NOVEL TREATMENTS FOR INDUCING CORTICAL PLASTICITY AND FUNCTIONAL RESTITUTION FOLLOWING MOTOR CORTEX STROKE

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Dedication

Mamusnak, Papusnak és Rékának.

Abstract

Stroke remains a leading cause of disability in the western world, with symptoms ranging in severity from mild congnitive or motor impairments, to severe impairments in both cognitive and motor domains. Despite ongoing research aimed at helping stroke patients the disease cannot be prevented or cured, therefore a large body of research has been aimed at identifying effective rehabilitative strategies. Based on our understanding of normal brain function, and the mechanisms mediating the limited spontaneous recovery that is observed following injury, factors that promote brain plasticity are likely to be effective treatments for stroke symptoms. The current thesis investigated three novel treatments (COX-2 inhibitor drug, vitamin supplement diet, and social experience) in a rat model of focal ischemia in the motor cortex. All three treatments have been previously shown to alter plasticity in the normal brain, however the current experiments show that the treatments have differential effects following stroke. The COX-2 inhibitors provided limited improvement in functional performance, whereas the vitamin supplement treatment had no effect. Social experience on the other hand was found to block the usually observed spontaneous improvements following the stroke. These results suggest that factors that alter dendritic plasticity may in fact serve as effective stroke treatments depending on the site and the mechanisms whereby the plastic changes are induced.

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General Introduction

Introduction to brain plasticity

The brain is the most complex organ within the human body. It is directly responsible for generating our everyday behaviour as well as our internal thoughts. Because of its complexity, many have attempted to study the brain by either carrying out empirical experiments, or recording detailed observations from Nature's experiments (brain injury, development, individual differences). Through these studies it was discovered that the brain has an inherent capacity to change over time. The capacity for the brain to reorganize in response to factors such as environmental demands, learning, or brain damage is commonly referred to as brain plasticity. The first use of the term plasticity in reference to the central nervous system comes from a thesis written in Romanian by Ioan Minea, where he described the morphological changes that occurred in spinal ganglion cells following trauma (Jones, 2000). Today, the term is often used as a blanket statement to encompass almost any form of cellular, molecular, or physiological change within the central nervous system. In fact, within the last year only (2004), over 19 000 papers were published that used the term "plasticity" in their title. These papers range from studies on computational models of plasticity to studies of limb amputees, and therefore it would be difficult for any one individual to keep up with all aspects of this enormous body of literature. On the other hand, completely focusing on a single aspect of brain plasticity would also limit one's perspective, as there are multiple areas of overlap among many of the subfields of brain plasticity. Drawing parallels among a small number of the most related subfields would be most beneficial in generating an understanding of the field as a whole. For example, the subfield of plasticity and

functional recovery shares many features in common with developmental plasticity, as well as plasticity during adult learning.

The current thesis investigated the effects of treatment-induced plastic changes in the cortex on functional restitution following motor cortex stroke. Based on the overwhelming evidence that the plastic changes following stroke injury parallel those observed during development, and adult learning, I precede my discussion of the injured brain with a brief introduction to plasticity in the intact brain. Specifically, I discuss the plastic changes that occur in development, during adult learning, and in the adult motor cortex.

Plasticity in Development

The development of the mammalian brain is marked by specific stages such as cell birth, cell differentiation, and synaptogenesis, which are associated with varied levels of plasticity. The events that characterize each of these stages have been well studied, and thus provide insight into the mechanisms that regulate developmental plasticity. Interestingly, some of these developmental stages are also recapitulated in the adult brain following injury (Cramer and Chopp, 2000). Studies of adult brain injury have found that specific features of brain function revert to those seen during development, with restitution of function being associated with a return to adult patterns. Specifically the processes of cellular differentiation and synapse formation show the highest degree of resemblance to developmental patterns.

During development, the cells that eventually make up the neocortex are generated in the center of the neural tube. From here, the immature neuroblasts migrate

along the processes of radialglia that stretch the distance between the inside wall of the neural tube and the outer most (cortical) surface (Kolb and Whishaw, 2003). It is only after the cells reach their final destination in the cortex that they undergo terminal differentiation into specific neuronal types and make dendritic and axonal connections with the appropriate targets. Currently there are two dominant theories that provide an explanation for how this occurs. The first is known as the protomap model and was proposed by Pasko Rakic (Rakic, 1988). Based on this theory the destination of the migrating cells is determined by a genetically controlled protomap in the ventricle (cortical primordium) that represents the spatial organization of the cerebral cortex. For example, the primary sensory and motor areas that neighbor each other in the cortex are represented in the ventricle by adjacently located sensory and motor cortex precursor cell populations. Based on this theory the genetic protomap in the developing ventricle dictates the area map formation within the cortex. The second theory, known as the protocortex model, states that the ventricle is essentially homogenious, and that the area maps in the developing cortex (protocortex) are patterned by cues from axons growing up from the thalamus (O'Leary, 1989). In contrast to the protomap model where the migrating neuroblasts are programmed to become part of a certain area map, according to the protocortex model the arrival of the thalamic afferents marks the beginning of cortical patterning.

Although we are currently unable to determine which of these two theories provides the more accurate description of cortical development, the important point here is that there is some sort of mechanism in place that directs the organization of the brain during development, and that this mechanism likely also limits brain reorganization after

injury. A more concrete way of stating this is that functional reorganization after injury will be constrained by the rules governing cortical patterning.

The process of synapse formation is an additional stage of development that has been found to be recapitulated following injury. In both cases the final pattern of synaptic connections is achieved by an initial overproduction in total synapse number, followed by extensive synaptic pruning (Cramer and Chopp, 2000). In contrast to human development where synapses start to form in the fetus (Huttenlocher, 2002), cortical synaptogenesis in the rat occurs almost completely postnatally (Kolb, 1995). During the period of maximal synapse formation (between birth and puberty) synapses are formed by both experience-expectant and experience-dependent mechanisms. Experience-expectant synapse development relies on the presence of certain sensory experiences, whereas the experience dependent mechanism refers to the generation of synapses that are unique to an individual organism (Kolb and Whishaw, 2003). Following puberty, synapse numbers continue to drop drastically throughout the cortex, therefore it seems fascinating that adult brain injury is able to induce such significant alterations in synaptic connections (see below).

Learning in the Adult Brain

In contrast to the stages of development that are associated with high levels of plasticity, the adult brain has historically been viewed as a static organ that is incapable of undergoing change. The current view, that the adult brain is in fact capable of change, has been largely facilitated by the ability to observe structural changes in the brain following events such as learning, or more generally, changes in experience.

The pioneering enrichment studies carried out Marian Diamond and other members of the Berkley Group demonstrated that environmental enrichment in adult rats induces a number of anatomical and behavioural changes. Enriched rats were found to have generally larger brains, a thicker cortex, and more available neurotransmitters such as acetylcholine (Rosenzweig et al., 1967). These anatomical alterations provided a mechanism for Donald Hebb's observation 30 years earlier that rats receiving similar enrichment showed improved learning ability relative to animals in standard housing (Hebb, 1947). More recent experiments have extended these findings by showing that localized anatomical changes occur following adult learning. For example, Kolb and colleagues carried out experiments demonstrating that the learning that occurs following behavioural training is associated with structural changes in specific cortical regions, and that normal activity alone in those regions does not induce the dendritic changes (Kolb, 1995). To show this, rats were trained to visually navigate to a hidden platform in a pool of water by using visual cues located on the walls of the room where the pool was located. Using this paradigm, one group of rats was required to learn the location of the hidden platform relative to the constellation of the extra-maze cues, whereas another group (control group) was allowed to swim in the pool for the same length of time, without having to learn anything about the visual cues. The learning that occurred in the visual navigation group was found to increase dendritic measures in the visual cortex, whereas the control group did not show any learning or any dendritic changes. These experiments demonstrate that specific forms of learning cause structural changes in the area of the brain that is mediating that specific behaviour. The fact that the visual cortex

is required for visual navigation has been confirmed by a recent study showing that animals with visual cortex lesions are impaired at learning the task (Whishaw, 2004).

Human studies have also found similar plastic changes in the adult brain following learning. Some of the most interesting examples of this phenomenon come from blind people who have had to learn to read Braille as adults. Braille reading requires running the fingertips across raised dot patterns and mentally converting these characters into useful information (language). Most people use two or three fingers to follow the text while some of the less experienced readers will use a single finger to read. Neuroimaging studies have shown that the representation of the reading hand in the somatosensory cortex is significantly larger in multi-finger Braille readers relative to those that read Braille with a single finger, or sighted control subjects who do not read Braille (Sterr et al., 1998). It is likely that this significant plastic change in the somatosensory cortex is induced by the hours of practice that Braille readers spend each day discriminating between the subtle differences in dot patterns that represent various words.

Imaging studies have also revealed a different form of plasticity in the visual cortex of blind people. The presentation of visual stimuli, such as written words, to the visual field of sighted people induces activity in the visual cortex (Joseph et al., 2001). Surprisingly, however, blind people engaged in Braille reading also show similar activation of the visual cortex (Burton, 2003), suggesting the induction of a form of cross-modal plasticity that likely allows blind people to experience a visual representation of Braille text. These studies are just a few examples from a large body of

literature showing the occurrence of changes in the adult brain following some sort of learning event.

Plasticity in the motor cortex

Next to the visual system, the motor cortex is the most thoroughly studied area of the nervous system in terms of the capacity to reorganize. This is because of the relative ease with which we can assess plasticity in the motor system using a combination of behavioural, anatomical, and electrophysiological measures in animal models, and because of the extensive human literature that has accumulated using non-invasive imaging techniques.

The fact that the motor cortex undergoes plastic changes following behavioural training has been demonstrated in animal studies where rats were trained to perform skilled motor behaviours using their forepaws (Kolb, 1995). These studies have shown that extensive training on a unilateral skilled reaching task that requires rats to retrieve a piece of food causes a significant increase in dendritic material in the motor cortex contralateral to the reaching paw. If, however, the rats are trained to retrieve a piece of food in a way that requires the skilled use of both forepaws, the dendritic alterations are observed bilaterally in the motor cortex. Additional studies have used intracortical microstimulation (ICMS) to investigate the physiological effects of motor skill acquisition in rats. This procedure allows for the visualization of the areal representations of various parts of the body in the motor cortex. Skilled reach training was found to increase the representation of the wrist and digits at the expense of decreasing shoulder and elbow representations (Kleim et al., 2002). The skilled reaching

task requires the animals to make complex wrist and digit movements in order to retrieve the food pellets whereas the elbow and shoulder movements are less complex. The changes in motor representations that are observed following reach training thus reflect the acquisition of new motor skills.

Skilled reach training has also been shown to induce changes in additional electrophysiological measures in the motor cortex, such as long-term potetiation (LTP). Although LTP can be induced artificially by applying a brief, high frequency burst of electrical stimulation, it shares many of the cellular characteristics of natural learning. Unilateral skilled reach training was found to potentiate synaptic efficacy (induce LTP) in the motor cortex contralateral to the reaching paw (Rioult-Pedotti et al., 1998), suggesting that the behavioural training induces plastic processes in the contralateral motor cortex. As is the case with most forms of motor learning, the acquisition of the skilled reaching task in rats occurs over a number of training sessions, and electrophysiological studies have provided insight into the mechanisms that characterize the various stages. When learning the reaching task, rats progress through three different stages that are characterized by varied levels of success. The first level represents the *acquisition of the task requirements* and is not associated with significant improvement in performance. Level two represents the *increase in task proficiency*, and is associated with improvement in performance, whereas level three represents the *maintenance of skill proficiency*, and is once again not associated with improvement in performance level. Electrophysiological studies have shown that only the second phase of training (increase in task proficiency) is positively correlated with an increase in neocortical polysynaptic efficacy (Monfils and Teskey, 2004). This finding indicates that the mechanisms

mediating the synaptic changes that occur during the increase in task proficiency are likely similar to those engaged during LTP.

In addition to the beneficial effects of motor plasticity, there are also examples where this process has detrimental effects. For example, musicians who engage in extensive and forceful use of the fingers will often develop a condition known as focal hand dystonia. This form of maladaptive plasticity is caused by the use dependent overlap of the sensorimotor representations of the individual fingers (Rosenkranz et al., 2005). The result of this pathological form of plasticity is that the affected individuals are unable to execute independent digit movements. Given that behavioural mechanisms contribute to the onset of focal hand dystonia, it was hypothesized that behavioural interventions would likely also be effective at ameliorating the symptoms (Elbert and Rockstroh, 2004). To test this hypothesis, Zeuner *et al* (Zeuner et al., 2005) performed a form of rehabilitative training where patients were required to perform unique movements with individual fingers while the remaining fingers were immobilized with a splint. Indeed, this form of therapy was found to significantly decrease the severity of the dystonia, showing that behavioural intervention may be used to reverse this form of maladaptive plasticity in the motor cortex.

The examples of motor plasticity that are discussed above are largely mediated by alterations at the postsynaptic end of the horizontal connections within the motor cortex. Although this appears to be the major form of plasticity following motor learning or cortical injury, behavioural adaptation may also occur through axonal sprouting and non-neuronal changes such as alterations in cerebrovasculature or the number of astrocytes within the brain (Grossman et al., 2002). In fact, certain treatments that are known to be

beneficial following brain injury act through these exact mechanisms. Voluntary exercise, for example is known to induce the formation of blood vessels in the brain and also result in better functional outcome following brain damage (Griesbach et al., 2004).

Our current understanding of motor cortex plasticity has also been greatly influenced by investigating the effects of lesions in the motor cortex. The next section, therefore will serve to introduce ablation as an experimental model for studying plasticity by providing a historical perspective of the experiments that have been carried out with the technique.

A Historical Perspective on Experimental Brain Damage and Plasticity

The clinical outcome from adult brain damage has been studied systematically in humans for over 100 years, but in addition, these observations were also paralleled by intensive experimental investigations in animals. The first of these studies was carried out in the 1850's by the French experimentalist Pierre Flourens (Kolb and Whishaw, 2003). Flourens developed a technique for inducing lesions in the brains of animals such as pigeons or chickens, and was thus able to observe the behavioural changes that resulted from these injuries. His primary intention in doing these experiments (as was the intention of most researchers at the time) was to investigate the idea that functions were localized within the cortex. He found that immediately after removing areas of the cortex, animals did not move, eat or drink as much as normal animals, however with time these behaviours recovered. Given that Gall and Spurzheim had recently proposed the idea that regions of the cortex are responsible for specific functions, Flourens' findings seemed counterintuitive at the time because the focal lesions did not induce permanent

disruptions of specific behaviours. He therefore proposed the novel idea that recovery from cortical injury was possible because the remaining cortex could do the same things that the missing cortex had done, and so could take over the lost functions. Our modern understanding of brain function would likely attribute these findings to brain plasticity, but at the time this phenomenon was mainly viewed as a hindrance to the efforts invested in studying localization of function.

In a further attempt to localize functions within the cortex, Friedrich Goltz made large cortical lesions in dogs and observed their behaviour for several months after the surgery. His observations also failed to support the argument of localization of function as the lesions did not induce any significant behavioural abnormalities (Kolb and Whishaw, 1988). For example, the dogs were able to walk on uneven ground, displayed normal patterns of activity, were able to thermoregulate and even learned to avoid an unpleasant stimulus that was presented in food. Additional experiments performed at the time confirmed that even larger lesions, such as hemidecortications, failed to produce the expected behavioural impairments. It was not until Gustav Fritsch and Edueard Hitzig performed their revolutionary stimulation studies in the 1870's that our modern understanding of cortical localization was established (Finger et al., 2000). By applying direct electrical stimulation to the surface of the cortex, they were able to localize motor function to the anterior part of the hemisphere in dogs (Boling et al., 2002). Follow up experiments by Otto Soltman extended these findings by performing ablation studies in dogs following the mapping of the motor cortex (Finger et al., 2000). His findings also indicated that adult motor cortex ablations caused motor problems that seemed to dissipate over time.

It is interesting to note that in the early days of experimentations with brain lesions it was fairly uncommon to use fully developed, adult animals. Most studies were carried out on young animals because it was found that they could endure the traumatic effects of the surgery better and were also more suitable for anatomical work than adults (Finger et al., 2000). The serendipitous finding that functional outcome varies depending on the age of the animal at the time of injury eventually prompted a systematic investigation into this phenomenon, and is still intensively studied today. Lesion studies today are less restricted by methodological constraints in that we are able to induce brain injury and ensure that the animals survive the procedure without any serious health complications. This has facilitated the development of several clinically relevant animal models that are used to study various disease states that affect humans. The remainder of this chapter will focus on one clinical condition in particular, that being adult stroke. I will first provide a general introduction to stroke in patients followed by an introduction to the various animal models that are used to study the disease. I will than describe the cellular hallmarks of stroke pathology before moving on to a discussion of the existing evidence for the idea that brain plasticity is a viable mechanism for stimulating improved outcome following stroke.

Introduction to Stroke

General Facts

Every ten minutes someone in Canada has a stroke (Vancouver Island Health Authority (2002). This number is even greater for the United States, where a stroke occurs every 45 seconds (American Heart Association(2005a)). The degree and rate of

recovery has been found to vary greatly. About 10% of stroke survivors recover almost completely, 25% recover with minor impairments, 40% experience moderate to severe impairments requiring special care, 10% require care in a nursing home or other long-term care facility, and the remaining 15% die shortly after the stroke. Recurrent strokes are also quite frequent, as about 25% of the people who recover from their first stroke will have another stroke within 5 years.

The risk factors of stroke vary with age, the trend being that adults over 65 years of age have a seven-fold greater risk of dying from stroke relative to the general population (NINDS, 2005b). The most powerful risk factor that has been identified to increase stroke risk is high blood pressure (hypertension). A systolic pressure of 120 mm of Hg over a diastolic pressure of 80 mm of Hg is generally considered normal. One third of the US population is hypertensive, meaning that they persistently have blood pressure greater than 140 over 90. Hypertension increases the risk of stroke by four to six times (NINDS, 2005b) . In addition to the unmodifiable risk factors of age and hypertension, cigarette smoking has emerged as a leading modifiable risk factor. Smoking doubles the risk of stroke (independent of other risk factors) by promoting atherosclerosis and weakening the wall of the cerebrovascular system. Alcohol consumption and the use of illicit drugs such as amphetamine and heroin represent additional significant modifiable risk factors.

Types of Strokes

A stroke occurs when the blood supply to part of the brain is suddenly interrupted. This can occur through either a blockage in a blood vessel, causing an ischemic stroke, or by a blood vessel bursting, causing a hemorrhagic stroke.

Ischemic stroke – Approximately 80% of strokes are ischemic, and are caused either by a cerebral embolism, a larger artery thrombosis, or a small artery thrombosis (referred to as a lacunar-type stroke). An embolus is a free-roaming clot in the blood that usually originates in the heart and eventually becomes lodged in a cerebral artery. A thrombotic stroke is caused by the formation of a blood clot in one of the cerebral arteries. In this case however, the clot stays attached to the wall of the artery and grows large enough to block blood flow. A blockage in a brain artery can also be caused by a process known as stenosis, or the narrowing of the artery due to the buildup of plaque and blood clots.

Hemorrhagic strokes – In the healthy functional brain, neurons do not come into direct contact with blood. Instead, a blood-brain barrier created by glial cells regulates the movement of molecules from the circulatory system to the neurons. In the event of a burst in a blood vessel, blood enters the surrounding tissue, thus upsetting the blood supply as well as the delicate chemical balance that neurons require to function. A blood vessel may burst at the site of an aneurysm (a weakening of the in the arterial wall), or at the site of plaque accumulation. Plaque-encrusted artery walls lose their elasticity and become thin and brittle, thus making them prone to breaking. When a blood vessel bursts directly in the brain it is referred to as an intracerebral hemorrhage, whereas if the vessel bursts in the outer coverings of the brain (the meninges), it is referred to as a subarachnoid hehemorrhage.

Transient Ischemic Attack – Minor strokes that are not associated with a chronic neurological impairment are referred to as transient ischemic attacks (TIAs). The average duration of a TIA is a few minutes, and usually the symptoms go away within an hour. The short-term risk of stroke, however, is substantially increased, with about 10% of TIA patients experiencing a stroke within 90 days and 5% experiencing a stroke within 2 days (NINDS, 2005b).

Clinical Condition

To be able to develop successful stroke treatments in animal models, one must have a thorough understanding of the clinical condition that characterizes the patients. The diagnosis of a stroke presents a significant change in the patients life, and all forms of subsequent interventions are intended to reverse these changes. The most obvious change will be the fact that the patient will have some sort of neurological impairment, and that he will find himself in a hospital setting being cared for by people he has never met before. Therefore, interventions that result in a shorter hospital stay and help the patients regain independence will be most beneficial.

The human brain is highly lateralized in function, therefore the neurological deficits that will manifest, in large part, depend on the side of the brain that is injured. Contralateral hemiparesis or hemiplegia is the most common symptom of stroke, however additional symptoms also occur depending on the site of injury. Right hemisphere stroke is known to cause significant deficits in visual-spatial perception as well as significant neglect of the left side of the world. These patients often ignore people or objects that are placed in the left visual field and often fail to attend to the left

side of their body when dressing or eating. In addition, patients are also often unaware of their disability (anosagnosia) and often fail to notice or deny the presence of their affected limbs. Although most right hemisphere patients have no trouble speaking or communicating, they often lack insight into their deficits, carry a flat affect and tend to be impulsive in their thinking (Teasell et al., 2005). In contrast, language disorders (aphasias) are the most common symptoms of left hemisphere stroke (Mulley, 1985). The most common form of aphasia, Broca's aphasia, results in patients not being able to generate speech, while retaining the ability to understand speech. In some cases the inability to speak can be caused by verbal apraxia, which is a specific form of a more general symptom characterized by the inability to execute voluntary movement. Apraxia, in the more general form, results from the inability to translate an aim (such as walking) into a desired action, in the absence of paralysis (Mulley, 1985).

In addition to the physical and neurological problems that stroke patients must deal with, statistics show that at least half of the patients will also become depressed (Bhogal et al., 2005). Depression in the first few weeks after the stroke is likely part of the normal grief reaction and should probably not be corrected pharmacologically for two reasons. First, altering the mood or cognitive state of patients through antidepressants, may interfere with the natural course that patients go through when coming to terms with their injury. Second, the neurogenic side effects of the SSRI class of antidepressants (Malberg et al., 2000) are thought to be detrimental when the brain is in a labile state (such as is the case immediately following a stroke). Depression occurring months after the stroke is believed to be different in nature and may respond well to pharmacological treatment. This delayed form of depression may be caused by the patient not being able

to adapt to the physical deficits following the stroke, or it may be due to a specific effect of the localized brain damage (Mulley, 1985). Support for the latter prediction comes from studies that have compared the level of depression between orthopedically-disabled patients and stroke patients that have a similar level of disability. The results show that stroke patients are about four times as likely to be depressed than the orthopedic patients (Folstein et al., 1977), suggesting that the stroke itself may cause a neurochemical imbalance that can lead to depression. The location of the injury does not appear to influence the likelihood of depression (Carson et al., 2000), however, depressed patients showed poorer functional (Kotila et al., 1999) and cognitive (Robinson et al., 1986) recovery. In addition, post-stroke depression was found to act negatively on social activity and also result in social withdrawal. This behaviour may in turn further exacerbate depression after stroke (Bhogal et al., 2005).

A large number of studies have now shown that the specific hospital setting where stroke patients recover also has a significant effect on recovery. Designated stroke units are known to improve short- and long-term survival, improve functional outcome, and increase the possibility of earlier discharge (Indredavik et al., 1999). The mechanisms mediating these beneficial effects however are still under investigation. Meta-analyses have shown that stroke units provide the greatest benefit to patients that are moderately impaired, probably because they are sufficiently functioning to be able to partake in the rehabilitation and at the same time have room for improvement (Teasell, 2005). Patients with severe stokes are better managed at more long-term, and less intensive rehabilitation facilities (Kosasih et al., 1998), whereas mild stroke patients can be successfully rehabilitated in an outpatient setting (Teasell, 2005). Attempts to identify the beneficial

components of designated stroke units are complicated by inconsistencies in the definition of a stroke unit. Teasell (Teasell, 2005) has identified three different forms of stroke units. Acute stroke units accept patients acutely but discharge early (usually about 7 days), rehabilitation stroke units accept patients after a delay of usually about 7 or more days, and finally, comprehensive stroke units accept patients acutely but also provide rehabilitation for at least several weeks. Despite of the great variability in the timeframe of treatment administration, both the acute and rehabilitative care units are associated with decreased mortality and dependency (Teasell, 2005). Additional studies are needed to further determine the positive aspects of stroke units, with the intention of adapting these positive components in smaller, less specialized centers of rehabilitation.

Animal models of ischemic stroke

Although stroke has also been modeled in higher organisms such as baboons (Symon et al., 1975), financial and ethical concerns have hindered the large-scale use of non-human primates in stroke models. As a result of this, most stroke studies have been carried out in rodents as the anatomy and behaviour of animals such as rats, mice and gerbils facilitates the study of this complex clinical condition. Despite of popular public opinion, the validity of a stroke model should not be determined based on the species *per se*, but rather on how the investigators use the species. Careful endpoint measures of behaviour should be guided by qualitative assessments of injury-induced changes in spontaneous behaviour (Cenci et al., 2002), and should be used in combination with detailed anatomical analyses such as the evaluation of infarct volume or the quantification of residual cell number. Animal models are often used for pre-clinical

screening of potential treatments, therefore the behavioural deficits and the anatomical pathology should be modifiable (either positively or negatively) by various interventions.

Ischemia in animals can be induced focally in restricted brain regions, or globally by reducing blood flow to the brain as a whole. During global ischemia, neuronal injury is selective to vulnerable areas of the brain, such as the CA1 region of the hippocampus (Davies et al., 2004), whereas focal ischemia can target any brain region. Reversible global ischemia is most commonly achieved through the four-vessel occlusion model (4-VO). For this procedure the vertebral arteries of the animal are permanently occluded under anesthesia while the common carotid arteries are temporarily occluded at a later time point, while the animals are awake. Neurological deficits only occur following combined vertebral and carotid artery occlusion, therefore this model has the advantage of allowing observation of the behavioural sequelae both during and after the ischemic event. A similar model, known as two-vessel occlusion (2-VO), involves bilateral occlusion of the common carotid artery coupled with systematic hypotension to produce reversible forebrain ischemia (Traystman, 2003).

Focal cerebral ischemia is commonly achieved by either permanent or temporary occlusion of the middle cerebral artery (MCA). Permanent MCA occlusion can be achieved by electrocoagulation of the vessel through a small subtemporal craniotomy, resulting in cortical and striatal infarction (Tamura et al., 1981). The MCA can also be permanently occluded by injecting the photochemical dye rose bengal into the blood stream and shining a laser light on the distal branches of the MCA to activate the compound (Yao et al., 2003). Alternatively, temporary MCA occlusion may be achieved through the luminal suture model or by a simple snare ligature that can be tightened

around the vessel. The luminal suture model involves inserting a coated suture into the internal carotid artery at the neck of the animal, and advancing it cranially to block the MCA (Longa et al., 1989). The advantage of this technique is that the procedure is performed without a craniotomy, thus providing a more accurate representation of the clinical condition. An even more realistic model is the embolization of blood clots that are injected into the carotid artery (Clark et al., 1991). Although the etiology of the ischemic injury is quite realistic in this model, there is no way of predicting where in the brain the stroke will occur, making the model highly impractical.

A more precise way of inducing cortical injury in rodents is to devascularize over a specific cortical region (Kolb et al., 1997). This technique, sometimes referred to as a pial-strip lesion, has many advantages over other lesion models. It is highly reproducible, produces mainly cortical damage, and can be performed quickly, with minimal discomfort to the animals. The surgery is performed by removing a skull-flap from an anesthetized rat, followed by the removal of the underlying dura (Gonzalez and Kolb, 2003). The pia-arachnoid vasculature is then surgically removed resulting in a cortical ischemic infarct that is restricted to the area of the craniotomy.

Pathophysiology of Focal Ischemic Stroke

The brain region where cells terminally lose membrane potentiality is referred to as the infarct core (Dirnagl et al., 1999), whereas the region of constrained blood supply but preserved energy metabolism is referred to as the penumbra (Hossmann, 1994). As can be seen in Figure 1-1, cells in the core of the injury die fairly quickly after the injury

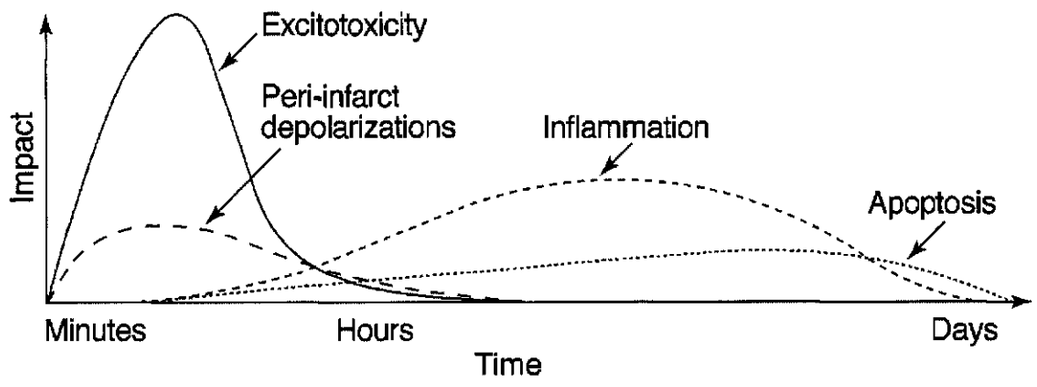

Figure 1-1. Indicates the time course of the pathological events that occur following ischemic stroke. Initially, cells die because of peri-infarct depolarizations and excitotoxicity. This is followed by inflammation and apoptotic cell death (From: *TINS* 22(9):391, 1999)

through necrotic mechanisms, whereas cells in the penumbral region die more slowly through programmed cell death (apoptosis) (Barber et al., 2001).

The initial pathological events following stroke are caused by the failure of cells to generate sufficient energy to maintain proper ionic gradients. With energy depletion, cells in the core of the stroke lose their membrane potential and depolarize, triggering massive calcium (Ca^{2+}) influx through voltage sensitive Ca^{2+} channels. The Ca^{2-} influx causes the release of excitatory amino acids (such as glutamate) into the synapse (Barber et al., 2001). The binding of glutamate to NMDA receptor channels causes additional Ca^{2-} to flow into the cell, resulting in the activation of Ca^{2+}-dependent proteases and phospholipases, which break up cellular proteins and lipids respectively (Kandel et al., 2000). Thus, glutamate neurotransmission indirectly causes excitotoxic cell death in the early stages of stroke pathology.

In contrast to the permanent depolarization that occurs in the core of the stroke, cells in the penumbra are able to repolarize and depolarize repeatedly in response to the metabolic fluctuations (Barber et al., 2001). This phenomenon is referred to as spreading depression or peri-infarct depolarization and can occur for more than eight hours after the ischemic event, with several repolarization/depolarization cycles per hour (Dirnagl et al., 1999). The number of cycles is a good predictor of final lesion volume, with more cycles resulting in larger lesions and *vice versa* (Mies et al., 1993). The fact that both NMDA and AMPA receptor antagonists block peri-infarct depolarization in rodents suggests that these pharmacological agents may provide effective neuroprotection following stroke. However, clinical trials of these agents have consistently failed (DeGraba and Pettigrew, 2000), suggesting that peri-infarct depolarization is not the main pathological mechanism

in the penumbra of human patients. An alternative explanation is that in the animal studies the beneficial effects were due to the drug inducing hypothermia, and serving as a neuroprotectant through this indirect mechanism.

Subsequent pathological events that are observed following ischemic stroke are inflammation and apoptosis. These events are likely initiated within a few hours of the insult and remain upregulated for several days or weeks after the incident (Dirnagl et al., 1999). The inflammatory pathway is initiated by cytokines that are released by astrocytes, microglia, leukocytes, and endothelial cells in response to the ischemia. The release of factors such as platelet-activating factor, tumor necrosis factor (TNF) alpha, and interleukin-1ß, results in the infiltration of the injured tissue by immune cells from the blood stream. This is achieved by leukocytes stimulating the production of adhesion molecules such as intercellular adhesion molecule 1, which in turn facilitate the crossing of neutrophils across the vascular wall to enter the brain parenchyma (Dirnagl et al., 1999). Additional immune cells such as macrophages and monocytes follow the neutrophils and target the injured cells. The leukocytes further promote infarction through the production of toxic byproducts and their phagocytic action (Barber et al., 2001). Resident brain cells also play an active role in post-ischemic inflammation. Astrocytes become hypertrophic while microglia retract their processes to assume an activated state (Wilhelmsson et al., 2004). Recent evidence suggests that these events are beneficial in the acute stages of the injury, however they serve as potent inhibitors of cellular reorganization during subsequent stages of recovery. Blocking the astrocytic hypertrophy through genetic manipulations results in a fourfold increase in the loss of

synapses in the lesion area 4 days after the injury, however by two weeks, the number of synapses in the same animals was restored to pre lesion levels (Wilhelmsson et al., 2004).

Ischemic neuronal cells also contribute to the inflammation pathway by upregulating the expression of cyclooxygenase 2 (COX-2) and TNF alpha, however the exact role of these molecules is still under investigation. Some studies have found TNF alpha to exacerbate ischemic injury (Barone et al., 1997), whereas others have suggested that the cytokine is beneficial due to its induction of antioxidant enzymes (Bruce et al., 1996). Similarly, the COX-2 enzyme was found to be highly upregulated following ischemia, causing increased levels of prostaglandins within the lesion area (Nogawa et al., 1997). However, it is still unknown whether these signaling molecules have a positive or negative effect on stroke outcome (Gobbo and O'Mara, 2004). It is possible that the specific function of these pro-inflammatory messages depends on the exact time at which they are upregulated. Gobbo and O'Mara (2004) found that blocking COX-2 induction pharmacologically after the injury provided functional benefits, whereas treatment with the drug immediately before the injury was not beneficial.

The final effect of these pathological events on cell function will depend largely on the intensity of the negative stimuli and the type of cell in question (Leist and Nicotera, 1998). Although an ischemic event is associated with significant cell loss through both apoptotic and necrotic mechanisms, the remaining cells also undergo structural changes that may facilitate functional restitution.

Plasticity as a Mechanism for Restitution of Function

At least part of the recovery process following stroke undoubtedly involves the resolution of the pathophysiological events that were described in the previous section, but most of the spontaneous behavioural restitution that will occur is likely mediated by plastic changes in remaining brain regions. The capacity of the cortex to reorganize following motor injury is often investigated by mapping the motor representations in the cortex either through intracortical microstimulation or (ICMS, in animal studies) or through transcranial magnetic stimulation (TMS, in humans). TMS involves applying a brief but intense magnetic field directly to the scalp (Cramer and Bastings, 2000). If the stimulation is applied to the motor cortex, recordable electromyographic responses can be observed in the corresponding muscles, and thus a motor map can be created in a way that is analogous to the ICMS technique that I described previously.

Using the ICMS technique, Frost *et al.* (2003) investigated the cortical reorganization that occurred following unilateral ischemic injury in the primary motor area (M1) of adult squirrel monkeys. They found that the observed spontaneous behavioural recovery was associated with increased distal forelimb representations in the ventral premotor cortex (PMV). This pattern of reorganization is typically seen in M1 following the acquisition of skilled motor behaviour. The finding that in the absence of M1 a distal motor region (PMV) undergoes plastic changes provides further evidence for the vicariance theory as a mechanism of restitution following cortical injury. The reorganization of a secondary cortical area following damage to a primary area has previously been observed in the somatosensory cortex (Pons et al., 1988) suggesting that this form of reorganization also occurs in non-motor areas as well. In addition, the fact

that in the motor system the degree of functional expansion in PMV is directly proportional to the amount of damage in M1 also suggests that the remote reorganization is directly related to the reciprocal connectivity of motor areas (Frost et al., 2003). In the primate motor system the premotor cortex (which includes PMV), the supplementary motor area and the cingulate motor area all have reciprocal connections with M1 and each other, therefore it is likely that the function of one area will be affected by damage to another.

The idea that lost functions can be replaced by remaining cortical regions provides an attractive explanation for the spontaneous functional restitution observed after stroke, however, what happens to the original function of the cortex that took over the lost function? One prediction is that these plastic changes will result in crowding of the reorganized region (Teuber, 1975), and would therefore interfere with the control of other behaviours not normally affected by lesions of the motor cortex. Experiments investigating this hypothesis in rats however have shown that this does not in fact occur. The plastic changes that were observed in rats following motor cortex lesions did not disrupt performance on a cognitive task that is sensitive to lesions in the area of the cortex that underwent the plastic changes (Kolb et al., 2000).

Mapping studies of clinical patients have also shown that there is substantial reorganization of motor regions in both the injured and the intact hemispheres following stroke in the primary motor cortex. The results indicate that good functional outcome is mediated by reorganization in the injured hemisphere, whereas reorganization in the intact (contralateral) hemisphere usually results in poorer functional outcome. TMS stimulation of the injured hemisphere elicits movement in the contralateral limb in

patients who show good recovery, (Palmer et al., 1992; Muellbacher and Mamoli, 1995) whereas in patients with poor functional outcome movements can be elicited from stimulation of the intact hemisphere (ipsilateral to the injury) (Turton et al., 1996; Netz et al., 1997). It appears that although there are substantial plastic changes in both hemispheres following stroke, the non-stroke hemisphere is not a direct substitute for the damaged motor areas in the stroke hemisphere. It is likely that the contribution of the non-stroke hemisphere occurs via polysynaptic pathways involving other motor areas or through interhemispheric pathways such as transcallosal connections and thus results in incomplete recovery (Cramer and Bastings, 2000). A recent functional Magnetic Resonance Imaging (fMRI) study of stroke patients with restricted damage to M1 demonstrated that the improved functional outcome resulting from rehabilitative training was due to neighbouring cortical regions taking over the lost functions (Jaillard et al., 2005). The authors argue that the motor impairments that were observed in the patients initially after the injury may have been caused in part by the involvement of the premotor areas of the undamaged hemisphere. The subsequent vicariance of the lost function to adjacent cortical areas may facilitate the subsequent improvement in function.

In addition to the vicariance of function following stroke, there is evidence from animal studies suggesting that the behavioural recovery can be facilitated by the resolution of diaschisis around the lesion area. The term diaschisis was first proposed by Constantin von Monakow as an explanation for the observation that there were disruptive effects of focal lesions in distal parts of the brain (ie areas that were not directly affected by the lesion). Earlier reports have suggested that factors such as edema, and pressure on the brain can cause distal effects that are secondary to the initial injury (Finger et al.,

2004). Edema has been shown to have negative effects on functional outcome following experimental stroke models, however these effects are believed to be transient and not the main cause of the impairment (Whishaw, 2000). Von Monakow's concept, however, can be distinguished from these phenomena in that it is neurally mediated and has specific focal consequences (Finger et al., 2004). An ischemic injury to part of the hand representation in the motor cortex of adult squirrel monkeys was found to result in the widespread reduction in the spared hand representations adjacent to the lesion and an increase in the adjacent proximal (ie arm and shoulder) representations (Nudo, 1997). If, however, the animals received daily rehabilitative training following the injury, there was a net expansion of the hand representations in the zone immediately surrounding the infarct (See Figure 1-2). Thus, in contrast to the spontaneously recovering monkeys, monkeys that received behavioural training post injury showed retention of the undamaged hand representations (Nudo et al., 1996).

Finally, extensive dendritic reorganization has also been observed following the induction of experimental stroke in rats. A recent study by Gonzalez *& Kolb (2003)* compared the effects of four different models of cortical injury on dendritic morphology in remaining cortical regions. Surprisingly, all four injury models were associated with unique patters of dendritic reorganization, despite of the fact that in most cases the behavioural outcome was similar. These results suggest that there is a strong need to characterize the stroke model both behaviourally and anatomically prior to drawing conclusions about the effects of further manipulations.

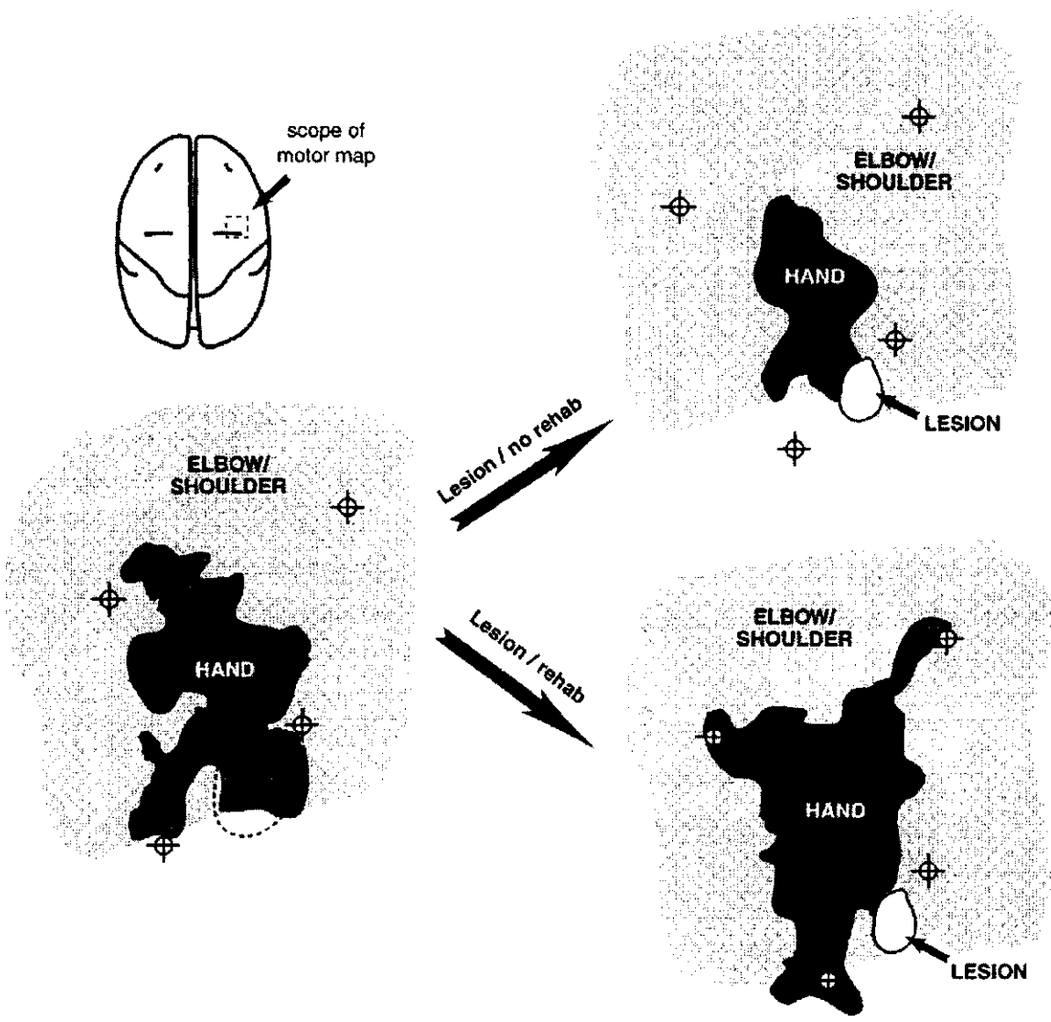

Figure 1-2. Functional remodeling of the hand representation in the motor cortex a few months following an ischemic infarct. In animals that did not receive any rehabilitative training the remaining hand representation was invaded by more proximal limb representation (elbow/shoulder). Animals that underwent intensive retraining of motor skill showed an expansion of the hand representation at the expense of the elbow/shoulder representations (From: *Mol. Psychiat.* 2:118, 1997).

Present Study: objectives and hypotheses

The main objective of the current thesis was to investigate the effects of factors that modulate brain plasticity on functional and structural restitution following stroke. Specifically, three different (and novel) treatments were selected based on previous observations that the administration of these treatments induced dendritic changes in intact animals. Given that other factors that stimulate dendritic changes also promote functional restitution after brain injury we predicted that these novel treatments would also improve functional outcome following stroke in rats.

The first treatment was a novel COX-2 inhibitor drug (NS398) that was administered to the rats through diet. The COX-2 enzyme is responsible for the conversion of arachidonic acid into prostaglandins within the brain, and its expression is significantly upregulated following stroke. In aged rats, chronic administration of NS398 has previously been shown to induce dendritic alterations that were also associated with a reversal of age-related cognitive decline. We therefore were interested in whether chronic NS398 administration would also induce dendritic changes following stroke, and whether this change would improve functional outcome. In order to confirm a correlational relationship between the dendritic changes and any behavioural effect of the chronic drug treatment, a second experiment was carried out where NS398 was given only acutely after the stroke. Acute treatment is insufficient to produce dendritic changes in the brain, however it did allow us to determine the effects of inhibiting the production of prostaglandins acutely after a stroke. In addition, prostaglandins serve as pro-inflammatory signaling molecules, therefore blocking the production of prostaglandins allowed us to investigate the role of acute inflammation on recovery from stroke.

The second novel treatment was a diet enriched in various vitamins and minerals. This diet has previously been shown to improve outcome following perinatal frontal cortex injury in rats (Halliwell, 2003), and is also known to ameliorate the symptoms of several psychiatric conditions in human patients (Kaplan et al., 2001; Kaplan et al., 2002). Based on the fact that the previously observed beneficial effects of the vitamin supplement were also associated with an increase in dendritic length of cortical neurons, we predicted that this same treatment would also be beneficial following stroke. Specifically, we predicted that the diet would cause an increase in dendritic length in animals both with and without motor cortex lesions, and that the treated animals would also show better functional improvement following a motor cortex lesion relative to untreated animals.

The third and final treatment was a form of social experience that we believe engages frontal brain circuitry. In previous experiments we found that if the cage-partners of rats are rotated every 48 hrs., the animals spend significantly more time engaged in social behaviour relative to animals that are always housed with the same cage partner. We also found this experience to cause significant dendritic changes in a frontal cortical region that is involved in social behaviour. Based on this finding we predicted that inducing dendritic changes in the frontal cortex through social experience would serve as a beneficial treatment for motor cortex stroke.

Although strokes are more prevalent in the aging population, the current set of experiments were carried out in rats that were approximately equivalent in age to a young adult. To carry out a similar set of experiments in aged rats would require the addition of

several control groups, as there are both anatomical and behavioural are-related changes that may interact with some of the treatment effects.

Behavioural and Anatomical Procedures

I) Schallert Cylinder Task

The cylinder task (also referred to as the forepaw asymmetry task) was developed by Tim Schallert (Schallert et al., 1997) to assess functional impairment following unilateral brain injury. To do this, rats are placed inside a clear cylinder for 5 minutes, and the exploratory behaviour of the animals is video recorded through a slanted mirror that is placed underneath the cylinder. Rats will usually explore the inside of the cylinder by tactile paw placements around the surface of the cylinder. Intact animals do not usually have a preferred paw on which to support their body weight, whereas animals with unilateral strokes will favor the non-damaged paw to support themselves.

Fugure 1-3. The Schallert cylinder task.

II) Forepaw Inhibition

While swimming, rats have been found to totally inhibit their forepaws and only use their hindpaws and their tail to propel themselves forwards. Unilateral lesions of the frontal cortex have been found to disrupt the inhibitory brain circuitry controlling this behaviour and therefore cause rats to commit unilateral forepaw strokes on the side opposite of the lesion (Kolb and Whishaw, 1981). To evaluate forepaw inhibition, rats were required to swim the length of an aquarium (approximately 1.5 m) to a visible platform located at the other end. The number of forepaw strokes that were committed with each paw were counted.

Figure 1-4. The forepaw inhibition task.

III) Whishaw Tray Reaching

The ability of the rats to use their forepaws to perform a skilled behaviour was quantified by training the animals on a reaching for food task. The rats were required to reach through a wall of vertical bars to retrieve granulated pieces of food from a trough placed on the other side of the bars (Whishaw and Kolb, 2005). In order for a reach to be successful, the rat had to be able to place the retrieved food directly into the mouth, or else it would fall irretrievably through the wire-mesh floor of the cage. An endpoint measure of reaching success was calculated by dividing the total number of successful reaches by the total number of reach attempts within a 5 minute testing session.

Figure 1-5. The Whishaw tray reaching task.

IV) Whishaw Single Pellet Reaching

This reaching task is analogous to the tray-reaching task in that the rats are required to use their forepaws to retrieve food. However, this version of the task requires more skilled prehension as the target is a single food pellet that is placed on a shelf located on

the outside of the testing box. The animals are trained to approach the front of the reaching box and use their preferred paw to retrieve and consume the single food pellet from the horizontal shelf in front of them. In addition to the endpoint measure of reaching success, the various components of the reaching movement may be analyzed through frame-by-frame video analysis, therefore providing a detailed description of the motor impairments of the animals (Whishaw et al., 1991).

Figure 1-6. The Whishaw single pellet reaching task.

V) Golgi-Cox Analysis

The Golgi technique, originally developed by Camilo Golgi in the late 1800's, allows for the visualization of cells in the brain through the deposition of a heavy metal (originally silver) on the surface of cells. With recent modifications, namely using mercury as a heavy metal, the procedure can be used to completely impregnate approximately 2-5% of the cells in the brain (Gibb and Kolb, 1998). The post-synaptic material of neurons can than be analyzed by first creating a two-dimensional representation of the dendritic structure through the *camera lucida* technique, and than

estimating the total dendritic length through the Scholl analysis. Although more recent techniques have been developed for visualizing the dendritic profile of cortical cells (Zuo et al., 2005), the Golgi technique is still advantageous because it facilitates the analysis of a large number of cells from many different cortical regions.

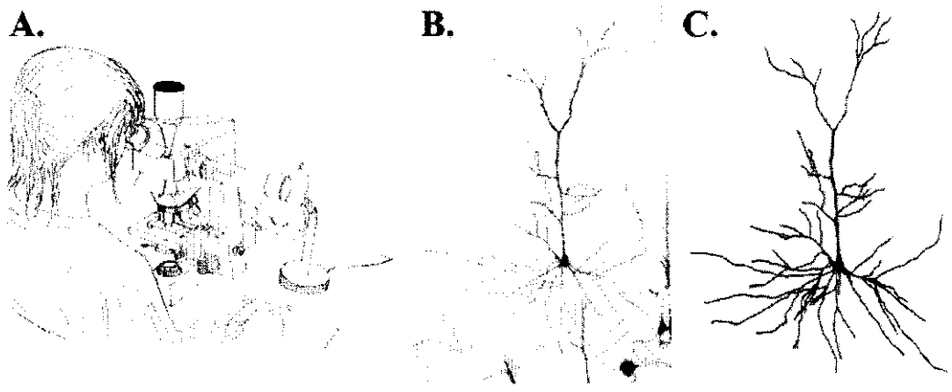

Figure 1-7. Through the use of a light microscope that is equipped with a drawing tube, a cell drawer can trace onto a piece of paper the dendritic structure of individual neurons, thus creating a two dimensional representation of the cell and all its processes (A). The drawing tube allows the cell drawer to view the image of the cell (B) and the drawn representation of the cell (C) at the same time.

VI) Assessment of Lesion Volume

Coronal sections through the lesion area were used to estimate lesion volume by comparing the area of the remaining tissue in the lesion hemisphere to that in the undamaged hemisphere. The resulting value was converted to percentage, and therefore served as an indirect measure of infarct volume relative to the undamaged hemisphere.

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Chapter 2

Chronic treatment with the COX-2 inhibitor NS398 induces cortical plasticity and limited functional improvement after motor cortex stroke

Abstract

The cyclooxygenase-2 (COX-2) enzyme is part of the inflammatory pathway and is induced within the brain by a variety of pathological events, including ischemia. Pharmacological agents that block the function of the COX-2 enzyme have been found to be neuroprotective in a number of injury models, and long-term administration of these drugs has been shown to induce plastic changes in the brain. In the current experiment, we investigated the effectiveness of stimulating cortical plasticity following stroke injury through the administration of a novel COX-2 inhibitor drug (NS398). Furthermore, we determined whether the induced plastic changes improved functional outcome following the motor cortex stroke. Chronic drug administration was found to induce dendritic hypertrophy in cells in the parietal cortex, and this anatomical change was associated with the animals making significantly more reach attempts, as well as successful reaches during a skilled reaching task. Additional motor tests however revealed that the treatment did not affect the level of motor recovery, as the animals showed chronic impairments in the Schallert cylinder, and the forepaw inhibition tasks. Short-term administration of the drug, immediately following the stroke did not induce any dendritic changes, nor was it found to influence behavioural performance on any of the motor tasks. Based on these results we conclude that the plastic changes that are induced by long term COX-2 inhibitor administration provide some benefit to functional outcome following ischemic cortical injury.

Introduction

Ischemic brain injury in clinical patients is often associated with chronic motor impairments. Although there is partial resolution of these symptoms, recovery is rarely complete, therefore studies have been aimed at investigating the mechanisms mediating the partial recovery, with hopes of improving it through the application of rehabilitative treatments. Recent evidence from both human imaging studies (Barber et al., 2001), as well as experimental stroke models (Frost et al., 2003) suggests that the resolution of the secondary effects of the injury (such as edema) as well as the initiation of plastic processes in remaining cortical regions facilitate functional improvement. Treatment strategies, therefore, have been targeted at enhancing these mechanisms with hopes of improving neurological function. One potential treatment that has recently been shown to affect both the inflammatory pathway as well as synaptic plasticity is the administration of cyclooxygenase-2 (COX-2) inhibitors.

COX-2 is the inducible form of the rate-limiting enzyme that catalyzes the biosynthesis of prostaglandins from arachidonic acid. The basal expression of COX-2 has been localized by immunolabeling techniques to the cell bodies, dendrites and spines of granule and pyramidal cells of the hippocampus, amygdala, and a small number of neurons in neocortical layers II/III (Kaufmann et al., 1996). Other, non-neuronal cells, such as microglia and endothelial cells of the brain vasculature also express the COX-2 gene when stimulated by cytokines such as interleukin-1ß (Cao et al., 1996; Bauer et al., 1997; Inoue et al., 2002). The induction of neuronal COX-2 by synaptic activity has been demonstrated in experiments where the infusion of tetrodotoxin (TTX) into one eye was found to significantly reduce COX-2 mRNA in the deafferented visual cortex (Yamagata

et al., 1993). Developmental studies indicate that the profile of COX-2 expression parallels the formation of excitatory synapses, and inhibiting cellular activity through administration of the *N*-methyl-*D*-aspartate (NMDA) antagonist MK-801 reduces COX-2 expression (Yamagata et al., 1993). Electrophysiological studies have shown that COX-2 is upregulated following hippocampal kindling (Tu and Bazan, 2003) and that selective COX-2 inhibitors block the induction of LTP (Shaw et al., 2003) and LTD (Murray and O'Connor, 2003). Administration of the selective COX-2 inhibitor NS398 to adult rats within two hours of behavioural training was also found to impair memory formation, although delaying the injections until two hours after the training had no effect on memory (Teather et al., 2002; Shaw et al., 2003). Additional studies have demonstrated that the long-term administration of NS398 to aged rats improved age-related spatial deficits and was also associated with hypertrophic dendritic changes in the neocortex and the hippocampus (Drott et al., 2002). There are thus converging lines of evidence suggesting that COX-2 is involved in the modulation of synaptic plasticity in the intact brain.

COX-2 is also strongly induced by pathological events such as spreading depression (Koistinaho and Chan, 2000), excitotoxic lesions (Adams et al., 1996), and cerebral ischemia (Sairanen et al., 1998). The mechanisms inducing COX-2 expression in complex pathologies, such as those following stroke, are driven by the activation of two temporally overlapping pathways: 1) the activation of NMDA receptors due to massive glutamate release (Koistinaho and Chan, 2000); and, 2) the production of inflammatory cytokines resulting in increased intracranial pressure and edema (Nogawa et al., 1997). The significance of this large-scale upregulation of COX-2 is currently the

focus of a large number of studies but the results appear to support conflicting theories. For example, (Nogawa et al., 1997) have shown that the up regulation of neuronal COX-2 contributes to ischemic brain damage following middle cerebral artery occlusion (MCAO) in rats, and Sugimoto and Iadecola (Sugimoto and Iadecola, 2003) demonstrated that COX-2 inhibition by NS398 reduced infarct volume and improved neurological deficits in ischemic mice. In contrast, studies by Hara *et al.* (Hara et al., 1998) found that NS398 had no effect on infarct volume following MCAO, and Baik *et al.* (Baik et al., 1999) showed that the treatment aggravated seizure activity and cell death following kainic acid-induced seizures.

These conflicting results may be partly accounted for by the fact that the above studies used different injury models and different measures of outcome (lesion volume vs. gross neurological assessments) to draw conclusions about the role of COX-2 induction. A recent study by Gonzalez & Kolb (Gonzalez and Kolb, 2003) has shown that even within the various stroke models, the distal cellular effects of the stroke may be completely different depending on the exact etiology of the injury. Thus, it is likely that the effect of COX-2 inhibitors will also wary depending on the injury model used. In addition, the time course of COX-2 inhibitor drug administration also has differential cellular and behavioural effects (Gobbo and O'Mara, 2004) and should therefore be systematically investigated for each injury model.

The current set of experiments investigated the effects of the selective COX-2 inhibitior NS398 on ischemic injury to the rat motor cortex. Specifically, experiments were carried out that: 1) described the profile of COX-2 induction following unilateral pial-strip lesions of the motor cortex 2) assessed the effects of chronic (pre- and

poststroke) and acute (poststroke) treatment with NS398 on functional outcome following stroke 3) determined the effects of NS398 treatment on dendritic morphology of cortical pyramidal cells as well as the effect of the drug on lesion volume.

Methods

The current experiment used 48 adult male Long-Evans rats that were born and raised at the University of Lethbridge vivarium. For the behavioural testing portion of the experiment, 40 animals were divided equally into 5 groups: 1) control and no drug, 2) stroke and no drug, 3) control and NS398, 4) stroke and NS398 (pre- and poststroke), and 5) post-stroke NS398. The remaining 8 rats did not undergo behavioural testing, and instead were used to investigate the expression of COX-2 following the ischemia. Animals were maintained on a 12 hr. dark/light cycle and except for the food restriction period, food and water were available *ad lib*. During food restriction, each animal received only 30g of food inside the home cage per day.

Drug administration

NS398 (Cayman Chemical No. 70590) was administered to the animals via the rodent medicated dosing system available from Bio-Serv (Frenchtown, New Jersey). The drug was incorporated into 5 g bacon flavoured tablets that were placed inside the home cage and were readily consumed by the animals. The administration of NS398 within two hours of behavioural training has been shown to interfere with consolidation of the recently learned information (Teather et al., 2002), therefore in the current experiment drug administration was regularly performed daily at a dose of 2 mg/kg at least two hours

following behavioural testing (See appendix 1 for a detailed experimental timeline of drug treatment).

Pial-strip lesions of the motor cortex

Following the pre-training period, half of the animals received motor cortex lesions in the hemisphere contralateral to the reaching paw (Gonzalez and Kolb, 2003). For this procedure, the animals were anesthetized with somnotol (65 mg/kg) and positioned in a stereotaxic apparatus that was equipped with an Isoflurane anesthetic machine. The level of anesthesia was maintained at a constant level throughout the procedure by varying the level of the gas anesthetic. A dental drill was then used to create a cranial window extending 3 mm anterior, 2 mm posterior and 3 mm lateral to bregma (1 mm lateral from the midline). The exposed dura was carefully removed and the underlying vasculature was wiped away with a saline-soaked cotton swab. The incision was sutured shut and the animals were allowed to recover overnight in individual cages before being returned to the colony with their original cage partners.

Behavioural Testing

Whishaw tray reaching. During the training period food-deprived animals were placed individually into the reaching boxes for 30 minutes a day for 14 days. The front wall of the boxes was constructed of 2 mm vertical bars spaced 9 mm apart while the floor of the cage was constructed of wire mesh. The rats were required to reach between the vertical bars and retrieve pieces of chicken feed that were available in a 4 cm wide and 0.5 cm deep tray on the outside of the cage. If the animals grasped food with their forepaws but

then failed to place it directly into their mouth, the food would fall irretrievably through the wire mesh flooring thereby preventing the accumulation of food on the floor of the cage. Following the two weeks of reach training, all subsequent testing sessions were limited to 5 minutes and were video recorded to allow for an accurate determination of reaching success. A reach attempt was defined as any forward reaching motion by the paw once the paw was inserted through the bars. Using this definition it was possible for an animal to make several reaching attempts while only inserting the paw once through the bars. A hit was recorded when an animal successfully grasped for food and was able to place at least some of the food into its mouth. The pre-lesion performance of all animals was determined the day prior to inducing the lesions, and post-lesion performance was monitored once a week for 6 weeks. Animals were forced to use the injured paw by wrapping a bracelet around the intact paw that prevented it from being inserted through the bars of the reaching cage.

Limb-use asymmetry test. Animals were individually placed in a transparent cylinder 20 cm in diameter and 30 cm in height for 5 minutes set on a transparent table. A mirror placed at an angle below the cylinder allowed for the video recording of the animals' vertical exploration patterns. When placed inside the cylinder, animals spontaneously reared and investigated the wall of the cylinder through tactile paw placements. A paw preference ratio was determined once a week during the post-lesion testing period by counting the number of initial wall contacts that were supported by the unaffected, the affected and both (simultaneous) limbs. An asymmetry score was calculated as the number of limb touches with the affected paw plus ½ the number of simultaneous

touches, divided by the total number of observations (unaffected plus affected plus both). Using this formula, a symmetry score that is about 0.5 indicates that the animal used both limbs to explore the cylinder, whereas a score of less than 0.5 indicates a decreased use of the affected limb. This scoring system has been shown to enhance sensitivity and reduce variability in the results (Woodlee et al., 2005).

Forepaw Inhibition. Animals were trained for 3 days to swim to a visible platform located at the end of a rectangular aquarium (120 x 43 x 50 cm). The water was maintained at a temperature of 27° C and by the third day the rats had learned to abandon exploring the aquarium walls and swam directly to the visible platform. When swimming in a straight line, rats exclusively use their hindpaws to achieve forward movement, and only use their forepaws for changing direction or stopping (e.g., Kolb and Whishaw, 1983). For each testing session at least three swimming trials were video recorded in which the animals did not make contact with any of the side walls during the swim. The number of paw strokes was counted for each of the forepaws before, as well as on a weekly basis for 6 weeks following the stroke.

Anatomical procedures

Golgi-Cox staining. All animals were sacrificed approximately 50 days after surgery by administering an overdose of sodium pentobarbital followed by an intracardiac perfusion with 0.9% saline. The brains were immediately removed from the skull and processed for Golgi-Cox staining (Gibb and Kolb, 1998). Pyramidal cells from layer III of the parietal cortex adjacent to the cortical injury (Zilles' Par 1) were traced onto paper at

250X through a drawing tube. The morphology of these cells has been shown to be related to functional recovery following such lesions (Rowntree and Kolb, 1997). In order for a cell to be considered for analysis: 1) it had to be well impregnated and unobstructed by other cells or blood vessels 2) both the apical and basilar trees had to be complete, with no broken or missing branches. The first five cells that fit the above criteria were drawn from each hemisphere. The cell drawings were than analyzed using the Sholl method (Sholl, 1956), and the total dendritic length of both the apical and basilar trees was calculated. Given that the animals received extensive unilateral skilled reach training that might influence dendritic arborization, initial analyses were carried out using hemisphere as a factor. In cases where there were no significant hemisphere effects, the data were collapsed across hemispheres.

Immunohistochemistry. The effect of the motor cortex stroke on COX-2 expression was investigated through immunohistochemical labeling. Animals were perfused with 0.9% buffered saline followed by fresh 4% buffered paraformaldahyde. The brains were removed and post-fixed overnight in perfusate. Free-floating Vibratome sections were incubated for 20 minutes in a 0.3% H_2O_2 solution, followed by an overnight incubation in a phosphate buffer solution (PBS) containing 0.3% Triton-X100, 3% goat serum and the primary antibody at a concentration of 1:1000 (COX-2 murine polyclonal antibody, Cayman Chemical, Cat. #160106). Following three PBS washes the sections were incubated in a biotinylated anti-Guinea Pig secondary antibody (1:1000, Vector Laboratories) for 1 hr, washed, and incubated for 45 minutes in the ABC reagent (1:1000, Vector Laboratories). Finally, the labeling was visualized with DAB (Sigma) and the

sections were mounted on subbed slides, dehydrated in progressive alcohol baths and coverslipped.

Lesion volume. To determine if NS398 had an effect on infarct size, lesion area was quantified at seven different planes in Golgi-Cox stained coronal sections of the brain. This was done by capturing digital images of the sections of interest and then outlining the injured and uninjured hemispheres on a computer screen. Using the NIH Image program (v1.62) the cross-sectional area of both the injured and uninjured hemispheres was measured at each plane of analysis. The measurements were summed across planes and the difference between the remaining area of the injured and the uninjured hemispheres was calculated and multiplied by 100. The resulting value served as an indirect measure of infarct volume, as it expresses the area of remaining tissue in the injured hemisphere as a percent of the contralateral, intact hemisphere.

Statistical analyses. Using group as a factor, one-way analyses of variance (ANOVAs) and repeated measures ANOVAs were used to identify group differences in the behavioural tasks and the anatomical measures. Where appropriate, Fisher's PLSD *post hoc* tests were carried out to investigate specific group comparisons.

Behavioural Results

Whishaw tray reaching. Following the two-week training period all animals learned to reach for food, and as a group, reaching success plateaued at around 60%. During the post-lesion testing period animals that did not receive lesions continued to perform at this

level, with little fluctuation. In contrast, animals that received strokes showed a marked impairment the first week after the surgery, dropping to about 20% accuracy, followed by slow improvement in function on subsequent testing sessions (Figure 2-1). Neither regimes of the COX-2 inhibitor administration had an affect on reaching success, although the long-term administration of the drug did affect the reaching habits of animals with lesions. Specifically, the stroke+NS398 group made significantly more reaching attempts and also succeeded in retrieving more food during the five minute testing session than untreated stroke animals (Figure 2-2). In fact, stroke animals that also received the long-term NS398 treatment were indistinguishable from control animals in terms of the number of times they were able to bring food to their mouth during the testing sessions.

A repeated-measures ANOVA of reaching success revealed that there was a main effect of Group $F(4,34) = 30.615$, $P < 0.0001$ and a significant group x time interaction ($P = 0.0002$) that was driven by the partial recovery in the lesion groups. *Post hoc* analyses showed that all of the stroke groups (regardless of treatment) were impaired relative to controls $F(4,34) = 30.615$, $P < 0.0001$. A repeated measures ANOVA of the number of reach attempts showed a significant main effect of Group ($F(4,34) = 8.162$, $P = 0.0001$) which resulted from the stroke+NS398 group making significantly more reaching attempts than each of the other groups (P's < 0.05 or better). The group x time interaction, however, was not significant ($P = 0.2818$). Analysis of the number of hits (the number of times the animals were able to bring food to the mouth) indicated a main effect of Group ($F(4,34) = 7.190$, $P < 0.0003$) and a significant group x time interaction ($P < 0.0001$). This interaction is driven by the fact that the increase in the number of hits

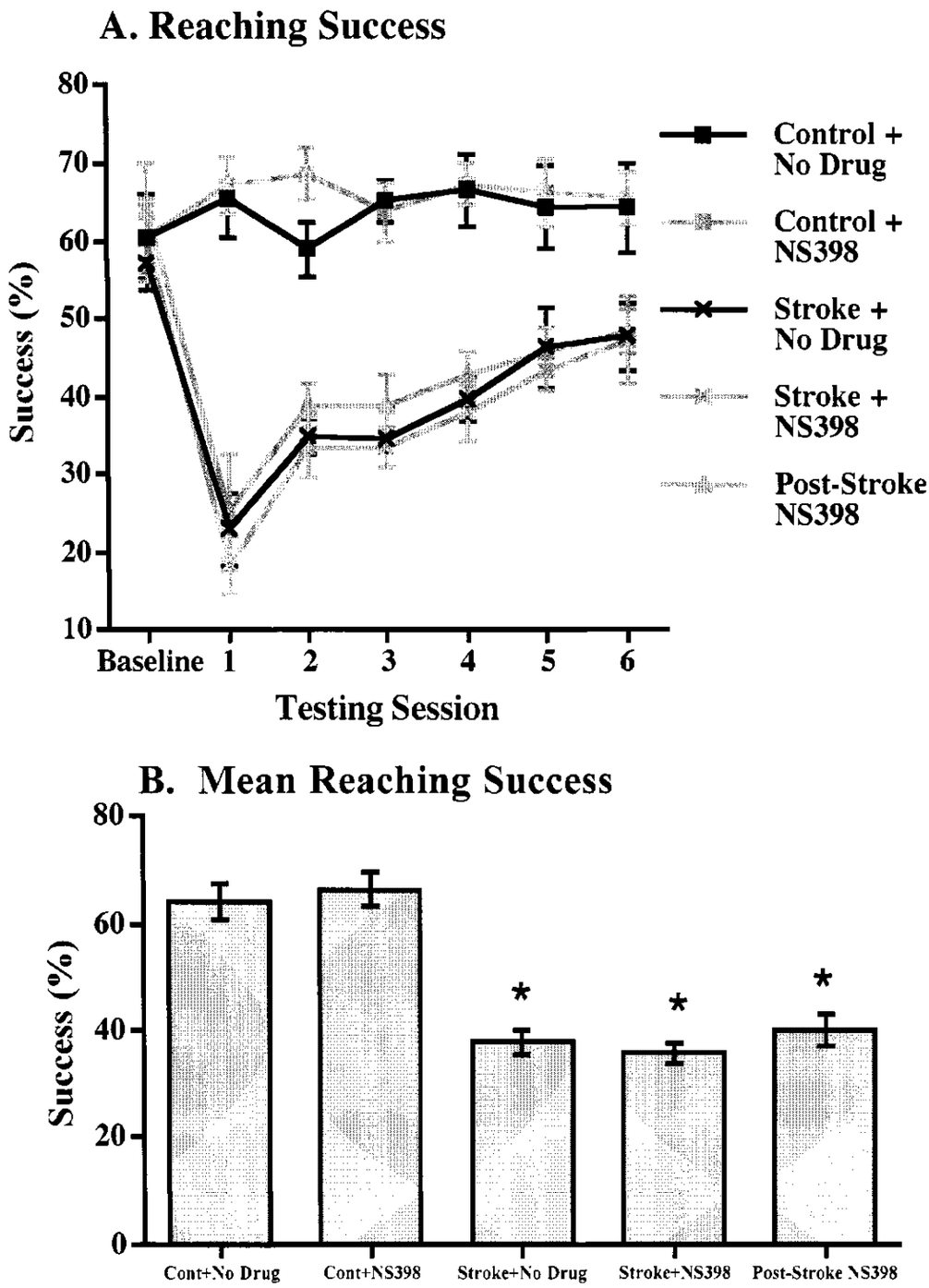

Figure 2-1. Reaching success in the Whishaw tray reaching task for individual testing sessions (A) and mean post-lesion performance (B). The lesion resulted in a significant deficit in all post lesion test sessions, and none of the treated groups showed a significant benefit of the treatment.

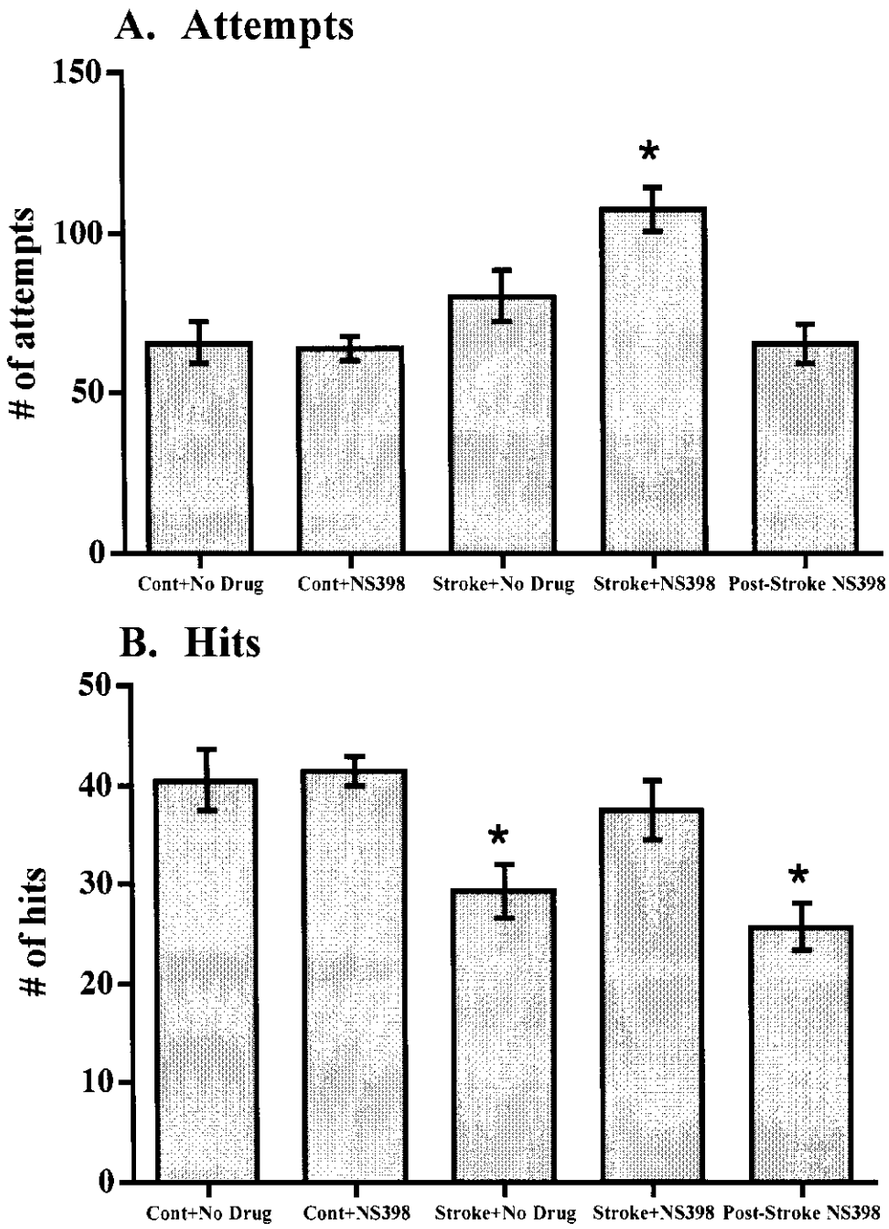

Figure 2-2. Indicates the mean number of reach attempts (A), and the mean number of successful reaches or hits (B) for each group. Only the stroke-NS398 group showed a significant increase in the number of reach attempts and the number of successful reaches. In fact, the stroke+NS398 group made the same number of successful reaches as the non-lesion control groups.

by the stroke+NS398 group was not present immediately after the stroke injury, but instead developed gradually over the treatment period. Post *hoc* analyses indicated that only the no treatment stroke, and the post-stroke NS398 groups made fewer hits (P = 0.0316 or better). The number of hits by the NS398+stroke group did not differ significantly from controls ($P > 0.2763$).

Limb-use asymmetry test. Animals that did not receive lesions explored the cylinder by tactile placements of both forepaws. Animals with lesions showed a significant decrease in use of the affected forepaw, and the drug treatment did not alter this behaviour (Figure 2-3).

A repeated-measures ANOVA indicated a main effect of Group ($F(4,34)$ = 12.773, $P < 0.0001$) and that the group x time interaction was not significant (P = 0.6075). *Post hoc* analyses showed that all lesion groups were impaired relative to controls ($P = 0.0012$ or better).

Forepaw inhibition. As can be seen in Figure 2-4, animals that did not receive lesions mostly inhibited their forepaws during swimming, whereas lesion animals made significantly more forepaw strokes with the impaired (contralateral) limb.

A repeated-measures ANOVA indicated a main effect of Group ($F(4,34)$ = 28.840, $P < 0.0001$), however, the group x time interaction was not significant (P = 0.2856). *Post hoc* analyses showed that all lesion groups were impaired relative to the controls ($P < 0.0002$ or better). Furthermore, the impairment in the post-stroke NS398 group was significantly greater relative to the stroke+NS398 group ($P = 0.0087$).

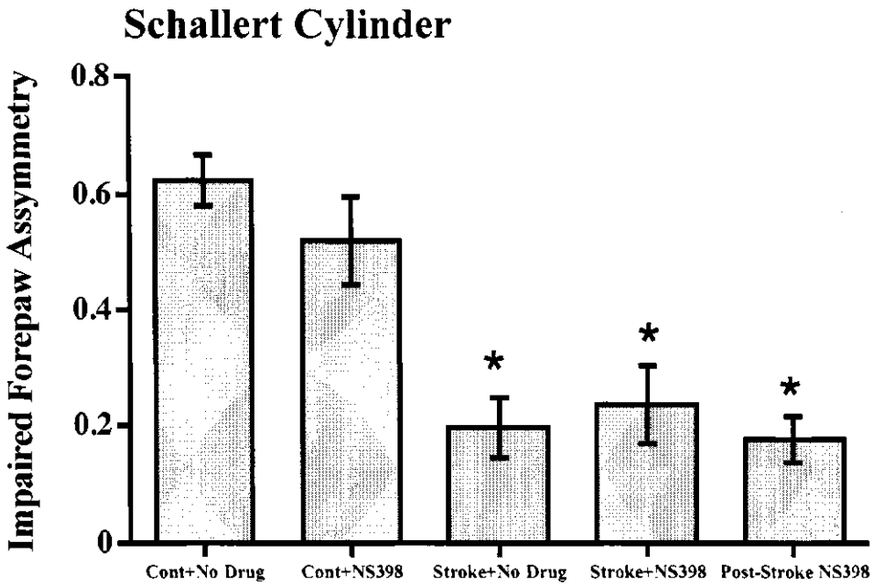

Figure 2-3. Mean post-surgical forepaw asymmetry in the Schallert cylinder task. The lesion induced a significant decrease in the use of the affected forepaw, and the treatments did not have an effect on this impairment.

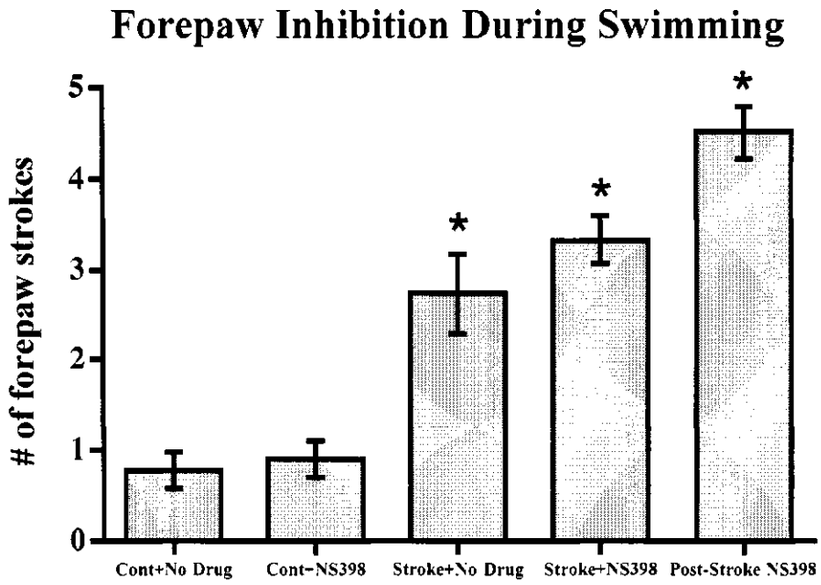

Figure 2-4. Indicates the mean number of paw strokes with the impaired forepaw. All stroke groups performed significantly more forepaw strokes relative to the non-lesion control groups, with the post-stroke NS398 group showing a significantly larger impairment than the other groups.

Anatomical Results

Lesion volume. The cross sectional area of the lesion hemisphere was about 90-95% of the area of the undamaged hemisphere across the different lesion groups (Figure 2-5). Long-term NS398 treatment was associated with a slight reduction in the area of the remaining tissue, whereas post-lesion NS398 treatment had no effect. An ANOVA indicated a main effect of group ($F(2,20) = 4.148$, $P < 0.0311$) and *post hoc* analyses showed a significant decrease in the cross-sectional area of the remaining tissue in the stroke+NS398 group ($P = 0.0095$).

Golgi-Cox dendritic analysis. The cortical cells of animals that received long-term NS398 treatment (stroke+NS 398) showed a significant increase in dendritic length compared to non-treated animals, or the post lesion NS398 group (Figure 2-6). The effect of the treatment was found to be similar between the apical and basilar dendrites as the area (apical or basilar) x treatment interaction was not significant ($F(4,39) = 4.006$, $P = 0.1936$). For subsequent analyses the values from the apical and basilar measures were summed to yield a measure of total dendritic length. An ANOVA on the total dendritic length indicated a main effect of Group ($F(4,39) = 4.006$, $P < 0.0081$) and *post hoc* analyses showed that the long-term use of NS398 in both the control and stroke animals increased dendritic length whereas the post stroke NS398 did not (P's <.05 or better).

Immunohistochemistry. – Previous experiments have shown that COX-2 expression in the normal brain is restricted mostly to the hippocampus, amygdala, piriform cortex and at minimal levels in other cortical regions (Kaufmann et al., 1996). Our results confirm this

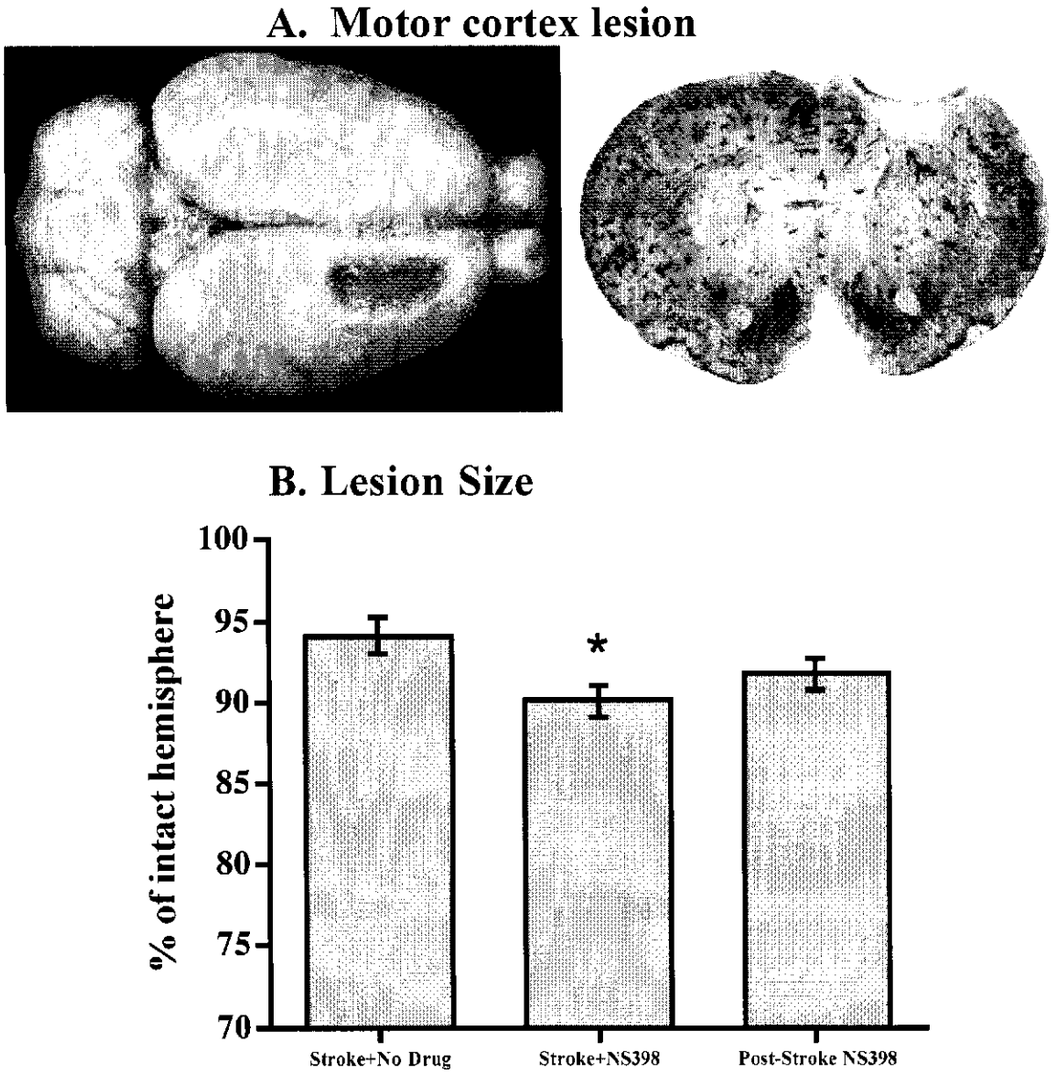

Figure 2-5. Representative picture of lesion size and location (A), and the quantification of the area of the remaining tissue in the injured hemisphere relative to the area of the intact hemisphere (B). This value is an indirect measure of lesion size, and the chronic NS398 treatment caused a slight but significant increase in lesion size.

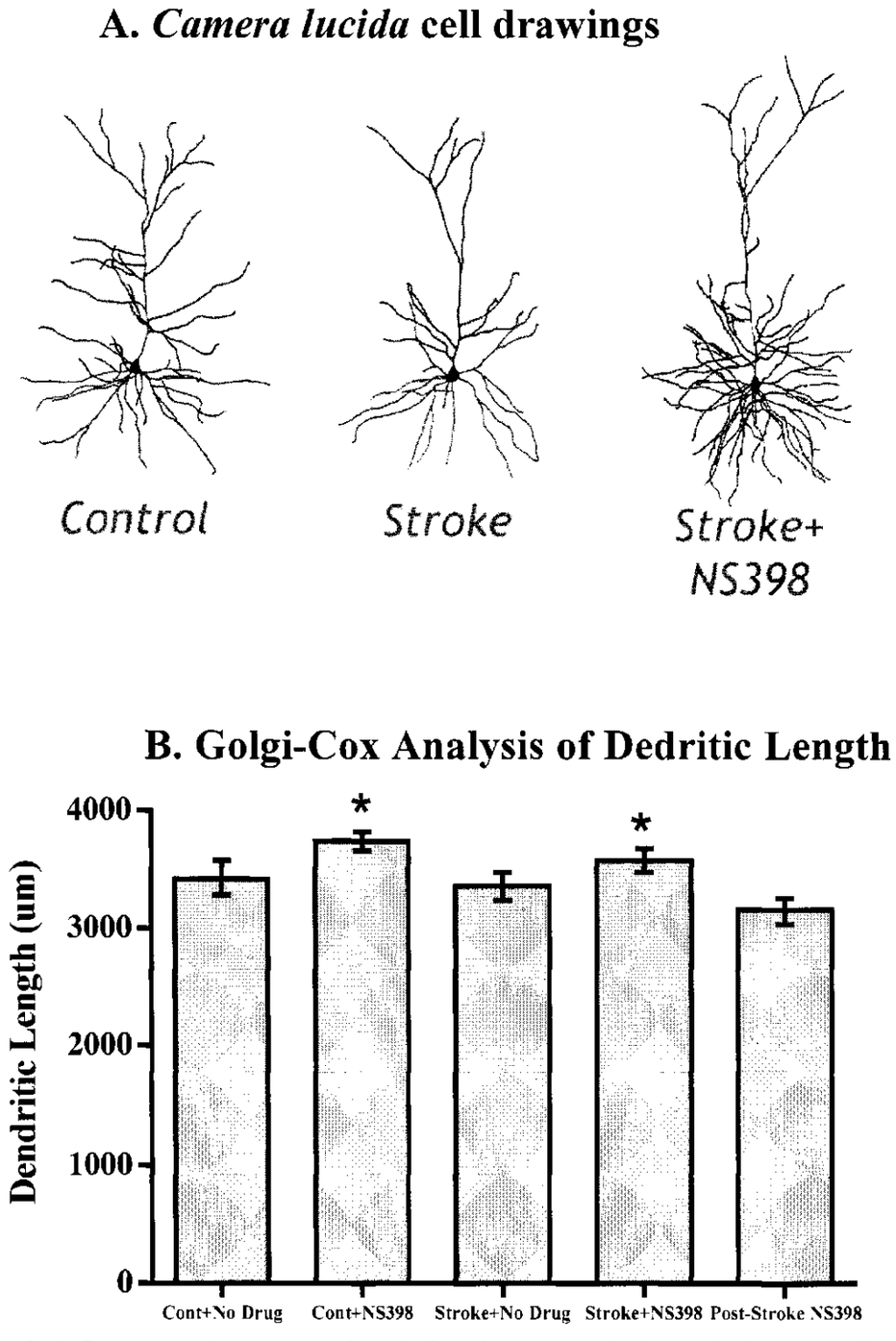

Figure 2-6. Representative *camera lucida* drawings of cortical cells in layer III of Par1 (A). NS398 treatment caused a significant increase in dendritc length in both control and stroke animals (B).

pattern of expression and provide additional findings regarding COX-2 expression following stroke. The upregulation of COX-2 was greatest in cortical regions neighboring the infarct, however distal cortical regions also showed COX-2 induction in the injured hemisphere. As can be seen in Figure 2-7, superficial layers of the piriform cortex, parietal cortex, and visual cortex showed the greatest expression among the distal ipsilateral cortical sites. COX-2 expression in the intact hemisphere as well as the hippocampus of the injured hemisphere was not altered by the injury, and there was no detectable immunoreactivity in the striatum and white matter.

Discussion

The current experiment investigated the effects of COX-2 inhibition on functional restitution, lesion volume, and dendritic arborization following unilateral motor cortex injury. Immunolabeling techniques demonstrated that COX-2 expression is significantly upregulated in the ischemic hemisphere following a unilateral lesion, however, blocking this upregulation through acute administration of NS398 did not induce motor recovery following the injury. Chronic drug treatment did produce significant changes in reaching behaviour that allowed animals to reach an end point performance that was similar to pre-lesion levels. Anatomically, the chronic treatment also produced dendritic changes in both the intact and the brain-injured animals.

The upregulation of COX-2 has been previously observed in animal models of cerebral ischemia (Dore et al., 2003; Yokota et al., 2004) as well as clinical patients (Sairanen et al., 1998), however, the function of this upregulation is still under investigation. Induction of COX-2 mRNA in animal models has been shown to occur in

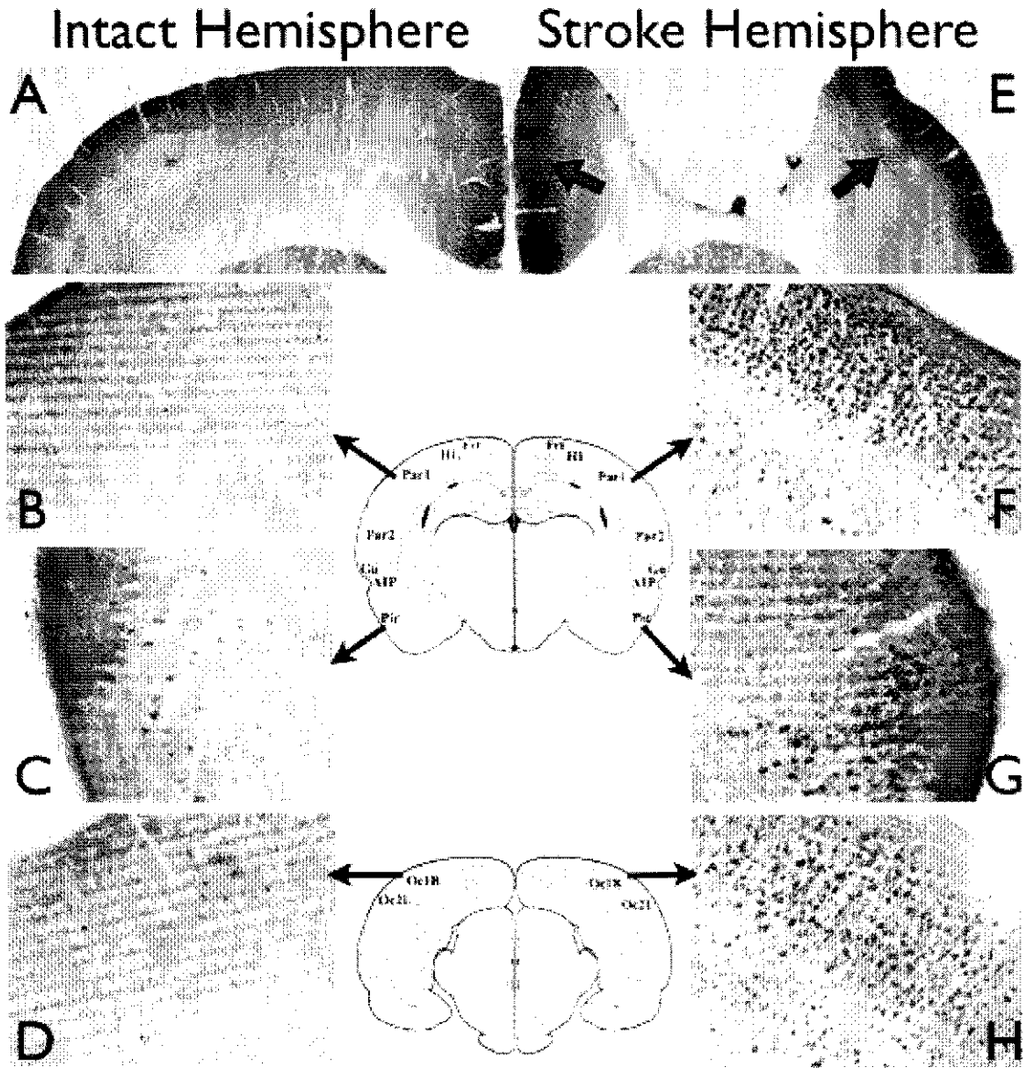

Figure 2-7. Immunolabeling of COX-2 in cortical regions of the intact (A-D) and the injured hemisphere (E-H). There was consistent induction of COX-2 immunoreactivity in the injured hemisphere around the lesion site (B), in parietal cortex (F), in piriform cortex (G) and in occipital cortex (H).

cases of both mild ischemia, where there were no signs of edema or cell death, as well as in models of severe stroke, where there was significant cell death and swelling (Nogawa et al., 1997). The fact that COX-2 was significantly upregulated in our current model of ischemia suggests that the pial-stip model is suitable for investigating the effects of COX-2 inhibitor drugs following stroke. Our finding of COX-2 induction in cortical regions distal from the lesion (such as posterior parietal, piriform, and occipitalcortex) as well as cortical regions neighboring the lesion suggests that the induction of COX-2 is unlikely to be directly related to ischemic cell death.

NS398 administration did not appear to affect the use of the impaired paw following stroke as the animals had chronic impairments in the cylinder task and also failed to inhibit the injured forepaw when swimming to a visible platform. The reaching task however, revealed that the drug treatment did indeed influence the motor behaviour of the animals, although in a surprising way. Animals that received the chronic drug treatment were able to execute significantly more successful reaches within the five-minute testing sessions relative to untreated stroke animals, by attempting significantly more reach attempts. If one considers the ability to consume the food pellets as the endpoint measure of the tray reaching task, the results of the current experiment demonstrate that: 1) stroke injury is associated with a significant deficit in endpoint measures during tray reaching and 2) chronic NS398 treatment is effective at ameliorating this deficit. Previous studies investigating the effects of other stroke treatments (such as NGF) found that the treatment-induced dendritic hypertrophy resulted in improved functional recovery in the same reaching task used here. In contrast to the current experiment, however, the dendritic changes induced by NGF were observed in

remaining motor regions as opposed to the posterior somatosensory cortex. These results suggest that dendritic plasticity in motor regions may support the recovery of lost functions, whereas plastic changes in non-motor regions will likely support behavioural adaptations such as those observed in the current experiment.

Our finding that the drug treatment did not prevent the loss of brain tissue following stroke was also surprising as previous studies have shown beneficial effects of the drug on lesion volume in models of temporary ischemia (Hara et al., 1998; Nakayama et al., 1998; Sugimoto and Iadecola, 2003; Candelario-Jalil et al., 2004). In addition, studies carried out *in vitro* have demonstrated that NS398 is neuroprotective in models of both anoxia/ischemia (Wu Chen et al., 2004) and models of hypoxia/reperfusion (Vartiainen et al., 2001). Our *in vivo* model of permanent focal ischemia involves the removal of blood vessels from the surface of the brain, thus creating an infarct that is restricted to the cerebral cortex. Other injury models, such as MCA occlusion, are often associated with significant striatal and white matter damage (Tamura et al., 1981). Given that COX-2 is not expressed in the striatum or white matter a model of restricted cortical injury may be suitable when investigating the effects of a COX-2 inhibitor treatment on cerebral ischemia.

One characteristic of the pial-strip model is that while it is effective at creating a highly reproducible cortical infarct, the removal of the blood vessels also inhibits the delivery of pharmacological treatments to cells in the injured area. In our experiment NS398 was administered through diet, and previous studies have demonstrated that NS398 is reabsorbed in the digestive tract and readily passes the blood brain barrier (Drott et al., 2002). Although we are unable to determine how much drug was available

to the penumbral tissue, the fact that we found treatment induced dendritic changes in nearby cortical tissue confirms the presence of the drug in the vicinity of the lesion cavity.

The unavailability of the drug to cells at the core of the infarct may explain why in the current experiment we failed to show a neuroprotective effect of NS398. In fact, we found that chronic NS398 treatment slightly increased lesion volume as determined by the ratio of the surface area of the injured and the intact hemispheres. The effect of the drug on lesion volume, however, does not account for the increase in the number of reach attempts that was associated with chronic drug treatment. A simple regression of lesion size onto the number of reach attempts indicated a non-significant relationship between the two variables ($R = 0.361$, $P = 0.379$), where the difference in lesion volume accounted for only 13.1% of the variance in the number of reach attempts.

The dendritic analysis demonstrated that chronic NS398 treatment had a hypertrophic effect on the dendrites of cells in layer III of the parietal cortex in both control and stroke animals. A similar treatment schedule with the same drug has previously been shown to cause dendritic changes in aged animals, and was also associated with improved performance on cognitive tests (Drott et al., 2002). Our current finding of only a modest effect on motor performance following the drug treatment was somewhat surprising given that an increase in dendritic plasticity is generally associated with significant functional restitution following motor cortex stroke (Kolb et al., 1997; Johansson and Belichenko, 2002; Frost et al., 2003). One possible explanation of the current result is that the drug induced dendritic changes occurred before the lesion, thus inducing a behavioural sequelae different from that observed when plastic changes are

induced after the injury. This possibility is supported by the fact that neither the dendritic changes nor the behavioural adaptation were observed in the post-lesion treated group.

We should note, however, that during reach testing, animals with stroke+NS398 made significantly more reach attempts and were able to place food into the mouth more often relative to untreated stroke animals or those that only received the post lesion treatment. The fact that this behavioural adaptation evolved over the treatment period (i.e. was not present during the first week of post-lesion testing), and occurred only in the group of animals that also showed dendritic hypertrophy in parietal cortex suggests that the treatment-induced dendritic changes may have been related to the behavioural adaptation.

In summary, the current set of experiments provide further evidence for the recent observation that very few pharmacological treatments are able to induce true functional recovery following stroke (Diguet et al., 2004). Our finding that a treatment that enhances cognitive functions in aged animals has only limited effects on motor performance following motor cortex stroke suggests that cognitive and motor domains may respond differently to treatments that alter dendritic plasticity.

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Chapter 3

Differential effects of vitamin supplement treatment on plasticity in the intact and the ischemic brain

Abstract

Plastic changes in remaining brain regions are believed to support the partial spontaneous recovery of function that is observed following stroke. Increasing brain plasticity through a variety of treatments has previously been shown to enhance recovery, therefore the aim of the current experiment was to use a diet that has previously been shown to alter brain plasticity as a therapeutic treatment for stroke. Adult rats receiving either the vitamin supplemented diet, or a control diet were trained on three different motor tasks (Whishaw tray reaching, Schallert cylinder and forepaw inhibition) prior to receiving a unilateral devascularizing lesion of the motor cortex. Behavioural performance was monitored for six weeks following the injury, at which point a subset of the animals were trained and tested on a novel motor task, the Whishaw single pellet reaching task. Following the completion of behavioural testing the animals were sacrificed and the brains processed for Golgi-Cox dendritic analysis. Behaviourally, the vitamin supplement did not induce significant improvement following the injury on any of the motor tasks examined, nor did it influence the acquisition of the novel behavioural task after the injury. Anatomically, the vitamin supplement was found to induce dendritc growth of cells in layer III of the parietal cortex of animals that did not receive lesions, however it had no effect on animals with strokes. The stroke itself also induced dendritic atrophy in the same cell population on the lesion hemisphere only. Based on these results we conclude that the vitamin supplement fails to reverse the lesion induced dendritic atrophy and therefore fails to improve functional outcome following motor cortex stroke.

Introduction

The link between dietary habits and the occurrence of cerebrovascular disease, such as stroke, has been well established by epidemiological investigations. As a direct consequence of these studies, it is now possible for high-risk patients to go on diets that will decrease the chances of the occurrence of stroke. For example, we know that a diet low in saturated fat and cholesterol, low in sodium, high in potassium and calcium, and containing lots of fruits and vegetables is effective at reducing blood pressure and decreasing the risk of cerebrovascular disease (Spence, 2003). In contrast to our understanding of the effects of diet on stroke prevention, not much is known about the effects of dietary manipulations on stroke recovery. Initial studies investigating this phenomenon have reported that about 16% of stroke patients receiving hospital care are malnourished at the time of stroke onset (Unosson et al., 1994). Even more surprising is the finding that this number increases to 35% by the second week of the hospital stay (Davalos et al., 1996). The fact that undernourishment immediately after stroke is associated with reduced survival and functional ability (2003) merits further investigation into the clinical effects of dietary manipulations on stroke recovery. Recent clinical trials have shown that malnourished patients who receive oral nutritional supplements show significant beneficial effects of the treatment, however, those patients that are well nourished during recovery do not experience any added benefits of the supplements (Dennis et al., 2005). The question that remains, therefore, is whether other forms of dietary manipulations can be used to positively influence stroke outcome in well-nourished patients.

Given our current understanding of the mechanisms that facilitate recovery from stroke, it is likely that a diet that alters dendritic plasticity would be effective at ameliorating some of the behavioural deficits following stroke. Plastic processes in remaining cortical regions are known to facilitate the limited spontaneous recovery that is observed following stroke (Frost et al., 2003). It is also known that treatments that increase cortical plasticity in rats (such as complex housing) further stimulate functional restitution following motor cortex stroke (Biernaskie and Corbett, 2001; Johansson and Belichenko, 2002). Additional experiments investigating factors that stimulate synaptic plasticity have shown that the administration of a diet enriched in omega-3 fatty acids restores the impairment in long-term potentiation that is usually observed in aged rats (Martin et al., 2002), and normalizes the levels of growth factors, such as BDNF, in a model of traumatic brain injury (Wu et al., 2004). Given the extensive evidence suggesting that an increase in synaptic plasticity facilitates functional recovery following stroke, it would be predicted that dietary alterations that increase synaptic plasticity would serve as effective treatments for brain injury.

Initial experiments carried out by Halliwell *et al.* (Halliwell, 2003) have shown that a diet called EMP^{+}, which is enriched in various vitamins and nutrients, improves anatomical and behavioural outcomes following perinatal brain injury in rats (for a complete list of the EMP+ ingredients see appendix 2). Based on these studies we wanted to investigate the effectiveness of this vitamin supplement diet in improving outcome following a different form of brain injury in adult animals – namely, motor cortex stroke. Rats were given either a control diet, or the vitamin supplement diet prior to receiving a unilateral motor cortex injury via the pial-strip technique. The dietary

manipulations continued for the duration of the experiment, during which time the behavioural performance of the animals was monitored on three different motor tasks. At the end of the experiment, the effect of the diet on synaptic plasticity was quantified by analyzing the dendritic morphology of pyramidal cells in the posterior parietal cortex – a region that is known to undergo plastic changes following a variety of treatments including the diet used in this study (Halliwell et al., 2005).

Methods

Thirty-two adult male Long-Evans rats were divided among the following four groups: I) control – no treatment (n=8) II) control + VS (vitamin supplement) (n=8) III) stroke + no treatment (n=8) IV) stroke + VS (n=8). The VS consisted of a specially formulated diet that also contained a cocktail of various vitamins and minerals (EMP^{+}). Animals in the no treatment groups received the specially formulated diet without the added vitamins and minerals. The dietary manipulations were initiated approximately 4 weeks prior to the stroke injury and continued for the duration of the experiment (approximately 11 weeks post-lesion). For a detailed experimental timeline, see appendix 3. The rats were maintained on a 12 hr. dark/light cycle and except for the food restriction period, food and water were available *ad lib*. During food restriction, each animal received only 30g of food per day inside the home cage.

Pial-strip lesions of the motor cortex

Following the pre-training period, half of the animals received motor cortex lesions in the hemisphere contralateral to the reaching paw (Gonzalez and Kolb, 2003).

For this procedure, the animals were anesthetized with somnotol (65 mg/kg) and positioned in a stereotaxic apparatus that was equipped with an Isoflurane anesthetic machine. The level of anesthesia was maintained at a constant level throughout the procedure by varying the level of the gas anesthetic. A dental drill was then used to create a cranial window extending 3 mm anterior, 2 mm posterior and 3 mm lateral to bregma (1 mm lateral from the midline). The exposed dura was carefully removed and the underlying vasculature was wiped away with a saline-soaked cotton swab. The incision was sutured shut and the animals were allowed to recover overnight in individual cages before being returned to the colony with their original cage partners.

Behavioural Tasks

Whishaw tray reaching. During the training period food deprived animals were placed individually into the reaching boxes for 30 minutes a day for 14 days. The front wall of the boxes was constructed of 2 mm vertical bars spaced 9 mm apart while the floor of the cage was constructed of wire mesh (Gharbawie et al., 2005). The rats were required to reach between the vertical bars and retrieve pieces of chicken feed that were available in a 4 cm wide and 0.5 cm deep tray on the outside of the cage. If the animals grasped food with their forepaws but then failed to place it directly into their mouth, the food would fall irretrievably through the wire mesh flooring thereby preventing the accumulation of food on the floor of the cage. Following the two weeks of reach training, all subsequent testing sessions were limited to 5 minutes and were video recorded to allow for an accurate determination of reaching success. An attempt was defined as any forward reaching motion by the paw once the paw was inserted through the bars. Using this

definition it was possible for an animal to make several reaching attempts while only inserting the paw once through the bars. A hit was recorded when an animal successfully grasped the food and was able to place at least some of the food into its mouth. The pre-lesion performance of all animals was determined the day prior to inducing the lesions, and post-lesion performance was monitored once a week for 6 weeks. Following a two week period of no behavioural testing the animals were once again reassessed on post-lesion week 9. This last testing session served to indicate the effects of long-term treatment with the supplement on behavioural performance. During post-lesion testing, the rats were prevented from using the unimpaired limb by wrapping a bracelet around the forearm that prevented it from being inserted through the bars of the reaching cage.

Limb-use asymmetry test. Animals were individually placed in a transparent cylinder 20 cm in diameter and 30 cm in height for 5 minutes set on a transparent table. A mirror placed at an angle below the cylinder allowed for the video recording of the animals' vertical exploration patterns (Gharbawie et al., 2004). When placed inside the cylinder, animals spontaneously reared and investigated the wall of the cylinder through tactile paw placements. A paw preference ratio was determined once a week during the post-lesion testing period by counting the number of initial wall contacts that were supported by the unaffected, the affected and both (simultaneous) limbs. An asymmetry score was calculated as the number of limb touches with the affected paw, divided by the total number of observations (unaffected plus affected plus both). Using this formula, a symmetry score that is about 0.5 indicates that the animal used both limbs equally to

explore the cylinder, whereas a score of less than 0.5 indicates a decreased use of the affected limb.

Forepaw Inhibition. Animals were trained for 3 days to swim to a visible platform located at the end of a rectangular aquarium (120 x 43 x 50 cm). The water was maintained at a temperature of 27°C, and by the third day the rats learned to abandon exploring the aquarium walls and swam directly to the visible platform. When swimming in a straight line, rats use their hind paws to achieve forward movement, and only use their forepaws for changing direction or stopping. Cortical lesions have previously been shown to disrupt this behaviour (Kolb and Whishaw, 1983), therefore allowing us to assess the effects of the vitamin supplement on the loss of inhibition in the effected forepaw. For each testing session at least three swimming trials were video recorded where the animals did not make contact with any of the sidewalls during the swim. The number of paw strokes was counted for each of the forepaws before the injury as well as on a weekly basis for 6 weeks following the stroke and on post-surgery week 9.

Whishaw single pellet reaching. A subset of the 32 animals that started the experiment were trained on the Whishaw single pellet reaching task (n= 18) in order to determine the effect of the VS treatment on the acquisition of a novel motor task following stroke injury. Food-deprived animals were trained for this task according to the protocol described by Gharbawie *et al.* (Gharbawie et al., 2005). The testing apparatus consisted of a Plexiglas box with the dimensions 45cm x 14cm x 35cm. The front panel of the box had a 1 cm wide vertical slit with a horizontal shelf positioned 3 cm above the floor on

the outside of the reaching box. Individual food pellets were placed in indentations spaced 1 cm away from the slit and centered on its edges. Given that paw preference had already been determined during training in the tray reaching task food pellets were only placed in the indentations contralateral to the animals preferred paw. The rats were trained daily for about 2 weeks, by which point there was no additional improvement in performance across days. Percent reaching accuracy was calculated as the total number of successful reaches divided by the total number of pellets presented (25), multiplied by 100. This two-week training session was followed by 5 days of testing, with each testing session lasting a maximum of 10 minutes. The testing sessions were filmed on a ZR 30 MC Camcorder set at 1000^{th} of a second shutter speed (Gharbawie et al., 2005).

Anatomical procedures

Golgi-Cox staining. All animals were sacrificed either 65 or 90 days after surgery by administering an overdose of sodium pentobarbital followed by an intracardiac perfusion with saline. The brains were removed from the skull and processed for Golgi-Cox staining (Gibb and Kolb, 1998). Pyramidal cells from layer III of the posterior parietal cortex (Zilles' Par 1 (Zilles, 1985)) were traced onto paper at 250X through a drawing tube. In order for a cell to be considered for analysis: I) it had to be well impregnated and unobstructed by other cells or blood vessels II) both the apical and basilar trees had to be complete, with no broken or missing branches. The first five cells that fit the above criteria were drawn from each hemisphere. The cell drawings were analyzed using the Sholl method (Sholl, 1956), and the total dendritic length of both the apical and basilar trees was calculated. Given that the animals received unilateral cortical lesions, initial

analyses were carried out using hemisphere as a factor. In cases where there were no significant hemisphere effects, the data were collapsed across hemispheres.

Lesion volume. To determine if the vitamin supplement had an effect on infarct volume, lesion area was quantified at five different planes in Golgi-Cox stained coronal sections. This was done by capturing digital images of the sections of interest and then outlining the injured and uninjured hemispheres on a computer screen. Using the NIH Image program (v1.62) the cross-sectional area of both the injured and uninjured hemispheres was measured at each plane of analysis. The measurements were summed across planes and the difference between the remaining area of the injured and the uninjured hemispheres was calculated and multiplied by 100. The resulting value served as an indirect measure of infarct volume, as it expresses the area of remaining tissue in the injured hemisphere as a percent of the contralateral, intact hemisphere.

Behavioural Results

Whishaw tray reaching

During the training phase, all animals developed a preferred reaching paw and the mean pre-lesion success rate was about 65% for all groups. As can be seen in Figure 3-1A, the stroke lesions caused a chronic impairment in reaching success and there was significant improvement in both the non-treated and the vitamin supplement-treated groups. There was no benefit of the vitamin supplement treatment on either reaching success (Figure 3-1B) or the number of reach attempts (Figure 3-1C).

A two-way, repeated-measures analysis of variance (ANOVA) using surgery and diet as factors revealed that there was a main effect of surgery ($F(1,28) = 37.368$, $P <$

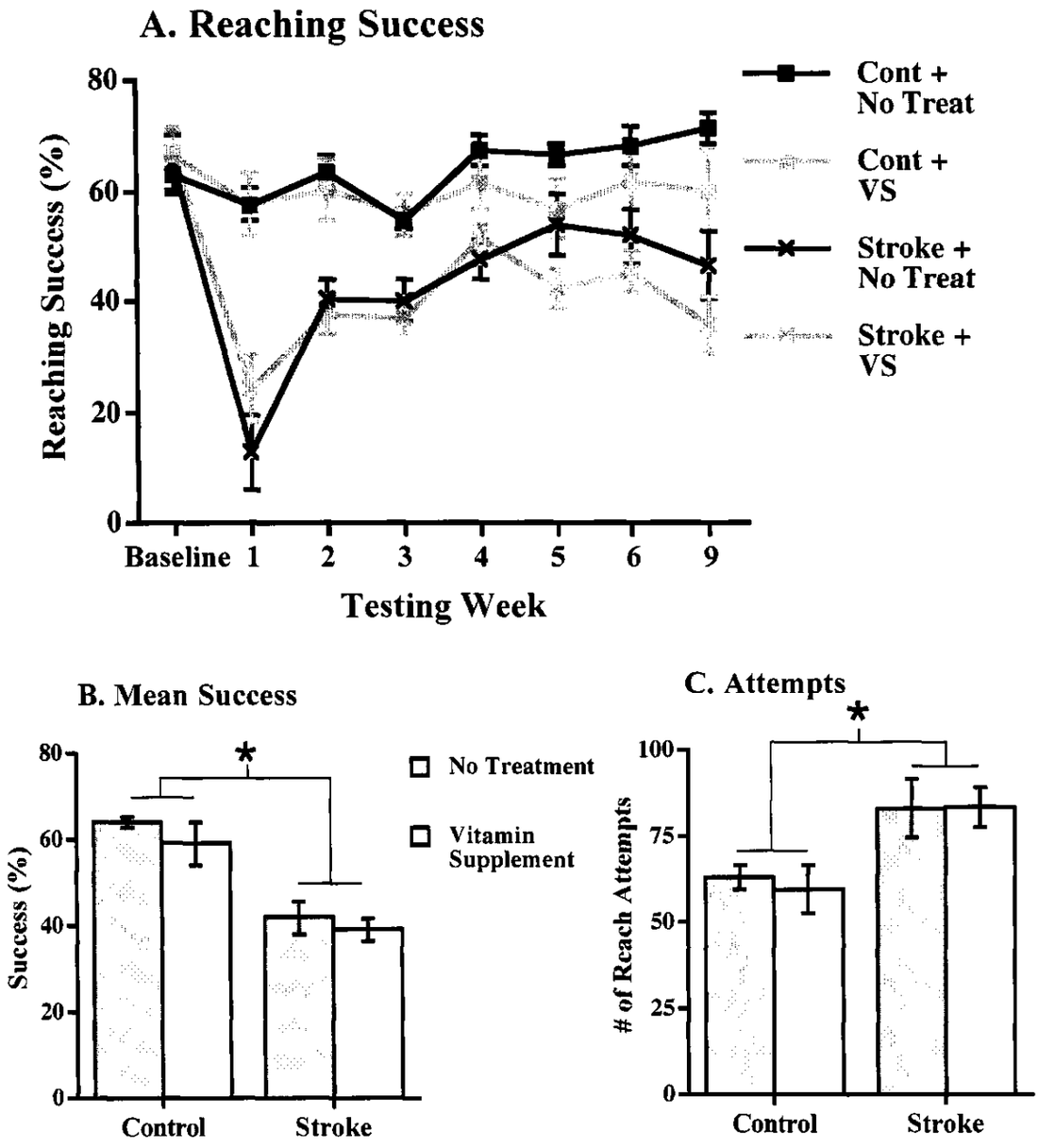

Figure 3-1. Reaching performance in the Whishaw tray reaching task. Stroke animals showed some improvement in reaching success during the nine weeks of post-lesion testing regardless of treatment (A). The mean post-lesion success rate however revealed a significant effect of stroke (B). Stroke animals also made significantly more reach attempts relative to the control group, however, the vitamin supplement treatment did not influence the number of attempts (C) or the number of successful reaches ($P = 0.3177$, data not shown).

0.0001), but not of diet ($F(1,28) = 1.295$, $P = 0.2647$) or the interaction ($F(1,28) = 0.110$, $P = 0.7427$) on reaching success. Although there was a significant week x surgery interaction ($F(7,196) = 0.12.193$, $P < 0.0001$), the week x surgery x diet interaction did not turn out to be significant ($F(2,196) = 0.754$, $P = 0.6262$). A two-way ANOVA of the number of reach attempts indicated that the stroke injury caused animals to make significantly more reach attempts ($F(1,28) = 11.806$, $P = 0.0019$), however neither the effect of the diet ($F(1,28) = 0.056$, $P = 0.8150$), nor the surgery x diet interaction were significant ($F(1,28) = 0.110$, $P = 0.7430$).

Limb-use asymmetry test

Animals that did not receive stroke lesions used both forepaws equally to support their body weight during exploratory rears inside the cylinder. The unilateral stroke lesion caused an acute impairment in this behaviour in that the animals used the affected limb significantly less following surgery (Figure 3-2). Although there was significant spontaneous improvement in the forepaw asymmetry score, neither the rate of recovery nor the final level of function was influenced by the vitamin supplement.

A two-way, repeated-measures ANOVA of the forepaw asymmetry score revealed that there was a main effect of stroke ($F(1,28) = 13.755$, $P = 0.0009$), but not diet ($F(1,28) = 0.607$, $P = 0.4424$). The surgery x diet interaction was not significant ($F(1,28) = 0.555$, $P = 0.4627$), however, the week x surgery interaction was ($F(6,168) = 6.718$, $P < 0.0001$), indicating that all stroke animals improved over time, regardless of treatment.

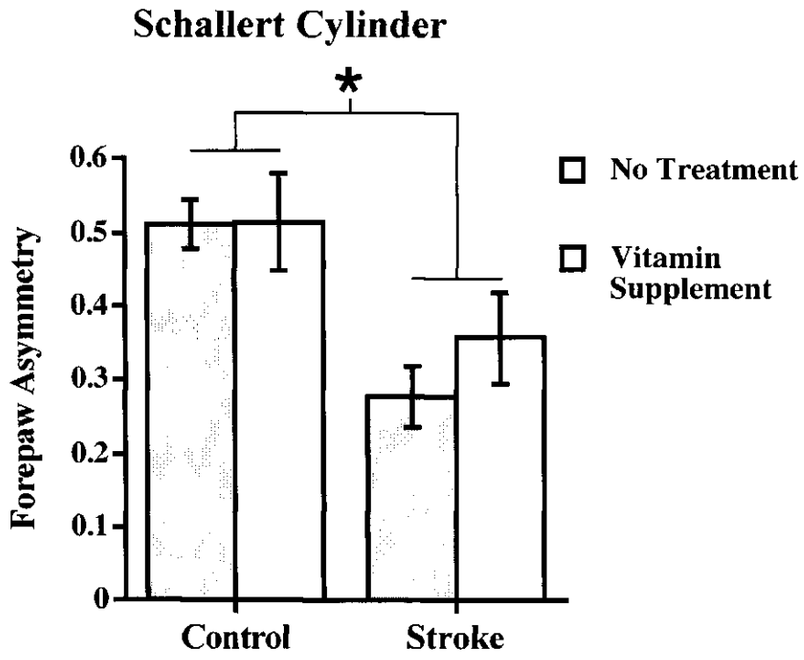

Figure 3-2. Mean post-surgical forepaw asymmetry in the Schallert cylinder. Stroke animals used the impaired forepaw significantly less than control animals, and the vitamin supplement did not have an effect on this difference.

Forepaw inhibition

During pre-lesion training, all animals learned to swim to the visible platform located at the other end of the aquarium. The rats used their hind paws to propel themselves through the water, while keeping the forepaws tucked in against the body, almost completely inhibited. Animals that received unilateral stroke injury lost inhibition of the forepaw contralateral to the injury, and made several forepaw strokes while swimming the length of the pool. The number of forepaw strokes decreased significantly with time during post-lesion testing, but the vitamin supplement did not influence the rate of recovery (Figure 3-3).

A two-way repeated measures ANOVA of the total number of forepaw strokes with the impaired paw revealed that there was a main effect of surgery ($F(1,28) = 12.726$, $P = 0.0013$), but not diet ($F(1,28) = 0.015$, $P = 0.9022$). The surgery x treatment interaction was also not significant ($F(1,28) = 0.68$, $P = 0.4167$), whereas the significant week x surgery interaction ($F(7,196) = 10.007$, $P < 0.0001$) indicated that there was some improvement in all stroke groups, regardless of treatment. The number of forepaw strokes with the intact paw was not affected by the lesion ($P = 0.1034$).

Whishaw single pellet reaching

Following the nine week post-lesion assessment in the Whishaw tray reaching task, a subset of the animals were trained on the Whishaw single pellet reaching task to assess the acquisition of a new motor skill following stroke injury. Control animals, as well as animals with stroke injury were capable of learning this task, however, the success rate of all of the groups was lower than was observed in the tray reaching task.

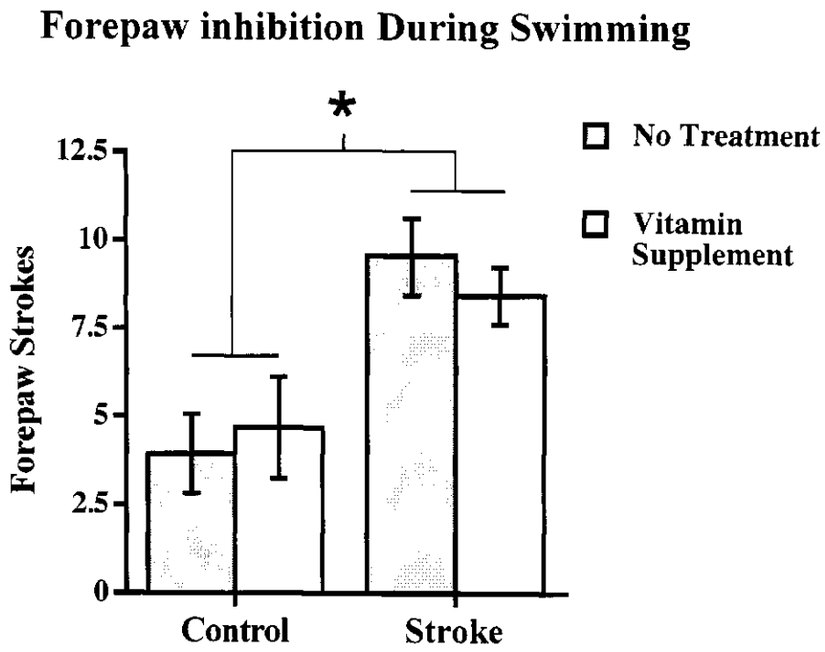

Figure 3-3. Stroke animals made significantly more forepaw strokes when swimming relative to control animals. The vitamin supplement did not have an effect on this difference.

There were no group differences in the rate of acquisition of the task, nor was there a difference in the mean success rate that was achieved at the end of training (Figure 3-4).

A repeated-measures ANOVA of reaching success, using Group as a factor revealed that there was no significant effect of group ($F(2,15) = 0.979$, $P = 0.3986$) and that the group x time interaction was also not significant ($F(6,45) = 1.141$, $P = 0.3543$).

Anatomical results

Lesion volume

As can be seen in Figure 3-5A, the pial-strip lesions performed in the current experiment produced mostly cortical damage in the region where forelimb movements are represented. The mean infarct volume was about 13 % of the total hemisphere, and this was not altered by the vitamin supplement treatment (Figure 3-5B). An ANOVA revealed that there was no significant difference in the volume of the remaining brain tissue between the vitamin supplement treated and the non-treated groups ($F(1,14) = 0.214$, $P = 0.6506$).

Golgi-Cox dendritic analyses

The vitamin supplement treatment caused dendritic hypertrophy of both the apical and basilar dendrites of layer III pyramidal cells in the parietal cortex (Zilles' Par1). This effect, however, was only observed in animals that did not receive strokes (Figure 3-6A). The stroke itself also caused dendritic changes with a decrease in dendritic length in the injured hemisphere (Figure 3-6B).

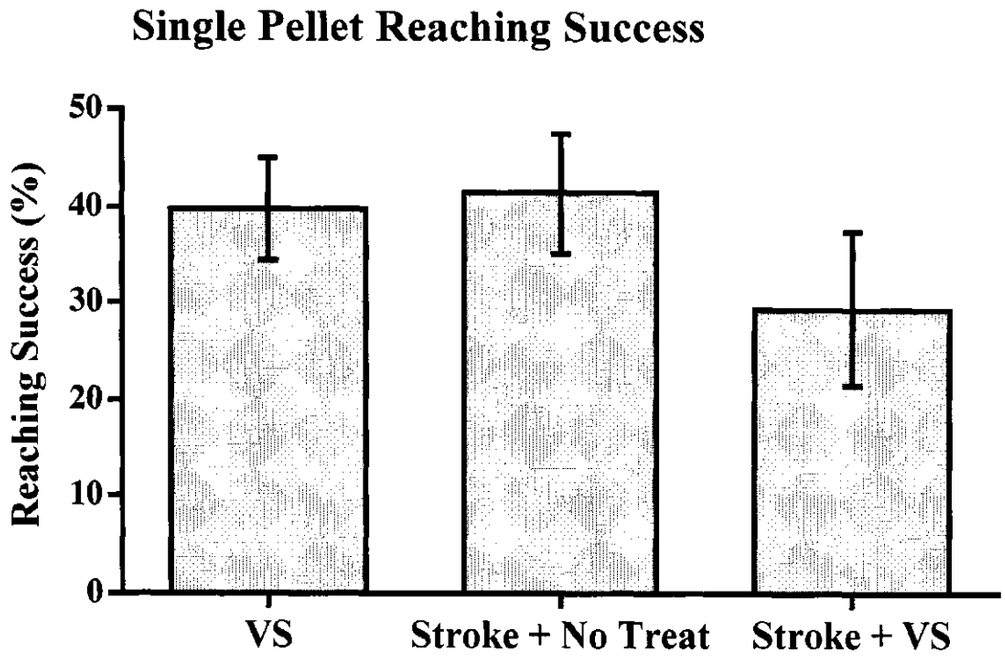

Figure 3-4. Reaching success in the Whishaw single pellet reaching task was not effected by the stroke injury. Although there was a trend for the Stroke + VS group to perform slightly worse relative to the Stroke + No Treatment group, the difference was not significant.

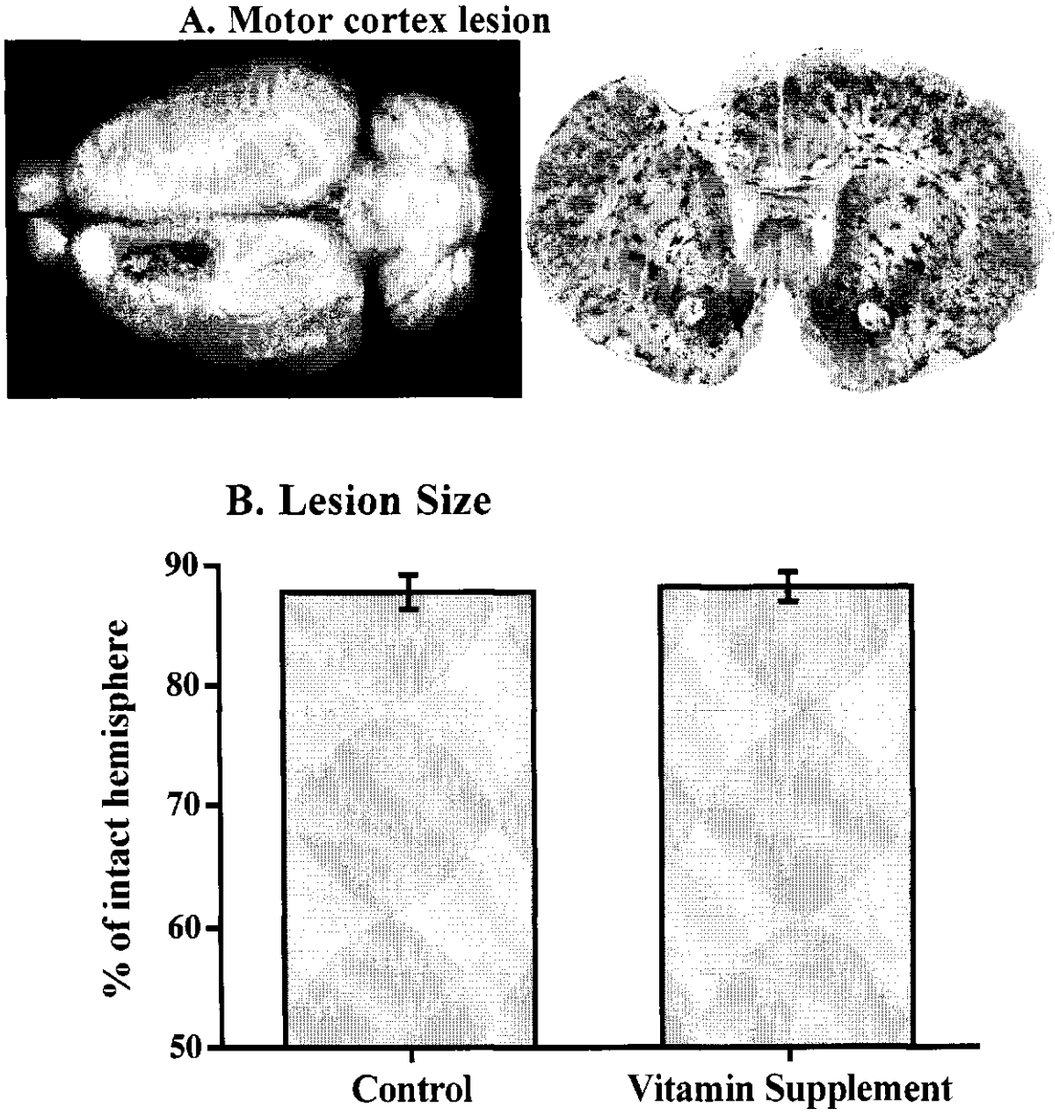

Figure 3-5. Representative picture of lesion size and location (A) and the quantification of the area of the remaining tissue in the injured hemisphere (B). This serves as an indirect measure of lesion size, and the vitamin supplement treatment did not have a significant effect on this measure.

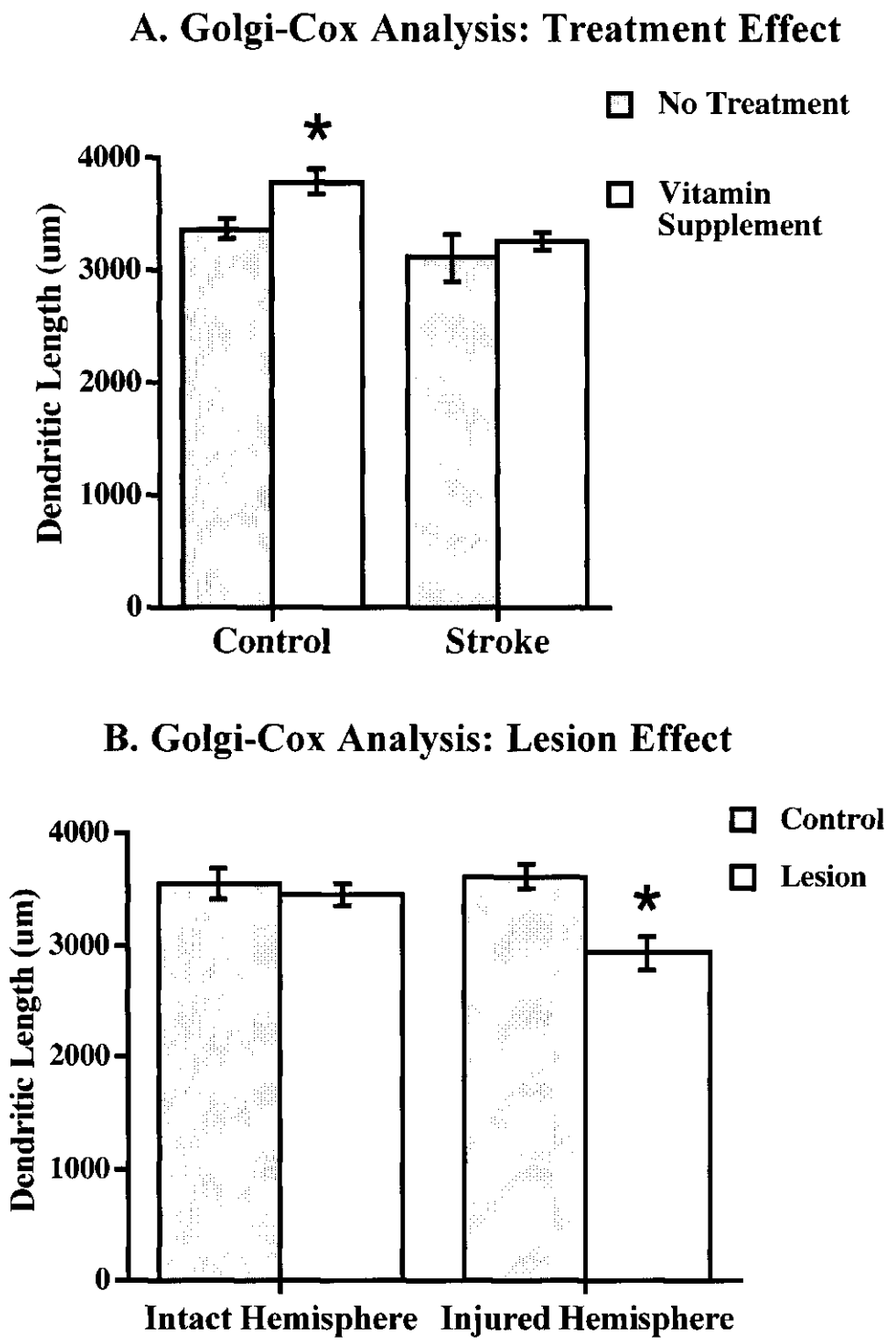

Figure 3-6. The vitamin supplement treatment caused a significant increase in dendritic length in the control animals, however the effect did not reach significance in the stroke group (A). The lesion itself was found to cause a significant decrease in dendritic length in the injured hemisphere only (B).

The dendritic effects were not significantly different between the apical and basilar dendrites ($F(1,28) = 1.768$, $P = 0.1943$), therefore the measures from the two fields were summed to yield a single value of total dendritic length. A two-way ANOVA of total dendritic length revealed that there was a main effect of surgery ($F(1,28) = 8.865$, $P = 0.0059$) and of diet ($F(1,28) = 4.723$, $P = 0.0384$), but the interaction was not significant ($F(1,28) = 1.032$, $P = 0.3183$). A visual inspection of the data in figure 5a prompted us to carry out a *post-hoc* analysis that demonstrated that the main effect of treatment is driven by the control + VS group being significantly different from all other groups ($P = 0.0321$ or better). To determine if the lesion-induced dendritc atrophy occurred in both hemispheres, we carried out a two-way ANOVA using hemisphere and surgery as factors. The analysis revealed a significant hemisphere x surgery interaction ($F(1,28) = 5.432$, $P = 0.0272$), indicating that the atrophy only occurred in the lesion hemisphere (Figure 3-6B).

Discussion

The main objective of the current study was to examine the effect of a vitamin supplement diet on dendritic plasticity and functional recovery following a unilateral ischemic motor cortex injury. Specifically, we were interested in whether the enriched diet would provide an added benefit in well-nourished adult rats that were maintained on a balanced lab diet prior to the experimental manipulations. We found that the enriched diet was effective at stimulating dendritic plasticity in animals without lesions, whereas animals that received stroke lesions did not show any anatomical or behavioural effects

of the treatment. Furthermore, we found that the unilateral pial-strip lesion caused significant dendritic atrophy of Par1 pyramidal cells in the lesion hemisphere only.

Most studies investigating the effects of diet on the brain manipulate the level of single nutrients or vitamins and assess the effect of this manipulation on behavioural and anatomical measures. For example, dietary administration of omega-3 fatty acids was found to reduce oxidative stress and counteract learning disability following cortical injury (Wu et al., 2004), whereas a diet supplemented with blueberries and other known antioxidants was found to significantly reduce lesion volume and increase locomotor activity following ischemic brain damage (Wang et al., 2005). The diet used in the current experiment contained 36 different items and therefore served as a broad-based nutritional supplement. In addition to containing high levels of a number of vitamins (A, B, C, D, E) and minerals, the diet also contained amino acid precursors such as choline, phenylalanine and glutamine and high doses of fish oil extract (Halliwell, 2003). Fish oil is a significant source of omega-3 fatty acids (Morris et al., 2003), whereas the amino acid precursors enhance the production of a number of different neurotransmitters (Halliwell, 2003). Together with the various vitamins and minerals, these components of the diet likely target a number of biochemical pathways that may facilitate the dendritic hypertrophy of cortical cells. Although we are unable to determine the exact pathway that is necessary and sufficient to produce dendritic hypertrophy, the fact that the changes occurred only in control animals allows us to speculate about the putative underlying mechanism.

One possibility is that the increased availability of amino acid precursors in the diet increased production of the neurotransmitters (or growth factors) that are necessary

for normal cortical function. Other treatments, such as complex housing, that are known to increase cortical levels of acetylcholine (Bennett et al., 1964) also cause an increase in dendritic length (Kolb and Gibb, 1991), suggesting that there may be a link between the biochemical changes and structural modifications of neurons. It is therefore possible that the greater availability of precursor molecules in animals that received the vitamin supplement caused increased production of cortical neurotransmitters such as acetylcholine, and thus induced the production of new dendrites to accommodate this increase. The lack of similar dendritic changes in animals with strokes may be due to the lesion-induced alterations of neurotransmitter and amino acid levels in the cortex (Molchanova et al., 2004). Experimental stroke models have shown that ischemic cortical injury is followed by the release of large quantities of excitatory neurotransmitters, such as glutamate, into the extracellular space (Guyot et al., 2001). In addition to causing excitotoxic cell death, this imbalance in neurotransmitter levels may also prevent the occurrence of the dendritic hypertrophy that was observed in the control animals.

Another possibility is that the vitamin supplement caused the dendritic hypertrophy by modifying the expression of genes within the cell nucleus or through the expression of various growth factors that stimulate dendritic growth. One component of the diet, vitamin E, has been shown to modulate gene expression in that it promotes the expression of a nuclear transcription factor and its downstream genes that are neuroprotective following ischemic injury (Zhang et al., 2004). It may be that other vitamins contained in the diet are acting through a similar mechanism to directly activate genes that are required for the production of dendritic processes. Growth factors such as

nerve growth factor (Kolb et al., 1997) and basic fibroblast growth factor (Rowntree and Kolb, 1997) are known to stimulate dendritic growth in cortical cells, therefore it may be the case that the vitamin supplement treatment is altering the expression of these proteins (Kolb et al., 1997).

Alternatively, it may be the case that the dendritic hypertrophy produced by the vitamin supplement occurred before the lesion, and that the lesion subsequently reversed these changes. Ischemic cortical injury is associated with the breakdown of membrane phospholipids (Dhillon et al., 1994), resulting in the release of free fatty acids (FFAs) into the blood stream and surrounding tissue (Zhang and Sun, 1995; Phillis and O'Regan, 2003). Besides being potent inhibitors of mitochondrial respiratory function, the accumulation of FFAs also interferes with intracellular signaling pathways that are involved in synaptic activity (Homayoun et al., 2000). Given that newly generated dendrites depend on synaptic activity to establish and maintain connections with other cells, it could be the case that the lesion induced accumulation of FFAs interfered with the synaptic activity that is required to maintain the newly formed dendritic processes.

The unilateral motor cortex lesions that were induced in the current experiment also caused significant atrophy of pyramidal cells in layer III of the parietal cortex in the lesion hemisphere only. A previous study by Kolb *et al.* has reported bilateral atrophy of the same cell population after unilateral motor cortex injury (Kolb et al., 2000), however, there is a key difference between these two studies that could account for the difference in results. In contrast to the pre-lesion motor training that was performed in the current experiment, the animals in the Kolb *et al.* study were only trained after having had the unilateral motor cortex lesions. Because of the lack of pre-training, the animals with

lesions required over four months of reach training to learn the task, as opposed to the two-week training period employed in the current experiment. Given that reach training has previously been shown to alter the morphology of sensorimotor neurons (Withers and Greenough, 1989) it is likely that the extensive reach training had different effects on cell morphology relative to the two-week training period.

The fact that the vitamin supplement did not induce any dendritic changes in animals with strokes complements the absence of a behavioral effect of the treatment. Previous experiments have shown that treatments that induce cortical plasticity either directly stimulate behavioural recovery (Kolb et al., 1997), or facilitate behavioural adaptations that ultimately also improve functional outcome (see COX-2 study). The treatment used in the current experiment did not appear to influence either of these mechanisms. Over the post-lesion testing period, all animals that received strokes showed significant improvement in the tray reaching task and were not significantly different from controls in learning a novel motor task (Whishaw single pellet reaching). Although similar lesions have been shown to cause chronic deficits in the single pellet reaching task (Whishaw, 2000), it is likely that the weekly testing in tray reaching facilitated the development of compensatory strategies that allowed the animals to perform well in the single pellet reaching task. This is supported by other experiments showing that training in the tray reaching task causes significant improvement in the single pellet reaching task following middle cerebral artery occlusion (Gharbawie et al., 2005).

Although the vitamin supplements did not prove to be beneficial in the current experiment it may be the case that the treatment would induce plastic processes when

administered in combination with other treatments, such as complex housing or behavioural rehabilitation. Witt-Lajeunese *et al.* (Witt-Lajeunesse et al., 2005) have previously shown that while the combined administration of basic Fibroblast Growth Factor (bFGF) and rehabilitative training enhanced functional recovery following adult brain injury in rats, neither factor served as a successful treatment when administered alone. Furthermore, studies investigating clinical patients have shown that the multidimensional care that is offered in dedicated stroke units is associated with superior outcome relative to standard hospital care (1997). There are thus multiple lines of evidence suggesting that some forms of therapy are only effective when combined with other treatments.

The vitamin supplement treatment used in the current experiment has previously been shown to improve outcome following neonatal brain injury in rats (Halliwell, 2003) and has also been shown to ameliorate the symptoms of several psychiatric conditions in humans as well (Kaplan et al., 2001; Kaplan et al., 2002). It was therefore surprising that the treatment did not have any beneficial effects following adult motor cortex strokes. The fact that the treatment produced plastic changes in intact animals, but not in animals with stroke injury, suggests that certain treatments may have differential effects depending on the state of the brain at the time of treatment administration. In addition, the lack of treatment induced behavioural improvement in the stroke animals further confirms the notion that the induction of plastic processes is necessary for the promotion of functional improvement following stroke injury.

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Chapter 4

Social experience blocks functional restitution following motor cortex stroke

Abstract

The current experiment investigated the effect of social experience on the recovery of function following unilateral motor cortex stroke. Adult rats were trained on the Whishaw single pellet reaching task prior to receiving a devascularizing lesion in the forepaw representation of the motor cortex. During the post-lesion testing period half of the rats were exposed to a form of social experience that has previously been shown to stimulate synaptic plasticity in frontal cortex circuitry, whereas the rest of the rats were housed in pairs in standard cages. At the end of the experiment the brains were processed for Golgi-Cox staining and dendritic length was measured in layer V of the intact forelimb area, layer III of Zilles' area Cg3 and layer II/III of Zilles' area AID. It was anticipated that the social experience would enhance dendritic reorganization and facilitate functional recovery. In contrast, social experience was found to completely block the normal spontaneous behavioural restitution in the lesion animals, let alone facilitate further recovery. Anatomically, whereas social experience selectively increased dendritic length in AID in normal rats, this was not seen in the lesion animals as the lesion alone produced an increase in dendritic length in both AID and Cg3. The findings are discussed in terms of the role of multiple experiences, including stress, on spontaneous plasticity that occurs following unilateral motor cortex strokes, and the effectiveness of altering synaptic plasticity in inducing behavioural recovery.

Introduction

It is now well established that the environment that rats are housed in following cerebral injury has a significant effect on the degree of functional recovery. For example, environmental enrichment or complex housing have consistently been found to aid recovery of both cognitive and motor impairments following a variety of injury models, including unilateral motor cortex stroke (Biernaskie et al., 2001; Johansson and Belichenko, 2002; Risedal et al., 2002; Dahlqvist et al., 2003). Although the exact mechanisms of these beneficial effects are still largely unknown, it is believed that a combination of physical exercise and social interactions facilitates a general increase in brain plasticity. Plastic processes in areas distal from the lesion have been found to play a major role in functional recovery following ischemic injury (Frost et al., 2003), therefore it is likely that environmental enrichment further promotes such plastic changes and thus improves functional outcome.

To further investigate the most beneficial components of environmental enrichment, a number of studies have now been carried out that compared the relative effectiveness of physical exercise and social interactions in enhancing functional outcome following experimental motor cortex stroke (Johansson and Ohlsson, 1996; DeVries et al., 2001; Dahlqvist et al., 2003). The results suggest that although rats housed together in a large cage show better functional recovery than animals housed individually with access to a running wheel, neither of these groups recovered as well as rats housed in an enriched environment. One possible explanation for this outcome is that neither the form of social manipulation used in these experiments nor the wheel running, provided sufficient novel stimulation for the animals. Rats housed in complex environments

receive novel stimulation by being transferred to an entirely new environment as well as through the regular rotation of the various toys and objects that are placed inside the environments. Even under these conditions though, the plastic changes that are induced by complex housing are restricted to sensory areas such as visual and somatosensory cortices, whereas more anterior cortical regions such as the prefrontal cortex remain unaltered (Greenough et al., 1973; Kolb et al., 2003). Given that cerebral injury frequently involves the frontal lobe, it is reasonable to wonder if this region might be altered using another form of experience.

In the current experiment we examined the effects of specifically engaging the orbital and medial prefrontal cortices of rats that received unilateral strokes of the motor cortex. To achieve this, rats were exposed to a form of social experience where the cage partners of the animals were rotated regularly within a group of six animals. We have previously shown this form of social experience to be associated with plastic changes similar to those observed in somatosensory cortex following complex housing, but in this case the dendritic reorganization and synaptogenesis was only observed in the orbital and medial prefrontal cortices (Hamilton et al., 2003). Because prefrontal regions show spontaneous dendritic growth after motor cortex injury, we hypothesized that our form of social experience would further change these cells and also facilitate functional recovery following motor cortex stroke.

Methods:

Subjects and experimental setup

The subjects used in this experiment were 28 adult male rats that were born and housed in the vivarium at the University of Lethbridge. Animals were maintained on a 12 hr. dark/light cycle and except for the food restriction period, food and water were available *ad lib*. During food restriction, each animal received only 30 g of food inside the home cage per day. Behavioural testing was carried out late in the afternoon and the social manipulation occurred after the behavioural testing, near the end of the light cycle. Twenty-four of the animals were divided into 4 weight-matched groups: Stroke+social experience (n=6), control+social experience (n=6), stroke+no treatment (n=6), no lesion+no treatment (n=6). Before initiating any experimental manipulations, the 6 animals in each group were housed together in a large cage for 2.5 weeks in order to become familiar with one another. Following this period of group housing, single pellet reach training was initiated, and the animals were housed in pairs in standard sized, hanging plexi-glass cages. The remaining animals (n=4) did not receive any behavioural training or surgical manipulations, but instead were used to quantify the dendritic effects of the social manipulation in naïve animals.

Single pellet reaching

Food-deprived animals were trained on the Whishaw single pellet reaching task according to the protocol described by (Gharbawie et al., 2005). The testing apparatus consisted of a Plexiglas box with the dimensions 45cm x 14cm x 35cm. The front panel of the box had a 1 cm wide vertical slit and a horizontal shelf 3 cm above the floor on the

outside of the reaching box. Individual food pellets were placed in indentations spaced 1 cm away from the slit and centered on its edges. The initial days of training served the purpose of habituating the animals to the box, developing a taste for the food pellets and also determining the preferred paw for each animal. Following this initial training period, the animals were encouraged to approach the front of the reaching box and retrieve a food pellet that was placed in the indentation contralateral to the animals preferred paw. The rats were trained for about 4 weeks, by which point they all had achieved a consistent level of accuracy. The reaching accuracy was calculated as the total number of successful reaches, divided by 25 (the total number of pellets presented) X 100 (to express as percent accuracy). Each testing session lasted a maximum of 10 minutes, therefore if an animal made reaching attempts at fewer than 25 pellets, than that number was used in calculating reaching success. All of the animals were filmed on the last training session before the surgery on a ZR 30 MC Camcorder set at 1000^{th} of a second shutter speed (Gharbawie et al., 2005).

Pial-strip lesion of the motor cortex

Following the pre-training period, half of the animals received motor cortex lesions in the hemisphere contralateral to the reaching paw (Gonzalez and Kolb, 2003). For this procedure, the animals were anesthetized with somnotol (65 mg/kg) and positioned in a stereotaxic apparatus that was equipped with an Isoflurane anesthetic machine. The level of anesthesia was maintained at a constant level throughout the procedure by varying the level of the gas anesthetic. A dental drill was used to create a cranial window extending 4 mm anterior, 2 mm posterior and 3 mm lateral to bregma (1

mm lateral from the midline). The exposed dura was carefully removed and the underlying vasculature was wiped away with a saline-soaked cotton swab. The incision was sutured shut and the animals were allowed to recover overnight in individual cages before being returned to the colony with their original cage partners.

Post lesion manipulation and assessment

Following a day of recovery, the social experience was initiated in half of the animals and was continued for 5 weeks. For this procedure, animals in the social group received a new cage and bedding as well as a new cage partner every 48 hrs. Animals in the no treatment groups only received the new cage and bedding. Although social animals received a different cage partner every 48 hrs. the animals were generally familiar with one another as they were housed together for 2.5 weeks prior to reach training. In addition, the social switches were carried out only amongst the six animals in that particular group, therefore, every fifth switch resulted in the original cage partners being matched, from which point the original switching cycle was repeated. This rotation paradigm further facilitated the animals becoming more familiar with one another throughout the duration of the experiment.

Reach testing was initiated on the fourth post-surgical day. In order to minimize the effect of repeated testing on the rate of spontaneous recovery, a testing pattern was used where the animals were tested for two consecutive days followed by three days with no reach testing. This pattern was repeated for 5 weeks and each reaching session was video recorded.

The group of animals that did not receive reach training were exposed to the social manipulations for the same length of time as the rest of the animals and were sacrificed with the rest of the animals.

Assessment of open field activity

We have previously investigated the effect of social experience on open field activity (Hamilton et al., 2005), therefore in the current experiment we were interested in whether social experience altered the activity of animals with motor cortex lesions. Following the last day of single pellet reach testing the general activity of animals that received lesions was assessed in an open field according to the protocol of Brown *et al.*, (Brown and Kolb, 2001). Briefly, the rats were placed in a box equipped with a series of infrared sensors, which allowed for both the vertical and the horizontal activity to be monitored. The measurements of the box were 40cm x 40cm x 30cm and locomotor activity was monitored for ten minutes by a Digiscan animal activity monitor (Omnitech, Columbus, OH). The horizontal and vertical activity were summed for each animal, thus providing a single measure of total activity within the ten minute testing session.

Histological preparation and analyses

All animals were sacrificed on approximately post-surgical day 35 by administering an overdose of sodium pentobarbital followed by an intracardiac perfusion with saline. In order to determine the chronic effect of the social manipulation on circulating stress hormones, a blood sample was collected directly from the heart of each animal just prior to the perfusion step.

To investigate the effects of unilateral motor cortex injury and social experience on dendritic morphology the brains were processed for Golgi-Cox analysis (Gibb and Kolb, 1998). Pyramidal cells from layer V of the forelimb area (FL), layer III of the anterior cingulate (Cg3) and layer II/III of the orbitofrontal (AID) cortices (Zilles, 1985) were traced on to paper at 250X through a *camera lucida* drawing tube. In order for a cell to be considered for analysis it had to be: I) well impregnated and unobstructed by other cells or blood vessels II) both the apical and basilar trees had to be complete, with no broken or missing branches. The first five cells that fit the above criteria were drawn from each hemisphere. The cell drawings were than analyzed using the Sholl method (Sholl, 1956), and the total dendritic length of both the apical and basilar trees was calculated. Given that the animals received extensive unilateral skilled reach training, as well as unilateral cortical injury, initial analyses were carried out using hemisphere as a factor. In cases where there were no significant effects, the data were collapsed across hemisheres. The apical trees of cells in layer V of FL are usually truncated during histological preparation, and for this reason, only the basial trees were analyzed from this area.

To determine if social manipulation had an effect on infarct size, lesion area was quantified at seven different planes in Golgi-Cox stained coronal sections. This was done by capturing digital images of the sections of interest and then outlining the injured and uninjured hemispheres on a computer screen. Using the NIH Image program (v1.62) the cross-sectional area of both the injured and uninjured hemispheres was measured at each plane of analysis. The measurements were summed across planes and the difference between the remaining area of the injured and the uninjured hemispheres was calculated

and multiplied by 100. The resulting value served as an indirect measure of infarct volume, as it expresses the area of remaining tissue in the injured hemisphere as a percent of the contralateral, intact hemisphere.

Quantification of serum corticosterone levels was carried out using the Coat-A-Count radioimmunoassay (available from Diagnostic Products Corporation, LA). To ensure maximal accuracy of the results, each sample was run twice, and cases where the variability was above the acceptable range were excluded from the analyses.

Behavioural Results

Single pellet reaching

By the end of pre-training all animals learned to reach for food in the reaching apparatus and the mean performance of all the animals was about 50%. Rats that received motor cortex strokes showed a significant drop in performance during the first post-operative testing session whereas the performance of the no-lesion groups did not change. Animals in the stroke no-treatment group showed a gradual increase in performance with subsequent testing sessions, although their performance on the last day was still impaired relative to the control group. Animals that received strokes as well as social experience had similar impairments as the stroke only group, but unexpectedly, they did not show the same spontaneous recovery with subsequent testing sessions. In fact, this group did not show significant improvement in performance at any point during post-surgical testing (see Figure 4-1A).

A two-way ANOVA (Surgery x Treatment) on the mean postsurgical performance indicated that there was a main effect of lesion ($F(1,19) = 35.909$, $P <$

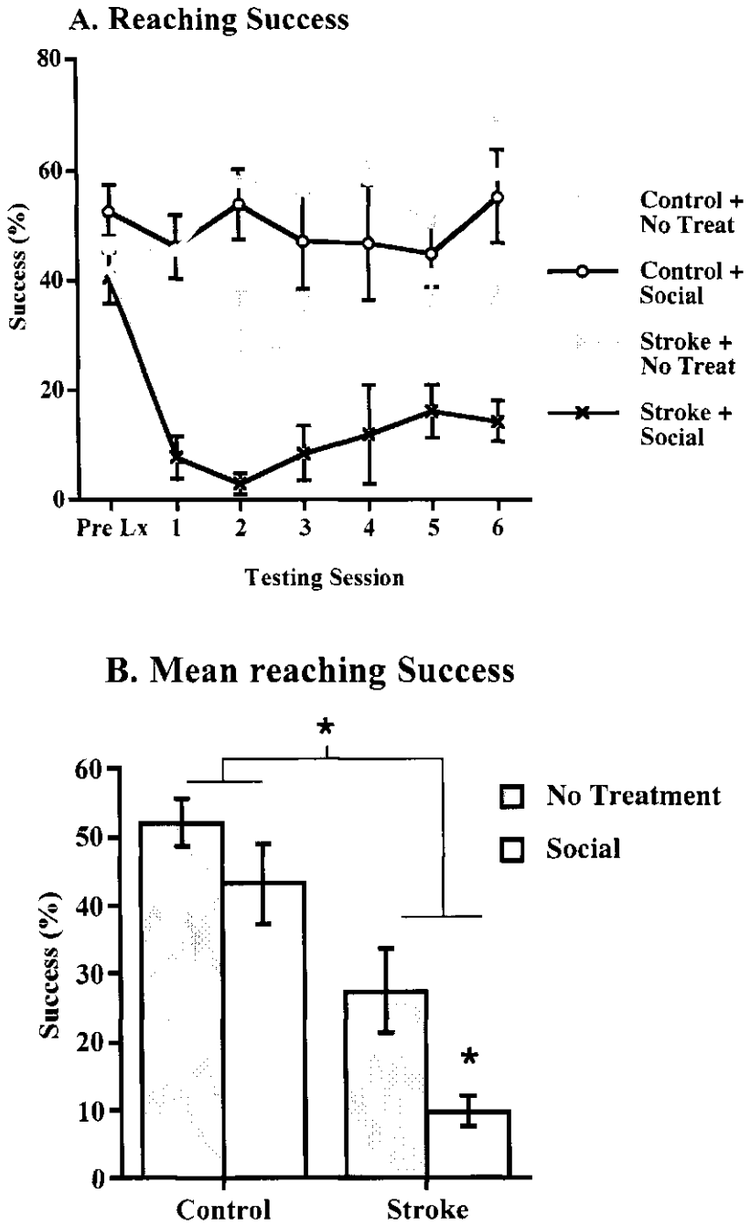

Figure 4-1. Reaching performance in the Whishaw single pellet reaching task. Both stroke groups showed a significant impairment following the injury, and the stroke + no treatment group showed significant improvement on subsequent testing sessions. The social experience completely blocked any improvement, as there was no significant difference in reaching success between the first and the last post surgical testing session (A). Looking at the mean post-stroke success rate, animals in the stroke + social group performed significantly worse than the stroke + no treatment group (B).

0.0001) and of treatment ($F(1,19) = 7.561$, $P = 0.0127$), but the interaction was not significant ($F(1,19) = 0.803$, $P < 0.3814$) because both the control and lesion animals with social experience showed a trend to poorer performance (Figure 4-1B). A *post hoc* analysis however, revealed that the difference between the lesion+control and lesion+social groups was significant ($P = 0.0161$).

Open field activity

To determine if social experience influenced the activity of animals with unilateral motor cortex strokes, the activity of animals with stroke lesions was measured in an open field. As Figure 4-2 illustrates, there were no observable differences in the total activity between the social and the control group. A one-way ANOVA confirmed that the treatment did not have a significant effect on activity in the open field ($F(1,10) = 0.106$, $P = 0.7515$).

Serum Corticosterone levels

A blood sample collected at the end of the experiment from each animal was used to quantify the stress levels at that point in time. A two-way ANOVA (Surgery x Treatment) demonstrated that neither the stroke injury ($F(1,19) = 0.552$, $P = 0.4667$), nor the social manipulation ($F(1,19) = 0.233$, $P = 0.6349$) had a significant effect on the levels of corticosterone. The surgery x treatment interaction was also not significant ($F(1,10) = 0.035$, $P = 0.8533$) (see Figure 4-3).

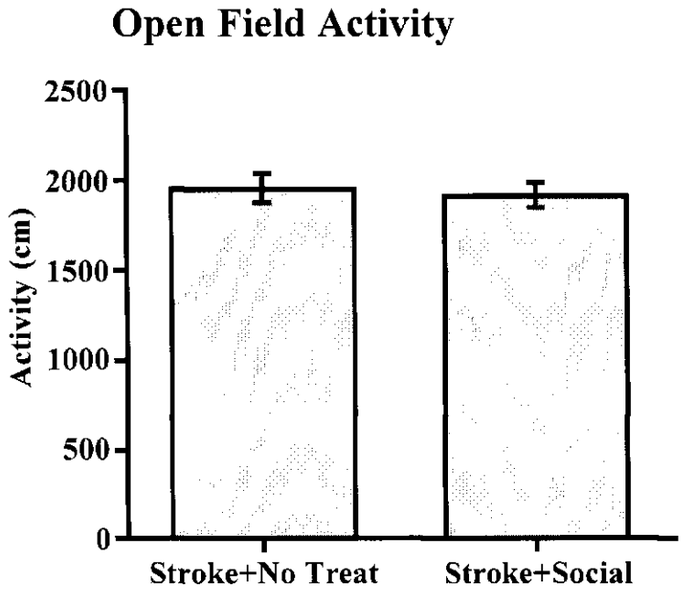

Figure 4-2. Total activity during the 10 minutes of open field activity testing. Social experience did not alter the activity of animals with strokes. In previous experiments (see Hamilton *et al.*, 2005) we have found that activity in intact animals is also not effected by social experience (data not shown).

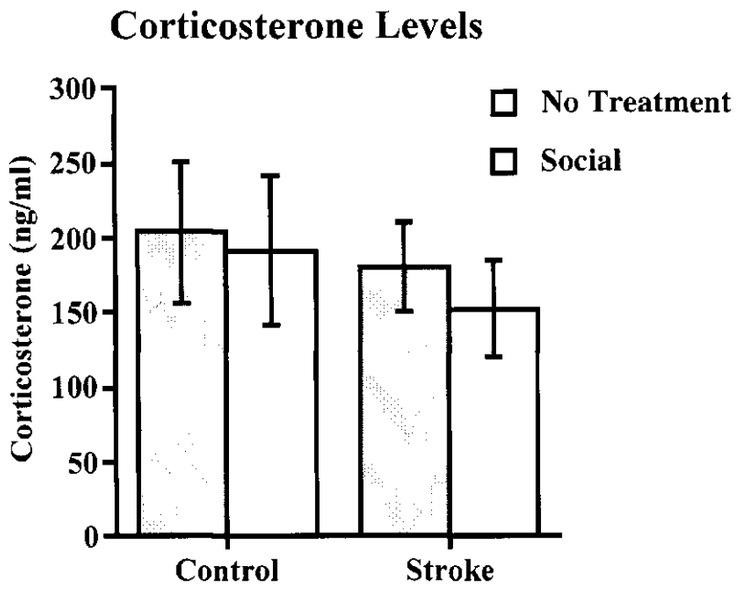

Figure 4-3. Serum corticosterone levels at the end of the experiment as determined by the Coat-A-Count radioimmunoassay. Neither the social experience nor the stroke had a significant effect on corticosterone levels.

Infarct Size

The stroke model used in the current experiment created an infarct that was mostly restricted to the cortex and removed about 13% of the tissue in that hemisphere. As can be seen in Figure 4-4B, social experience did not alter the volume of the lesion in that there was no significant difference between the area of the remaining hemisphere between the two groups. A one-way ANOVA indicated that the area of the remaining hemisphere did not differ significantly between the social and the no-treatment groups ($F(1,10) = 0.023$ $P = 0.8829$).

Dendritic Morphology

Area FL

The stroke injury induced in the current experiment completely destroyed the FL area of the motor cortex on the side contralateral to the reaching paw and therefore no cells could be drawn from this area. In analyzing the length of the basilar dendrites in cells in the intact (non-reaching) hemisphere it was found that neither the injury itself nor the social experience produced any changes relative to control animals (Figure 4-5). A two-way ANOVA (Surgery x Treatment) indicated that there was no significant effect of lesion ($F(1,18) = 0.515$ $P = 0.4820$), treatment ($F(1,18) = 2.160$ $P = 0.1589$) nor the interaction ($F(1,18) = 0.160$ $P = 0.6942$).

Area AID in untrained rats

Animals that did not receive training on the single pellet reaching task showed significant dendritic changes in response to the social manipulation. As we have previously demonstrated in female animals, social experience in male rats also causes

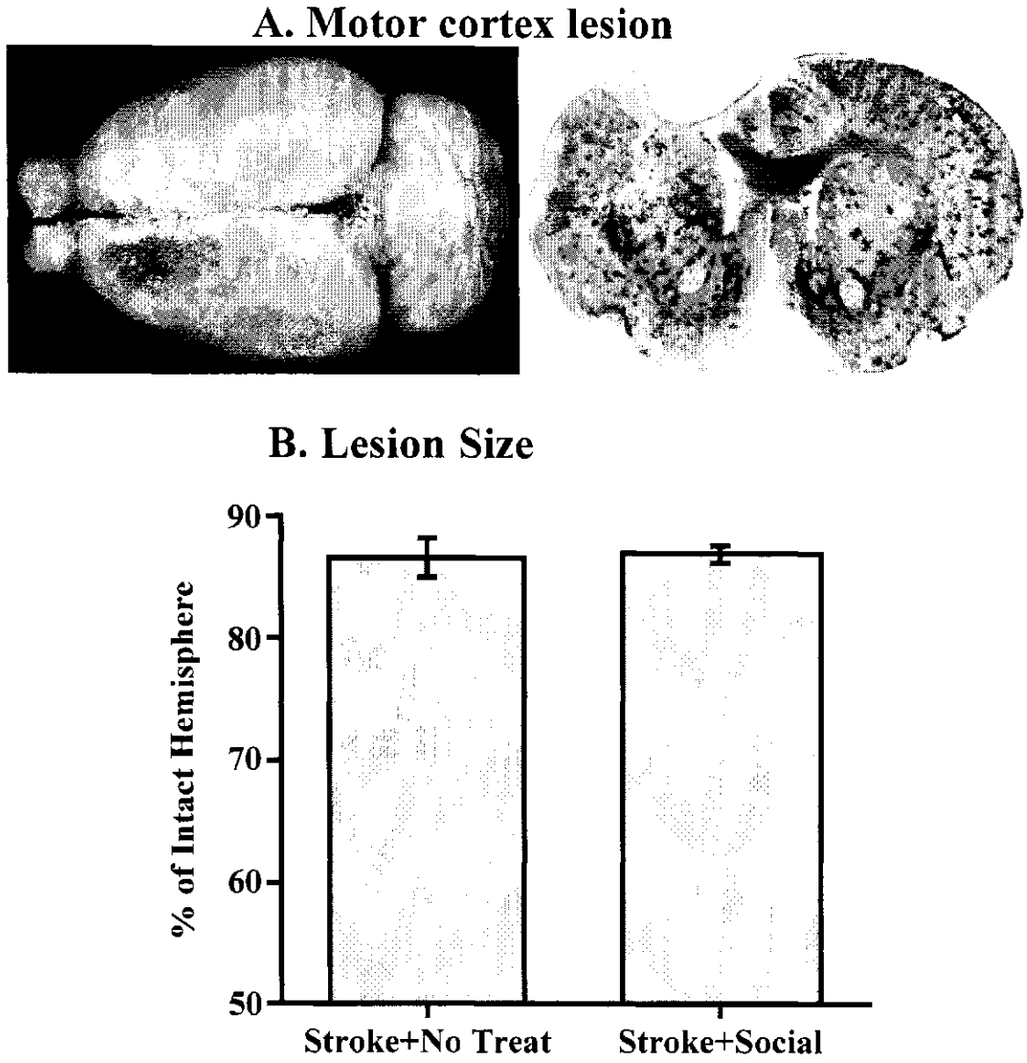

Figure 4-4. A representative image of the lesion size and location (A). Lesion size did not differ between the No treatment and the social groups (B).

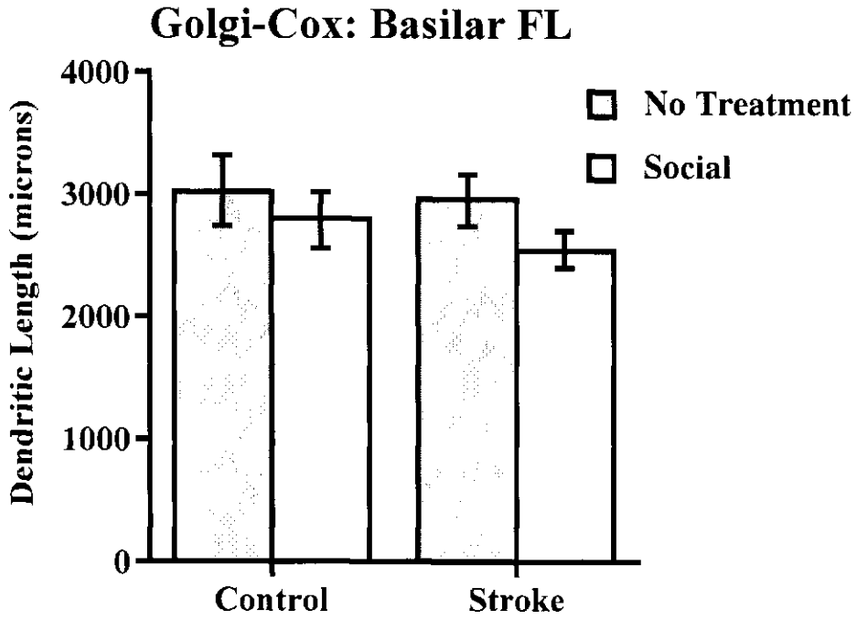

Figure 4-5. Total dendritic length of the basilar field of layer V neurons in the contralateral (to the stroke) motor cortex. Although there was a strong trend for the atrophy of cells in the social group, this was not significant.

significant hypertrophy of the basilar dendrites of cells in AID (see Figure 4-6). A one-way ANOVA of basilar dendritic length indicated a main effect of group ($F(1,4)$ = 26.041 $P = 0.0070$), however, the effect in the apical tree was not significant ($F(1,4)$ = 0.670 $P = 0.4589$).

Area AID in trained rats

Animals that received strokes had significantly longer apical and basilar dendrites relative to the un-operated group, but social experience did not have a significant effect on dendritic length (Figure 4-7). There was a strong trend for an increase in basilar dendritic length in the un-operated social group, however this did not reach significance.

A two-way ANOVA (Surgery x Treatment) of the apical branches indicated a main effect of lesion ($F(1,41) = 5.151$ $P = 0.0286$), but not treatment ($F(1,41) = 0.025$ $P = 0.8740$), nor the interaction ($F(1,41) = 0.116$ $P = 0.7349$). Similarly, the analysis of the basilar branches also showed a significant lesion effect ($F(1,41) = 9.515$ $P = 0.0036$), and no effect of treatment ($F(1,41) = 0.331$ $P = 0.5680$), nor interaction ($F(1,41) = 0.869$ $P = 0.3567$).

Area Cg3

There was no effect of social experience or stroke injury on dendritic length in the apical tree, although there was a significant effect of injury on the basilar tree (Figure 4-8A). Compared to the un-operated group, animals that received strokes had a 15% increase in the length of the basilar dendrites, regardless of whether they received social experience or not (Figure 4-8B). There was also a trend towards a decrease in basilar

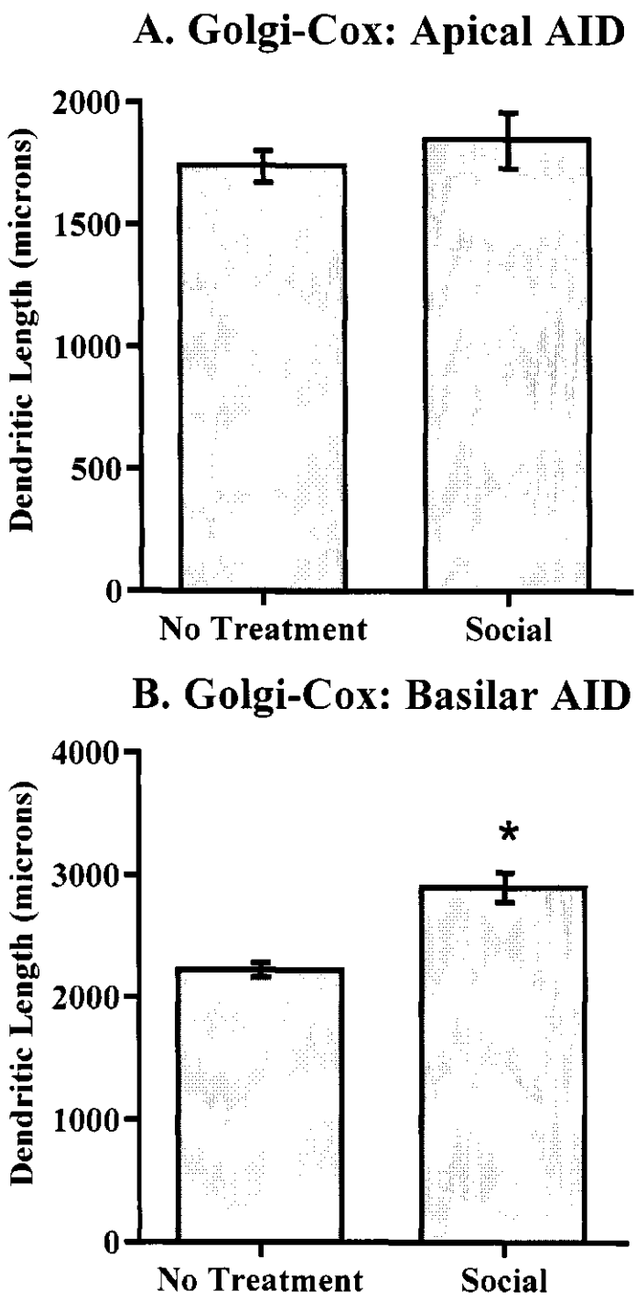

Figure 4-6. Dendritic length of the apical (A) and basilar (B) fields of layer II/III cells in the AID region of the cortex in animals that did not receive reach training. Social experience was found to significantly increase dendritic length in the basilar field.

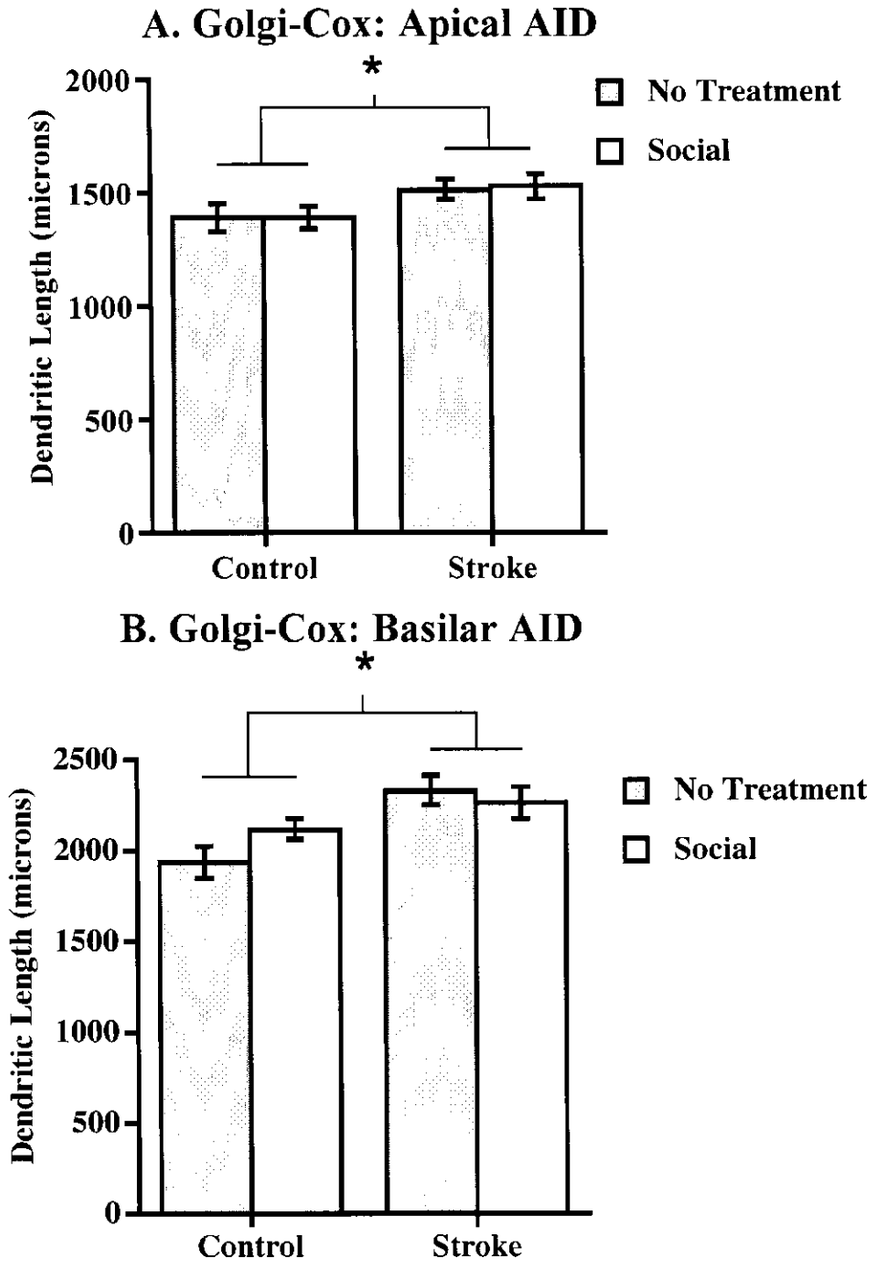

Figure 4-7. Dendritic length of the apical (A) and basilar (B) fields of layer II/III cells in the AID region of the cortex in animals that received reach training. Although there was a strong trend for the social animals in the control groups to have longer basilar dendrites, this effect did not reach significance. The stroke injury, however was associated with a significant increase in dendritic length of both apical and basilar fields.

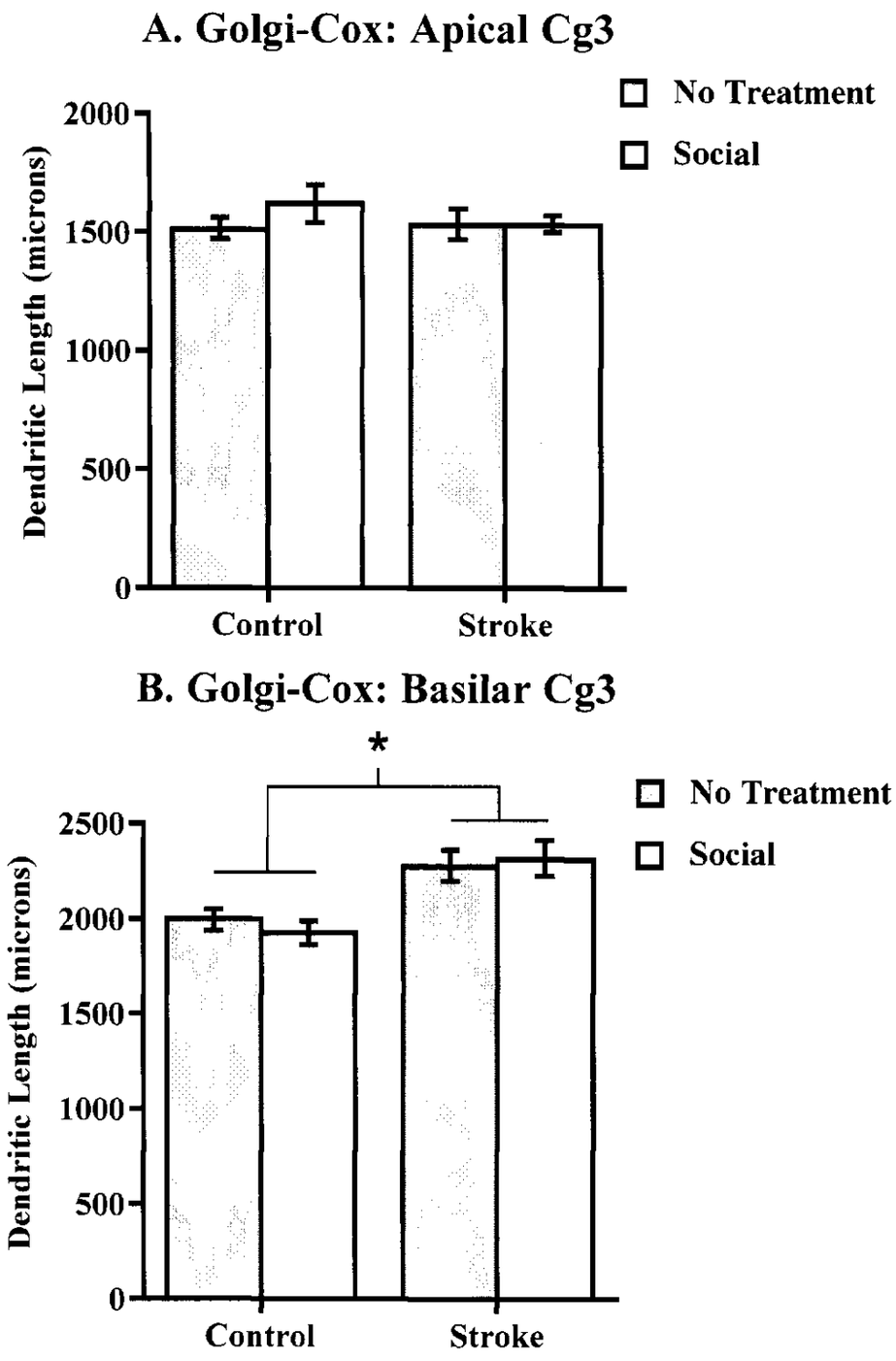

Figure 4-8. Dendritic length of the apical (A) and basilar (B) fields of layer III cells in the Cg3 region. The stroke injury was found to significantly increase basilar dendritic length regardless of social condition.

dendritic length in un-operated animals that also received social experience, although this did not reach statistical significance.

A two-way ANOVA (Surgery x Treatment) for the apical tree found no effect of injury ($F(1,44) = 0.274$ $P = 0.6030$), or social experience ($F(1,44) = 0.838$ $P = 0.3649$). The identical analysis of the basilar tree indicated a significant effect of surgery ($F(1,44) = 20.541$ $P < 0.0001$), but not treatment ($F(1,44) = 0.068$ $P = 0.7961$), nor the interaction ($F(1,44) = 0.527$ $P = 0.4719$).

Discussion

The results of the current experiment demonstrate that there are permanent structural changes in the orbital and medial prefrontal cortices of rats following unilateral motor cortex stroke. In addition, we also show that the spontaneous behavioural recovery that occurs after such injury can be completely blocked by exposing the animals to a form of social experience that presumably engages the frontal brain circuitry.

The alteration of neural networks following cortical injury has been well documented in previous experiments (eg. Gonzalez and Kolb, 2003)) but the exact causes and details of the dendritic changes are still poorly understood. One explanation of lesion-induced dendritic plasticity is that the behavioural adaptations following the injury are directly responsible for the anatomical changes. For example, it has been observed that after a unilateral motor cortex stroke in rats there is significant dendritic hypertrophy in the intact motor cortex as well as an increase in the use of the non-effected paw (Allred and Jones, 2004). Based on the timecourse of this behavioural and anatomical reorganization it is believed that the behavioural compensation is most likely driving the

structural changes in the intact hemisphere. It should be emphasized that dendritic changes that are induced by the emergence of behavioural compensation may be different in nature from the plastic processes that directly facilitate the recovery of function. In the current experiment we observed significant dendritic changes in two cortical regions and based on the known function of these areas we are able to draw certain conclusions abut the nature of these changes.

The layer 2/3 cells of the orbital cortex showed significant hypertrophy following lesions. It is very unlikely though that these dendritic changes are directly related to the behavioural improvement that was observed following the injury as this area does not have any motor projections to the spinal cord or other motor areas. A more likely explanation is that this change is driven by the increased reliance of the animals on olfactory input following the lesion induced sensory-motor impairments. Given that rats spend a large portion of their time exploring their environment through tactile forepaw placements (Gharbawie et al., 2005), the emergence of heightened sensory abilities in a different sensory domain might serve as an effective compensatory strategy following sensory-motor cortex lesions. The orbital prefrontal cortex is known to subserve such olfactory function through extensive afferent projections that it receives from the olfactory bulb (Alvarez and Eichenbaum, 2002).

The medial frontal cortex also shows dendritic changes following motor cortex stroke and there are at least two compelling arguments that suggest that the plastic changes in this region are actually facilitating the motor recovery. First, tracing studies have shown that projections arising from Cg3 that terminate in the striatum and the spinal cord subserve some motor function in the intact brain (Levesque and Parent, 1998).

Given that the motor recovery following injury is likely facilitated by plastic processes in remaining cortical regions, the Cg3 would be the most likely region to overtake this function based on its existing motor connections. The second line of evidence comes from previous experiments that have reported dendritic reorganization of Cg3 neurons in conjunction with limited spontaneous recovery following motor cortex lesions. The exact details of these studies are somewhat inconsistent in that some studies report hypertrophy in Cg3 following injury (Kolb et al., 1997) whereas others found the exact opposite, or no change (Gonzalez and Kolb, 2003). A more recent study provides a possible explanation for the conflicting results by showing that the exact details of the dendritic changes are dependent on the precise etiology of the injury (Gonzalez and Kolb, 2003). For example MCAO lesions were associated with atrophy of Cg3 neurons whereas devascularization caused hypertrophy in only the apical branches in the same neurons. The fact that in the current study we found hypertrophy of the basilar tree might be explained by differences in the amount of behavioural training as well as differences in lesion size between the two studies. Gonzalez *et al* trained animals on a number of different behavioural tasks that assessed gross motor abilities, whereas in the current experiment behavioural training was limited to single pellet reaching and therefore required the practice of more fixed or limited forelimb movements. The lesions produced in the current study were also significantly smaller that those of Gonzalez *et al*, and lesion size has previously been shown to be a factor in the degree of dendritic reorganization following injury.

Effects of Social Experience

In previous experiments we have investigated the effects of the social experience paradigm used here on dendritic and synaptic reorganization in both the AID and Cg3 cortical regions of female Long-Evans rats (Hamilton et al., 2003). In the current experiment we conducted a pilot study on male rats of the same species to investigate the effects of social experience on dendritic morphology in AID. We found significant hypertrophy of AID cells in these animals as well, but surprisingly, when the social experience was administered in conjunction with behavioural training the dendritic changes were no longer present. The behavioural training alone did not induce any dendritic changes in AID, suggesting that the region does not play a direct role in the acquisition of the motor skill. It is possible however, that the olfactory component of the reach training influenced the cells in AID in a way to block the subsequent effect of social experience. Specifically, the reach training may have altered the distribution of dendritic spines, which would likely alter the dendritic response of the cells to subsequent experience.

Even more surprising was the finding that the social experience completely blocked the behavioural recovery following the stroke lesion. We would anticipate that if the lesion induced dendritic changes in the socially-stable animals are somehow related to the behavioral improvements, then the rats in the changing social environment ought to have improved functional outcome as well. It is possible that the dendritic length measurements are misleading here as they mask more subtle changes in synaptic reorganization. That is, the combination of injury- and experience-related synaptic reorganization may produce a different pattern of synaptic organization than the injury-

related changes alone. The idea that synaptic growth might interfere with motor behaviour has at least one precedent. Gonzalez *et al.*, (Gonzalez et al., 2005) have shown that modifying dendritic plasticity through the administration of nicotine also interferes with the learning of the same single pellet reaching task used here, and repeated doses of nicotine produce dendritic changes in both AID and Cg3 (e.g., Robinson and Kolb, 2004).

An alternate explanation of the behavioural results is that the poor behavioural recovery following the injury resulted from the effect of stress that may be associated with the social instability. It has been shown that stress can inhibit the skilled motor performance of intact animals as well as animals with brain injury (Metz et al., 2001). In our experiment we quantified stress through open field activity as well as through the analysis of circulating corticosterone levels at the end of the experiment. Although these measures indicated that the social experience did not induce a chronic stress response, these measures did not tell us anything about the acute levels of stress, especially immediately following the injury. Nonetheless, if the animals were experiencing acute stress as a result of the social experience, it would be predicted that the behavioural performance of the control animals would decease and that the performance of the lesion animals would slowly recover, as the experience became less stressful. However, neither of these trends were observed in the current experiment. It is also possible that the acute stress of social instability may have altered regions that we did not investigate here, such as amygdala or hippocampus, and it is the changes in these regions that affected the motor behaviour.

Another possible explanation for our unexpected negative effect of social experience on functional recovery after stroke is that social experience somehow affects the production of neurotrophic factors that are important for functional recovery. The expression of factors such as Fibroblast Growth Factor-2 (FGF-2) and Brain-Derived Neurotrophic Factor (BDNF) is influenced by various forms of experience (Branchi et al., 2004; Berchtold et al., 2005). It is not known, however, how social experience might influence neurotrophic factor production. If social experience (or possibly associated stress) acted to suppress such production then we might expect some interference with spontaneous recovery.

Finally, we would be remiss if we did not comment on the relevance of our results to the treatment of stroke patients. Hospitals are characterized by social instability. There are new physicians, nurses, orderlies, and with the exception of private rooms, there is often considerable turnover in roommates. We can only wonder how these social factors might influence recovery from stroke.

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Chapter 5

General Discussion

Discussion

The current thesis investigated the effectiveness of three novel treatments in promoting functional and structural restitution following motor cortex stroke in rats. The treatments were selected because of their ability to induce dendritic plasticity in the cerebral cortex of uninjured animals. Stroke in humans is often associated with significant motor and cognitive deficits, and recovery is rarely complete. Although a large number of clinical trials are currently underway to investigate the effectiveness of various stroke treatments, the results thus far have provided conflicting results. In fact, a recent meta-analysis evaluating the effectiveness of all stroke treatments currently in use in the clinical population revealed that the evidence for beneficial effects of treatment versus no treatment is limited (Teasell et al., 2004). It is argued therefore, that the strategy for identifying new effective treatments should be founded on evidence-based practice. In other words, only those treatments that have been shown to have beneficial effects on recovery should be adapted to clinical practice. Any deviation from this practice would run the risk of implementing treatments based on anecdotal experiences, and are likely to be ineffective at improving patient outcome and in some cases can even be harmful to patients (Hart et al., 2000).

A review of both the human and animal literature shows that there are at least two lines of evidence indicating that synaptic plasticity mediates behavioural recovery following stroke injury. The first comes from studies that have consistently shown that spontaneous behavioural recovery is mediated by remaining brain regions undergoing plastic changes (Frost et al., 2003). The second line of evidence comes from initial experiments showing that increasing dendritic measures through various treatments (such

as environmental enrichment) further improves functional outcome following stroke (Biernaskie and Corbett, 2001). Taken together, these findings provide strong support for the general idea that brain plasticity influences functional restitution following stroke. Although we now know that a large number of treatments influence plasticity in the normal brain, the mechanisms of these treatments are quite variable and therefore their effectiveness must be investigated in the injured brain as well. Such investigations should include a comprehensive behavioural and anatomical evaluation, as the treatment may have variable effects on these measures.

In the current thesis, the induction of dendritic reorganization through dietary, pharmacological, and environmental treatments allowed us to draw four different conclusions about plastic changes and stroke. First, we found that unilateral pial strip lesions induce significant dendritic changes in the prefrontal and posterior parietal cortices of rats. Second, we found that inducing plasticity in the posterior parietal cortex (chronic NS398 administration) did not induce true recovery of motor function, but was still effective at promoting motor improvement. Third, we found that a factor that is known to alter dendritic plasticity in the intact brain (vitamin supplements) does not necessarily do so following stroke. Finally, we found that a behavioural manipulation (social experience) that induces dendritic reorganization in frontal cortical areas blocks all of the spontaneously occurring behavioural recovery following stroke.

Lesion Induced Dendritic Changes

Our extensive investigation of the effects of a unilateral motor cortex injury on the dendritic profile of cells in the remaining cortex indicated that there were significant

dendritic changes in both anterior and posterior cortical regions. More specifically, an analysis of total dendritic length indicated that following the unilateral lesion, layer III cells in the medial frontal, and layer II/III cells of the orbitofrontal cortices showed a significant increase in both hemispheres relative to controls. In contrast, layer III cells in the primary somatosensory cortex (Par 1) showed a significant decrease in dendritic length in the injured hemisphere only.

The increase in dendritic material in the cingulate cortex is likely a compensatory mechanism for the loss of the primary motor cortex. The motor projections that originate from this region are not the primary corticospinal projections that control skilled motor behaviour in an intact animal, as they reach the spinal cord through intermediate striatal and thalamic circuits (Levesque and Parent, 1998). The dendritic changes that are observed following a motor cortex lesion, however, suggest that the area partially supports the spontaneous motor recovery (Gonzalez and Kolb, 2003). In contrast, the orbitofrontal cortex does not project to the spinal cord, therefore it is likely that the dendritic changes observed in this region are different in nature from the changes in the cingulate cortex. In fact, it may be the case that the changes in the orbital cortex result from the over-reliance of the animals on olfactory sensory input following the forepaw sensorimotor impairments. More specifically, training on the single pellet reaching task, where the rats use olfaction to guide skilled limb movements toward a food pellet, may have a larger effect in the injured animals and thus induce dendritic hypertrophy. The orbital cortex is known to contain significant afferent olfactory input, so it is possible that engaging the olfactory system through repeated reach testing would cause permanent dendritic reorganization in this region. This idea is further supported by the fact that

animals with orbitofrontal lesions are impaired at the skilled reaching task used here, whereas they show no impairments in other measures of motor behaviours that are not dependant on olfaction (Kolb, personal communication).

In contrast to the dendritic hypertrophy observed in frontal brain regions, the atrophy that was observed in the posterior somatosensory cortex only occurred in the lesion hemisphere. The somatosensory cortex has abundant reciprocal connections with the primary motor cortex (Kolb and Whishaw, 2003), therefore it is possible that this area undergoes atrophy similar to that observed in thalamic nuclei following frontal cortical lesions (e.g., Vicedomini et al., 1982) It is possible that the cellular atrophy that was observed in the primary somatosensory cortex was caused by the loss of input from the motor cortex.

A curious finding of the current study was that the dendritic changes were bilateral in the anterior cortical regions and unilateral in posterior cortex. This may be due to the presence of significantly more cross-hemisphere connections in anterior brain regions relative to posterior areas. Both the corpus callosum and the commissural connections contain significantly more fibers in the anterior part of the brain (Paxinos and Watson, 1998) relative to posterior brain regions, and a large portion of these fibers connect homotopic brain regions of the two hemispheres. Given this anatomical organization, it makes sense for unilateral lesions to cause bilateral reorganization in brain regions containing significantly more inter-hemispheric connections.

A second possibility is that the atrophy resulted from the animals using their injured paw less for things such as exploration and the manipulation of food within their home cage. This unilateral decrease in activity would likely cause an atrophy of cells in

the somatosensory regions of the cortex. The fact that cortical regions in the intact hemisphere reorganize following unilateral motor cortex stroke has previously been observed by Allred and Jones (2004) who reported that animals with unilateral motor cortex strokes showed an increased reliance on the intact paw and a correlated hypertrophy of cells in motor regions of the intact hemisphere (Allred and Jones, 2004). In contrast to our current findings, this structural change found by Allred and Jones was found to be transient as it disappeared when the animals started using the injured paw again. Although the design of our current experiments did not allow for the investigation of transient dendritic changes following the unilateral stroke, the fact that the observed changes were present at least 7 weeks after the surgery suggests that they are permanent in nature.

Improved Behavioural Performance Without Recovery of Function

The goal of most therapeutic interventions is to reverse the behavioural impairments that emerge following brain injury. In the case of focal damage, such as stroke, this can theoretically be achieved in two different ways. First, efforts may be directed at reinstating the lost functions by replacing the lost tissue either through the recruitment of endogenous stem cells, or through the transplantation of precursor cells to the site of injury. In theory, the cells that repopulate the lesion cavity would eventually replace the brain circuitry that was lost due to the injury and thus allow the animals to truly recover the lost functions. Studies employing this strategy have been successful at promoting cells to repopulate the lesion area and have also shown significant behavioural

improvements that are dependent on the new cells. The strictest definition of functional recovery, however, states that the behavioural improvement must be mediated by the replacement of the exact same synapses that mediated the behaviour before the injury (Whishaw, 2000). Currently, evidence is lacking for the direct role of these cells in the behavioural recovery, as the cells have yet to be proven to form active connections with the remaining neural tissue. It is likely that the positive effects are mediated by chemical factors that are secreted by the cells rather than the direct wiring of the new cells into the remaining brain circuits.

The second strategy for promoting functional improvement following stroke is to stimulate anatomical changes in remaining brain regions that could potentially support behavioural compensation. Superficial behavioural analyses often fail to differentiate between these two phenomena (recovery and compensation), as the end point measures are the same in both cases. For example, in skilled reaching tasks the end point measure of reaching success often returns to pre-lesion levels as time passes, suggesting that the behaviour has recovered. More detailed analyses however have consistently shown that the movement patterns and the motor strategies used by the animals following the "recovery" are completely different from the pre-lesion movements (Whishaw, 2000). It is therefore more appropriate to attribute the behavioural improvements to compensatory mechanisms. Anatomical reorganizations in remaining motor regions (such as synaptogenesis or axonal sprouting) are likely responsible for the development of the compensatory movements, whereas regenerating the lost tissue may potentially support true behavioural recovery.

Factors that induce plastic processes in remaining motor regions would therefore be expected to stimulate behavioural compensation. The findings of the current set of experiments demonstrated that plastic processes in non-motor cortical regions can also support behavioural compensation in motor tasks. Specifically, rats receiving chronic NS398 treatment were found to make significantly more reach attempts and thus also made significantly more successful reaches in the reaching task. In contrast, the impairments in the Schallert cylinder and the forepaw inhibition tasks were not affected by the treatment. The key difference here is that during the reaching task the animals were forced to use the impaired limb, whereas the other tasks measured the spontaneous use of either fore-paws. These results suggest that the treatment may be inducing improved motor function when the animals are required to use the impaired forepaw, but when given a choice, the rats still prefer to use the intact forepaw (Cylinder task). This behavioural profile was also associated with dendritic hypertrophy, relative to untreated animals, in layer III pyramidal cells of the posterior parietal cortex. This cortical region contains very few neurons that have corticospinal projections (Paxinos, 2004), suggesting that the treatment did not improve the animals' motor abilities *per se*, but rather facilitated a more efficient use of the existing motor abilities. Animals that only received lesions (without any treatments) also showed an increase in reach attempts, indicating that this is a natural strategy used to compensate for the impaired limb. The fact that the treatment significantly increased the number of successful reaches (relative to animals with lesions only) indicates that the treatment is able to facilitate behavioural adaptations that may be mediated by dendritic hypertrophy in the posterior parietal cortex.

Treatments Affect the Injured and the Intact Brain Differently

Although all three treatments that were studied in the current experiment induced plastic changes in the intact brain, the vitamin supplement was found to be ineffective at altering dendritic structure following stroke injury. This finding is curious, especially given the fact that the cortical area where the measurements were taken (Par 1) shows dendritic atrophy following stroke, and dendritic hypertrophy following vitamin supplement treatment in intact animals. The results indicate that the vitamin supplement was ineffective at restoring the level of pre-lesion dendritic arborization in the posterior parietal cortex. It thus appears that some treatments that affect the uninjured brain may have different effects in the uninjured brain. There appears to be some existing evidence in support of this conclusion. For example, Biernaskie & Corbett (2001) found that although neither ischemia nor complex housing induced dendritic reorganization in motor cortex, the combination of treatments did induce significant dendritic hypertrophy. Similarly, (Gibb, 2005) has shown that whereas prenatal exposure to nicotine does little to the intact brain, this exposure facilitates recovery from perinatal injury. Thus, it appears that the injured brain may sometimes react differently to treatments than the uninjured brain.

It may be the case that that the vitamin supplement would have been effective had it been administered in conjunction with another form of intervention, as has been found to be the case with some previous treatments. For example, bFGF treatment alone following motor cortex injury was found to be ineffective at stimulating recovery,

whereas pairing it with behavioural rehabilitation stimulated significant behavioural improvements (Witt-Lajeunesse et al., 2005). Although the vitamin supplement caused dendritic changes in the intact brain, it is likely that the treatment would have to be administered in combination with additional interventions such as complex housing or behavioural rehabilitation in order to be effective in the injured brain

Can Plastic Changes Have a Detrimental Effect on Functional Outcome?

The general premise of most investigators studying the effects of plasticity on recovery from adult brain injury is that an increase in cortical plasticity is beneficial. This belief is based on several experiments that have shown that housing animals in a complex environment results in improved outcome from cortical injury (Ohlsson and Johansson, 1995; Dahlqvist et al., 2004; Johansson, 2004). Complex housing induces dendritic hypertrophy in cells of every area of the cortex examined, except the prefrontal cortex (Kolb et al., 2003). More recent experiments that investigated the timecourse of the enrichment induced plasticity revealed that there is in fact an initial hypertrophy of cells in the orbitofrontal cortex in the first two weeks of enrichment, but the effect disappears by the third week of the enrichment period (Comeau, 2005, personal communication). In contrast to environmental enrichment, the social manipulation that was employed in the current set of experiments causes permanent dendritic changes in the prefrontal cortex and therefore allowed us to investigate the role of plastic changes in this region on functional restitution. In contrast to the beneficial effects of structural

plasticity in posterior brain regions, we found that animals receiving the social manipulation performed significantly worse relative to untreated rats.

The fact that the prefrontal cortex does not readily undergo experience-dependent change has made it difficult to study the role of this region in facilitating recovery following cortical injury. The current experiments, however, have shown that the stroke injury induces what appear to be permanent dendritic changes in these regions. Therefore, it is possible that the detrimental effects of social experience result from further engaging this area on top of the lesion induced changes. The fact that plastic changes following cortical injury may in some cases hinder functional outcome has been reported previously. For example, reorganization of the intact hemisphere has been shown to be associated with significantly worse functional outcome following stroke relative to patients where the reorganization occurs in the injured hemisphere (Turton et al., 1996). Although the social experience used in the current experiments also induced bilateral plastic changes that were associated with poor functional outcome, it is unlikely that there is a direct and causal relationship between these two observations. A detailed examination of the cognitive effects of the social manipulation provides support for this view.

Similar to the human expereince, a stroke in the motor cortex of the rat also presents a significant change in that animals' life. In humans, the two most obvious changes that the patients must deal with are their neurological impairments, and the fact that they will be surrounded and cared for mostly by people that they have never met before. In the rat model used in the current set of experiments, although the neurological impairments are quite severe immediately after the stroke, there was substantial

spontaneous improvement in the following weeks. In fact, the impairments are only apparent when the rats are performing complex reaching movements during behavioural testing, and are unnoticeable during the performance of regular behaviours in the home cage such as eating or walking. Given this significant improvement in function, it is likely that the presence of a novel cage partner in the social group presents a more salient cognitive challenge for the animals than the neurological impairments. As a consequence, it is likely that the social rats were forced to commit more of their "cognitive resources" to mechanisms that mediate proper social interactions with their rotating cage partners as opposed to committing resources to mechanisms that would improve motor skills. This allocation of cognitive resources is reflected as plastic changes in the prefrontal cortex of social animals, and it is unlikely that this plasticity is directly responsible for the impaired motor behaviour. Instead, it may be the additional components of the social experience (such as stress or dendritic alterations in other brain regions) that caused the impairment in motor outcome. In sum, therefore, the current experiments fail to provide evidence for the idea that the dendritic changes in the frontal cortical regions are directly responsible for the impaired behavioural improvement following the injury. The possibility that neuronal changes induced by treatments that alter frontal circuitry could interfere with functional recovery could be examined in future studies that use psychomotor stimulants as treatments for stroke because these drugs produce profound changes in neurons in both medial frontal (hypertrophy) and orbitofrontal (atrophy) cortex (e.g., Robinson and Kolb, 2004).

Relevance to Clinical condition

The current thesis was aimed at investigating the role of plastic processes in stimulating functional restitution following stroke. The three treatments that were administered to induce the dendritic reorganization are part of the standard care that some stroke patients receive while at the hospital, however their efficacy in stimulating improved outcome are largely unknown. For example, stroke patients are often given non-steroidal anti-inflammatory drugs after their injury to reduce swelling, but the effects of this treatment on dendritic plasticity have remained largely uninvestigated. Given the results of the current experiments it would be instructive to investigate the correlation between the level of functional outcome and the administration of COX-2 inhibitor drugs (such as Celebrex) in the clinical population.

Similarly, the effects of the vitamin supplement used in the current experiments should also be investigated. The fact that the supplement was found to increase cortical plasticity in the intact brain, and the previous finding that the treatment is effective at ameliorating the symptoms of several psychiatric disorders in patients (Kaplan et al., 2001; Kaplan et al., 2002) suggests that this treatment would be worthwhile investigating in the clinical stroke population. Although we did not find it to be beneficial in our animal model of stroke it may be that combining the vitamin supplement with rehabilitative therapy would be beneficial.

Perhaps the most relevant portion of the current thesis to the clinical population was the investigation of the role of social experiences in influencing functional restitution following stroke injury. Stroke patient care in general hospital settings is often provided by caregivers that work various shifts throughout the work week and see a large number

of patients each day. This rotation of caregivers does not facilitate the development of stable relationships between the patients and the nurses, and thus likely causes patient discomfort and also higher levels of stress. In contrast to the rat studies, where the stroke symptoms were mainly motor impairments, clinical patients often also exhibit a wide range of cognitive symptoms such as depression, agnosia or dysphasia, and therefore require more intensive nursing care. Nurses that would be more familiar with the personalities and the individual needs of patients would likely prove to be more beneficial relative to the rotating nurses. In fact, the significantly improved outcome of patients cared for in dedicated stroke units is believed to be partly mediated by the more individualized care that patients typically receive in these settings (Lincoln et al., 1996).

Limitations of Current Experiments and Future Directions

The current thesis is limited by the fact that only three different treatments were used to modulate brain plasticity and that our measure of plastic change was restricted to dendritic branch analyses using the Golgi-Cox technique. In order to generate a more complete picture of the role of cortical plasticity in functional restitution it would be necessary to investigate a larger number of treatments that would either potentiate or inhibit brain plasticity. Furthermore, when using pharmacological agents to increase plasticity (such as the COX-2 inhibitors used here) it would also be beneficial to determine the direct effects of the drug on brain placticity versus the potential side effects that the drugs may have. This can be achieved by blocking the plasticity inducing effect of the drug and thus allowing for the side effects to be investigated.

In addition, it would also be instructive to investigate plasticity using additional techniques such as electron microscopy, or the quantification of various gene products and transcription factors. A recent study has demonstrated that the Golgi-Cox technique may be applied to analyze the same brains that are used for electron microscopy (Monfils et al., 2005), however, the two techniques yield different quantitative measures of synapse numbers. A complementary analysis using the two different techniques of synapse analysis would therefore be most instructive. In addition, the ultrastructural analyses would provide additional information about synapse type and density, as well as information about non-neuronal changes such as blood vessel density. The use of molecular techniques to investigate plastic changes may be more sensitive as they quantify changes at the initial stage of the chain of events leading to the structural modifications. Such sensitive analyses would perhaps have provided a more detailed picture of the plastic changes induced by the treatments, and may also have provided more detailed insight into the mechanism of action for the various treatments.

With these limitations in mind, there are several interesting experiments that follow logically from the current thesis. First, it would be interesting to investigate the effects of the treatments following larger unilateral lesions. These larger lesions would more accurately represent the clinical condition as the behavioural symptoms would include cognitive deficits in addition to the motor impairments. To properly assess the effects of the larger lesions the animals would have to be also tested on a battery of cognitive tasks as well. It would be predicted that treatments that stimulate plastic processes in the posterior brain regions (such as the COX-2 inhibitors) would be more effective at enhancing cognitive deficits relative to the motor impairments. Additional

experiments should also be carried out to investigate in greater detail the effects of the controlled social interactions on behavioural outcome following stroke. In the current set of experiments animals that had a stroke injury were only housed with additional stroke animals, whereas intact animals were only housed with other intact animals. It would be interesting to observe the effects of the interaction of intact animals with stroke animals. It is likely that a dominance hierarchy would be established in each pair, with the injured animal most likely assuming the subordinate role. The social interactions between these pairs would probably be drastically different from what was observed in the previous experiments, and therefore it would be predicted that the anatomical and behavioural effects of this treatment would also be different.

Conclusion

The results of this thesis indicate that brain plasticity may have differential effects on functional recovery from cortical damage depending on the site of the induced plasticity and the mechanisms whereby these plastic processes are induced. More generally, the experiments also demonstrate the need for the combination of detailed behavioural and anatomical analyses when assessing the effectiveness of potential stroke treatments. In carrying out such experiments, we showed that there is a correlative qrelationship between plastic changes in posterior cortical regions and an improvement in behavioural function – in the absence of such plastic changes the behavioural effects are not present. Furthermore, we also showed that an experience that continually engages the frontal brain circuitry inhibits any spontaneous recovery following brain injury. This

experiment may be used as an example for how the methods whereby the plastic changes are induced can also have a significant effect on the level of functional improvement following brain injury.

The ultimate goal of studies that model human disease in animals is to be able to learn something that could eventually help the clinical patients. By showing that certain treatments that increase cortical plasticity improve functional outcome following stroke, the current experiments demonstrate the feasibility of using animal models for guiding evidence-based practice in the clinical population.

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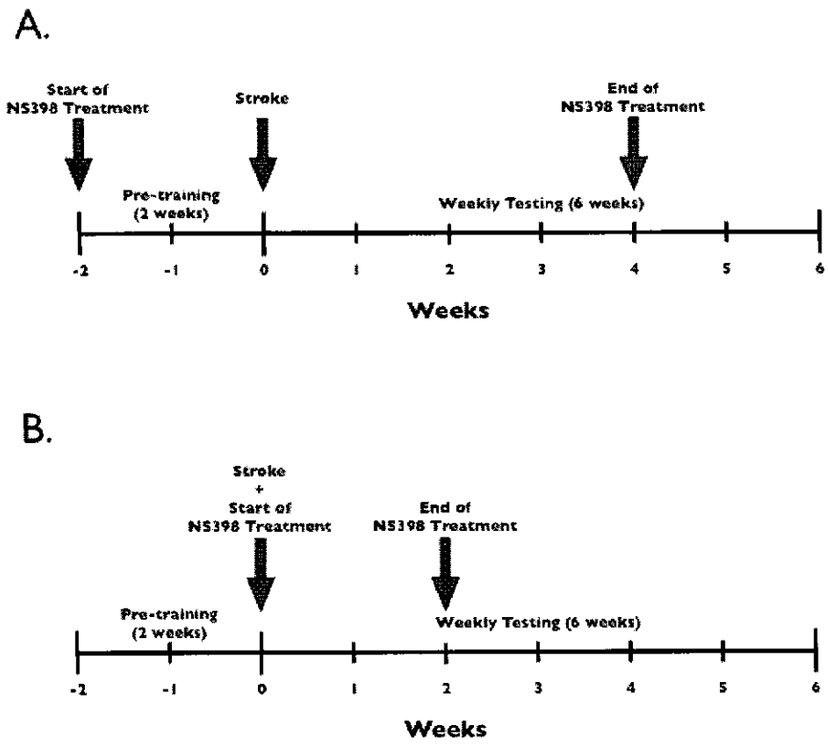
Appendix 1 – Experimental timelines for COX-2 experiments

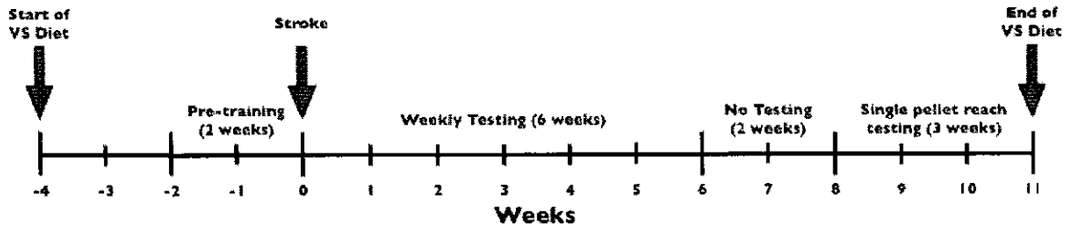


For the chronic drug treatment (A), NS398 was administered for 2 weeks prior, and 4 weeks after the stroke injury. For the post-lesion NS398 treatment, the drug was administered for 2 weeks after the injury.

Appendix 2 – EMP^{+} Ingredients

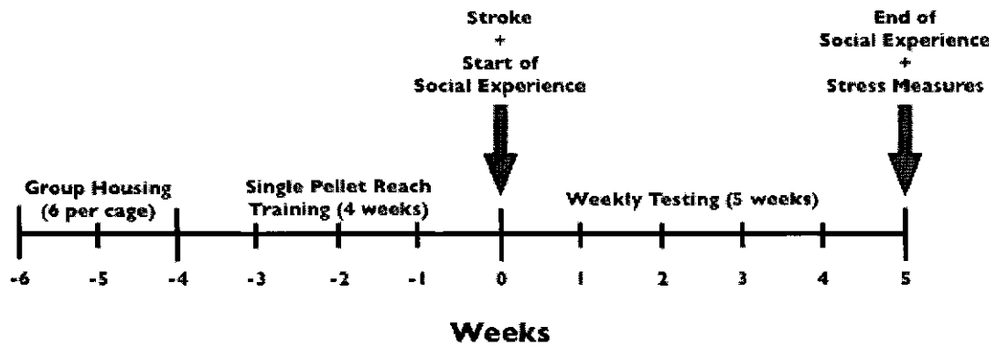
Nutrient	Amount
Vitamin A	960 IU
Vitamin C	100 mg
Vitamin D	240 IU
Vitamin E	60 IU
Vitamin B1	3 mg
Vitamin B2	2.25 mg
Vitamin B3	15 mg
Vitamin B5	3.6 mg
Vitamin B6	6 mg
Vitamin B9	240 mcg
Vitamin B12	150 mcg
Vitamin H	180 mcg
Calcium	220 mg
Phosphorus	140 mg
Magnesium	100 mg
Potassioum	40 mg
Iodine	34 mcg
Zinc	8 mg
Selenium	34 mcg
Copper	1.2 mg
Manganese	1.6 mg
Chromium	104 mcg
Molybdenum	24 mcg
Iron	2.29 mg
CNS Proprietary Blend di-phenylanine, glutamine, citrus bioflavanoids, grape seed, choline bitartarate, inositol, ginko biloba, methionin, germanium sesquioxide, boron, vanadium, nickel	277 mg

Appendix 3 – Experimental timeline for vitamin supplement experiment



The vitamin supplement treatment was initiated 4 weeks prior to the stroke lesion and continued for 11 weeks post-stroke.

Appendix 4 – Experimental timeline for social experience experiment



Social experience was initiated the day following post-surgical recovery, and was continued until the end of the experiment, at which point the stress levels of the animals was also quantified.