GM-CSF PROMOTES NEUROBEHAVIOURAL IMPROVEMENTS FOLLOWING SUBCORTICAL WHITE MATTER STROKE IN MICE

by

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Abstract

Neurobehavioural deficits caused by subcortical white matter stroke in humans often result in difficulty performing daily tasks. Due to the relative inaccessibility of the subcortical white matter arterial supply to induce focal ischemia through conventional methods, the use of the potent vasoconstrictor endothelin-1 was injected into the subcortical white matter directly to produce a localized infarct in the mouse. The objective of the present thesis was to analyze the unique neurobehavioural deficits that accompanied subcortical white matter stroke. It was hypothesized that post-stroke administration of the proinflammatory cytokine GM-CSF would promote functional recovery of the corresponding motor deficits. Behavioural analysis revealed that post-stroke injections of GM-CSF led to significant recovery of motor deficits, signifying a role for GM-CSF in subcortical white matter stroke induced neurobehavioural improvements. These results demonstrate that post-stroke administration of GM-CSF activates neuroprotective mechanisms within the subcortical white matter infarct region to enhance functional recovery.

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Results-Experiment 2:

List of Abbreviations

6-ODHA- 6-hydroxydopamine

AMPA- 2-amino-3- (3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid

ANOVA- analysis of variance

AP- anterioposterior

Apaf-1- Apoptotic protease activating factor 1

BBB- blood brain barrier

Ca^{2+}- calcium ion

CBF- cerebral blood flow

CNS- central nervous system

CSF- cerebral spinal fluid

CT- computed tomography

CV- Cresyl violet

DPBS- Dulbecco's phosphate buffer solution

DR4/5- death receptor 4/5

DV- dorsoventral

EAA- excitatory amino acid

ET-1- endothelin-1

EWMN- Eshkol-Wachmann Movement Notation

G-CSF- granulocyte colony-stimulating factor

GFAP- glial fibrillary acidic protein

GM-CSF- granulocyte macrophage- colony stimulating factor

ICAD- inhibitor of caspase-3– activated DNase

IL-1- interleukin-1

I.P.- intraperitoneal

LFB- Luxol fast blue

NeuN- neuronal nuclei

NF- neurofilament

MCA- middle cerebral artery

MCAo- Middle cerebral artery occlusion

ML- mediolateral

MRI- magnetic resonance imaging

NMDA- N-Methyl-D-aspartate

NOS- nitric oxide synthase

RNS- reactive nitrogen species

ROS- reactive oxygen species

rTPA- recombinant tissue plasminogen activation

OLG- oligodendrocyte

OGD- oxygen-glucose deprivation

PBS- phosphate buffer solution

PFA- paraformaldehyde

PNS- peripheral nervous system

PSI-protein synthesis inhibition

ROS- reactive species of oxygen

SEM- standard error of the mean

SWMS- subcortical white matter stroke

TUNNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling

TNF-α- tumor necrosis factor alpha

TNFR- tumor necrosis factor receptor

KO- knockout

WT- wild-type

GM-CSF promotes neurobehavioural improvements following subcortical white matter stroke in mice

Stroke

Disturbances in the blood supply to the brain results in an incapacitating neurological condition termed stroke (Hossmann, 2006). Disturbances can be a consequence of blood vessel blockage (thrombosis, arterial embolism) resulting in a lack of blood flow, called ischemia; or a leakage of blood from a ruptured vessel called a hemorrhage. Stroke is the principal cause of chronic adult disability, and the third leading cause of death in Canada, killing nearly 14,000 Canadians annually (2008 statistic, Heart and Stroke Foundation). Amongst the 50,000 strokes in Canada each year, 50% of patients are left with severe impairment and chronic disability, costing the Canadian economy $3.6 billion a year in physician services, hospital care, lost wages and decreased productivity (2000 statistic, Heart and Stroke Foundation).

Over 80% of strokes are ischemic, and the resulting symptoms and deficits depend on the type of ischemia. Focal ischemia occurs when cerebral blood flow (CBF) is reduced to a specific brain region and will therefore present symptoms related to the affected area. For example, unilateral paralysis, weakness or inability to walk suggests the occurrence of a stroke within the primary motor cortex of the contralateral (opposite side) hemisphere of the brain. Whereas, global brain ischemia occurs when CBF to the brain is drastically reduced or halted affecting several areas of the brain and resulting in multiple symptoms such as blindness, dizziness, slurred speech, weakness in arms or legs and loss of coordination.

Immediately following the onset of ischemia, a central "core" infarct zone develops within the brain tissue of severely compromised CBF (>10-25% of original flow). The ischemic core endures irreversible injury due to the loss of much needed oxygen and glucose to brain cells called neurons. The drastic decline in CBF results in the rapid depletion of energy stores required for metabolic processes vital for neuronal survival. The failure to maintain energy-dependent processes causes neurons to lose their normal transmembrane ionic gradients, resulting in an ion imbalance that ultimately leads to apoptotic and necrotic death signaling cascades with corresponding sensory and motor dysfunction (Hossmann, 2006; Zhang & Murphy, 2007). Surrounding the stroke core is a rim of moderately affected tissue called the penumbra. The penumbral zone is supplied with blood from collateral arteries within the brain resulting in a preservation of cellular metabolism and viability accompanied by impaired electrical activity (Astrup, Siesjo, & Symon, 1981; Astrup, Symon, Branston, & Lassen, 1977; Hossmann, 1994). Unfortunately, collateral circulation is inadequate to maintain neuronal demands for oxygen and glucose for a prolonged period of time, thus to salvage these cells, reperfusion (restoration of blood flow) must be established by 6-8 h following the initial onset of stroke symptoms (Baron, Vonkummer, & Delzoppo, 1995).

For every minute that passes following stroke onset, an average patient loses an astonishing 1.9 million brain cells, 13.8 billion synapses and 12km of axonal fibers; consequently losing as many neurons per hour as a person would in 3.6 years of normal aging (Saver, 2006). Therefore, many post-stroke therapies are aimed at limiting the extent of brain injury by targeting brain cells within the penumbra.

Unfortunately, as of yet, there are limited clinical treatments approved by health care professional that successfully treat neurological consequences of stroke following an incident. In fact, the lone accurate ischemic stroke treatment is the intravenous administration of recombinant tissue plasminogen activation (rtPA). RtPA promotes reperfusion to the blood deprived stroke region (Montaner et al., 2003) by promoting thrombolysis, the breakdown of blood clots. Unfortunately, to be effective, rtPA must be administered prior to a 4-hour deadline following the onset of stroke symptoms. Regrettably, this restriction results in a limited population (3-8.5%) of eligible stroke patients for rtPA treatment because most patients do not seek treatment quickly enough. It is therefore, imperative to uncover new therapeutic strategies to limit the spread of post-stroke brain damage in a larger population of stroke patients. The objective of this research is to evaluate the functional behavioural deficits characterized in a subcortical white matter ischemic mouse model and administer a pharmacological agent to promote neurobehavioural recovery of corresponding functional deficits.

Stroke Research: Animal Models

A major implication to stroke injury is the inherent inability for central nervous system (CNS) cells to survive or regenerate following an ischemic insult. Numerous experimental models have been developed to study the specific attributes involved in the complex array of post-stroke responses within CNS cells that may be involved in this phenomenon. Due to the extensive economic burden created by permanent post-stroke care for patients with functional deficits

impairing the performance of daily tasks, researchers are keen in the development of therapeutic strategies to explore potential mechanisms targeting apoptosis, neuroprotection and survival. The characterization of these mechanisms could lead to the development of pharmacological agents with therapeutic capabilities to salvage the unfavorable tissue damage that persists following the onset of stroke symptoms.

Most rodent models of focal ischemia involve the manipulation of blood flow through one of the three major paired arteries that supply blood to the brain called the middle cerebral artery (MCA). In these models, the MCA is occluded transiently or permanently using either a coated intraluminal suture or microvascular clips to prevent CBF. In both cases, the suture or clip may be removed following an allotted period of time to allow reperfusion to ischemic tissue, resulting in a large infarct that often occupies much of the cerebral hemisphere including the striatum and cortex (Longa, Weinstein, Carlson, & Cummins, 1989). Unfortunately, with permanent or prolonged periods of occlusion, the subsequent cerebral damage is often accompanied by hypothalamic injury that impairs motivation and temperature regulation thereby obscuring the interpretation of behavioural outcomes. In addition, such widespread damage is typically fatal or untreatable in humans (Carmichael, 2005) and thus, does not provide a representative model for exploring survival and neuroprotective strategies for long-term functional deficits. Therefore, to generate a stroke that creates less extensive cerebral damage and results in specific behavioural outcomes to represent those observed following stroke in

humans, researchers use a potent cerebrovascular vasoconstrictor called endothelin-1 (ET-1) (Yanagisawa et al., 1988).

Endothelin-1

ET-1 is a 21 amino acid peptide, first recognized by Yanagisawa et al in 1988 as a potent and long lasting cerebrovascular vasoconstrictor. There are three identified isoforms of endothelin (ET-1, ET-2 and ET-3), however, ET-1 is the only isoform that is synthesized and stored within the endothelium of cerebral microvessels (Yoshimoto et al., 1990) and has been found in both neurons and glia (Giaid et al., 1989; MacCumber, Ross, & Snyder, 1990) throughout the cortex, the striatum, hippocampus, amygdala, pituitary gland, the hypothalamus, cerebellar Purkinje cells, raphe nuclei and the intermediolateral cell column of the spinal cord (Giaid et al., 1989; Lee, de la Monte, Ng, Bloch, & Quertermous, 1990; Takahashi et al., 1991; Yoshizawa et al., 1990). The expression patterns and effects on the release of several neuropeptides of ET-1 within the CNS suggest it may function as a neurotransmitter and/or a neuromodulator (Masaki, 1995; Webb, Monge, Rabelink, & Yanagisawa, 1998; Yoshizawa et al., 1990).

The vascular effects of the endothelin bioactive peptides are mediated through two different G protein-coupled receptors: the endothelin-1 selective endothelin receptor (ET_A) and the non-selective receptor subtype (ET_B) (Arai, Hori, Aramori, Ohkubo, & Nakanishi, 1990; Sakurai et al., 1990). ET-1 binds to ET_A receptors with a much higher affinity than ET-2 and ET-3, and appear primarily on vascular smooth muscle, mediating the vasoconstrictor effects of ET-1 (Dohi &

Luscher, 1991) with the peripheral nervous system (PNS). In contrast, the ET_B receptor subtype has the same affinity for all three endothelin isoforms (Sakurai et al., 1990) but appears on the vascular endothelium to mediate the transient vasodilation effects response of ET-1 through the release of nitric oxide (de Nucci et al., 1988). Furthermore, ET_B has also been shown to mediate vasoconstriction in response to ET-1 (Moreland, McMullen, Delaney, Lee, & Hunt, 1992).

In research, ET-1 is used as a pharmacological tool capable of inducing severe, sustained and even reversible occlusion of cerebral vessels *in vivo*. ET-1 can be stereotaxically injected into any brain region of interest or to the surface of the brain to constrict local arterioles (Sharkey, Ritchie, & Kelly, 1993; Windle et al., 2006) in varying concentration to affect a specified region and infarct size. ET-1 application reduces local CBF gradually (Macrae et al., 1993) and for a prolonged period of time followed by a steady progression of reperfusion over several hours to the compromised brain tissue (Biernaskie & Corbett, 2001; Macrae, Robinson, Graham, Reid, & McCulloch, 1993). The characteristics of gradual reperfusion following ET-1 administration accurately represent human stroke evolution in comparison to the rapid, abrupt reperfusion that occurs following the removal of luminal sutures or microvascular clips in MCA occlusion models.

Within 4h of ET-1 application, robust neuropathological tissue damage is evident within the injected region (Macrae et al., 1993). Varying the concentration or volume of intracerebral ET-1 injections correlates to the lesion size produced within the inoculated region, thereby creating an association between damage and behavioural deficits, in an attempt to a represent models of human stroke. For

example, the intracranial application of exogenous 0.5µl of 10^{-3} M ET-1 is capable of reducing striatal blood flow to the pathologically low level of 60% within 20 minutes lasting up to 3 h (Fuxe et al., 1992). Lower doses of ET-1 (0.15µl of 10^{-5} M) result in a 40% reduction in CBF (Willette, Sauermelch, Ezekiel, Feuerstein, & Ohlstein, 1990) leading to metabolic breakdown and eventual cell death (Biernaskie, Corbett, Peeling, Wells, & Lei, 2001).

Due to the limited research that investigates stroke induced axonal injury and behavioural deficits as a result of subcortical white matter stroke (SWMS) in mice, generating damage within a targeted region of the corpus callosum can provide an excellent means to investigate potential therapeutic intervention. A reliable method to create targeted focal damage within the brain is with the use of ET-1.

Subcortical White Matter Stroke

Strokes affecting the subcortical white matter regions of the brain such as the corpus callosum accounts for approximately 30% of all strokes in humans. What is most intriguing about subcortical white matter stroke lesions is the prevalence of "silent" infarcts that do not correlate to neurological clinical pathology. In fact, white matter lesions are common in healthy elderly people in addition to stroke patients. Little is know about the relevance of subcortical white matter lesions, however it has been shown that people with white matter lesions are at an increased risk of stroke to more than 3 times, independent of other stroke risk factors such as cerebrovascular disease (Vermeer et al., 2003). Subcortical white matter lesions

also result in cognitive decline in executive functioning and processing speeds, dementia and even death (Debette & Markus, 2010).

Subcortical white matter lesions observed in computed tomography (CT) and magnetic resonance imaging (MRI) in first time stroke patients and in neurologically normal elders are likely caused by cerebral ischemia due to pathologically consistent results of demyelination, gliosis, and necrosis (Munoz et al., 1993; van Sweiten et al., 1991). SWMS has had inadequate pre-clinical modeling due to the relative isolation of the arterial supply; however, using ET-1 to target the arterial supply of the corpus callosum has shown promising biochemical indications of stroke pathology (Carmichael et al., 2009).

Post-Ischemic Cell Death

Following ischemia, neurons undergo one of two types of cell death depending on the severity of CBF reductions and consequent decreases in metabolic energy. For example, energy depletion occurs within a few hours in permanent MCA occlusion models in the rat (Shiraishi, Sharp, & Simon, 1989), resulting in protein synthesis inhibition (PSI) leading to the mode of cell death called necrosis. Whereas, transient ischemia models resulting in temporary energy failure result in the fragmentation of genomic DNA (Y. Li et al., 1995; MacManus, Buchan, Hill, Rasquinha, & Preston, 1993) demonstrating a slower form of neuronal death with features of apoptotic cell death. The mechanisms of necrosis and apoptosis following stroke have been studied extensively, indicating that necrosis typically occurs within the stroke core and apoptosis within the penumbra.

Although the primary pathologic mechanism of stroke is energy depletion, there has been a considerable amount of evidence demonstrating a role for the excitatory amino acid (EAA), glutamate, in contributing to post-ischemic cell death. Glutamate is released by approximately 40% of all synapses in the CNS (Fonnum, 1984), and activates several types of pre- and post-synaptic glutamate receptors. While glutamate participates in a host of neurological functions including memory, movement, sensation, cognition and synaptic plasticity (Gasic & Hollmann, 1992; Lipton & Kater, 1989), it also has a pathological effect by inducing excitotoxicity (Olney, Ho, & Rhee, 1971). Excitotoxicity is the excessive activation of neuronal glutamate receptors caused by high concentrations of glutamate within the synapse, leading to a drastic increase in calcium ion (Ca^{2+}) influx (Beckman & Koppenol, 1996; Kitagawa et al., 1999; Velier et al., 1999). Extreme increase in Ca^{2+} causes mitochondrial calcium overload, termination of ATP production and breakdown of phospholipids, proteins and nucleic acids by activation of calcium-dependent phospholipases, proteases and endonucleases. In addition, increased intracellular Ca^{2+} surges extracellular glutamate concentrations thereby propagating excitotoxicity (Moro, Cardenas, Hurtado, Leza, & Lizasoain, 2004). Amongst the 3 known ionotropic glutamate receptors; the *N*-methyl-D-aspartate (NMDA) receptor, the AMPA receptor, and the kainate receptor; NMDA receptors are the most permeable to Ca^{2+} ions, and are thought to play an important role in the development of excitotoxicity (Hazell, 2007). Moreover, NMDA induced excitotoxicity has shown to trigger tissue damage in both experimental (Arundine & Tymianski, 2004; D. W. Choi, 1992) and clinical focal ischemia (Nakanishi, 1992). In

fact, it is the excessive rise in intracellular Ca^{2+} caused by NMDA induced excitotoxicity that leads to many downstream neurotoxic cascades (Hara, Fink, et al., 1997; Hara, Friedlander, et al., 1997; Lawrence et al., 1997) that ultimately cause necrosis and apoptosis.

Necrosis

Necrosis is characterized by cellular energy failure resulting in rapid PSI within the ischemic core. The mechanics of necrosis work in contrast to those of apoptosis. In fact, apoptosis relies on protein synthesis for the upregulation of proteins such as caspase-3, -9 and blc-2 to influence programmed cell death. Therefore, the proteins that implement apoptotic cell death may not be expressed due to rapid PSI presented in necrosis. Characteristics of necrosis and stroke, including cytoplasmic swelling, inflammation, dissolution of organelles and plasma membranes, indicating that both the consequences of ischemia and necrosis may be one in the same.

Necrosis occurs mainly within the first minutes following the onset of ischemia within the core region (Bonfoco, Krainc, Ankarcrona, Nicotera, & Lipton, 1995; Dirnagl, Iadecola, & Moskowitz, 1999; Nicotera, Leist, & Manzo, 1999; Unal-Cevik, Kilinc, Can, Gursoy-Ozdemir, & Dalkara, 2004). Unfortunately, necrotic tissue within the ischemic core is irreversibly damaged regardless if blood flow is re-established. Necrosis affects large numbers of contiguous cells that undergo swelling of the cytoplasm and organelles including the mitochondria. These events lead to the rupture of the plasma membrane and lysis of the cell to cause an

inflammatory reaction (Love, 2003). The consequential mechanisms of increased NMDA receptor-mediated intracellular Ca^{2+} involves the activation of calcium-dependent or induced enzymes that mediate toxic effects including nitric oxide synthase, cyclooxygenase, and calpain 1. Calpain 1, a calcium-dependent protease, contributes to the production of the reactive oxygen species, superoxide (Stark, Seubert, Lynch, & Baudry, 1989). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are chemically reactive molecules that contain oxygen or nitrogen respectively, that produce cellular damage to macromolecules such as lipids, proteins, and nucleic acids, leading to cell death (Hengartner & Horvitz, 1994; Thompson, 1995). Isoforms of nitric oxide synthase (NOS) produce the toxic nitric oxide that combines with superoxide to form highly reactive peroxynitrite species that exacerbate ischemic tissue damage (Beckman & Koppenol, 1996)There is strong evidence showing that NOS are involved in the mechanisms mediating neurotoxicity after cerebral ischemia Evidence provided by both in vitro (brain slices and OGD cell cultures) and in vivo models of MCOA cerebral ischemia in rodents show that intracellular ROS contribute to brain damage following ischemia development (Moro et al., 2004).

Apoptosis

In contrast to necrosis, apoptotic mechanisms begin hours and can last up to several days following the onset of the initial ischemic insult (Broughton, Reutens, & Sobey, 2009). Characteristic morphological changes of apoptosis include: cell shrinkage, condensation of chromatin, formation of cytoplasmic protrubences at the

cell surface, and fragmentation of the cell into multiple small membrane-bound bodies that contain intact organelles (Wyllie, Beattie, & Hargreaves, 1981). The delayed initiation of apoptotic mechanisms following stroke make apoptosis an appealing target for new forms of therapeutic intervention. Apoptosis occurs mainly in the penumbra region and is both spatially and temporally different than the rapid necrotic death that occurs within the ischemic core. Additionally, apoptotic cells are rapidly removed by phagocytosis resulting in neuronal death with little to no inflammation thereby avoiding the collateral damage of neighbouring neurons. Fortunately, reintroducing CBF within an allotted period of time, and/or interfering with the ischemic cascade can rescue the neurons within penumbra.

Ischemic-induced apoptosis is commonly referred to as caspase-mediated apoptosis due to the prominent role of caspase activity in response to ischemic neuronal damage. Caspases are a family of cysteine proteases that catalyze proteins that binding to their substrate cleavage site (Cohen, 1997; Earnshaw, Martins, & Kaufmann, 1999; Hengartner, 2000; Kaufmann & Hengartner, 2001; Strasser, O'Connor, & Dixit, 2000). There are two types of apoptotic caspases, initiator and effector caspases. The initiator caspases cleave inactive pro-forms of effector caspases thus activating them. The effector caspases are responsible for the morphological and biochemical feature of apoptosis and function by cleaving cellular substrates such as cytoskeletal proteins, structural nuclear proteins, anti-apoptotic proteins and the inhibitor of caspase-activated DNase (ICAD) (Love, 2003).

There are two pathways of caspase activation: the extrinsic and intrinsic

pathways. The extrinsic pathway is also known as the death-receptor mediated pathway where death receptors such as Fas, tumor necrosis factor-alpha receptor 1 (TNFR1) and death receptors 4 and 5 (DR4, DR5) (Ashkenazi & Dixit, 1999; Budihardjo, Oliver, Lutter, Luo, & Wang, 1999; Cohen, 1997; Strasser et al., 2000) are activated in response to molecular signals released during an ischemic-attack. Research indicates an upregulation of the transmembrane TNFR1 and the Fas receptor/ligand following cerebral ischemia (Felderhoff-Mueser et al., 2000; X. Wang et al., 1994). Additionally, a corresponding increase in caspase-8 that is activated by both the Fas and TNF receptors has shown a corresponding increase in expression and activation following ischemia (Velier et al., 1999). This increase in the initiator caspase-8 results in downstream significant and direct cleavage and activation of the effector pro-caspase-3 (Scaffidi et al., 1998) resulting in apoptotic death. A large body of evidence shows that brain ischemia can cause the activation and upregulation of caspases, especially caspase-3 in the hippocampus and caudate-putamen in transient focal ischemic models (Hermann, Kilic, Hata, Hossmann, & Mies, 2001; Namura et al., 1998; Niwa et al., 2001). One report indicated that transient global ischemia in the rat caused caspase-3 mediated cleavage of ICAD, resulting in apoptotic degradation of DNA by CAD (Cao et al., 2001).

The main culprits shown to trigger the intrinsic (mitochondrial) apoptotic pathway are oxidative stress and the cytotoxic accumulation of intracellular Ca^{2+} (Dirnagl et al., 1999; Niizuma et al., 2010). Increased reactive oxygen/nitrogen species (ROS/RNS) and Ca^{2+} mediate the induction and activation of pro-apoptotic proteins including Bcl-2 members Bax and Bad, leading to changes in the

mitochondrial membrane permeability (Green & Kroemer, 2004). Typically, the anti-apoptotic Bcl-2 family members protect mitochondrial integrity by inhibiting pro-apoptotic proteins. However, following ischemia, pro-apoptotic proteins may antagonize the anti-apoptotic Bcl-2 proteins leading to mitochondrial damage. Research has shown an increase in levels of pro-apoptotic proteins Bax and Bad along with decreases in anti-apoptotic Bcl-2 proteins within the stroke core and penumbra following an ischemia attack (Ferrer & Planas, 2003). Following both focal and global cerebral ischemia, apoptotic mitochondrion releases the pro-apoptotic protein cytochrome c. Cytochrome c promotes oligomerization of apoptosis activating factor-1 (Apaf-1), binding of the initiator procaspase-9 to the Apaf-1 oligomer, and the assembly of the apoptosome. The apoptosome comprises of molecules of cytochrome c, Apaf-1, and caspase-9 that function to activate and upregulate pro-caspase-3, the key effector of caspase-mediated cell death (Broughton et al., 2009; Love, 2003).

Ultimately, the synergistic effects of free radical generation (Beckman & Koppenol, 1996; Bond et al., 1999; Pellegrini-Giampietro, Zukin, Bennett, Cho, & Pulsinelli, 1992) death receptor activation and mitochondrial dysfunction (Lam et al., 1998; Miller, Sarantis, Traynelis, & Attwell, 1992) leads to deterioration of scaffolding and signaling by the destruction of cellular proteins (Ying, Han, Miller, & Swanson, 1999), lipids (Murphy, Miyamoto, Sastre, Schnaar, & Coyle, 1989), and DNA (Snape, Baldwin, Cross, & Green, 1993; Wahlgren et al., 1999). These effects result in necrosis, apoptosis or both depending on the severity of the infarct and the

speed of each pathophysiological process (Boxer et al., 1990; Graham, Chen, Sharp, & Simon, 1993; S. E. Smith, Lekieffre, Sowinski, & Meldrum, 1993).

Ischemic White Matter Injury

In addition to the detrimental ischemic effects of glutamate to neuronal integrity, the cellular elements of white matter are also significantly affected. Excessive glutamate production leads oligodendrocytes (OLG) and myelin damage (Alberdi, Sanchez-Gomez, Marino, & Matute, 2002; Matute, Sanchez-Gomez, Martinez-Millan, & Miledi, 1997; Sanchez-Gomez & Matute, 1999) whereas axons are injured by ionic mechanisms leading to the accumulation of intracellular calcium (Fern, Ransom, & Waxman, 1995; Stys, Ransom, Waxman, & Davis, 1990). In fact, excitotoxic white matter injury has been shown to correlate with glutamate release following oxygen glucose deprivation (OGD) *in vitro* (Tekkok, Ye, & Ransom, 2007), an *in vitro* model developed to represent conditions following cerebral ischemia *in vivo.* Studies have shown that blocking the glutamate receptor by using antagonists protect the white matter from ischemic injury (Agrawal & Fehlings, 1997; S. Li & Stys, 2000; Tekkok & Goldberg, 2001), demonstrating that glutamate-induced excitotoxicity is a possible mechanism contributing to overall cerebral damage following stroke. These effects are due to overactivation of the ionotropic glutamate receptors AMPA and kainate that found in abundance on oligodendrocytes (Patneau, Wright, Winters, Mayer, & Gallo, 1994). However, more recent studies have demonstrated that OLGs also express NMDA receptors and thus may participate in glutamate-induced OLG damage (Karadottir, Cavelier, Bergersen, & Attwell, 2005;

Micu et al., 2006; Salter & Fern, 2005). The downstream pathways of apoptosis and necrosis elicited by excitotoxicity within white matter have been demonstrated to display the same patterns as grey matter neurons within the core and penumbra following ischemia.

Behavioural Measures of Functional Recovery following Ischemia

It is commonly believed that neuronal death leads to the irreversible loss of cognitive, sensory and motor function due to a lack of regenerative capacity within the CNS. Surprisingly, many stroke patients demonstrate significant functional improvement over weeks, months and even years, following an initial infarct. This phenomenon is termed functional recovery and is defined by scientists and clinicians as the enhancement of sensory and motor deficits initially caused by stroke. Unfortunately, the recovered behaviour frequently differs from the pre-stroke patterns due to the loss of highly specific neuronal networks required to perform highly coordinated tasks. Most of the commonly used human and animal behavioural tests are used to measure functional recovery following ischemia but can rarely determine if the behaviour reflects true recovery, behavioural compensation or a combination of both. In this thesis, the term recovery is used as a means of improved performance without distinguishing between the degree of compensation and pure recovery.

Studies in rodents, investigating post-ischemic kinematics of skilled reaching components show impairments in range of motion, grasping and supination of contralesional forelimb. Impairments are typically caused by the offset of postural

adjustments such as changing body angle, and rotations in shoulder and body movement that allows for compensation of motor performance to levels demonstrated prior to stroke injury (Whishaw, 2000). Similar kinematic tests and electromyography recording in human stroke patients demonstrate similar post-stroke compensatory mechanisms during reaching and grasping tasks (Levin, Kleim, & Wolf, 2009; Levin, Michaelsen, Cirstea, & Roby-Brami, 2002).

Therefore, to assess these impaired motor movements resulting as consequences of stroke, researchers have developed a variety of behavioural protocols to assess functional recovery post-stroke animal models. Some tests include, but not limited to, the cylinder test, reaching test, staircase test, rotarod test, balance beam, climbing test, corner test, inverted cage lid test and descriptive paw test. For the purpose of this thesis, the cylinder test and reaching test will be used to assess the different components of post-stroke deficits and functional recovery.

Cylinder Test- Forelimb Asymmetry

The cylinder test is primarily used to measure unilateral deficits in voluntary forelimb placement. This test was first developed to examine forelimb deficits in the rat Parkinson's disease model induced by unilateral 6-hydroxydopamine (6-OHDA) lesions (Shallert & Tillerson, 2000; Cenci & Lundblad, 2005). It was observed that 6-OHDA lesions reflected a decrease in contralesional paw use upon rearing and exploration (Iancu, Mohapel, Brundin, & Paul, 2005). Thus, based on the innate exploratory behaviour in the rodents, the investigation of the neural basis of spatial

and motor behaviour is used as a tool to assess brain function (Gharbawie, Whishaw, & Whishaw, 2004) by evaluating a rodent's spontaneous forelimb use upon vertical wall exploration when rearing on their hindlimbs.

The cylinder test has been widely used as a measure of contralesional forelimb deficits in motor system injury models of stroke (Bland et al., 2000; Markgraf et al., 1992; Schallert, Fleming, Leasure, Tillerson, & Bland, 2000), to assess functional deficits related to unilateral brain damage caused by middle cerebral artery occlusion (MCAO) or ET-1 injections. The test begins when a rodent is placed in a transparent, Plexiglas cylinder and an experimenter counts the number of independent wall placements with the affected and unaffected forelimb or the number of simultaneous placement of both forelimbs on the wall of the cylinder while rearing. Unilateral brain damage caused by stroke, results in the asymmetric use of the ipsilesional (unaffected) forelimb during exploration (Schallert, 2006).

Advantages to using the cylinder test include its ease in use and scoring (Schallert & Woodlee, 2005). Rodents do not require pre-training for the task; however, baseline data are generally recorded to assess pre-surgical biases in paw preference (Schallert et al., 2000). In addition, no more than 5 minutes of observation is often required to make an accurate assessment. However, the tests most prominent advantage involves its sensitivity, due to its ability to detect even mild neurological impairments (Hua et al., 2002), providing a tool to measure forelimb functional deficits in focal sensorimotor ischemia.

Reaching Task- Skilled Forelimb Use

Recent studies show that rodent skilled forelimb movements resemble primate hand movement (Lin et al., 2001) therefore; the assessment of potential therapeutic strategies to improve fine motor deficits in rodents has gained much attention. Once such assessment is the reaching test, designed to measure skilled forepaw use and motor function deficits after unilateral stroke in rodents (Allred & Jones, 2008; Miklyaeva & Whishaw, 1996; Whishaw, Piecharka, Zeeb, & Stein, 2004). Animals are placed into a reaching chamber that comprises of a Plexiglas apparatus with a platform located on the outside a wall slit that holds food. A single pellet of food is placed in one of two indentations located to the right or the left of a 1cm slit. To measure contralesional (for example, right forelimb) forelimb reaching components, a pellet is placed in the left indentation, forcing the rodent to use the injured forelimb to grasp the pellet. The animal must reach through the slit, grasp and retract the food pellet to the mouth for a successful reach. Failures, successes and attempts to grasp the food pellet are measured to determine the severity of impairment (Gharbawie, Gonzalez, & Whishaw, 2005; Metz, Antonow-Schlorke, & Witte, 2005).

Stroke induced damage to the motor cortex results in impairments of the components of reaching as described by Wishaw and Pellis in 1990. Using the conceptual framework of movement developed in 1958 by Noa Eshkol and Avraham Wachman, termed the Eshkol-Wachman Movement Notation (EWMN), Wishaw developed a 10 component system of movements presented by rodents while reaching, grasping and retracting their forelimb for a food pellet in a reaching box.

Briefly, the EWNM system expresses relations and changes of relation between body parts. The body is viewed as a system of articulated axes of body and limb segments. The polar coordinates of each horizontal space that represent the body determines the movement in relation to the environment, to the animal's body midline axis, and to the next proximal or distal limb or body segment (Eskhol & Wachman, 1958). The 10 components of movement, as described by Wishaw, include: digits to midline, digits semiflexed, aim, advance, digits extend, pronation, grasp, supination I, supination II, and release (Farr & Whishaw, 2002). In this study, reaching sequences were recorded from the lateral view providing vertical movements, and from the ventral view providing horizontal movements. The combination of these two views gave complete analysis of reaching movements.

Due to the complexity of the reaching task, a pre-training period of approximately 2-4 weeks is essential for the animals to acquire the skills required to complete the task (Schaar, Brenneman, & Savitz, 2010). Furthermore, animals must be food restricted for sufficient motivation to acquire and participate in the task. Despite the considerable amount of time required for pre-training, paw reaching is an extremely valuable way to assess fine motor deficits due to it's sensitivity to assess movements similar to those impaired in humans following stroke (Whishaw, Pellis, & Gorny, 1992). Notably, the reaching task can detect motor deficits for up to three months following the induction of stroke (Grabowski, Brundin, & Johansson, 1993) and is thus a valuable tool to measure short term as well as long-term effects of stroke or potential therapeutic strategies.

Factors Contributing to Stroke Recovery

Plasticity

Several mechanisms contributing to stroke recovery parallel those involved with plasticity (Kleim & Jones, 2008). Plasticity within the CNS, also known as neuroplasticity, refers to alterations in the strength of synaptic connections in response to changes in behaviour, the environment or neural processes (Pascual-Leone et al., 2011). There are two factors that enable plasticity in the post-stroke adult brain: the diffuse, redundant connectivity in the CNS and remapping related cortical regions to create new structural and functional neuronal circuits.

Neurons involved in complex tasks such as memory trace are not localized to a single region of the brain, but instead are distributed as networks throughout the cortex. Therefore, the brain functions as a spatially allocating computational organ, routing signals along multiple pathways, with the innate ability to adapt transmission to changes in processing. These processing changes are enabled by both the diffuse and redundant connectivity of the CNS, that may be a culprit in facilitating recovery from stroke damage (Murphy & Corbett, 2009). Significant functional improvement in the human stroke-injured brain may be a result of the upstream and downstream hierarchal distribution of neural networks affected by infarction (Chollet et al., 1991; Cramer, 2008). These networks can include the concept of lateralization, where the intact contralesional hemisphere can contribute to the recovery of a motor or sensory function of the injured behaviour. This concept applies if there are ipsilateral pathways also present; for example, when the right hemisphere controls the right side of the body (Brus-Ramer, Carmel, & Martin,

2009; Gonzalez et al., 2004). Contrary to this seemingly advantageous concept, human imaging studies demonstrate that the most successful post-stroke recovery occurs when relatively normal lateralized patterns of sensorimotor activation are seen within the infarct affected hemisphere compared to patients with bilateral cortical activation (Ward, Brown, Thompson, & Frackowiak, 2003). It appears that the complexity of lateralization and function reflect the degree of injury and therefore, the extent of recovery (Hsu & Jones, 2006; Stinear et al., 2007).

Cortical remapping, the second factor contributing to post-stroke recovery, involves the transfer of incoming sensory or motor output signals from one cortical region to another and is not necessarily involved with new structural circuits (Murphy & Corbett, 2009). Following reperfusion, surviving neurons within the recovering peri-infarct area, endure structural and functional remodeling that permits attempts at recovery. For example, the compromised circuits in animals affected by small stroke (only 5-15% of hemisphere) compete for adjacent healthy tissue to consume compromised map territory (Cramer, Shah, Juranek, Crafton, & Le, 2006; Nudo, Wise, SiFuentes, & Milliken, 1996; Winship & Murphy, 2008). Thus, small infarcts likely result in recovery due to remapping of adjacent cortical peri-infarct tissues with similar functions. In contrast, tissues with similar functions to those affect by large stroke may only be found at more distant sites or within the contralateral hemisphere and thus recovery is less likely.

Critical Periods

Critical periods are limited episodes of time when an organism is more sensitive to environmental influences or stimulation than other times throughout its life. The critical period for cortical reorganization following stroke can be compared to similar processes involved in normal fetal development. Animal studies reveal many genes and proteins vital for neuronal growth, synaptogenesis and proliferation of dendritic spines are highly expressed during early brain development and decline substantially with age (Hattiangady & K., 2005). A second, limited increase in expression of these genes has been documented following stroke (Carmichael, 2006; Carmichael et al., 2005; Cramer, 2008; Hattiangady & K., 2005). Increasing evidence suggest that cortical plasticity following stroke can be modulated by both rehabilitative training and pharmacological therapy (Johansson, 2000; Nudo et al., 1996; Stroemer, Kent, & Hulsebosch, 1998), however these therapeutic interventions must take place within critical period of time following the onset of stroke to elicit beneficial results. An elegant study, where rats were exposed to enriched rehabilitation starting at either 5, 14 or 30 days following MCAO, revealed that rats given early rehabilitation (5 or 14 days) displayed significant performance recovery compared to rats given delayed treatment (30 days) (Biernaskie, Chernenko, & Corbett, 2004). Furthermore, the early enrichment resulted in increased dendritic branching in layer V of cortical neurons. Additional speculation has shown that activity-dependent cortical changes promoted by enhanced rehabilitation can be further facilitated when combined with suitable pharmacological intervention during a critical post-injury period (Dobkin, 1998).

Stroemer et al provide strong indication for enhanced neuroplasticity as a result of amphetamine administration and physical therapy post-stroke in rats following cortical infarction (Stroemer, Kent, & Hulsebosch, 1995; Stroemer et al., 1998). Similar functional recovery has been documented following intracisternal injection of neurotrophic growth factors: basic fibroblast growth factor and osteogenic protein-1 (Kawamata, Alexis, Dietrich, & Finklestein, 1996; Kawamata, Ren, Chan, Charette, & Finklestein, 1998). The intrinsic neuronal signaling pathways elicited to modify transcription factors that contribute as activators of gene expression to promote cortical plasticity and functional recovery are yet to be established. However, there is substantial research indicating post-stroke modifications of certain signaling pathways that could provide clues to potential therapeutic strategies.

Granulocyte-macrophage colony-stimulating factor: GM-CSF

Granulocyte macrophage stimulating factor (GM-CSF) functions as a hematopoietic factor and a proinflammatory cytokine expressed in a variety of differentiated and non-differentiated cell types throughout the body, including, T-cells, monocytes, macrophages and endothelial cells (Bussolino et al., 1991). Currently, there are several hematopoietic/cytokine factors identified in hematopoiesis, angiogenesis (new blood vessels form from pre-existing vessels) and arteriogenesis (increased diameter of arterial vessels) considered as therapeutic agents in various neurological diseases, mainly neurodegenerative disorders like Parkinson's Disease, amyotrophic lateral sclerosis (ALS) or stroke. Amongst the

most prominent are GM-CSF, granulocyte colony-stimulating growth factor (G-CSF) and erythropoietin (EPO) (Maurer et al., 2008). An important function of GM-CSF is it's role in promoting growth and differentiation throughout the development of hematopoietic progenitor cells into macrophages, dendritic cells and granulocytes. Although GM-CSF was initially characterized in the hematopoietic system and traditionally used in clinical onco-hematological disorders to reconstitute hematopoiesis following chemo/radiation therapy (Antman et al., 1988) it has a second prominent role in the CNS. GM-CSF mRNA, protein, and receptors have been detected in the human CNS as early as the 8-16 weeks gestation (Dame, Christensen, & Juul, 1999; Sawada, Itoh, Suzumura, & Marunouchi, 1993) and has been noted to play a role in the post-injury inflammatory response to potentially play a role in promoting neuroprotection and regeneration following injury (Mangano et al., 2011; Schabitz et al., 2008).

GM-CSF in the injured CNS

Injury within the CNS promotes a complex array of inflammatory reactions including the recruitment of blood-derived macrophages, and monocytes, and the activation of local astrocytes and microglia. The exact role of GM-CSF upon injury is yet to be thoroughly identified, however research shows potential roles in regeneration and neuroprotection. The administration of GM-CSF in culture composed of sympathetic neurons obtained from mouse superior cervical ganglia stimulates neurite growth (Kannan et al., 2000). The action of GM-CSF within the CNS is related to presence of the transmembrane GM-CSF receptors on microglia,

oligodendrocytes, astrocytes and to some extent, neurons (Sawada et al., 1993). In the normal CNS, astrocytes appear to be a distinctive source of GM-CSF that regulates microglial proliferation, morphological changes and antigen presentation (Malipiero, Frei, & Fontana, 1990; Re et al., 2002; Schermer & Humpel, 2002; Suzumura, Sawada, & Marunouchi, 1996). However, in the injured CNS, GM-CSF is secreted by activated vascular endothelial cells, and acts as an anti-apoptotic factor that delays the sequence of cell death of recruited neutrophils, allowing prolonged phagocytosis of cellular debris (Coxon, Tang, & Mayadas, 1999). GM-CSF binding is considered to be a critical mediator in the development of chronic inflammation following injury. GM-CSF adopts the role of a pro-inflammatory cytokine by binding with low affinity to the alpha subunit of the GM-CSF receptor. GM-CSF binding plays a crucial role in IL-3 and IL-5 cytokine binding to the beta subunit to activate the receptor and confer signal transduction (Mirza, Walker, Chen, Murphy, & Young, 2010). The half-life of exogenous GM-CSF in vivo is more than 4 hours (Sainathan et al., 2005; Burgess et al., 1977). GM-CSF is cleared from the blood of C57BL m ice in 7.3 ± 1.3 hours. Radiolabeled GM-CSF appeared to be distributed through most tissues six hours following intravenous injection. In addition, the GM-CSF detected in the urine appeared to result from the degradation of GM-CSF as opposed to the macromolecular protein (Burgess et al., 1977). The duration of the effects of GM-CSF on tissue is unknown and is likely correlated to the type and pathological state of the tissues involved.

Consistent with the notion of GM-CSF's influence of microglial function, and since microglia are known to remove debris within the CNS following injury, it is

logical to assume that GM-CSF may be a key factor in promoting axonal regeneration. The lack of an innate intrinsic ability for the CNS to actively achieve regeneration of damaged neurons is in part due to an extrinsic phenomenon termed astrogliosis. Astrogliosis is caused by the accumulation of astrocytes and microglia that form a 'glial scar' within the injured area; as a result GM-CSF regulates the composition of the glial scar and may contribute to neuroprotection by conferring survival and function of neighbouring neurons (Giulian, Li, Li, George, & Rutecki, 1994). Unfortunately, this action by GM-CSF alone is not sufficient to promote regeneration.

Several studies have however shown CNS neuroprotective properties of GM-CSF *in vivo* (Mangano et al., 2011; Schabitz et al., 2008) in different neurodegenerative and injury models including Parkinson's disease and stroke. One possible mechanism to explain neuroprotection again points to the role GM-CSF and microglia. Since microglia are the macrophage cells of the CNS and are responsible for scavenging myelin debris and infectious agents, it seems to reason that GM-CSF can improve cell survival through the enhancement of microglial activity.

Rationale for Present Thesis

There are several stroke models presented in the literature, however a controlled infarct is vital to the evaluation of explicit behavioural deficits associated with targeted damage. Thus, using the traditional MCAO model to analyze the neurobehavioural effects of subcortical white matter stroke does not suffice due to

its propensity to affect a combination of white matter and gray matter by damaging most of the cerebral hemisphere (Carmichael, 2005). This type of damage can cause varying multifaceted behavioural deficits, with the subsequent inability to compare behaviour and rehabilitation between animals. Conversely, ET-1 can be used to promote focal infarcts within a targeted region of the brain including the white matter tracts of the corpus callosum (Sharkey & Butcher, 1995; Sozmen, Kolekar, Havton, & Carmichael, 2009).

Several animal models of ischemia have demonstrated neuroprotective and angiogenic properties of GM-CSF providing rationale for potential post-stroke treatment to ameliorate physiological impairments. In fact, Shabitz and colleagues have identified GM-CSF as a neuronal growth factor that counteracts apoptosis and reduces ischemic infarct size *in vivo,* thereby reducing negative pathophysiological consequences of stroke. The presence of GM-CSF within the CNS has also been shown to increase GM-CSF alpha-receptor expression on neurons following ischemia in a rodent MCAO model (Schabitz et al., 2008), implicating possible effects of GM-CSF on neuronal intracellular activity following stroke. Perhaps one of the most important features of GM-CSF in becoming a potential post-stroke treatment is that it crosses the blood brain barrier (BBB) and blood-spinal cord barrier, allowing for easy access to the CNS to elicit its effects (McLay, Kimura, Banks, & Kastin, 1997).

Experiment 1: Subcortical White Matter Stroke Mouse Model

The first objective was to generate a reproducible ischemic infarct within the subcortical white matter region of the mouse by injecting the potent vasoconstrictor ET-1 and examine forelimb fine motor movement deficits.

Adult female C57BL/6 mice were initially trained to retrieve M&M's from a platform with their right forelimb in a Plexiglas reaching box. Following training, animals underwent 6 days of pre-surgical behavioural testing involving the reaching box test, cylinder test, to measure baseline scores for forelimb fine motor movements and forelimb asymmetry. Animals then underwent intracranial surgery and received three 120nl unilateral injections of ET-1 into the corpus callosum of the left hemisphere. Seven consecutive days of post-surgical behavioural testing analyzed contralesional forelimb fine motor movements, grasping proficiency and forelimb asymmetry. Succeeding behavioural testing, animals were sacrificed, and brain tissue was examined using Luxol Fast Blue staining and immunofluorescence of neurofilament to examine the size and location of the infarct within the corpus callosum caused by ET-1 injections. Further immunohistochemical analysis included GFAP staining to investigate inflammatory responses within the corpus callosum and NeuN staining to evaluate cell body integrity within the adjacent motor cortex. It was hypothesized that ET-1 injections would create a reproducible infarct within the corpus callosum resulting in measurable contralesional forelimb motor movement deficits in stroke mice.

Experiment 2: GM-CSF and Forelimb Motor Function Improvements

The second objective was to examine the effects of GM-CSF on contralesional forelimb fine motor movement and forelimb asymmetry in ET-1 induced stroke mice. The same training and pre-surgical regimen from experiment 1 was used involving the reaching box and cylinder test. Animals underwent intracranial surgery on the fourteenth day of pre-surgical training and received three 120nl unilateral injections of ET-1 into the corpus callosum of the left hemisphere. Previous research has shown locomotor improvements in rats following post-MCAO injections of GM-CSF; however the injections were given once a day for 5 consecutive days post-stroke at a concentration of 60ug/kg (Kong et al., 2009). In this thesis, a randomly selected subset of stroke mice received a GM-CSF injection (100ug/kg) immediately following surgery. These same animals received two additional i.p. injections of GM-CSF (100ug/kg) on day 5 and 10 following ET-1 induced ischemia. Post-surgical behavioural testing in the reaching box, cylinder and inverted cage lid began the day after surgery for seven consecutive days, and on days 9, 11, 14, 21 and 28. It was hypothesized that the administration of GM-CSF would produce measurable functional recovery in contralesional forelimb fine motor movements involved in grasping and forelimb asymmetry in stroke animals.

Materials and Methods

All procedures were conducted in accordance with guidelines provided by the Canadian Council on Animal Care and were approved by the Carleton University Animal Care Committee.

Animals

Female wild type C57BL/6 mice (n=62) were obtained from Charles River Laboratories, (Montreal, Quebec, Canada) at approximately 5-6 weeks of age (20-25g) and acclimated in the laboratory for 1 week prior to experiments. Animals were housed in standard (27x21x14cm) polypropylene cages. Animals used for behavioural experiments were house in pairs and animals used for biochemical experiments were housed in fours. All animals were accommodated in standard conditions on a 12:12 h light/dark cycle (lights on at 08h00). Food (Roulston Purina mouse chow) and water were provided *ad libitum* for animals used in biochemical experiments (n=30), whereas animals in behavioural experiments (n=32) were moderately food restricted (4g) during behavioural training, pre- and post-surgical testing.

Intracranial Surgery

Animals were anesthetized with isofluorane (4% in O_2 for induction). Their heads were clean shaven and disinfected. Animals were mounted to a stereotaxic frame (Harvard Apparatus, Canada) and anesthetization was maintained at 1.5%-2% in O_2 delivered through a facemask attached to the stereotaxic frame. Tear gel

was applied to the eyes to prevent drying and body temperature was maintained with a heating pad to prevent hypothermia. Animals received a subcutaneous injection of 0.2ml of buprenorphine (0.01mg/kg subcutaneously) prior to surgery for pain relief.

Unilateral lesions were applied to the corpus callosum within the left hemisphere. A midline incision was made in the scalp from behind the eyes to the ears, and the sensorimotor cortex was exposed by drilling a hole (Circuit Medic, Micro-Drill System) in the skull with a 1mm drill bit. The dura mater was removed using a 23-gauge needle.

Three 120nl injections of ET-1 (Calbiochem, 1ug/ul) in sterile Dulbecco's phosphate buffer solution (DPBS) were delivered via a metal needle connected to a 10µl Hamilton syringe mounted onto an injection pump (Harvard Apparatus, Pump11Elite). The needle was inserted into the brain to the corpus callosum at an angle of 36° at three separate coordinates:

1. Anterioposterior (AP) + 0.52mm, Mediolateral (ML)+ 0.15mm, Dorsoventral (DV)−2.3mm;
2. AP + 0.88mm, ML+ 0.15mm, DV−2.3mm;
3. AP + 1.24mm, ML+ 0.15mm, DV−2.3mm, from Bregma, midline and brain surface respectively (Figure 1).

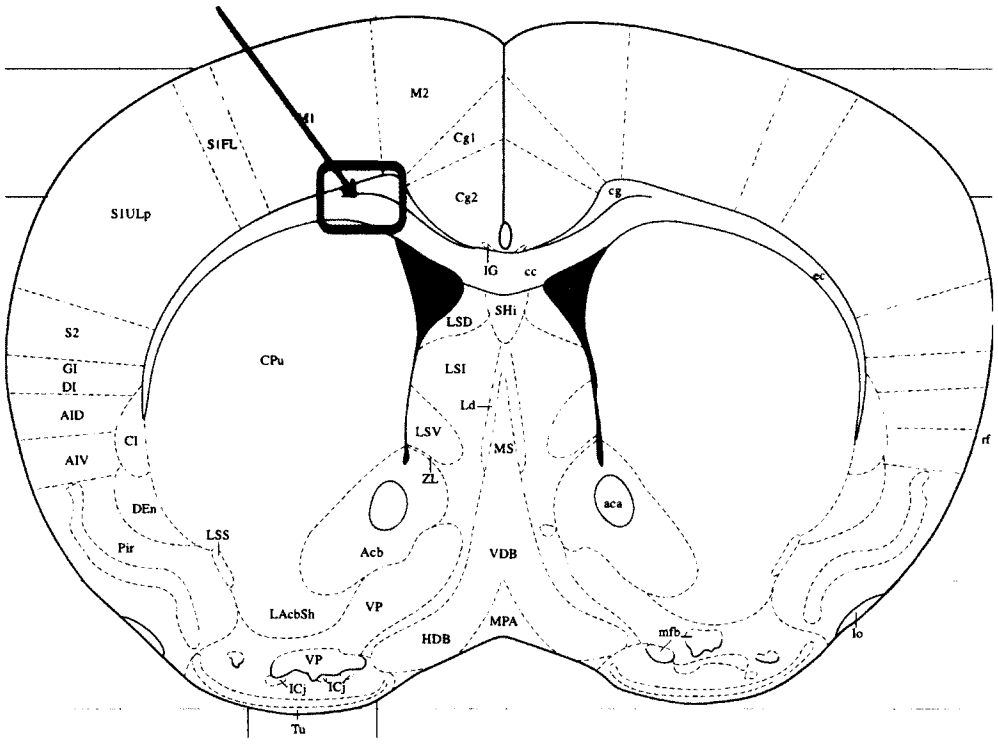

Figure 1. Schematic representation of a coronal brain section depicting ET-1 injection sites. The box in the left hemisphere indicates the location of the 3x120nl ET-1 injection sites into the corpus callosum (cc) below the motor cortices (M1, M2) and adjacent to the cingulate cortices (Cg1, Cg2). The arrow represents the surgical needle insertion through the primary motor cortex at an angle of 36°.

ET-1 was delivered at a rate of 120nl/min for each injection. The location, dose and delivery were adapted from a study conducted by Carmichael and colleagues (2009). Following each injection, the needle was left in the brain for 3 minutes to allow for proper diffusion of ET-1 solution into the brain tissue. Following the third injection, the scalp was sealed with glued (Vetbond tissue adhesive). Control mice underwent all the above procedures, but received three injections of DPBS. Mice were housed singly on a heating pad immediately following surgery for a minimum of 1 hour to recover from anesthetic. Once recovered (grooming, eating and drinking), mice were re-housed with the same pre-surgery cage mate(s). All mice within a cage received the same experimental treatment. All surgeries lasted 30-50 minutes and surgical instruments were autoclaved prior to the first surgery and sterilized after each surgery.

Animals received a subcutaneous injection of 0.2ml of buprenorphine (0.01mg/kg subcutaneously) once every 24 hours for 3 days following surgery for postoperative pain relief.

GM-CSF Treatment

Experimental group (ET-1 + GM-CSF) animals received 0.2ml i.p. injections of GM-CSF (R&D Systems; 100ug/kg) immediately following the last intracranial injection of GM-CSF and again on the 5th and 10th days following surgery at 8:00am. Control animals (ET-1 only) received 0.2ml i.p. injections of 0.9% saline following the same schedule as the GM-CSF injections.

Behavioural Testing

Animals participated in a battery of behavioural tests designed to measure forelimb fine motor movement deficits and forelimb asymmetry. A Plexiglas reaching box was used to measure reaching success and forelimb fine motor movement deficits and the cylinder test to measure forelimb asymmetry.

Reaching Box

The Plexiglas reaching box measured 19.8cm long, 8.3cm wide, and 20.3cm high. The front of the box had a 1cm wide vertical slot for the mice to reach M&M's located on a 0.4cm thick plastic shelf (8.3 cm long and 3.8 cm wide). The shelf contained two indentations 1cm way from the slot where an M&M was placed for testing (Figure 2).

Animals were habituated to the apparatus for 3 days by placing them into the box for 10 minutes each day, followed by a moderately food restricted training period to provide motivation to learn the reaching task. The mice were trained by being placed them into the box for up to 30 minutes. M&M's were initially available on the cage floor and within tongue distance on the shelf. M&M's were gradually removed from the apparatus floor and placed farther away on the shelf until the animals were forced to reach through the slot with their right forepaw to retrieve the M&M placed in the left indentation on the platform. The training period was complete when the mice were able to perform the reaching task with the right forelimb comfortably. This procedure was accommodated from (Farr & Whishaw, 2002).

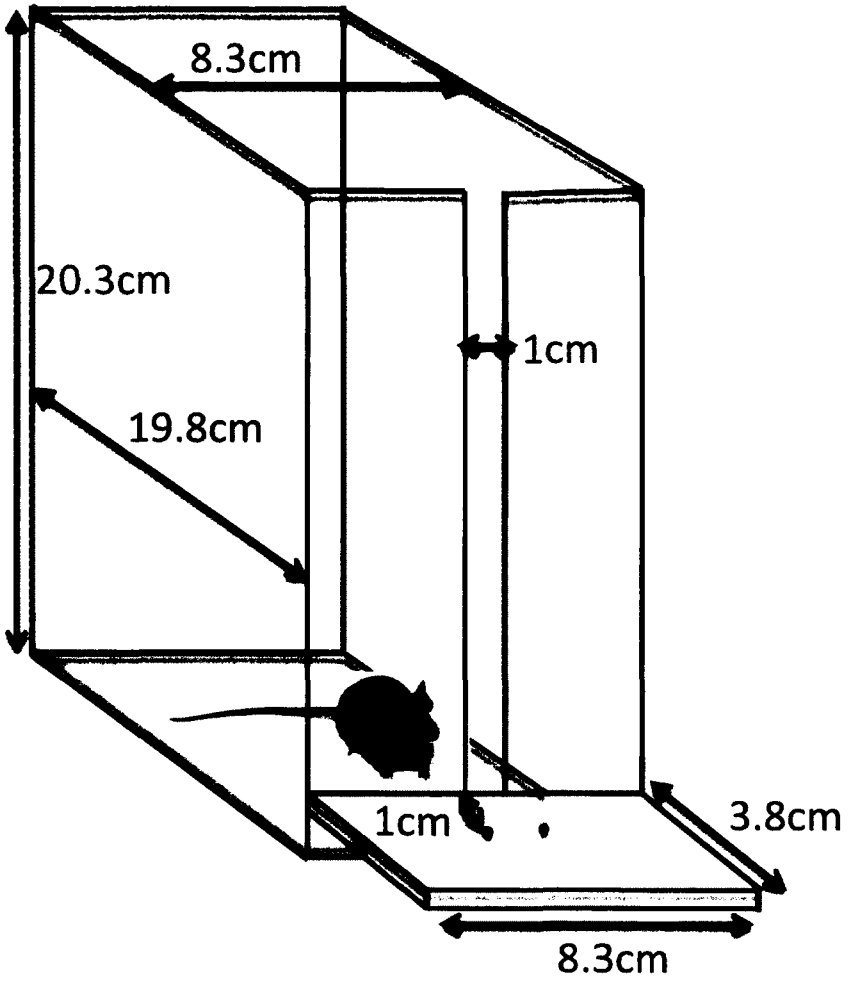

Figure 2. Plexiglas Reaching box. Transparent apparatus used to measure contralesional forelimb fine motor movements in ET-1 injected ischemic mice. Mice were trained and tested retrieving M&M's from an indentation located 1cm on the right of a platform. The reaching box test measures skilled forelimb use and detects functional behavioural deficits in post-stroke injury models.

Following training, animals were food restricted to 90% of body weight, and received 6 consecutive days of pre-surgical testing within the reaching box. Reaching performance was video recorded on the last 2 days prior to surgery to analyze the qualitative components of movement prior to surgery.

On the seventh day preceding the termination of the pre-surgical training, mice were allocated to a control or treatment group in a randomized manner to ensure there was no difference in preoperative performance on the behavioural tests prior to surgery. Following surgery animals underwent a battery of behavioural tests and was video recorded for 7 consecutive days in experiments 1 and 2 (Figures 3 and 4), and on additional days (8, 10, 11, 14, 21 and 28) in experiment 2 (Figure 4). Motor performance was video recorded from a frontal view (above an inclined mirror to capture the ventral side of the mice to score for qualitative movements).

Animals were required to reach for 10 M&M's to satisfy the reaching box paradigm. Since animals were food restricted, they were more than sufficiently motivated to attempt to obtain 10 M&M's. To measure percent reach success, a success was measured as an animal reached through the slot and obtained an M&M. However, if the animal knocked the M&M away or dropped the M&M after grasping it, the reach was scored as a miss. Performance was defined as percent success (number of successful retrievals/10)*100 (Farr & Whishaw, 2002).

Qualitative movement was adapted from the conceptual framework adapted from Eshkol-Wachmann Movement Notation (EWMN). EWMN is a system of movement analysis designed to express relations and changes of relation between

body parts throughout movement. On the basis of descriptions provided in EWNM, rating scales of movements were created. Each qualitative movement was rated using a 3-point scale. A score of 0 was given if the movement was normal; a score of 1 was given in cases of ambiguity concerning the movement or if the movement was present but incomplete; and a score of 2 was given if the movement was absent. Animals were omitted for data analysis if they did not perform 5 successes for the day of measurement.

Five pre-surgical and 5 post-surgical reaches for each animal in experiment 1 and 5 pre-surgical and 5 post-surgical reaches on days 1, 7, 14, 21 and 28 for each animal in experiment 2 were analyzed and rated for qualitative features of movement according to the EWMN. Ten components of reach were rated according to guidelines established by Farr and Wishaw (2002): (1) Digits to midline- using mainly the upper arm, the reaching limb is lifted from the floor and the tips of the digits are aligned with the midline of the body. (2) Digits semiflexed- as the limb is lifted, the digits are flexed and the paw is supinated so that the palm of the paw is aligned almost vertically. (3) Aim: Using an upper arm movement, the elbow is adducted to the midline while the tips of the digits remain aligned with the midline. (4) Advance: The limb is advanced directly through the slot toward the food target using an upper arm movement, and during advancement the snout is raised to allow passage of the paw into the slot. (5) Digits extend: The digits extend during the advance. (6) Pronation: When the paw is over the target, the paw pronates and digit 5 (the outer digit) through to digit 2 touches the surface in succession, mainly by abduction of the elbow and also by a rotational movement around the wrist. During

pronation, the digits open. (7) Grasp: The digits flex over the food and close around it. The paw remains in place and the wrist is slightly extended to lift the food. (8) Supination I: As the paw is withdrawn, it supinates by almost 90°. (9) Supination II: Once the paw is withdrawn from the slot the paw further supinates by 45° to present the food to the mouth. (10) Release: The mouth contacts the paw, and the digits open to release the food (directly from Farr & Wishaw, 2002).

Cylinder Test

The cylinder test was used to assess forelimb use and asymmetry in postural weight support during exploratory activity. The animal was placed in a glass cylinder for 10 minutes and video recorded from above. Forelimb contact against the wall of the box during rearing (animal stood completely erect on hind legs) and lateral exploration was recorded by the following criteria: (1) Simultaneous use of both forelimbs by contacting the wall during a full rear and for lateral movements along the wall was recorded as "both". (2) When a mouse explored the wall laterally, alternating evenly between both forelimbs was recorded as "both". (3) The first forelimb to contact the wall during a full rear was recorded as an independent wall placement for that limb. (4) After the first forelimb contacted the wall to and then other forelimb was placed on or made several contacts to the wall, but the first forelimb was not removed, a "left or right forelimb independent" and a "both" were recorded.

The first 20 movements were recorded during the 10-minute test. The final score was calculated as follows: Final score= (number of nonimpaired forelimb

(left) movement - number of impaired forelimb (right) movement)/ (number of nonimpaired forelimb (left) movement + number of impaired forelimb (right) movement + number of "both" movements). A positive score indicated favoured use of the contralesional (stroke affected-right) forelimb, a negative scores indicated favoured use of the ipsilesional (non-affected-left) forelimb and a score of zero indicated equal use of both left and right forelimbs upon rearing and exploration of the cylinder.

All behavioural tests were video recorded with a Nikon D3100 DSLR camera. Videos were viewed on a MacBook Pro. Representative movements for each test were captured using Quicktime Player. Figures were prepared using Adobe Photoshop CS5 and Microsoft PowerPoint for Mac.

Histology

Luxol Fast Blue

Animals were deeply anesthetized with an i.p. injection of Euthanyl (active ingredient sodium pentobarbital- 70mg/kg) and perfused transcardially with 0.9% saline followed by 10ml of 4% paraformaldehyde (4% PFA) in 0.1M phosphate buffer solution (1xPBS). Brains were extracted and post fixed for 72hours at 4°C then transferred to 10% sucrose in PBS for 24 hours and 30% sucrose for a minimum of 48 hours. Several series of 20μm coronal, rostral to caudal tissue samples were serial sectioned with a Leica CM 1900 cryostat, placed onto frosted polarized slides and stored at -20°C.

Fixed brain sections were used to assess the presence of myelinated axons within the corpus callosum (Luxol fast blue-LFB). Samples were incubated in LFB

solution (Electron Microscope Sciences- EMS- LFB 0.1%w/v, ethyl alcohol 94.25%v/v, methanol 4.75%v/v and glacial acetic acid 0.05%v/v) for 12 hours at 37°C. Samples were rinsed in distilled water and differentiated in methanol, lithium carbonate then 70% alcohol. Samples were again rinsed in distilled water then counterstained in a CV solution containing acetic acid (EMS- CV acetate 0.1%w/v, water 100% v/v). CV staining was differentiated in 95% ethyl alcohol and 100% anhydrous alcohol. Lastly, samples were incubated in histology grade xylenes (Fisher Scientific) for 10 minutes and coverslipped using Permount (Fisher Scientific).

Immunofluorescence

The brains of the mice were extracted following a lethal dose of Euthanyl and subsequent transcardial perfusion with 0.9% saline followed by 10ml of 4% PFA in 0.1M phosphate buffer solution (1XPBS). Serial coronal sections were sectioned with a Leica CM 1900 cryostat, placed onto polarized slides ad stored in -20°C.

Prepared slides were defrosted and washed with 0.01M phosphate buffered saline (1XPBS) three times at 10 minutes. Sections were then incubated in a primary antibody solution containing a primary diluent (0.01M PBS, 0.3% Triton x100, 0.02% sodium azide) and a primary antibody of either Ms-NeuN MAB377 (1:2000), Anti-Glial Fibrillary Acidic Protein clone GA5 (1:1000), (Millipore) or rabbit anti-NF200 (1:400) (Sigma) and incubated at 4°C for 24-48 hours.

Following incubation the samples were washed with 1XPBS three times at 10 minutes then treated with a 1:200 concentration of secondary antibody solution consisting of a secondary diluent (0.01M PBS and 0.03% Triton x100) and a

secondary antibody of Alexa Fluor 488 specific for species (anti-mouse or anti-rabbit) and incubated for 2 hours at room temperature (23°C). Sections were washed following the same saline procedure indicated above. Coverslips were mounted with fluoromount (Sigma-Aldrich) and analyzed under ZEISS Axiovert 40-C Inverted Microscope. Photographs of tissue samples were taken with an Infinity 3 microscope camera using Infinity Analyze software from Lumenera Corporation. Figures were prepared using Adobe Photoshop CS5.

Experiment 1: Subcortical White Matter Stroke Mouse Model

Experiment 1 developed a reproducible subcortical white matter stroke model with a specific functional motor deficit. First, intracranial surgery was performed on two cohorts of 10 animals to determine if the adapted protocol from Carmichael and colleagues resulted in a reproducible subcortical white matter infarct with limited cortical damage. In each cohort, mice received either three 120nl stereotaxic injections of ET-1 (n=5) or DPBS (n=5) into the left hemisphere of the brain.

Histological Identification of Infarct

Post-mortem analysis on brain tissue aimed to ensure a reproducible focal infarct within the corpus callosum with limited damage to adjacent cell bodies within the cortex. Damage to the corresponding cell bodies would interfere with molecular signaling pathway analysis. Luxol Fast Blue staining was used to analyze myelinated axons within the corpus callosum. Immunofluorescent staining with

neurofilament was used to label axons within the corpus callosum to ensure axonal degeneration; NeuN was used to evaluate cell body integrity of neurons within the adjacent motor cortex whose axons project into the infarct zone within the corpus callosum; and GFAP was used to analyze inflammatory responses within the targeted stroke region.

Behavioural Training

Second, 16 animals were used to ensure functional motor deficits following ET-1 injection into the corpus callosum below the sensorimotor cortex. Animals were habituated in a Plexiglas reaching box for 3 consecutive days, immediately followed by a period of reach training. Training resulted in the animals successfully using the right forelimb to reach for an M&M placed within the left indentation on the platform of the apparatus. Following training, 6 days of pre-surgical testing within the reaching apparatus and one day of cylinder testing were completed to measure baseline performance (Figure 3). Not all animals were able to perform the reaching task efficiently and were thus omitted from the Plexiglas reaching box task, however, they remained in the study to perform the cylinder task. In addition, one animal was removed from the study due to behavioural problems involving over activity at inappropriate times throughout testing.

Intracranial Surgery

Immediately following pre-surgical testing, intracranial surgery was implemented and each animal received three 120nl unilateral injections of either

ET-1 (n=8) or DPBS (n=7). Injections were given on a 36° angle into the subcortical white matter region below the sensorimotor cortex of the left hemisphere. For 7 consecutive days following surgery, behavioural testing for contralesional forelimb grasping proficiency and forelimb asymmetry were video recorded and scored.

Post-Surgical Analysis

Following post-surgical testing subsamples of animals from control (n=4) and stroke (n=4) groups were deeply anesthetized with a lethal dose of Euthanyl and perfused transcardially. All 8 brains were extracted, fixed, dehydrated and then serial sectioned in a rostral to caudal manner on a cryostat. Sections were mounted onto frosted polarized slides and were either treated with Luxol Fast Blue to examine damage to the corpus callosum or immunohistochemically stained for neurofilament, NeuN and GFAP. Additional subsamples of animals from control (n=3) and stroke (n=4) groups were decapitated to isolate separate cortical samples from the ipsilesional and contralesional hemispheres for future Western Blot analysis of molecular signaling proteins.

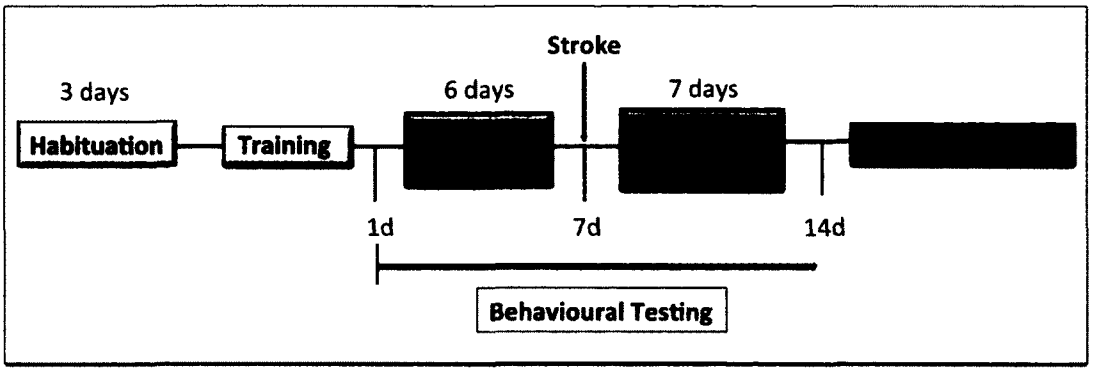

Figure 3. Experimental timeline used to analyze neurobehavioural behavioural deficits of ET-induced subcortical white matter stroke compared to DPBS injected controls.

Experiment 2: GM-CSF and forelimb motor function recovery

Experiment 2 assessed the effects of i.p. injections of GM-CSF on contralesional forelimb functional recovery in ET-1 induced ischemic mice. Behavioural testing and GM-CSF administration followed a strict paradigm outlined in Figure 4.

Behavioural Training

Sixteen animals were habituated for 3 days in the Plexiglas reaching box, followed by a 14-day training period. Training resulted in animals successfully using the right forelimb to attain M&M's placed on the platform of the apparatus. Animals were trained to accomplish a baseline percent reaching success of at least 50% on a daily basis before proceeding to pre-surgical testing. A total of four animals were unable to learn the reaching task efficiently and were removed from the reaching box test but remained in the study to perform the cylinder test. In addition, one animal displayed behavioural problems involving over activity at inappropriate times throughout testing and was thus removed from the study. Pre-surgical testing consisted of 6 days of reaching box testing and one day of cylinder testing prior to surgery.

Intracranial Surgery

All fifteen animals received three 120nl stereotaxic injections of ET-1 into the corpus callosum of the left hemisphere. A subset of animals (n=8) was selected for experimental GM-CSF treatment in a random manner on the day of surgery. The

remaining subset of animals (n=7) was designated as control mice that received 0.2ml injections of 0.9% saline following the same regimen as the GM-CSF injections. Experimental animals (ET-1 + GM-CSF) received a 100ug/kg dose i.p. injection of GM-CSF immediately following the final ET-1 injection, and on post-surgical day 5 and 10 (Figure 4).

Post-Surgical Analysis

Post-surgical behavioural testing consisted of 7 days of reaching box, and cylinder testing. Additional days of testing included day 8, 10, 11, 14, 21 and 28 following surgery. The experimenter recorded scores blind to experimental groups to avoid biased results.

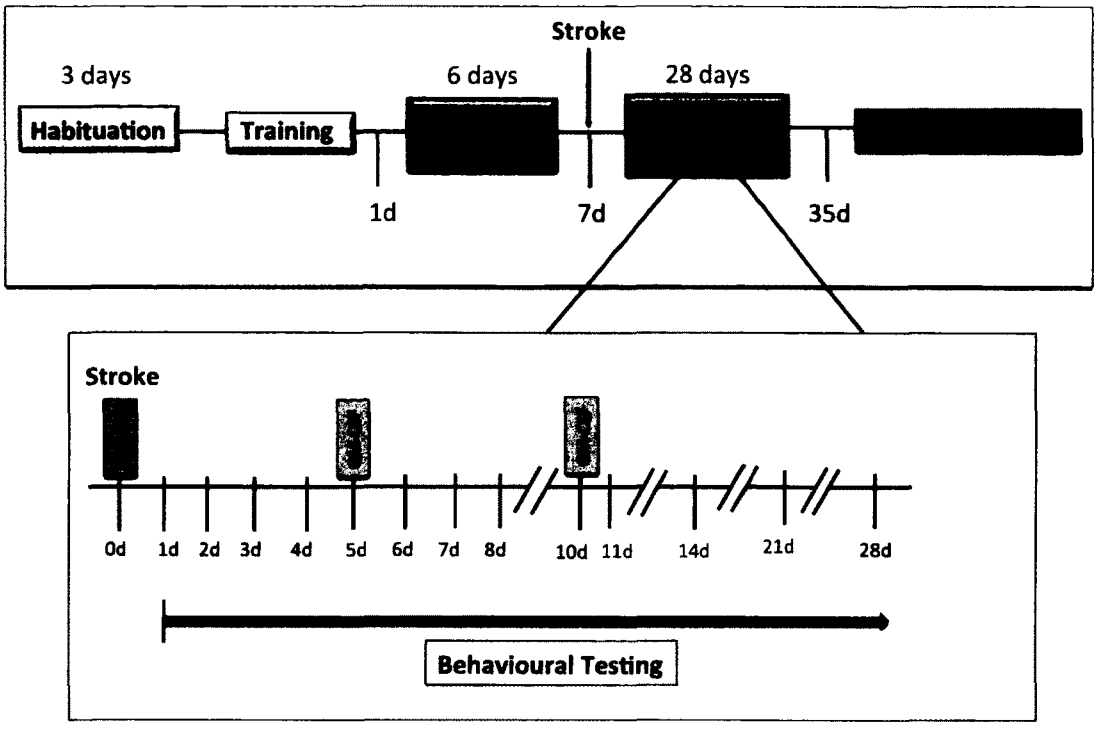

Figure 4. Experimental timeline used to analyze the effects of post-stroke administration of GM-CSF on the behavioural deficits caused by ET-1 induced subcortical white matter stroke.

Statistical Analysis

All data are presented as mean ± SEM. Differences between control and experimental groups and pre- and post-surgical time points were compared by two-way (group x day) repeated measures analysis of variance (ANOVA). Either non-stroke/control and stroke or stroke and stroke +GM-CSF was used as the between subjects factor and pre-and post surgical days as the within subject factor. The 10 qualitative components of movement data were analyzed using the same two-way repeated measures ANOVA but with an additional within subjects factor (10 movements).

A One-way ANOVA was used to compare post-surgical movements between stroke and GM-CSF treated groups. Paired t-tests were performed to evaluate differences across days within one group (ex: pre-and post surgical performance differences in stroke animals). And unpaired, two-tailed t-tests were used to compare differences in means between groups on specific days of testing.

In all statistical tests, a *p*-value of 0.05 or less was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics (Ver. 19).

Results

Subcortical White Matter Stroke

The main goal of experiment 1 was to generate a reproducible mouse model of focal SWMS within the corpus callosum below the sensorimotor cortex, resulting in measurable forelimb fine motor movement deficits. It was hypothesized that stroke animals would demonstrate a decline in skilled contralesional forelimb movement as a result of ET-1 induced ischemia within the corpus callosum, while the adjacent cortical tissue within the motor cortex remained intact.

Intracranial Surgery

Mice received three 120nl unilateral intracranial injections of ET-1 following reach training and a pre-surgical testing period for reach and forelimb asymmetry. Intracranial injections were performed on a 36° angle to evade damage to the sensorimotor cortex above the ischemic region of the corpus callosum. Surgical procedures including skull drilling and needle insertion resulted in minimal damage to the surface of the motor cortex (Figure 5), eluding possible contributions to subsequent locomotor deficits in stroke mice.

Figure 5. Dorsal view of a typical needle insertion site within the cortex of subcortical white matter stroke mice. The arrows in the left hemisphere denote area of ET-1 needle insertion through the sensorimotor cortex.

Histology

Luxol Fast Blue

To further determine if the 3 ET-1 injections resulted in reproducible, focal infarcts within the corpus callosum, histological staining using Luxol fast blue was performed to visualize myelinated axons of the corpus callosum. ET-1 injections resulted in an extensive loss of myelination along the axons within the corpus callosum 7 days following the administration of ET-1 (Figure 6). There was no change in the distribution or staining intensity of LFB within the corpus callosum of the contralateral hemisphere.

Figure 6. Luxol fast blue staining of ischemic brain sections shows a decrease in myelination within the corpus callosum. Fixed brain sections (20μm) taken 7 days following 3X120nl ET-1 injections demonstrate extensive white matter fiber loss (arrow) within the infarct region of the corpus callosum of the left hemisphere compared to the contralateral (right) hemisphere.

Immunofluorescence

NeuN

Cell body integrity within the motor cortex, adjacent to the infarct zone within the corpus callosum was confirmed with NeuN (neuronal nuclei) immunofluorescence 7 days following ET-1 injection (Figure 7). There was a decrease in surviving cell bodies superior to the infarct, compared to the contralesional hemisphere; however, the remaining cell bodies appear viable under increased magnification.

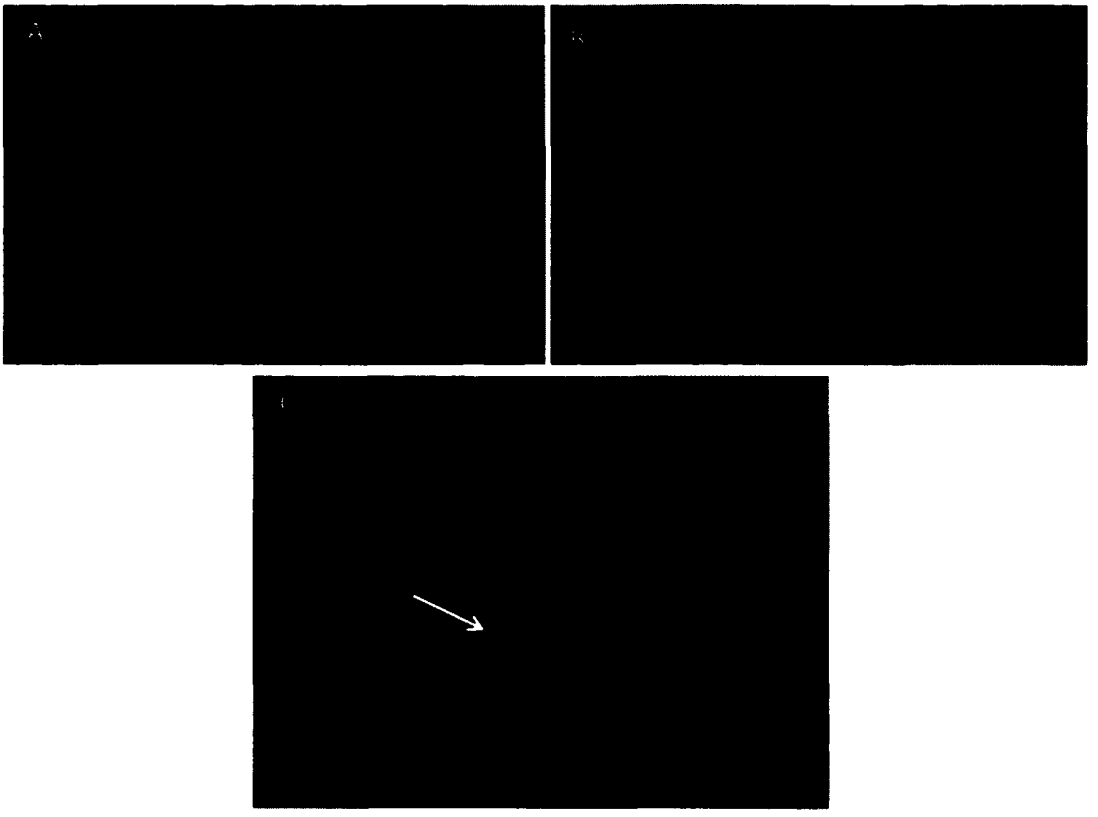

Figure 7. NeuN positive cell bodies within the motor cortex, superior to the subcortical white matter infarct region. A decrease in the number of cell bodies was found superior to the infarct zone in 20μm brain sections taken 7 days following ET-1 injections (box in A), compared to the contralateral hemisphere (box in B). The remaining cell bodies stained with NeuN immunofluorescence appear viable under 40X magnification (arrow in C).

Neurofilament

Immunofluorescent staining of neurofilament was performed to ensure that reduced LFB staining of myelinated axons within the corpus callosum are reminiscent of axonal degeneration and not demyelination alone. Neurofilament staining revealed a difference in abundance and morphology between the ipsilesional (iCC) and contralesional (cCC) sides of the corpus callosum. The intensity of neurofilament staining is drastically increased compared to the contralesional hemisphere at 7 days post stroke (Figure 8A and B). The densely stained neurofilament positive cells fill the subcortical white matter ischemic space and have a drastically altered morphology compared to the contralesional hemisphere. Neurofilament positive cells within the infarct zone appear round with little to no extensions, representative of a lack of axonal processes, whereas the neurofilament positive cells located within the corpus callosum of the contralesional hemisphere are fewer in number and comprise of long axonal processes (Figure 8C and D). These results are consistent with demyelination and axonal degeneration within the infarct area of the corpus callosum.

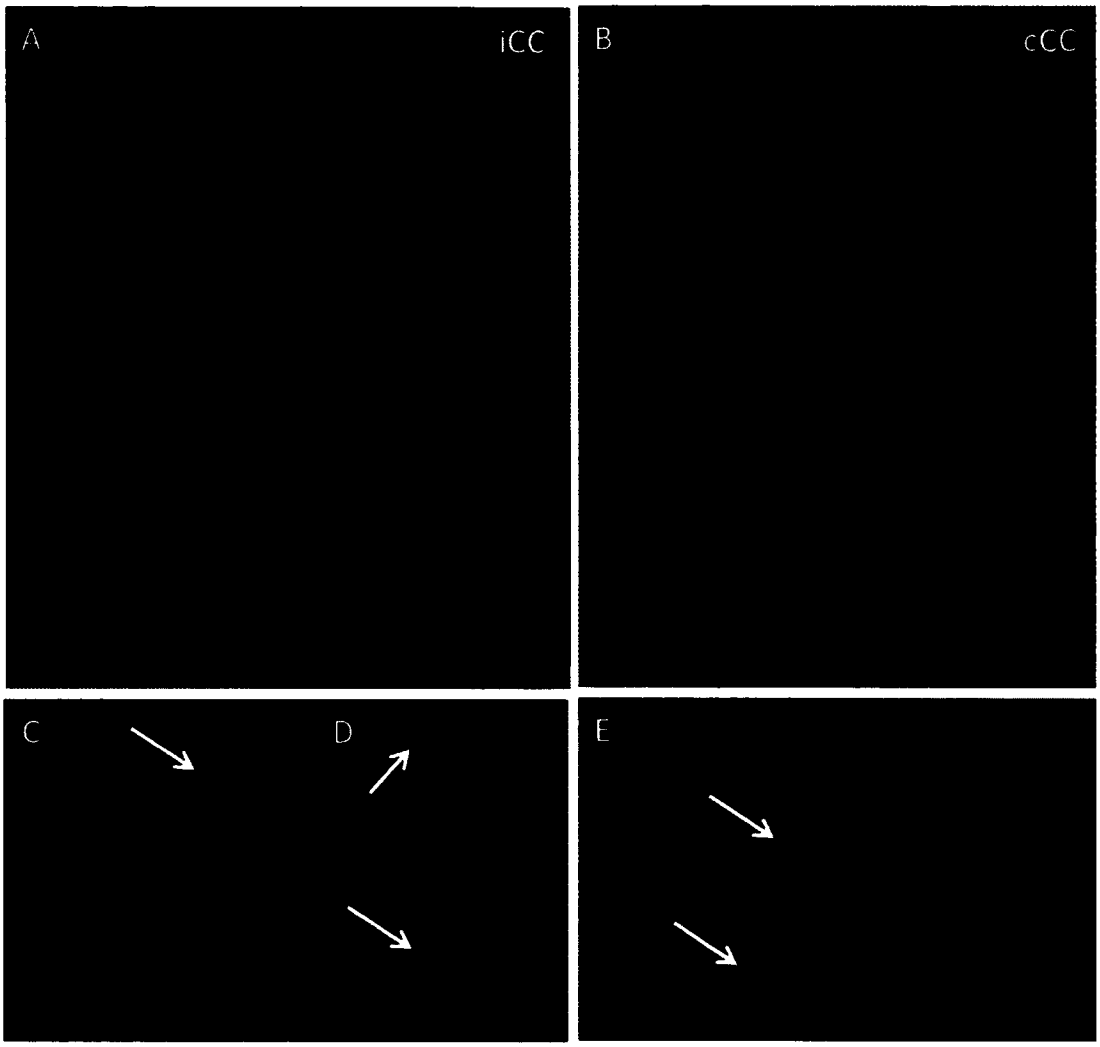

Figure 8. Neurofilament positive cells within the corpus callosum of stroke mice confirm axonal degeneration. A. Neurofilament positive cells in subcortical white matter 7 days following ET-1 injections. (iCC=ipsilesional corpus callosum) B. Neurofilament positive cells in subcortical white matter in contralesional hemisphere (cCC=contralesional corpus callosum). C, D. Magnification of box in A. Axonal processes in the infarct region of the corpus callosum demonstrate characteristics of degeneration and appear short in length with minimal axonal extensions (arrows). E. Magnification of box in B, representative of normal axonal process within the contralesional corpus callosum. Neurofilament positive cell reveal long axonal processes with collateral branching (arrows).

GFAP

To evaluate if SWMS caused inflammatory responses reminiscent to stroke pathology, fixed 20 μm brain sections were stained with glial fibrillary acidic protein (GFAP) to identify reactive astrocytes within the corpus callosum. Coronal brain sections taken 9 hours following ET-1 injection began to show an increase in GFAP expression within the ipsilesional corpus callosum (iCC) compared to the contralesional hemisphere (cCC) (Figure 9A, B). The number and morphological profiles of the GFAP positive astrocytes differ greatly between iCC and the cCC. GFAP-positive astrocytes within the stroke region are abundant in number and have a short spiny appearance (Figure 9C); whereas, the GFAP-positive astrocytes within the cCC are relatively few in number and are morphologically distinct with longer processes (Figure 9D).

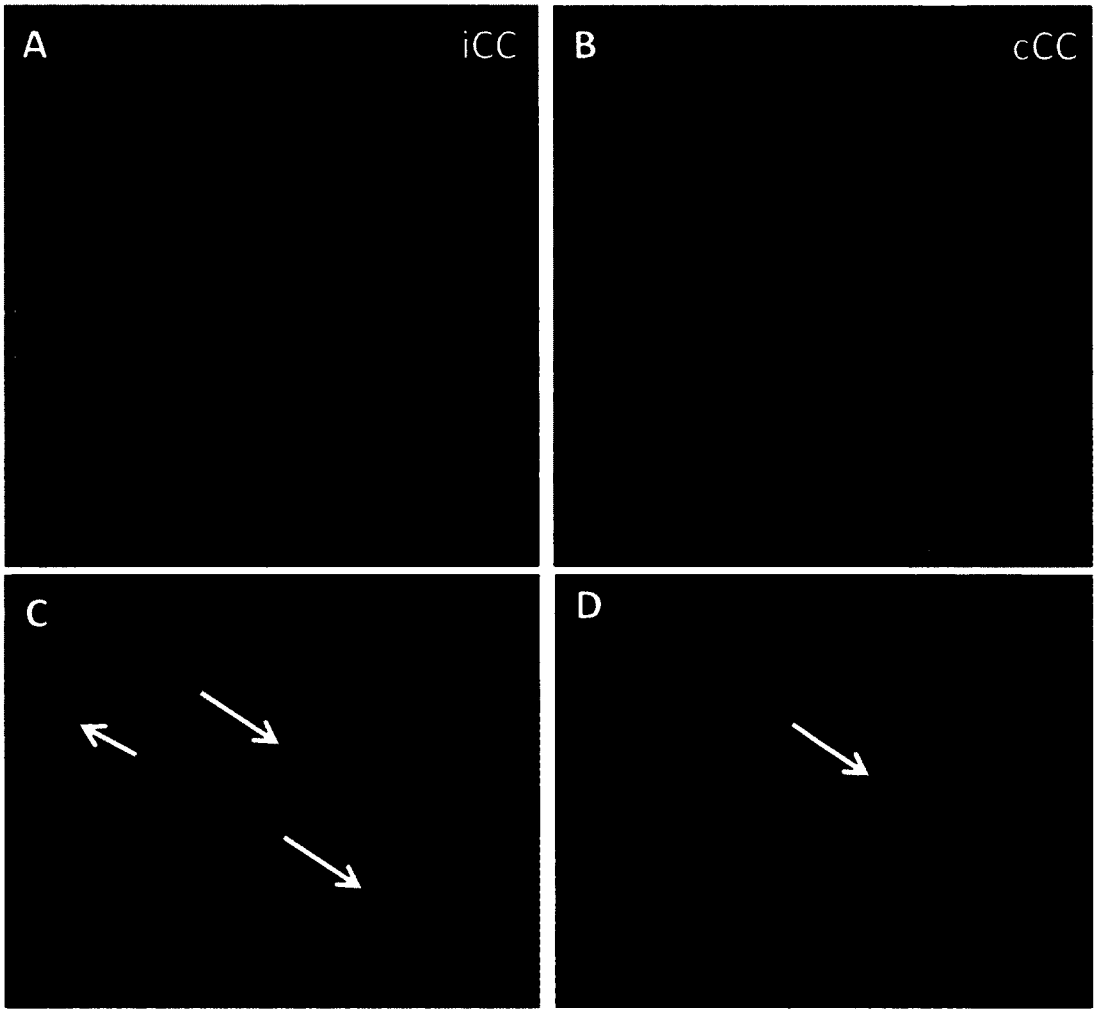

Figure 9. Glial responses to white matter stroke. A. GFAP immunoreactivity in subcortical white matter 9 hours following ET-1 injections. (iCC=ipsilesional corpus callosum). B. GFAP immunoreactivity in subcortical white matter in contralesional corpus hemisphere (cCC=contralesional corpus callosum). C. Magnification of box in A, demonstrating the increased abundance of reactive astrocytes with morphological features of short, spiny processes (arrows). D. Magnification of box in B, the corpus callosum of the contralesional hemisphere has very few GFAP positive cells with long processes (arrow).

Behavioural Analysis

Quantitative Changes in Reaching

Reaching Success

The effects of subcortical white matter stroke on contralesional forelimb fine motor movements measured by percent success (Figure 10) was examined using a two-way (group x day) mixed design ANOVA with stroke and non-stroke/control groups as a between subjects factor and pre-and post surgical days as a within subject factor. The ANOVA revealed a significant main effect ($F(1,10)=50.209$, $p<0.001$) for group, validating that control animals achieved a significantly higher post-surgery reaching percent success (60.5 ± 1.9) compared to post-surgery percent success in ET-1 induced stroke mice (26.2 ± 2.5). No significant effect of days ($F(7,70)=1.053$, $p=0.403$) was found, however there was a highly significant stroke by day interaction ($F(7,70)=5.154$, $p<0.001$), indicating an upward linear trend towards improving functional use of contralesional forelimb grasping by day 7. Notably, the improvement remained considerably lower compared to forelimb function displayed by control animals and thus, does not reflect a trend towards regaining full functional recovery.

Paired t-tests between pre- and post-surgical percent success scores for stroke animals revealed significant differences between the pre-surgical scores and post-surgery scores on day 1 ($p=0.005$), 2 ($p=0.017$), 3 ($p=0.042$) and 5 ($p=0.041$). In addition, percent success for the 6 stroke animals dropped from a pre-surgical average of 38.1 ± 2.6 to a post-surgical score 26.2 ± 2.5, indicating that stroke animals achieved higher reaching scores before ET-1 induced ischemia.

Paired t-tests between pre- and post-surgical percent success for control animals revealed significant differences between pre-surgical scores and post-surgical days 2 ($p=0.001$), 3 ($p<0.001$) and 5 ($p=006$). Control scores increased significantly from 44.2 ± 2.4 percent success to 60.5 ± 1.9; however, these results likely represent a consequence of further experience in the reaching apparatus and do not reflect a treatment effect of the surgical injection of DPBS.

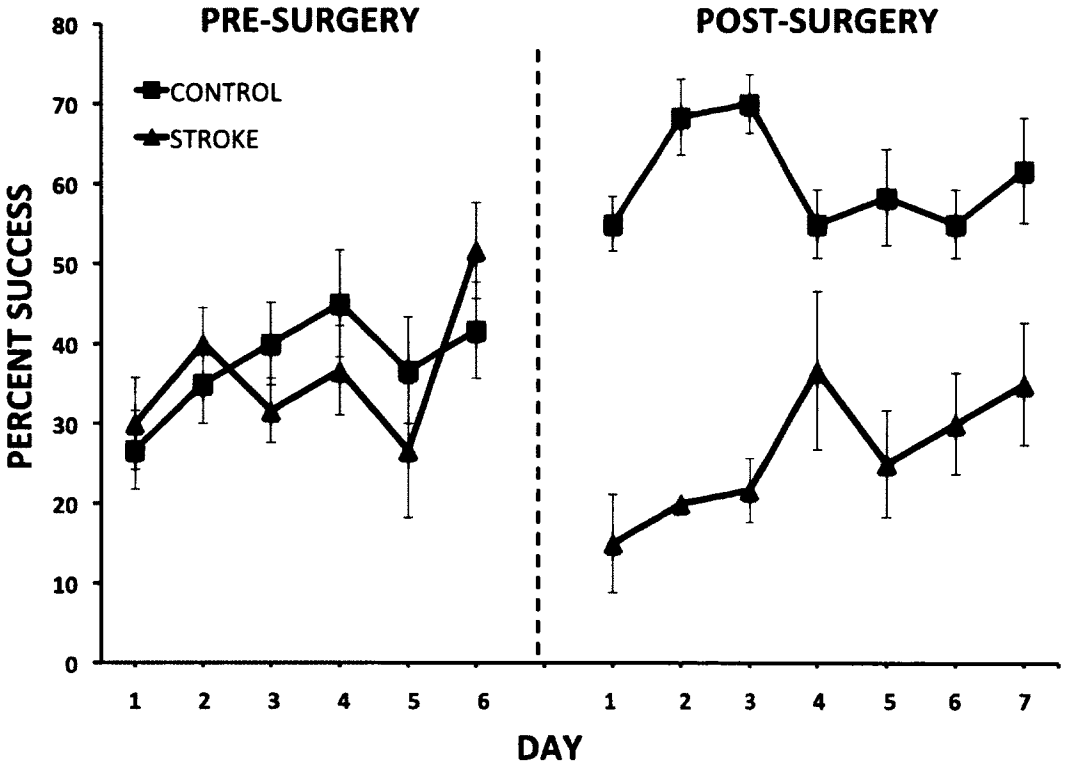

Figure 10. Post-Ischemic Decline in Reaching Success (means ± SEM). Pre- and post-surgical scores for percent success reveal a significant difference between stroke and control animals. There is no difference in pre-surgical scores between groups. The dotted line represents the administration of ET-1 (or DPBS) to promote subcortical white matter stroke. Post-surgical performance of control (DPBS-injected) (n=6) compared to stroke (ET-1 injected) animals (n=6) were significantly different. Stroke scores dropped from an average of 38.1±2.6% to 26.1± 2.5%. Control animals scores increased from 44.2±2.4% to 60.5±1.9%.

Qualitative Components of Movement

Ten components of reach as defined by Farr and Whishaw (2002), evaluated the effects of subcortical white matter ischemia on forelimb fine motor movement. The ability for an animal to fully perform a specified component of movement was examined using a two-way mixed design ANOVA with stroke and non-stroke/control as a between subjects factor and pre- and post-surgical performance as a within subject factor. The ANOVA of 5 pre-surgical and 5 post-surgical successful reaches revealed a significant main effect between groups ($F(1, 10)=20.6$, $p=0.001$), indicating that the mice achieved higher scores for at least one component of movement following subcortical white matter stroke. Additionally, there was a main effect of movement ($F(9,10)=2.1$, $p=0.037$), signifying that some movements were more impaired than others.

A one-way ANOVA for post-surgical movements between groups indicated significant differences between stroke and control performances in the digits to midline, aim, advance, pronation, grasp, supination I and release components of reach (Figure 11). The impaired movements in stroke mice were significantly compromised compared to control mice.

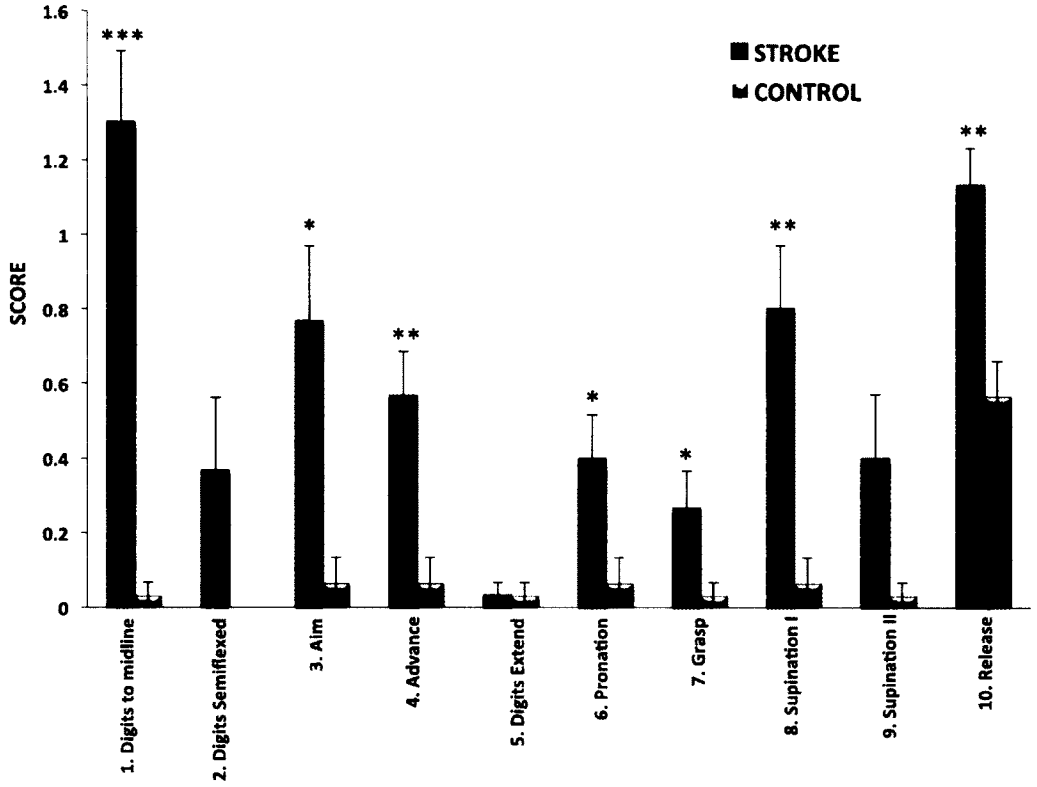

Figure 11. Stroke Induced Reaching Component Deficits in Skilled Forelimb Test. Scores (mean ± SEM) for each of the 10 movement components of reach (1, digits to midline; 2, digits semiflexed; 3, aim; 4, advance; 5, digits extend; 6, pronation; 7, grasp; 8, supination I; 9, supination II; 10, release). A score of 0 represents normal movement and a score of 2 represents the complete absence of the movement. A significant difference between stroke and control groups was found in the following movements: 1, 3, 4, 6, 7, 8, and 10 (*P<0.05, **P<0.01, ***P<0.001).

Qualitative Changes in Reach

All the following descriptions and ratings were acquired from reaches that were successful. The altered reaching components displayed in stroke mice on successful reaches likely contributed to a decline in successful reaching compared to control mice.

Digits to Midline and Aim

Both control and stroke mice raised the right reaching forelimb from the floor, semiflexed the digits in a distinct single movement. Control mice then adducted the elbow to the midline of the body and the forelimb aligned with the midline in an aiming position (Figure 12A). However, stroke mice displaced their forelimb laterally, in an upward position (Figure 12B). Upon lifting the right forelimb to the aiming position, the shoulder of control mice remained elevated and parallel to the left shoulder, whereas the right shoulder of the stroke mice dropped to better aim the stroke affected forelimb through the slot. In addition, many of the stroke mice compensated for unusual body placement and aim by placing the left forelimb against the wall of the reaching box (Figure 12B).

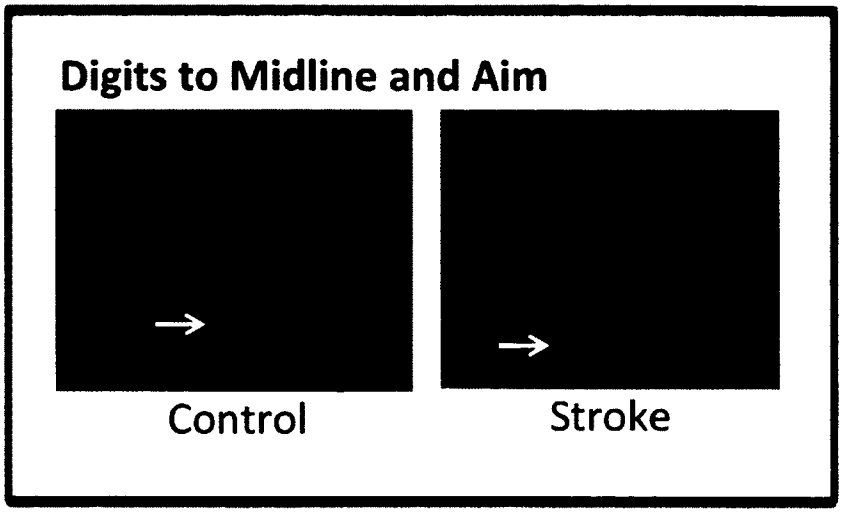

Figure 12. Digits to Midline and Aim. Control mouse raised reaching limb and aligned its digit tips with the midline of the body (arrow in A) and proceeded to aim the paw by bringing the elbow towards the midline. The stroke mouse aligned digit tips in an upward position (arrow in B), shifted the right shoulder downwards, and placed the contralateral forelimb onto the wall of the reaching box to aim its right paw.

Advance and Digits Extend

Control mice advanced the paw directly towards (Figure 13A, top) and over the M&M, while extending their digits (Figure 13B, top), whereas, stroke mice displayed one of two actions of advancement and digit extension. One consisted of dragging the paw on the surface of the platform towards the M&M (Figure 13A, bottom) while extending their digits (Figure 13B, bottom). The other, consistent with the control mice, did not drag the paw along the surface of the platform; instead the paw was raised while advancing towards the M&M (Figure 13C). Though, the paw was raised much higher and preceded to slap the paw down onto the M&M (Figure 13D) as opposed to gracefully placing the extended digits over the M&M.

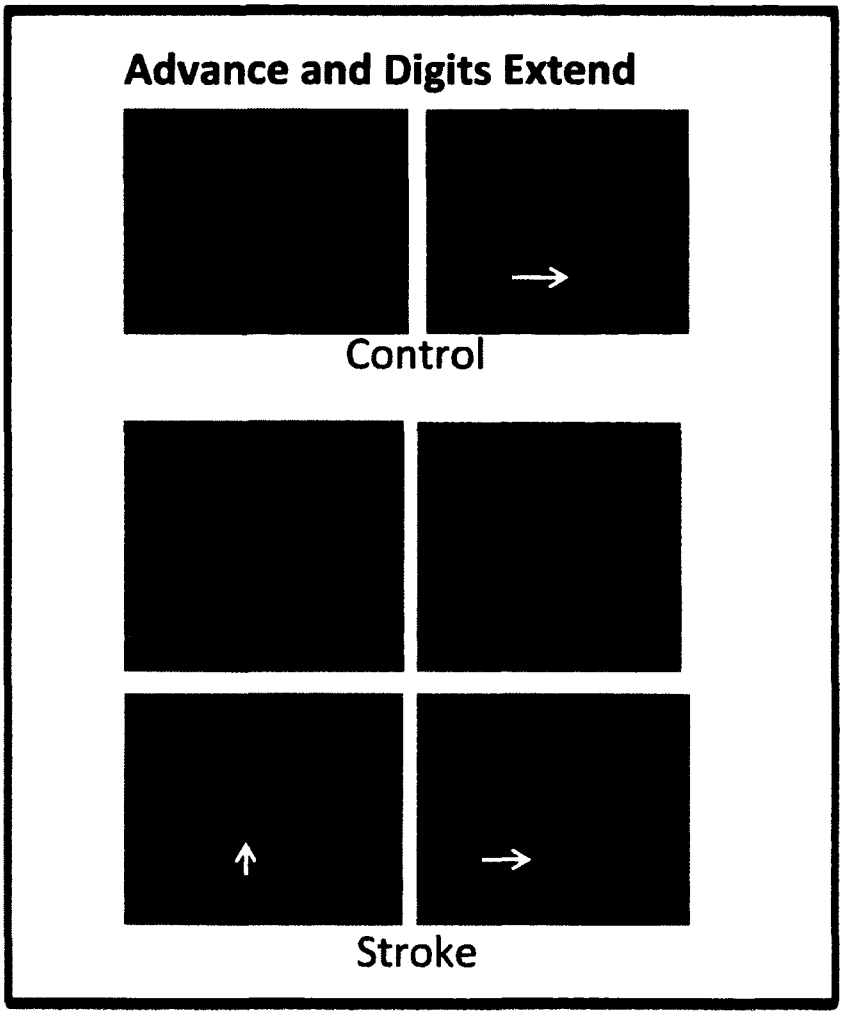

Figure 13. Advance and Digits Extend. A and B, Control. The control mice advanced the reaching forelimb directly towards (A) and over the M&M. The digits extended while initiating rotation of the paw over the M&M (arrow in B). A-D, Stroke. The stroke mice either dragged their paw across the platform surface towards the M&M (A, B), or the paw was raised higher than the control mice (arrow in C) and slapped laterally to contact the M&M while extending the digits (arrow in D).

Pronation

The paw of the control mice was pronated over the M&M as the digits opened and contacted the platform from digit 5 (pinky) to digit 2 (pointer). This sequential was movement demonstrated when food was and was not present in the indentation on the platform (Figure 14 A-C, top). The stroke mice failed to demonstrate a sequential pattern of pronation and either slapped the paw laterally onto the M&M or approached the M&M from the side. The stroke mice that performed the side approach opened their digits sequentially, however digit 5 (pinky) slid forward and remained in contact with the platform and the paw failed to pronate over the M&M (Figure 14 A-B, bottom).

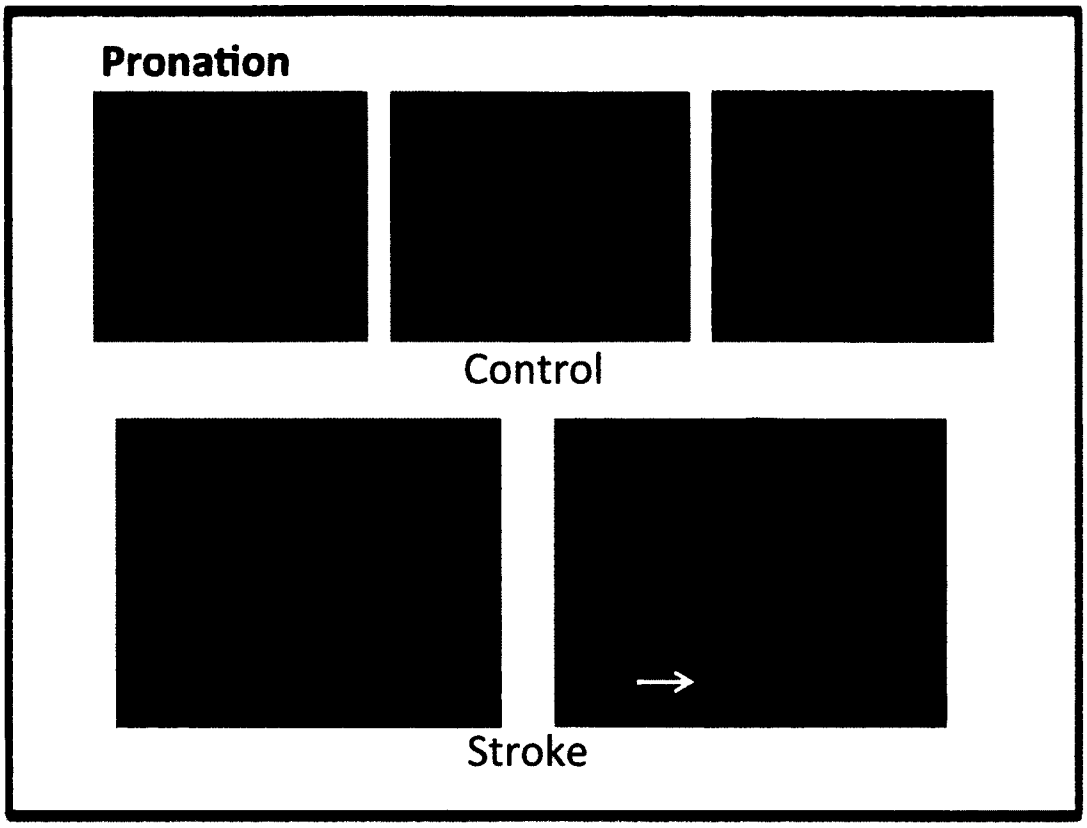

Figure 14. Pronation. A-C, Control. The digits contacted the platform/M&M surface in a series beginning with the most medial (pinky) to the most lateral (pointer). The digits opened and the wrist pronated to cover the M&M. A and B, Stroke. The reaching paw either approached the M&M from the side (arrow in B) or slapped down onto the M&M and the digits failed to contact the platform/M&M in a series.

Grasp and Supination I

Once the M&M was contacted, the control mice closed their digits around the M&M and closed the paw so the palm was oriented in a vertical position relative to the platform (Figure 15A). The paw remained in this position as the paw was withdrawn towards the slot. The stroke mice grasped at the M&M whether the paw made contact with the M&M or not. Several stroke mice had to make numerous attempts at grasping the M&M before they were able to successfully attain it. They showed little to no supination after successfully grasping the M&M and dragged the M&M towards the slot while keeping the paw horizontal to the platform (Figure 15B).

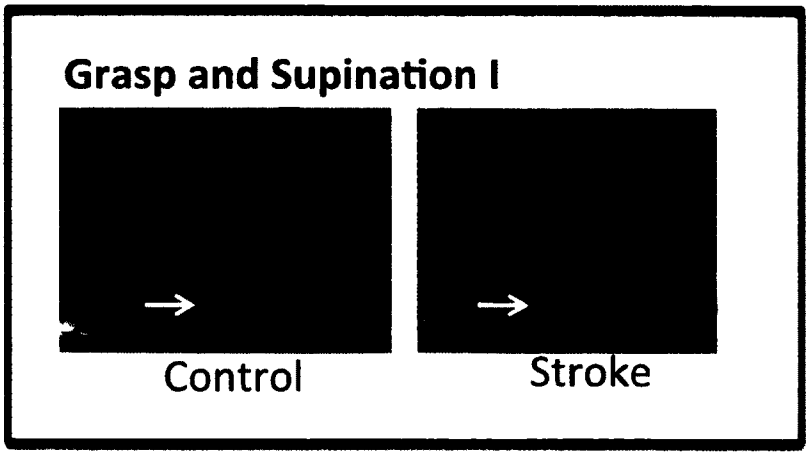

Figure 15. Grasp and Supination I. The control mouse closed the digits around the M&M (A) and supinated the paw while withdrawing the food to the slot. The stroke mouse closed the digits around the M&M and dragged the M&M across the platform towards the slot without supination (B).

Supination II and Release

Once the M&M was withdrawn through the slot, the control mice supinated the paw to orient the palm upwards to present the M&M to the mouth (Figure 16A, top). The M&M was presented and released into the mouth with the reaching paw only, whereas, stroke mice used the non-reaching paw to assist with supination to present and release the M&M to the mouth (Figure 16A, bottom). In addition, stroke mice tilted the head downward to chase the paw(s) to retrieve the M&M (Figure 16B, bottom), whereas the control mice presented the paw to the mouth at the same level as the platform.

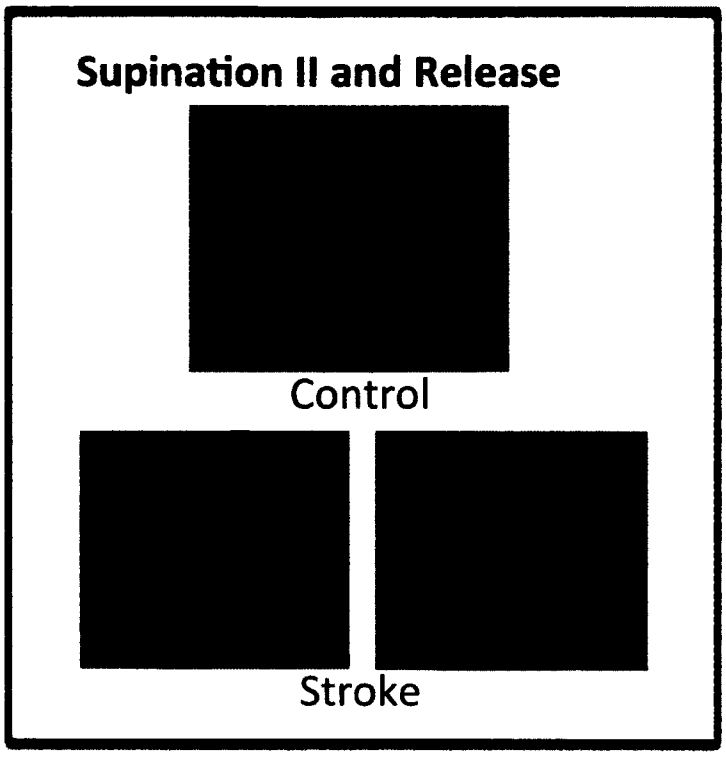

Figure 16. Supination II and Release. The control mouse supinated the paw, presented the M&M to the mouth (A) and opened the digits for release to the mouth. The stroke mouse tilted the head downward towards the paw (B) and required the assistance of the other paw before releasing the M&M into the mouth.

Forelimb Asymmetry

The effects of subcortical white matter stroke on forelimb asymmetry (Figure 17) were examined using a two-way (group x day) mixed design ANOVA. The ANOVA revealed a highly significant main effect for group ($F(1,13)=31.094$, $p<0.001$), indicating that the control animal's forelimb asymmetry scores (-0.02 ± 0.02) differed significantly compared to ET-1 induced stroke animal scores (0.32 ± 0.02). Control animals used both left and right forelimbs to contact the wall of the cylinder more equally compared to stroke animals that favoured the ipsilesional (left) forelimb upon rearing. In addition, a highly significant group by day interaction was found ($F(7,91)=4.187$, $p<0.001$), representing a difference in pre- and post-surgical asymmetry scores based on whether the animal received a stroke or not. In addition, a significant main effect of days was found ($F(7,91)=2.776$, $p=0.012$), indicating that there is a significant difference in forelimb asymmetry within specific days.

Paired t-tests between pre- and post-surgical forelimb asymmetry scores for stroke animals revealed significant differences on all 7 days, indicating that stroke animals had greater forelimb asymmetry scores following surgery. Stroke asymmetry scores increased from -0.027 ± 0.07 to 0.284 ± 0.002.

Paired t-tests between pre- and post-surgical forelimb asymmetry scores for control treated animals did not reveal any significant differences, indicating no significant change in forelimb asymmetry scores as a result of DPBS injections. Two tailed, unpaired, t-tests for individual days revealed significant differences between control and stroke animals on post-surgical days 1-7.

The stroke mice remained motionless for a greater period of time within the cylinder throughout the testing period compared to control mice. In addition, the contralesional forepaw of the stroke mice was raised slightly from the ground with digits curled towards the palm in an attempt to avoid applying pressure to the limb. Whereas, the control mice were more proactive and had the tendency to explore the cylinder more and use both forepaws upon rearing. Several control mice showed minimal preference for one paw or another throughout testing, however these observations were consistent with pre-surgical testing, and did not influence the significance between the 2 groups post-surgical scores.

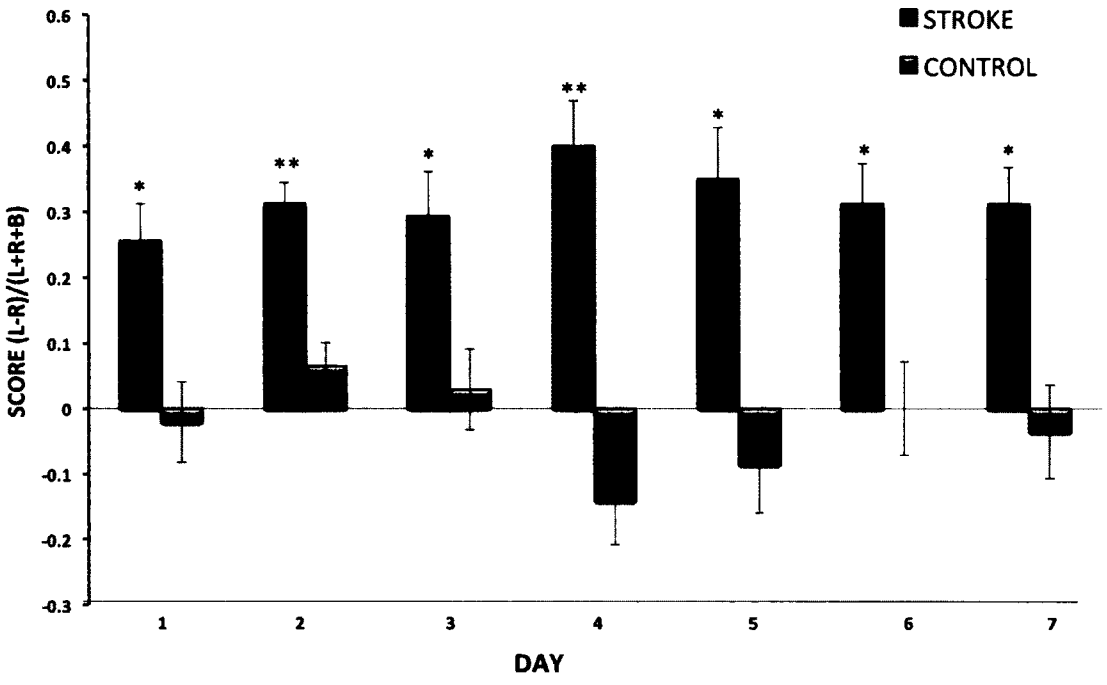

Figure 17. Subcortical White Matter Stroke Contributes to Forelimb Asymmetry. Cylinder testing in control (DPBS injected) vs. stroke (ET-1 injected) animals daily for 7 days following surgery. Stroke animals had significant asymmetrical use of the forelimbs, favouring the ipsilesional (left) forelimb over the contralesional (right) forelimb (*P<0.01, **P<0.001). A positive score indicated favoured use of the contralesional forelimb, a negative score indicated favoured use of the ipsilesional forelimb, and a score of zero indicated equal use of both forelimbs upon rearing and exploration of the cylinder.

GM-CSF Mediated Functional Improvements

The main goal of experiment 2 was to examine the behavioural effects of GM-CSF administration in subcortical white matter stroke mice. Mice received 3 post-stroke injections of GM-CSF on specified days (0, 5 and 10). Contralesional forelimb motor function was evaluates using three behavioural tests designed to explore different components of performance. It was hypothesized that GM-CSF administration would show enhanced behavioural performance compared to stroke mice.

Behavioural Analysis

Reaching success

A two-way (group x day) mixed design ANOVA was conducted to analyze percent reaching success between stroke and stroke + GM-CSF animals (Figure 18). The ANOVA revealed a significant main effect ($F(1, 9)=53.9$, $p<0.001$) for group, signifying a difference in percent reaching success between stroke and stroke + GM-CSF groups. Indeed, stroke + GM-CSF animals achieved a significantly higher percent success (69.1 ± 1.3) compared to stroke animals (41.3 ± 2.6). An interaction effect between group and day ($F(13, 117)=5.1$, $p<0.001$) was revealed; showing the pre-surgical mean for reach success from the stroke animals was higher than the GM-CSF treated animals. Following surgery, the mean percent success for stroke animals dropped below the mean percent of GM-CSF thereby representing an interaction effect. An upward linear relationship ($F(13,11)=6.8$, $p<0.001$) was found for stroke

+ GM-CSF animals representing an improvement in percent success across testing days.

Paired t-tests between pre- and post-surgical percent success scores for stroke animals revealed significant differences ($p<0.05$) between pre-surgical scores and all post-surgery days. The percent success for the 6 stroke induced animals dropped from a pre-surgical average of 70.3 ± 1.7 to a post-surgical average of 27.8 ± 2.1, indicating that stroke mice had significantly fewer successful reaches post-surgery.

Paired t-tests between pre- and post-surgical percent success scores for stroke + GM-CSF animals revealed significant differences on day 1 ($p=0.009$), 4 ($p=0.009$) and 6 ($p=0.002$) and day 7 ($p<0.001$). On these four days, GM-CSF treated mice had significantly fewer successful reaches compared to pre-surgical scores. However, beginning on day 8, post-surgical scores do not differ significantly compared to pre-surgical scores, indicating that they performed equally as well. Stroke + GM-CSF scores increased from an overall pre-surgical score of 65.0 ± 1.8 percent success to an overall post-surgical score of 71.4 ± 1.7 percent success.

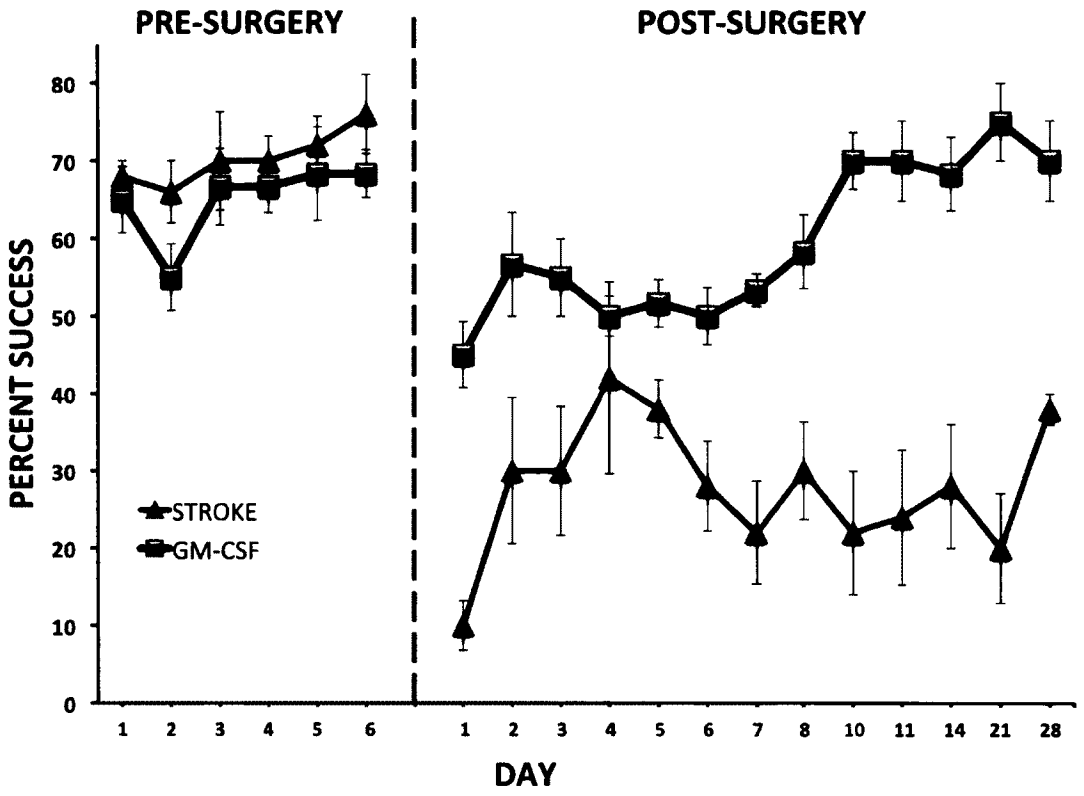

Figure 18. Post-Ischemic Administration of GM-CSF Enhances Reaching Success. Pre- and post-surgical scores for percent success reveal a significant difference between stroke and GM-CSF treated animals. There is no difference in pre-surgical scores between groups. The dotted line represents the administration of ET-1 to promote subcortical white matter stroke. Post-surgical performance of stroke animals (n=5) were significantly different compared to GM-CSF treated stroke animals (n=6). Stroke scores dropped from an average of 70.3±1.7% to 27.8± 2.1%. GM-CSF treated animal scores initially decreased following stroke, but gradually increased to pre-surgical scores.

Qualitative Components of Movement

A two-way mixed design ANOVA was performed, with group as between subject's factors and pre-/post-surgical days, and components of movements as two within factors to evaluate the effect of post-stroke administration of GM-CSF on the average score of the 10 components of movement during a reach (Figure 19). An interaction was found between stroke and GM-CSF groups across different days of reach performance testing ($F(5, 40)=3.9$, $p=0.005$). Similarly, there was a main effect of days ($F(5, 40)=8.3$, $p<0.001$) and groups ($F(1,8)=19.7$, $p=0.002$). No interaction was found between group and the average score of the 10 components of movement combined ($F(9, 72)=1.3$, $p=0.3$), however, a significant main effect for movement was found ($F(9, 72)=11.9$, $p<0.001$), indicating that there is a significant difference between one or more of the 10 different components of movement scores. An interaction was found between movement component scores across days; ($F(45, 360)=2.8$, $p<0.001$) and a three-way interaction was found between movement components, days and group ($F(45, 360)=1.7$, $p=0.006$).

A one-way ANOVA for group and days revealed no significant differences between groups in pre- and post-surgical days 1 and 7 test scores; however, significant differences were found on post-surgical days 14 ($F(1,19)=6.1$, $p=0.02$), 21 ($F(1,19)=23.6$, $p<0.001$) and 28 ($F(1,19)=22.6$, $p<0.001$). These results indicate that following 3 post-stroke injections of GM-CSF, animals had enhanced fine motor movement. Individual one-way ANOVAs for the 10 components of movement for each pre-and post-surgical day revealed significant differences ($p<0.05$) (Figure 19) in pronation, grasp, supination I and release for post-surgical day 14; digits

semiflexed, aim, advance and grasp for post-surgical day 21; and digits to midline, digits semiflexed, aim, advance, digits extend, pronation, grasp, and release for day 28.

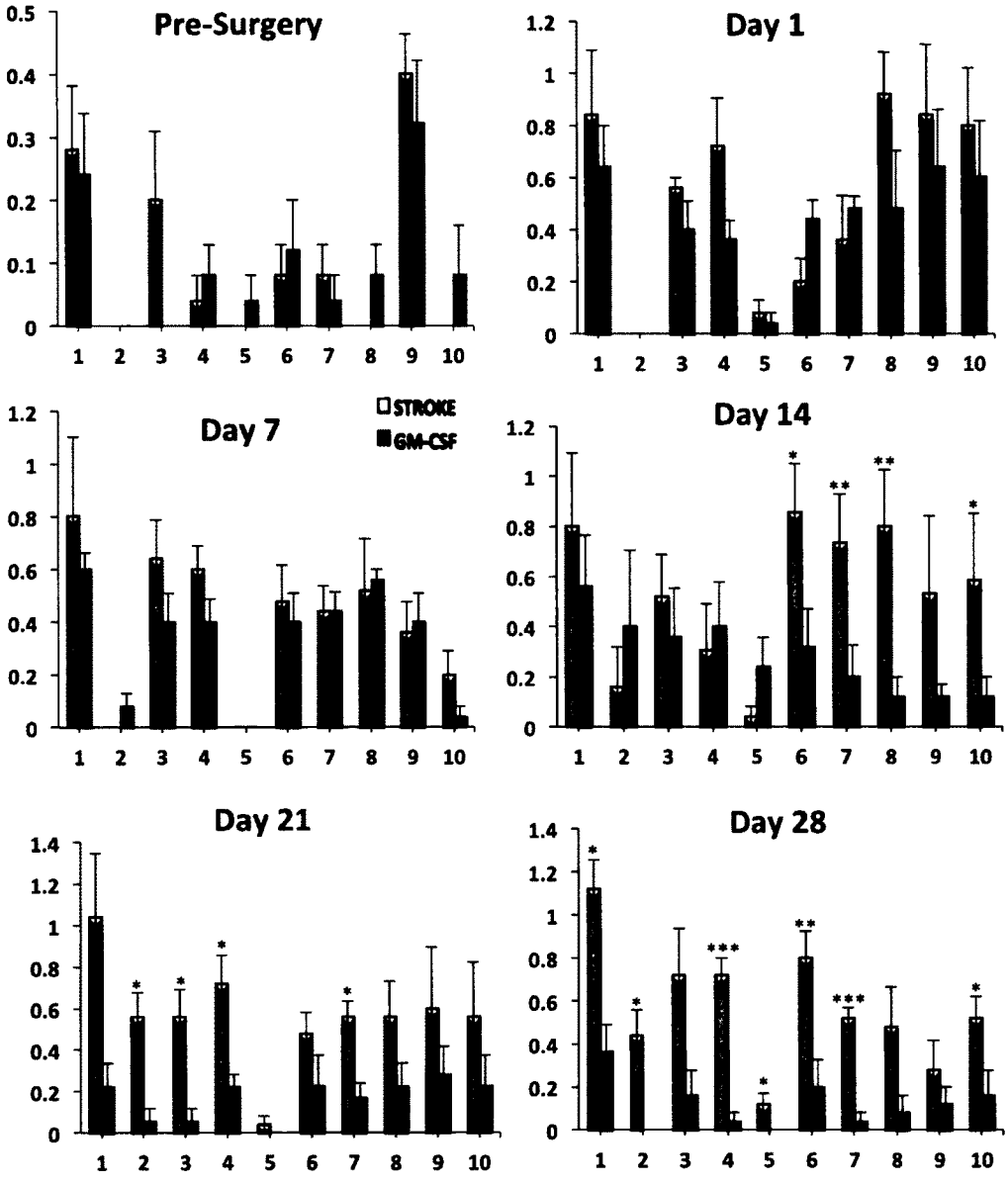

Figure 19. GM-CSF Administration Improves Reaching Deficits Following Stroke. Pre-surgical and post-surgical performance scores (means ± SEM) for each of the 10 movements of reach. Stroke, grey bars; stroke + GM-CSF treatment, black bars. Numbers 1 to 10 on x-axis correspond to the 10 reaching components (1, digits to midline; 2, digits semiflexed; 3, aim; 4, advance; 5, digits extend; 6, pronation; 7, grasp; 8, supination I; 9, supination II; 10, release). Mean score for 5 pre or post-surgical reaches on y-axis correspond to a score of 0 indicates normal movement and a score of 2 indicates the complete absence of the movement (***P<0.001, **P<0.01, *P<0.05).

Unpaired, student *t*-tests were calculated for GM-CSF treated animals on post-surgical days 1 and 28 to examine which components of movement improved over time following the administration of 3 doses of GM-CSF (Figure 20). Significant differences were found for advance, grasp, supination I, supination II and release. Following the 3 injections of GM-CSF on days 0, 5 and 10, the deficits in these movements improved significantly ($p<0.05$) by day 28 likely contributing to the increase in successful reaching percent reported in Figure 18.

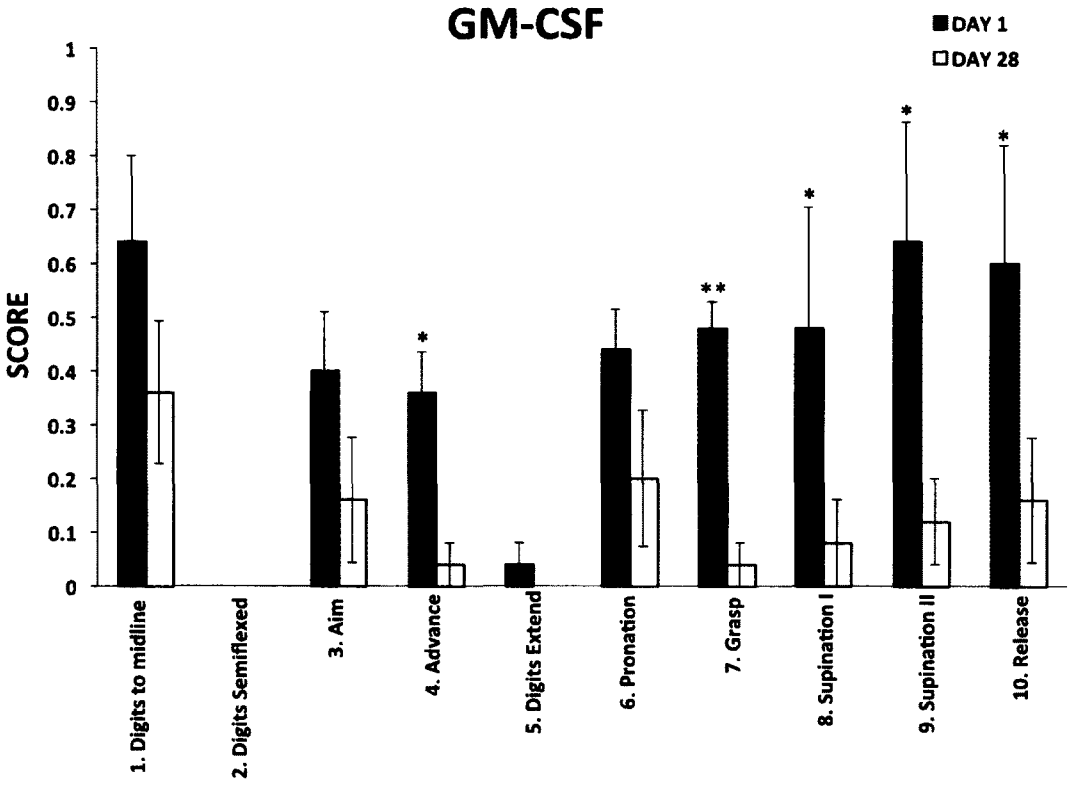

Figure 20. GM-CSF Improves Reaching Deficits in Ischemic Mice Over Time. Performance scores (mean ± SEM) for each of the 10 components of movement in GM-CSF treated animals on day 1 and day 28 following stroke ($^{**}P<0.01$, $^{*}P<0.05$). Performance for advance, grasp, supination I, supination II and release improved between day 1 post stroke to day 28 with GM-CSF treatment.

Advance

At day 1 post-stroke, GM-CSF treated mice advanced their forelimb along the surface of the platform (Figure 21A) in the same manner as many of the untreated stroke mice. By day 28, GM-CSF treated mice advanced their forelimb to the M&M much the same way as the control mice in experiment 1 (Figure 21B). The reaching forelimb was raised and in a vertical position relative to the platform and moved directly towards the M&M.

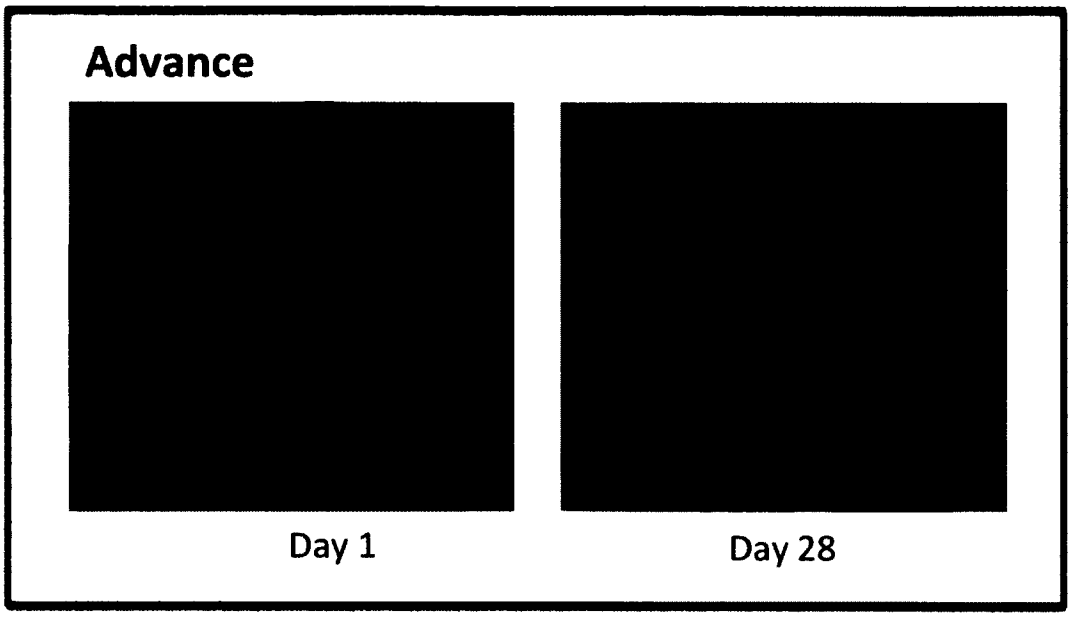

Figure 21. Advance. A. Day 1 post-stroke animals advanced the reaching forelimb towards the M&M while dragging the paw along the surface of the platform. B. By day 28 post-stroke, GM-CSF treated mice displayed advance movement similar to control mice in experiment 1, with the paw raised vertically and directly towards the M&M.

Grasp and Supination I

At day 1 post-stroke, GM-CSF treated mice grasped the M&M from the side of as opposed to the top (Figure 22A), therefore, supination I movement was absent and the paw was dragged back towards the slot (Figure 23). By day 28, GM-CSF treated animals grasped the M&M from the top while extending digits (Figure 23B). The animals withdrew the M&M towards the slot by rotating the paw to a vertical position (Figure 23B).

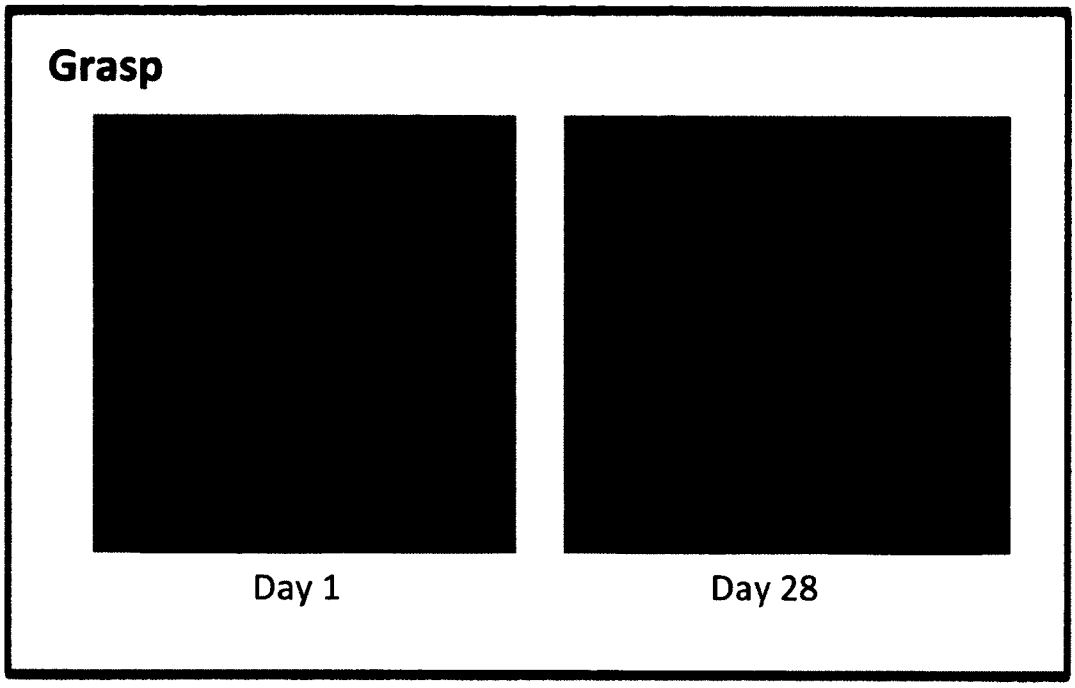

Figure 22. Grasp. A. Day 1 post-stroke animals enclosed digits and grasped the M&M from the side. B. By day 28 post-stroke, GM-CSF treated animals enclosed digits and grasp M&M from the top.

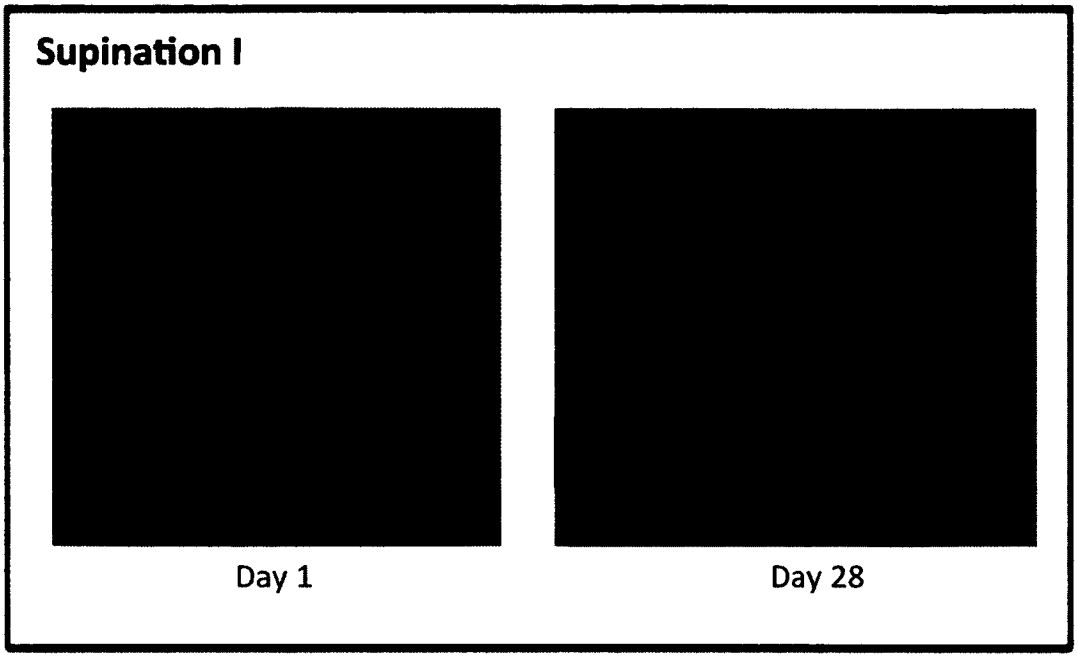

Figure 23. Supination I. A. Day 1 post-stroke animals did not supinate the paw and grasped the M&M from the side and dragged it to the slot. B. By day 28 post-stroke, GM-CSF treated animals supinated the reaching paw vertically and retrieved M&M directly through the slot.

Supination II and Release

At day 1 post-stroke, GM-CSF treated animals required the aid of the non-reaching forelimb in presenting and releasing the M&M to the mouth (Figure 24A). However, at day 28, the animals no longer required the aid of the non-reaching forelimb and presented and released the M&M with the reaching paw alone (Figure 24B).

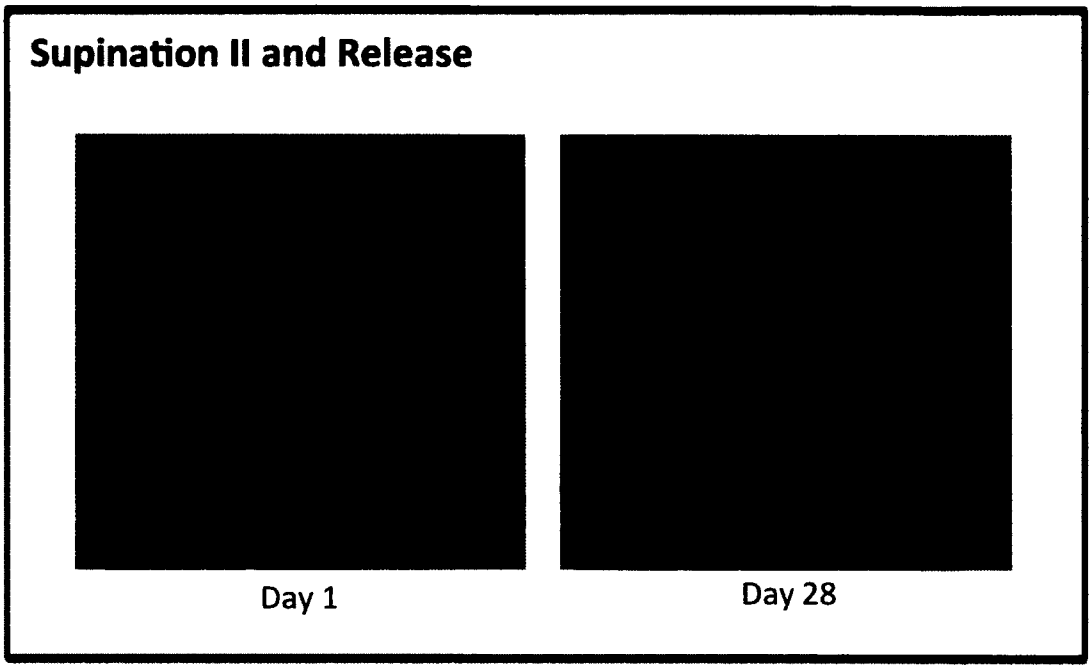

Figure 24. Supination II and Release. A. Day 1 GM-CSF treated animals used the non-reaching paw to present and release the M&M to the mouth. B. By day 28 GM-CSF treated animals presented and released the M&M with the reaching paw alone.

Forelimb asymmetry-cylinder test

The effects of GM-CSF treatment following subcortical white matter stroke on forelimb asymmetry (Figure 25) were examined using a two-way (group x day) mixed ANOVA. The ANOVA revealed a highly significant main effect for group ($F(1,12)=19.053$, $p=0.001$), indicating that the stroke animal forelimb asymmetry scores (0.28 ± 0.02) differed significantly compared to the stroke + GM-CSF treated animal scores (0.006 ± 0.03). GM-CSF treated animals used both left and right forelimbs to contact the wall of the cylinder more equally compared to stroke animals that favoured the ipsilesional (left) forelimb upon rearing. Furthermore, a significant group by day interaction was found ($F(8,96)=2.606$, $p=0.013$), indicating a difference in pre- and post-surgical asymmetry scores based on whether the animal received post-stroke GM-CSF treatment or not. In addition, a significant main effect of days ($F(8,96)=2.046$, $p=0.049$) indicated a difference in forelimb asymmetry between specific days.

Paired t-tests between pre- and post-surgical forelimb asymmetry scores for stroke animals revealed significant differences ($p<0.05$) between pre-surgery and all post-surgical days, indicating that the stroke animals had greater forelimb asymmetry scores immediately following surgery. Stroke asymmetry scores increased from -0.03 ± 0.07 to 0.28 ± 0.02, inferring stroke mice used the ipsilesional (left) forelimb more often upon rearing and vertical exploration following subcortical white matter stroke.

Paired t-tests between pre- and post-surgical forelimb asymmetry scores for GM-CSF treated animals revealed no significant differences, indicating no significant

change in forelimb asymmetry scores prior to stroke surgery and post-stroke treatment with GM-CSF. Stroke + GM-CSF forelimb asymmetry scores changed from a pre-surgical score of 0.00 ± 0.06 to 0.006 ± 0.030 following surgery and GM-CSF treatment.

Two tailed, unpaired, t-tests for individual days revealed significant differences between stroke and GM-CSF treated animals on post-surgical days 5, 7, 11, 14, 21 and 28. The significant difference increases over time from a p-value of 0.018 to <0.001.

Throughout the post-surgical testing period, GM-CSF treated animals developed a preference to use the stroke affected forelimb in the cylinder task upon rearing.

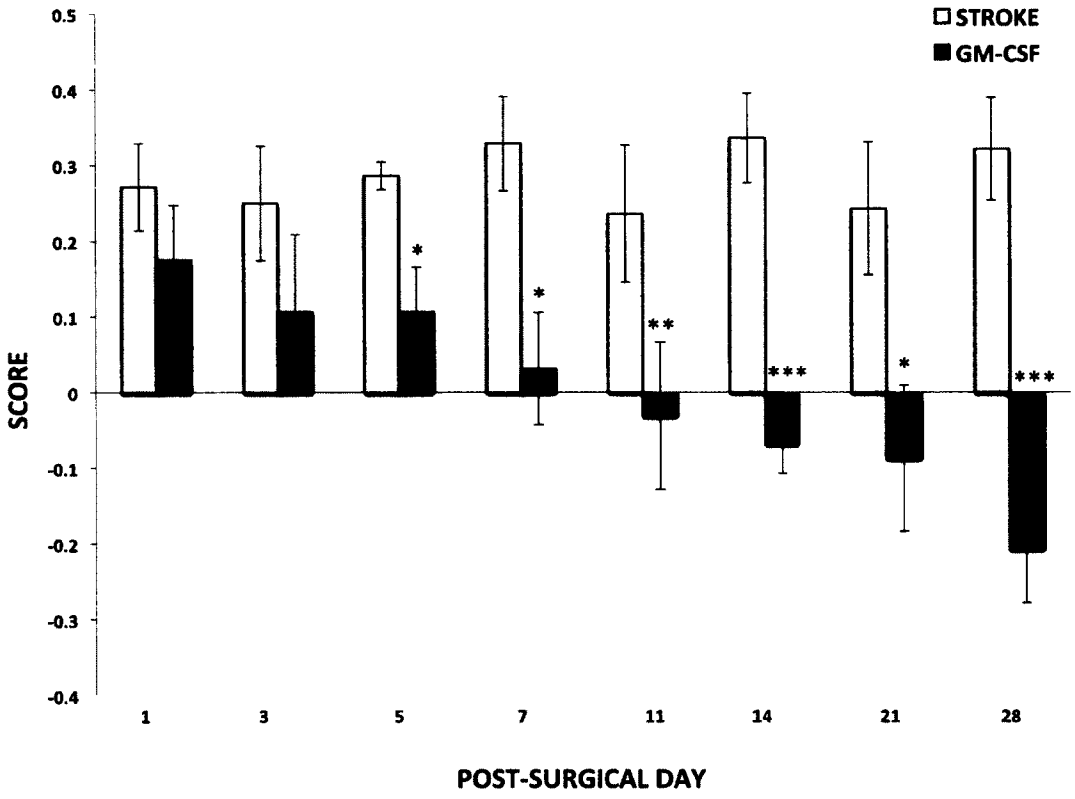

Figure 25. Forelimb Asymmetry is Improved Over Time with Post-Stroke GM-CSF Administration. Cylinder testing in stroke vs. stroke + GM-CSF treated animals on post-surgical days 1, 3, 5, 7, 11, 14, 21 and 28. Significant differences in forelimb symmetry scores (mean ± SEM) were found on post-surgical days 5, 7, 11, 14, 21 and 28. A positive score indicated favoured use of the contralesional (right) forelimb, a negative score indicated favoured use of the ipsilesional (left) forelimb, and a score of zero indicated equal use of both forelimbs upon rearing and exploration of the cylinder.

Discussion

These experiments demonstrated that ET-1 induced subcortical white matter stroke in mice resulted in neurobehavioural functional deficits in forelimb symmetry, reaching and grasping. In addition, the post-stroke administration of GM-CSF on days 0, 5, and 10 resulted in significant functional recovery of subcortical white matter associated deficits over a period of 28 days.

ET-1 Induced Subcortical White Matter Stroke

ET-1 induced SWMS in adult female C57BL/6 mice had significant molecular and behavioural alterations compared to saline injected controls (experiment 1). The post-mortem analysis of brain tissue, 7 days following stroke, confirmed several neuropathological indicators of axonal degeneration of demyelinated axons within the infarct region. Immunofluorescence staining revealed axonal swelling, and microtubule disassembly in the demyelinated axons, signs of cell death and neuroinflammatory responses. The upregulation and morphological changes in GFAP expression found 9 hours following stroke is consistent with previous research and indicates that an increase of reactive astrocytes following stroke (Shibata, Ohtani, Ihara, & Tomimoto, 2004) contributes to the process of glial scar formation. Glial scar formation is a principal physiological component that contributes to the inhibition of neuronal survival and regeneration following stroke. Further investigation into post-stroke mechanisms involving caspase-3, BCl2, and bax activity would stipulate mechanisms of necrosis and/or apoptosis within the infarct region.

Efficacy of ET-1 to Induce Subcortical White Matter Stroke in Mice

The technical simplicity of using ET-1 to induce stroke in rats has made it appealing for use in mice. However, due to mixed success reported in the literature concerning the efficacy of ET-1 producing measurable infarcts in mice, many conclude that it is not useful in generating mouse stroke models. Although ET-1 has previously been reported to be ineffective in producing lesions following intracerebral injection in C57BL/6 strain of mice (Horie et al., 2008; Y. Wang, Jin, & Greenberg, 2007), these experiments clearly dispute ET-1's ineffectiveness by providing evidence focal lesion within the corpus callosum. Consistent with ET-1's ability to produce measurable lesions within the corpus callosum, a recent publication reported decreases in CBF 4hours post-ET-1 injection (1ug) resulting in cortical infarct lesions (Soylu et al., 2012). It is unclear why some studies have succeeded in producing lesions while others have failed. Speculation into the concentration, supplier, brand and methods of administration of ET-1, and the age, strain and sex of experimental mice may indicate possible contributing factors. The use of ET-1 itself as a vasoconstrictor is not likely to be a culprit behind the mixed results due to the abundance of ET-1 receptors widely distributed throughout the CNS on smooth muscle cells, neurons, astrocytes, microglia and endothelia (Hughes et al., 2003). Both this study in conjunction with the study performed by Carmichael and colleagues (2009) clearly advocate the competence of ET-1 injection in mice to provide a proficient model for further investigation into the molecular signaling mechanisms, behavioural effects and possible therapeutic interventions for SWMS.

Behavioural Consequences of Subcortical White Matter Stroke

In addition to the neuropathological indicators of stroke, this experiment also demonstrated that ET-1 injections into the subcortical white matter resulted in considerably significant deficits in forelimb motor function in a variety of tasks. These results indicate that stroke mice suffered unilateral brain damage by presenting an asymmetric reliance on the unaffected (left) limb, and deficits in reaching and grasping with the contralesional forelimb.

Stroke mice displayed significant behavioural deficits in both percent reaching success and in several components of movement in the reaching task. It is important to note that the post-stroke trend towards improvement in percent reach success demonstrated throughout the week were likely attributed by the development of physical compensation in the mechanics of reaching. Specifically, stroke mice learned to deal with their functional deficits and devised different approaches to successfully attain the M&M's. Although there was a trend towards improved percent success in stroke mice, the control mice performed significantly better on a daily basis across the 7 days of post-surgical testing.

The 10 components of movement were quantified by frame-by-frame video analysis and 5 post-surgical successful reaches were compared between stroke and control mice. SWMS revealed deficits in digits to midline, aim, advance, pronation, grasp, supination I and release. These results are consistent with work done in rats exhibiting behavioural deficits in reaching and grasping following lesions affecting various areas of the brain including the sensorimotor cortex (Gharbawie et al., 2005; Whishaw, Pellis, Gorny, & Pellis, 1991), the lateral front cortex (Gharbawie et al.,

2005) and the caudate putamen, globus pallidus and substantia nigra (Gharbawie & Whishaw, 2003; Miklyaeva, Castaneda, & Whishaw, 1994; Pisa, 1988; Whishaw, Castaneda, & Gorny, 1992). Until this study, these behavioural deficits have not been described in a subcortical white matter stroke mouse model. However, previous studies in mice have reported deficits in the aim, advance, pronation, supination II and release components of movement following a pial strip (removal of pial vasculature) over the motor cortex (Farr & Whishaw, 2002). The fact that the presented SWMS model generated behavioural deficits similar to those created by cortical stroke within the primary motor region is a strong indication that the penumbral region of the stroke extends to the motor cortex resulting in apoptotic mechanisms mediating extended neuronal cell loss. In the presented experiments, NeuN immunofluorescence revealed viable neuronal cell bodies superior to the infarct region compared to the contralesional hemisphere, consistent with the evasion of mechanical damage induced by needle insertion during surgery. However, the state of these cells remain unknown and further investigation into the molecular constituents of these neurons could indicate if they are undergoing pre-apoptotic processes or if they are in fact surviving and functioning properly.

Post-surgical scores for the components of movement deficits in stroke mice provide a mechanical explanation for the differences in percent reach success seen between stroke and control mice. The method of analysis offers a description of the complexity of movements involved in the phases of reaching and grasping. It is clear that poor percent success scores in stroke mice was attributed by the impaired ability to properly position themselves within the reaching box, lift their forelimb

through the slot directly towards the M&M with proper pronation and supination of digits and paw to withdraw the food and release it into the mouth.

Post-surgical behavioural impairments were not due to a lack of training, loss of motivation, or an inability to move digits. Mice received extensive pre-surgical training in the reaching box, all mice attained baseline measures that did not significantly differ from one another, and mice were assigned to receive strokes at random. In addition, mice that were unable to perform the task efficiently were omitted for pre-surgical and post-surgical testing. Mice were food restricted to 90% baseline weight to maintain motivation to attain M&M's throughout the testing period. Results were not due to paralysis of the digits because functional deficits following stroke did show a slight positive linear trend towards improvement between day 1 and day 7, and several other components of movement were not significantly impaired.

It is important however to note that post-surgical control mice did achieve higher percent reaching success compared to pre-surgical scores. This does not reflect a treatment effect due to injections of DPBS, but was attributed by further experience in the reaching box. In experiment 3, pre-surgical reach training was extended for an additional week and resulted in an overall mean of 67.4$\pm$1.3% compared to 36.8$\pm$1.8% in experiment 1.

This experiment is the first to evaluate the behavioural and functional aspects of SWMS. Furthermore, it is the first to evaluate these aspects using the controversial method of ET-1 induced focal ischemia in C57BL/6 mice. The importance of these findings can contribute substantial and significant work to

classifying molecular mechanisms involved in behavioural deficits that plague subcortical white matter injuries including stroke, and neurodegenerative diseases such as demyelination disease, Alzheimer's and Parkinson's. This model creates numerous opportunities to classify molecular mechanisms contributing to behavioural deficits and potential genetic manipulation to discover therapeutic intervention strategies.

Subcortical White Matter Stroke Disrupts Neural Networks within the Motor Cortex

The SWMS model presented in this thesis directly targets the corpus callosum, an area of the brain rich in axons used to transmit information between thousands of neurons within both hemispheres. Axons and dendrites are the pillars for communication between neurons, creating complex neural networks throughout the brain. Damage to these neuronal components leads to vast functional implications depending on the brain region and networks affected. Therefore, it is expected that the neurobehavioural deficits displayed in stroke mice are a result of damage in the neural networks originating from the motor cortices that extend into the corpus callosum. The presence of surviving cell bodies, demonstrated with NeuN staining 7 days post-stroke, suggests that the associated cell bodies of damaged axons within the infarct region take a longer time to die through apoptotic mechanisms, thereby providing a time frame to inspire neuroprotection.

GM-CSF Enhances Contralesional Forelimb Deficits Caused by Subcortical White Matter Stroke

This study demonstrated that administration of exogenous GM-CSF following SWMS significantly improved contralesional forelimb performance of reaching and grasping. In addition, functional improvements emerged throughout the testing period and with additional injections of GM-CSF, reflected by improved scores in reach percent success and components of movement between post-stroke day 1 and day 28 (experiment 2).

The behavioural analysis of forelimb asymmetry based on postural support during exploratory behaviour in the cylinder test revealed influences of GM-CSF administration on performance beginning at post-stroke day 5. The increased usage of the contralesional forelimb in vertical exploratory behaviour indicates that the unilateral damage to the motor cortex (penumbra) is significantly reduced. Therefore, fewer lost neurons within the penumbra means neurons can be "saved" by the activation of neuroprotective mechanisms associated with GM-CSF administration that can inhibit apoptosis signaling.

Behavioural assessments following post-stroke administration of GM-CSF on reaching and grasping movements revealed significantly improved performances. Moreover, additional injections of GM-CSF on days 5 and 10 lead to further behavioural improvements. Post-stroke administration of GM-CSF resulted in significant differences in advance, grasp, supination I, supination II and release between day 1 and day 28, and are likely what contributed to increased reaching success. The early post-stroke deficits in advance, grasp, supination and release in

stroke mice gradually improved with GM-CSF treatments resulting in movements comparable to those displayed in control mice. The exact mechanisms contributing to improved performance of these movements are yet to be established in this model, although speculation into the neuroprotective properties of GM-CSF is a promising area to explore.

It is important to note that in both experiments, there appears to be an improvement in post-stroke reaching percent in stroke mice at day 4, followed by a gradual decline in in performance over subsequent days. In the days following stroke, damaged regions undergo a series of molecular cascades that lead to the "self-destruction" of neurons. Clinical evidence suggests that stroke patients exhibit a slow evolution of brain injury that occurs over a period of hours to days indicating that many neurons in the ischemic penumbra (peri-infarct zone) may undergo apoptosis several days following the onset of stroke (Woodruff et al., 2011). Therefore, the apparent increase in percent success reflects the use of the remaining functioning neurons within the penumbra that begin to undergo apoptosis by day 5.

Plasticity

The concept of neuroplasticity mechanisms of activity-dependent rewiring and synapse strengthening in conjunction with the possible neuroprotective effects of GM-CSF treatment likely play a role in the gradual improvement in reaching performance. In fact, animal models have demonstrated a month of heightened plasticity with associated recovery from impairment following stroke. Additionally, enriched rehabilitation including environmental enrichment and skilled reaching

paradigms improves recovery in MCAO in rats (MacLellan et al., 2011). Therefore, the testing regimen of reach success in GM-CSF treated post-stroke mice in this thesis is considered an enriched rehabilitation factor contributing to improved performance.

Another indication that plasticity may be playing a role in improved functional performance in stroke mice following GM-CSF administration is the results for forelimb asymmetry 14 days following stroke. GM-CSF treated mice begin to favour the contralesional (right) forelimb over the use of the ipsilesional or both forelimbs upon rearing within the cylinder. Interestingly, between 14 and 28 days, GM-CSF mice continue to increase right forelimb use while stroke mice continued to favour ipsilesional forelimb use. Investigation into the molecular expression of factors that promote plasticity in this model of subcortical white matter stroke in mice would provide insight into the possible mechanisms mediating these effects.

Neuroprotection through Bcl-2 Activation

Another contributing factor that must be taken into consideration when exploring the behavioural improvements revealed in this thesis is the role of GM-CSF. The effects of GM-CSF within the CNS have been found to mimic the effects it exerts in bone marrow stem cell proliferation and inhibition of hematopoietic cell apoptosis. GM-CSF had been shown to stimulate intracellular signal transduction pathways to induce proliferation of neural progenitor cells in vitro (Kim et al., 2004), and therefore, GM-CSF may act as a mediator of neurogenesis following stroke. However in this study it is unlikely due to improved neurobehavioural

outcomes immediately following the first exogenous post-stroke injection. GM-CSF induced neurogenesis may mediate further neurobehavioural improvement in the weeks following stroke, however this remains to be investigated. The early improvements in this study implies a neuroprotective effect of GM-CSF, which requires a much shorter period of time to produce neurobehavioural improvements compared to neurogenesis.

In ischemia, glutamate accumulates in the extracellular space to cause excessive activation of NMDA receptors resulting in excitotoxicity caused by excessive accumulation of sodium, calcium producing cellular swelling and death. Therefore, attenuating the damaging effects of excitotoxicity is a concern for developing new therapeutic interventions to treat ischemia. GM-CSF has been characterized as neuroprotective in both glutamate-induced excitotoxic cell culture models and in an *in vivo* model of focal ischemic injury (Kong et al., 2009). An antiapoptotic role of GM-CSF has been characterized by several studies, revealing its effects on the expression of genes within the CNS (J. K. Choi et al., 2007; Huang et al., 2007; Nakagawa, Suga, Kawase, & Toda, 2006; Schabitz et al., 2008) including Bcl-2 (antiapoptotic) and Bax (proapoptotic). Studies have shown that GM-CSF administration results in an increase in Bcl-2 expression and decreased the expression of Bax and caspase 3 both in vitro and in vivo (Kong et al., 2009) likely indicating that more cells survived apoptosis in the periinfarct region. It has also been shown that the expression of Bcl-2 through the JAK2/Stat3 cytokine signaling pathway inhibits apoptosis by blocking caspase-3 activation in a murine model of stroke (Shyu et al., 2008), providing a possible explanation to the behavioural

improvements seen in this study. Additional investigation into the intracellular targets of post-stroke administration of GM-CSF can provide a better understanding of the mechanisms responsible for attenuation of glutamate-mediated excitotoxicity and its role in neuroprotection.

In this thesis, GM-CSF was administered systemically through intraperitoneal injections. An important feature of GM-CSF as a therapeutic agent is its ability to cross the blood brain barrier to produce its effects within the CNS. A few studies have shown that a single injection of GM-CSF provides neuroprotective effects within 24 hours following stroke (Kong et al., 2009); however long term neuroprotection is yet to be investigated. In addition, there are several studies that investigate short-term molecular and pathophysiological effects of multiple GM-CSF injections across 5 days (Kong et al., 2009; Shyu, Lin, Lee, Liu, & Li, 2006), yet none have investigated the effects of fewer injections spaced days apart. To demonstrate long term (28 days) neurobehavioural improvements in fine motor movements with 3 injections of exogenous GM-CSF spaced 5 days apart has never been reported until now.

Another feature by which GM-CSF may mediate neuroprotective effects is through arteriogenesis. Arteriogenesis is the remodeling of pre-existing collateral pathways (Buschmann et al., 2001) to areas of damage produced by stroke. GM-CSF has been identified as a factor involved in increasing the diameter of existing arterial vessels and promoting vascular growth in the penumbral area following ischemia (Buschmann, Busch, Mies, & Hossmann, 2003; Schneeloch, Mies, Busch, Buschmann, & Hossmann, 2004). Arteriogenesis supports restored perfusion in the

ischemic regions of the brain to improve long-term functional outcome by supplying the region with much needed energy and resources for survival and sustained function.

There are currently no clinical trials using GM-CSF as a treatment for stroke although GM-CSF has been used in treating leukemia and to reconstitute hematopoiesis following radiation and chemotherapy (Antman et al., 1988). In addition there are several clinical trials using GM-CSF as a potential treatment for sepsis and injury/acute respiratory distress syndrome indicating the safety and potential effectiveness in human care. GM-CSF is a good candidate for stroke treatment for numerous reasons including its ability to cross the BBB (McLay, Kimura, Banks, & Kastin, 1997), the long term effects seen in rodents with relatively few doses, GM-CSFR are expressed in the brain in a similar pattern as the rodent and GM-CSF has shown antiapoptotic effects on human neuroblastoma cells.

Conclusion

The presented experiments demonstrate that ET-1 induced subcortical white matter stroke in C57BL/6 mice produces measurable functional deficits in forelimb movements, and that post-stroke administration of exogenous GM-CSF facilitates functional recovery in several components of movement. Given that these experiments were the first to identify functional deficits associated with subcortical white matter in mice, further studies are required to clarify the role of exogenous administration of GM-CSF on the phenomenon, whether it be plasticity or neuroprotection or a combination of both, contributing to functional recovery and the molecular mechanisms mediating these effects. Discovery of these mechanisms will provide insight into how to integrate GM-CSF into rehabilitation regimens in experiments involving post-subcortical white matter stroke models.

References

Agrawal, S. K., & Fehlings, M. G. (1997). Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. *J Neurosci, 17*(3), 1055-1063.

Alberdi, E., Sanchez-Gomez, M. V., Marino, A., & Matute, C. (2002). Ca(2+) influx through AMPA or kainate receptors alone is sufficient to initiate excitotoxicity in cultured oligodendrocytes. *Neurobiol Dis, 9*(2), 234-243.

Allred, R. P., & Jones, T. A. (2008). Maladaptive effects of learning with the less-affected forelimb after focal cortical infarcts in rats. *Exp Neurol, 210*(1), 172-181.

Antman, K. S., Griffin, J. D., Elias, A., Socinski, M. A., Ryan, L., Cannistra, S. A., . . . Schnipper, L. E. (1988). Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. *N Engl J Med, 319*(10), 593-598.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., & Nakanishi, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature, 348*(6303), 730-732.

Arundine, M., & Tymianski, M. (2004). Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol Life Sci, 61*(6), 657-668.

Ashkenazi, A., & Dixit, V. M. (1999). Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol, 11*(2), 255-260.

Astrup, J., Siesjo, B. K., & Symon, L. (1981). Thresholds in cerebral ischemia - the ischemic penumbra. *Stroke, 12*(6), 723-725.

Astrup, J., Symon, L., Branston, N. M., & Lassen, N. A. (1977). Cortical Evoked-Potential and Extracellular K+ and H+ at Critical Levels of Brain Ischemia. *Stroke, 8*(1), 51-57.

Baron, J. C., Vonkummer, R., & Delzoppo, G. J. (1995). Treatment of Acute Ischemic Stroke - Challenging the Concept of a Rigid and Universal Time Window. *Stroke, 26*(12), 2219-2221.

Beckman, J. S., & Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol, 271*(5 Pt 1), C1424-1437.

Biernaskie, J., Chernenko, G., & Corbett, D. (2004). Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. [Research Support, Non-U.S. Gov't]. *J Neurosci, 24*(5), 1245-1254.

Biernaskie, J., & Corbett, D. (2001). Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury. *J Neurosci, 21*(14), 5272-5280.

Biernaskie, J., Corbett, D., Peeling, J., Wells, J., & Lei, H. (2001). A serial MR study of cerebral blood flow changes and lesion development following endothelin-1-induced ischemia in rats. *Magn Reson Med, 46*(4), 827-830.

Bland, S. T., Schallert, T., Strong, R., Aronowski, J., Grotta, J. C., & Feeney, D. M. (2000). Early exclusive use of the affected forelimb after moderate transient focal ischemia in rats : functional and anatomic outcome. *Stroke, 31*(5), 1144-1152.

Bond, A., Ragumoorthy, N., Monn, J. A., Hicks, C. A., Ward, M. A., Lodge, D., & O'Neill, M. J. (1999). LY379268, a potent and selective Group II metabotropic glutamate receptor agonist, is neuroprotective in gerbil global, but not focal, cerebral ischaemia. *Neurosci Lett, 273*(3), 191-194.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., & Lipton, S. A. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A, 92*(16), 7162-7166.

Boxer, P. A., Cordon, J. J., Mann, M. E., Rodolosi, L. C., Vartanian, M. G., Rock, D. M., . . . Marcoux, F. W. (1990). Comparison of phenytoin with noncompetitive N-methyl-D-aspartate antagonists in a model of focal brain ischemia in rat. *Stroke, 21*(11 Suppl), III47-51.

Broughton, B. R., Reutens, D. C., & Sobey, C. G. (2009). Apoptotic mechanisms after cerebral ischemia. *Stroke, 40*(5), e331-339. doi: 10.1161/STROKEAHA.108.531632

Brus-Ramer, M., Carmel, J. B., & Martin, J. H. (2009). Motor cortex bilateral motor representation depends on subcortical and interhemispheric interactions. *J Neurosci, 29*(19), 6196-6206.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., & Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol, 15*, 269-290.

Burgess, A.W., & Metcalf D. (1977). Serum half-life and organ distribution of radiolabeled colony stimulating factor in mice. *Exp Hematol, 5*:456-464.

Buschmann, I. R., Busch, H. J., Mies, G., & Hossmann, K. A. (2003). Therapeutic induction of arteriogenesis in hypoperfused rat brain via granulocyte-macrophage colony-stimulating factor. *Circulation, 108*(5), 610-615.

Buschmann, I. R., Hoefer, I. E., van Royen, N., Katzer, E., Braun-Dulleaus, R., Heil, M., . . Schaper, W. (2001). GM-CSF: a strong arteriogenic factor acting by amplification of monocyte function. *Atherosclerosis, 159*(2), 343-356.

Bussolino, F., Ziche, M., Wang, J. M., Alessi, D., Morbidelli, L., Cremona, O., . . . Mantovani, A. (1991). In vitro and in vivo activation of endothelial cells by colony-stimulating factors. *J Clin Invest, 87*(3), 986-995.

Cao, G., Pei, W., Lan, J., Stetler, R. A., Luo, Y., Nagayama, T., . . . Chen, J. (2001). Caspase-activated DNase/DNA fragmentation factor 40 mediates apoptotic DNA fragmentation in transient cerebral ischemia and in neuronal cultures. *J Neurosci, 21*(13), 4678-4690.

Carmichael, S. T. (2005). Rodent models of focal stroke: size, mechanism, and purpose. *NeuroRx, 2*(3), 396-409.

Carmichael, S. T. (2006). Cellular and molecular mechanisms of neural repair after stroke: making waves. *Ann Neurol, 59*(5), 735-742.

Carmichael, S. T., Archibeque, I., Luke, L., Nolan, T., Momiy, J., & Li, S. (2005). Growth-associated gene expression after stroke: evidence for a growth-promoting region in peri-infarct cortex. *Exp Neurol, 193*(2), 291-311.

Cenci, M. A. & Lundblad, M. in Animal Models of Movement Disorders (ed. LeDoux, M.) 193–209 (Elsevier, Amsterdam, 2005).

Choi, D. W. (1992). Excitotoxic cell death. *J Neurobiol, 23*(9), 1261-1276.

Choi, J. K., Choi, B. H., Ha, Y., Park, H., Yoon, S. H., Park, H. C., & Park, S. R. (2007). Signal transduction pathways of GM-CSF in neural cell lines. *Neurosci Lett, 420*(3), 217-222.

Chollet, F., DiPiero, V., Wise, R. J., Brooks, D. J., Dolan, R. J., & Frackowiak, R. S. (1991). The functional anatomy of motor recovery after stroke in humans: a study with positron emission tomography. [Comparative Study Research Support, Non-U.S. Gov't]. *Ann Neurol, 29*(1), 63-71. doi: 10.1002/ana.410290112

Cohen, G. M. (1997). Caspases: the executioners of apoptosis. *Biochem J, 326 (Pt 1)*, 1-16.

Coxon, A., Tang, T., & Mayadas, T. N. (1999). Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo. A role for granulocyte/macrophage colony-stimulating factor. *J Exp Med, 190*(7), 923-934.

Cramer, S. C. (2008). Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. *Ann Neurol, 63*(3), 272-287.

Cramer, S. C., Shah, R., Juranek, J., Crafton, K. R., & Le, V. (2006). Activity in the peri-infarct rim in relation to recovery from stroke. *Stroke, 37*(1), 111-115.

Dame, J. B., Christensen, R. D., & Juul, S. E. (1999). The distribution of granulocyte-macrophage colony-stimulating factor and its receptor in the developing human fetus. *Pediatr Res, 46*(4), 358-366.

Debette, S., & Markus, H.S. (2010). The clinical importance of white matter hyperintensities on brain magnetic resonance imaging: systematic review and meta-analysis. *BMJ, 341*, 3666.

de Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T. D., & Vane, J. R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci U S A, 85*(24), 9797-9800.

Dirnagl, U., Iadecola, C., & Moskowitz, M. A. (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci, 22*(9), 391-397.

Dobkin, B. H. (1998). Driving cognitive and motor gains with rehabilitation after brain and spinal cord injury. *Curr Opin Neurol, 11*(6), 639-641.

Dohi, Y., & Luscher, T. F. (1991). Endothelin in hypertensive resistance arteries. Intraluminal and extraluminal dysfunction. *Hypertension, 18*(4), 543-549.

Earnshaw, W. C., Martins, L. M., & Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem, 68*, 383-424.

Eshkol, N. and Wachman, A., *Movement Notation.* Weidenfeld and Nicholson, London, 1958.

Farr, T. D., & Whishaw, I. Q. (2002). Quantitative and qualitative impairments in skilled reaching in the mouse (Mus musculus) after a focal motor cortex stroke. *Stroke, 33*(7), 1869-1875.

Felderhoff-Mueser, U., Taylor, D. L., Greenwood, K., Kozma, M., Stibenz, D., Joashi, U. C., . . . Mehmet, H. (2000). Fas/CD95/APO-1 can function as a death receptor for neuronal cells in vitro and in vivo and is upregulated following cerebral hypoxic-ischemic injury to the developing rat brain. *Brain Pathol, 10*(1), 17-29.

Fern, R., Ransom, B. R., & Waxman, S. G. (1995). Voltage-gated calcium channels in CNS white matter: role in anoxic injury. *J Neurophysiol, 74*(1), 369-377.

Ferrer, I., & Planas, A. M. (2003). Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *J Neuropathol Exp Neurol, 62*(4), 329-339.

Fonnum, F. (1984). Glutamate: a neurotransmitter in mammalian brain. *J Neurochem, 42*(1), 1-11.

Fuxe, K., Kurosawa, N., Cintra, A., Hallstrom, A., Goiny, M., Rosen, L., . . . Ungerstedt, U. (1992). Involvement of local ischemia in endothelin-1 induced lesions of the neostriatum of the anaesthetized rat. *Exp Brain Res, 88*(1), 131-139.

Gasic, G. P., & Hollmann, M. (1992). Molecular neurobiology of glutamate receptors. *Annu Rev Physiol, 54*, 507-536.

Gharbawie, O. A., Gonzalez, C. L., & Whishaw, I. Q. (2005). Skilled reaching impairments from the lateral frontal cortex component of middle cerebral artery stroke: a qualitative and quantitative comparison to focal motor cortex lesions in rats. *Behav Brain Res, 156*(1), 125-137.

Gharbawie, O. A., & Whishaw, I. Q. (2003). Cholinergic and serotonergic neocortical projection lesions given singly or in combination cause only mild impairments on tests of skilled movement in rats: evaluation of a model of dementia. *Brain Res, 970*(1-2), 97-109.

Gharbawie, O. A., Whishaw, P. A., & Whishaw, I. Q. (2004). The topography of three-dimensional exploration: a new quantification of vertical and horizontal exploration, postural support, and exploratory bouts in the cylinder test. *Behav Brain Res, 151*(1-2), 125-135.

Giaid, A., Gibson, S. J., Ibrahim, B. N., Legon, S., Bloom, S. R., Yanagisawa, M., . . . Polak, J. M. (1989). Endothelin 1, an endothelium-derived peptide, is expressed in neurons of the human spinal cord and dorsal root ganglia. *Proc Natl Acad Sci U S A, 86*(19), 7634-7638.

Giulian, D., Li, J., Li, X., George, J., & Rutecki, P. A. (1994). The impact of microglia-derived cytokines upon gliosis in the CNS. *Dev Neurosci, 16*(3-4), 128-136.

Gonzalez, C. L., Gharbawie, O. A., Williams, P. T., Kleim, J. A., Kolb, B., & Whishaw, I. Q. (2004). Evidence for bilateral control of skilled movements: ipsilateral skilled forelimb reaching deficits and functional recovery in rats follow motor cortex and lateral frontal cortex lesions. *Eur J Neurosci, 20*(12), 3442-3452.

Grabowski, M., Brundin, P., & Johansson, B. B. (1993). Paw-reaching, sensorimotor, and rotational behavior after brain infarction in rats. *Stroke, 24*(6), 889-895.

Graham, S. H., Chen, J., Sharp, F. R., & Simon, R. P. (1993). Limiting ischemic injury by inhibition of excitatory amino acid release. *J Cereb Blood Flow Metab, 13*(1), 88-97.

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629.

Hara, H., Fink, K., Endres, M., Friedlander, R. M., Gagliardini, V., Yuan, J., & Moskowitz, M. A. (1997). Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein. *J Cereb Blood Flow Metab, 17*(4), 370-375.

Hara, H., Friedlander, R. M., Gagliardini, V., Ayata, C., Fink, K., Huang, Z., . . . Moskowitz, M. A. (1997). Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc Natl Acad Sci U S A, 94*(5), 2007-2012.

Hattiangady, B., Rao, M. S., Shetty, G. A. & Shetty,, & K., A. (2005). Brain-derived neurotrophic factor, phosphorylated cyclic AMP response element binding protein and neuropeptide Y decline as early as middle age in the dentate gyrus and CA1 and CA3 subfields of the hippocampus. *Experimental Neurology, 195*, 353-371.

Hazell, A. S. (2007). Excitotoxic mechanisms in stroke: an update of concepts and treatment strategies. *Neurochem Int, 50*(7-8), 941-953.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Hengartner, M. O., & Horvitz, H. R. (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell, 76*(4), 665-676.

Hermann, D. M., Kilic, E., Hata, R., Hossmann, K. A., & Mies, G. (2001). Relationship between metabolic dysfunctions, gene responses and delayed cell death after mild focal cerebral ischemia in mice. *Neuroscience, 104*(4), 947-955.

Horie, N., Maag, A. L., Hamilton, S. A., Shichinohe, H., Bliss, T. M., & Steinberg, G. K. (2008). Mouse model of focal cerebral ischemia using endothelin-1. *J Neurosci Methods, 173*(2), 286-290.

Hossmann, K. A. (1994). Viability Thresholds and the Penumbra of Focal Ischemia. *Annals of Neurology, 36*(4), 557-565.

Hossmann, K. A. (2006). Pathophysiology and therapy of experimental stroke. [Review]. *Cell Mol Neurobiol, 26*(7-8), 1057-1083. doi: 10.1007/s10571-006-9008-1

Hsu, J. E., & Jones, T. A. (2006). Contralesional neural plasticity and functional changes in the less-affected forelimb after large and small cortical infarcts in rats. [Research Support, N.I.H., Extramural]. *Exp Neurol, 201*(2), 479-494.

Hua, Y., Schallert, T., Keep, R. F., Wu, J., Hoff, J. T., & Xi, G. (2002). Behavioral tests after intracerebral hemorrhage in the rat. *Stroke, 33*(10), 2478-2484.

Huang, X., Choi, J. K., Park, S. R., Ha, Y., Park, H., Yoon, S. H., . . . Choi, B. H. (2007). GM-CSF inhibits apoptosis of neural cells via regulating the expression of apoptosis-related proteins. *Neurosci Res, 58*(1), 50-57. doi: 10.1016/j.neures.2007.01.015

Hughes, P. M., Anthony, D. C., Ruddin, M., Botham, M. S., Rankine, E. L., Sablone, M., . . . Perry, V. H. (2003). Focal lesions in the rat central nervous system induced by endothelin-1. *J Neuropathol Exp Neurol, 62*(12), 1276-1286.

Iancu, R., Mohapel, P., Brundin, P., & Paul, G. (2005). Behavioral characterization of a unilateral 6-OHDA-lesion model of Parkinson's disease in mice. *Behav Brain Res, 162*(1), 1-10.

Johansson, B. B. (2000). Brain plasticity and stroke rehabilitation. The Willis lecture. *Stroke, 31*(1), 223-230.

Kannan, Y., Moriyama, M., Sugano, T., Yamate, J., Kuwamura, M., Kagaya, A., & Kiso, Y. (2000). Neurotrophic action of interleukin 3 and granulocyte-macrophage colony-stimulating factor on murine sympathetic neurons. *Neuroimmunomodulation, 8*(3), 132-141. doi: 54273

Karadottir, R., Cavelier, P., Bergersen, L. H., & Attwell, D. (2005). NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature, 438*(7071), 1162-1166.

Kaufmann, S. H., & Hengartner, M. O. (2001). Programmed cell death: alive and well in the new millennium. *Trends Cell Biol, 11*(12), 526-534.

Kawamata, T., Alexis, N. E., Dietrich, W. D., & Finklestein, S. P. (1996). Intracisternal basic fibroblast growth factor (bFGF) enhances behavioral recovery following focal cerebral infarction in the rat. *J Cereb Blood Flow Metab, 16*(4), 542-547.

Kawamata, T., Ren, J., Chan, T. C., Charette, M., & Finklestein, S. P. (1998). Intracisternal osteogenic protein-1 enhances functional recovery following focal stroke. *Neuroreport, 9*(7), 1441-1445.

Kim, J. K., Choi, B. H., Park, H. C., Park, S. R., Kim, Y. S., Yoon, S. H., . . . Ha, Y. (2004). Effects of GM-CSF on the neural progenitor cells. *Neuroreport, 15*(14), 2161-2165.

Kitagawa, H., Warita, H., Sasaki, C., Zhang, W. R., Sakai, K., Shiro, Y., . . . Abe, K. (1999). Immunoreactive Akt, PI3-K and ERK protein kinase expression in ischemic rat brain. *Neurosci Lett, 274*(1), 45-48.

Kleim, J. A., & Jones, T. A. (2008). Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. [Consensus Development Conference]. *J Speech Lang Hear Res, 51*(1), S225-239.

Kong, T., Choi, J. K., Park, H., Choi, B. H., Snyder, B. J., Bukhari, S., . . . Ha, Y. (2009). Reduction in programmed cell death and improvement in functional outcome of transient focal cerebral ischemia after administration of granulocyte-macrophage colony-stimulating factor in rats. Laboratory investigation. *J Neurosurg, 111*(1), 155-163.

Lam, A. G., Soriano, M. A., Monn, J. A., Schoepp, D. D., Lodge, D., & McCulloch, J. (1998). Effects of the selective metabotropic glutamate agonist LY354740 in a rat model of permanent ischaemia. *Neurosci Lett, 254*(2), 121-123.

Lawrence, M. S., McLaughlin, J. R., Sun, G. H., Ho, D. Y., McIntosh, L., Kunis, D. M., . . . Steinberg, G. K. (1997). Herpes simplex viral vectors expressing Bcl-2 are neuroprotective when delivered after a stroke. *J Cereb Blood Flow Metab, 17*(7), 740-744.

Lee, M. E., de la Monte, S. M., Ng, S. C., Bloch, K. D., & Quertermous, T. (1990). Expression of the potent vasoconstrictor endothelin in the human central nervous system. *J Clin Invest, 86*(1), 141-147.

Levin, M. F., Kleim, J. A., & Wolf, S. L. (2009). What do motor "recovery" and "compensation" mean in patients following stroke? *Neurorehabil Neural Repair, 23*(4), 313-319.

Levin, M. F., Michaelsen, S. M., Cirstea, C. M., & Roby-Brami, A. (2002). Use of the trunk for reaching targets placed within and beyond the reach in adult hemiparesis. *Exp Brain Res, 143*(2), 171-180.

Li, S., & Stys, P. K. (2000). Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter. *J Neurosci, 20*(3), 1190-1198.

Li, Y., Sharov, V. G., Jiang, N., Zaloga, C., Sabbah, H. N., & Chopp, M. (1995). Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. *Am J Pathol, 146*(5), 1045-1051.

Lin, C. H., Tallaksen-Greene, S., Chien, W. M., Cearley, J. A., Jackson, W. S., Crouse, A. B., . . . Detloff, P. J. (2001). Neurological abnormalities in a knock-in mouse model of Huntington's disease. *Hum Mol Genet, 10*(2), 137-144.

Lipton, S. A., & Kater, S. B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci, 12*(7), 265-270.

Longa, E. Z., Weinstein, P. R., Carlson, S., & Cummins, R. (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke, 20*(1), 84-91.

Love, S. (2003). Apoptosis and brain ischaemia. *Prog Neuropsychopharmacol Biol Psychiatry, 27*(2), 267-282. doi: 10.1016/S0278-5846(03)00022-8

MacCumber, M. W., Ross, C. A., & Snyder, S. H. (1990). Endothelin in brain: receptors, mitogenesis, and biosynthesis in glial cells. *Proc Natl Acad Sci U S A, 87*(6), 2359-2363.

MacManus, J. P., Buchan, A. M., Hill, I. E., Rasquinha, I., & Preston, E. (1993). Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett, 164*(1-2), 89-92.

Macrae, I. M., Robinson, M. J., Graham, D. I., Reid, J. L., & McCulloch, J. (1993). Endothelin-1-induced reductions in cerebral blood flow: dose dependency, time course, and neuropathological consequences. *J Cereb Blood Flow Metab, 13*(2), 276-284.

Malipiero, U. V., Frei, K., & Fontana, A. (1990). Production of hemopoietic colony-stimulating factors by astrocytes. *J Immunol, 144*(10), 3816-3821.

Mangano, E. N., Peters, S., Litteljohn, D., So, R., Bethune, C., Bobyn, J., . . . Hayley, S. (2011). Granulocyte macrophage-colony stimulating factor protects against substantia nigra dopaminergic cell loss in an environmental toxin model of Parkinson's disease. *Neurobiol Dis, 43*(1), 99-112.

Markgraf, C. G., Green, E. J., Hurwitz, B. E., Morikawa, E., Dietrich, W. D., McCabe, P. M., . . . Schneiderman, N. (1992). Sensorimotor and cognitive consequences of middle cerebral artery occlusion in rats. *Brain Res, 575*(2), 238-246.

Masaki, T. (1995). Possible role of endothelin in endothelial regulation of vascular tone. *Annu Rev Pharmacol Toxicol, 35*, 235-255.

Matute, C., Sanchez-Gomez, M. V., Martinez-Millan, L., & Miledi, R. (1997). Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci U S A, 94*(16), 8830-8835.

Maurer, M. H., Schabitz, W.R. & Schneider, A. (2008). Old friends in new constellations- the hematopoetic growth factors G-CSF, GM-CSF and EPO for the treatment of neurological diseases. *Curr Med Chem, 15*(14), 1407-1411.

McLay, R. N., Kimura, M., Banks, W. A., & Kastin, A. J. (1997). Granulocyte-macrophage colony-stimulating factor crosses the blood--brain and blood--spinal cord barriers. *Brain, 120 (Pt 11)*, 2083-2091.

Metz, G. A., Antonow-Schlorke, I., & Witte, O. W. (2005). Motor improvements after focal cortical ischemia in adult rats are mediated by compensatory mechanisms. *Behav Brain Res, 162*(1), 71-82.

Micu, I., Jiang, Q., Coderre, E., Ridsdale, A., Zhang, L., Woulfe, J., . . . Stys, P. K. (2006). NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. *Nature, 439*(7079), 988-992. doi: 10.1038/nature04474

Miklyaeva, E. I., Castaneda, E., & Whishaw, I. Q. (1994). Skilled reaching deficits in unilateral dopamine-depleted rats: impairments in movement and posture and compensatory adjustments. *J Neurosci, 14*(11 Pt 2), 7148-7158.

Miklyaeva, E. I., & Whishaw, I. Q. (1996). HemiParkinson analogue rats display active support in good limbs versus passive support in bad limbs on a skilled reaching task of variable height. *Behav Neurosci, 110*(1), 117-125.

Miller, B., Sarantis, M., Traynelis, S. F., & Attwell, D. (1992). Potentiation of NMDA receptor currents by arachidonic acid. *Nature, 355*(6362), 722-725.

Mirza, S., Walker, A., Chen, J., Murphy, J. M., & Young, I. G. (2010). The Ig-like domain of human GM-CSF receptor alpha plays a critical role in cytokine binding and receptor activation. *Biochem J, 426*(3), 307-317.

Montaner, J., Molina, C. A., Monasterio, J., Abilleira, S., Arenillas, J. F., Ribo, M., . . . Alvarez-Sabin, J. (2003). Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation, 107*(4), 598-603.

Moreland, S., McMullen, D. M., Delaney, C. L., Lee, V. G., & Hunt, J. T. (1992). Venous smooth muscle contains vasoconstrictor ETB-like receptors. *Biochem Biophys Res Commun, 184*(1), 100-106.

Moro, M. A., Cardenas, A., Hurtado, O., Leza, J. C., & Lizasoain, I. (2004). Role of nitric oxide after brain ischaemia. *Cell Calcium, 36*(3-4), 265-275.

Munoz, D.G., Hastak, S.M., Harper B. (1993). Pathologic correlates of increased signals of the centrum ovale on magnetic resonance imaging. *Arch Neurol, 50*:492–497.

Murphy, T. H., & Corbett, D. (2009). Plasticity during stroke recovery: from synapse to behaviour. *Nat Rev Neurosci, 10*(12), 861-872.

Murphy, T. H., Miyamoto, M., Sastre, A., Schnaar, R. L., & Coyle, J. T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron, 2*(6), 1547-1558.

Nakagawa, T., Suga, S., Kawase, T., & Toda, M. (2006). Intracarotid injection of granulocyte-macrophage colony-stimulating factor induces neuroprotection in a rat transient middle cerebral artery occlusion model. *Brain Res, 1089*(1), 179-185.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science, 258*(5082), 597-603.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., . . . Moskowitz, M. A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci, 18*(10), 3659-3668.

Nicotera, P., Leist, M., & Manzo, L. (1999). Neuronal cell death: a demise with different shapes. *Trends Pharmacol Sci, 20*(2), 46-51.

Niizuma, K., Yoshioka, H., Chen, H., Kim, G. S., Jung, J. E., Katsu, M., . . . Chan, P. H. (2010). Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. *Biochim Biophys Acta, 1802*(1), 92-99.

Niwa, M., Hara, A., Iwai, T., Wang, S., Hotta, K., Mori, H., & Uematsu, T. (2001). Caspase activation as an apoptotic evidence in the gerbil hippocampal CA1 pyramidal cells following transient forebrain ischemia. *Neurosci Lett, 300*(2), 103-106.

Nudo, R. J., Wise, B. M., SiFuentes, F., & Milliken, G. W. (1996). Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. *Science, 272*(5269), 1791-1794.

Olney, J. W., Ho, O. L., & Rhee, V. (1971). Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. *Exp Brain Res, 14*(1), 61-76.

Pascual-Leone, A., Freitas, C., Oberman, L., Horvath, J. C., Halko, M., Eldaief, M., . . . Rotenberg, A. (2011). Characterizing brain cortical plasticity and network dynamics across the age-span in health and disease with TMS-EEG and TMS-fMRI. *Brain Topogr, 24*(3-4), 302-315.

Patneau, D. K., Wright, P. W., Winters, C., Mayer, M. L., & Gallo, V. (1994). Glial cells of the oligodendrocyte lineage express both kainate- and AMPA-preferring subtypes of glutamate receptor. *Neuron, 12*(2), 357-371.

Pellegrini-Giampietro, D. E., Zukin, R. S., Bennett, M. V., Cho, S., & Pulsinelli, W. A. (1992). Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc Natl Acad Sci U S A, 89*(21), 10499-10503.

Pisa, M. (1988). Motor functions of the striatum in the rat: critical role of the lateral region in tongue and forelimb reaching. *Neuroscience, 24*(2), 453-463.

Re, F., Belyanskaya, S. L., Riese, R. J., Cipriani, B., Fischer, F. R., Granucci, F., . . . Santambrogio, L. (2002). Granulocyte-macrophage colony-stimulating factor induces an expression program in neonatal microglia that primes them for antigen presentation. *J Immunol, 169*(5), 2264-2273.

Sainathan, S.K., Tu, L., Bishnupuri, K.S. (2005). PEGylated murine granulocyte-macrophage colony stimulating factor: production, purification, and characterization. *Protein Expr Purif, 44*, 94-103.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., & Masaki, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature, 348*(6303), 732-735.

Salter, M. G., & Fern, R. (2005). NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature, 438*(7071), 1167-1171.

Sanchez-Gomez, M. V., & Matute, C. (1999). AMPA and kainate receptors each mediate excitotoxicity in oligodendroglial cultures. *Neurobiol Dis, 6*(6), 475-485.

Saver, J. L. (2006). Time is brain--quantified. *Stroke, 37*(1), 263-266.

Sawada, M., Itoh, Y., Suzumura, A., & Marunouchi, T. (1993). Expression of cytokine receptors in cultured neuronal and glial cells. *Neurosci Lett, 160*(2), 131-134.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., . . . Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO J, 17*(6), 1675-1687.

Schaar, K. L., Brenneman, M. M., & Savitz, S. I. (2010). Functional assessments in the rodent stroke model. *Exp Transl Stroke Med, 2*(1), 13.

Schabitz, W. R., Kruger, C., Pitzer, C., Weber, D., Laage, R., Gassler, N., . . . Schneider, A. (2008). A neuroprotective function for the hematopoietic protein granulocyte-macrophage colony stimulating factor (GM-CSF). *J Cereb Blood Flow Metab, 28*(1), 29-43.

Schallert, T. (2006). Behavioral tests for preclinical intervention assessment. *NeuroRx, 3*(4), 497-504.

Schallert T, Woodlee MT: Orienting and placing. In the Behavior of the Laboratory Rat: A Handbook with Tests Oxford: Oxford University Press Whishaw I, Kolb B 2005, 129-140.

Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L., & Bland, S. T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology, 39*(5), 777-787.

Schallert, T. & Tillerson, J. L. in Central Nervous System Diseases: Innovative Animal Models from Lab to Clinic (eds Emerich, D. F., Dean, R. L. & Sanberg, P. R.) 131–151 (Humana, Totowa, New Jersey, 2000).

Schermer, C., & Humpel, C. (2002). Granulocyte macrophage-colony stimulating factor activates microglia in rat cortex organotypic brain slices. *Neurosci Lett, 328*(2), 180-184.

Schneeloch, E., Mies, G., Busch, H. J., Buschmann, I. R., & Hossmann, K. A. (2004). Granulocyte-macrophage colony-stimulating factor-induced arteriogenesis reduces energy failure in hemodynamic stroke. *Proc Natl Acad Sci U S A, 101*(34), 12730-12735.

Sharkey, J., & Butcher, S. P. (1995). Characterisation of an experimental model of stroke produced by intracerebral microinjection of endothelin-1 adjacent to the rat middle cerebral artery. *J Neurosci Methods, 60*(1-2), 125-131.

Sharkey, J., Ritchie, I. M., & Kelly, P. A. (1993). Perivascular microapplication of endothelin-1: a new model of focal cerebral ischaemia in the rat. *J Cereb Blood Flow Metab, 13*(5), 865-871.

Shibata, M., Ohtani, R., Ihara, M., & Tomimoto, H. (2004). White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. *Stroke, 35*(11), 2598-2603.

Shiraishi, K., Sharp, F. R., & Simon, R. P. (1989). Sequential metabolic changes in rat brain following middle cerebral artery occlusion: a 2-deoxyglucose study. *J Cereb Blood Flow Metab, 9*(6), 765-773.

Shyu, W. C., Lin, S. Z., Chiang, M. F., Chen, D. C., Su, C. Y., Wang, H. J., . . . Li, H. (2008). Secretoneurin promotes neuroprotection and neuronal plasticity via the Jak2/Stat3 pathway in murine models of stroke. *J Clin Invest, 118*(1), 133-148.

Shyu, W. C., Lin, S. Z., Lee, C. C., Liu, D. D., & Li, H. (2006). Granulocyte colony-stimulating factor for acute ischemic stroke: a randomized controlled trial. *CMAJ, 174*(7), 927-933.

Smith, S. E., Lekieffre, D., Sowinski, P., & Meldrum, B. S. (1993). Cerebroprotective effect of BW619C89 after focal or global cerebral ischaemia in the rat. *Neuroreport, 4*(12), 1339-1342.

Snape, M. F., Baldwin, H. A., Cross, A. J., & Green, A. R. (1993). The effects of chlormethiazole and nimodipine on cortical infarct area after focal cerebral ischaemia in the rat. *Neuroscience, 53*(3), 837-844.

Soylu, H., Zhang, D., Buist, R., Martin, M., Albensi, B. C., & Parkinson, F. E. (2012). Intracortical injection of endothelin-1 induces cortical infarcts in mice: effect of neuronal expression of an adenosine transporter. *Exp Transl Stroke Med, 4*(1), 4.

Sozmen, E. G., Kolekar, A., Havton, L. A., & Carmichael, S. T. (2009). A white matter stroke model in the mouse: axonal damage, progenitor responses and MRI correlates. *J Neurosci Methods, 180*(2), 261-272.

Stark, K., Seubert, P., Lynch, G., & Baudry, M. (1989). Proteolytic conversion of xanthine dehydrogenase to xanthine oxidase: evidence against a role for calcium-activated protease (calpain). *Biochem Biophys Res Commun, 165*(2), 858-864.

Stinear, C. M., Barber, P. A., Smale, P. R., Coxon, J. P., Fleming, M. K., & Byblow, W. D. (2007). Functional potential in chronic stroke patients depends on corticospinal tract integrity. *Brain, 130*(Pt 1), 170-180.

Strasser, A., O'Connor, L., & Dixit, V. M. (2000). Apoptosis signaling. *Annu Rev Biochem, 69*, 217-245.

Stroemer, R. P., Kent, T. A., & Hulsebosch, C. E. (1995). Neocortical neural sprouting, synaptogenesis, and behavioral recovery after neocortical infarction in rats. *Stroke, 26*(11), 2135-2144.

Stroemer, R. P., Kent, T. A., & Hulsebosch, C. E. (1998). Enhanced neocortical neural sprouting, synaptogenesis, and behavioral recovery with D-amphetamine therapy after neocortical infarction in rats. *Stroke, 29*(11), 2381-2393; discussion 2393-2385.

Stys, P. K., Ransom, B. R., Waxman, S. G., & Davis, P. K. (1990). Role of extracellular calcium in anoxic injury of mammalian central white matter. *Proc Natl Acad Sci U S A, 87*(11), 4212-4216.

Suzumura, A., Sawada, M., & Marunouchi, T. (1996). Selective induction of interleukin-6 in mouse microglia by granulocyte-macrophage colony-stimulating factor. *Brain Res, 713*(1-2), 192-198.

Takahashi, K., Ghatei, M. A., Jones, P. M., Murphy, J. K., Lam, H. C., O'Halloran, D. J., & Bloom, S. R. (1991). Endothelin in human brain and pituitary gland: presence of immunoreactive endothelin, endothelin messenger ribonucleic acid, and endothelin receptors. *J Clin Endocrinol Metab, 72*(3), 693-699.

Tekkok, S. B., & Goldberg, M. P. (2001). Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. *J Neurosci, 21*(12), 4237-4248.

Tekkok, S. B., Ye, Z., & Ransom, B. R. (2007). Excitotoxic mechanisms of ischemic injury in myelinated white matter. *J Cereb Blood Flow Metab, 27*(9), 1540-1552.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science, 267*(5203), 1456-1462.

Unal-Cevik, I., Kilinc, M., Can, A., Gursoy-Ozdemir, Y., & Dalkara, T. (2004). Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia. *Stroke, 35*(9), 2189-2194.

van Swieten, J.C., van den Hout, J.H., van Ketel, B.A. (1991). Periventricular lesions in the white matter on magnetic resonance imaging in the elderly. A morphometric correlation with arteriolosclerosis and dilated perivascular spaces. *Brain, 114*, 761.

Velier, J. J., Ellison, J. A., Kikly, K. K., Spera, P. A., Barone, F. C., & Feuerstein, G. Z. (1999). Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after focal stroke in the rat. *J Neurosci, 19*(14), 5932-5941.

Vermeer, S.E., Hollander, van Dijk, M.E.J., Hofman, A., Koudstaal, P.J., Breteler, M.B. (2003). Silent Brain Infarcts and White Matter Lesions Increase Stroke Risk I n the General Population. *Stroke, 34*, 1126-1129.

Wahlgren, N. G., Ranasinha, K. W., Rosolacci, T., Franke, C. L., van Erven, P. M., Ashwood, T., & Claesson, L. (1999). Clomethiazole acute stroke study (CLASS): results of a randomized, controlled trial of clomethiazole versus placebo in 1360 acute stroke patients. *Stroke, 30*(1), 21-28.

Wang, X., Yue, T. L., Barone, F. C., White, R. F., Gagnon, R. C., & Feuerstein, G. Z. (1994). Concomitant cortical expression of TNF-alpha and IL-1 beta mRNAs follows early response gene expression in transient focal ischemia. *Mol Chem Neuropathol, 23*(2-3), 103-114.

Wang, Y., Jin, K., & Greenberg, D. A. (2007). Neurogenesis associated with endothelin-induced cortical infarction in the mouse. *Brain Res, 1167*, 118-122.

Ward, N. S., Brown, M. M., Thompson, A. J., & Frackowiak, R. S. (2003). Neural correlates of motor recovery after stroke: a longitudinal fMRI study. [Research Support, Non-U.S. Gov't]. *Brain, 126*(Pt 11), 2476-2496.

Webb, D. J., Monge, J. C., Rabelink, T. J., & Yanagisawa, M. (1998). Endothelin: new discoveries and rapid progress in the clinic. *Trends Pharmacol Sci, 19*(1), 5-8.

Whishaw, I. Q. (2000). Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. *Neuropharmacology, 39*(5), 788-805.

Whishaw, I. Q., Castaneda, E., & Gorny, B. P. (1992). Dopamine and skilled limb use in the rat: more severe bilateral impairments follow substantia nigra than sensorimotor cortex 6-hydroxydopamine injection. *Behav Brain Res, 47*(1), 89-92.

Whishaw, I. Q., Pellis, S. M., & Gorny, B. P. (1992). Skilled reaching in rats and humans: evidence for parallel development or homology. *Behav Brain Res, 47*(1), 59-70.

Whishaw, I. Q., Pellis, S. M., Gorny, B. P., & Pellis, V. C. (1991). The impairments in reaching and the movements of compensation in rats with motor cortex lesions: an endpoint, videorecording, and movement notation analysis. *Behav Brain Res, 42*(1), 77-91.

Whishaw, I. Q., Piecharka, D. M., Zeeb, F., & Stein, D. G. (2004). Unilateral frontal lobe contusion and forelimb function: chronic quantitative and qualitative impairments in reflexive and skilled forelimb movements in rats. *J Neurotrauma, 21*(11), 1584-1600.

Willette, R. N., Sauermelch, C., Ezekiel, M., Feuerstein, G., & Ohlstein, E. H. (1990). Effect of endothelin on cortical microvascular perfusion in rats. *Stroke, 21*(3), 451-458.

Windle, V., Szymanska, A., Granter-Button, S., White, C., Buist, R., Peeling, J., & Corbett, D. (2006). An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. *Exp Neurol, 201*(2), 324-334.

Winship, I. R., & Murphy, T. H. (2008). In vivo calcium imaging reveals functional rewiring of single somatosensory neurons after stroke. [Research Support, Non-U.S. Gov't]. *J Neurosci, 28*(26), 6592-6606.

Woodruff, T. M., Thundyil, J., Tang, S. C., Sobey, C. G., Taylor, S. M., & Arumugam, T. V. (2011). Pathophysiology, treatment, and animal and cellular models of human ischemic stroke. *Mol Neurodegener, 6*(1), 11.

Wyllie, A. H., Beattie, G. J., & Hargreaves, A. D. (1981). Chromatin changes in apoptosis. *Histochem J, 13*(4), 681-692.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., . . . Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature, 332*(6163), 411-415.

Ying, W., Han, S. K., Miller, J. W., & Swanson, R. A. (1999). Acidosis potentiates oxidative neuronal death by multiple mechanisms. *J Neurochem, 73*(4), 1549-1556.

Yoshimoto, S., Ishizaki, Y., Kurihara, H., Sasaki, T., Yoshizumi, M., Yanagisawa, M., . . . Murota, S. (1990). Cerebral microvessel endothelium is producing endothelin. *Brain Res, 508*(2), 283-285.

Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S. J., Kimura, S., . . . Kanazawa, I. (1990). Endothelin: a novel peptide in the posterior pituitary system. *Science, 247*(4941), 462-464.

Zhang, S., & Murphy, T. H. (2007). Imaging the impact of cortical microcirculation on synaptic structure and sensory-evoked hemodynamic responses in vivo. [Research Support, Non-U.S. Gov't]. *PLoS Biol, 5*(5), e119.