Endogenous Cell-Based Therapy Using Cyclosporine A Promotes Cognitive Recovery in a Mouse Model of Stroke

By

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ABSTRACT

Post-stroke executive function (EF) deficits can severely impact the quality of life. Current treatments provide limited motor or cognitive recovery. Recently, an endogenous repair strategy using Cyclosporine A (CsA) promoted neural stem and progenitor cells [collectively termed neural precursor cells (NPCs)] migration to the infarct site, tissue regeneration and functional recovery following sensorimotor stroke. Herein, we tested the efficacy of CsA in improving EF deficits in a cognitive stroke model in mice. The medial prefrontal cortical (mPFC) stroke produced long-term EF impairments in the Puzzle Box Task. CsA treatment increased the size of the NPC pool at day 7 and promoted NPC migration to the infarct site at day 60 post-stroke. Early CsA administration was not neuroprotective and did not affect neural degeneration or lesion volume. Long-term CsA treatment improved post-stroke cognitive performance at 3 and 5 weeks. We suggest CsA is a promising therapeutic candidate for post-stroke cognitive recovery.
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There is no failure except in no longer trying. There is no defeat except from within, no really insurmountable barrier save our own inherent weakness of purpose.

Kin Hubbard
CONTRIBUTIONS

Vaakiny Raguthevan imaged and counted fluorojade C positive cells to assess the role of CsA on neural degeneration (Figure 3.2C and 3.2D) and imaged and analyzed the lesion volume of cresyl violet stained sections (Figure 3.2 E and 3.2F) on Day 1, 4 and 10 post-stroke; Ilan Vonderwalde assisted in conducting the adhesive tape removal test and subsequent data analysis (Figure 3.1E and 3.3B); Kelsey Adams assisted in performing the short- and long-term neurosphere assays post-stroke (Figure 3.2B and 3.5B).
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LIST OF ABBREVIATIONS

α  alpha
β  beta
ACA anterior cerebral artery
ACg anterior cingulate
AdpNSCs adult derived primitive neural stem cells
AID dorsal anterior insular
AIV ventral anterior insular
BDNF brain derived neurotrophic factor
BLBP brain lipid binding protein
BMC bone marrow cell
BrdU 5-bromo-2’-deoxyuridine
CsA cyclosporine A
CyP cyclophilin
DCX doublecortin
DG dentate gyrus
EF executive function
EFH epidermal growth factor, fibroblast growth factor, and heparin
EGF epidermal growth factor
EPO erythropoietin
ET-1 endothelin-1
ER estrogen receptor
FBS foetal bovine serum
FGF fibroblast growth factor
GFAP glial fibrillary acidic protein
GLAST glutamate aspartate transporter
IL infralimbic
IGF insulin growth factor
LIF leukemia inhibitory factor
LO lateral orbital
LPFC lateral prefrontal cortex
LV lateral ventricles
MCAO middle cerebral artery occlusion
Met metformin
MO medial orbital
mPFC medial prefrontal cortex
MPTP mitochondrial permeability transition pore
NPCs neural precursor cells
NSCs neural stem cells
OB olfactory bulb
PB puzzle box
PFC prefrontal cortex
PrC precentral
PrL prelimbic
PSA-NCAM polysialylated-neural cell adhesion molecule
PVD pial vessel disruption
SAP spontaneous alternation percentage
SE subependyma
SFM serum-free media
SGZ subgranular zone
STAIR stroke therapy academic industry roundtable
STEPS stem cell therapies as an emerging paradigm in stroke
TAM tamoxifen
TGF transforming growth factor
tPA tissue plasminogen activator
VEGF vascular endothelial growth factor
VLO ventrolateral orbital
VO ventral orbital
VPFC ventral prefrontal cortex
YFP yellow fluorescent protein
CHAPTER 1: INTRODUCTION

1.1 STROKE

1.1.1 ETIOLOGY

Stroke is the second leading cause of death and chronic disability, resulting in nearly 5 million deaths and an additional 6 million disabilities worldwide. In Canada, an estimated 62,000 new cases of stroke occur every year, and two thirds of these cases occur in population over 65 years of age. Approximately, 80% of all stroke cases are ischemic with the remainder being hemorrhagic. Nearly, 400,000 people live with long-term neurological sequelae following stroke. Not only do post-stroke neurological deficits, both motor and cognitive, adversely affect functional independence and the quality of life of patients, but supporting a stroke-affected population with disabilities is a tremendous economic burden to society. Each year, Canada spends an average of $3.6 billion on direct and indirect healthcare expenses pertaining to a stroke affected population. These estimates do not consider long-term expenses from personal support worker, nursing homes and indirect expenses resulting from unemployment, which are additional contributors. These facts highlight the need for development of new and effective therapeutic strategies that will contribute towards sustainable long-term recovery, following stroke.

1.1.2 PATHOPHYSIOLOGY

Stroke results from an interruption of blood flow to, or within, a brain region. Reduced brain perfusion to the affected region results in inadequate delivery of oxygen, sugar, and essential nutrients that are quintessential for normal brain function. Stroke can be
categorized into two major types: ischemic and hemorrhagic. Ischemia is the most common form of stroke and is caused by transient disruption of blood flow due to occlusion of a blood vessel from embolism (extracranial) or thrombosis (intracranial).\textsuperscript{3,11} Hemorrhagic stroke, either within the brain (intracerebral) or on the surface (subarachnoid), results from the rupture of blood vessels.\textsuperscript{3,11}

At the acute phase of an ischemic stroke, a series of complex pathophysiological processes, including energy failure, occurs within minutes (Figure 1.1). These ischemic cascades create a gradient within the affected region, generating two zones of injury: the core and the penumbra. The ischemic core experiences a disruption of ionic gradients, resulting in excitotoxicity. Cellular membrane depolarization causes glutamate release, resulting in an influx of calcium (\(\text{Ca}^{2+}\)) and build up of free radicals.\textsuperscript{2,3,11} Build up of nitric oxide, peroxynitrite and oxygen free radicals further exacerbates the ischemic cascade. Simultaneously, intracellular \(\text{Ca}^{2+}\) induces endoplasmic reticulum stress, which affects protein synthesis and folding, disrupting proper cellular function. The rapid homeostatic and metabolic failure disrupts mitochondrial integrity, causes cell membrane disintegration and activation of pathways that create oxidative stress by increasing the production of reactive oxygen species.\textsuperscript{12} Simultaneously, the immune system rapidly reacts to assume a pro-inflammatory role, resulting in an infiltration of peripheral immune cells, such as leukocytes, neutrophils, monocytes, dendritic and T-cells.\textsuperscript{12–15} Resident microglia are activated in response to an amplified cytokinetic profile resulting in a robust inflammatory response, leading to necrosis, apoptosis, and phagocytosis of cells.\textsuperscript{11,12,16,17}
The penumbral region surrounding the ischemic core comprises tissue that remains hypoperfused and functionally compromised; viable and distinguishable from the core with neuroimaging tools.\textsuperscript{18} Cell death in this region is not as rapid as observed in the core and occurs over hours to days after the insult. This time window is thought to provide a therapeutic opportunity for the rescue of the penumbral tissue, potentially reversing damage and salvaging neurological function.\textsuperscript{11,19,20} Evidently, neurological sequelae that ensue following ischemia depend on a number of factors: the size of the ischemic zone (proportion of penumbra with respect to the core), the region of the brain that is affected, and how fast treatment is implemented to restore blood flow.

1.1.3 CURRENT THERAPEUTIC STRATEGIES IN ACUTE ISCHEMIC STROKE

RESTORING BLOOD FLOW

Despite being the leading cause of neurological disability and a major cause of mortality, there are limited treatments available for stroke. Tissue plasminogen activator (tPA) is recognized as one of the most common, clinically approved, targeted treatments for acute ischemic stroke.\textsuperscript{21} Shortly following the onset of an ischemic attack, tPA is intravenously administered to promote recanalization. This removes clots that occlude blood vessels, thereby restoring normal blood circulation. Seminal work by the National Institute of Neurological Disorder and Stroke demonstrated that tPA administration is 30 percent more likely to improve patient outcome on a range of assessment scales (i.e. Barthel, Rankin, Glasgow, and NIHSS) at three months compared to placebo.\textsuperscript{22} These findings fuelled further research, which were supportive of the safety and efficacy of tPA as an acute therapeutic approach for stroke patients. While it is typically suggested that tPA is beneficial if administered within 4.5 hours from the onset of stroke,\textsuperscript{21,23–25}
experimentation with treatment windows varying between 4.5 and 6 hours resulted in no differences in patient outcomes during short-term followups.25

A recurring limitation in these studies involves short-term (less than one year post-stroke) assessments of tPA, while the long-term aftermaths of tPA are not addressed. Although an abundance of studies have supported safety and effectiveness of tPA, this drug remains heavily underutilized due to its strict patient criteria.27 Unfortunately, <10% of stroke patients are eligible to receive tPA due a number of reasons, the most obvious being that the majority of patients fail to recognize the signs of stroke and arrive to the hospital after the window of tPA treatment.27 In addition, appropriate pre-screening of patients using neuroimaging is required to determine if tPA is a suitable treatment and these tools are not available in all clinical settings.11 A number of variables affect the efficacy of thrombolysis, such as the type of thrombus, a patient’s age, co-morbidities and the location and extent of damage.28 Arteries in proximity or distal to the common carotid arteries may not be responsive to recanalization, resulting in a significant drop in blood flow rates.29,30 In fact, 60 to 80% of patients fail to survive the first 90 days post-stroke or fail to regain functional independence following occlusion of proximal anterior circulation.31,32 Clot composition, for example, fibrin-rich versus atherosclerotic thrombus, also affects the effectiveness of recanalization.33 Moreover, tPA has associated risks in causing intracerebral hemorrhage and mortality, which outweigh the benefits of tPA in reducing infarction.2,7,11 In 2008, despite a significant improvement on reperfusion rate (i.e. 56% versus 26%; tPA versus placebo) the randomized controlled Echoplanar Imaging Thrombolytic Evaluation Trial demonstrated a non-significant association of intravenous
tPA on clinical outcome at 90 days following stroke compared to placebo. Therefore, patient samples need to be selectively chosen for thrombolysis treatments. Understanding the limitations resulted in the consideration of other acute treatment interventions, such as endovascular thrombectomy, which has now become a routine therapy. In 2015, the ESCAPE trial reported that endovascular treatments improve workflow and outcome effectiveness in acute stroke. The study showed that endovascular treatment improved clinical outcome and reduced mortality rate of patients with occluded proximal and anterior circulation at 90 days. Alternative, but not widely used, interventions include anti-platelets and anti-coagulants, vasodilators, surgical and endovascular interventions. Although manual endovascular clot retrieval interventions have been successful, efficacy of noninvasive strategies of clot removal using focused ultrasound is currently being assessed.

**NEUROPROTECTION**

Following stroke, the penumbral region shows the greatest potential for plasticity. As discussed above, damage to this area is not as rapid as in the ischemic core; hence, this area is a target for neuroprotective agents, which can potentially rescue viable tissue and limit further impairment. Many neurotrophic, anti-inflammatory, antihypertensive, anti-apoptotic, anti-oxidative, channel-blocking, and membrane-stabilizing agents have been examined for their neuroprotective benefits in stroke in pre-clinical settings. However, none have been clinically effective thus far. Some have provided insight into the development of novel approaches. For example, the neuroprotective agent erythropoietin (EPO) has shown to improve outcome in a number of different brain injury models; however, its long-term benefits for patients are
yet to be determined. Mild brain hypothermia has been successful as a therapeutic strategy after cardiac arrest and is now being tested for its efficacy as an intervention for patients affected with cerebral ischemia. Post-synaptic density (PSD)-95, which inhibits excitatory signaling cascades, has also shown promise following acute ischemic stroke. Most recent and ongoing clinical trials are evaluating effectiveness of existing and novel strategies important for both diagnostics and therapeutics. Some examples of interventions currently being tested in clinical trials include transcranial direct current stimulation, fluid therapy, acupuncture and endovascular therapies. However, neuroprotective benefits in clinical population following stroke are yet to be determined.

1.1.4 Current Strategies To Promote Long-term Recovery

To date, strategies that were devised to promote recanalization or neuroprotection have targeted mechanisms at the acute phase of stroke to limit ischemic cascades and further brain damage, but were not aimed to prioritize long-term recovery. Most patients suffer from some form of post-stroke disability (i.e. deficits in cognition, language, or motor function), the severity of which is largely dependent on the nature of stroke and the extent of brain damage. However, spontaneous recovery is a significant contributor of final patient outcome and is heavily dependent on the capacity of unaffected penumbral tissue around the ischemic core. A number of recent studies have proposed a model of “fixed proportional recovery” that suggests that a patient’s extent of spontaneous recovery can be predicted within the first 72 hours. Spontaneous recovery is proportionally fixed and is not restricted to motor function. Generally, patients reach 70% to 80% of their maximum potential within the first 3 to 6 months. Remaining
deficits persist long-term and only a small percentage of patients recover completely to match their activity level to that of community dwellers who are not affected by stroke.\textsuperscript{51,55} Therefore, it is necessary to target treatments to enhance recovery of remaining deficits that persist long-term to ensure that patients can regain functional independence.

Rehabilitation does not cure or rescue ischemic-dependent brain damage. However, it is currently the most common form of treatment that allows patients to relearn skills and compensatory strategies to reintegrate back into society, perform activities of daily living, and improve their quality of life.\textsuperscript{51,56} Rehabilitation interventions can be broadly categorized as inpatient or outpatient interventions, depending on the patient's physical strength, functional independence, and the regularity of supervision or assistance required in carrying out day-to-day activities. Rehabilitation is generally targeted towards improving specific aspects, such as speech and language\textsuperscript{57}, functional usage of arm\textsuperscript{58,59}, self management\textsuperscript{56}, strength and balance\textsuperscript{60}, and walking.\textsuperscript{61} These strategies are usually integrated through language, physical, cognitive or occupational therapies or robotic devices.\textsuperscript{56} Although there have been advancements in devising effective, task-specific, and function-based rehabilitation strategies that renew promise for improved outcomes to enhance independence, there are a number of limitations, including the fact that improvements plateau a few months after rehabilitation regimes are stopped.\textsuperscript{56} Further, heterogeneous ischemic lesions in patient population make it difficult to devise universal strategies that would be effective for all. Also, there is a lack of supporting evidence that consolidates the efficacy of various rehabilitative strategies.\textsuperscript{51,62} This has led to the development of adjunctive
therapies that can be implemented in combination with rehabilitation to aid in maximizing gains.\textsuperscript{12,56} The therapeutic strategies of these adjuncts involve targeting underlying various factors (e.g. growth factors) that contribute to diaschisis. Diaschisis usually takes places in the first weeks or even months following stroke. This results in cortical map restructuring through neurite growth, neurogenesis, synaptogenesis, and axonal sprouting.\textsuperscript{52} The post-stroke structural remodeling during this process drives functional reorganization, which includes dysregulation of neurotransmission, disruption of neurocircuitry due to the loss of cells, and reorganization of neurocircuitry to compensate for damage.\textsuperscript{12,56,63,64}

\textbf{PHARMACOLOGICAL ADJUNCTS}

There is incentive to combining neuropharmacological agents at the acute phase of stroke along with intensive rehabilitation to enhance recovery.\textsuperscript{65,66} Mechanisms that disrupt neurotransmitter (e.g. dopamine, acetylcholine, serotonin, and norepinephrine) regulation following an ischemia have been previously targeted. However, the most desirable option involves achieving a balance in excitatory-inhibitory cortical signaling that contributes to diaschisis. As learning and plasticity are dependent on neurotransmitter regulation, it is thought that stimulating pathways that optimize long-term potentiation, task-specific or activity-dependent signaling can support motor learning during an ongoing rehabilitation post-stroke, and promote recovery.\textsuperscript{56} Brain damage after stroke is primarily dependent on glutamate-dependent excitotoxicity, hence regulation (agonistic and antagonistic) of NMDA receptor activation is considered a promising target for modulation.\textsuperscript{67} Many NMDA antagonists such as Selfotel, Aptiganel and Gavestinel, have not shown success in clinical trials.\textsuperscript{68} However,
the drug Memantine, which is used to treat Alzheimer’s patients, has shown promising results in promoting post-stroke recovery in aphasia as a standalone or combinatorial treatment with constraint-induced aphasia therapy.\textsuperscript{69} Levodopa, a dopamine precursor, has also been shown to improve motor learning in chronic stroke patients.\textsuperscript{70} Similarly, early administration (i.e. 5 to 10 days post-stroke) of fluoxetine, a selective serotonin reuptake inhibitor (SSRI), in combination with physiotherapy has also demonstrated enhanced functional recovery compared to placebo at 3 months post-stroke.\textsuperscript{71,72} It is thought that the benefits of SSRIs may be facilitated via excitation of corticospinal circuitry.\textsuperscript{73} A number of other drugs which have been used to modify neurotransmitter availability such as amphetamines, have been tested in combination with task-specific training, but many have been underpowered.\textsuperscript{66,74} However, a randomized, double blinded, placebo-controlled study in 2006 using 71 stroke patients, showed that implementation of a combinatorial therapeutic strategy, such as physiotherapy coupled with Dextroamphetamine, showed no additional benefits on motor or functional recovery compared to physiotherapy alone.\textsuperscript{75} Further studies using pharmacological adjuncts require appropriate evaluation of population samples along with mode and duration of treatment plan to validate findings.

**Non-Invasive Neural Stimulation**

Cortical stimulation using repetitive transcranial direct current stimulation or transcranial magnetic stimulation has received significant attention as a therapeutic approach to augment post-stroke recovery and restore function. Recent advancements in focal stimulation techniques, such as optogenetic stimulation, magnetic resonance-guided focused ultrasound, and stereotactic radiotherapy, may also offer benefits in
stroke recovery. Optogenetics offers a form of therapy that precisely targets small regions of affected brain and relevant circuitry to promote functional recovery as opposed to providing gross stimulation that feeds into other areas. Magnetic resonance guided focal ultrasound, coupled with advanced neuroimaging techniques, can permit acoustic energy transmission through the intact skull and is considered a potential neurotherapeutic candidate. However, stimulation techniques to date reveal no significant improvement in the stroke patient population with respect to functional recovery.

Peripheral motor and sensory nerve fibers can also be stimulated as a means to promote functional recovery. Peripheral stimulation has been shown to alter neural connectivity in the human motor cortex that regulates hand muscles. Functional electrical stimulation is usually offered through electromyography whereby voluntary movement evokes activity-based electrical stimulation in the muscles. This has been shown to promote reorganization of cortical structure and enhance re-learning of skills post-stroke. A number of important aspects of the stimulation paradigm, such as frequency (allows to control temporal summation of current), intensity (i.e. pulse amplitude and width which allow regulation of spatial summation of current) and duration of treatment need to be considered when devising treatment interventions to enhance post-stroke recovery. Indeed, stimulation can be used to both enhance and inhibit signaling, hence implementation strategies have to be carefully evaluated, based on desired patient outcomes, to derive robust and positive results in patient populations.
ROBOTIC AND VIRTUAL REALITY TRAINING DEVICES

Given the isolated and static nature of stroke-related injury, spared or unaffected circuits can be recruited and harnessed to restore function. Technological advancements in brain-computer interface using functional electrical stimulation, orthosis, or robotics have demonstrated effectiveness in post-stroke rehabilitation. Improvements in pattern recognition based on activity-based surface electromyography has also allowed for better control of upper limb prosthesis. Apart from robotic devices, virtual reality is an emerging and interesting approach offering therapeutic interventions for stroke patients. A virtual-reality system is essentially dependent on the recruitment of mirror neurons to assist patients in acquiring motor skills. Virtual reality training involves a virtual environment (usually portrayed through a computerized screen) where a patient is able to engage in simulated activities (e.g. games) that require various motor skills (i.e. grasp and reach). Performing such activities repetitively help to improve directionality and speed of arm movement. Brain-computer interface offers promise as a potential therapeutic strategy, however, understanding of cortical inputs and how neural network patterns change post-stroke is essential to tailor this technology to functionally benefit post-stroke patients.

CELL-BASED THERAPIES

Cell-based therapies have been explored as a therapeutic strategy to enhance neuroplasticity and recovery following stroke. Cell-based therapies can be categorized into two main types: 1) *exogenous* therapy, which involves transplantation of cells to replace those lost after injury and/or provide trophic support, and 2) *endogenous* therapy, which involves exploitation of resident precursor populations by inducing proliferation,
migration, and differentiation of neural precursor cells. To date, clinical trials have mostly focused on exogenous transplantation to treat stroke, employing a variety of cell types that can provide trophic support rather than replacing cells. Some examples include human embryonic stem cells, neural stem cells, immortalized cells, and bone marrow derived cells (BMCs). However, with the goal of cell replacement, it has been important to consider cells with neurogenic potential. While stroke is known to result in the loss of all neurogenic cell types, as well as vascular cells, the cell type that will lead to recovery is not well established.

Bone marrow derived cells (BMCs) have been most widely tested in clinical settings and have proven to be a safe and feasible treatment option for stroke patients in promoting functional recovery in both acute and chronic phases. Emerging evidence also suggests the increasing transplantation of neural stem and progenitor cells, however, the mechanism of action of transplanted neural stem and progenitor cells is unknown. This is further complicated by the fact that stroke lesions are not similar across patients, resulting in different levels of severity and persistence of disability, with both motor and cognitive performance to consider. Other important factors with respect to cell transplantation include the type and dosage of cells, mode of treatment delivery, application and assessment time points. Understanding immune rejection and tumorigenicity following cell transplantation have received growing attention, but long-term physiological effects of cell lines chosen for transplantation requires further evaluation. Taking the limitations into consideration, it is important to design studies that are measurable, feasible and replicable, while maintaining overall patient safety.
Figure 1.1 Pathophysiological cascades following acute stroke.
An overview of various biochemical cascades that occur within the first few hours following an ischemic insult.

1.2 Adult Neural Stem Cells

Neural stem cells (NSCs) persist in the adult central nervous system and contribute to ongoing neurogenesis throughout life. They possess the ability to proliferate, self-renew, and give rise to more than one type of cell in the brain. NSCs and their progeny, collectively termed neural precursor cells (NPCs), are found within the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subependyma (SE) lining the lateral ventricles of the adult forebrain.

1.2.1 The Adult Neural Stem Cell Lineage

Much of what we know about the adult neural stem cell lineage has been established predominantly from investigations in the adult rodent brain. Early in vitro work by Reynolds and Weiss (1992), involving crude dissections of the striatum, revealed presence of neural stem cells that were able to proliferate to form cluster of cells in the presence of epidermal growth factor (EGF), and give rise to neurons and astrocytes in appropriate culture conditions. In 1994, Morshead and colleagues demonstrated that this population of stem cells was found specifically within the periventricular region of the adult forebrain and more specifically, in the SE.

In the adult rodent brain, the periventricular region of the lateral ventricles contains a single layer of ciliated ependymal cells, which separates the lateral ventricle (LV) from the SE. The SE is a few cell layers thick and serves as a niche for NPCs which comprises of a relatively quiescent, neural stem cell population and its more abundant constitutively proliferating progenitors. The adult NSC population is rare and small subpopulation of SE cells (<0.5%) with a long cell cycle time of 15 days.
in vivo. Adult NSCs have morphological characteristics of astroglial cells that remain in direct ventricular contact via extended apical processes. The NSCs have processes that extend through ependymal cells into the lumen of the LV to contact the cerebrospinal fluid. NSCs express markers like the glial fibrillary acidic protein (GFAP, a mature astrocyte marker), brain lipid-binding protein (BLBP) and glutamate aspartate transporter (GLAST) in their quiescent state and an intermediate filament protein nestin in their activated state. Single cell clonal analyses reveal that in an activated state, NSCs undergo slow, asymmetric division to self-renew and also give rise to transient amplifying cells, which generate polysialylated neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX) expressing neuroblasts. These progenitors constitute approximately 10% of the SE and proliferate rapidly with a cell cycle time of 12.7 hours in the mouse. In the adult brain, the majority of neuroblasts (60%) undergo cell death under baseline conditions, with a fraction (15%) of the precursors remaining confined in SE, while the rest (25%) migrate towards the olfactory bulb (OB). These neuroblasts undergo several divisions and coalesce to form aggregated chains, creating the rostral migratory stream (RMS) that helps them to move towards the OB where they differentiate into interneurons (i.e. granule and periglomerular neurons; Figure 1.2). In addition to ongoing OB neurogenesis, SE NSCs also produce NG2 expressing oligodendrocytes progenitor cells that migrate and contribute to limited oligodendrogenesis in the striatum, corpus callosum and the fimbria fornix. Hence, ongoing neurogenesis and gliogenesis occurs throughout adulthood.
The SGZ is found between the hilus and the granule cell layer of the dentate gyrus in the hippocampus and is also a neurogenic niche in the adult brain. This region provides a supportive environment for ongoing neurogenesis. Similar to the lineage of SE NSCs, NSCs in the SGZ express GFAP, SOX-2 and nestin. These cells divide and form rapidly dividing intermediate progenitor cells, which are characteristic of SE transient amplifying progenitors, giving rise to neuroblasts that give rise to mature dentate granule neurons which integrate as a functionally viable part of the neural circuitry within the hippocampus.

1.2.2 In Vitro Isolation of Adult Neural Stem Cells: Neurosphere Assay

In order to study the behaviour of adult NSCs under different conditions in vitro, the “neurosphere assay” can be employed. The neurosphere assay involves the dissection of, tissue from the forebrain neurogenic regions (i.e. SE or SGZ), dissociation into single cells and plating in culture media that is supplemented with the mitogens EGF, fibroblast growth factor (FGF) and heparin (collectively referred to as EFH). NSCs proliferate and form clonally derived colonies, called neurospheres, in 7 to 10 days.100,111 Individual neurospheres in vitro are comprised of nestin expressing NPCs (<1% stem cells and >99% progenitors) and upon plating in the presence of fetal bovine serum (FBS) the progenitors have the capacity to differentiate into neurons, astrocytes and oligodendrocytes.100,111 Moreover, individual neurospheres can be dissociated into single cells and replated in growth factors to form secondary neurospheres.112 Thus, the neurosphere assay is a simple and robust way to demonstrate the cardinal properties of self-renewal and multilineage potential from adult derived NSCs (Figure 1.3). Further,
this assay can be used to study how adult NSCs respond to changes in different factors or various biological manipulations.
Figure 1.2: Adult neural stem cell lineage in vivo.

A schematic representation of adult neural stem cell (NSC) lineage in vivo in the mouse brain. A coronal brain section highlights the SE (red) lining the walls of the lateral ventricles located right beneath the corpus callosum. Within the SE, a rare population of NSCs (GFAP+, GLAST+, BLBP+) proliferate to self-renew and also give rise to transient amplifying progenitor cells (dlx2+, Ascl1 or Mash1+), which divide to become neuroblasts. The majority (60%) of the neuroblasts undergo apoptosis and some migrate along the RMS and differentiate into OB interneurons. GFAP = glial fibrillary acidic protein, LIF = leukemia inhibitory factor, BLBP = brain lipid binding protein, SE = subependyma, RMS = rostral migratory stream, OB = olfactory bulb.
Figure 1.3: The in vitro neurosphere assay.

A neurosphere assay depicting the formation of primary neurospheres from a single neural stem cell in the presence of EFH. A neurosphere is made of neural precursor cells comprised of both neural stem (<1%) and progenitor (>99%) cells. Primary neurospheres, dissociated into single cells and replated in the presence of EFH, give rise to secondary neurospheres, demonstrating their self-renewal capacity. Neurospheres can be dissociated and plated in the presence of FBS to induce differentiation of spheres into neurons (βIII-tubulin+), oligodendrocytes (O4+) and astrocytes (GFAP+), demonstrating multipotentiality. EFH = epidermal growth factor, fibroblast growth factor, and heparin, FBS = fetal bovine serum, NSC = neural stem cell.
1.3 Modulating Endogenous NPC Behaviour in vivo

1.3.1 Growth Factors

In order to harness the potential of endogenous NPCs for use in regenerative strategies, the role of a number of growth factors have been studied in modulating NPC behaviour. In assays, EGF and FGF have been the primary mitogens used to enhance proliferation to expand the size of the NPC pool.\textsuperscript{100,102} NPCs \textit{in vivo} express EGF receptors and intraventricular EGF infusion into the adult brain results in a significant expansion of the NPC pool and migration away from the SE.\textsuperscript{113,114} FGF regulates adult NPCs behaviour as seen using an FGF knockout mouse model which decreases the size of the NPC population by 50\%.\textsuperscript{115} EGF and FGF-2 promote NPC proliferation, as removal of both results in decreased proliferation, a shift towards endogenous expression of pro-survival factors insulin-like growth factor-1 and platelet derived growth factor beta and differentiation.\textsuperscript{116}

The \textit{in vivo} niche contains cells and factors that are permissive for NPC proliferation and neurogenesis. For example, cortical astrocytes have been shown to express EGF and FGF-2 suggesting that they provide endogenous signals for SE NPC proliferation.\textsuperscript{117} Besides EGF and FGF, a plethora of alternative growth and neurotrophic factors have been shown to play a role in NPC proliferation. Brain derived growth factor (BDNF) interacts through the truncated TRK-B receptors to induce proliferation of nestin expressing, BrdU-positive NPCs\textsuperscript{118}, and intraventricular infusion of BDNF increases the number of OB neurons in the adult brain.\textsuperscript{119} Mice lacking transforming growth factor alpha (TGF-\textalpha) show decreased NPC proliferation in SE without affecting the number of NSCs, suggesting TGF-\textalpha is important for
proliferation of SE-derived NPCs.\textsuperscript{120} Other examples of factors that promote NPC proliferation and neurogenesis include, vascular endothelial growth factor (VEGF), ciliary neurotrophic factor, stromal- and pigment epithelium derived growth factors.\textsuperscript{107,114,121–123}

### 1.3.2 Injury

Adult NPCs are responsive to injury. Following stroke, there is marked increase in NPC proliferation within the SE.\textsuperscript{124,125} In addition to enhanced proliferation which leads to an increase in the size of the NPC pool, a number of studies reveal that SE derived precursors migrate to the injury site following stroke. In 2002, Arvidsson and colleagues reported post-stroke neurogenesis by labeling NPCs with 5-bromo-2’deoxyuridine (BrdU; a cell proliferation marker) at 2 weeks post-stroke. They observed many DCX expressing cells co-labeled with BrdU that exhibited migratory morphologies, suggesting that these cells were migrating from the SE to the infarct region.\textsuperscript{124} DCX immunoreactivity also colocalized with nestin-expressing cells and were found in the ischemic penumbra 72 hours after a 90 minute focal ischemia.\textsuperscript{126} Further, the activation and migration of BrdU/DCX co-expressing cells was directly proportionate to the extent of striatal lesion volume.\textsuperscript{127} Since then, others have relied on genetic tools and pre-labeling strategies to demonstrate NPC migration to sites of injury.\textsuperscript{128,129} Further, recent work has demonstrated that stem cells, not just neuroblasts, migrate to the site of a stroke lesion where they differentiate into reactive astrocytes.\textsuperscript{130} Other forms of brain injury, such as traumatic brain injury, have also been shown to promote proliferation of GFAP expressing adult NPCs.\textsuperscript{131} Taken together, these findings reveal that SE derived NPCs proliferate, migrate towards the injury site.
and differentiate into mature phenotypes. SGZ derived NPC proliferation has also been reported in focal and global models of ischemia. Overall, adult NPCs are activated following injury, however whether this activation is not sufficient to promote self-repair is unclear.

1.3.2 Promoting cell survival: Cyclosporine A

Apart from growth factors, other small molecules have shown to regulate NPC behaviour. Cyclosporine A (CsA), a commonly used immunosuppressant, is an 11 amino acid cyclic polypeptide that is derived from a soil fungus, Tolypocladium inflatum (Figure 1.4). With respect to its immunosuppressive mode of action within a cell, CsA binds to cyclophilins (CyP) which are cis-trans conforming peptidyl-prolyl isomeric immunophilins. CyPs are in the cytosol (i.e. CyP A), endoplasmic reticulum (i.e., CyP B or C), mitochondria (i.e. CyP D), and cell nucleus (i.e. CyP E). CsA exerts its pharmacological effects through i) a calcineurin-dependent pathway or ii) a calcineurin-independent pathway, which are downstream of the binding of CsA to a cyclophilin binding protein.

Preclinical work by Jean Borel and colleagues in the 1970s led to the discovery and development of CsA as an immunosuppressant. Their group demonstrated that CsA selectively inhibited lymphocyte proliferation without affecting the proliferation kinetics of other somatic cells in rats. This garnered an interest towards an understanding of the suitability of this compound for clinical applications. Success in kidney, bone marrow transplantations, rheumatoid arthritis, and psoriasis followed by more appropriate clinical trials, leading to approval of its utilization for prophylaxis of immunorejection that ensues following transplantation of tissues and organs in clinical
The immunosuppressive effects of CsA is mediated through the blockage of the calcineurin-dependent pathway when CsA binds to CyP A, for which it the highest affinity.\textsuperscript{112} Inhibition of Ca\textsuperscript{2+}/calmodulin mediated signaling cascades, which are required to dephosphorylate and translocate nuclear factor of T cell into the nucleus, prevents interleukin-2 (a cytokine) transcription and ultimately inhibits T-cell activation and immunosuppression.\textsuperscript{139}

CsA has also shown to act through a non-immunosuppressive, calcineurin-independent pathway. This involves binding of CsA to CyP D in the mitochondria.\textsuperscript{140} Blocking CyP D inhibits mitochondrial permeability transient pore (MPTP) formation and the release of cytochrome C, which effectively enhances cell survival. Hence, CsA prevents cell death of neural precursor cells without engaging immunomodulation pathways (Figure 1.5).\textsuperscript{140} We have demonstrated that this pro-survival effect of CsA leads to an expansion in the size of the NPC population, both \textit{in vitro} and \textit{in vivo}.\textsuperscript{112} In culture, CsA treatment for 7 days significantly increases the number of neurospheres by 1.7 fold. Systemic administration of CsA also expanded neurosphere numbers by a significant 2.6 fold, compared to control conditions. The effect of CsA on cell survival was also observed in the adult hippocampus using both \textit{in vitro} and \textit{in vivo} assays.\textsuperscript{141} Furthermore, administration of CsA for 7 days resulted in a significant increase in the numbers of newborn neurons that survived within the SGZ. Thus, the pro-survival effects can not only promote NPC survival in both SE and SGZ but also results in increased neurogenesis in SGZ.\textsuperscript{141}
CsA is an 11 amino acid cyclic polypeptide, with a molecular formula of $\text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{12}$.

Figure 1.5: CsA pathways that regulate cell-survival.

In pathway 1, CsA localizes into the cytoplasm to bind to CyP A, a complex that inhibits calcineurin and inhibits dephosphorylation of BAD and nNOS. Inhibiting dephosphorylation of BAD and nNOS result in the prevention of 1) Bcl-2 and Bcl-xL mediated signaling cascades, and 2) decreased NO and subsequent signaling molecules, respectively, that induce cellular death. In pathway 2, CsA blocks CyP D and prevents the formation of a CyP D-Ant-VDAC-PiC complex, which is involved promoting cellular death via MPTP formation. MPTP formation results in swelling of the mitochondrial matrix and release of cytochrome C release and eventual death of cells. CsA = cyclosporine A, CyP A = cyclophilin A, BAD = Bcl-2 Associated Death Promoter, nNOS = neural nitric oxide synthase, CyP D = cyclophilin D, MPTP = mitochondrial permeability pore formation.

1.4 Recruiting Endogenous NPCs for Therapeutic Applications to Promote Stroke Recovery

1.4.1 Stroke Injury Models in Adult Rodents

The translation of preclinical findings to the clinical setting is challenging for a number of reasons some of which include the following: narrow set of preclinical experimental conditions, in-vitro to in vivo correlations, and the manifestation of diseases in animals versus humans, in general. In order to produce more clinically relevant outcomes, recommendations were set out by the stroke therapy academic industry roundtable (STAIR) in 1999 to ensure scientific rigor in preclinical stroke research. Although the guidelines were directed towards acute ischemic stroke and neuroprotective studies, it emphasized the importance of controlled experimental settings, replicability of findings in different species and laboratories, highlighting inclusion/exclusion criteria, and considerations regarding appropriate sample size, sex differences, and model development which is important to scientific research in a broader capacity. Certainly, key considerations include appropriate selection of species and injury models. Rodents (i.e. mice and rats) have most frequently been studied to evaluate treatment efficacies to promote stroke recovery, for both financial and practical reasons. Currently, focal stroke models have been used more readily in preclinical studies for the following reasons: i) the ability to induce stroke in specific brain regions, ii) the resulting neurological deficits can be controlled based on regional specificity, and iii) the realistic evaluation of therapeutic outcomes is possible through increased understanding of how targeting specific anatomical regions respond to therapeutic interventions.
One of the most widely used models of focal ischemia is the intraluminal suture middle cerebral artery occlusion (MCAO) model, where a monofilament or suture is inserted intraluminally into the internal carotid artery and further advanced to block blood flow in the middle cerebral artery for minutes to hours, thereby creating a transient ischemic insult. MCAO is able to reproduce a penumbra similar to human stroke conditions and mostly creates prominent and extensive lesions in the ipsilateral hemisphere, including most of the cortex and striatum, temporal and frontoparietal lobes, thalamic and hypothalamic regions. The extent of lesions impacting the hypothalamus can cause hyper or hypothermia, which must be managed by post-stroke external temperature manipulation. From a functional efficacy standpoint, MCAO can produce measurable neurological deficits in rodents, which can be assessed using a variety of basic neurological scoring scales or a battery of motor, sensory, and cognitive tests. Some of the concerns related to the uses of this model are that it is quite variable between individual rodents. There is a higher risk of mortality. Regions affected do not directly correlate with extent of damage observed in humans post-stroke. Further, the large lesions are not necessarily clinically relevant for regenerative strategies in particular, because they mimic malignant infarction in humans, which is untreatable.

The photothrombosis model of stroke creates a cerebral infarct by activating previously delivered light-sensitive dye present in the blood circulation using a light beam. Upon photon activation, energy is released to produce intermediary oxygen radicals, disrupting endothelial membrane integrity via peroxidation and platelet activation and aggregation surrounding the area of insult. The benefit of the model is its simplicity and it can be used to target circumscribed cortical regions. Moreover, it
produces lesions that are highly reproducible in the absence of mortality. Drawbacks of this model are that the lesion lacks a penumbra and is not suitable for subcortical lesions. This poses concerns for neuroprotective strategies and restricts the regions of injury that can be studied.  

The endothelin (ET)-1 model of stroke is also used in preclinical animal studies. ET-1, the most widely expressed isopeptide of the endothelin family, is a secretory peptide comprised of 21 amino acids (Figure 1.5). It is chiefly responsible for prolonged vasoconstriction, and as a result, takes on an active role in promoting hypertension. ET-1 is secreted by various tissues including endothelial and neural cells, and mediates its effects essentially through two receptors subtypes: ET\textsubscript{A} and ET\textsubscript{B}. These G protein-coupled receptors are found in various regions of the rodent brain. The differential regulation of vasoconstriction and vasodilatory effects of ET-1 is mediated through ET\textsubscript{A} receptors primarily expressed in vascular smooth muscle and ET\textsubscript{B} receptors expressed in endothelial cells. In the rat brain, ET-1 administration results in a rapid decrease of blood flow by 70-90\% followed by reperfusion over a period of hours. It has been utilized in different models of ischemia. ET-1 was first used to produce MCAO by direct application to the exposed artery. In a modified model, which involved occluding the anterior cerebral artery (ACA) with ET-1 resulted in models of cortical ischemia and white matter damage. This ACA occlusion model was established to create cognitive deficits in rats for preclinical studies. However, it is primarily used as a model of sensorimotor stroke by direct stereotaxic injections into the sensorimotor cortex.
Focal ischemia model by *pial vessel disruption* (PVD) permanently devascularizes by removing blood vessels through physical manipulation. However, PVD is considered a neurotrauma model as the induction by physical removal of the vessels is not clinically relevant to ischemic stroke. However, studies have used it for stroke research as it mimics aspects of lacunar (small vessel occlusion) stroke, such as cavitation. PVD results in focal cortical damage resulting in cell death in the cortex with a lesion cavity that often extends to the corpus callosum. Other less common, preclinical stroke injury models include i) the *embolic stroke model*, which involves introducing tiny clots into major arteries via a microcatheter, which then passively flow through the blood circulation to occlude vessels, and 2) *craniectomy*, a modified method of MCAO involving direct surgical occlusion using microaneurysm clips, ligatures, or electrocoagulation methods. The latter models create reproducible focal ischemic insults, without creating lesions in unwanted areas (i.e. hypothalamus), but are invasive and/or technically challenging.

**1.4.2 POST-STROKE NEUROLOGICAL ASSESSMENTS IN RODENTS**

The successful development of a stroke injury model is highly dependent on outcomes in two major aspects: 1) anatomy, and 2) neurological (motor or cognitive) function. Assessing neurological functions using appropriate behavioural tests following stroke has become a demanding aspect of evaluating therapeutic efficacy. Traditionally in preclinical stroke research, post-stroke assessments focus on motor and/or sensory function, reflex, and balance. Some common motor/sensorimotor tests include the cylinder task (forelimb function), foot fault task (fore- and hindlimb function), staircase or pellet-reaching task (skilled reaching), and rotarod task (motor coordination and
balance).\(^{165}\) Others include, ledged tapered beam task (fore- and hindlimb function), corner test (sensorimotor/postural asymmetry), forelimb flexion (reflex) and adhesive removal test (stimulus-driven movement asymmetry).\(^{165}\) Very little attention has been geared towards understanding cognitive performance following stroke. The Morris water maze\(^{166–168}\) and the radial arm maze\(^{169–171}\) tasks have been most commonly used to assess post-stroke deficits in cognitive aspects such as flexibility, spatial, working and reference memory. Careful employment of neurological assessment is critical to determining the success of a model, therapeutic benefits of treatments, or the underlying cellular mechanisms or anatomical changes that may be responsible in promoting functional recovery.

1.4.3 **Using NPCs to Promote Motor Recovery**

Current treatments (neuroprotective and rehabilitation) are not sufficient to promote complete recovery in stroke survivors who continue to have residual impairments long after their initial insult.\(^{44,56}\) This has created a strong impetus to look for additional therapeutic interventions to enhance recovery. One of the most promising strategies is stem-cell based approaches. Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) II published guidelines that were updated to optimize experimentation procedures.\(^{172}\) Key factors included cell characterization, animal species, stroke models, appropriate vehicle or controls, cell based dose-response curves for optimal dosage and mode of treatment, proper behavioural tests and assessment time points. Also, these guidelines encouraged *in vivo* experiments to assess underlying cellular mechanisms and developing mechanism-based treatments based on their long-term efficacy and side effect profile.\(^{172}\)
The ultimate goal of developing therapeutic interventions to treat stroke is to promote recovery and improve the quality of life of stroke patients. Cell-based strategies have generated considerable excitement since the discovery of NPCs in the adult CNS. The hope is that transplantation and/or neurorestorative approaches using NPCs and other stem cell populations will further advance the field of regenerative medicine. This section focuses on the approaches utilizing resident neural stem cells to promote neural repair and functional recovery, while maintaining rigor based on STEPS recommendations.

Work by Kolb and colleagues in 2007 was one of the first successful attempts in demonstrating that endogenous repair mechanisms could be modulated to promote functional recovery. They demonstrated that the sequential intraventricular administration of EGF and erythropoietin (EPO) following PVD in the sensorimotor cortex of rats promoted functional recovery. The basis for the strategy was to utilize EGF to increase the size of the NPC pool\textsuperscript{113} and erythropoietin (EPO) to promote neural differentiation of the progeny \textit{in vivo}.\textsuperscript{175} Indeed, 7 days of EGF administration beginning at 3 days post-PVD, followed by EPO administration for 7 days, led to cortical tissue regeneration and recovery of motor impairments up to 6 weeks after PVD in rats. A number of behavior tests were used to demonstrate the recovery including the cylinder test, swimming test, and single pellet reaching.\textsuperscript{164} Similar observations were also observed post-PVD in mice, where sequential administration of EGF and EPO resulted in functional recovery in a motor task.\textsuperscript{98} These findings were also supported in a more clinically relevant ET-1 model of sensorimotor cortex stroke,
where sequential administration of EGF and EPO, when combined with rehabilitation (i.e. enriched environment) resulted in accelerated functional recovery in the staircase pellet reaching task in 4 weeks.\textsuperscript{174} Interestingly, EGF+EPO alone was not effective in promoting recovery in the rat ET-1 stroke model; however, the combination of NPC activation and rehabilitation was more effective than either strategy alone.

While growth factors are effective molecules to expand the NPC pool, they may not be suitable for therapeutic considerations in clinical practice if the main mechanism of action involves proliferation, with the potential outcome of tumorigenesis.\textsuperscript{175} Factors that can enhance NPC activation are worthy candidates for consideration when developing clinically relevant interventions. Previous work has shown that metformin, a drug commonly used to treat type II diabetes, is able to activate NPCs in the neonatal rodent brain and promote neurogenesis and gliogenesis from NPCs.\textsuperscript{134,176} Indeed, metformin administration following neonatal stroke was effective in promoting behavioural recovery in motor tasks.\textsuperscript{134} Another example of a factor that promotes NPC activation is CsA. As mentioned earlier, CsA acts directly on NPCs to promote survival, without directly affecting NPC proliferation.\textsuperscript{98,112} The daily administration of CsA following PVD in mice leads to NPC proliferation and migration, cortical tissue regeneration and functional recovery in the foot fault task, which assesses the number of slips by the contralateral paw while walking on a wire grid.\textsuperscript{98} Further, a focal ET-1 lesion in the sensorimotor cortex, followed by CsA treatment, beginning at the time of or 3 days following stroke, until time of sacrifice, also promoted motor recovery in the foot fault task.\textsuperscript{140} Together, these studies encourage further exploration of CsA's
potential to promote tissue repair and more thorough assessment of functional recovery in other models of stroke.

1.4.4 Cognitive Recovery After Stroke

Cognitive deficits are common following stroke and have been shown to interfere or reduce recovery in patients. Most studies deal with motor impairments. Limited effort has been directed towards therapeutic strategies to promote cognitive recovery. However, there are justifiable reasons as to why this has been the case. In preclinical studies, cognitive models of stroke have been difficult to develop in comparison to sensorimotor models of stroke. In rodents, lesions in the sensorimotor cortex create obvious post-stroke sensorimotor impairments, making it easier to evaluate deficits and recovery. In contrast, cognitive deficits are complex and are not immediately apparent. In humans, unilateral stroke is sufficient to create a plethora of cognitive and EF impairments ranging from attention, language, memory, and orientation. The rodent brain is less lateralized than the human brain and as a result unilateral lesions in rodents rarely lead to cognitive impairment. Whereas, bilateral lesions have produced pronounced EF deficits. Although MCAO model of stroke can often have motor and cognitive outcomes, it is difficult to assess cognitive recovery in the stroke model where motor deficits are a confounding variable. The suture model of MCAO can often damage the hippocampus, which is not typically affected in humans. Therefore, consideration of factors, such as selection of species, stroke models that target specific and relevant brain regions, and behavioral assays that separately assess motor and cognitive functioning, is crucial.
<table>
<thead>
<tr>
<th>Model</th>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Middle Cerebral Artery Occlusion</td>
<td>Insertion of microfilament or suture to block artery</td>
<td>Highly reproducible lesions</td>
<td>Invasive; uncontrolled damages of various brain regions often not damaged in humans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Create various degrees of transient or permanent ischemia</td>
<td>Accidental hemorrhage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clinically relevant</td>
<td>Hypothermia due to extensive damage to hypothalamus</td>
</tr>
<tr>
<td>Photothrombosis</td>
<td>Cerebral infarction using light to activate photo-sensitive dye</td>
<td>Highly focal lesions</td>
<td>No penumbra, restricted to cortical injury</td>
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<td></td>
<td></td>
<td>Low mortality</td>
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<td></td>
<td></td>
<td>Less invasive</td>
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<tr>
<td>Endothelin-1</td>
<td>Temporary vasoconstriction followed by reperfusion</td>
<td>Creates clinically relevant focal lesions</td>
<td>Invasive; can alter regenerative niche as ET receptors are expressed on glial cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low mortality</td>
<td></td>
</tr>
<tr>
<td>Pial Vessel Disruption</td>
<td>Permanent devascularization by wiping the pial and associated blood vessels</td>
<td>Focal cortical damage</td>
<td>Invasive, clinically irrelevant</td>
</tr>
</tbody>
</table>

Table 1.1: Rodent models of focal ischemia.

1.5 THE PREFRONTAL CORTEX

1.5.1 ANATOMY AND CYTOARCHITECTURE

In humans, the PFC (the anterior portion of the frontal lobe) is a highly evolved area, constituting 30 percent of the brain.\(^{186}\) It can be subdivided into medial, lateral, and orbital regions\(^{187}\). Similar to humans, the rodent PFC is comprised of three major components: medial, lateral and ventral segments. The *medial PFC* (mPFC) can be further divided into dorsal and ventral subregions. Dorsal mPFC consists of precentral (PrC) and anterior cingulate (ACg) cortices and ventral mPFC consists of the prelimbic (PrL), infralimbic (IL), and medial orbital (MO) cortices. The *lateral PFC* (LPFC) is comprised of dorsal and ventral agranular insular (AID and AIV, respectively) and lateral orbital (LO) cortices. The *ventral PFC* (VPFC) is comprised of ventral and ventrolateral orbital (VO and VLO, respectively) cortices. In humans and rodents, these anatomical areas can be characterized based on cytoarchitecture\(^{188}\). PFC is comprised of agranular layers I, II, III, V and VI which vary in the way they are organized within the different subregions of PFC (see summary in Table 1.2). It is important to note that in rodents, the ACA, a diverging branch of the internal carotid artery, supplies blood to the prefrontal cortical regions.\(^{189}\) Whereas, human PFC receives blood supply from both the ACA and MCA, with MCA covering most of the territory.\(^{190}\)

1.5.2 ROLE OF PFC IN EXECUTIVE FUNCTION BEHAVIOUR

The PFC is important in establishing an integrative network with various cortical and subcortical regions through numerous direct, indirect and reciprocal connections.\(^{191}\) The extensive connectivity between PFC and the rest of the brain highlights its importance in regulating a variety of higher-order and complex activities in both
humans and rodents. The prefrontal cortex is heavily implicated in the regulation of executive function (EF). EF is a broadly conceived umbrella term for an array of complex cognitive behaviours, which include attentional regulation, initiation, shifting and termination of tasks, sequencing and planning, decision making, problem solving, and similar goal-oriented behaviour. Working memory, governed by the frontal lobe and other regions of the brain, is also considered to be an executive behaviour regulated in part by the PFC. In humans, various components of EF behaviour are regulated by different regions of the PFC. LPFC is responsible for monitoring, attention, orientation, and active information processing in working memory, managing current goals and predicting future behaviour. The mPFC region is involved in management of motivation, conflicts, regulation of pain perception and spatial memory. Further, ventral mPFC plays an important role in metacognition, decision-making, information retrieval, and long-term memory processing. Finally, the orbitofrontal cortex is responsible in behavioural components, such as, initiation, reward, taste, emotion, and social cognition, and most importantly, self-regulation.

In rodents, EF is governed primarily by the mPFC which is analogous to the primate dorsolateral prefrontal and anterior cingulate cortices. Cytotoxic lesion of the mPFC prelimbic, infralimbic and anterior cingulate area resulted in attentional and inhibitory dysregulation but did not affect novel object recognition. Asymmetrical outcomes in exploratory behavior and taste aversion resulted following hemisphere-dependent infralimbic lesions. Further, impairments in problem solving, and slower task acquisition due to deficits in attentional and behavioural flexibility were reported. As EF deficits are common following stroke, development of
clinically relevant models of stroke that mimic human deficits are important and needed. Accordingly, development of an ET-1 model of mPFC stroke in rats have resulted in attentional set-shifting deficits, anxiolytic behaviour, impaired object recognition, and cognitive inflexibility, without affecting locomotion.183,195,202 Taken together, these findings highlight the role of PFC in regulating EF behaviour.

1.5.3 Post-Stroke Cognitive Deficits: Available Treatments
Nearly 75% of stroke cases include impairment in EF which can significantly impact quality of life depending on the region and extent of tissue damage.178,195 Currently, the only form of treatment available to target cognitive improvement involves rehabilitative strategies that help with social integration and participation in community living.203,204 The rehabilitative strategies have focused on metacognitive strategies (i.e. “thinking about your thinking”), which can be generalizable and applicable to everyday goal-oriented tasks (e.g. grocery shopping, meal preparation, and weekly budgeting).205 The ability to generate goal-subgoal lists and stepwise strategies, maintain self-awareness, self-control, and self-monitor while performing goal-oriented tasks are predominantly regulated by EF processes, and are commonly impaired in stroke patients.206,207 Further complicating recovery efforts, diminished cognitive abilities affect patient progress in motor rehabilitation.178 For example, stroke patients with executive dysfunction can have problems following guidelines, processing information, and understanding a sequence of events to complete tasks during rehabilitative training, contributing to an impediment in functional recovery and increasing the risk of falls.6 Therefore, devising effective therapeutic (i.e. neurorestorative strategies) strategies to enhance cognitive recovery requires more attention and resources.
### Table 1.2: Divisions and cytoarchitecture of PFC in rodents.

<table>
<thead>
<tr>
<th>Subregional Divisions of the Prefrontal Cortex</th>
<th>Cytoarchitecture</th>
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<tbody>
<tr>
<td><strong>Medial</strong></td>
<td></td>
</tr>
<tr>
<td>Dorsal</td>
<td>Precentral (PrC)</td>
</tr>
<tr>
<td></td>
<td>Anterior cingulate (ACg)</td>
</tr>
<tr>
<td>Ventral</td>
<td>Prelimbic cortex (PrL)</td>
</tr>
<tr>
<td></td>
<td>Infralimbic cortex (IL)</td>
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<tr>
<td></td>
<td>Medial Orbital (MO)</td>
</tr>
<tr>
<td><strong>Lateral</strong></td>
<td></td>
</tr>
<tr>
<td>Dorsal</td>
<td>Agranular insular (AID)</td>
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<tr>
<td>Ventral</td>
<td>Agranular insular (AIV)</td>
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<tr>
<td>Lateral</td>
<td>Orbital (LO)</td>
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<tr>
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<tr>
<td>Ventral</td>
<td>Orbital (VO)</td>
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<tr>
<td>Lateral</td>
<td>Orbital (VLO)</td>
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1.6 **RATIONALE, HYPOTHESIS AND OBJECTIVES**

To date, research has primarily focused on understanding the effects of neural repair strategies on post-stroke motor impairments with little attention devoted to neural repair strategies to promote cognitive recovery. Herein, I propose to study the potential of CsA to promote endogenous NPC activation and enhance cognitive recovery following stroke in the medial prefrontal cortex (mPFC) of adult mice.

### 1.6.1 HYPOTHESIS

CsA treatment following mPFC stroke will result in an activation and expansion of the endogenous NPC population and promote cognitive recovery in adult mice.

### 1.6.2 OBJECTIVES

- **Objective 1. Establish a cognitive model of stroke**

To develop a cognitive model of stroke in mice, ET-1 will be used to induce lesions into the mPFC region of adult animals. The cellular and behaviour outcomes will be examined to evaluate the model.

- **Objective 2. Examine effects of stroke on SE derived NPCs, in the presence or absence of CsA**

To examine the effects of mPFC lesions on SE derived NPC behaviour, in the presence or absence of CsA, short- and long-term *in vitro* neurosphere assays will be performed.

- **Objective 3. Examine the effects of CsA on cognitive recovery**

To examine the effects of CsA treatment and its effects on cognition at short- and long-term points post-stroke, using assessments that measure task acquisition as well as short and long-term memory.
CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMALS

All procedures adhered to the University of Toronto Animal Care Committee and Canadian Council on Animal Care guidelines. Adult male C57BL/6J of 6-8 weeks of age (20-25g; Charles River) were housed on a 12-hour light/dark cycle with food and water provided ad libitum. All animals were acclimatized to the institutional facility for one week prior to surgeries or any behavioural testing.

2.2 NEUROSPHERE ASSAY

The adult SE was dissected and cultured as previously described. Briefly, tissue was collected and treated with enzymes (1.33 mg/ml trypsin, 0/67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid; Sigma-Aldrich) for 30 minutes at 37°C. Trypsin inhibitor (0.67 mg/ml) was added to prevent further enzymatic activity and tissue was tritured to dissociate into a single cell suspension. In 24 well plates (VWR, Mississauga, ON), cells were plated at a clonal density of 10 cells/µl in serum free media (SFM) that was supplemented with epidermal growth factor (20ng/ml; Sigma-Aldrich), fibroblast growth factor (10 ng/ml; Sigma-Aldrich) and heparin (7.35 ng/ml; Sigma-Aldrich). Neurospheres were grown for 7 days and counted. Assays were performed 7 or 60 days following stroke to assess the effects of mPFC stroke, with or without CsA treatment, on the size of the NPC pool. Animals were grouped into three groups: Stroke-alone, Stroke+CsA, and uninjured controls. For neurosphere assays cultured from the mPFC lesioned tissue on day 60, cortical tissue was removed from the injured portion of the cortical layer above the corpus callosum. Tissue sample from both hemispheres were combined together.
2.3 Stroke Injury and CSA Administration

Mice were anesthetized with Isofluorane (5% induction and 2% maintenance) and given Ketoprofen (0.1ml/10g of body weight; 0.5mg/ml in dosage) prior to surgery. Skin was wiped with ethanol and betadine prior to incision. The sensorimotor stroke was unilaterally induced using endothelin-1 (800 picomolar) according to methods reported previously.\textsuperscript{140} For the mPFC stroke, mice received bilateral injections of ET-1 though burr holes overlying the mPFC, with two injections per hemisphere. Each injection of ET-1 (0.76 µl; 800 picomolar) was injected at a rate of 0.2 µl/min using a 1 µl Hamilton syringe at the following coordinates: (1) anterior-posterior (AP)= +2.2 mm from Bregma; mediolateral (ML)= ±0.4 mm from midline; dorsoventral (DV)=2.4 mm, and (2) AP= +1.5 mm from Bregma, ML= 0.4 mm from midline, DV=2.6 mm below the skull surface. The needle was kept in place for 3 minutes after injection and then slowly withdrawn. The lesions were observed using cresyl violet staining 4 days post-stroke. For long-term behavioural and culture studies post-stroke, mini osmotic pumps (0.25 µl/hr for 2 weeks; Alzet, Cupertino, CA; model 1002) with CsA (15mg/kg/day; dissolved in 65% ethanol and 35% cremaphor) were implanted subcutaneously and replaced every 2 weeks until the time of sacrifice. \textit{In vitro} studies were performed on animals 7 days after mPFC stroke with or without CsA administered subcutaneously using 0.5 µl/hr mini osmotic pumps (1007D; ALZET, Cupertino, CA).

2.4 Behavioural Testing

With the exception of the training phase for the adhesive removal test (below), all behavioural testing took place after surgery. Prior to testing, all animals were acclimatized to the testing room for 10 minutes. Animals were housed in isolation for
the entire duration of behavioural testing. Outcomes for the Puzzle Box Task and the Y-maze Task were analyzed using Viewer (Biobserve, Bonn, Germany). Animals were grouped into three groups: Stroke-alone, Stroke+CsA, and uninjured controls.

2.4.1 The Adhesive Removal Test

This test assesses sensorimotor behaviour in mice. Animals are habituated to a clean and empty mouse cage for 1 minute after which a 0.3 cm x 0.4 cm piece of tape is placed on the central hairless portion of each of the forepaws. The “time to contact” and the “time to remove” are measured to assess paw and mouth sensitivity and dexterity correctness, respectively. The forepaw on which tape was placed first was alternated every day. Each day of training consists of one trial per animal and maximum of 5 minutes are given per trial for both tapes to be removed. The task consists of 2 days of pre-training (exposure to tape), 5 days of training to ensure that they can perform the task in less than 1 minute by the 5th day, and 2 days of testing on day 4 and day 5 after stroke with or without CsA administration. To evaluate task performance, we assigned a functional score using the following formula: \([\text{time to remove}] - [\text{time to contact}]\). An average functional score was derived following the two days of testing.

2.4.2 The Puzzle Box Task

The puzzle box task is a problem-solving task that assesses performance in general cognition and EF, including acquisition and problem-solving, short-term memory, and long-term memory. The puzzle box comprises an empty, brightly lit start zone and a dark, covered goal zone filled with stimulating materials and objects (i.e. bedding and igloo) (Figure 2A). Mice are introduced to a box with a divider that has an underpass
connecting the empty, brightly lit start zone to the dark, more enriched goal zone. Mice go through the underpass to get from the start zone to the goal zone and the difficulty of getting to the goal zone increases across trials over days. The task was implemented as previously described by Abdallah and colleagues (2011). Mice performed 3 trials per day (300 seconds/trial), over a span of 3 days. The difficulty of the task increases each day. Day 1, trial 1 allowed easy access to the goal zone through an open door and underpass. Trial 2 and 3 only gave access to the underpass. On day 2, trial 4 was a repeat of day 1, trial 2/3, but new rules were implemented on trial 5, where accessibility was made more difficult by covering the underpass with bedding which mice needed to remove to enter the goal zone. On day 3, trial 7 was a repeat of trial 5/6. Trial 8 required the removal of a cardboard plug that was blocking the underpass and this was repeated in trial 9 (Figure 2B and C). Mice were tested at three different time points following stroke: day 4-5, day 22-24 and day 45-47. Animals that failed to complete the training trials (i.e. Trial 1, 2 and 3 on day 1) in less than 5 minutes were excluded from further analysis.
Figure 2.1: The Puzzle Box Task

A) A puzzle box separated into a dark (goal zone) and bright (start zone). B) Highlighted problem-solving tasks of puzzle box over 3 consecutive days. Day 1 involves training, which simply requires animals to pass through an underpassage to reach goal zone. On day 2, a problem-solving task is introduced that requires digging bedding to reach goal zone. Day 3 involves a new problem-solving task of removing a cardboard plug to reach goal zone. C) A complete summary of the puzzle box task, which includes trials, tasks, and the cognitive functions tested.
2.4.3 The Y-Maze Task

The Y-maze spontaneous alternation task evaluates spatial working and reference memory in rodents.\textsuperscript{211} The Y-maze consists of 3 arms defined as A, B, and C. Mice were placed in the maze and allowed to explore freely for 8 minutes. The numbers of times the mice spontaneously alternate and visit three different arms consecutively (i.e. make the choices ABC, BAC, or CAB) were measured. Mice were given a score below 50% if they made a choice to return to a previously visited arm and failed to explore all three different arms in a row. Long-term performances were evaluated on Day 49 following stroke.

2.4.4 The Open Field Task

The Open Field Task is an assay used to screen for anxiety related behaviour and general locomotion, by allowing animals to explore a novel environment.\textsuperscript{212,213} The open field consists of a 60 cm x 60 cm chamber with 16 x 16 squares, referred to as ‘zones’ that fill the area. The central most 8 x 8 zones constitute the ‘central zone’. Animals were allowed to explore the novel area for 10 minutes and various activities, such as locomotor activity and time spent in the corners versus the central zone of the field were evaluated at 1, 5 and 10-minute time points.

2.5 Tissue Preparation and Histology

Animals were deeply anesthetized with Avertin (Sigma-Aldrich) and transcardially perfused with cold 0.01M phosphate buffered saline (PBS), followed by 4\% paraformaldehyde. Brains were collected and stored in 4\% paraformaldehyde overnight,
followed by a transfer to 20% sucrose solution until sectioning. All brains were coronally sectioned (20 µm) using a cryostat and collected in series.

*Lesion Volume and Neural Degeneration Analyses:* Sections were collected from brains at day 1, 4 and 10 after stroke ± CsA treatment and stained with cresyl violet (Sigma-Aldrich) for lesion volume analysis. Sections were imaged at 240 µm intervals and the lesion volume was calculated as follows: (surface area of lesion using ImageJ) X (distance between the sections). Fluoro Jade (FJ) C (stock solution: 25 mg FJC in 250 mL dH₂O; Millipore) was used to assess neuronal degeneration. Slides were immersed in 1% NaOH in 80% ethanol for 5 minutes, rinsed in 70% and dH₂O for 2 min each, and incubated in 0.06% KMnO₄ for 10 minutes and then rinsed again in dH₂O for 2 min. The next day slides were incubated in a working solution (2 ml of stock solution in 198 ml dH₂O and 198 mL acetic acid) for 30 minutes and washed in dH₂O prior to cover slipping. The total numbers of FJC+ cells were counted from every 10th section (200 µm apart) between bregma +2.34 mm and 1.10 mm (rostro-caudal). FJC+ cell counts were derived from a pre-calculated “reference” area. Within a brain, coronal sections were divided into two clusters based on morphological characteristics (i.e. anterior and posterior to the genu of the corpus callosum). The total volume of the reference area was calculated to be 4.64 mm³, a sum of cluster 1 (2.55mm x 1.0 mm x 1.16 mm) and cluster 2 (1.5mm x 1.0mm x 1.16mm) volumes. The reference area was kept consistent between all brains. A representative sample of FJC+ (bright green cells) cells was counted from the reference area.
2.6 **Statistical Analysis**

The Graphpad Prism 6.0 and/or Microsoft Excel 2011 were used for all statistical analyses. Two-tailed *t*-test was used for two-group comparisons and repeated measures ANOVA was used to assess performance across multiple groups for each more than one trial. For multiple group comparisons, the appropriate post-hoc test was used (i.e. Tukey or Dunnett) based on whether all groups were compared to each other, irrespective of the control group, or if all groups were compared to one fixed control group. All data are reported as means ± s.e.m and statistical significance was evaluated at a probability level of *p* ≤ 0.05.
CHAPTER 3: RESULTS

3.1 A COGNITIVE STROKE MODEL THAT PRODUCES COGNITIVE DEFICITS AT EARLY TIME POINTS WITHOUT AFFECTING MOTOR FUNCTION

With the goal of determining whether endogenous NPC activation would lead to recovery of cognitive impairments post-stroke, similar to what is observed following sensorimotor stroke, I sought to establish a reliable and reproducible model of stroke in mice that would result in cognitive impairments, as previously established in rats. I used endothelin-1 (ET-1), a spasmodic agent, which produces ischemia by inducing vasoconstriction followed by reperfusion. Bilateral focal injections of ET-1 (2 injections per hemisphere) into the mPFC region generated lesions in the second frontal area, prelimbic, infralimbic and anterior cingulate cortices as schematically represented in Figure 3.1A and B. Bilateral lesions extended 800um rostrocaudally between 2.34 mm and 1.10 mm anterior to bregma, producing an average lesion volume of 4.98 ± 0.92 mm$^3$ (mean ± standard error) on day 4 post-stroke as demonstrated using cresyl violet staining (Figure 3.1C).

To rule out sensorimotor deficits resulting from stroke in the mPFC region, I used a modified version of the adhesive removal test. In this task, adhesive tapes were placed on forepaws (Figure 3.1D) and the time (seconds, s) of i) tape contact and ii) tape removal were measured. A functional score was assigned per forepaw, which was calculated as the time of tape removal minus the time of tape contact. Mice that received unilateral ET-1 sensorimotor cortical stroke (right hemisphere) showed a significant difference in their tape test performance between the contralateral (injured, left) forepaw and the ipsilateral (uninjured, right) forepaw (125.67 ± 43.44 s (left forepaw) vs 6.25 ±
0.39 s (right forepaw); F(1,8)=18.48, p=0.0026, ANOVA). As expected, mice that received ET-1 induced bilateral mPFC stroke did not exhibit asymmetrical sensorimotor impairments between the left and right paw motor score (5.68 ± 3.89 s (left forepaw) vs 7.32 ± 2.72 s (right forepaw); p > 0.05; Figure 3.1E). More importantly, their performance in the tape test did not significantly differ from uninjured (right) forepaw of animals that received unilateral sensorimotor stroke. Hence, the bilateral mPFC stroke does not result in forepaw sensorimotor impairment.

To assess post-stroke cognitive outcome, performance in the puzzle box (PB) task was measured. The PB task has been used to assess general cognition and EF impairments in several mouse injury models.\textsuperscript{199} The task examines a number of behavioural components including problem-solving, short-term memory, and long-term memory. Mice were tested from day 4–6 following injury (Figure 3.1F). Performance that was significantly different (i.e. increase in latency) from uninjured controls was defined as impairment. The length of time for mice to complete each of the 9 trials was measured. At this early time, stroke-alone mice were significantly impaired (i.e. took longer to complete the task or failed to complete the task; increased average latency) on trial 5 and trial 6 (trial 5: 249.94 ± 14 s and trial 6: 164.39 ± 27 s; p\textsubscript{trial5}=0.010, p\textsubscript{trial6}=0.0291, Dunnett’s multiple comparisons test, ANOVA) compared to uninjured controls (trial 5: 185.67 ± 21 s and trial 6: 90.13 ± 21 s) on day 2 of PB. On day 3, the stroke-alone mice were able to complete trial 8 (i.e. physically removing a plug) within the given time (Figure 3.1F) and with similar pass rates (control: 60%; stroke-alone: 55.6%). This provided further support of the absence of motor impairments. Hence it was unlikely that motor deficits accounted for the failures on trials 5 and 6. Notably,
stroke-alone mice also showed significant impairment on trial 9 compared to uninjured controls (control: 145.67 ± 31 s; stroke-alone: 208.39 ± 22 s; \( p_{\text{trial9}}=0.0339 \), Dunnett’s multiple comparisons test, ANOVA) suggesting a general impairment in short-term memory compared to uninjured controls (Figure 3.1F). Analysis of the pass rate (the percentage of mice that completed each trial in 300 seconds) revealed no difference between control and stroke-alone mice (Table 3.1). Overall, these data showed that bilateral ET-1 mPFC stroke produced significant cognitive impairments in problem-solving and short-term memory at early times post-stroke.
Figure 3.1. Establishing an endothelin-1 cognitive model of stroke in mice by producing lesions in the mPFC region.

A) Sagittal representation of lesions in the brain region (in red). B) Coronal representation of the mPFC regions (in red). C) Cresyl violet stained 20μm thick coronal tissue sections highlighting mPFC lesions 4 days after surgery (in red). D) Schematic representation of adhesive tape placement on left forepaw (hashed box). E) Post-stroke functional scores (in seconds) 4 days following stroke in the adhesive tape removal test. Unilateral sensorimotor stroke in right hemisphere resulted in contralateral (left) forepaw deficits (n=3). Bilateral mPFC stroke did not result in sensorimotor impairments (n=7/group). F) Short-term cognitive assessment in the PB task. Deficits in problem-solving, short and long-term memory are observed as early as 4 days following mPFC stroke. Significant impairments are observed on Trial 5, 6, and 9 in stroke-alone group (n=18) compared to uninjured controls (n=15). FA2 = frontal area 2, AC = anterior cingulate cortex, PR = prelimbic, IL = infralimbic. *p<0.05, ** p<0.01
Table 3.1. Performance of animals on various behavioural components in the Puzzle Box Task on day 4 to 6 following mPFC

<table>
<thead>
<tr>
<th></th>
<th>Total Animals</th>
<th>% Passed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acquisition (i.e. Problem solving)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 1:</strong> Control</td>
<td>15</td>
<td>100%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>100%</td>
</tr>
<tr>
<td>Trial 2 Stroke + CsA</td>
<td>17</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Day 2:</strong> Control</td>
<td>15</td>
<td>87%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>61%</td>
</tr>
<tr>
<td>Trial 5 Stroke + CsA</td>
<td>17</td>
<td>53%</td>
</tr>
<tr>
<td><strong>Day 3:</strong> Control</td>
<td>15</td>
<td>60%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>56%</td>
</tr>
<tr>
<td>Trial 8 Stroke + CsA</td>
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<td>47%</td>
</tr>
<tr>
<td><strong>Working or Short-Term Memory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 1:</strong> Control</td>
<td>15</td>
<td>100%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>100%</td>
</tr>
<tr>
<td>Trial 3 Stroke + CsA</td>
<td>17</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Day 2:</strong> Control</td>
<td>15</td>
<td>93%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>61%</td>
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<tr>
<td>Trial 6 Stroke + CsA</td>
<td>17</td>
<td>65%</td>
</tr>
<tr>
<td><strong>Day 3:</strong> Control</td>
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<td>67%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>56%</td>
</tr>
<tr>
<td>Trial 9 Stroke + CsA</td>
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<tr>
<td><strong>Long-Term Memory</strong></td>
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<td><strong>Day 2:</strong> Control</td>
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<td>100%</td>
</tr>
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<td>Stroke Alone</td>
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<td>100%</td>
</tr>
<tr>
<td>Trial 4 Stroke + CsA</td>
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<td>100%</td>
</tr>
<tr>
<td><strong>Day 3:</strong> Control</td>
<td>15</td>
<td>93%</td>
</tr>
<tr>
<td>Stroke Alone</td>
<td>18</td>
<td>61%</td>
</tr>
<tr>
<td>Trial 7 Stroke + CsA</td>
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<td>59%</td>
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3.2 CsA administration increases the size of the NPC pool

Next, I asked how mPFC stroke and/or early administration of CsA following mPFC stroke affected the NPC pool using the in vitro colony-forming assay (neurosphere assay). Neurospheres are clonally derived colonies of NPCs that originate from a single stem cell; hence, the number of neurospheres reflects the size of the NPC pool. Mice were given an ET-1 mPFC stroke, with or without CsA treatment, and were sacrificed 7 days post-stroke. Tissue was cultured and the numbers of SE derived neurospheres was assessed after 7 days in vitro (see timeline on Figure 3.2A). A 1.4 fold and a significant 1.7 fold increase in the numbers of neurospheres was observed in stroke-alone and stroke+CsA group, respectively, compared to uninjured controls (control: 39 ± 8 spheres; stroke-alone: 54 ± 2 spheres; stroke+CsA: 65 ± 16 spheres per 5000 cells; F(2,6)=7.473, p=0.0235, ANOVA; Figure 3.2B). Thus, CsA treatment following mPFC stroke significantly increases the NPC population.

3.3 Cyclosporine A does not provide neuroprotection following mPFC stroke

CsA has previously been reported to be a neuroprotective agent. Since I delivered CsA starting at the time of stroke, I sought to determine whether CsA was providing neuroprotection in the stroke-alone brain. Following ischemic stroke, a gradient of damage occurs within the affected region, comprising the core and the penumbra, respectively. The core region undergoes irreversible damage (i.e. cell death) within minutes after insult. The surrounding penumbra contains functionally viable cells for several hours to days after the insult. I examined (i) neural degeneration and (ii) the lesion volume in the stroke only and stroke+CsA groups on day 1, 4 and 10 post-
mPFC stroke. FJC, a polyanionic fluorochrome, was used to label degenerating neurons (Figure 3.2C). The numbers of FJC+ cells were counted in stroke-alone and stroke+CsA groups within the lesion (±0.64mm³). No significant difference was found in the numbers of FJC+ cells between stroke-alone versus (vs) stroke+CsA on day 1 (stroke-alone: 268,799 ± 24,878 cells; stroke+CsA: 280,089 ± 47,471 cells), day 4 (stroke-alone: 134,071 ± 36,015 cells; stroke+CsA: 189,036 ± 71,746 cells), or day 10 (stroke-alone: 18,480 ± 2,372 cells; stroke+CsA: 6,209 ± 655 cells) in the same volume (F(1,20)=0.2132, p=0.6492, ANOVA; Figure 3.2D). The total numbers of FJC+ cells decreased over time with significantly fewer FJC+ cells on day 10 post-stroke in both the stroke-alone and stroke+CsA groups compared to day 1 (p_{Day1-10:stroke-aloneVSstroke-alone}=0.0212, p_{Day1-10:stroke-aloneVSstroke+CsA}=0.0146, p_{Day1-10:stroke+CsAVSstroke-alone}=0.0150, p_{Day1-10:stroke+CsAVSstroke+CsA}=0.0102, Tukey’s multiple comparison test, ANOVA; Figure 3.2D). Importantly, there was no significant difference observed between stroke-alone and stroke+CsA groups at any time examined. Consistent with these findings, the lesion volume analysis (Figure 3.2E) showed no significant differences in relative fold change between the stroke-alone and stroke+CsA groups at any of the times examined (F(1,24)=0.3347, p=0.5683, ANOVA; Figure 3.2F). Taken together, these findings reveal that CsA treatment beginning at the time of stroke did not impact the lesion volume or neural degeneration post-stroke.
Figure 3.2. CsA treatment significantly increases the size of the endogenous NPC pool but is not neuroprotective

A) Experimental paradigm for evaluating in vitro NPC activation following mPFC stroke. B) Post-stroke CsA treatment results in a significant 1.7 fold (n=5) in the number of neurospheres compared to controls (n=7). C) FJC+ cells in a 5x magnified image with an inset of 20x magnification image highlighting FJC+ cells 4 days post-stroke (white arrows) on the infarct site. D) FJC+ cell counts on day 1 (n=5/group), 4 (n=5/group) and 10 (n=3/group) post-stroke compared to controls (n=7). A significant decrease in the number of FJC+ cells are observed on day 10 in stroke-alone and stroke+CsA compared to day 1. E) A schematic representation of lesion (in red outline) that was calculated for lesion volume using cresyl violet staining. F) No significant difference is stroke-alone vs stroke+CsA is observed at any times examined (day 1, n=5/group; day 4, n=5/group; day 10, n=5/group). *p<0.05, **p<0.01
3.4 LONG-TERM CSA ADMINISTRATION PROMOTES RECOVERY OF PERSISTING COGNITIVE DEFICITS FOLLOWING MPFC STROKE

Consistent with the lack of neuroprotective benefits from CsA following mPFC stroke, short-term behavioural deficits observed in the stroke+CsA group in the PB task were not improved at early times post-stroke. Similar to stroke-alone, stroke+CsA had deficits on trials 5 and 6 (trial 5: 256.94 ± 13 s; trial 6: 185.59 ± 26 s; p<0.01) compared to uninjured controls. There were no significant differences between stroke-alone and stroke+CsA across trials (Figure 3.3A). Hence, CsA administration beginning at the time of mPFC stroke was not sufficient to improve deficits in problem solving, short-term memory, or long-term memory at early times post-stroke (day 4-6). Similar to mPFC stroke-alone, stroke+CsA treated mice did not show motor impairments on the adhesive removal test (F(1,11)=0.1945, p=0.6677, ANOVA; Figure 3.3B).

Next, we asked whether long-term administration of CsA would promote cognitive recovery post-stroke as defined by mice behaving similar to uninjured controls. The same cohorts of mice were tested in the PB task at early (day 4-6) and late (day 22-24 and day 45-47) times post-stroke (Figure 3.4A). Two long-term time points were chosen based on previous work demonstrating that EF deficits are often revealed at later times post stroke. At both late time points the stroke-alone group, but not stroke+CsA group, demonstrated persistent behavioural deficits compared to uninjured controls (Figure 3.4B). At day 22-24, stroke-alone exhibited significant impairments on trial 6 (stroke-alone: 230.22 ± 20 s; control: 142.22 ± 26; p_{trial6}=0.0285, Dunnett’s multiple comparison test, ANOVA) and trial 7 (stroke-alone: 263.67 ± 18 s; control: 180.33 ± 42; p_{trial7}=0.0398, Dunnett’s multiple comparison test, ANOVA), whereas,
stroke+CsA showed no significant difference from controls in either trial (trial 6: 183.91 ± 24 s; trial 7: 236.64 ± 16 s). Trial 6 (on day 2) and Trial 7 (on day 3) are repetitions of trial 5 (on day 2), which involves digging bedding to get to goal zone. Hence, the stroke-alone group showed deficits in both short-term and long-term memory (Figure 3.4B) and this was not observed with stroke+CsA, which were not significantly different from controls.

By day 45–47, the stroke-alone group displayed a significant impairment on trial 8 (new task acquisition involving the removal of a cardboard plug from the underpass to reach the goal zone) compared to uninjured controls (stroke-alone: 171.44 ± 21 s; control: 102.64 ± 24 s; \( p_{\text{trials}} = 0.0180 \), Dunnett’s multiple comparison test, ANOVA; Figure 3.4B). Hence EF impairments persist over time leading to impaired problem-solving abilities. No impairment was observed in short-term memory or long-term memory by day 45–47 post-stroke (Figure 3.4C). Notably, while CsA treatment did not significantly improve cognitive recovery compared to the stroke-alone group at the long-term time points (day 22-24 and day 45-47), CsA treated mice did not show any persisting cognitive deficits compared to uninjured controls on trial 6, 7 or 8 either. Hence, long-term CsA treatment administration demonstrated beneficial effects in promoting cognitive recovery.

In an attempt to confirm that the increased latency to complete the tasks in the PB task was due to impaired EF and not the result of anxiety-like behavior, we performed the open field task at early (day 7) and late (day 48) time points post-stroke (Figure 3.4A). Mice were allowed to explore a square 60 cm x 60 cm box for 10
minutes and the amount of time spent in the corner zones was assessed at 1, 5 and 10 minutes (Figure 3.4D). No significant differences were observed in the amount of time (duration) spent in corner zones amongst stroke-alone, stroke+CsA and uninjured controls at any time examined (F(2,27)=1.243, p=0.3045, ANOVA). In addition, stroke-alone and stroke+CsA did not significantly differ in locomotor activity in this task compared to uninjured controls, supporting our finding that motor function was not impaired with mPFC stroke.

To assess whether mPFC stroke resulted in deficits in frontal lobe mediated spatial working memory, a separate set of experiments were performed where mice were tested on the Y-maze spontaneous alternation task on day 49 post-stroke. The Y-maze task assesses spontaneous alternations percentage (i.e. the number of times animals chose to visit a novel arm as opposed to returning to the previously visited arm divided by the total number of alternations; SAP, %). There were no significant differences observed in the performance of the Y-maze task between groups (stroke-alone: 56.3% ± 2%; stroke+CsA: 60.5% ± 4%; control: 50.4% ± 5%; F(2,17)=2.052, p=0.1591, ANOVA; Figure 3.4E). Hence, mPFC stroke did not result in deficits in spatial working memory.
Figure 3.3. Short-term CsA treatment following mPFC stroke does not affect cognitive or sensorimotor function.

A) No significant differences observed between stroke alone (n=18) and stroke+CsA (n=17) on day 4-6 in the Puzzle Box Task. B) No significant differences observed between stroke-alone (n=7) and stroke+CsA (n=6) or within groups in left and right forepaw performance in the Adhesive Tape Removal Task.
Figure 3.4. Long-term CsA treatment shows cognitive recovery following mPFC stroke in the Puzzle Box task, but stroke ± CsA treatment does not produce in spatial memory deficits or anxiety-like behaviour.

A) Experimental paradigm for long-term behavioural assays. B) Long-term behavioural assessment on the PB task on Day 22-24. Stroke-alone (n=9), but not stroke+CsA (n=11), shows persisting deficits on trial 6 and 7 (short-term and long-term memory) compared to controls (n=9). C) Long-term behavioural assessment on the PB task on Day 45-47. Stroke alone (n=18), but not stroke+CsA (n=16) show deficit in task acquisition (trial 8) compared to controls (n=14). D) No significant difference between groups (stroke alone, n=11; stroke+CsA, n=11; control, n=8) in time spent in corner zones (in seconds) with 10 minutes in the Open Field task in short-term (day 7) or long-term (day 48). E) Percentage of spontaneous alternations in the Y-maze task. No differences are observed between the stroke-alone (n=8), stroke+CsA (n=8) and control (n=4) groups in their spatial memory functioning. PB = puzzle box; OF = open field; YM = Y-maze. *p<0.05, **p<0.01
3.5 NPCs migrate to the site of injury following mPFC stroke

Previous findings have shown NPC migration to the site of the injury at early times following sensorimotor stroke, in the presence or absence of CsA treatment.\textsuperscript{98,130} We asked whether similar NPC migration was seen following mPFC stroke. On day 60 post-stroke (4 days after the end of CsA treatment), neurosphere cultures were prepared from uninjured controls, stroke-alone and stroke+CsA treated mice. We observed no significant differences in the number of SE derived neurospheres between the groups (F(2,27)=1.369, p=0.2715, ANOVA). However, there was a trend towards decreased NPC pool in the SE following long-term CsA treatment (Figure 3.5). Interestingly, when the neurosphere assay was performed from the cortical injury site, both the stroke-alone and stroke+CsA had cortical neurospheres (14% versus 43%, respectively). These finding suggest that CsA administration following mPFC stroke promotes NPC migration to the injury site.
Figure 3.5. Long-term CsA treatment does not expand NPC pool following mPFC stroke.

A) Experimental timeline of long-term neurosphere assay performed 60 days following Stroke ± CsA treatment. B) There were no significant differences observed between stroke-alone, stroke + CsA and controls (n=6 per group).
CHAPTER 4 DISCUSSION

4.1 SUMMARY OF CURRENT FINDINGS

In this study, we investigated the efficacy of endogenous NPC activation using CsA to promote cognitive recovery following stroke. We validated the reproducibility of a cognitive model of stroke in mice by inducing bilateral ET-1 lesions in the mPFC region. The stroke lesion resulted in cognitive deficits as early as 4 days post-stroke without affecting motor function, similar to findings previously shown in rats. We demonstrated that administration of CsA following mPFC stroke expanded the SE derived NPC pool at early times post-stroke, but this expansion was not maintained long-term. CsA administration beginning at the time of stroke did not affect cell death or decrease lesion volume, and did not improve short-term cognitive deficits in the PB task. Long-term behavioural assessment showed persisting deficits in the PB task in stroke-alone mice, whereas, long-term CsA treatment reduced these deficits such that stroke+CsA mice were not significantly impaired compared to uninjured controls. Hence, our findings demonstrated that activation strategy using CsA has potential to enhance recovery following mPFC stroke.

4.2 MODULATING NPC POOL BEHAVIOUR: SHORT AND LONG-TERM STUDIES

Previous work has demonstrated through the neurosphere assay that sensorimotor cortical stroke alone is sufficient to expand the NPC pool at 7 days post-injury. The same form of significant activation was not observed following mPFC stroke. This could be due to a number of reasons. First, in comparison to the sensorimotor cortex, the mPFC is more rostral to the SE where the resident NPCs are found. As such, the increased distance between injury and NSC niche caused reduced NPC activation.
Second, the injury induced expansion of the NPC pool could have occurred at an earlier or later time than we examined. The longer distance between injury and the NPC niche, as well as the fact that the lesions were bilateral in our study (and are unilateral in most models) may influence the time of activation. If NPC activation within the SE was highest on day 4 post-stroke, similar to previous observations, then it is possible that the cells started to die or migrate away from SE by day 7. This could result in the decreased number of neurospheres from SE as observed in our findings. To test our hypothesis on NPC migration, we performed the neurosphere assay on day 7 from the cortical mPFC injury site. No cortical neurosphere formation on day 7 post-stroke with or without early CsA administration suggested that cells were not reaching the mPFC injury site within 7 days. However, CsA administration for 7 days post-stroke resulted in an increase in the number of neurospheres from SE. These findings support previous work showing that CsA promotes cell survival.

During long-term (day 60 post-stroke) assessment, post-stroke NPC activation within the SE was not maintained in the presence or absence of CsA treatment. Similar to previous observations following sensorimotor stroke, where the numbers of NPCs returns to control levels at later times, we found no change in the numbers of neurospheres between groups following mPFC stroke in vitro. Interestingly, we found that stroke+CsA treated mice had a decreasing trend in the number of SE derived neurospheres, which suggested that NPCs either underwent cell death or migrated away from the SE. Indeed, cortical neurospheres were never observed in control (non-stroke) cortical tissue however, cortical neurosphere were derived from the mPFC injury site from both stroke-alone and stroke+CsA groups, demonstrated NPC
migration. Other possible explanations could include severity of the injury resulting in maximum activation of SE-derived NPCs at early time points, which plateaued long-term. This is supported by previous work which has demonstrated that stem cells that undergo extensive proliferation become “exhausted” and are unable to maintain long-term proliferation. Transplantation studies by Piccin and colleagues (2014) showed that the rodent neurogenic niches in old and young mice affect the proliferative status of NPCs. Changes in the cytoarchitecture and cell signaling in the young neurogenic niche is lost in old age, contributing to a significant decrease in proliferative and migratory capacity of NPCs following stroke. Although the possibility of stem cells becoming more “quiescent” with age is valid, this was not applicable to the current study. Experiments utilizing transgenic animal models that allow direct tracking of NPCs would help to clearly elucidate underlying cell behaviour following stroke in vivo.

### 4.3 Short and Long-term Post-Stroke Cognitive Performances

Following mPFC stroke, short-term deficits were observed as early as day 4. Generally, deficits were found as early as day 4 following sensorimotor stroke as well. However, early administration of CsA did not help rescue these early cognitive deficits, similar to findings in sensorimotor stroke models. Following our short-term neurosphere assay within the mPFC cortical region, no neurospheres were found. This was suggestive of the possibility that NPCs did not migrate to the injury site as early as day 7. This finding explains why early cognitive recovery was not observed. Alternatively, if cells did migrate close to the injury region, it is possible that appropriate circuitry needs to be reestablished to improve cognitive performance, which is not possible as early as day
4. Following stroke, the ischemic lesion is a hostile area that continues to undergo cell death from minutes to months, severely disrupting vascular and neural circuitry. This results in unpredictable, and often severe, cognitive impairments. These may not be directly regulated by the affected region, but may be an unwanted outcome of the damaged neural circuitry. Indeed, studies have shown that specificity or localization of cognitive functions do not directly correlate with restricted anatomical areas. Rather, it is the collective processing of a vast neural network, connected by cortical and subcortical regions that underlies the behaviour. Furthermore, it is suggested that behavioural analyses should take place at least a week after injury and/or intervention to allow animals to recovery from surgical procedures. Hence, it is difficult to regain performance abilities so early following injury.

In humans, cognitive impairments persist long-term after stroke. This was supported by current findings as we continued to observe long-term cognitive deficits in the stroke-alone group. Accordingly, we examined the therapeutic benefits of long-term CsA administration on cognitive recovery, which also allowed time for animals to recover completely from physical weakness or complications resulting from surgeries. Long-term CsA administration reduced cognitive deficits but did not significantly promote recovery compared to the control group. The inability of CsA to significantly promote long-term cognitive recovery could have a few explanations. First, CsA may exert its benefits in a time-dependent manner, such that CsA is important for an early NPC expansion, which is insufficient in rescuing gradually declining cognitive function. Previously, our group has shown that CsA alone is able to maintain cell survival long-term. Therefore, it is worthwhile to investigate if
extending the duration of CsA administration can significantly promote cognitive recovery.

Second, it is possible that CsA alone is not sufficient to reverse cognitive impairments, as the expansion of the NPC pool is not actively contributing to reestablishment of the neural circuitry, which is essential for complex cognitive processing. Thus, strategies that promote plasticity, combined with the increased numbers of NPCs, may be more effective. Recent findings have shown that metformin (Met) a drug used to treat type II diabetes, can enhance neurogenesis in the adult brain and enhance spatial memory function in the water maze task.\textsuperscript{176} Sequential administration of EGF and EPO has also shown to promote recovery in the adult brain and this is thought to be the result of EGF promoting NPC expansion and EPO promoting neuronal differentiation and angiogenesis.\textsuperscript{228} Further, rehabilitation (i.e. enriched environment) in combination with growth factor infusion substantially accelerates functional recovery following motor stroke.\textsuperscript{174} Based on these findings, integrating adjunct therapies, such as sequential administration of Met, EPO or introducing rehabilitation in combination with our neural repair strategy using CsA to promote post-stroke cognitive recovery is worth investigating. In addition to using combinatorial therapeutic strategies, efforts must be directed towards understanding the contribution of underlying neural mechanisms in cognitive recovery, such as axonal sprouting, dendritic arborization, and synaptogenesis.

Third, animals were exposed to the PB task on a number of occasions (4 days, 22 days and 45 days post-stroke), which could encourage learning of the task. This could
potentially confound and mask the extent of impairments observed, and improve performance. However, cognitive deficits did persist at the later time points. We found that animals exposed to the PB task on day 4 and 45 (twice) versus those exposed to the task on day 4, 22 and 45 (three times) had no differences in their performance ability at day 45–47. Hence, multiple exposures to the PB task did not rescue cognitive impairments this study.

Lastly, a single behavioural task was used to assess cognitive deficits in the study. However, the task was time-efficient, easy to conduct and tested various cognitive aspects. More importantly, the task produced consistent, and consequently, reliable results. It has been a continuous challenge to develop sensitive cognitive assays that will highlight EF deficits in rodents. As mentioned earlier, assays like the water maze and radial maze have been used, but differences in reported outcomes have made it difficult to rely on these tasks as a measure of success for therapeutic interventions. One of the advantages to using the water maze task is that it eliminates the motivation component in performance since the task relies on survival behaviour. Other promising tasks that can be used to measure EF include a relatively new touch screen task that can sensitively assess a battery of cognitive components (i.e. attention, inhibition and memory).229,230 The complexity of EF warrants selection of proper behavioural assays which will be sensitive to high-level cognitive components that drive EF prior to moving forward with studies where efficacy of therapeutic interventions are examined. Ideally, the efficacy of the model must be tested in more than one behavioural assay prior to testing the benefits of CsA as a treatment intervention.
4.4. Drug Toxicity and Administration

Clinically, CsA has been used to treat a wide range of disorders where immunological dysregulation is a common etiological factor. Apart from prophylaxis, CsA treatment has shown to be effective for many diseases, such as, nephritis, thyroiditis, multiple sclerosis, pancreatitis, arthritis and psoriasis. With its extensive application in clinical settings, toxicological effects have also been well documented. CsA can have profound effects on renal functioning by altering release of substances such as angiotensin II, prostaglandins and endothelin. As a result hypertension has been a common side effect in CsA users. Neurologically, CsA has been shown to cause mental dysfunction, cortical blindness, headache and seizures, tremor, peripheral neuropathy, diffuse encephalopathy, and others. CsA has also shown to be hepatotoxic. Mild elevations in serum bilirubin, aminotransferase and alkaline phosphatase levels have been observed, although downstream levels are self-limiting and do not require considerable dose adjustment. Thus, to avoid relapse, CsA is suggested to be administered at the lowest effective dosage (oral or intravenous) as it is rapidly absorbed by blood cells and plasma and has a bioavailability of 90% in healthy subjects.

In the present study a single and continuous dosage of CsA was systemically administered in animals. A clinically suitable dosage was used that has previously shown to be effective and safe. CsA was diluted in a cremaphor-based vehicle, as CsA is not an effective water-soluble molecule and cannot freely pass the blood-brain barrier. Although, in our current study, usage of cremaphor as a vehicle did not have detrimental or apparent physiological effects on animals, alternative and more organic (i.e. olive oil) drug formulation vehicles should be considered to have better control of
toxicological implications since cremaphor has been shown to exert a wide range of adverse effects.\textsuperscript{236}

It is acknowledged that systemic delivery of CsA may pose potential risks of unwanted toxicity, which may not be suitable for clinical settings. Therefore devising safe and effective delivery strategies has become an important consideration. Recent findings demonstrated that CsA can be directly administered into the brain thereby circumventing the blood brain barrier.\textsuperscript{237} Following sensorimotor stroke, CsA was locally delivered into a site of interest via a hydrogel encapsulation comprising of hyaluronan, methylcellulose and poly(lactic-co-glycolic acid) microparticles. CsA was effectively delivered for 14 days. Combination of hydrogel and CsA provided dual benefits of endogenous NPC activation and tissue protection which has been previously observed following systemic delivery.\textsuperscript{140} Therefore, this local delivery strategy provides a new avenue of safe CsA administration without incurring unnecessary risk of systemic toxicity and global immunosuppression.

4.5. \textbf{Sexual Dimorphism}
In this study, only male mice were used. There is potential for this to impact the outcome of our studies. Indeed, treatment outcome, stroke severity, and epidemiology varies between male and female stroke patients.\textsuperscript{238} For example, although women have lower incidences of stroke in comparison to men, stroke-affected women have more severe disabilities.\textsuperscript{239} Sex differences are also observed in animal models of injury as recently reviewed.\textsuperscript{240} Similar to humans, cell death following ischemia varies in male and female.\textsuperscript{238,241} With respect to stem cell-based therapies, stemness is also influenced
by sexual dimorphism. For example, bone-marrow derived stem cells are more abundant in response to traumatic injuries in males than females\textsuperscript{242} Variances in neurogenic properties in male rodents promote NSCs to adopt more neuronal or oligodendroglial lineages, where females are more prone to astrocytic fates.\textsuperscript{243} It is important to address the sex and age differences that will impact the development of novel therapeutic interventions using stem cell based approaches.

4.6 Future Directions

To advance knowledge of therapeutic benefits of an endogenous stem cell based strategy in promoting post-stroke cognitive recovery a number of factors should be considered. First, to devise effective cell replacement strategies, it is important to use tools that will facilitate an understanding of NPC behaviour and the underlying mechanisms \textit{in vivo} following injury. In this current study, much of our understanding of NPC behaviour following mPFC stroke depended on studies that examined NPC behaviour using \textit{in vitro} assays. Towards the goal of \textit{in vivo} analysis, lineage tracking of adult NPCs following mPFC stroke, with or without CsA treatment, could be achieved using transgenic animal models that help us directly track NSCs as well as NPCs. For example, an inducible Nestin-CreER\textsuperscript{T2} X ROSA/YFP transgenic mouse model can permanently label a cohort of Nestin-expressing cells by expressing yellow fluorescent protein (YFP) following tamoxifen (TAM) administration.\textsuperscript{140} Using this tool, one could elucidate the migratory behaviour of NPCs at different time points post stroke and/or treatment. Moreover, co-labeling nestin-expressing NPCs with different neural phenotypes will help us determine the fate of NPCs. This allows us to assess whether CsA is effective as a stand-alone treatment, or whether sequential administration of
drugs that induce neurogenesis or gliogenesis (for example) can help reestablish neural circuitry.

While working with in vivo transgenic models, it is important to consider their advantages and disadvantages. Many transgenic lines have been designed that use nestin as the promoter, however, Nestin-expressing NPCs is one amongst many types of NPCs. In addition, direct NSC behaviour is difficult to track using nestin transgenic lines. NSCs along with their end-progeny are a heterogenous population of cells which cannot be classified using one marker or morphological characteristic. GFAP is undeniably one of the more common markers used to identify “astrocytic” NSCs within the SE and have been used to study the cell population in activated or quiescent stages, independently. Selective ablation of GFAP-expressing cells in a transgenic line expresses thymidine kinase, following exposure to ganciclovir, has been imperative in in the understanding of a primitive NSC population upstream of adult NSCs. Further, GFAP provides us an alternative way to mark NPCs of different cellular fates by co-labeling the population with differentiated precursor markers, such as DCX for neuronal precursors. Some other NSC markers include Yamanaka factor SOX2, ASCL1, and GLAST.

Second, understanding how CsA treatment with adjunct therapies can affect long-term cognitive recovery is important. In 2016, Schuch et al. demonstrated the efficacy of a combinatorial treatment strategy using CsA with enriched rehabilitation, which comprised of environmental enrichment along with motor training (i.e. daily reach). Following neonatal hypoxic ischemic injury, rats received various treatment
combinations for 4 weeks post-stroke, which included CsA or vehicle with or without rehabilitation. Enriched rehabilitation alone improved performance in skilled-reaching task and exploratory behaviour in the open field task, although no direct effects on cortical tissue recovery. It would be important to test out the combinatorial efficacy of CsA treatment with enriched rehabilitation relevant to behavioural recovery. As delayed CsA treatment has previously showed no negative effects on recovery following sensorimotor stroke, it would be important to ask the same question following mPFC stroke. With this in mind, CsA treatment would start 3 days following mPFC stroke. CsA can be given in adjunction to rehabilitation for varying lengths of time (4 and 6 weeks). Behavioural tests must be administered at an early time point (to address existence of deficits) and at long-term (2, 4 and 6 week) time points to address long-term cognitive recovery. It is predicted that animals that with enriched rehabilitation in combination with CsA will show enhanced cognitive recovery compared to CsA as a stand-alone treatment.

In the current study, cognitive performance was measured primarily using the PB task. In future, it will be important to examine cognitive recovery by assessing performance on more than one behavioural assay (i.e. PB task + attentional set-shifting task using touch screen). Simultaneously, an appropriate motor task must be implemented to rule out impairments (e.g. digging behaviour for the PB task). With these considerations in mind, a better understanding of the efficacy of cell replacement strategies in promoting cognitive recovery in the mPFC mouse model of stroke can be determined.
CONCLUSION

In summary, the development of this reproducible cognitive model of stroke provides a foundation to examine the efficacy of novel therapeutics to enhance recovery in mice. Collective understanding based on previous\textsuperscript{140,215,228} and current findings by our group reveal that cell based intervention efficacy may depend on the location of injury (with respect to neurogenic niche) and the resulting impairments. While CsA has been shown to activate NPCs in the \textit{SE}\textsuperscript{235} and \textit{DG}\textsuperscript{141} of the adult forebrain and promote recovery after sensorimotor cortical stroke\textsuperscript{140}, there is room for improvement in terms of its efficacy as a therapeutic agent to promote cognitive recovery.
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