A MOUSE MODEL FOR STUDYING STROKE INDUCED IMPAIRMENTS, RECOVERY, AND COMPENSATION IN THE MOTOR CORTEX.

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"A mouse is an animal that, if killed in sufficiently many and creative ways, will generate a PhD." *unknown*

Thesis Abstract

Stroke is the third leading cause of death and survivors suffer motor impairments. The rodent sensorimotor system is similar to the human's, making rodents a good model to study the effects of stroke. Transgenic technology makes the mouse a desirable stroke model, however, there are few behavioural tests to assess behavioural outcome. This thesis evaluates mice subjected to permanent or temporary occlusion focal motor cortex strokes in a skilled reaching task. The first experiment documents changes in skilled movements in mice with a permanent occlusion focal motor cortex stroke. The second experiment is identical but uses a temporary occlusion focal motor cortex stroke. The third experiment compares the two strokes. The results indicate permanent occlusion mice suffer greater impairments, and a larger injury, than temporarily occluded animals. The mice with the largest insults were most impaired. Mice make an excellent behavioural and genetic model for studying motor system stroke.

Acknowledgements

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List of Abbreviations

ADP- adenosine diphosphate
AMPA- alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Apo A-1- apolipoprotein A-1
Ca^{2+}- calcium
CFA- caudal forelimb area
DAG- diacylglycerol
DNA- deoxyribonucleic acid
ER- endoplasmic reticulum
ET- endothelin
fMRI- functional magnetic resonance imaging
HDL- high density lipoproteins
HLA- hindlimb area
IP_3- inositol triphosphate
K^+- potassium
MCA- middle cerebral artery
MCAO-middle cerebral artery occlusion
MRI- magnetic resonance imaging
Na^+- sodium
NAD- nicotinamide adenine dinucleotide
NGF- nerve growth factor
NMDA- N-methyl-D-aspartate
NO- nitric oxide
NOS- nitrous oxide synthase
PARP- poly ADP-ribose polymerase
PIP_2- phosphatidyl inositol
PLC- phospholipase C
PKC- protein kinase C
RFA- rostral forelimb area
ROS- reactive oxygen species
SI- primary somatosensory cortex
SII- secondary somatosensory cortex

1. Introduction

1.1. Introduction to the thesis.

"In forty years I plan to have a decent job. I will have a nice house with furnishings and will drive a quality car to work every day."

Isn't this how many people picture their future? What if one day, in my future, I had a headache that developed into nausea? Would I stay home, thinking I have the flu? What if that headache doesn't go away and I suddenly have trouble speaking, my vision becomes blurry, and I can't move one side of my body. By the time I would make it to the emergency room I would have already suffered neurological damage from a stroke. What if I had to stay in the hospital because I required assistance dressing and eating? What would my family have to sacrifice to help me live? This isn't how I picture my future; I don't think anyone does. Every year approximately 50 000 Canadians live this future. Experimental research into stroke is essential because it may help reduce the impact of the disease on the lives and economy of Canadians.

It is not ethical to conduct invasive procedures in humans for the purpose of experimental research. Therefore most stroke research is conducted in animals. Rodents make a good model for studying the pathology of stroke for a variety of reasons (for a review see Cenci, *et al.*, 2002). One reason is that it is possible to control many extraneous factors in the rodent environment that may influence experiments; these include atmosphere, temperature, activity, diet, experience and mating. In addition, the rodent motor system is remarkably similar to humans. Studying the motor system has important clinical implications because impaired motor function is one of the most common and frustrating symptoms exhibited by stroke patients.

This thesis describes a focal model of stroke to the motor cortex of mice. The motor cortex stroke will be described to have drastic effects on the movements of these animals. The following eight sections comprise the introduction portion of this thesis.

The first section of the introduction defines and describes stroke. The second section emphasizes the differences that exist between the human and rodent motor system. Because the second section portrays a fixed view of the motor system, the third section will emphasize the phenomenon of plasticity. Plasticity is a general term used to describe experience induced changes in the brain, including the motor system. The fourth section discusses the various rodent models of stroke, and highlights some considerations that should be made when choosing a model of stroke. Since the pathology of stroke is an important factor when choosing a model, the fourth section leads into the fifth section, which is a discussion about the mechanisms of cell death associated with stroke. The sixth section describes behavioural testing in the rodent. The seventh section briefly describes the use of transgenic mice to study stroke. The final section of the introduction outlines the experiments.

1.2. Introduction to stroke.

Stroke is a cardiovascular disease that affects the vessels that supply the brain with blood. A stroke occurs when a vessel bursts or is clogged. Stroke is the fourth leading cause of death in Canada, claiming 16 000 lives each year. There are approximately 50 000 strokes annually in Canada, making stroke the leading cause of disability for those who survive. Stroke survivors have a twenty percent chance of suffering a second stroke within two years of the initial event. Stroke is primarily a disease of the elderly as the risk of stroke doubles after the age of fifty-five. Out of every hundred stroke victims twenty will die, fifty will return home, ten will undergo hospital rehabilitation and fifteen will require long term care. It is estimated that stroke costs the Canadian economy 2.7 billion dollars each year (Heart and Stroke Foundation of Canada; The Canadian Stroke Network).

There are two types of strokes: twenty percent are hemorrhagic and eighty percent are ischemic. A hemorrhage is uncontrolled bleeding in the brain. A subarachnoid hemorrhage is bleeding between the brain surface and the skull. An intracerebral hemorrhage occurs when a vessel within the brain ruptures. Hemorrhages are often

caused by structural defects in the walls of the brains blood vessels. Hemorrhages can occur at an aneurysm, which is a small weak section of the vessel wall, or at an arteriovenous malformation, which are areas of the brain where there are many thin-walled blood vessels.

Ischemic stroke refers to a reduction in blood supply to the brain. The reduction is typically produced by a blood clot or the buildup of plaques formed from atherosclerosis (hardening of the arteries). The best treatment for an ischemic stroke is immediate administration of anticoagulants to break up the clots. Clots can be either thrombotic, formed in an artery of the brain, or embolic, formed in the body and carried through the bloodstream to the brain. Depending on their size, clots can enter any of the numerous vessels of the brain (Netter and Dalley, 1998). Clots are particularly dangerous for a patient when they block one of the major artery systems that supply a large area of the brain.

One of the main brain blood vessels is the middle cerebral artery, depicted in Figure 1.21.

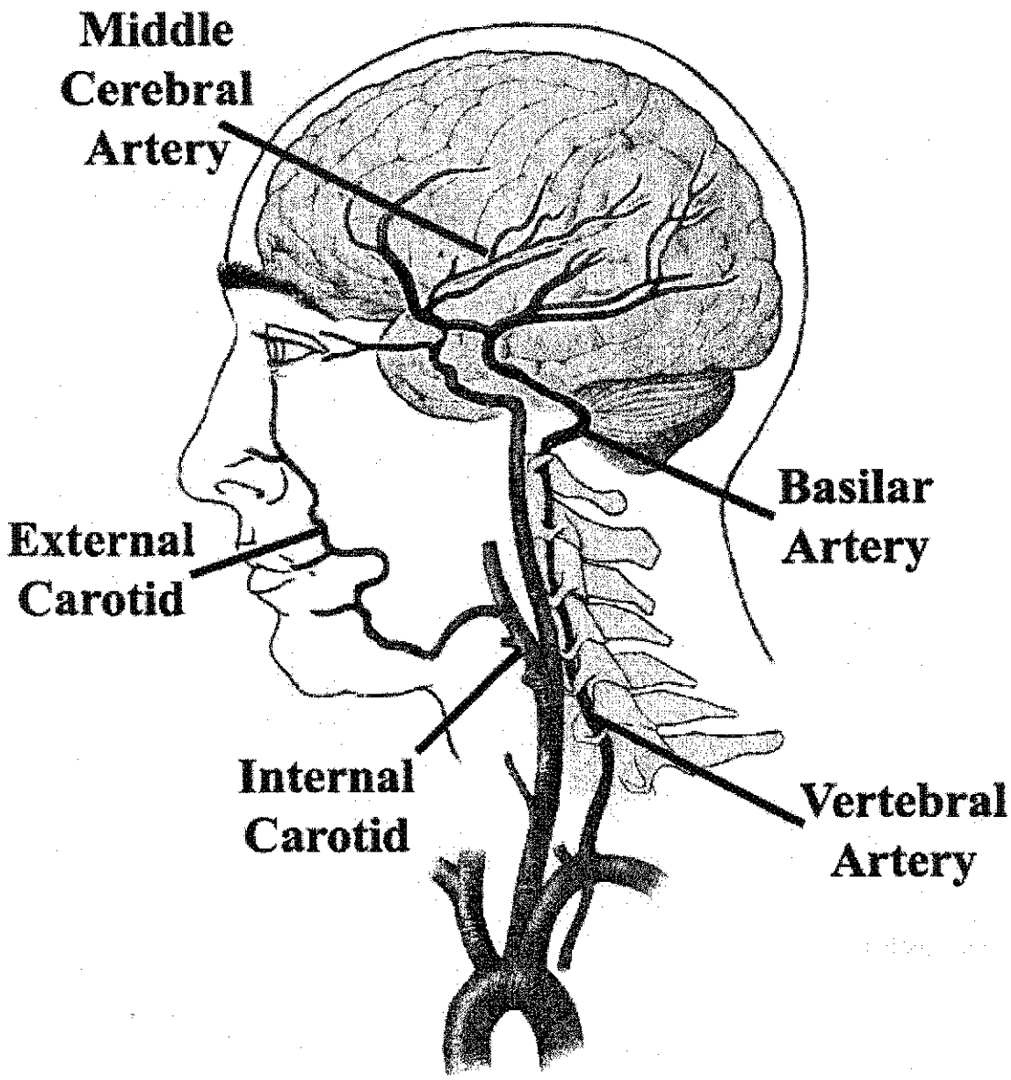

Figure 1.21. A diagram depicting the middle cerebral artery in the brain. The carotid artery branches into the middle and anterior cerebral arteries while the vertebral artery bifurcates into the posterior cerebral and basilar arteries. This Figure was modified from Blumenfeld, 2002.

Humans have four main arteries that supply the brain with blood. The two vertebral arteries supply the hindbrain by bifurcating into the posterior cerebral artery, which supplies the occipital and temporal lobes, and the basilar artery, which extends into the cerebellum and brainstem. The other two arteries entering the brain, the carotids, originate in the aorta and are located on each side of the neck. They bifurcate below the jaw line into the internal and external carotids. The external carotid supplies the facial muscles. The internal carotid also bifurcates near the eye into the anterior cerebral artery and the middle cerebral artery (MCA) both of which supply the forebrain. The MCA is a frequent destination for clots in stroke patients (Hossmann, 1998). Because it supplies the majority of the lateral cortex, thalamic structures, and the basal ganglia, ischemia to the forebrain can result in many types of motor impairments (for a review see Slater and Johns, 2003).

The nature of the impairment a patient suffers depends on the location and severity of the stroke. People with cognitive impairments can lose the ability to speak or understand speech or become confused about their environment (Kalaria and Ballard, 2001). Motor impairments affect a patient's ability to move and perform every day tasks such as dressing or eating. These impairments are often unilateral, meaning one side is more affected. This is usually the side opposite or contralateral to the location of the insult in the brain (for a review see Hendricks, *et al.*, 2002). Often a patient will fail to recognize the disabled side of the body; this phenomenon is referred to as neglect (Swan, 2001). In order to understand the process of movement, and impairments that arise from injury, it is helpful to have an appreciation of the structure of the motor system.

1.3. Introduction to the motor system.

The motor system is a complex system that includes many regions of the brain and areas of the body, all of which contribute to the production of movement. Because the motor system is closely connected to the sensory system, the two are often collectively referred to as the sensorimotor system. This section is designed to provide a brief description of the sensorimotor system by describing, in sequence, the sensory input

to the cortex, the layers of the cortex, cortical topography, and the motor output pathway. Throughout this section the similarities and differences between the rodent and human will be emphasized.

Movement begins with sensation. The epidermis, or outer layer of the skin, contains many receptors, whose neurons carry sensory information into the spinal cord. These neurons are called the dorsal root ganglion neurons because their cell bodies lie beside the spinal cord (Kolb and Whishaw, 2001). The dorsal root ganglion neurons extend upwards into the spinal cord, forming the dorsal column (Nieuwenhuys, *et al.*, 1981). The dorsal column is depicted in a cross section of the spinal cord in Figure 1.31.

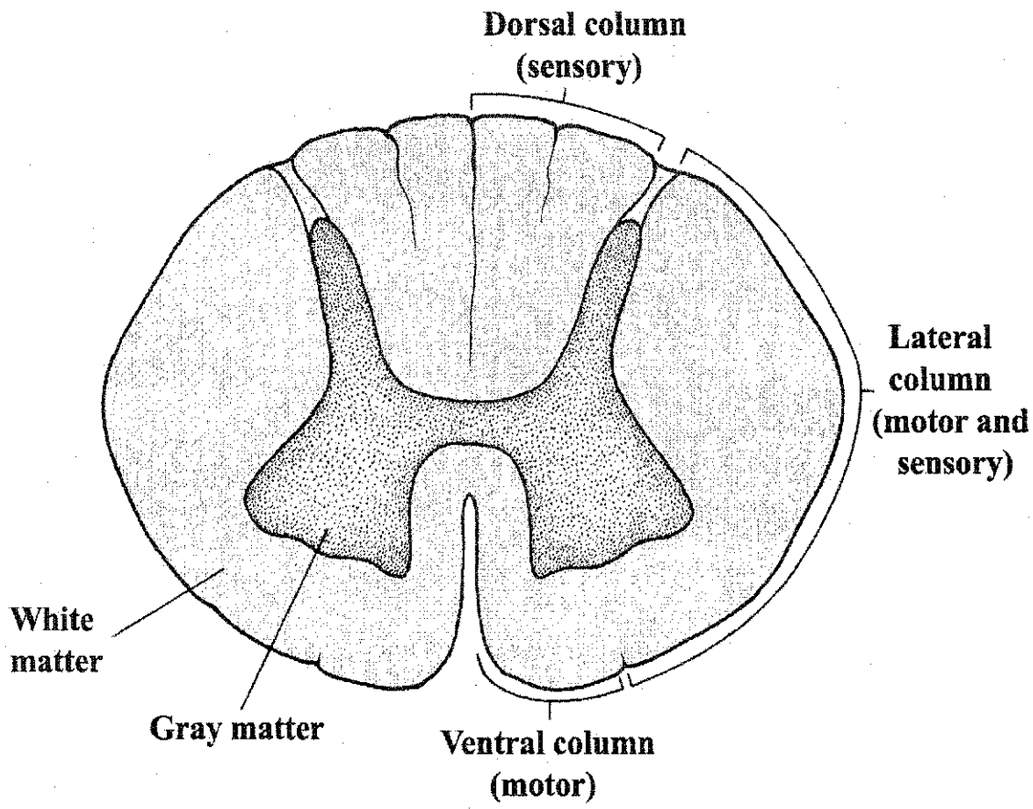

Figure 1.31. A cross section of the spinal cord indicating the gray and white matter, as well as the column organization. The cross section background was modified from Kandel, *et al.*, 2000.

The fibres of the dorsal column enter the brain and cross over to the other hemisphere to form the medial lemniscus. The medial lemniscus synapses with the ventrolateral thalamus and the thalamus projects to Layer IV of the primary somatosensory cortex. This system is similar in humans and rats (Paxinos, 1994; Burstein and Giesler, 1989).

The neo cortex of both humans and rats is composed of six layers (Hepp-Reymond, 1988; Kandel, *et al.*, 2000; Kolb and Whishaw, 2001). The six layers of the motor and sensory cortex are depicted respectively in Figure 1.32.

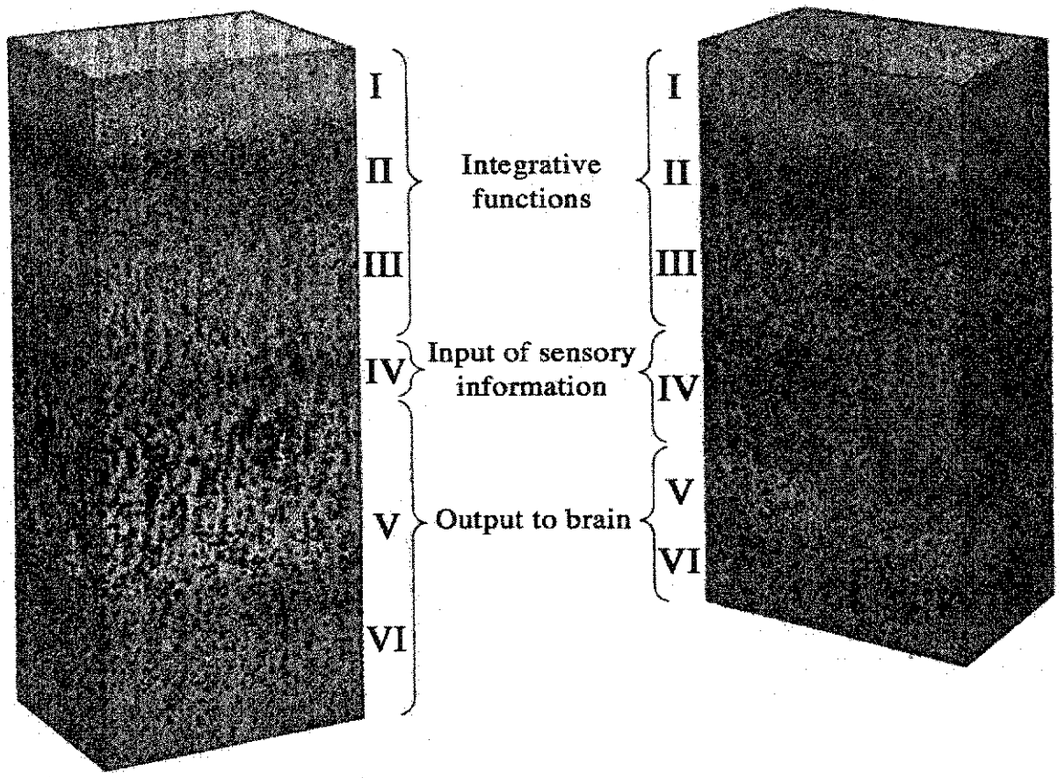

Figure 1.32. The six layers of the motor and sensory cortices respectively. This figure was modified from Kolb and Whishaw, 2001.

Layer I contains few projections and many glial cells that have primarily a supportive role. Layers II and III contain some pyramidal neurons, named such because they have pyramid shaped cell bodies (Hepp-Reymond, 1988) that are involved primarily in information processing. Layer IV is dense in the sensory cortex (Figure 1.32) as it contains many non-pyramidal, stellate-shaped, neuron cell bodies that receive sensory input. A large concentration of pyramidal cell bodies exists in Layer V of the motor cortex (Figure 1.32). These cells form the output pathway of the cortex.

The divisions of the cortex are illustrated in a lateral view of the human brain in Figure 1.33 (Kolb and Whishaw, 2001).

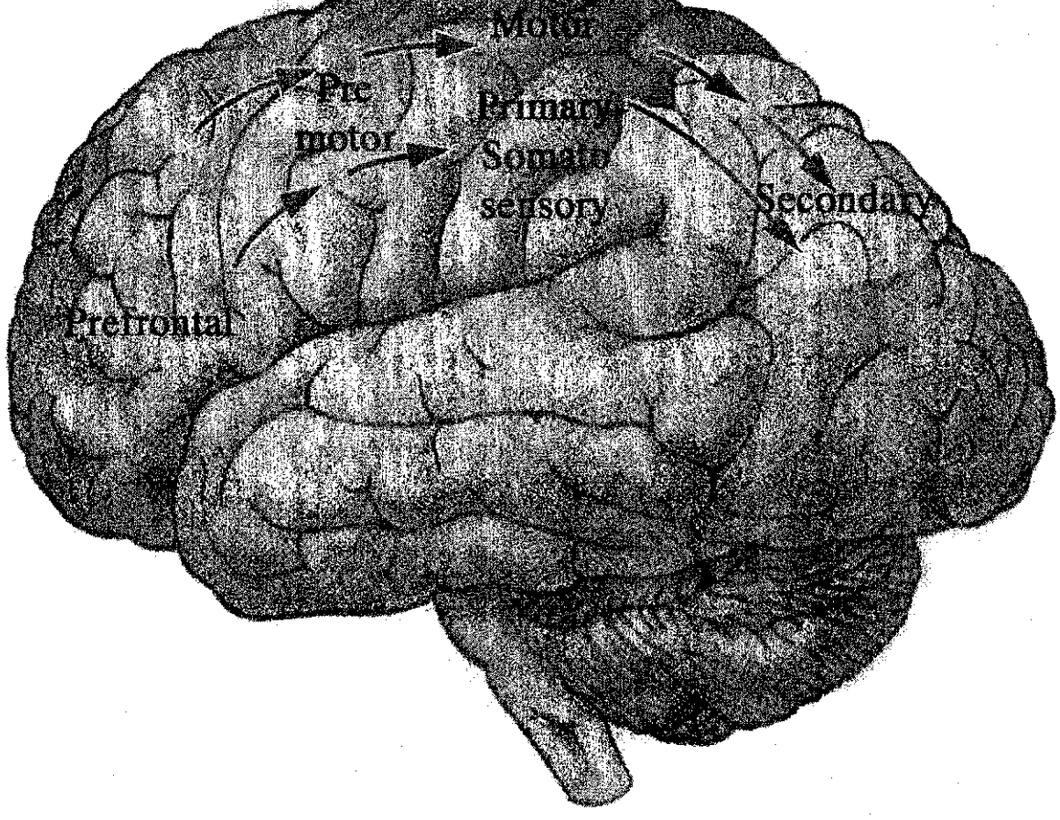

Figure 1.33. The cortical organization of the human brain. The prefrontal cortex is indicated in green, the premotor in purple, the motor in blue, the primary somatosensory in red and the secondary somatosensory in yellow. The arrows indicate the flow of information. This figure is adapted from Kolb and Whishaw, 2001.

After information is received in Layer IV of the primary somatosensory cortex it travels to the secondary somatosensory cortex. The secondary somatosensory cortex, in turn, sends information forward to the prefrontal cortex, which is involved in planning movements. Subsequently, information is received by the premotor area, which is involved in coordinating and sequencing movements, and then sent to the motor cortex to begin the execution of movement.

The cortical division in a rat is illustrated in a dorsal photograph of a rat brain in Figure 1.34. Though the rat brain may look different from that of the human, there are many similarities.

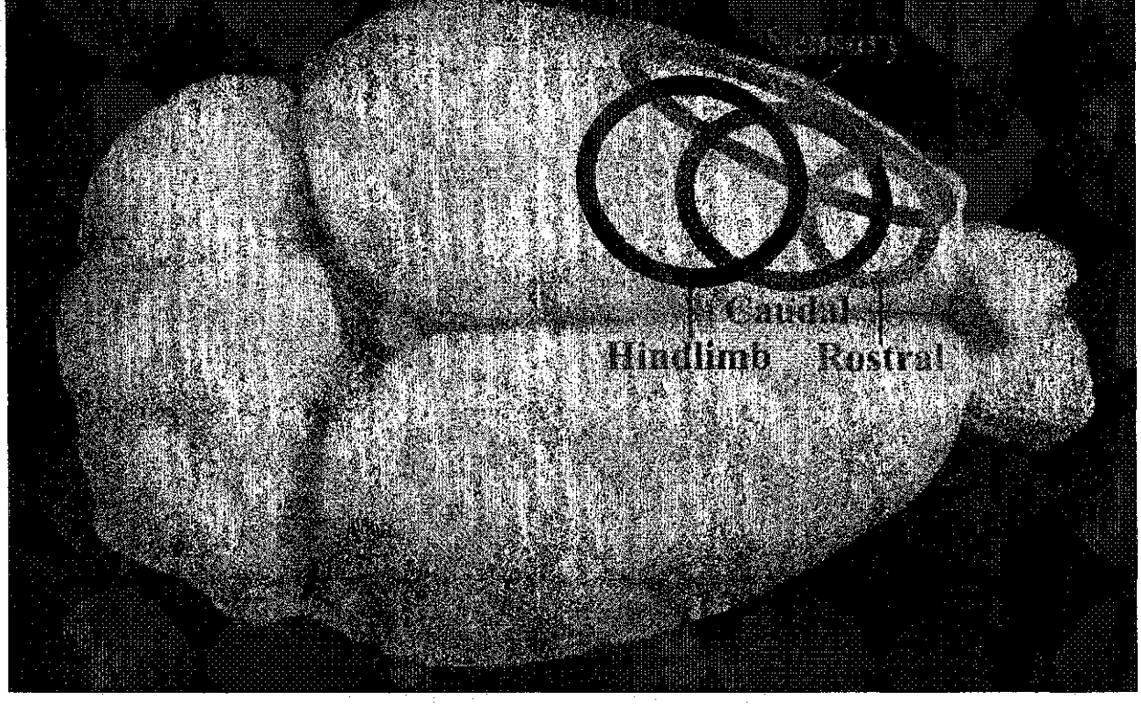

Figure 1.34. The cortical organization of the rat brain. The big structure at the back of the brain is the cerebellum and the two tiny structures on the front of the brain are the olfactory bulbs. The motor cortex is divided into the rostral forelimb area (RFA) represented in green, the caudal forelimb area (CFA) represented in blue, and the hindlimb area (HLA) is represented in red. The purple region indicates the sensory cortex.

As in the human, sensory information from the body and face is received in Layer IV of the rat primary sensory cortex (SI). SI stretches down the side of the rat brain and overlaps extensively with the motor cortex. As in humans, sensory information is transferred from the sensory to the motor cortex. The rat motor cortex, similar to that of the human, is composed of many sub areas, each of which can elicit movement from different body parts. Specifically the rat has three motor regions that overlap, the most anterior being the rostral forelimb area (RFA), the middle being the caudal forelimb area (CFA) and the most posterior being the hindlimb area (HLA). Despite the topographical differences, Layer V of the motor cortex is responsible for the initiation of movement from all three regions.

In the rat, as in humans, Layer V of the motor cortex consists of many pyramidal cells whose axons project downwards into the spinal cord. These descending fibres form the corticospinal tract, which later divides into the lateral and ventromedial systems (Kandel, *et al.*, 2000; Miller, 1987; Nieuwenhuys, *et al.*, 1981). These tracts are depicted in Figure 1.35.

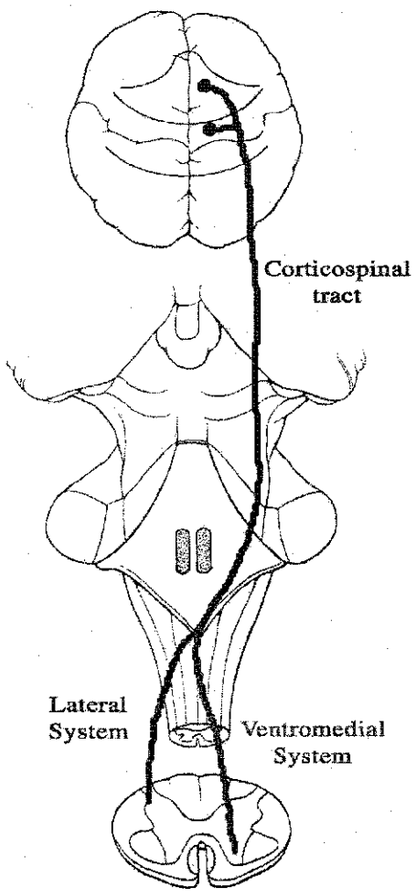

Figure 1.35. The corticospinal tract originates in the motor cortex and projects to the pyramidal decussation where it becomes the lateral and ventromedial tracts. This figure was modified from Kandel, *et al.*, 2000.

The corticospinal tract regroups in the medullary pyramid where some of the projections exit to terminate in the brain stem nuclei and the others travel down to the pyramidal decussation. It is here that seventy five percent of the corticospinal fibres cross over to form the lateral system, which innervates the contralateral side of the body. The lateral system controls distal limb, or hand and foot movements. The uncrossed fibres that continue to innervate the ipsilateral side of the spinal cord are called the ventromedial system. The ventromedial neurons control the proximal musculature near the midline such as the shoulders and pelvis. (For a depiction of the lateral and ventral columns of the spinal cord, see Figure 1.31).

Movement is not solely dependent on somatosensory information. Other sensory systems including the vestibular, visual, and auditory systems, as well as brain stem tracts and nuclei, and other brain structures like the cerebellum (Thach, *et al.*, 1991), and basal ganglia (Alexander, *et al.*, 1990) are also important in producing movement. The diversity of structures emphasizes the complexity that goes into producing movement. The complexity is made worse by the fact that the system is subject to change, a phenomenon called plasticity.

1.4. The phenomenon of plasticity.

Plasticity refers to the ability of the nervous system to reorganize and change in response to environmental influences or injury. This thesis is particularly concerned with motor cortex plasticity that results from injury. Historically, it has been believed that the areas of the motor cortex were very specific and discrete and that the adult brain was incapable of change. The idea that a different cortical area represented each part of the body was first hypothesized in the late seventeenth century (Fritsch and Hiitzig, 1870). Studies conducted in dogs (Ferrier, 1875) and primates (Leyton and Sherrington, 1917) indicated that stimulation of discrete brain regions elicited movements of one area of the body. Similar research in humans (Jackson, 1958) led to the coining of the term homunculus (Penfield and Boldrey, 1937). The homunculus is a size-specific pictorial representation of the body parts associated with each area of the cortex. Today it is

believed, not only that the motor cortex is not so discretely organized, but also that the cortical representations for each body part are constantly changing (Strick, 1988; Nudo, *et al.*, 2001; Schieber, 2001). The following is an example of motor cortex plasticity in the rat.

The rat motor cortex is organized into the two forelimb areas, rostral and caudal, and a hindlimb area. Electrostimulation of the motor cortex results in the generation of movements in limbs contralateral to the side of the brain being stimulated. It is possible to assess the total cortical area that elicits movement from each body part in each new animal. As it turns out, the RFA and the CFA are often indistinguishable in that they are both able to produce proximal (elbow and shoulder) and distal (wrist and digit) movements. A control rat would have an approximately equal distribution of proximal and distal musculature in the motor cortex. A rat trained to perform skilled movements will develop more distal movement representation in the forelimb areas. This change in relation between areas committed to proximal and distal movement is an example of plasticity (Kleim, *et al.*, 1998). A representative surface map of the motor cortex of a trained rat is indicated in Figure 1.41.

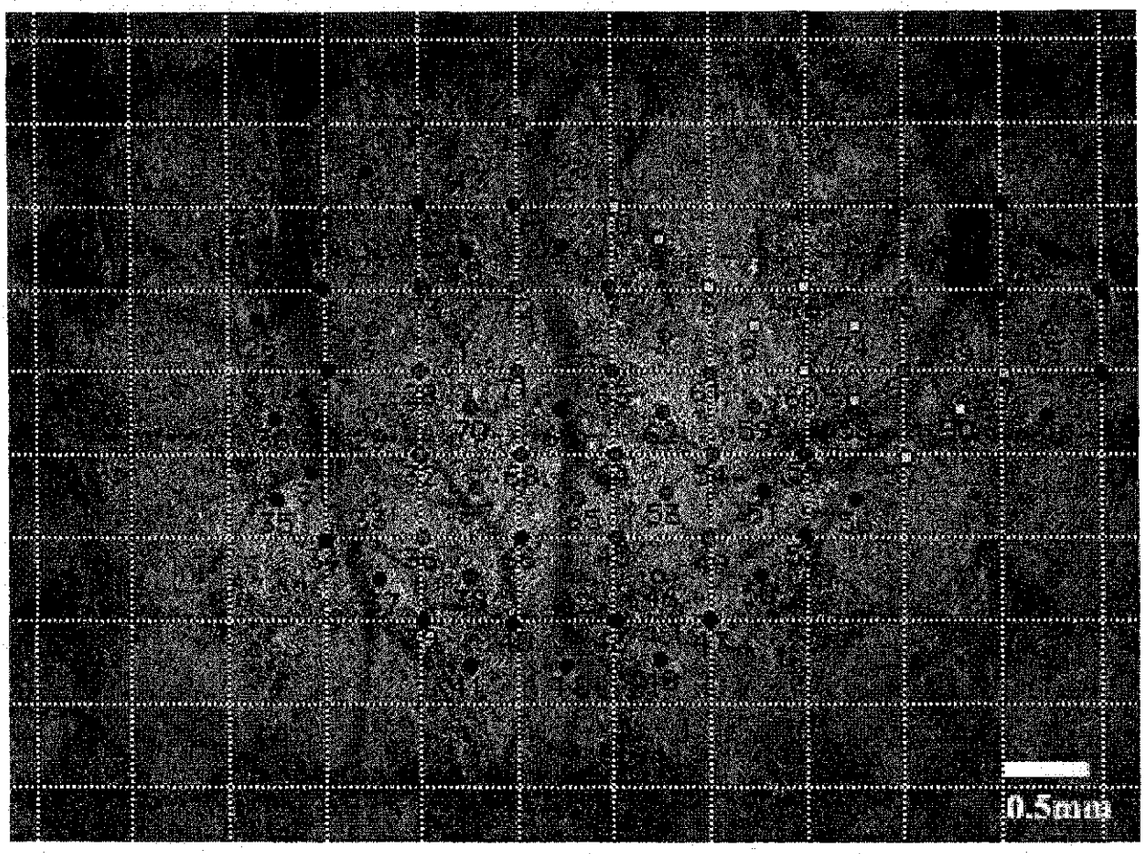

Figure 1.41. A motor cortex electrostimulation map of a control rat trained in the single pellet reaching task. Green dots represent the distal movements, blue the proximal, yellow the head and whiskers, pink the neck, and black is non responsive. This figure was donated by Dionne Piecharka at the University of Lethbridge.

There are many green dots and each represents a site that elicits distal wrist and digit movements. The green dots outnumber the blue dots that represent proximal elbow and shoulder movements. The yellow and red dots respectively indicate head, vibrissae, and neck movements. The black dots indicate non-responsive areas where no movements were elicited.

Schieber (2001) describes the underlying complexity of the cortex that allows the process of plasticity and subsequently movement to occur. He proposes that there are many different areas of cortex that elicit movement from one muscle, thus the territories for each muscle experience substantial dispersal across the cortex. In addition, the motor output of one corticospinal neuron diverges and innervates many other corticospinal neurons. Multiple collateral projections between cortical neurons allow for horizontal triggering of action potentials between motor neurons. Schieber (2001) uses a piano keyboard in Figure 1.42 to illustrate his more modern view of how the complex and plastic motor cortex produces movement.

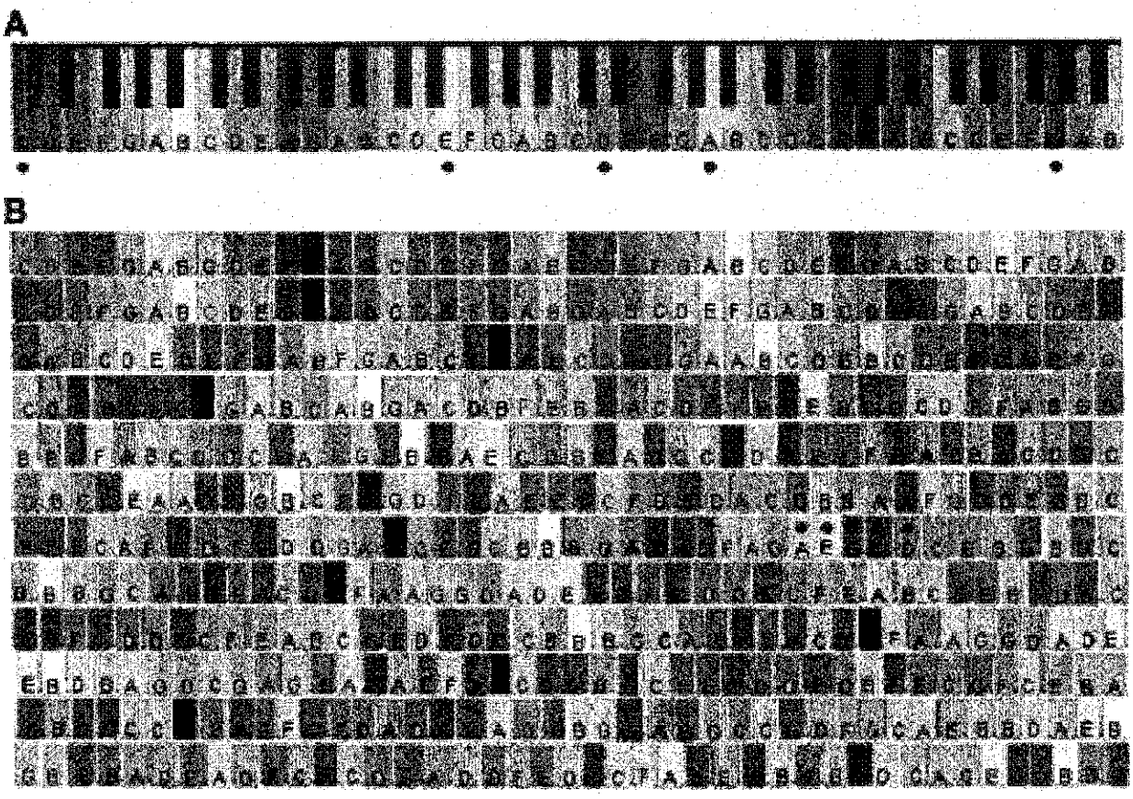

Figure 1.42. A: The 42 white keys on a standard piano keyboard were colored in order to distinguish them from one another. Each note can be located by its placement in the standard keyboard. It is impossible to play certain combinations of notes at the same time, such as the keys indicated by the black dots, because they are so far apart. B: A larger, nonstandard keyboard was created by placing each note at many possible locations, thus each note is represented over many larger areas that overlap with other notes. It is easy to see that there are many more possible note combinations than what is available on the standard keyboard, including the impossible to combine black dotted notes on the standard keyboard in A. This is Figure 10 from Schieber, 2001.

Each key in a standard piano keyboard is colored in Figure 1.42 A. It is impossible to play the combination of notes indicated by the black dots because they are distant. Figure 1.42 B shows a complex keyboard composed of many different combinations of the original keys. With the new arrangement there are many more possible combinations of notes. One of these combinations allows the notes indicated in Figure 1.42 A to be played. Thus, by analogy, motor movements can be produced by simultaneous activation of various combinations of neurons that do not always lie in sequential relation to one another.

Because movement is so complex, it is necessary to choose an appropriate model in order to study the effects of stroke on the motor system. The primate would be an ideal model, because their motor system most closely resembles ours. Rodents make a more cost effective alternative, while sharing many similarities to the human motor system. The following section describes the most popular rodent models in stroke research, along with justification for choosing the mouse.

1.5. Modeling stroke in the rodent.

When modeling stroke it is important to choose a model that will be best suited to the types of questions being asked. Rodents are a good model system in which to study stroke because they are relatively inexpensive, they have a short lifespan, and are similar to humans. There are essentially two general methods of modeling stroke in rodents (for a review see Hossmann, 1998 and Nedergaard, 1988). The first is a global ischemia model, which will temporarily impair blood flow to the entire brain. It is accomplished by occluding various combinations of the four arteries that service the brain. This method is most commonly used to mimic the clinical condition of cardiac arrest and will result in more extensive damage in the periphery of the brain where the vasculature is branched and more diffuse (Hossmann, 1998). The second method of modeling stroke is a focal ischemia model that impairs blood flow to a smaller region of the brain damaging primarily that area. This can be accomplished using a middle cerebral artery occlusion

(MCAO)(Tamura, *et al.*, 1981). The middle cerebral artery in the rat is indicated in a dorsal photograph of the rat brain in Figure 1.51.

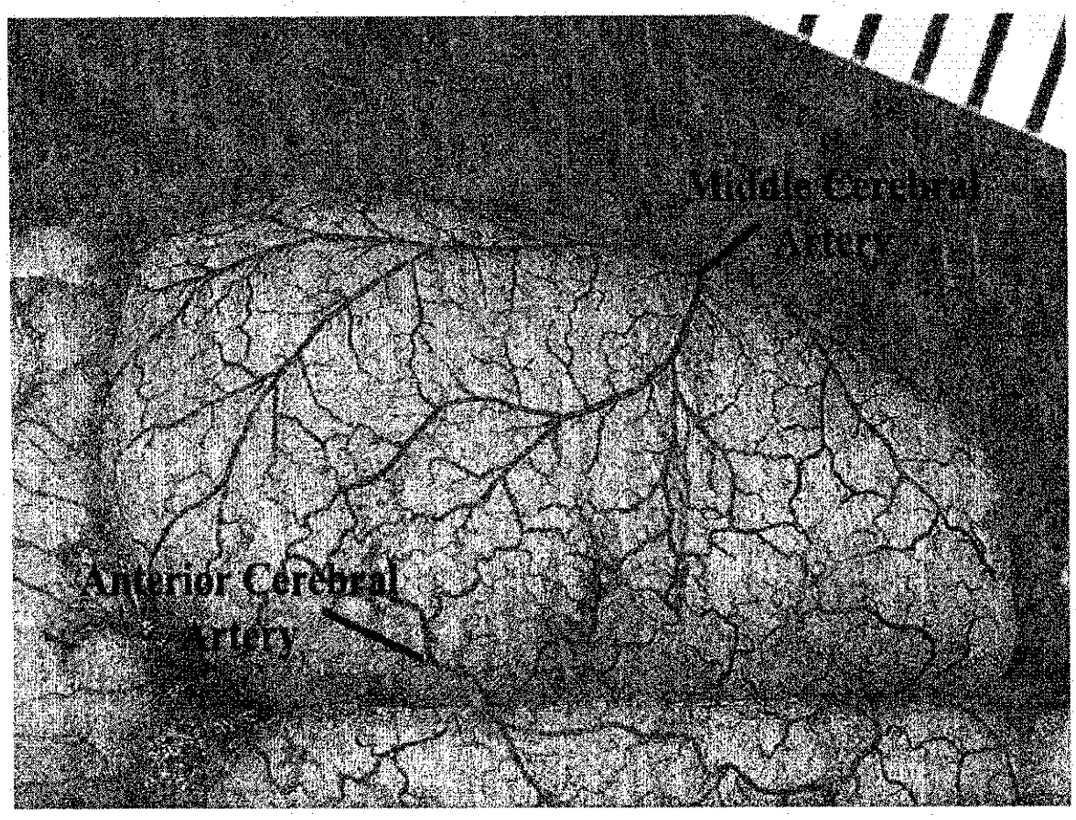

Figure 1.51. The surface cerebrovasculature of the rat. The divisions in the corner represent 1 mm. This photograph is courtesy of Richard Dyck at the University of Calgary.

There are two main variations of the MCAO method. The first requires feeding a thread through the internal carotid until it reaches the base of the MCA; the thread can either permanently or temporarily occlude the MCA. In the second variation, the MCA itself can be accessed via a craniotomy and permanently occluded via electro-coagulation or temporarily occluded with a knot or a clip. Both methods have two primary disadvantages. The first is that infarct sizes are variable because of collateral blood flow. Both the Circle of Willis and the pial network, which connects the anterior, posterior and middle cerebral arteries (Hossmann, 1998), can maintain blood flow to some degree when the MCA is occluded. Figure 1.51 depicts the numerous tiny vessels that interconnect the main vessels in the cortex. The second disadvantage is that an MCAO produces massive damage to numerous brain structures: the white matter, striatum, hippocampus and the motor cortex.

Because this thesis is concerned primarily with the motor system, an alternative stroke that affects primarily the motor cortex was chosen. This alternative model devascularizes the motor cortex and it is known as the pial strip. A pial strip removes the pial vasculature over a designated surface of the motor cortex, ensuring that the stroke is localized primarily to that area. The cerebrovasculature on the surface of the motor cortex is also illustrated Figure 1.51.

When choosing a model, it is also important to take into account the unique cellular pathology stroke produces. The next section will describe the affects of a stroke at a cellular level.

1.6. Molecular mechanisms of cell death.

Stroke produces a distinctive pattern of tissue damage. The site of the stroke leaves an area of complete cell death that is called the infarct. Typically the tissue surrounding the infarct will also experience some degree of damage due to the impaired blood flow. This area is referred to as the penumbra. The penumbra is of specific interest because cell death is often delayed and thus the penumbral tissue may be salvageable.

The pathology associated with a focal and global model of rodent ischemia is reviewed elsewhere (Lipton, 1999; Hossmann, 1994; Nedergaard, 1988).

There are many mechanisms that may contribute to cell death. Because the primary focus of this thesis is with motor impairments and not cellular pathology, this section will briefly outline the complex process of cell death. The prevailing idea related to stroke induced cell death is called the Excitotoxicity or Excessive Neuronal Excitation Hypothesis. Cell death begins when the reduction of oxygen and glucose results in a decrease in energy production in the affected area. The cell needs energy to maintain ion gradients. Sodium (Na^+) and toxic calcium (Ca^{2+}) ions (Fleckenstein, *et al.*, 1974; for a review see Kristián and Siesjö, 1998) are present in large quantities outside the cell primarily due to pumps that ensure these ions are expelled (for a review see Carafoli, 1987). If ion gradients are disrupted K^+ flows out, voltage gated ion channels open, and Na^+ and Ca^{2+} flow in (Nicholson, *et al.*, 1977).

Calcium entry results in release of the primary excitatory neurotransmitter glutamate; it had been hypothesized that glutamate release is toxic to the cell (Olney, *et al.*, 1971). This is confirmed by studies that show glutamate causes a Ca^{2+} mediated cell death (for a review see Choi, 1987). Glutamate activates the N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors on the cell surface (Pelligrino-Giampietro, *et al.*, 1992). Activation of the AMPA and NMDA receptors will allow Na^+ translocation into the cell, which will depolarize the postsynaptic membrane. Excessive glutamate will cause excessive depolarization, which will then displace the magnesium (Mg^{2+}) ion inside the NMDA Ca^{2+} channel. Additional Ca^{2+} will then flow into the cell via the NMDA receptor. This hypothesis is attractive because the CA1 and CA4 neurons of the hippocampus have high concentrations of NMDA receptors and they are particularly vulnerable to ischemic damage.

Calcium is also released from the intracellular endoplasmic reticulum (ER). Cell surface receptors activate a membrane bound G protein, which in turn activates the intramembrane phospholipase C. Phospholipase C contributes to membrane destruction and also cleaves the intramembrane phosphatidyl inositol (PIP_2) into the two secondary messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 can translocate into the cytoplasm where it acts on the ER promoting Ca^{2+} release (for a review Berridge, 1993).

Ca^{2+} also activates a number of cellular cascades and products including: phospholipases, endonucleases, proteases, lipases, and protein kinases involved in phosphorylation. Ca^{2+} also promotes the formation of enzymes that produce free radicals such as nitric oxide (NO) and reactive oxygen species (ROS) (Choi, 1987; Kristián and Siesjö, 1998). ROS, also called free radicals, are groups of atoms that have unpaired electrons. The unpaired electron makes the radicals unstable so they react quickly with other molecules to obtain an additional electron, often destroying them in the process. Common cellular targets are cell membranes because they have many exposed hydrogen atoms with available electrons. Ischemic cells experiencing an energy crisis will also exhibit enhanced ROS production that contributes to cell death. Figure 1.61 summarizes the excitotoxicity hypothesis.

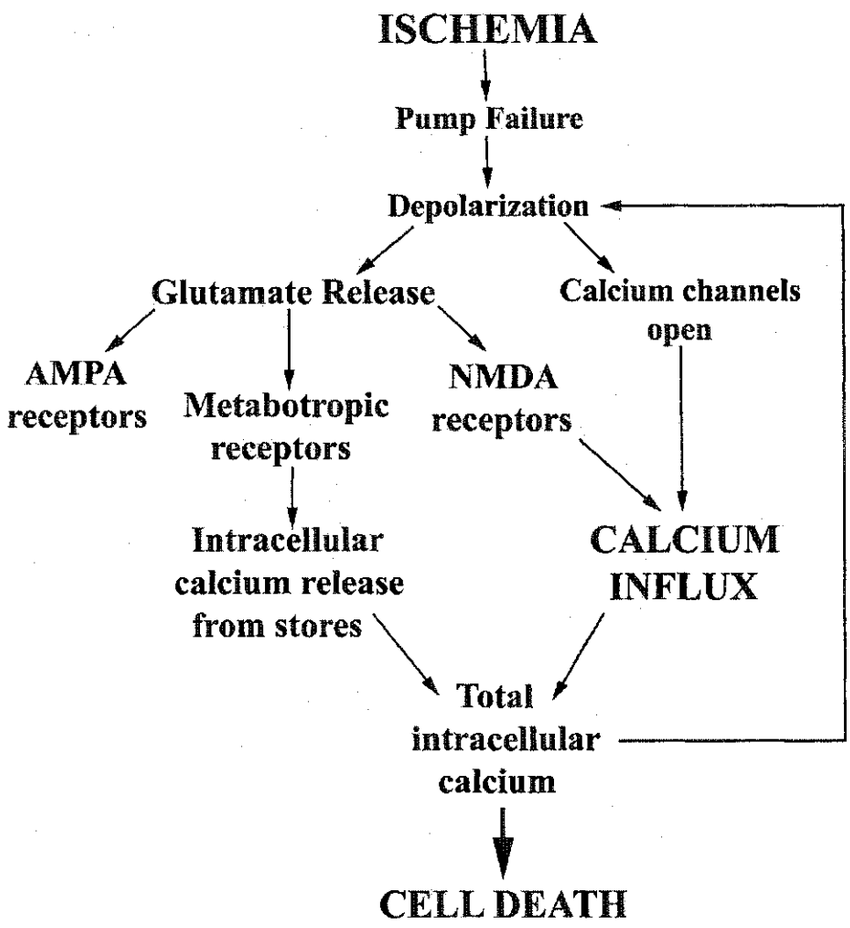

Figure 1.61. A summary of the processes that are hypothesized to result in cell death. This figure is adapted from the Lyden and Wahlgren, 2000 review.

Understanding the complex process of cell death may lead to the ability to salvage the injured tissue. This is because there are many possible mechanisms involved in the complex cascade that can potentially be manipulated. These manipulations that are thought to be neuroprotective can be tested for functional outcome using behavioural studies with measurable parameters in standardized tests. The following section will describe some aspects of behavioural testing useful for assessing motor function.

1.6. Testing behaviour in the rodent.

There are a variety of behavioural tests available for testing sensorimotor function in rats, but there are very few available for mice. Immediately following a motor system injury, a rat's ability to walk may be affected and these deficits can be measured by performance on the rotarod, a rotating cylinder, or a rung-walking test (Metz and Whishaw, 2002). Because the sensorimotor deficits a stroke animal suffers are often unilateral (Yamamoto, *et al.*, 1988), most tests measure forelimb asymmetry. For example, if an animal is lowered towards a surface it will extend its forelimb in anticipation of contact; this response is impaired in the injured forelimb (Hua, *et al.*, 2002; Schallert, *et al.*, 2000). The injured forelimb is also relatively inactive during spontaneous vertical exploration (Schallert, *et al.*, 1997; Schallert, *et al.*, 2000). In addition, control rats do not use their forelimbs while swimming, except to turn, but this forelimb inhibition may be lost following an ischemic insult causing the rat to paddle with all four limbs (Stoltz, *et al.*, 1999). Most of these tests provide a quantitative score of performance. The animal often appears to recover quickly on these measures after the insult. It is therefore necessary to have a detailed qualitative assessment of impairments to distinguish between recovery and compensation.

The single pellet reaching task has the advantage of providing both a quantitative and a qualitative measure of performance (Whishaw, 2000; Whishaw, *et al.*, 1992; Whishaw, *et al.*, 1992a). It has been standardized for both rats and mice (Farr and Whishaw, 2002, Whishaw, 1996). When a laboratory mouse is reaching for a single piece of food in the single pellet reaching task, the entire sequence can be subdivided into ten

stereotypical movements (Farr and Whishaw, 2002). These movements can be evaluated using a scale adapted from a movement notation that expresses the limbs as axis and documents movement by observing the changes in the limb and body axis (Eshkol and Wachmann, 1958). A reach occurs as follows, the paw is lifted so that the semiflexed digits are aligned with the body's midline. Then the elbow is brought to the midline. This is called the "aiming" position. During the advance the digits extend as the paw is moved through the slot in anticipation of contact with the food. The digits then pronate over the food item so that each digit contacts the surface of the shelf in succession. This movement is called the arpeggio. The digits close around the food item during the grasp. The paw is supinated for the first time as it is withdrawn through the slot and supinated further to present the food to the mouth. Aiming, pronation and supination are performed mainly by upper arm movements.

Mice, like rats and humans, are also impaired at skilled reaching following injury to the motor system (for a review see Whishaw, 2000; Whishaw and Coles, 1996; Whishaw and Gorny, 1994, Whishaw, *et al.*, 1992a; Whishaw, *et al.*, 1992b; Whishaw, *et al.*, 1991; Castro, 1972). Injury to the motor cortex affects various aspects of the reach, specifically the aiming and rotatory movements of the forelimb (Whishaw, 2000; Whishaw and Coles, 1996; Whishaw, *et al.*, 1992a; Whishaw, *et al.*, 1992b; Whishaw, *et al.*, 1991) and often recovery is minimal and the animals learn to compensate for the deficits (Whishaw, 2000; Friel and Nudo, 1998; Whishaw, *et al.*, 1991).

The most commonly used mouse behavioural examinations are neurological rating scales (Rogers, *et al.*, 1997; Hunter, *et al.*, 2000; Sampei, *et al.*, 2000). Cognitive and sensory tests, such as passive avoidance, that incorporate learning and memory are also commonly used (Hattori, *et al.*, 2001; Zhang, *et al.*, 2002). They are not specific for the motor system, however. Tasks that are commonly used for testing the motor system in mice are the rotarod, in which mice are required to walk on a rotating cylinder (Jones and Roberts, 1968) or the raised beam where the footsteps are counted. These tests do not often reflect any long term deficit because mice recover the ability to walk quite quickly. The Montoya staircase task (Montoya, *et al.*, 1991) places the animal in an enclosure

with a divided staircase, ideally the impaired limb will be unable to retrieve the food source placed on that side of the divided staircase; this task has been adapted for mice (Baird, *et al.*, 2001). Nevertheless, the mice exhibit unusual strategies when retrieving the food, making it difficult to assess the degree of impairment.

Because there are few ways to assess functional outcome in mice, one might ask, why use mice at all? There is one strong advantage to using mice and it will be described in the following section.

1.6. Why use mice?

The recent development of transgenic technology has made it possible to create many strains of mice, which is revolutionizing genetic research (for an excellent review see Wagner, *et al.*, 1995 or Jaenisch, 1988). Transgenic technology involves specific manipulations of the genome, making it possible to assess the function of individual genes to the animal. Because transgenics have been developed in the mouse, it is possible to remove or insert genes from any living organism including humans, as well as promoters and reporters into the transgenic genome. Stroke research has benefited from transgenic technology by using genetic manipulations to answer some molecular concepts (for relevant papers see Sampei, *et al.*, 2000; Guégan, *et al.*, 1998; Eliasson, *et al.*, 1997; Chan, *et al.*, 1995). The contributions of transgenics to stroke research will be discussed later. For the present it is worth making note of the most important transgenic contributions to stroke research. Knocking out the gene that codes for nitrous oxide synthase (NOS) in mice prevented the production of nitric oxide, which is known to be a powerful and disastrous free radical (Chan, *et al.*, 1995). Thus, these "knockouts" suffered minimal tissue damage from a stroke when compared to the wild type controls.

This advantage of using mice makes it necessary to develop mouse appropriate behavioural tests. Therefore, the first goal of the thesis was to adapt the single pellet skilled reaching task to mice with a stroke in order to obtain a quantitative and qualitative functional measure of behavioural outcome, recovery and compensation.

1.7. Introduction to the Experiments.

Because the single pellet reaching task is fairly specific to the sensorimotor system, the focal model, pial stripping, was chosen to localize the stroke to the motor cortex. The first experiment documents the single pellet reaching task in C57/Black/6 mice that have received a stroke. The C57/Black/6 mice are one of the most common strains of mice (Fujii, *et al.*, 2003). Testing took place both before and after a focal motor cortex stroke produced by pial stripping. Post stroke, the animals are severely impaired both quantitatively in the number of pellets they were able to retrieve, and qualitatively, in the way the pellets were retrieved.

The second experiment was designed in much the same way. It was apparent that the mice were not able to easily grasp 20mg food pellets, so a smaller food item, dehulled millet seeds, was obtained. In addition, the method of producing stroke was altered. The focal motor cortex stroke was produced by applying the potent vasoconstrictor Endothelin-1 (ET-1) (Yanagishawa, *et al.*, 1988) to the motor cortex; this compound has been used to produce reproducible temporary occlusions (Sharkey, *et al.*, 1993). An endothelin application does not damage the pial vasculature and it's affects are temporary. The endothelin-type stroke did not result in a large quantitative deficit, but the animals were impaired qualitatively.

The third experiment compared the effects of the two stroke types. Post surgery, the ET-1 group was not significantly different from the control group in their quantitative success rates; the mice that received the pial strip were impaired and gradually recovered to the level of the endothelin mice over the remainder of the testing period. The detailed qualitative analysis revealed that both groups of mice suffered impairments in the same types of movements, though the pial strip animals were more severely impaired. A correlation analysis indicated that the larger the stroke insult the more impaired the mice were at many of the movements. The brain tissue of the endothelin group showed little infarct damage but microscopic observation indicated that the underlying cell

morphology was abnormal. Thus, it seems possible to titrate the endothelin model to produce varying degrees of damage.

2. Experiment 1:

Skilled movements in a mouse are impaired after a focal motor cortex stroke.

Abstract

Background and Purpose - Skilled reaching movements are an important aspect of human motor behavior, but are impaired following motor system stroke. The purpose of this study was to document skilled movements in mice before and after a focal motor cortex stroke for the purpose of developing a mouse model of human stroke.

Methods - Male C57/BL6 mice were trained to reach with a forelimb for food pellets and then given a motor cortex stroke, induced by pial stripping, contralateral to their preferred reaching limb. Reaching success and the movements used in reaching were analyzed by frame-by-frame inspection of presurgical and postsurgical video records.

Results - Reaching success was severely impaired following the stroke. Improvement in success over two postsurgical weeks was moderate. Analysis of ten movement components, comprising reaches pre- and postsurgically, indicated that most of the rotatory movements of the limb used for aiming, advancing, pronating, and supinating the paw were impaired. When successful reaches did occur, body movements that compensated for the impairments in limb rotatory movements aided them.

Conclusions - The results indicate that skilled reaching in the mouse is impaired by focal motor cortex stroke and they suggest that the mouse, and the skilled reaching task, provides an excellent model for studying impairments, compensation, and recovery after motor system stroke.

Introduction

Comparative analyses of food handling and skilled reaching by laboratory rats (*Rattus norvegicus*) and mice (*Mus musculus*) show that the forelimb movements used by the two species are similar (Whishaw, 2000; Iwaniuk and Whishaw, 2000; Whishaw, *et al.*, 1998; Whishaw, 1996; Whishaw and Coles, 1996). To eat they use five movements in sequence. They identify the food via olfaction, pick up the food using the mouth, sit back on their haunches, grasp the food with the forepaws by bringing the elbows inward, and manipulate the food with the digit tips.

Similarly, while performing in a formal laboratory skilled reaching task, requiring extension of a forelimb through an opening in a cage in order to obtain a food pellet, rat and mouse movements are similar (Whishaw, 1996; Whishaw, *et al.*, 1992). The paw is lifted so that the digits, semiflexed, are aligned with the midline of the body, and then the elbow is also brought to the midline, to an "aiming" position. As the limb is advanced through the slot, the digits are extended. As the limb is pronated, the digits are opened thus placing the paw over the food. The food item is grasped by digit closing, the wrist is extended to lift the paw, and the paw is withdrawn through the slot. The paw is then supinated further so that the food can be presented to the mouth. Aiming, pronation and supination are performed mainly by upper arm movements (Whishaw and Pellis, 1990; Castro, 1972). Pronation consists of rotating the paw so that digits 5 (outermost) through 2 (innermost) contact the surface on which the food pellet is placed, and this "arpeggio" movement is assisted by movements around the wrist (Whishaw and Gorny, 1994).

Studies using rats show both spontaneous and skilled limb use are impaired following damage to the motor system (Stein, 2001; Whishaw, 2000; Hunter, *et al.*, 2000; Kolb, *et al.*, 2000; Zhang, *et al.*, 2000; Modo, *et al.*, 2000; McKenna and Whishaw, 1999; Petullo, *et al.*, 1999; Whishaw, 1996; Whishaw, *et al.*, 1992a; Whishaw, *et al.*, 1992b; Montoya, *et al.*, 1991; Whishaw, *et al.*, 1991; Gonzalez, *et al.*, 1986; Castro, 1972). Impairments produced by damage to different motor regions are also distinctive. For example, following a motor cortex injury, a rat still retrieves food by reaching with the

affected forelimb but success is reduced (Biernaskie and Corbett, 2001; Whishaw, 2000; Kolb, *et al.*, 2000). In addition, most of the movement components of the reach are abnormal. Compensatory body movements provide the rotatory movements to assist pronation and supination (Whishaw, 2000). Thus, the species has provided a primate substitute for studying the neural basis of skilled movements.

Given recent interest in using transgenic mice for stroke research (Hattori, *et al.*, 2001; Sampei, *et al.*, 2000; Guégan, *et al.*, 1998; Rogers, *et al.*, 1997; Eliasson, *et al.*, 1997; Chan, *et al.*, 1995) the present study investigated the effects of motor cortex stroke on skilled reaching in mice. The mice were trained to reach through a slot for food and their performance was video recorded before and after a cortex stroke produced by pial stripping.

Materials and Methods

Subjects

Eight five-month old male C57/Black 6 mice (*Mus musculus*) weighing between 25-30g were raised in the University of Lethbridge animal colony from animals originally obtained at Jackson Laboratories, Bar Harbor, ME. Animals were housed individually in standard plastic mouse cages (lights on a 12:12 hour cycle beginning at 08.00; room temperature 22°C) and given one piece of Lab Chow (4g) after the testing period each day. Two of the eight animals consistently had less than half of a gram of food remaining in the morning that was removed at 8:00. Because testing began at 1:00, even the mice with remaining food had to wait five hours before regaining access to food. Each mouse was thus sufficiently restricted and motivated to perform the task. The experiment was conducted according to the Canadian Council on Animal Care code.

Surgery

After adaptation to reaching and six days of training the animals were anaesthetized using isoflurane (BIMEDA-MTC Animal Health Inc.). Eight mice, 5 left,

and 3 right paw preference, received a pial strip stroke to the motor cortex contralateral to the preferred forelimb. Using stereotactic surgery, a 3mm by 3mm region of the skull was removed, 0.5mm behind bregma to 3mm anterior to bregma, and from 0.5mm lateral to the midline to 3.5mm lateral to the midline. The dura was removed within the trephination and the underlying pia, arachnoid, and vasculature was wiped with a cotton swab until no vasculature was visible. Shams were not performed because the removal of the skull and dura did not result in any motor deficits in similar experiments in rats (Kleim, *et al.*, 1998). Vetropolycin (Janssen) gel was applied to the eyes and the incisions were closed. The animals were returned to their home cages and postsurgical testing began the following day.

Reaching Task

The Plexiglas reaching box (Whishaw, 1996) was 19.5cm long, 8cm wide, and 20cm high (Figure 2.11).

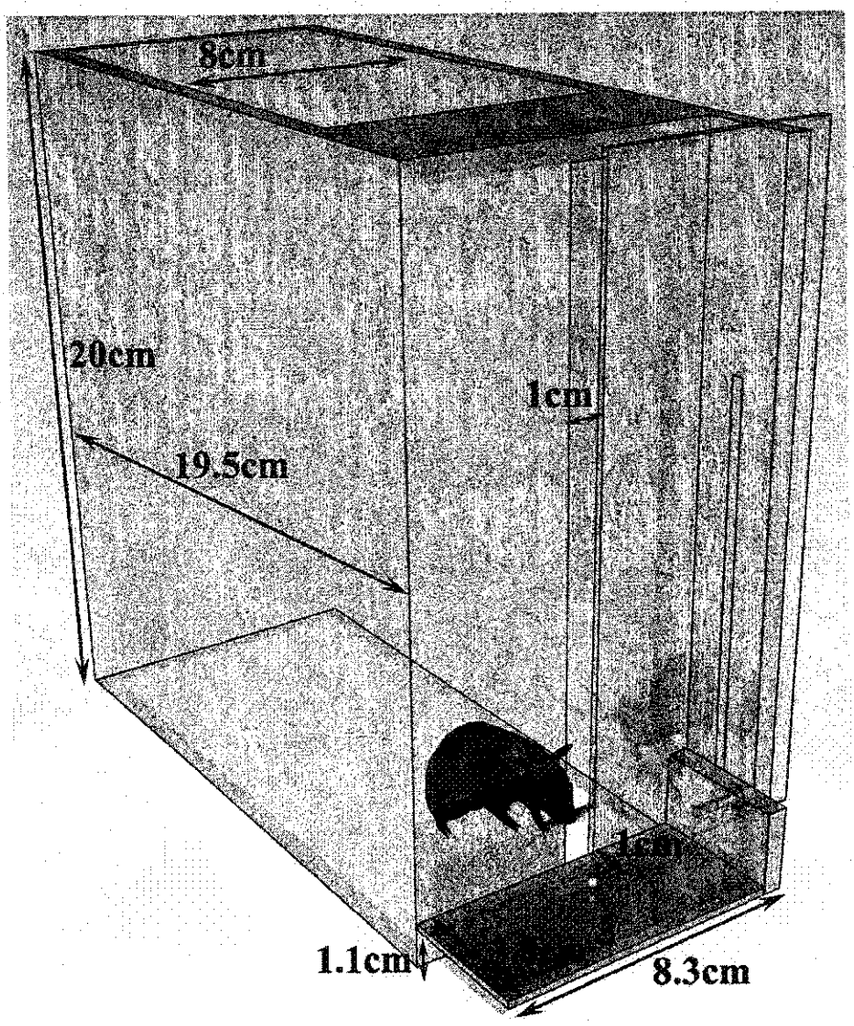

Figure. 2.11. The single pellet reaching apparatus. The shelf in front of the box contains two divots for food.

A 1cm wide vertical slit ran up the front of the box. A 0.2cm thick plastic shelf (8.3cm long and 3.8cm wide) was mounted 1.1cm from the floor on the front of the box. Twenty-milligram food pellets (Bioserve Inc.) were placed in indentations spaced 1cm away from the slit and centered on its edges. Animals were habituated for one week by placing them in the cages for ten minutes. Pellets were initially available on the cage floor and within tongue distance on the shelf. Pellets were gradually removed from the floor and placed farther away on the shelf until the mice were forced to reach to retrieve the food. The pellets were placed in both indentations allowing the mice to display which paw they preferred to use after which the food was placed in the indentation contralateral to the preferred paw. As the animal pronates the paw medially, this placement allows the mouse to obtain the pellet with a paw and not with the tongue (Whishaw, *et al.*, 1991).

Videorecording

Filming was done on the last two pre- and postsurgical days of training with a Sony DSRPD100 digital camcorder (30 frames/sec; shutter speed of 1000). Illumination was provided with a 2 arm Nikon MKII 150W fiber optic light. The animals were filmed from a frontal and ventral view (by placing the apparatus over an inclined mirror table). The tapes were viewed on a Sony DV cam DSR-20 player and Trinitron monitor. Representative movements were captured using Final Cut Pro frame grabbing software on a Macintosh G3 computer.

Reaching Success

Each animal reached for twenty pellets each day during the testing period. All animals were more than sufficiently motivated to attempt to obtain 20 pellets. If an animal reached through the slot and obtained a food pellet, the reach was scored as a success. If an animal knocked the food away, or dropped the food after grasping it, the reach was scored as a miss. Performance was defined by Percent Success = (number of successful retrievals/ 20)*100

Qualitative Analysis

Qualitative movement scoring was derived from a conceptual framework adapted from Eshkol-Wachmann Movement Notation (Eshkol and Wachmann, 1958). EWMN is designed to express relations and changes of relation between the parts of the body. The body is treated as a system of articulated axes (i.e., body and limb segments). A limb is any part of the body that either lies between two joints or has a joint and a free extremity. These are imagined as straight lines (axes), of a constant length, which move with one end fixed to the center of a sphere. On the basis of descriptions obtained from EWMN, rating scales of movements were derived (Whishaw and Pellis, 1990).

Five presurgical and five postsurgical reaches from each mouse were rated for qualitative features of the movement (Whishaw, *et al.*, 1991). Ten components of a reach were rated:

1) *Digits to the midline.* Using mainly the upper arm, the reaching limb is lifted from the floor so that the tips of the digits are aligned with the midline of the body.

2) *Digits semiflexed.* As the limb is lifted, the digits are flexed and the paw is supinated so that the palm of the paw is aligned almost vertically.

3) *Aim.* Using an upper arm movement, the elbow is adducted to the midline while the tips of the digits remain aligned with the midline.

4) *Advance.* The limb is advanced directly through the slot toward the food target using an upper arm movement and during advancement the snout is raised to allow passage of the paw into the slot.

5) *Digits extend.* The digits extend during the advance.

6) *Pronation.* When the paw is over the target, the paw pronates and digit 5 (the outer digit) through to digit 2 touches the surface in succession, mainly by abduction of the elbow and also by a rotational movement around the wrist. During pronation, the digits open.

7) *Grasp.* The digits flex over the food and close around it. The paw remains in place and the wrist is slightly extended to lift the food.

8) *Supination I.* As the paw is withdrawn, it supinates by almost 90 degrees.

9) *Supination II.* Once the paw is withdrawn from the slot the paw further supinates by 45 degrees to present the food to the mouth.

(10) *Release.* The mouth contacts the paw and the digits open to release the food.

Each movement was rated on a 3-point scale. If the movement was normal, a score of 0 was given. In cases where there was some ambiguity concerning the occurrence of a movement, or the movement was present but incomplete, a score of 1 was given. If the movement was absent, a score of 2 was given.

Histology

The mice were deeply anaesthetized and perfused through the heart with 0.9% saline followed by 4% para-formaldehyde/0.9% saline. Brains were removed and cryo-protected in 30% sucrose/4% para-formaldehyde for one week. The brains were sectioned (50μm) on a 2800 Frigocut E cryostat (Reichert-Jung), mounted on 1% gelatin and 0.2% chromalin dipped slides, stained with Cresyl violet, and cover slipped using Permount (Fisher-Scientific).

Results

Histology

All of the brains had damage in the motor cortex. Figure 2.12 indicates representative dorsal (Figure 2.12A-B) and coronal views (Figure 2.12C-E) of the lesion.

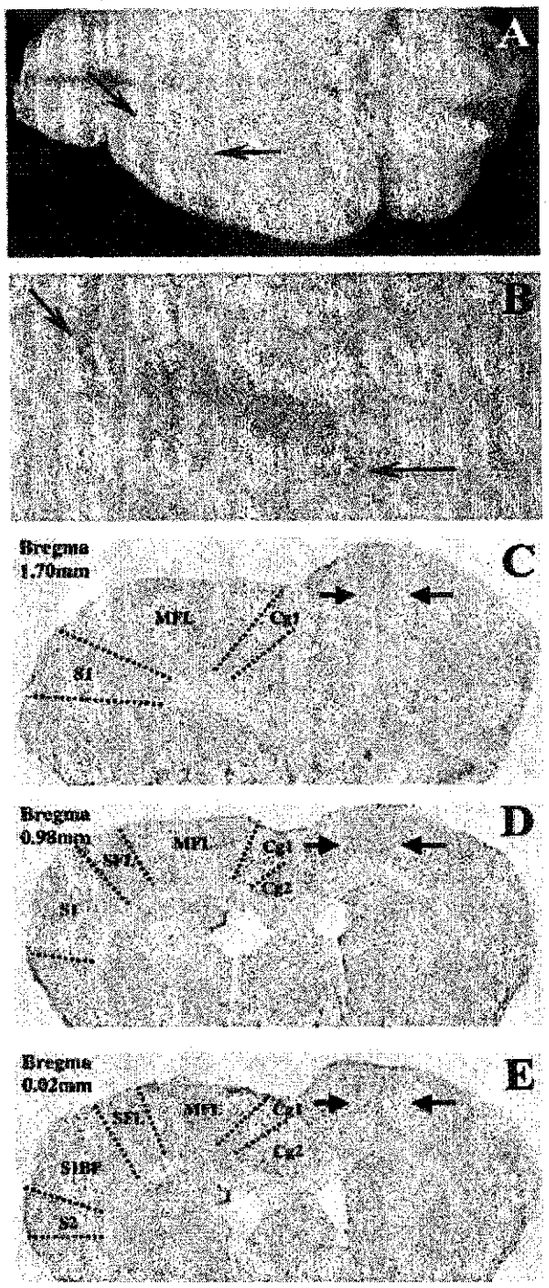

Figure 2.12. (A-B) Dorsal view of a typical motor cortex stroke. (C-E) Photomicrographs (Cresyl violet) of a representation of the motor cortex stroke at three coronal levels. The arrows in the right hemisphere indicate the location of the lesion in the motor cortex. The intact left hemisphere indicates the brain regions present (MFL- Motor Forelimb Area, Cg1 and Cg2- Cingulate Cortices 1 and 2, SFL- Sensory Forelimb Area, S1- Primary Sensory Area, S2- Secondary Sensory Area, S1BF- Primary Sensory Barrel Forelimb Area).

Pial stripping produced a conical cavity beneath the area of pial removal, and the cortex adjacent to the lesion evaginated and filled in the cavity produced by the lesion, making quantification of the actual lesion size difficult. Nevertheless, based on a comparison of the lesion location relative to the large layer V cells in the contralateral hemisphere, the lesions appeared restricted to the caudal and rostral forelimb areas as defined by electrophysiological stimulation studies in the rat (Kleim, *et al.*, 1998).

Quantitative changes in reaching following stroke

Reaching Success

The ANOVA on success revealed a significant Treatment effect, $F(1, 15)= 38.962$, $P< 0.001$, indicating that the mice achieved higher reaching scores before the motor cortex lesion. There was a significant effect of Days, $F(5, 75)=3.48$, $P< 0.007$, but there was no significant Group by Days interaction, $F(5, 75)= 2.114$, $P= 0.0729$. The mice achieved an average percent success of 40±10 prior to the lesion and the average percent success dropped to 10±10 following the lesion (Figure 2.13). An ANOVA on postsurgical performance was not significant, although the animals displayed a trend toward improving.

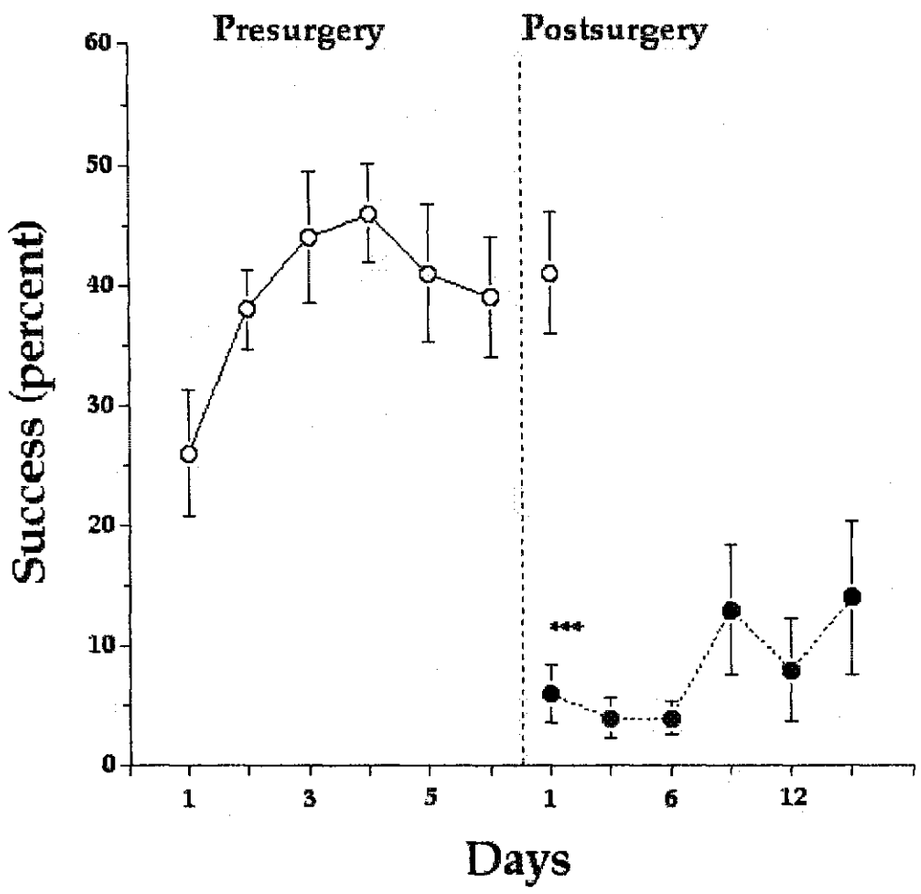

Figure 2.13. Reaching success (means ± SEM). The dotted line represents the administration of a motor cortex stroke. The single point post surgery averages the control performance. (*** $P < 0.001$).

Movement Components

The ANOVA of five presurgical and postsurgical reaches revealed a significant main effect of Group, $F(1,16)= 65.01$, $P < 0.001$, indicating that the mice accumulated higher scores for the ten movement components following cortical injury. There was also a main effect of Movement, $F(9, 144)=14.68$, $P < 0.001$, indicating that some movements were more impaired than others. More importantly, there was a significant Group by Movement interaction for the Postsurgery stroke group, $F(9, 144)= 8.232$, $P < 0.001$, as some movements in the stroke group were more impaired relative to control scores. Follow-up students unpaired t-tests indicated significant group differences for the aim, advance, pronation, supination II and release components of the reach, but not for the digits to the midline, digits semiflexed, digits extend, grasp and supination I (Figure 2.14).

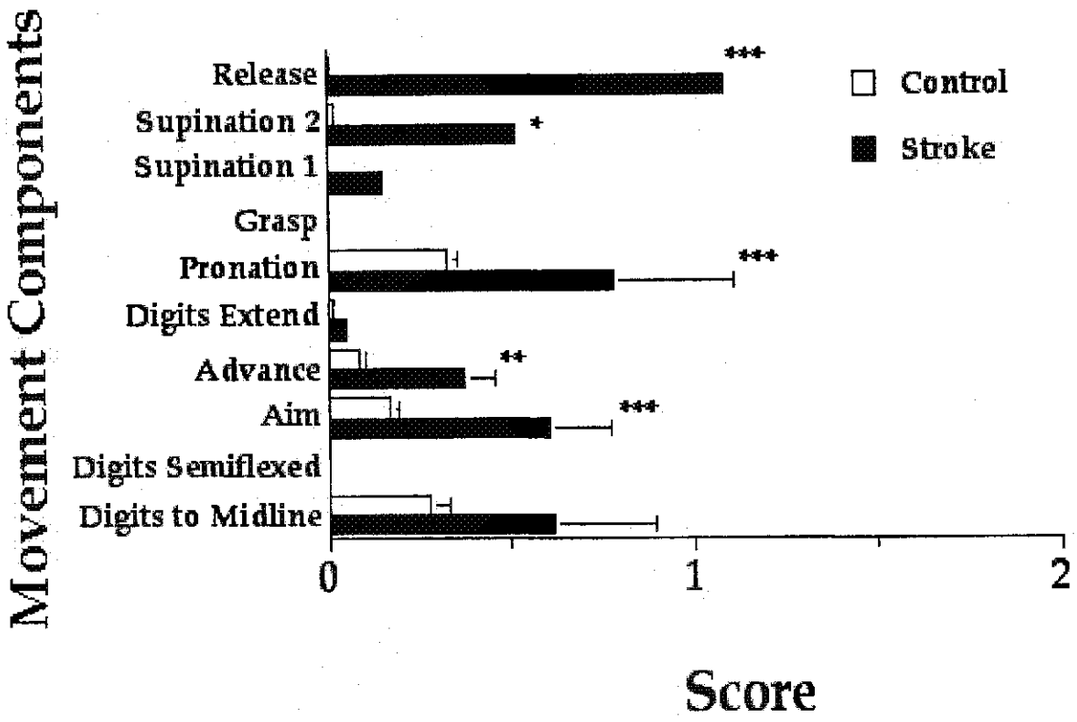

Figure 2.14. Scores (means ± SEM) for each of the ten movement components of the reach. Control white bars, stroke solid bars. A score of 0 indicates a normal movement while a score of 2 indicates complete absence of the movement. (*** $P < 0.001$)(** $P < 0.01$)(* $P < 0.05$).

Qualitative changes in movement following stroke

The following descriptions and ratings were all taken from reaches that were successful. Nevertheless, it is likely that the abnormalities displayed by the stroke mice on successful reaches did contribute to the lower incidence of successful reaches.

Digits to the Midline and Aim

Control mice lifted the reaching limb from the surface, semiflexed the digits, and aligned the digit tips with the midline of the body with a single movement (Figure 2.15A).

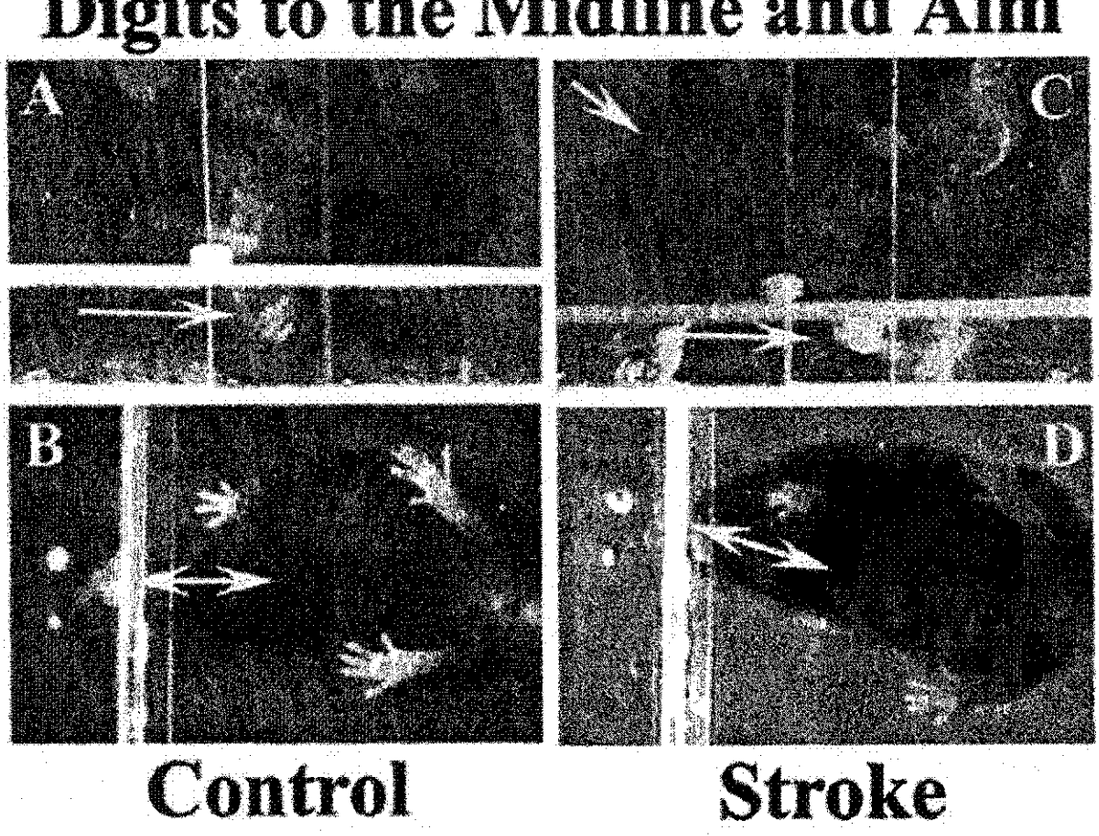

Figure 2.15. Digits to Midline and Aim. A, B: Control. The control mouse lifts the reaching limb and aligns its digit tips with the midline of the body (arrow in A) and then aims the limb by bringing the elbow to the midline (double sided arrow in B). C, D: Stroke. The stroke mouse also aligns the digit tips with the body but must shift the shoulders to achieve this (arrows in C). Note: The non-reaching forelimb is supporting the unusual posture. Note: The double-sided arrow in B and D indicates the angle of the paw-elbow segment relative to the angle of the body.

They then adducted the elbow to the midline of the body so that the forelimb was aligned with the body midline in an "aiming" position (Figure 2.15B). Stroke mice also lifted the limb and semiflexed the digits, but the digit tips were displaced more laterally. Then, rather than adducting the elbow to the midline, to bring the paw to a position from which it could enter the slot, the animals made an ipsiversive rotatory movement of the body to bring the limb to an aiming position. Thus, the comparison in Figure 2.15A (control) and Figure 2.15C (stroke) indicate that although the digits seem aligned with the body in both animals, the stroke animal has achieved alignment in part by using body rotation. The end result was that the control mice were able to advance the paw from an "aiming" position directly through the slot toward the food, whereas the stroke mice directed the paw through the slot diagonally.

Advance and Digits Extend

The control mice advanced the paw directly forward over the food item, while at the same time extending the digits (Figure 2.16A), leaving the paw in a position to pronate over the food pellet (Figure 2.16B).

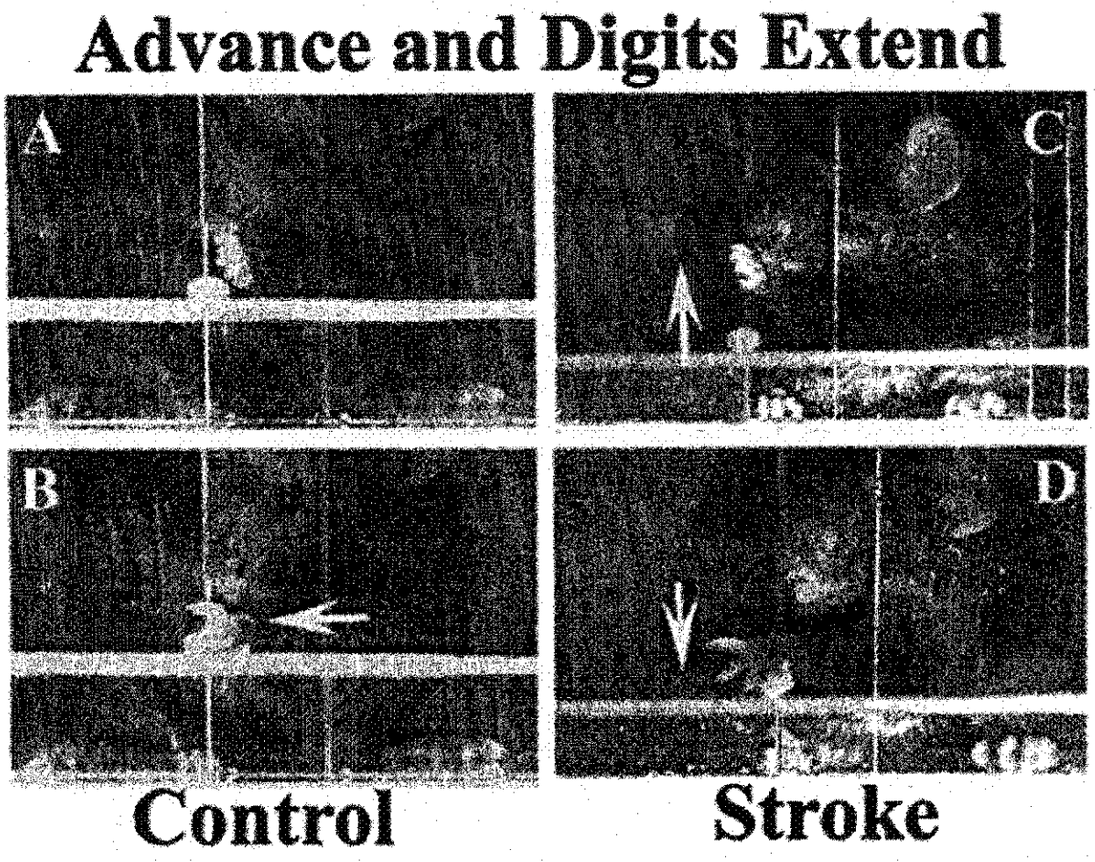

Figure 2.16. Advance and Digits Extend. A, B: Control. The control animal can advance the reaching forelimb directly over the food pellet (A) and extend the digits while beginning to rotate the paw over the food pellet (arrow in B). C, D: Stroke. The stroke animal lifts the reaching forelimb with the shoulders during the advance (arrow in C) and slaps laterally to contact the pellet while extending the digits (arrow in D).

The stroke mice also opened the digits as the paw advanced, but the paw entered the slot diagonally so that it was located dorsally and medially relative to the food pellet (Figure 2.16C,D).

Pronation

Once the limb is advanced and the digits are extended, the paw is pronated over the food pellets as the digits are opened from digit 5 through digit 2. The digits also contact the shelf in the sequence digit 5 through digit 2. This arpeggio movement is illustrated for a control mouse reaching in an instance in which no food pellet was located in the slot (Figure 2.17A-D top).

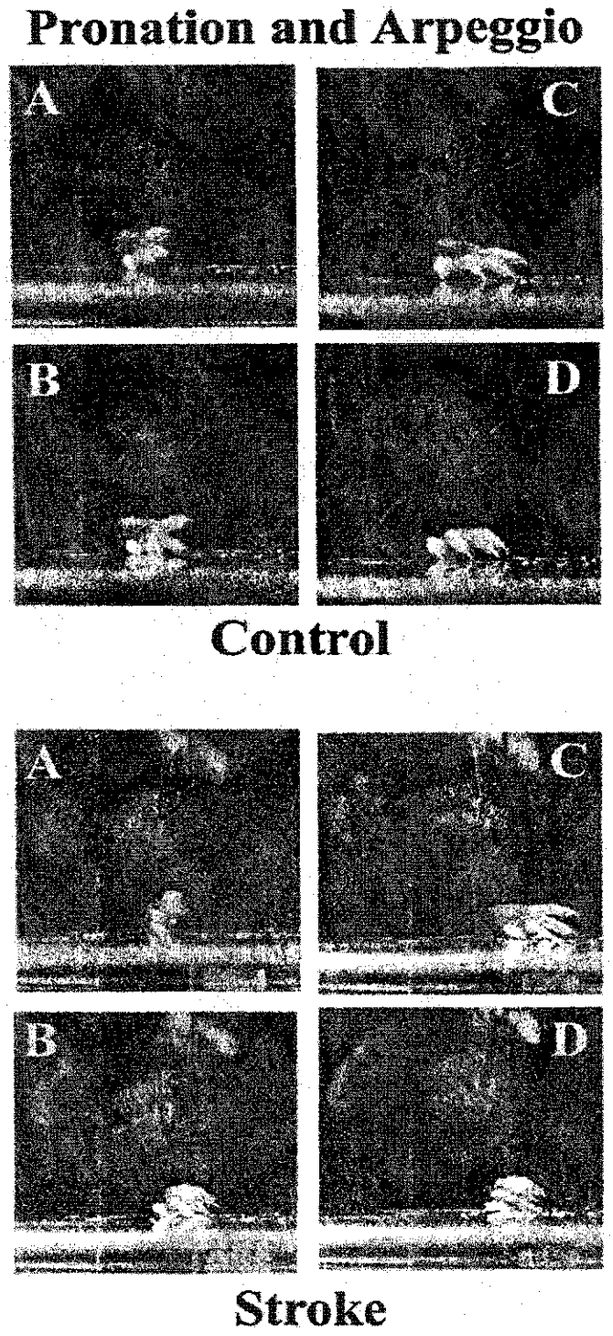

Figure 2.17. Pronation. A-D, top: Control. The digits contact the surface of the shelf in succession from the most medial (pinky) to the most lateral (pointer). The digits extend and open. A-D, bottom: Stroke. The paw comes in from the side, or slaps laterally, and digits do not contact the surface in succession.

Typically, when the paw and digits contact a food pellet, grasping occurs. If the paw fails to contact a food pellet, grasping does not occur. The stroke mice slapped the paw laterally, instead of using a rotatory wrist movement, and grasped whether or not a food pellet was contacted (Figure 2.17A-D bottom).

Grasp and Supination I

Once the food was contacted, the control mice closed the digits around the pellet and supinated the closed paw so that the palm of the paw was oriented vertically (Figure 2.18A), from which position the paw was withdrawn through the slot (Figure 2.18B).

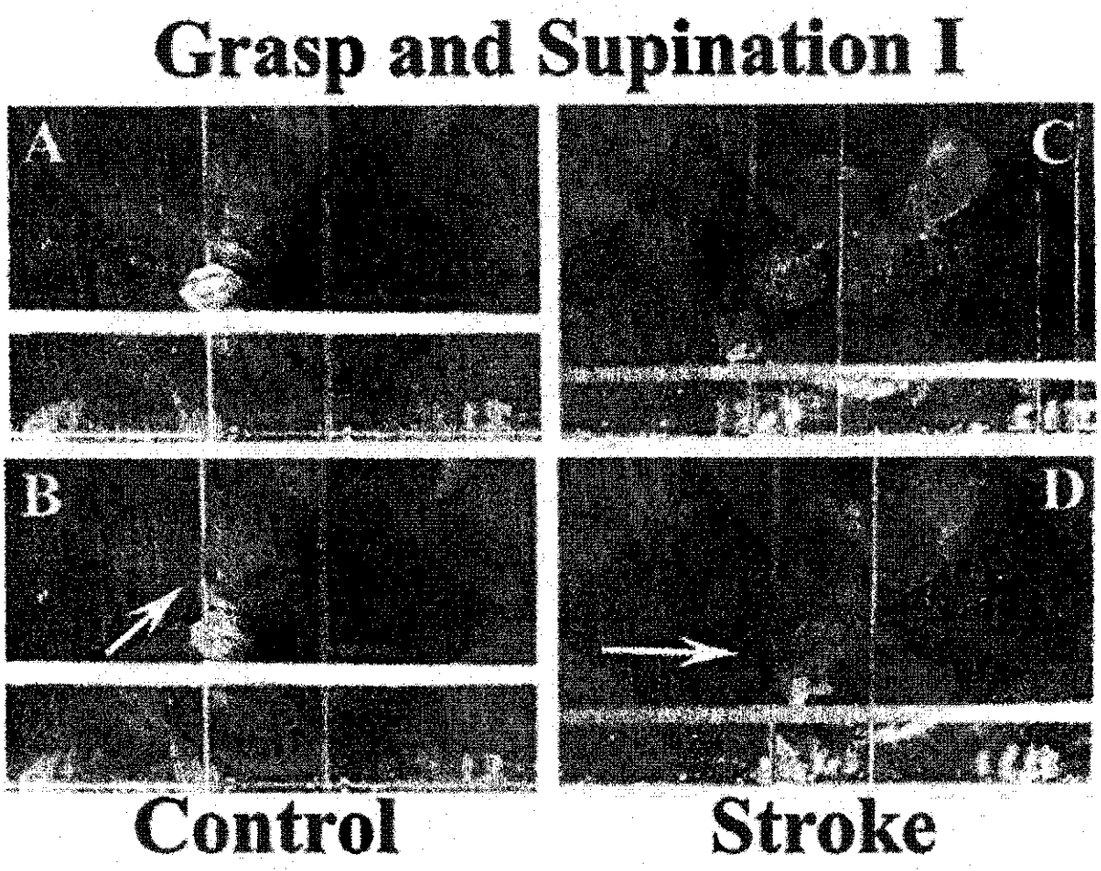

Figure 2.18. Grasp and Supination I. A, B: Control. The control animal closes the digits around the food item (A) and exhibits more supination during the withdrawal (arrow in B). C, D: Stroke: The stroke animal also closes the digits around the food item (C) and degree of supination is less than that of the control, using a dragging motion to obtain the food (arrow in D).

Stroke animals displayed little supination after grasping the food (Figure 2.18C), and whatever supination they did achieve occurred by leaning on the pellet with the forelimb as they withdrew the paw from the slot (Figure 2.18D).

Supination II and Release

Once the paw with food was withdrawn, the control mice further supinated the paw, so that the palm faced upward to present the food to the mouth (Figure 2.19A).

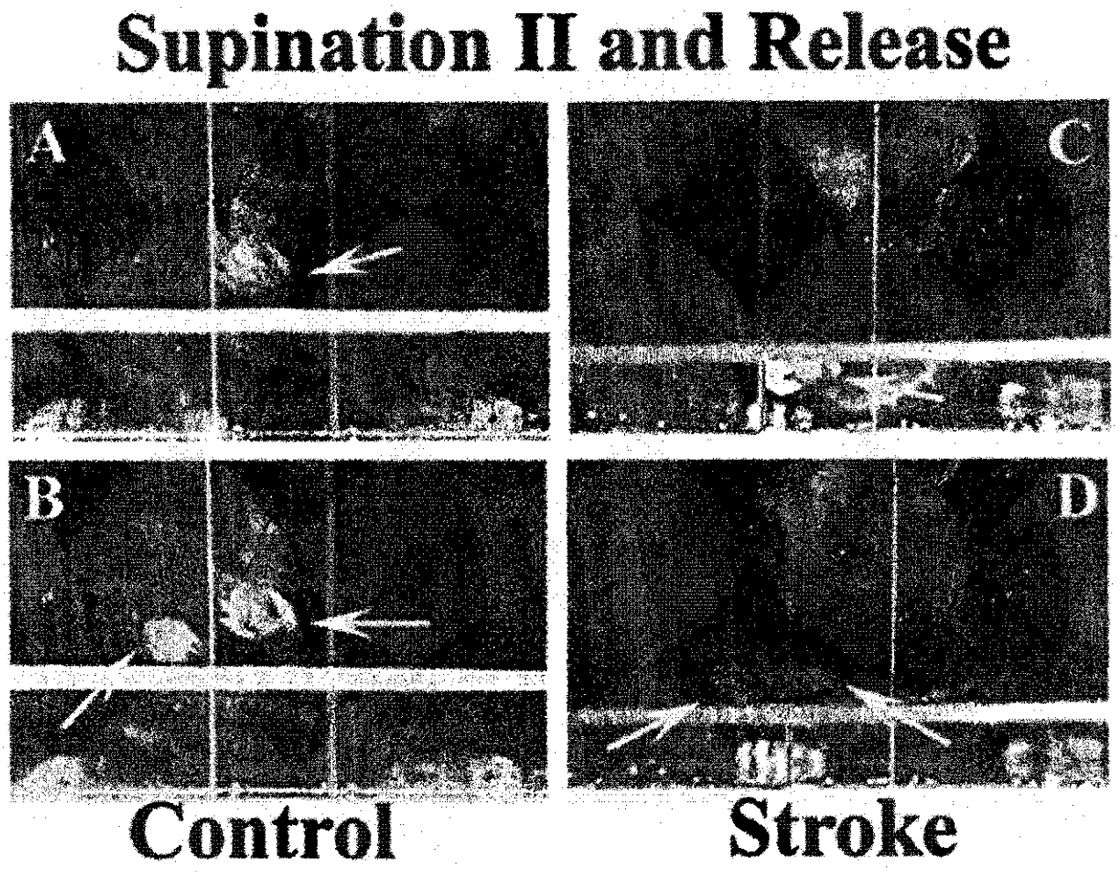

Figure 2.19. Supination II and Release. A, B: Control. A control animal supinates the paw to present the food to the mouth (arrow in A) and opens the digits to release it (arrow in B). C, D: Stroke. A stroke animal chases the paw downward (arrow in C) and the other paw provides assistance while releasing the pellet (arrow in D).

Supination II and release of the food was frequently accomplished without assistance from the non-reaching paw (Figure 2.19B). Stroke mice typically used the non-reaching forepaw to grasp the reaching forepaw and assist in Supination II and Release (Figure 2.19C,D).

Discussion

This study examined the effect of motor cortex stroke on quantitative and qualitative measures of skilled reaching in mice. Following a focal motor cortex stroke, reaching success by the paw contralateral to the lesion was impaired and did not recover over two postsurgical weeks of testing. At this time many component movements used for reaching remained abnormal, especially the rotatory movements of the limb associated with aiming, pronating, and supinating the paw. When reaches were successful, they were achieved using compensatory body movements. This is the first study to describe qualitative impairments in skilled reaching in the mouse and the results suggest that this species provides a good model for analysis of motor skills, plasticity, and recovery processes.

The mice were trained and tested pre-and postsurgically in an apparatus in which they reached through a slot in a test box for single pieces of food located on a shelf just outside the slot. Prior to the lesion, the animals were able to orient to the slot, advance a paw through the slot, grasp and retrieve the food pellets for eating. Performance presurgically was reasonably accurate but following the lesion the mice were extremely impaired, consistent with findings in mice subjected to prefrontal damage on another motor task (Baird, *et al.*, 2001).

Prior to surgery, the mice used a distinctive pattern of movement when reaching for food. They located the food by sniffing for the food pellet, oriented their body, and then made a characteristic series of limb movements in order to retrieve a food pellet. The paw was lifted so that the slightly flexed digits were aligned with the tips to the midline of the body. The elbow was then adducted to the midline of the body so that the limb was

aimed to enter the slot. As the paw was advanced through the slot to the food the digits were extended, and then the paw was pronated while opening the digits to grasp the food. The paw was then supinated to withdraw the food through the slot, and then once through, was supinated further to place the food in the mouth.

Following the stroke reaching movements were altered, even when the animals successfully obtained food. The paw and elbow were not aligned to the aiming position along the midline of the body, but rather proceeded toward the food diagonally. Some aiming was obtained by an ipsiversive rotation of the fore portion of the trunk. Pronation was incomplete as the animals swiped at the food pellet with a sideways motion of the paw. Finally, the movements of supination during withdrawal and release were abnormal as the paw was incompletely supinated. Again, food retrieval was assisted by rotatory movements of the trunk and through the assistance of the non-reaching paw.

Some of the stroke animals' movements were not impaired. Stroke animals remained able to semiflex and extend the digits as well as grasp the food. Again, these findings are consistent with the impairments described for the rat following a focal motor cortex injury (Whishaw, 2000; Whishaw, *et al.*, 1992a; Whishaw, *et al.*, 1992b; Whishaw, *et al.*, 1991; Gonzalez, *et al.*, 1986).

These changes in movements post stroke suggest that the function of the motor cortex is in part to produce rotatory movements of the limb (Evarts, 1979). This conclusion is consistent with other findings of impaired limb use (Palmer, *et al.*, 2001; Nudo, *et al.*, 2001; Biernaskie and Corbett, 2001; Whishaw, 2000; Borlongan, 2000; Friel and Nudo, 1998) with recovery likely due to compensatory movements.

In conclusion, this is the first study to document a detailed qualitative impairment in skilled movements following a motor cortex stroke in mice. The main findings are that mice perform skilled reaching movements quite well and the skilled reaching test is sensitive to damage to the motor cortex. A strength of the model is that it is able to

provide a quantitative score and a qualitative assessment of deficits, compensation and recovery.

3. Experiment 2:

The effects of a new stroke model on mouse skilled reaching.

Abstract

Background and Purpose – Focal ischemia via permanent occlusion of the motor cortex vasculature results in massive motor impairments in the mouse, similar to the rat and comparable to what is seen in humans. There are many methods of modeling stroke in rodents and each is capable of producing different pathological outcomes that may correlate with behavioural outcome. The purpose of this study was to document skilled movements in mice subjected to a temporary reduction in blood flow in the motor cortex followed by tissue reperfusion.

Methods - Male C57/BL6 mice were trained in the single pellet reaching task to obtain seeds with their preferred forelimb. Following baseline training the mice received a focal motor cortex stroke, induced by topical application of the vasoconstrictor Endothelin-1, in the hemisphere contralateral to their reaching limb. Video recordings were made pre and post-surgically to examine the movements.

Results - Reaching success was not affected following the stroke, but an analysis of the ten movement components of the reach indicated that most of the rotatory movements of the limb used for aligning, aiming, and supinating the paw were abnormal. The histology showed no evidence of infarct or swelling although irregular cellular morphology directly below the endothelin application site was evident.

Conclusions - The results indicate that certain aspects of skilled reaching in the mouse are impaired by a temporary focal motor cortex stroke, in contrast to the large degree of impairment that is seen following a permanent focal motor cortex stroke.

Introduction

The popularity and availability of transgenic mice for stroke research (Hattori, *et al.*, 2001; Baird, *et al.*, 2001; Hunter, *et al.*, 2000; Sampei, *et al.*, 2000; Rogers, *et al.*, 1997) has created a demand for behavioural assessment in mice. A detailed analysis of laboratory mice (*Mus musculus*) indicates that they perform skilled reaching in a manner similar to laboratory rats (*Rattus norvegicus*) (Farr and Whishaw, 2002; Iwaniuk and Whishaw, 2000; Whishaw, 1996; Whishaw and Coles, 1996; Whishaw, *et al.*, 1992; Whishaw and Pellis, 1990). Mice also experience the same deficits following a permanent focal motor cortex stroke (Farr and Whishaw, 2002).

There are many methods of producing a stroke, each resulting in complex cellular and behavioural changes. Therefore, it is attractive to hypothesize that a different model of stroke can result in different behavioural impairments in the mouse. Endothelin-1 is a potent vasoconstrictor peptide (Yanagisawa, *et al.*, 1988) that is synthesized in endothelial cells (Russell and Davenport, 1999). It can be used to create a temporary reduction of blood flow in rats both globally via intracarotid injection (Willette, *et al.*, 1990), and focally via intracerebral injection (Sharkey, *et al.*, 1993; Macrae, *et al.*, 1993; Sharkey, *et al.*, 1992). MRI imaging confirmed a focal injection of endothelin adjacent to the MCA can reduce striatal and cortical blood flow by 30-50%, which persists for 7-16 hours respectively (Biernaskie, *et al.*, 2001). Other studies with a slightly larger dose of endothelin result in a 70-96% reduction in cerebral blood flow (CBF) with effects lasting up to four hours (Macrae, *et al.*, 1993).

There are three types of endothelin: ET-1, ET-2 and ET-3. Endothelin-1 is found primarily in vascular endothelial cells while 2 and 3 are located primarily in the kidneys, lungs and adrenal glands (Sakurai, *et al.*, 1992). There are two identified types of endothelin receptors, ET_A and ET_B (for a review see Sakurai, *et al.*, 1992). The receptors have different affinities for the endothelins. ET_B, located primarily in endothelial cells, binds the three endothelin subtypes equally. ET_B induces a vasodilatory response via the

release of endothelium derived relaxing factor (EDRF) from endothelial cells. ET_A binds ET-1 and 2 preferentially to ET-3 and results in vasoconstriction. The ET_A receptors are located primarily on smooth muscles, as well as vascular tissue, neurons and glia (MacCumber, *et al.*, 1990). Both receptors are coupled via a G protein to phospholipase C (PLC) (Kawai, *et al.*, 1997; Stanimirovic, *et al.*, 1994). Endothelin binding to the receptor releases the G protein, which in turn activates the membrane bound PLC. Activated PLC hydrolyses the major membrane component phosphatidylinositol 4,5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) (for a review see Berridge, 1996). DAG will bind to protein kinase C (PKC). PKC phosphorylates many proteins, some of which are responsible for the vasoconstriction (He, *et al.*, 2000). In addition to inducing a vasoconstrictive response ET-1 has been shown to increase intracellular Ca^{2+} (Koizumi, *et al.*, 1994) via the L Type Ca^{2+} channel that is activated by PKC phosphorylation (He, *et al.*, 2000). This elevated calcium may be linked to enhanced glutamate release that contributes to neurotoxicity (Shihara, *et al.*, 1998; Koizumi, *et al.*, 1994). Regardless, endothelin has the advantage of producing a temporary reduction in blood flow that results in reperfusion.

Motor impairments have been documented in mice subjected to a permanent focal motor cortex stroke produced by pial stripping. The present study investigated the effects of a temporary, perfusion-reperfusion focal motor cortex stroke, produced by topical application of ET-1, on skilled reaching in mice. The mice were trained to perform skilled reaching through a slot for food and their performance was video recorded before and after the focal motor cortex stroke.

Materials and Methods

Subjects

Sixteen four-month old male C57/BL6 mice (Charles River, Montreal, Canada) weighing between 20-30g were housed individually. The 12:12 hour light cycle began at

08:00 and room temperature was constant at 22°C. Testing was conducted at 1:00pm and the animals were given one piece of Lab Chow (4g) after the testing period each day. The experiment was conducted according to the Canadian Council on Animal Care code.

Surgery

After habituation the mice were given ten days of baseline training. On day eleven they were anaesthetized with isoflurane (BIMEDA-MTC Animal Health Inc.). Vetropolycin (Janssen) ointment was applied to the eyes to prevent drying. The mice received a stroke to the motor cortex contralateral to the preferred forelimb. Using stereotactic surgery the following area of skull was removed. From 0.5mm anterior to bregma to 2.5mm posterior to bregma, and from 0.5mm lateral to the midline to 2.5mm lateral to the midline. The dura was removed within the trephination and 5uL of 40pmol/uL Endothelin-1 (Calbiochem) was applied to the surface of the brain. Five minutes later any remaining endothelin was wicked away and the incision was closed. The mice were injected with a 0.2mg/kg dose of Metacam (Boehringer Ingelheim) to control post operative pain and inflammation. The animals were returned to their home cages and ten days of postsurgical testing began the following day.

Reaching Task

A Plexiglas reaching box (Farr and Whishaw, 2002) was modified to be 20cm long, 9cm wide, and 20cm high (Figure 3.11).

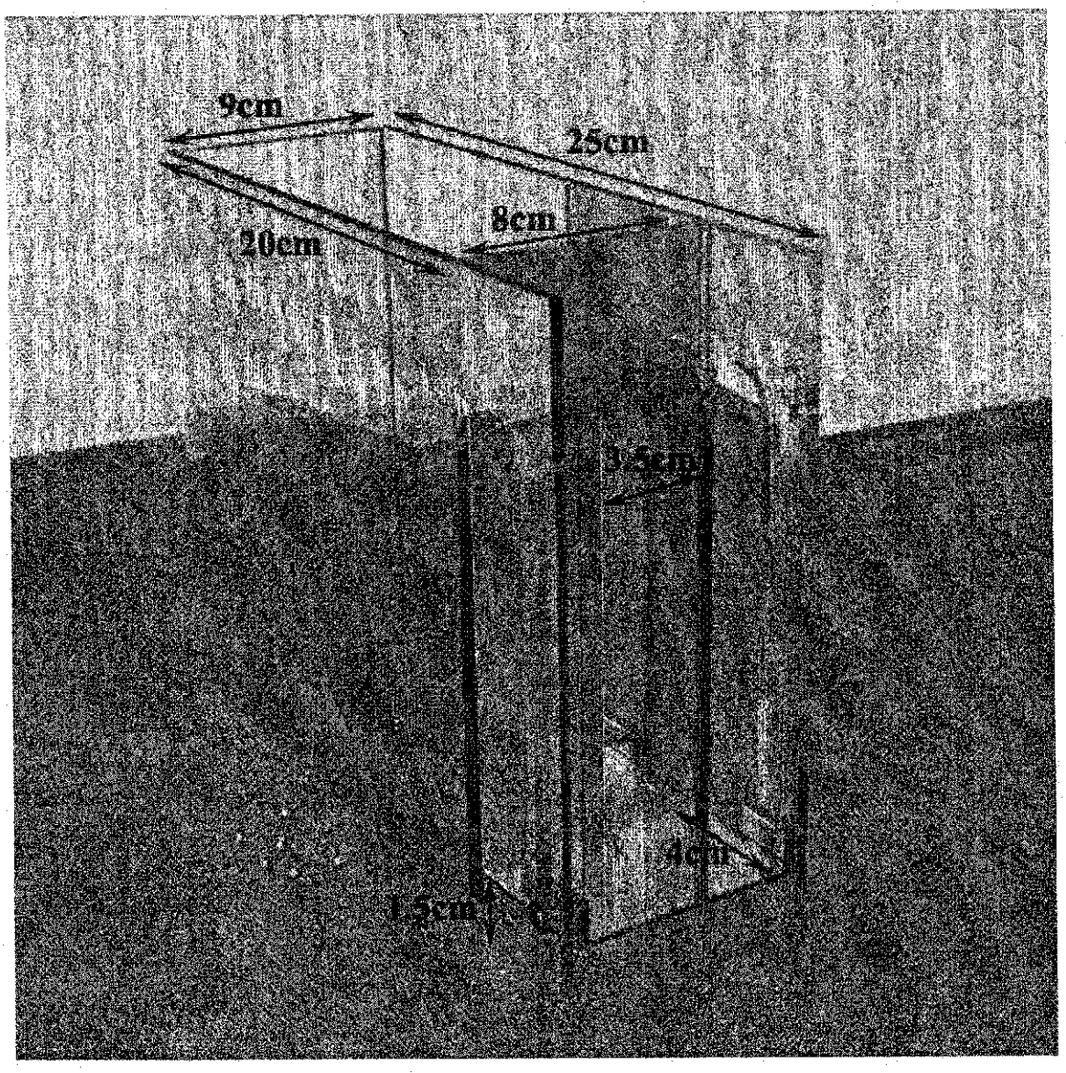

Figure. 3.11. The modified mouse reaching apparatus. The shelf in front of the box contains two indentations for food.

A 0.2cm thick plastic shelf (8cm long and 4cm wide) was mounted on the front of the box 1.5cm from the floor. Dehulled millet seeds, obtainable at any grocery store, were placed in indentations (depth < 1mm) spaced 1cm away from the slit and centered on its edges. A 1cm wide vertical slit ran up the front of the box to allow access to the food. Mice were habituated for one week by placing them in the cages for ten minutes each day. Seeds were initially available within tongue distance, and in both indentations. They were gradually moved away until the mice were forced to use their paws. The mice displayed the paw they preferred to use after which the food was placed in the indentation contralateral to the preferred paw.

Videorecording

Filming occurred on the last day of baseline training as well as two weeks post surgery for each animal. The camera was a Sony DSRPD100 digital camcorder (30 frames/sec; shutter speed of 1000). Illumination was provided with a 2 arm Nikon MKII 150W fiber optic light and a cold source Caselite (Lowel). The tapes were analyzed on a Sony DV cam DSR-20 player and Trinitron monitor.

Reaching Success

Only successful reaches were counted from a daily total of twenty seeds. If the mouse knocked the food away, dropped it, or required more than one attempt to obtain the food the reach was scored as a miss. Performance was defined by the formula:
Percent Success = (number of successful retrievals/ 20)*100

Qualitative Analysis

The ten movement components were evaluated using a variation of the Eshkol-Wachmann movement notation, as described previously. Each movement was rated on a 3-point scale. If the movement was normal, a score of 0 was given. In cases where there was some ambiguity concerning the occurrence of a movement, or the movement was

present but incomplete, a score of 1 was given. If the movement was absent, a score of 2 was given.

Histology

The mice were deeply anaesthetized and perfused through the heart with phosphate buffered 0.9% saline followed by 4% para-formaldehyde. Brains were cryo-protected in 30% sucrose/4% para-formaldehyde for three days and cut into 40μm sections on a 2800 Frigocut E cryostat (Reichert-Jung). The sections were mounted on 1% gelatin and 0.2% chromalin dipped slides, stained with Cresyl violet, and cover slipped using Permount (Fisher-Scientific).

Results

Histology

Figure 3.12 depicts representative photographs of stroke insults.

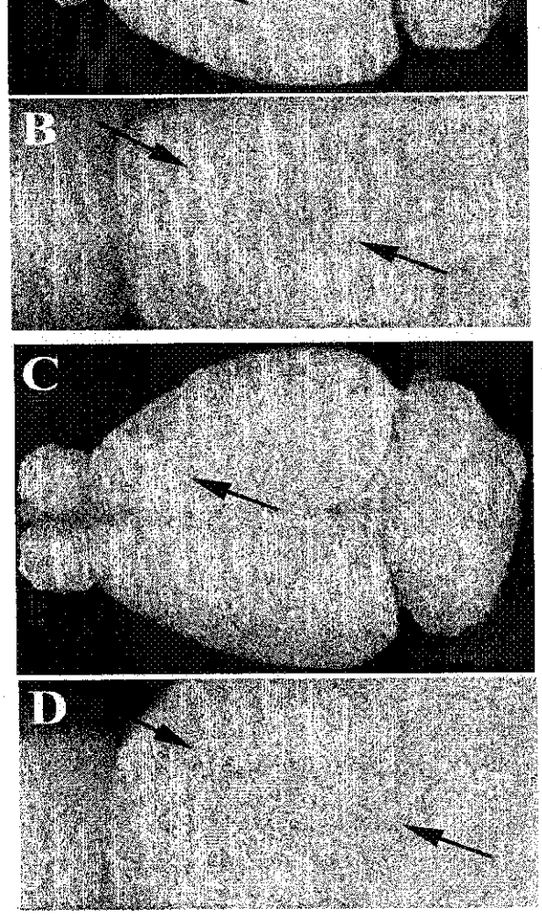

Figure 3.12. (A-D) Dorsal views of typical motor cortex strokes. (A-B) An example of the smallest stroke, the lesion site is clearly indicated in between the arrows in B. (C-D) An example of the largest stroke, the lesion site is indicated in D.

The visible damage on the surface of the cortex produced very small infarcts that were not easily visible in the sections. Figures 3.13 A and B depict representative sections through the lesion site. C and E depict cells within the intact hemisphere while D and F illustrate cells in the stroke hemisphere.

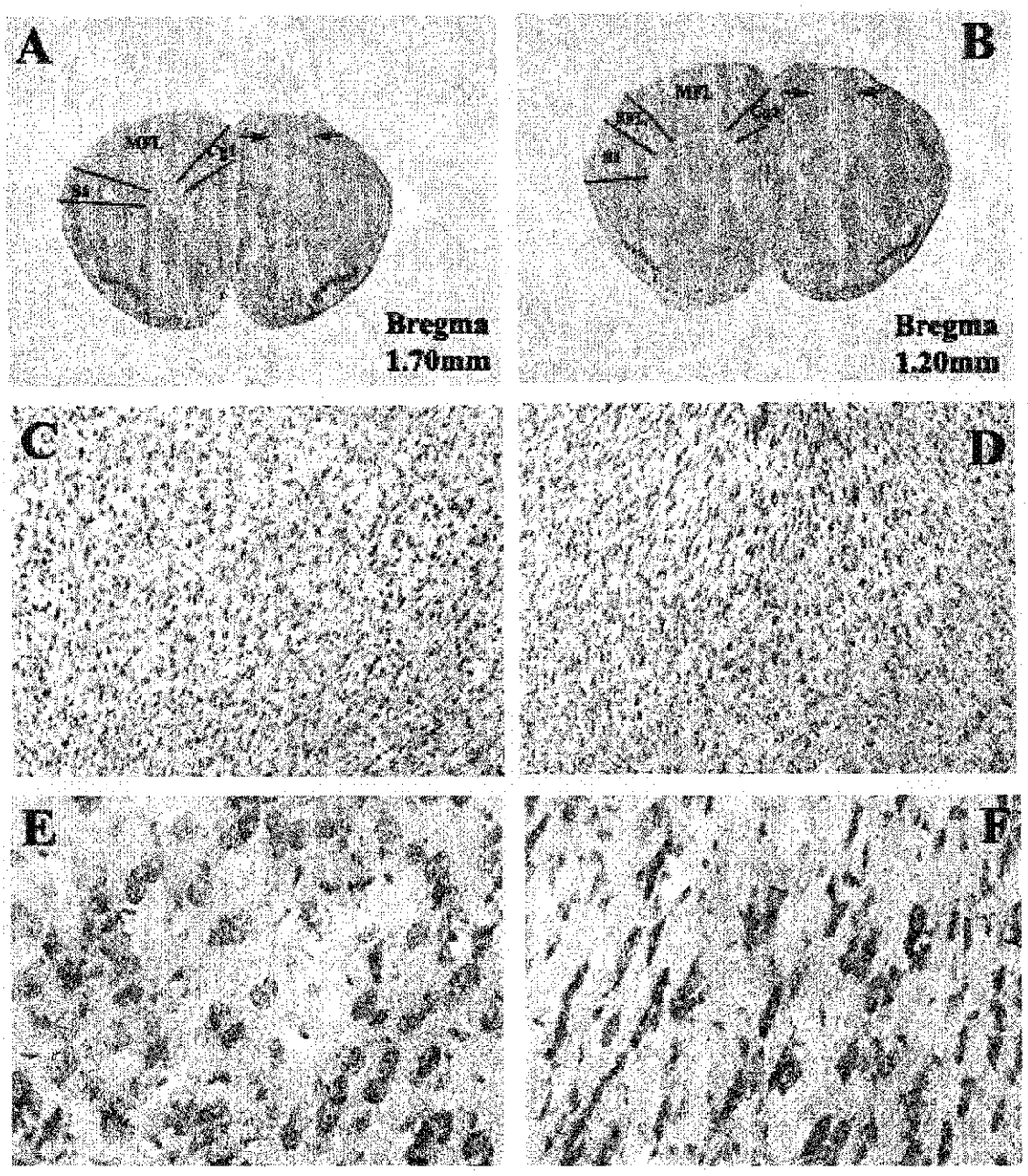

Figure 3.13. (A-B) Coronal photomicrographs of a representation of a motor cortex stroke through the lesion site (Cresyl violet). The intact hemisphere contains labeling for the appropriate areas: S1-sensorimotor cortex, SFL- sensoriforelimb area, MFL-Motor forelimb area and Cg1-cingulate cortex). The stroke site is indicated within the arrows. (C-D) Representations of the cellular morphology of the control (C) and stroke (D) motor cortex hemispheres at 100X magnification. (E-F) Representations of the cellular morphology of the control (E) and stroke (F) hemispheres at 400X magnification. Note: The abnormal cells present in the stroke hemisphere in the area immediately below the site of endothelin application.

Endothelin-1 did not produce a large infarct below the area of application. Instead there is a conical area below the lesion site with abnormal cell morphology. There are fewer cells and the ones that are present exhibit abnormal structure. The nucleus is not clearly visible and the cell staining is much darker, possibly indicating ruptured nuclei (Figure 3.13D,F). In addition, the tissue below the endothelin application did not swell. The damage appears to be restricted to the motor forelimb area.

Quantitative changes in reaching following stroke

Reaching Success

The percent success ANOVA, with days as a repeated measure, indicated that there was no treatment effect (Figure 3.14).

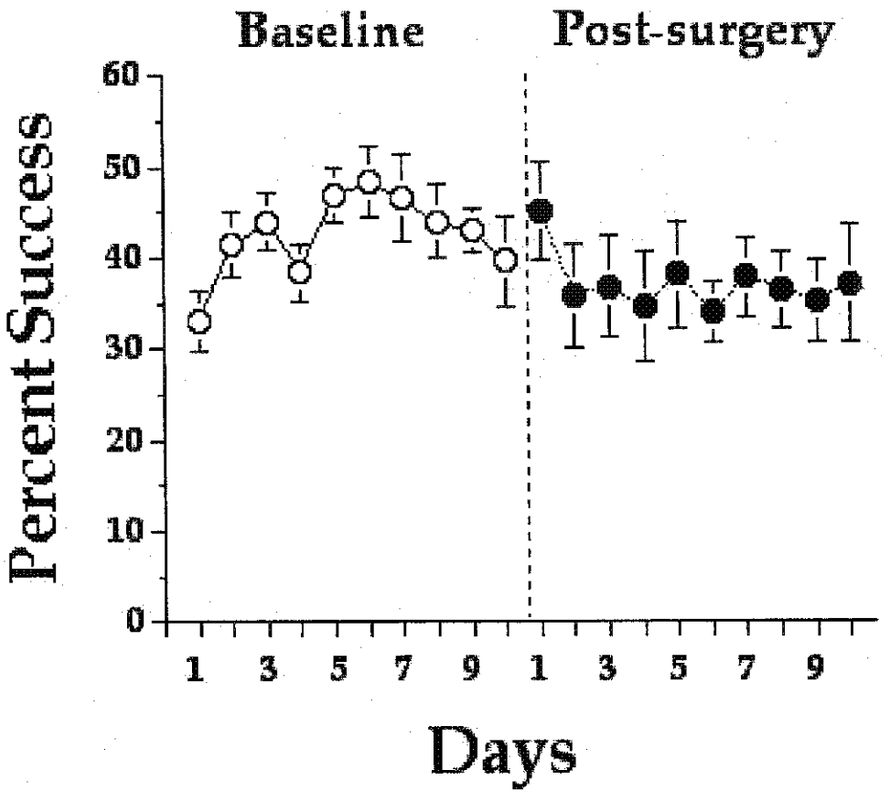

Figure 3.14. Reaching success (means ± SEM). The dotted line represents the administration of a motor cortex stroke.

The lack of statistical difference indicated that the animals' performance was not significantly affected by the stroke.

Movement Components

The qualitative ANOVA of five presurgical and postsurgical reaches, evaluated for the 10 different movement components, revealed a significant main effect of Group, $F(1,29)= 17.883$, $P < 0.001$. This indicated that the mice accumulated higher scores for the ten movement components following the stroke. There was also a main effect of Movement, $F(9, 261)=46.787$, $P < 0.001$, indicating that some movements were more affected than others. Finally, there was a significant Group by Movement interaction for the stroke group, $F(9, 261)= 8.739$, $P < 0.001$. This indicated that some movements in the stroke group were more impaired than the baseline components. Follow-up students unpaired t-tests were performed and indicated significant group differences for the digits to the midline, aim, and supination II components of the reach, but not for the digits semiflexed, advance, digits extend, pronation, grasp, supination I and release (Figure 3.15).

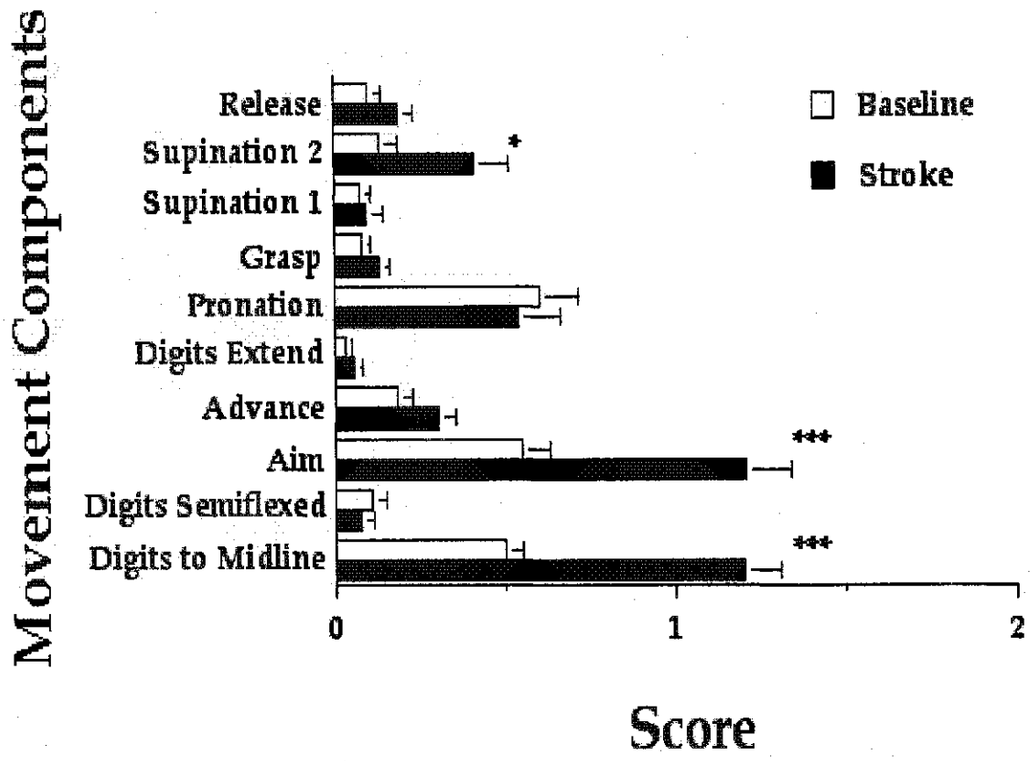

Figure 3.15. Scores for each of the ten movement components of the reach (means ± SEM). Clear bars indicate baseline performance and solid bars indicate post surgical performance. A score of 0 indicates a normal movement while a score of 2 indicates complete absence of the movement. (*** $P < 0.001$)(** $P < 0.01$)(* $P < 0.05$).

Qualitative changes in movement following stroke

The following descriptions were all taken from successful reaches.

Digits to the Midline and Aim

During baseline testing, mice lifted the semiflexed digits of the reaching paw and quickly aligned them with the midline of the body (Figure 3.16A).

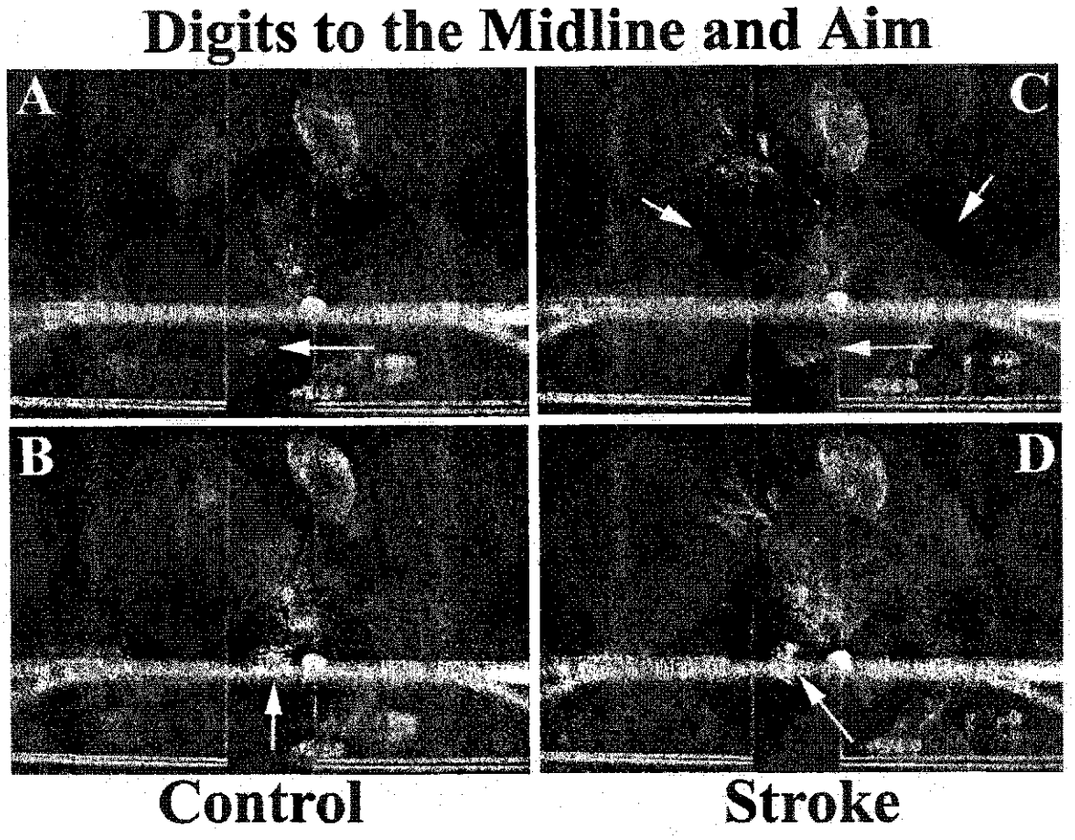

Figure 3.16. Digits to Midline and Aim. A, B: Baseline. During presurgical testing the mouse lifted the semiflexed digits to align them with the bodies midline (arrow in A). The limb was then aimed by bringing the elbow to the midline, this is visible by the position of the digits (arrow in B) forcing the elbow to be beneath them in the same axis as the paw. C, D: Stroke. The same mouse following stroke surgery shifted the shoulders to align the digits with the midline (arrows in C) and the elbow is not brought into the aim, indicated by the misplaced, diagonal paw and additional shoulder shifting.

The elbow was then aligned bringing the reaching limb into an "aiming" position (Figure 3.16B). Following surgery the mice lifted the limb and semiflexed the digits to a lesser extent (Figure 3.16C). The animals shifted their shoulders to bring the elbow into an aiming position, indicated by the position of the paw and angle of the trunk (Figure 3.16D).

Advance and Pronation

Prior to surgery the mice were able to advance the paw and extend the digits directly over the food item (Figure 3.17A) and begin pronation (Figure 3.17B).

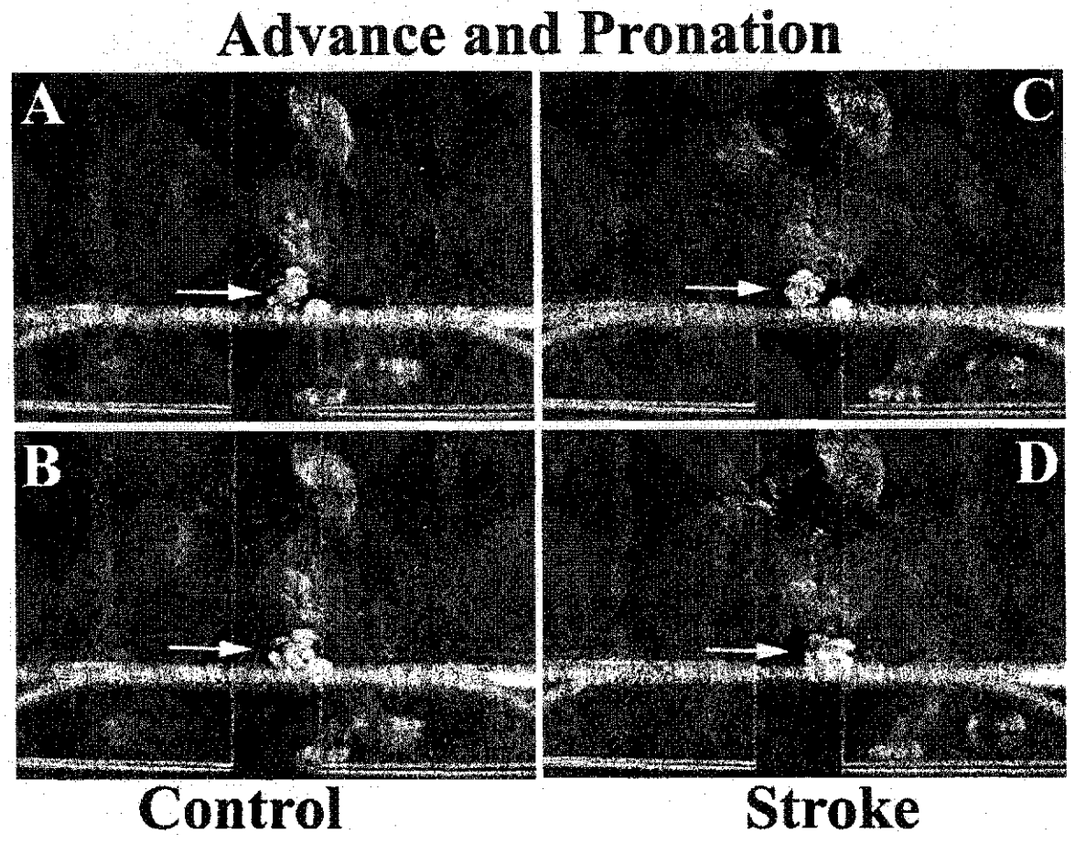

Figure 3.17. Advance and Pronation. A, B: Baseline. The baseline animals advanced the reaching forelimb directly over the food pellet and extend the digits (A) while beginning to rotate the paw over the food pellet (arrow in B). C, D: Stroke. After surgery the mice were still able to advance and extend the digits (arrow in C), though to a lesser extent, but can not fully pronate indicated by the paw (D) coming straight down, rather than rotating over.

Post surgery the mice were still somewhat able to advance and opened the digits (Figure 3.17C) but slapped down rather than rotating over the seed (Figure 3.17D).

Grasp and Supination I

Once the food was contacted, baseline mice closed the digits around the pellet (Figure 3.18A) and supinated the closed paw in order to begin withdrawl (Figure 3.18B).

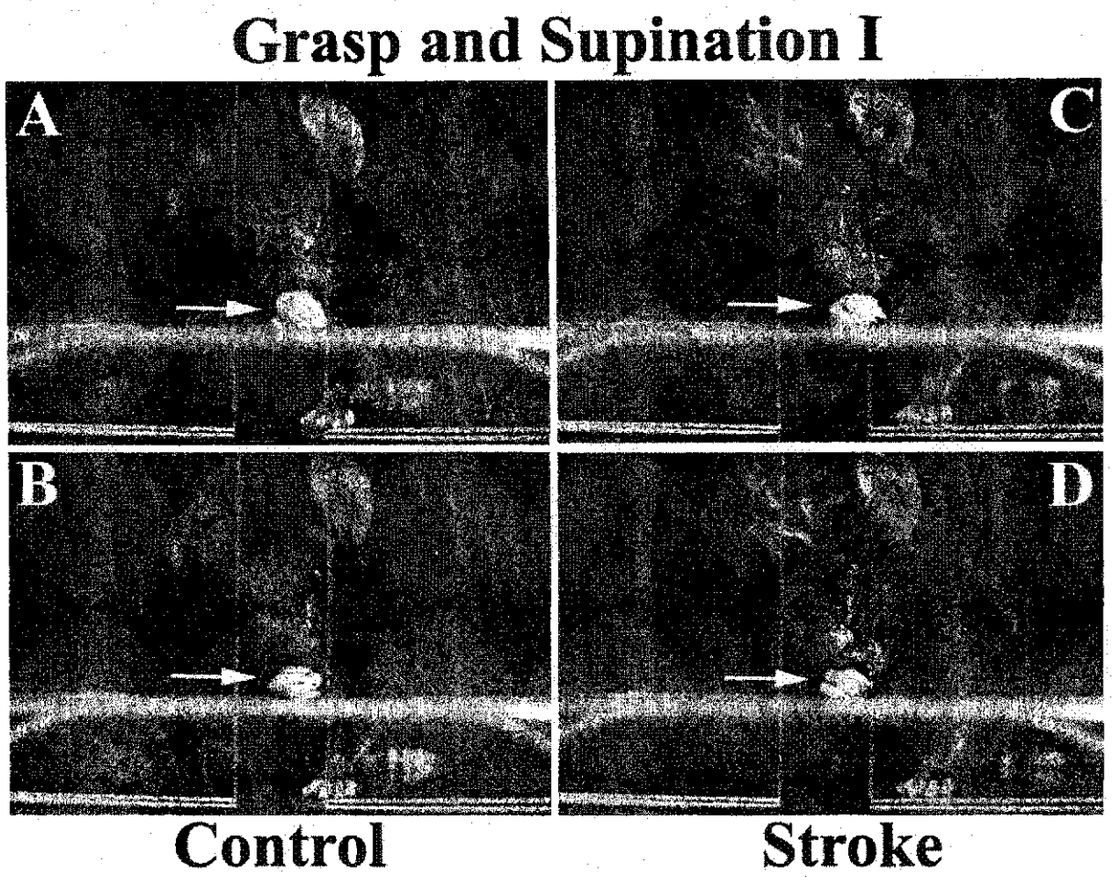

Figure 3.18. Grasp and Supination I. A, B: Baseline. The presurgical animal can close the digits around the food item (A) and supinate the paw during withdrawl (arrow in B). C, D: Stroke: The post surgical animal is not able to fully close the digits (C) and the degree of supination is less than that of the control, using a dragging motion to obtain the food (arrow in D).

Stroke animals displayed less grasping when compared to the their baseline scores (Figure3.18C) and food retrieval was begun by dragging the item backward (Figure 3.18D). In addition, the stroke animals would continue body rotation in order to rotate the limb.

Supination II and Release

Once the food was withdrawn, the baseline mice further supinated the paw to present the food to the mouth (Figure 3.19A).

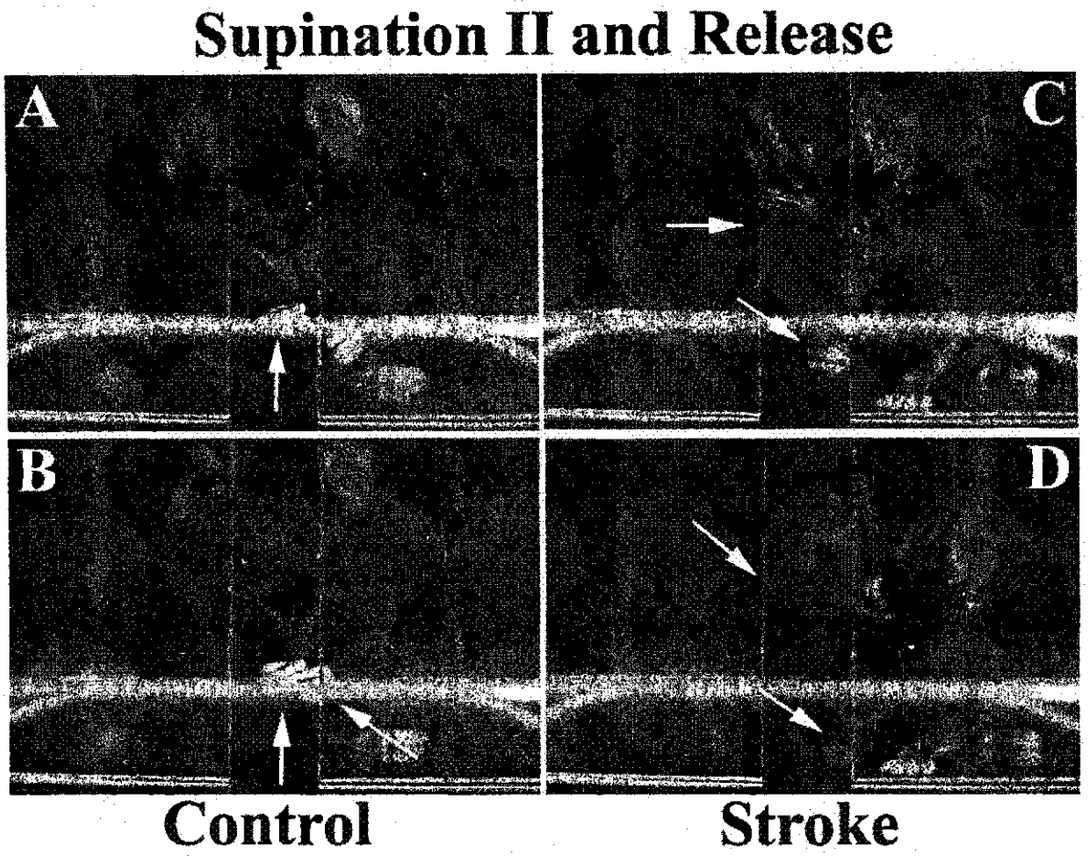

Figure 3.19. Supination II and Release. A, B: Baseline. A presurgical animal supinated the paw to present the food to the mouth (arrow in A), opened the digits to release it, and brought the non-reaching limb up to aid in food manipulation (arrows in B). C, D: Stroke. A post surgical animal chased the paw downward, unable to complete the second supination (arrow in C) and forced the paw to the ground to release the food (D).

Supination II and release of the food was frequently accomplished without assistance from the non-reaching paw (Figure 3.19B). Following surgery the mice typically exhibited difficulty with the second supination and chased the paw down to force release with the help of the floor (Figure 3.19 C,D).

Discussion

This study documented skilled reaching in mice subjected to a focal motor cortex stroke produced by a temporary occlusion of the cortical blood vessels. Surprisingly, the stroke did not produce a quantitative deficit in reaching success, though a qualitative analysis revealed the mice exhibited difficulty with some of the movements in the reach. The findings are particularly important especially when considering the method employed to produce the insult. This is the first study to describe a subtle lesion that has no effect on the quantitative performance but effects the qualitative performance in mice.

The mice were trained to perform skilled movements by reaching through a slot to obtain single seeds rewards. Following the focal motor cortex stroke the mice were still able to retrieve a comparable number of seeds. This finding contrasts what is normally seen in rats and mice subjected to a permanent focal motor cortex stroke. Animals with such a lesion suffer a large quantitative impairment that improves with training but does not completely return to baseline level (Whishaw, 1996; Whishaw, 2000; Whishaw, *et al.*, 1992; Castro, 1972; McKenna, *et al.*, 1999; Modo, *et al.*, 2000; Petullo, *et al.*, 1999; Whishaw, *et al.*, 1992; Whishaw, *et al.*, 1991).

The stroke insult caused the mice to exhibit abnormalities in the different components used during a reach. Specifically, the mice had difficulty aligning the digit tips to the body midline and subsequently with bringing the elbow into midline position. The mice were able to partially achieve the aim by shifting the shoulders and rotating the body. Due to the misplaced aim the paw advanced diagonally through the slot and often minor adjustments were required to get the paw over the food. The animals also had difficulty with the second supination that requires another rotation of the paw to present

the food to the mouth. Rotatory movements of the trunk assisted food retrieval. The stroke mice also exhibited subtle impairments in the ability to extend the digits and grasp. These findings are consistent with the impairments described for the rat following a focal motor cortex injury (Whishaw, 1996; Whishaw, 2000; Whishaw, *et al.*, 1992; Castro, 1972; McKenna, *et al.*, 1999; Whishaw, *et al.*, 1992; Whishaw, *et al.*, 1991).

These findings suggest that the small damage produced by the endothelin application may only produce subtle impairments with rotatory movements of the limb. This impairment may contribute only to deficits in qualitative assessments; quantitatively the mice are able to compensate quickly. It is possible that a stronger concentration of endothelin would result in increased damage that may mimic what is commonly seen in permanent focal stroke models.

In conclusion, this is the first study to document the impairments in skilled reaching in mice subjected to a temporary perfusion-reperfusion stroke. The main findings are that mice exhibit qualitative impairments but not quantitative impairments following the endothelin produced lesion. The method of producing the stroke may be influential on the types of impairments exhibited.

4. Experiment 3:

The effects of a permanent and temporary occlusion stroke on skilled reaching in the mouse.

Abstract

Background and Purpose - Stroke can be modeled in rodents using a variety of methods and each is capable of producing different histological damage. The previous experiments revealed that permanent removal of the cortical vasculature in mice results in massive quantitative and qualitative impairment whereas a temporary constriction of the same vessels results in only a subtle qualitative impairment. Thus, the purpose of this study was to compare the effects of the two models of stroke on skilled movements and histological outcome in the mouse.

Methods - Male C57/BL6 mice were trained to reach for seeds with their preferred limb. They were subdivided into three groups. One group remained a control. The other two groups received a focal motor cortex stroke in the hemisphere contralateral to their preferred limb, either via a pial strip or the topical application of Endothelin-1. Reaching success and the movements used in reaching were analyzed by pre and post-surgical video records.

Results - Reaching success indicated that the pial strip animals were significantly affected by the stroke, whereas the endothelin animals were not. An analysis of the ten movement components of the reach indicated that both groups were impaired with many of the same rotatory movements but the pial strip animals experienced a greater degree of impairment. A histological comparison indicated that the pial strip strokes were significantly larger than the endothelin strokes and the degree of impairment for certain movements an animal exhibited was positively correlated with the size of the lesion.

Conclusions - The results indicate that certain aspects of skilled reaching in the mouse are impaired following focal motor cortex stroke and that larger permanent occlusion lesions produce a greater degree of impairment. This may have implications for the choice of a stroke model.

Introduction

There are many methods of modeling stroke in rodents and the resulting pathology from each can be very different. Most focal motor cortex models result in striatal and cortical damage and animals exhibit a behavioural deficit in the contralateral limb. The previous experiments have indicated that variations in stroke methodology produce different histological and behavioural outcomes. The purpose of this study is to compare the permanent pial strip to the temporary endothelin application.

The first experiment disrupted skilled reaching movements in the mice by using a permanent occlusion model of focal motor cortex stroke. The vasculature over the motor cortex was removed, eliminating the possibility of tissue reperfusion. Histological analysis revealed that the tissue surrounding the motor cortex swelled up into the area of the infarct. Behavioural analysis revealed that the mice were impaired quantitatively in the ability to obtain the food and qualitatively, meaning the movements used to obtain the food were altered. This is consistent with what is seen in rats (Whishaw, 1996; Whishaw, 2000; Whishaw, *et al.*, 1992; Castro, 1972; McKenna, *et al.*, 1999; Whishaw, *et al.*, 1992; Whishaw, *et al.*, 1991). The second experiment made use of a temporary model of focal motor cortex stroke. A potent vasoconstrictor, ET-1 (Yanagisawa, *et al.*, 1988), was applied to the surface of the motor cortex to reduce blood flow for a period of time (Biernaskie, *et al.*, 2001; Sharkey, et al., 1993; Macrae, *et al.*, 1993; Sharkey, *et al.*, 1992; Willette, *et al.*, 1990). Histological analysis of the ET-1 tissue revealed that there was a conical area of abnormal cellular morphology directly beneath the application site, though there was no visible infarct. Behavioural analysis indicated that the mice were not impaired quantitatively, though they did exhibit abnormal movements in order to retrieve the food.

It is possible that the extent of tissue damage is directly correlated with the degree of impairment. The purpose of this experiment was to increase the dose of the Endothelin-1 application and compare it's effects to that of the permanent pial strip.

Materials and Methods

Subjects

Nine four-month old male C57/BL6 mice (Charles River, Montreal, Canada) weighing between 20-30g were housed individually in a 22°C room where the lights were on a 12:12 hour cycle beginning at 08:00. Testing was conducted at 9:00am and the animals were given one piece of Lab Chow (4g) after the testing period each day. The experiment was conducted according to the Canadian Council on Animal Care code.

Surgery

The mice were habituated to the testers and testing apparatus for three days and tested for ten baseline days prior to surgery. Six mice were anaesthetized with isoflurane (BIMEDA-MTC Animal Health Inc.), and Vetropolycin gel (Janssen) was applied to the eyes to prevent drying. The skull over the motor cortex was removed, from 0.5mm anterior to bregma to 2.5mm posterior to bregma, and from 0.5mm lateral to the midline to 2.5mm lateral to the midline. The dura was removed within the trephination. Three mice, all with right paw dexterity, received a pial strip to the motor cortex contralateral to the preferred forelimb, essentially the pial vasculature was wiped away with a sterilized cotton swab. The other three mice, all left paw preference, received a 5 minute topical endothelin application of 5uL of 80pmol/uL Endothelin-1 (Calbiochem), the pial vasculature was left intact. After the incision was closed the mice received a 0.2mg/kg dose of Metacam (Boehringer Ingelheim) to aid with post operative pain and inflammation. The post surgical testing began the following day.

Reaching Task

The same Plexiglas reaching box described in the previous experiment was utilized. Briefly, the mice reached through a 1cm vertical slit to obtain millet seeds that were placed in divots 1cm away from the front of the box. Animals were habituated for three days as seeds were gradually moved further away until the mice indicated their preferred paw.

Videorecording

Filming occurred on the tenth day of baseline training as well as the tenth day of post surgical training. The filming set up from the previous experiment was duplicated. Briefly, the camera was a Sony DSRPD100 digital (30 frames/sec; shutter speed of 1000), the lights were a 2 arm Nikon MKII 150W fiber optic, and a cold source Caselite (Lowel). The tapes were analyzed on a Sony DV cam DSR-20 player and Trinitron monitor.

Reaching Success

Daily scores for percent success were calculated from twenty seeds with the following formula:
Percent Success = (number of successful retrievals/ 20)*100

Qualitative Analysis

The ten movement components were evaluated using a variation of the Eshkol-Wachmann movement notation, as described previously. Briefly, a score of 0 indicated normal whereas a score of 2 indicated complete movement absence.

Histology

The mice were deeply anaesthetized and perfused through the heart with phosphate buffered 0.9% saline followed by 4% para-formaldehyde. Brains were photographed with a Kodak DCS 410 digital camera, cryo-protected in 30% sucrose/4% para-formaldehyde solution and sectioned (40μm) on a 2800 Frigocut E cryostat (Reichert-Jung). The sections were mounted on 1% gelatin and 0.2% chromalin dipped slides, stained with Cresyl violet, and cover slipped using Permount (Fisher-Scientific). Four sections from each animal were digitized with a Polaroid DMC-3 live feed digital camera and analyzed using NIH image software (Bethesda, MD., U.S.A.). Lesion volumes were estimated by measuring the stroke area in each of the four sections, and multiplying it by the area between each of the sections.

Results

Histology

All of the brains had damage in the motor cortex (Figure 4.11). The first two panels (A-F) represent a pial strip stroke and the second two panels (G-L) represent an endothelin induced stroke.

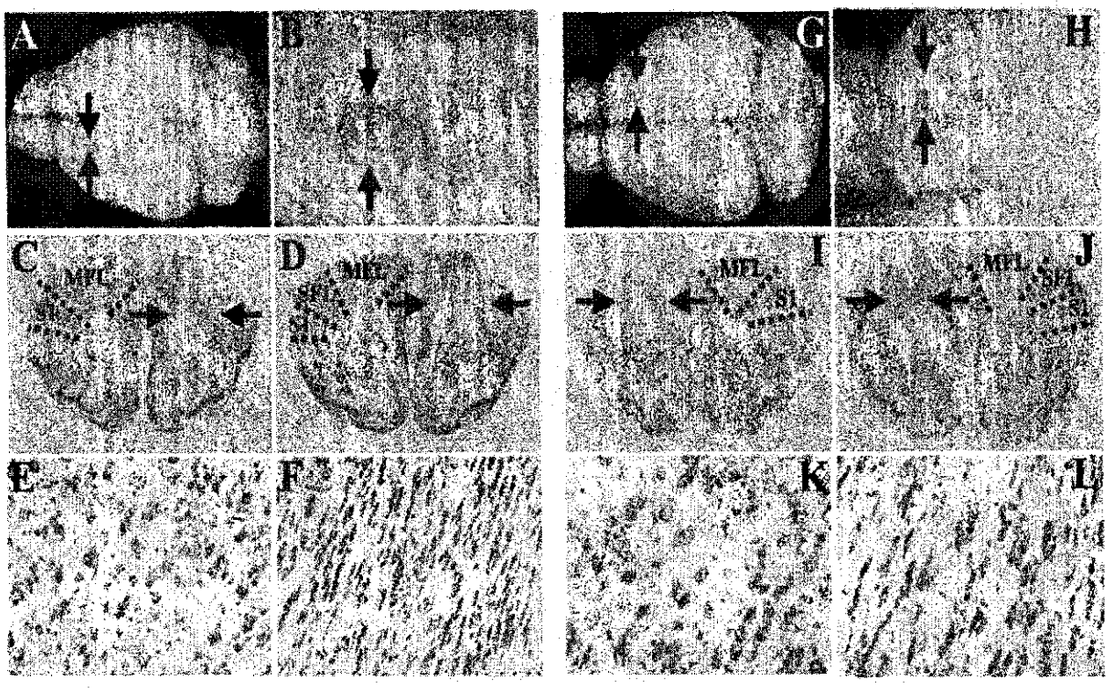

Figure 4.11. Pial strip. (A-B) Photomicrographs of a representative pial strip stroke, the infarct is indicated between the arrows. (C-D) Representative coronal sections through the stroke site (Cresyl violet). The intact hemisphere contains labeling for the appropriate areas: S1-sensorimotor cortex, SFL- sensoriforelimb area, MFL-Motor forelimb area. (E-F) Representations of the cellular morphology in the motor cortex of the control (E) and stroke (F) hemispheres at 400X magnification. Endothelin. (G-H) Photomicrographs of a representative endothelin stroke. (I-J) Representative coronal sections through the stroke site (Cresyl violet). (K-L) Representations of the cellular morphology in the motor cortex of the control (K) and stroke (L) hemispheres at 400X magnification. Note: the swelling of the stroke site in the pial strip sections (C-D) and the presence of abnormal cells in the stroke hemispheres of both stroke models (F,L).

The pial strip stroke produced a noticeable infarct (Figure 4.11A-B) and tissue swelling around the infarct site (Figure 4.11C-D). Detailed examination of the motor cortex cells indicated that the tissue in the intact hemisphere is normal, whereas the cells in the stroke hemisphere exhibit abnormal morphology. Abnormal cellular morphology includes cells with abnormal shape, and darker colors, as well as a reduction in the number of cells in general. Endothelin-1 did not produce a large infarct below the area of application. When superficially studying the sections it is often difficult to determine which hemisphere suffered the stroke (Figure 4.11I-J). Cellular examination reveals that there is a conical area below the endothelin application site with abnormal cell morphology (Figure 4.11L). Lesion volumes for each group are indicated in Table 4.11.

Table 4.11. The stroke volumes for each mouse in millimeters cubed. Average± SEM is indicated for each group.

Group	Stroke Volume (mm^3)
Pial Strip	
Mouse 1	10.03
Mouse 2	10.73
Mouse 3	12.36
Average	11.04± 0.69
Endothelin	
Mouse 4	5.42
Mouse 6	6.02
Mouse 9	7.53
Average	6.32± 0.63

The pial strip animals' average lesion volume was almost double the size of the endothelin animals. An unpaired students t-test revealed a t statistic of 5.056 and a corresponding probability of 0.0072, indicating that this difference in size is not simply due to chance.

Quantitative changes in reaching following stroke

Reaching Success

A repeated measures ANOVA for the last five pre-surgical days and the first five postsurgical days indicated that there was a significant effect of treatment ($F(1,6)= 11.172$, $P < 0.05$) and a significant group effect ($F(2,6)= 6.137$, $P < 0.05$) (Figure 4.12).

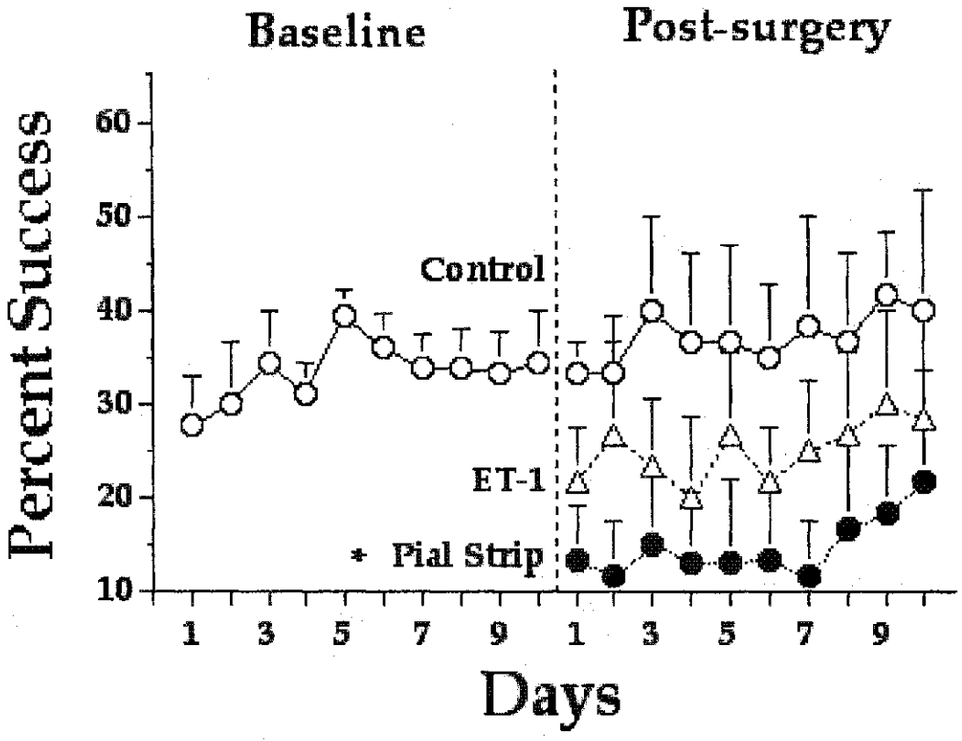

Figure 4.12. Reaching success (means ± SEM) of the three groups: Control, ET-1 and Pial Strip. The dotted line represents the administration of a focal motor cortex stroke. (* $P < 0.05$).

The first five post surgical days of the mice in the pial strip group were outside of the confidence interval when pairwise compared to the final five days of the control baseline performance. This indicates that the pial strip group's performance was statistically different from that of the controls during this time period. The pial strip mice had begun recovery by the eighth post surgical day and there is no statistical difference from the control group at this time. There was no statistical difference in performance between the endothelin and control groups, though the endothelin animals scored slightly lower than the controls following the stroke.

Movement Components

Five pre and post surgical reaches from each animal were used to provide one score for each of the ten movement components. There were no significant differences between any of the groups prior to surgery. The post surgical scores for the stroke treated groups and average for the control group are plotted in Figure 4.13.

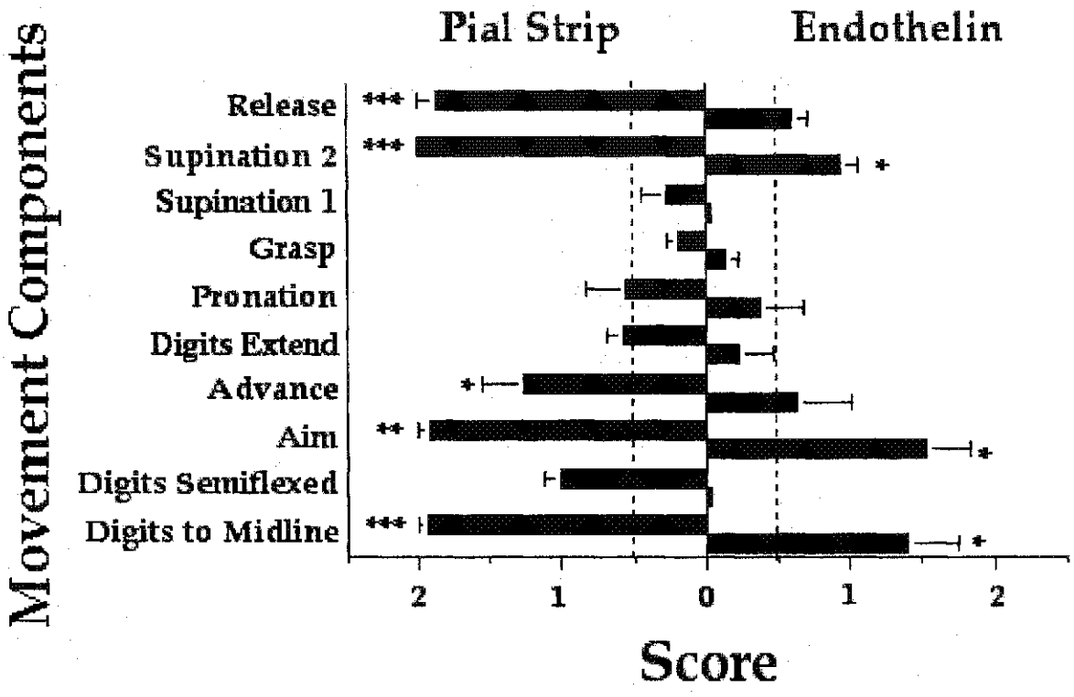

Figure 4.13. Scores for each of the ten movement components of the reach (means ± SEM) for the two stroke groups, Pial Strip and Endothelin-1. A score of 0 indicates a normal movement while a score of 2 indicates complete absence of the movement. The dotted lines represent an average score for all of the ten movement components in the control group. (*** $P < 0.001$)(** $P < 0.01$)(* $P < 0.05$).

A repeated measures ANOVA evaluated for the 10 different movement components post surgery, revealed a significant main effect of Group, $F(2,9)= 20.295$, $P < 0.001$. This indicates that the stroke mice accumulated significantly higher scores than the control group. There was also an effect of Movement, $F(9, 18)=24.126$, $P < 0.001$, indicating that some movements were more affected than others. Finally, there was a significant Group by Movement interaction $F(18, 81)= 4.388$, $P < 0.001$, indicating that there were differences between groups for certain movements. Follow-up students unpaired t-tests indicated that the pial strip group was significantly impaired when compared to the controls for digits to the midline, aim, advance, supination II, and release components of the reach. The Endothelin mice were only impaired at digits to the midline, aim and supination II.

Qualitative changes in movement following stroke

Both stroke groups exhibited difficulties aligning the digit tips and elbow with the bodies midline, as well as the second supination. In addition, the pial strip mice experienced difficulties with the advance and release. Because the pial strip animals exhibited a larger lesion volume than the endothelin group (Table 4.11) it is attractive to hypothesize that a larger degree of damage will impair a larger variety of movements. A correlation analysis between lesion volume and degree of impairment was performed for each of the ten movement components. A positive correlation was found for some of the movement components, meaning the larger the lesion volume the greater the impairment. Table 4.12 reports the corresponding r statistic and probability value for the 10 different movement components.

Table 4.12. The r correlation statistic value and corresponding probability for the Fisher's r to z test for the ten different movement components of the reach versus lesion volume.

Movement Components	r value	P value
Digits to the Midline	0.758	0.085
Digits Semiflexed	0.9	0.011
Aim	0.625	0.204
Advance	0.654	0.175
Digits Extend	0.620	0.210
Pronation	0.379	0.490
Grasp	0.329	0.554
Supination I	0.424	0.432
Supination II	0.959	0.0008
Release	0.978	0.0001

An r statistic of greater than 0.5 but less than 0.8 is indicative of a moderate positive correlation while a value greater than 0.8 indicates a strong correlation. Thus, digits to the midline, aim, advance, and digits extend exhibit a moderate correlation with lesion volume. Digits semiflexed, supination II and release exhibit a strong correlation with lesion volume. The corresponding probabilities indicate that only digits semiflexed, supination II, and release were statistically correlated. Stroke animals with a larger lesion did receive larger scores on most of the ten components. The two mice with the largest lesion volume also exhibited impairments with digits semiflexed, advance and digits extend.

Discussion

This study compared the effects of two different models of focal motor cortex stroke. One was a permanent removal of the pial vasculature (pial strip) and the other a temporary constriction of the same vessels (Endothelin-1 application). The results indicate that the pial strip has drastic affects on the ability of the mice to obtain food, as well as impairing the way in which they obtain the food during skilled reaching. The endothelin model of stroke did not significantly impair the mice quantitatively, though the animals did exhibit some abnormal movements in the qualitative analysis. Both types of stroke produce impairments in the same rotatory types of movements such as: digits to the midline, aiming, and supinating the paw. This indicates that a stroke to the motor cortex always results in certain movement abnormalities. Because mice with a larger lesion volume, such as the pial strip group, exhibited a larger degree of impairment, it is attractive to hypothesize that the differences in impairments are a result of the extent of the injury. The findings are particularly relevant when considering the method employed to produce the insult.

There are three main differences in the stroke models that could account for the differences in behavioural outcome. The first is that the pial strip permanently removes

the pia vasculature, irreversibly impairing blood flow in the motor cortex. Endothelin-1 is a vasoconstrictor that will eventually result in reperfusion of the intact motor cortex vessels. The second is that the removal of the pia disrupts the meninges of the brain during the pial strip. This membrane is left intact during a topical endothelin application. The third is that the histology indicated the pial strip caused the tissue surrounding the stroke to swell and fill in the infarct, whereas there was little displacement of the tissue in the hemisphere of the stroke after the endothelin insult. It is likely these differences contribute to the complexities observed in the behaviour.

The mice in both groups exhibited abnormalities in some of the same components used during a reach. Following the stroke all animals had difficulty with the initial alignment of the digits and the aim, they used body rotation to achieve these goals. All animals were also unable to perform the second supination often forcing the limb down to the ground to present the food to the mouth. All of these findings are consistent with the impairments from a focal motor cortex stroke described in the rat (Whishaw, 1996; Whishaw, 2000; Whishaw, *et al.*, 1992; Castro, 1972; McKenna, *et al.*, 1999; Whishaw, *et al.*, 1992; Whishaw, *et al.*, 1991). This suggests that the motor cortex has substantial contribution to the rotatory movements involved in a reach. In addition, the pial strip group exhibited lesion volumes that were significantly larger than that of the endothelin group. In addition, the pial strip animals experienced difficulties with other movements such as the advance and release. In extreme cases the mice with the largest pial strip lesion also exhibited subtle impairments in the ability to flex and extend the digits. This could mean that larger injuries can result in a greater degree of impairment. This is supported by the fact that there were moderate to strong positive correlations between lesion volume and degree of impairment for some of the ten movement components such as: digits to the midline, digits semiflexed, aim, advance, digits extend, supination II and release.

Interestingly, the larger dose of endothelin used in this experiment did produce a slightly lower quantitative percent success score in the stroke animals when compared to the controls, though it was not statistically different. The fact that endothelin has the

ability to make subtle lesions that affect only certain types of movements and do not affect reaching performance makes it an attractive model because it can be titrated to produce strokes of various sizes. It may be possible to provide an application of endothelin strong enough to mimic what is seen in the pial strip animals.

In conclusion, this is the first study to document the impairments in skilled reaching in mice subjected to a permanent and temporary focal motor cortex stroke. The main finding is that the permanent model results in a larger degree of behavioural impairment and histological damage than the temporary model.

5. Discussion

5.1. Outline of the discussion.

This thesis described skilled motor movements in mice and the alterations in these movements following both a permanent removal, and a temporary occlusion of the blood vessels in the motor cortex. The conclusions were that mice perform skilled motor movements and that these movements are impaired following a stroke to the motor cortex. Different stroke models can result in different types of behavioural impairments, and the degree of impairment is correlated with the size of the insult.

This thesis encourages the use of mice as an animal model for stroke research because of the current availability of transgenic mouse technology. Transgenic mouse technology can help evaluate the contribution of genetics to brain injury, specifically stroke. The discussion is therefore not intended to provide a summary of the results of the experiments but rather offer a rationale for, as well as a review of, the use of mice as an animal model for stroke research. The discussion is composed of five sections. The first section reviews the definition and description of a focal stroke. It will emphasize the most widely used method of modeling focal stroke in rodents, the MCAO. The second section will describe the current methods of modeling MCAO stroke in mice and some considerations that should be accounted for when choosing a mouse model. The third section describes the creation of transgenic mice, which will lead into a discussion of the contributions of transgenic technology to stroke research in the fourth section. The final section will summarize the thesis.

5.2. A review of stroke.

Stroke is a cardiovascular disease that occurs when there is an obstruction in the blood flow of the brain. Most strokes occur when a blood clot or atherosclerotic plaque forms in the brain, or breaks off from a buildup in the body, and travels through the blood

stream to lodge itself in a brain vessel. Once the supply of oxygenated blood is reduced the tissue that is irrigated by the blocked vessel often dies or suffers injury that can lead to death. The most commonly occluded vessel in human stroke patients is the MCA, which services a large portion of the forebrain.

An MCA stroke can result in a variety of clinical disabilities for a patient (for a review see Slater and Johns, 2003). The loss of motor function is one of the most frustrating impairments a stroke patient can suffer. The sudden inability to use the upper and lower contralateral limbs can mean that the patient will be unable to dress, eat, or even move. Skilled motor movements such as writing or picking up small objects can also be disrupted. An MCAO in the mouse mimics the clinical situation by causing motor impairments.

5.3. Mice as a model for stroke.

There are not many models of MCAO in the mouse. The most common method involves the insertion of a thin nylon coated filament into the internal carotid until it reaches the origin of the MCA, there-bye preventing blood flow through the MCA. This technique results in a 50% reduction in cortical blood flow (Clark, *et al.*, 1997). The thread can be left in place to create a permanent occlusion, which often results in an average infarct volume of $80mm^3$(Clark, *et al.*, 1997). The MCA can also be temporarily occluded. Removal of the thread produces a smaller average infarct size of $55mm^3$(Clark, *et al.*, 1997). The murine model of MCA focal ischemia in the mouse is very similar. In addition to the filament insertion, the external carotid artery is cauterized (Sander Connolly, *et al.*, 1996). This technique is able to produce extremely reproducible infarct volumes that are approximately 30% of the ipsilateral hemispheric volume (Sander Connolly, *et al.*, 1996). Both MCAO techniques offer the advantage of being reproducible and they do not require a craniotomy to access the small MCA of the mouse. Despite the advantages, the main drawback of the thread insertion models is that there is a large degree of damage to many subthalamic brain structures as well as the cortex and striatum. Functional motor assessment of these animals would be difficult because of the

variety of impairments produced by the MCAO. A focal motor cortex stroke model, such as the pial strip or endothelin application, affects only the sensorimotor cortices, which makes it possible to study the contribution of the motor cortex to skilled movements.

There is another factor that needs to be considered when choosing the appropriate mouse model. Because genetic background can influence stroke outcome, the choice of mouse strain should be evaluated carefully. The C57/BL6 mouse is one of the most characterized and widely used strains for stroke research; they are often used as background strains for the development of transgenic lines. Recent evidence indicates that the C57/BL6 strain is more susceptible to ischemic injury than other commonly used strains of mice (Fujii, *et al.*, 1997). Detailed studies of vascular anatomy indicated that C57/BL6 mice have an incomplete Circle of Willis. The Circle of Willis is a circular structure attached to the base of the brain that encircles the brainstem. Both basilar and carotid arteries terminate in the circle, thus the loop gives off the anterior, middle and posterior cerebral arteries. Since blood flow can go in either direction, the Circle of Willis can provide collateral blood flow when one of the protruding arteries becomes occluded.

Other sources of collateral blood flow in the brain are the pial anastomoses, which are tiny vessels that interconnect the major cerebral arteries. The anastomoses that connect the MCA to the anterior cerebral artery in C57/BL6 mice are located close to the midline. This means that the distal branches of the MCA must stretch further to reach the anastomoses, thus the MCA in C57/BL6 animals supplies a larger area than it would in other strains (Maeda, *et al.*, 1998). Despite the variables in vascular anatomy that must be taken into consideration, mice are still a good model because transgenic technology allows the examination of genetic contributions to stroke.

5.4. An introduction to transgenics.

A literature search of the keywords "transgenic mice" in Pub Med results in over 20 000 matches, most of which have been published in the last 20 years. Transgenics began in the early 1970's when it was found that injection if viral deoxyribonucleic acid

(DNA) into a mouse embryo produced an adult animal that exhibited viral DNA expression in many types of somatic tissue (Jaenisch and Mintz, 1974). Less than a decade later microinjection of foreign DNA into the pronucleus of a single celled embryo resulted in germline expression (Gordon, *et al.*, 1980). Germline transmission refers to incorporation of the foreign DNA into reproductive cells so that it can be passed on to progeny. The first transgenic mouse with confirmed overexpression of a rat transgene encoding a growth protein was unveiled a short time later (Palmiter, *et al.*, 1982). Pronucleus injection is used to obtain widespread transgene expression, however, the disadvantage is that it is hard to determine the incorporation site of the transgene in the genome of the generated animals. It is possible to target specific DNA regions of the genome with constructs that recognize specific DNA sequences. The constructs can be injected into embryonic stem cells (ES). ES are derived from blastocysts and have the ability to become any cell type, and they can be returned to the embryo following DNA injection. Both the adult mouse and any progeny must be screened for homozygous expression of the transgene. Homozygotes can then be used to produce a true breeding line. Transgene insertion is often accompanied by the insertion of promoters to drive transcription in specific tissue types, or reporters to imply transcription of other DNA sequences. The addition or removal of a gene in a mouse embryo is thought to produce an adult animal that displays the phenotype produced by that gene, making it possible to assess the contributions of various genes. For a review of these techniques see Jaenisch, 1988

Transgenic technology has had immense impact on the study of genetic contributions toward human disease (for a review see Wagner, *et al.*, 1995 and Koretsky 1992) particularly with regards to stroke research.

5.5. The contribution of transgenics to stroke.

Stroke research has benefited from transgenic insights into molecular pathology (for a review see Ahmed, *et al.*, 2000). The contribution of various genes and their products towards cardiovascular hypertension is reviewed in Bader, *et al.*, 2000. This

section will cover only a few important examples from the available literature. For example, high density lipoproteins (HDL) bind to cholesterol to transport it to the liver for metabolization. Lecithin-cholesterol acyltransferase is an enzyme that induces HDL and cholesterol binding by causing the two compounds to form an ester. Apolipoprotein A 1 (Apo A-1) is one protein that activates lecithin-cholesterol acyltransferase. Apo A-1 knockout mice are no longer able to produce Apo A-1 because they lack the gene that codes for it. As a result, the animals exhibit reduced cholesterol HDL complex levels in the blood plasma; cholesterol is more available. The knockouts have more atherosclerotic plaques and are hypertensive. Understanding the genetic contributions to hypertension can lead to a therapy that may help reduce the risk of stroke.

A large number of transgenic models are dedicated to reducing the number of reactive oxygen species present in stroke injured tissue (for a review see Chan, *et al.*, 1995). For example, nitrous oxide synthase (NOS) is an enzyme that produces the free radical NO, and NOS is activated during an ischemic insult. Knocking out the gene that codes for NOS resulted in reduced NO production in the mice and they exhibited a reduction in infarct size when compared to the wild type reference strain (Huang, *et al.*, 1993). Sampei, *et al.*, 2000 reported that mice overexpressing a copper superoxide dismutase (CuSOD) (SOD's are free radical scavengers or antioxidants) exhibited a reduced infarct volume that was up to 30% smaller in the transgenics when compared to the wild type (for a review see Chan, *et al.*, 1995). This can have important clinical implications because antioxidants can easily be administered orally or via injection.

Neurite growth factors are often produced in response to a stroke and many studies have looked at growth factor application as a potential treatment for ischemic injury. Guégan, *et al.*, 1998 used a nerve growth factor (NGF) transgene to test the effects of endogenous NGF synthesis on infarct volume. They inserted the NGF transgene downstream of the *c-fos* promoter, which activates many early response genes immediately following an ischemic insult. The resulting adult animals were subjected to a focal ischemic insult via electrocoagulation of the MCA. The results indicated that both the promoter and the transgene exhibited peak expression 6 hours post ischemia and the

transgenic mice experienced a 40% reduction in infarct volume when compared to the wild type.

Other research looked into the minimization of cell death post stroke. The poly ADP-ribose polymerase gene (PARP) is a nuclear enzyme that transfers adenosine diphosphate (ADP) to a variety of ribose groups by using the substrate nicotinamide adenine dinucleotide (NAD). NAD is an important energy source for the cell. During times of stress PARP is overactivated and NAD is rapidly depleted. PARP is also thought to be involved in the production of nitric oxide synthase. Transgenic mice, in which the PARP gene is disrupted and can no longer produce a viable protein, experience an 80% reduction in infarct volume as compared to the wild type (Eliasson, *et al.*, 1997).

This molecular research has contributed to the knowledge of the pathology of stroke and may in fact have clinical implications for treatments. There are many other genes whose effects can be examined with this type of technology.

5.6. A thesis summary.

Despite the fact that transgenic technology can provide insight into the genetic contributions to stroke, a reduction in infarct size does not necessarily mean an animal will exhibit improved functional outcome. Behavioural testing in stroke research is essential in order to evaluate the efficacy of any treatment. The purpose of this thesis was not only to demonstrate the usefulness of mice, but also to provide a detailed analysis of mouse motor behaviour that can be readily applied to any mouse strain in any experimental setting. The results of the experiments indicate that the single pellet reaching task is applicable to mice and that a focal motor cortex insult produces quantifiable impairments. There are also implications from the experiments for the choice of a stroke model. A larger focal motor cortex stroke produced by permanent removal of the cortical vasculature results in massive quantitative and qualitative impairments. Mice subjected to a stroke produced by temporary occlusion of the same vasculature only exhibit qualitative deficits and the area of tissue damage is much smaller. The fact that

both groups of mice exhibited impairments in the same rotatory limb movements indicates that even small lesions disrupt certain functions. The positive correlation between infarct size and degree of impairment for certain movement components indicates that as the lesion gets larger more of the movements become affected. This is particularly true for movements that are relatively stable. For example, mice rarely exhibit impairments in the movements that require fine digit manipulation such as digits semiflexed and digits extend, but animals with the largest insult did indicate impairments with these movements. In conclusion, the mouse makes an excellent model for studying stroke induced motor impairments, recovery and compensation.

6. References

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