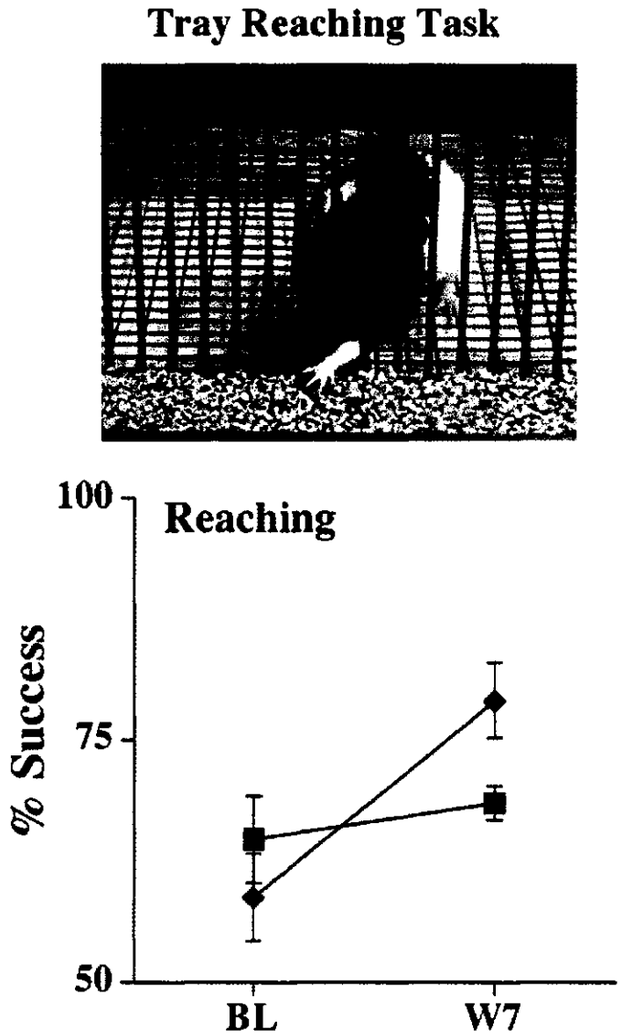

Figure A2: Top panel shows an animal in the reaching apparatus, arrow points at the animal's grasp for food. Bottom panels show the performance of a control and

a nicotine treated animal before the administration of nicotine (BL) and on the seventh (last) week of testing (W7) (mean ±SE). Note that by the last weeks of testing stroke animals that received nicotine performed significantly better when compared to their baseline.

Single Pellet Reaching

All the animals learned to retrieve 20 pellets from the shelf within few days and after a week their performance was recorded and analyzed for a total of thirteen days. Two kinds of analyses were performed: a) total success (sometimes animals have to perform more than one reaching movement before they retrieve the pellet successfully), and 2) success on first reach. Analyses showed marked impairments in animals that receive nicotine and this impairment was greater when success on first reach was analyzed. A repeated measures ANOVA on the total success showed (Figure 3) a significant effect of group ($F(1,8) = 8.45$, $P < 0.05$), a significant effect of test day, ($F(12,96) = 2.28$, $P < 0.05$), but no interaction ($F(12,96) = 0.84$, $P = 0.60$). When success on first reach analyzed, a repeated measures ANOVA showed (Figure 3) a significant effect of group ($F(1,8) = 14.56$, $P < 0.01$), a significant effect of test day, ($F(12,96) = 3.85$, $P < 0.001$), but no significant interaction ($F(12,96) = 0.94$, $P = 0.50$).

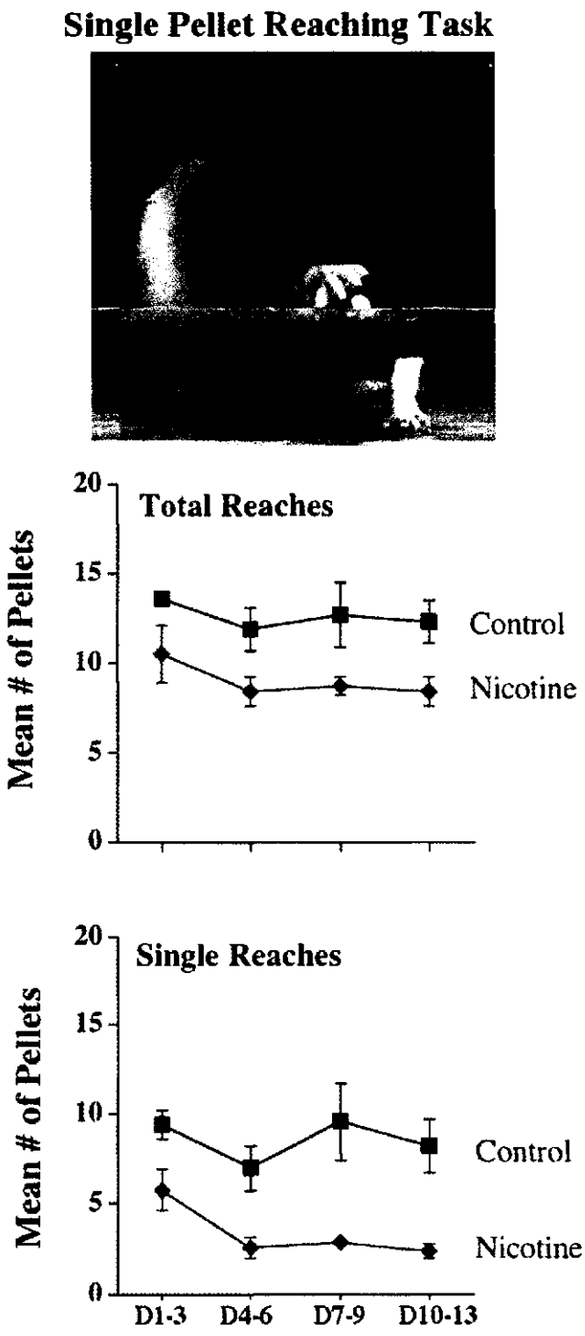

Figure A.3: Top panel shows an animal in the single-pellet reaching task: a rat reaches through a slot for a single food pellet located on a shelf. Middle panel

shows the total reaching success and bottom panel shows first reach success in the single pellet task (number of pellets retrieved out of 20 ±SE) for control and nicotine groups during for days (D) 1-3, 4-6, 7-9, and 10-13. Note that animals treated with nicotine were impaired in both measures at all time points.

Qualitative analysis of single pellet

The ten movement components of five successful reaches for the last day (day 13) were carefully examined frame by frame. The analysis showed that animals that received nicotine were severely impaired in most of the components of the reach. A repeated measures ANOVA showed a significant effect of group ($F(1,8) = 22.29$, $P < 0.01$), test day, ($F(9,72) = 11.6$, $P < 0.001$), and the interaction ($F(9,72) = 4.67$, $P < 0.001$) (Figure 4). Illustrations of the early and late components of the reach for both control and nicotine groups are presented in figures 5 and 6 respectively. Control animals that received nicotine were impaired in aiming, in the pronation, both supinations and in the release of the pellet to the mouth. During the aiming, nicotine animals showed an exaggerated adduction of the elbow that did not align with the midline of the body (Figure 5). The advance of the forelimb to the slot was usually short and thus the reach would be incomplete and unsuccessful in retrieving the pellets. When the nicotine rats successfully grasp the food, they displayed a partial supination I to withdraw the food through the slot, but then they failed to displayed a supination II to bring the food to the mouth (Figure 6). Rather the paw dropped to the floor of the cage at which point the other paw and snout became in contact with the food pellet.

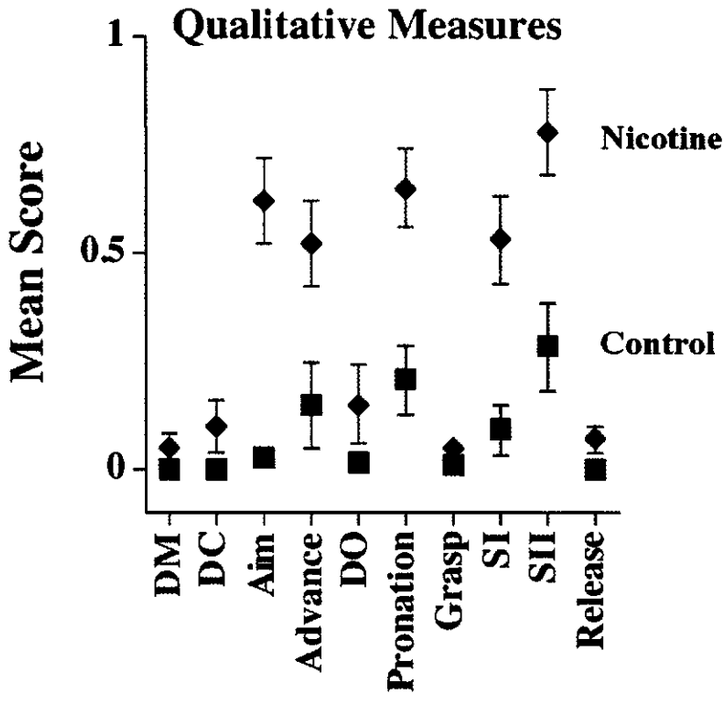

Figure A.4: Qualitative scores of ten elements. Note that animals that received nicotine displayed large deficits (higher scores) on several elements. Digits to the midline (DM); Digits close (DC); Aim; Advance; Digits open (DO); (6) Pronation; Grasp; Supination I (SI); Supination II (SII); Release. Note that animals treated with nicotine displayed impairments in the aim, advance, pronation, supination I and supination II.

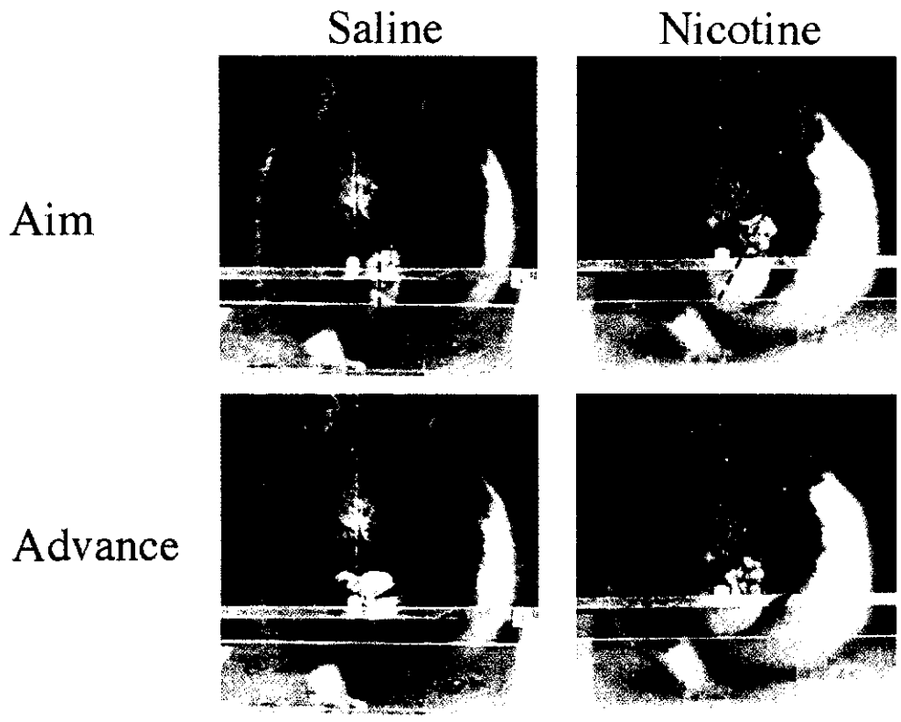

Figure A.5: Illustration of some of the early components of the reach (Aim and Advance) by a control and a nicotine-treated rat. Note (arrows) that nicotine-treated animals rather than aiming the limb they advanced the limb diagonally through the slot making many short attempts.

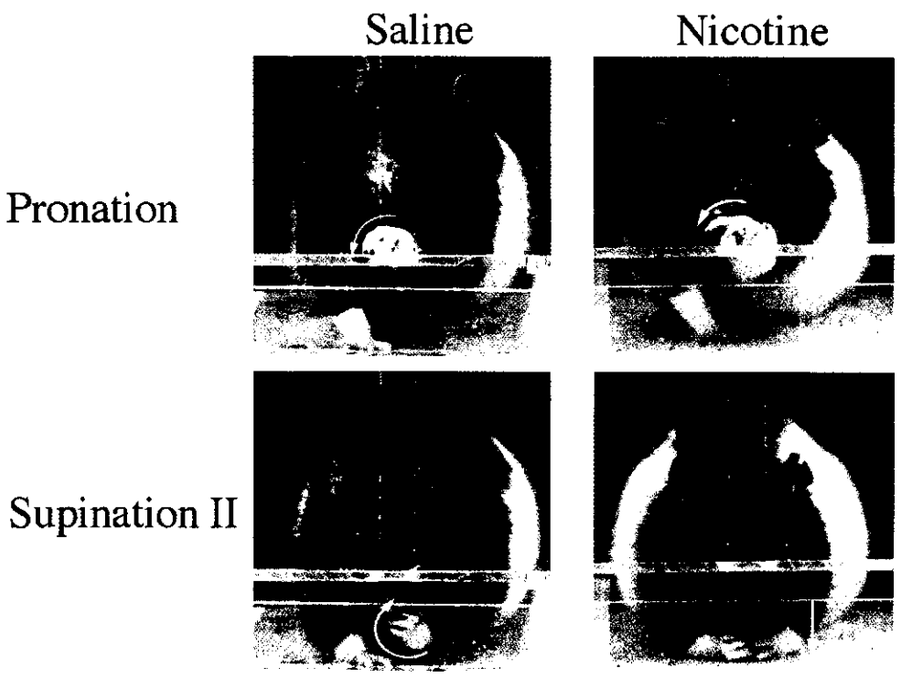

Figure A.6: Illustration of the late components of the reach (Pronation and Supination II) by a control rat and a nicotine-treated animal. Note (arrows) that animals treated with nicotine rather than fully pronating the paw they grasped the food with an incomplete rotation, and rather than supinating the paw to present the food to the mouth they dragged their limb through the slot and dropped the limb down to the floor of the cage.

Anatomical Results

Gross Anatomy

Brain Weight: When the brains were analyzed for weight with a simple ANOVA, a marginal increase was observed in the brains of animals that receive nicotine although it did not reach significance: ($F(1,8) = 4.62$, $P = 0.063$)

Brain Measurements: Because of the strong trend in the nicotine group to have heavier brains, a closer examination was conducted by capturing digital images of mounted Golgi-Cox impregnated sections at standardized levels (6 different planes). The cross-sectional area of the entire brain hemispheres was measured with the NIH IMAGE software, Ver.1.62. Animals that received nicotine overall had a 5% increase in hemispheric area. A repeated measures ANOVA showed a significant effect of group ($F(1,8) = 6.86$ $P < 0.05$), a significant effect of plane ($F(5,40) = 458.1$, $P < 0.0001$), but no significant interaction ($F(5,40) = 0.56$, $P = 0.72$).

Dendritic Analyses

The basilar fields of pyramidal cells of layer V in the forelimb area were analyzed for both hemispheres. Analyses of length and branching showed marked increases in the nicotine group (Figure 7). In order to elucidate if training had an effect on the morphology of the cells contralateral to the preferred paw, independent analyses with the side of the hemisphere as a factor were included. Control animals showed an effect of training as the length of the dendritic arbor of the cells contralateral to the preferred paw was enhanced relative to the ipsilateral arbors. Although there was a general increase on dendritic morphology in animals treated with nicotine, there was no differential effect of experience on the contralateral versus ipsilateral hemisphere in the nicotine-treated brains (Figure 8).

A two-way ANOVA on dendritic length with drug treatment and hemisphere as factors showed a main effect of drug treatment ($F(1,24) = 19.41$, $P < 0.001$), but not of training ($F(1,24) = 1.02$, $P = 0.32$), nor the interaction ($F(1,24) = 3.29$, $P = 0.08$). Similarly, A two-way ANOVA on dendritic branching showed a main effect of drug

treatment ($F(1,24) = 4.80$, $P < 0.05$), but not of training ($F(1,24) = 0.29$, $P = 0.59$), nor the interaction ($F(1,24) = 1.45$, $P = 0.23$). Although there was no main effect of training, inspection of Figure 7 suggests that training did have an effect. Thus we elected to conduct further analysis on the trained and untrained hemispheres.

Effects of training dependent on training condition

Figure 7 illustrates the effects of training on dendritic morphology and it shows that in control animals training enhanced dendritic length in the contralateral hemisphere.

Control animals: The effects of training were studied on the control animals by comparing the contralateral versus the ipsilateral hemisphere and a significant effect on dendritic length ($P < 0.05$ by unpaired Student's t-test) but not on dendritic branching ($P = 0.17$) were found. Dendritic arbors on the cells contralateral to the preferred paw for reaching were longer than the ones of the ipsilateral hemisphere.

Nicotine-treated animals: Training had no effect on the dendritic length ($P = 0.68$ by unpaired Student's t-test) or branching ($P = 0.82$) on animals that were exposed to nicotine. The size of the cells in the contralateral and ipsilateral hemisphere thus was equivalent.

Effects of nicotine are dependent on hemisphere

Figure 8 illustrates the effects of nicotine on the ipsilateral and contralateral to the preferred paw for reaching hemispheres and shows that dendritic arborization was enhanced in both hemispheres. Statistically significant differences were not found on cells of the contralateral hemisphere as training increased length and branching only in the control animals.

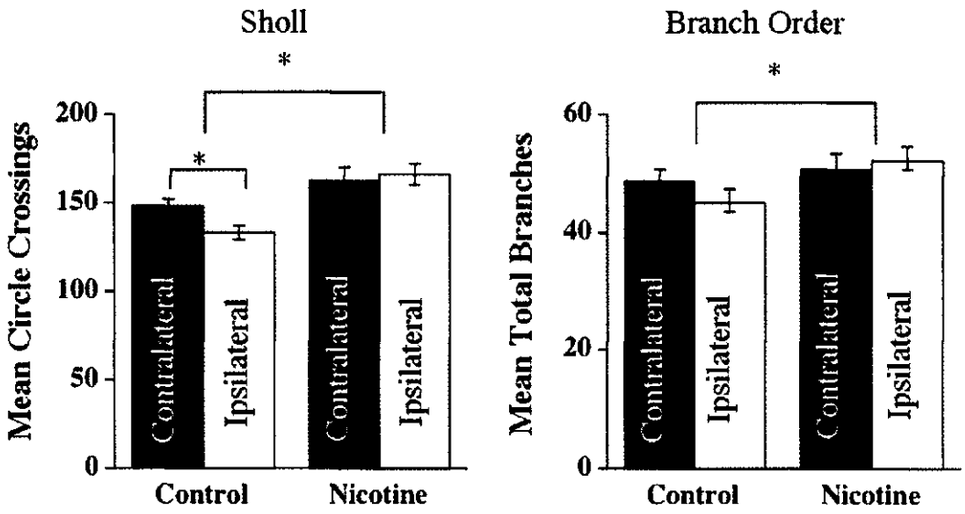

Figure A.7: Summary of Sholl and branch order analyses of pyramidal cells layer V of the forelimb area. Note that nicotine increased both, Sholl and branch order measures. Also note that in measures of dendritic length, there was an effect of training in the hemisphere contralateral to the preferred paw for reaching in control animals. Similar trend (although it was not significant) was observed in the branch order analysis.

Ipsilateral to the preferred paw: The ipsilateral hemisphere was analyzed for effects of nicotine and a significant effect of group was found on dendritic length ($P < 0.001$ by unpaired Student's t-test) and branching ($P < 0.05$).

Contralateral to the preferred paw: When the contrlateral hemisphere was analyzed for effects of nicotine, no significant effect of group was found on dendritic length ($P = 0.10$ by unpaired Student's t-test) nor on dendritic branching ($P = 0.56$).

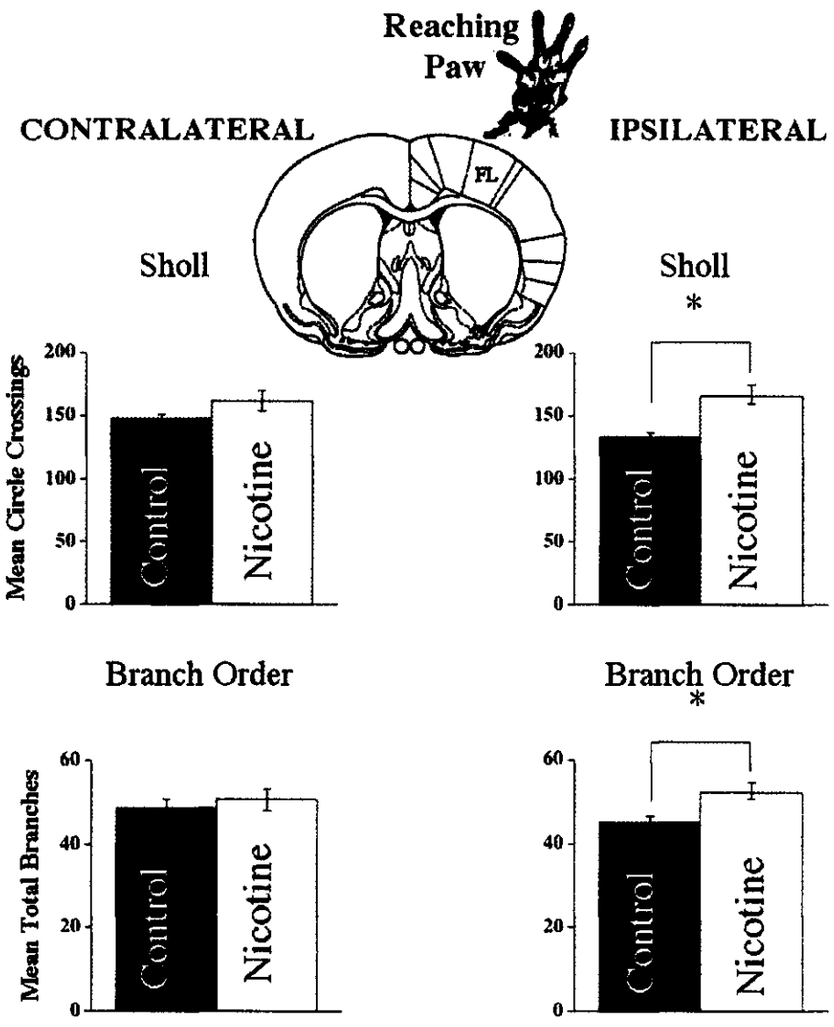

Figure A.8: Summary of Sholl and branch order analyses of pyramidal cells layer V of the forelimb area grouped according to the hemisphere (contralateral or ipsilateral) with respect to the reaching paw. Note the effect of nicotine on the ipsilateral hemisphere but not on the contralateral one. This effect is due to the increase in dendritic measures induced by training in control animals and not in nicotine-treated rats.

A.5. Discussion

Administration of nicotine can enhance performance on cognitive tasks (for a review see Rezvani and Levin, 2001) and motor tasks (for review see Heishman, 1999). The objective of the present study was to confirm that administration of nicotine can enhance motor skills in the rat and then to examine if prior exposure to nicotine also facilitates learning of new motor skills. Two skilled reaching tasks were used. Nicotine was administered after animals had acquired the first skilled reaching task, to examine its effects on asymptotic performance. Then, the animals were given the second skilled reaching task without further nicotine treatment, to assess the effects of preexposure on new motor learning. At the completion of behavioral testing, changes in the motor cortex dentritic morphology were assessed in Golgi-Cox stained tissue. Nicotine improved performance when given concurrently with training but impaired subsequent learning success and motor movements in the new motor task. The nicotine treatment enhanced motor cortex pyramidal cell dendritic branching and length of pyramidal cells. It is proposed that premature commitment of plasticity induced by nicotine in the normal brain may interfere with subsequent acquisition of new motor learning.

The present experiment was designed to first confirmed previous work that nicotine can enhance motor performance and then secondly, to evaluate whether previous nicotine administration would affect novel motor learning. For the experiment, animals were pre-trained in a tray-reaching task and then were given low doses of nicotine and further reach training for two weeks. This aspect of the experiment evaluated the effects of nicotine on ongoing motor performance. Two months after the last injection of nicotine, animals were given a novel motor skill task in which they had to learn to reach

for single food pellets. The second phase of the experiment evaluated the effect of prior exposure of nicotine on new motor skill learning. Thus, the experimental design assessed the potential effects of nicotine on motor performance as well as on new learning.

The two motor tasks, tray reaching task and the single pellet task, were chosen both because of their similarities and because are widely used in the assessment of motor deficits on a variety of neurological disorders. In the tray-reaching task, a rat is trained to reach through bars to retrieve food from a tray at the front of the cage and performance is measured by the success of the animal to retrieve and ultimately consume food. The task is relatively simple because no special limb targeting is required. The single pellet-reaching requires a rat to make a targeted reach and is more difficult in that the rat must first locate the food pellet and then make an accurate reach in order to retrieve it. Both tasks provide an end-point measure of reaching success, but in addition they also allow examination of the movements used for reaching from frame-by-frame inspection of the video records. Because one of the goals of the present study was to examine the effects of prior exposure to nicotine on motor performance, the presentation of the tasks (e.g. tray reaching first and then single pellet) was thus chosen in order to assess the effects of nicotine on concurrent performance as well as subsequently administered new motor learning on a similar task. It was expected that any facilitation of performance in the tray-reaching task would generalize to the single pellet task (Vergara-Aragon et al., 2003).

The results obtained on the first phase of the experiments confirmed previous findings that nicotine can enhance motor performance if given concurrently with a motor task. Previous studies have shown that finger tapping rate and motor reaction time during tests of attention can be enhanced by nicotine (for review see Heishman, 1999). A recent

study investigating the effects of nicotine administration on a handwriting task has also shown that after chewing gum containing nicotine, subjects, reduced movement times, increased velocities and showed more fluent handwriting movements (Tucha and Lange, 2004). In the present study animals treated with nicotine improved in hit percent from asymptotic base line performance. Hit percent is a measure of success that is sensitive to many kinds of motor system injury (for a review see Whishaw, 2000) Examination of the video records of reaching did not reveal any obvious differences in the way that the rats reached prior to and following nicotine administration. Thus, the fist phase of the study confirmed that concurrent administration of nicotine can enhance motor success.

In contrast to the beneficial effect of concurrent administration of nicotine in facilitating tray reaching performance, the prior exposure to nicotine had a detrimental effect on the subsequent acquisition and performance of single pellet reaching. This finding is novel and it was unexpected. What was especially surprising was that the animals displayed severe impairments not only in reaching success but also in the movements that they used. The movements that were most severely affected in the rats treated with nicotine were aiming, advancing, the pronating, and the supination of the limb. Rather than aiming the limb, nicotine-treated rats advanced the limb diagonally through the slot making many short attempts, rather than fully pronating the paw they grasped the food with an incomplete rotation, and rather than supinating the paw to present the food to the mouth they dragged their limb through the slot and dropped the limb down to the floor of the cage. These impairments are reminiscent of those displayed by rats with motor cortex injury (Whishaw et al., 1986; Whishaw, 2000). Although the finding of impaired skilled movements induced by nicotine is novel, there is substantial

evidence suggesting that extensive behavioral training can produce similar results. Extensive motor training in primates (Byl et al., 1996; Byl, 2004) or in humans (musicians, professional athletes, etc) can lead to dystonias, which are characterized by enlarged but unusual patterns of cortical organization (Elbert et al., 1998; Candia et al., 2003 for a review see Nudo, 2003).

It is puzzling that nicotine would improve performance on a reaching task but disrupt the acquisition of a second one. It is unlikely that the order in which the tasks were given had anything to do with this result. It has been shown previously that previous training on the tray-reaching task enhances subsequently performance on the single-pellet reaching task (Vergara-Aragon et al., 2003). That was our expectation in the design of the experiment. Although both reaching tasks share similar components (e.g. reaching for food) they differ in some fundamental features (e.g. precision and finesse). Reaching on the single pellet task makes a greater demand upon rotatotory movements of the limb than does tray reaching, and it appeared that it was upon these aspects of reaching that prior exposure to nicotine had the most detrimental effect. Unfortunately, the design of the experiment did not allow us to distinguish between prior training combined with nicotine vs nicotine alone, and this will be the subject of further studies.

One possible explanation of the detrimental effects of nicotine on the acquisition of a novel skilled motor task is that nicotine changed the dendritic structure of motor cortex neurons. The finding that nicotine produced changes in dendritic arbor confirms previous studies that show that chronic administration of nicotine leads to increases in dendritic arborization and spine density in the nucleus accumbens and prefrontal cortex (Brown and Kolb 2001). Although neuronal plasticity is usually associated with enhanced

functional outcome, some studies have shown that it could be associated with pathological changes (Fiala et al., 2002; Purpura, 1974). In the present study animals treated with nicotine showed an overall increase in dendritic arborization in the motor cortex but did not show an increase in the hemisphere contralateral to the preferred paw for reaching relative to the ipsilateral hemisphere. It is thus possible to propose that enhancement in dendritic arborization by nicotine, or combined nicotine and skilled training, used up or blocked the experience dependent plasticity required for new motor learning (Greenough et al., 1985; Withers and Greenough, 1989; Rioult-Pedotti et al., 1998; Kleim et al., 1998; Plautz et al., 2000).

One prediction derived from the finding that nicotine had no effect if given after the animals had learned a skilled motor task but impaired the acquisition of a new one would be that there is a limit to neuronal plasticity and once this limit is reached, further behavioral modifications become difficult. That is, because nicotine stimulated dendritic arborization, there was little room for further dendritic changes for acquiring a new task. The idea that saturation of plastic processes systems can follow behavioral training has been addressed by Rioult-Pedotti and colleagues (2000). They have shown that learning-induce enhancement can place limits on further synaptic potentiation. Training animals on a skilled reaching task markedly reduced long-term potentiation, which led authors to suggest that that synapses in the motor cortex of a trained animal were near the ceiling of their modification range. These findings present an immediate puzzle as discussed by Martin and Morris (2001). If the learning of one skill uses up almost all of the available capacity for synaptic enhancement, how can additional skills ever be learned? It is possible that exposing subjects to nicotine expends plasticity without allowing for

pruning that may be associated with new learning. In this respect, the loss of motor skill associated with aging, that is itself associated with enhanced dendritic growth, may be similar to the acute effects of nicotine.

In conclusion, it is surprising that given the popular notion that nicotine has negative impacts on athletic ability there have been no studies of the effects of nicotine upon on subsequently acquired motor ability. In the present study it was found that nicotine treatment could improve performance when administered concurrently with training but nevertheless impair subsequent skill acquisition. Because the animals displayed increasing branching of motor cortex pyramidal cell dendrites, it is proposed that nicotine may saturate dendritic plasticity thus reducing motor cortex function. Future work will be required to determine the extent to which nicotine alone or nicotine in conjunction with motor performance is associated with reduced motor skill.

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