Characterisation of endogenous repair mechanisms following endothelin-1 induced stroke in rats and the effects of human adult stem cell transplant to support brain reconstruction

Hima Charika Senanayaka Abeysinghe

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Department of Surgery (St. Vincent’s Hospital Campus)
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In memory of Dr Nao Kobayashi, March 20th 2014
Abstract

Brain injury from stroke often results in permanent damage and disability due to neurons failing to re-establish lost connections. Potential for brain regeneration relies heavily on the surrounding microenvironment. Contributions from inflammatory cells, angiogenesis and stem cells all require collective consideration when investigating treatment options. Understanding this paradigm is critical to developing therapies that promote recovery. Cell-based therapies offer hope in rescuing the stroke affected brain and restoring function. However, differentiation of transplanted cells into significant neuronal populations is yet to be realised. The work presented in this thesis investigates cellular responses to brain injury and repair mechanisms activated following stroke using the endothelin-1 model of focal cerebral ischemia in conscious rats. Additionally these studies explore the potential of human adult neural progenitor cells to support brain repair.

First, we investigated aspects of brain remodelling initiated following stroke, including the impact of lesion size on angiogenesis, cell responses within the subventricular zone (SVZ), inflammation, and scar formation. Immunohistochemical analysis revealed a positive correlation between stroke severity and the degree of pathological responses to recovery after stroke. Stroke severity was found to increase cell proliferation and migration from the SVZ, with many of these cells positive for GFAP and incorporated into the glial scar. Therefore, we highlight this as an important factor to consider when developing treatment strategies that stimulate cell responses within the neurogenic niche.

Long term survival and success of non-autologous stem cell transplants requires use of immunosuppressive agents such as cyclosporine A (CsA). The influence of CsA on stroke outcome required investigation prior to commencing the intended stem cell transplant studies. We explored the effects of CsA administration on neurological and histological outcomes 7 days after stroke. Findings indicated CsA treatment significantly reduced the development of neurological deficits after stroke but did not affect infarct volume, activation of microglia/macrophages, or events within the neurogenic niche. CsA treatment did however attenuate reactive gliosis after stroke and retained pro-survival astrocytic phenotypes important for supporting neuronal rescue.
Finally, we investigated the use of cell-based therapies to promote brain repair. SVZ-derived human neural progenitor cells (hNPCs) were isolated, characterized, and differentiated into GABAergic neurons. Pre-differentiated GABAergic neurons, undifferentiated SVZ-hNPCs or media alone were transplanted into the rat brain 7 days after stroke during angiogenesis as it was hoped exogenous transplants would benefit from a redeveloped microvascular bed. GABAergic cell transplants were observed to accelerate functional recovery, showed evidence of maturation and promoted endogenous neurogenesis 28 days post-transplant. Undifferentiated hNPC transplants were predominantly GFAP positive and incorporated into the glial scar. These results suggest techniques aimed at differentiating cells into a neural lineage prior to transplant may be a favourable alternative for stroke treatment. Furthermore, targeting angiogenesis for cell-based treatments may offer greater survival of cells and support graft maturation for functional recovery.

In conclusion, this work contributes significantly towards characterising endogenous cellular responses to stroke injury and recovery, and provides preclinical evidence for the benefits of directing exogenous stem cell differentiation towards neural lineage cells prior to transplant, resulting in accelerated recovery.
Declaration

This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Hima Abeysinghe

December 2014
Preface

Chapter 3 of this thesis represents work that has been published (Abeysinghe et al. 2014) and Chapter 4 represents work currently under review. I independently carried out all *in vivo* experiments described, analysed the data, wrote the initial manuscript draft and performed subsequent editing in response to supervisors’ suggestions and comments. Dr Carli Roulston, my principal supervisor, co-ordinated and supervised all aspects of these projects where *in vivo* experiments are described and was involved in the analysis and interpretation of data. Dr Roulston jointly conceived these projects with Prof Gregory Dusting and funding was provided through National Stroke Foundation, The Bethlehem Griffiths Foundation, and National Health and Medical Research Council Australia grants which covered all research expenditures. Both Dr Roulston and Prof Dusting were involved in editing and the final approval of these chapters.

Chapter 5 describes work that has been jointly conceived by Dr Roulston and Prof Greg Dusting and funding was provided through the Brain Foundation Research and National Health and Medical Research Council Australia grants. A/Prof Jeremy Crook and Dr Nao Kobayashi provided human subventricular-zone derived neural progenitor cells used for the study and were involved in the development and refinement of the project. Due to the study design, I independently carried out all *in vivo* experiments while Dr Nao Kobayashi, my co-supervisor, co-ordinated and performed all *in vitro* cell culture experiments and co-supervised the study design. Ms Laita Bokhari greatly assisted in the collection and assembly of data. Dr Carli Roulston supervised all aspects of the project where *in vivo* experiments are described. Dr Carli Roulston, Dr Nao Kobayashi, Ms Laita Bokhari and I were involved in the analysis and interpretation of the results. I wrote the initial manuscript draft and performed the subsequent editing as required based on Dr Carli Roulston and Dr Nao Kobayashi’s comments. Dr Nao Kobayashi passed away 20th March 2014, and unfortunately could not review this chapter or thesis. Dr Anita Quigley assisted in the final assembly of in vitro data in Dr Kobayashi’s absence. Dr Roulston, Prof Dusting and A/Prof Jeremy Crook were involved in editing the manuscript, and final approval of this thesis.

My contribution to experiments in each of the results chapters involved collection and assembly of data, statistical analysis and interpretation, and manuscript writing.
Therefore, I estimate that my contribution to the work and data presented in this thesis is greater than 80%.

I acknowledge the important contribution from others to experiments conducted and presented in this thesis:

Chapter 3:
- Dr Carli Roulston: Conception and design, provision of study material, assembly of data, data analysis and interpretation, manuscript editing, final approval of manuscript, financial support.
- Prof Gregory Dusting: Conception, final approval of manuscript, financial support.
- Laita Bokhari: Collection and assembly of data, data analysis and interpretation.

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- Dr Carli Roulston: Conception and design, provision of study material, assembly of data, data analysis and interpretation, manuscript editing, final approval of manuscript, financial support.
- Prof Gregory Dusting: Conception, final approval of manuscript, financial support.
- Laita Bokhari: Collection and assembly of data, data analysis and interpretation.

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- Dr Carli Roulston: Conception and design, provision of study material, assembly of data, data analysis and interpretation, manuscript editing, final approval of manuscript, financial support.
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- Prof Gregory Dusting: Conception, final approval of manuscript, financial support.
- A/Prof Jeremy Crook: Provision of study material, final approval of manuscript.
- Dr Anita Quigley: Collection of data.
- Laita Bokhari: Collection and assembly of data, data analysis and interpretation.
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Special thanks to my fellow postgraduate students Aaron Dingle, Damián Hernández, Cletus Pinto and Mark Waddingham, as well as the staff from the O’Brien Institute, namely Yi-wen Gerrand, Jason Palmer and Effie Keramidaris for their friendship and for teaching me to stress less over events that are out of my control. I would like to thank Zerina Lokmic, for her remarkable and broad intellect and mentorship, as well as Sarah McCann for her friendship and moral support. In addition, a thank you to the wonderfully cheerful ladies of the EMSU, Anna Deftereos, Amanda Rixon, Lilianna Pepe, and Sue Pierce for their kindness, assistance and friendship throughout these past few years.

Finally, I’d like to thank my beautiful family, my parents Tilak and Kumari Abeysinghe and brother Chatu Abeysinghe, for their unconditional love and support (financial and non-financial), and of course their persistent requests to keep in touch and ensure I’m taking care of my health, for which I am extremely grateful. A special thank you to Annette Webb for helping organise ‘Secret Sunday’s’ and culturing my life with the beautiful art of ballet. I especially would like to thank my dearest friends (and counsellors) Kim Steegh, for going above and beyond our friendship to help me countless number of times, and Sarah Buxton for her love and keeping me level headed throughout the years. To my loving boyfriend, Phil Neckers, words cannot express my boundless gratitude for your day to day support, encouragement, and enviable patience, you helped me finish this journey and forged my desire to achieve in my career and most importantly, life. I hold you all very close to my heart and would not be where I am today without you. Thank you.
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)</td>
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<tr>
<td>hNPCs</td>
<td>Human neural precursor/progenitor cells</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>LV</td>
<td>Lateral ventricle</td>
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<tr>
<td>BCSFB</td>
<td>Blood-cerebrospinal fluid barrier</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MCAo</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenosine dinucleotide phosphate</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>NINDS</td>
<td>National Institute of Neurological Disorders and Stroke</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>NMEDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthases</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural precursor/progenitor cells</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
<tr>
<td>OE</td>
<td>Olfactory epithelium</td>
</tr>
<tr>
<td>ORNs</td>
<td>Olfactory receptor neurons</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RM</td>
<td>Repeated measures</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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## Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>rt-PA</td>
<td>recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal derived factor-1</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
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<tr>
<td>STAIR</td>
<td>Stroke Therapy Academic Industry Roundtable</td>
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<tr>
<td>STEPS</td>
<td>Stem Cell Therapies as an Emerging Paradigm in Stroke</td>
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<tr>
<td>SVZ</td>
<td>subventricular zone</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>µm</td>
<td>micrometres</td>
</tr>
<tr>
<td>µl</td>
<td>microlitres</td>
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Peer reviewed research article
Abeysinghe H, Bokhari L, Dusting GJ, Roulston, CL. *Treatment with cyclosporine A reduces overactivation of astrocytes and averts deficits following transient focal ischemia in rats.* Submitted to Journal of Neurology and Neurophysiology.

Research article in preparation

Conference abstracts
*Oral:*

Abeysinghe H, Malakooti N, Crook J, Dusting GJ, Roulston CL, Kobayashi NR (2012) *Pre-differentiated subventricular-zone derived human neural stem cells repopulate the stroke damaged brain.* 22nd Annual St. Vincent’s Research Week; Melbourne, Australia, p23

Poster:

Abeysinghe H, Malakooti N, Dusting GJ, Roulston CL (2013) Lesion size affects angiogenesis, neural stem cell proliferation and glial scar formation following endothelin-1 induced stroke. The Aikenhead Centre for Medical Discovery Research Week; Melbourne, Australia, p75


Abeysinghe H, Malakooti N, Bokhari L, Crook J, Dusting GJ, Roulston CL, Kobayashi NR (2012) Neural progenitor cells pre-differentiated prior to transplant promote neuron repopulation within the stroke damaged brain. 8th World Stroke Congress; Brasilia, Brazil, p302

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1.1 Introduction

Cerebrovascular disease, including ischemic or hemorrhagic brain injury (stroke), is the third leading cause of death in the industrialised world (Thom et al. 2006). The current treatment for ischemic stroke is thrombolytic therapy (recombinant tissue plasminogen activator) which has a limited therapeutic window due to feared complications associated with symptomatic intracerebral haemorrhage (Wardlaw et al. 1997). As such less than 5% of patients are eligible for treatment. Although new studies suggest this treatment window might be extended (Donnan and Davis 2008) this too requires careful evaluation of both risks and potential benefits. Without alternative treatment options and with an aging population more prone to stroke, the burden suffered both socially and economically will continue to accelerate.

The relative paucity of curative approaches for stroke has led to widespread evaluation of alternative treatment modalities. Currently there are at least two broad categories of therapies under investigation which are; neuroprotection and neurorestorative approaches. Decades of research have focused on discovering and developing neuroprotective agents that may intervene in the biochemical cascade of events initiated by cerebral ischemia. With over 50 neuroprotective compounds developed that showed success in preclinical animal models (Green and Shuaib 2006), none have improved outcomes in phase III clinical trials. Most patients end up with an expanding lesion and functional loss. Despite this, many sufferers also experience some degree of functional recovery in the weeks after a stroke event (spontaneously or with rehabilitation) and this is in part due to reactivation of dormant “silent” pathways, and/or reorganisation of existing active neural circuits (Carmichael and Chesselet 2002, Cramer 2008b, Jacobs and Donoghue 1991). Furthermore, regenerative events within the brain are also triggered in the days after stroke and this provides a new treatment window of opportunity (Thored et al. 2006).

There now exists clear evidence supporting environmental enrichment strategies through rehabilitation with tasks including constraint-induced movement therapy, physical therapy and exercise to substantially improve neurological recovery after stroke (Nelles 2004, Nudo and Friel 1998, Pascual-Leone et al. 2005). Unfortunately, many stroke survivors are often unable to participate in meaningful physiotherapy until
weeks after stroke, and delayed rehabilitation can often result in suboptimal or worse outcomes (Lang et al. 2013, Wahl and Schwab 2012). In addition, such approaches are limited and can take years to achieve a beneficial result with no guarantee of positive outcomes. Hence the development of therapeutic treatments to compliment current rehabilitation methods may result in better long-term functional outcomes.

Neuro-restorative strategies focusing on cell-based therapies to facilitate brain repair and restoration of function represent a new and promising field for stroke treatment during the subacute and chronic phase of ischemic stroke (Abe 2000). To facilitate the translational development of cellular therapies as a novel and emerging form of stroke treatment, the meeting ‘Stem Cell Therapies as an Emerging Paradigm in Stroke’ (STEPS) was held in 2007 that modelled the format of earlier STAIR (Stroke Therapy Academic Industry Roundtable) proposals that were primarily concerned with the development of neuroprotective approaches (STAIR 1999, The STEPS Participants 2009). Investigators from academia, industry leaders, members of the National Institutes of Health, and the Food and Drug Administration convened in 2007 to discuss research guidelines of investigating cell therapies for stroke (The STEPS Participants 2009). As such preclinical and clinical cell-based studies commenced and demonstrated tremendous potential to improve clinical outcome in stroke patients. Based on this progress, the STEPs participants reconvened in 2009 and then again in 2014, to discuss emerging data, barriers to successful translation, and create an efficient framework to guide future investigations (Savitz et al. 2011, Savitz et al. 2014). In particular, imaging in clinical trials has been encouraged to assess cell viability, infarct size, and pathological responses such as inflammation to provide information for stratification of patients to trial in order to optimise treatment effects (Savitz et al. 2014). Based on these recommendations this thesis aims to address some of the essential criteria outlined above, by addressing changes within the brain microenvironment according to stroke severity, and subsequent use of stem cell transplants to support brain repair.

1.2 Classification and epidemiology

Focal stroke falls into two categories: ischemic strokes, which are the most common, and responsible for 85% of stroke cases resulting from interruption of blood supply to a certain region/s of the brain; and hemorrhagic strokes, which account for
15% of cases and occurs when a cerebral vessel bursts and bleeds into the brain parenchyma (intracerebral haemorrhage), or within the space between the arachnoid membrane and pia mater (subarachnoid hemorrhage) (AIHW 2013, NSF 2012b). Intracerebral hemorrhage constitutes 10% of all strokes, and aneurysmal rupture and subarachnoid hemorrhage represent the remaining 5-6% (Felberg and Naidech 2003). Cerebral ischemic strokes can either be a consequence of a transient occlusion of a cerebral blood vessel with and without some degree of reperfusion. Ischemic strokes can be caused by either thromboses (approximately 25% of stroke cases) or embolisms (more than 75% of stroke cases) that confines the damage to a specific region of the brain depending on the artery occluded (Adams et al. 1993). Middle cerebral artery (MCA) occlusion is the most common form of stroke that affects humans, with the majority being embolic occlusions either from an unstable plaque (arthromobolism), cardiac sources (cardioembolism), or spontaneous thrombosis. Cerebral ischemia can also occur in the form of global stroke, although less common this type of stroke involves a dramatic reduction in cerebral blood flow (CBF) to most or all of the brain affecting widespread areas of brain tissue and results from a number of mechanisms including cardiac arrest or occlusion of common carotid arteries. As cerebral ischemia is mainly a disease of vascular occlusion, it has been determined that the size of the infarcted tissue correlates with the severity and duration of reduced CBF (Astrup et al. 1981, Jones et al. 1981).

Every year at least 60,000 Australians suffer new and recurrent strokes (more than 1000 Australians sustaining a stroke weekly) with the annual cost estimated to be over $2.2 billion in Australia (NSF 2012a, NSF 2013). In 2012 the cost of stroke in Australia was estimated to be 5 billion dollars. However, when factoring in ongoing costs of disabilities, healthcare and removing individuals from the workforce the real burden of stroke is estimated at 49.3 billion dollars (NSF 2013).

Stroke is a major cause of morbidity particularly in the middle-aged and elderly population (de Freitas and Bogousslavsky 2001). Despite this, in 2012, 130,000 (20%) stroke survivors were under the age of 65 (NSF 2013) and stroke is often more disabling than fatal with 40% of sufferers dying within 12 months and the remaining survivors making incomplete recoveries and requiring assistance in daily activities (Bonita et al. 1997, NSF 2012a). Functions disabled by stroke include weakness or lack of movement
in legs and/or arms, incontinence, alterations in personality and problems with vision, sensation, communication, memory, and perception (AIHW 2013). Stroke therefore results in a considerable amount of suffering for stroke victims, their families and caretakers, in addition to associated economic costs (NSF 2013). There are multiple risk factors associated with this disease including hypertension, atherosclerosis, obesity, tobacco smoking, diabetes, high alcohol consumption, atrial fibrillation and other heart diseases, age, gender, and family history (AIHW 2013, Thom et al. 2006).

In 2008-2009, the total health-care expenditure alone for stroke in Australia equated to $606 million, which included hospitalisation for acute care, out-of-hospital medical services, and prescription pharmaceuticals. This estimate exclude the cost for rehabilitation, nursing home care, carers, residential aged-care facilities, out of pocket expenses and lost earnings for stroke survivors, families and businesses (AIHW 2013). However, the biggest impact of stroke is not the financial costs, but the loss of healthy life. The prevalence of stroke is expected to rise from 420,000 Australians in 2012 to approximately 709,000 Australians in 2032 which equates to 2.4% of the population (NSF 2013). If nothing is done to affect the incidence of stroke the prevalence will skyrocket, thus highlighting the pressing need to develop treatment options to reduce the great burden of stroke and improve the quality of life for all stroke survivors.

### 1.3 Ischemic core and the penumbra

An ischemic attack results in two distinguishable areas: the infarcted core, which is the region supplied by the occluded vessel; and the penumbra, which is the area between the lethally damaged core and normally perfused territory, and which receives some collateral blood flow from unaffected vessels (Astrup et al. 1981). A dramatic reduction in CBF occurs in the ischemic core to less than 10% of normal (Fisher and Garcia 1996), leading to failure of cellular energy supply, ion homeostasis, metabolic processes, and subsequently breakdown of cellular integrity resulting in necrotic cell death within minutes (Mergenthaler et al. 2004). Tissue within the ischemic core is often irreversibly damaged even if blood flow is re-established. The ischemic penumbra however can be defined by a moderate reduction in CBF approximately 30% of normal (Fisher and Garcia 1996). CBF thresholds that drop below 25% of normal have a 95% chance of developing an infarction. On the other hand, CBF values greater than 50% of
normal have less than 5% chance of infarction, and normally accounts for the penumbral area (Mergenthaler et al. 2004). Importantly, similar thresholds of CBF within the core and penumbral regions have been demonstrated in both humans (Heiss et al. 2001) and experimental animal stroke models (Ginsberg 2003). Although, this correlation is only valid based on the premise that no early therapeutic or spontaneous reperfusion has occurred (Mergenthaler et al. 2004).

Astrup et al. (1981) first suggested the concept of the ischemic penumbra, and defined it as a region surrounding the ischemic core that is functionally impaired but still metabolically active. Residual blood flow from collateral blood vessels provides cells within the penumbra limited metabolic nutrients required to temporarily maintain homeostasis during the initial stages of ischemia (Heiss et al. 2004). Despite this, interruption of cellular homeostasis in the penumbral region leads to slow cell death where penumbral tissue will be recruited into the infarcted core with a step-by-step spread of damage if treatment to restore blood flow is delayed (Busch et al. 1996). Due to this slow rate of neuronal death within the penumbra the prime goal for therapeutic intervention in the past has been to salvage cells within the penumbra and prevent further irreversible neuronal damage post-stroke.

1.4 Pathophysiological basis of stroke

Experimental and clinical findings have led to the characterisation of the pathophysiological basis for stroke that varies based on the location of vascular occlusion, severity, and duration of reduced CBF (Dirnagl et al. 1999, Lo et al. 2003, Moustafa and Baron 2008). A multitude of complex events triggered following cerebral ischemia include physiological, biochemical, molecular and genetic mechanisms that underlie neuronal damage and impair neurological functions. The major pathological mechanisms include energy failure leading to ionic perturbations required to maintain osmotic homeostasis, impairment of cellular transporters and pumps, intracellular calcium overload, excitotoxicity and increased release of toxic free radicals, blood brain barrier (BBB) disruption, inflammation and apoptosis (see Figure 1.1) (Dirnagl et al. 1999, Martin et al. 1994). These mediate injury to neuronal cells, glial cells and vascular networks through disrupting the function of important cellular organelles including mitochondria, nuclei, cellular membranes, endoplasmic reticula and
lysosomes. The result is cell death by either necrotic (acute) or apoptotic (delayed) mechanisms. Along with pathophysiological cascades of tissue damage, concurrent protective mechanisms are also initiated from stroke onset where genes involved in anti-apoptotic, anti-inflammatory, and anti-oxidative processes (hypoxia-inducible-factor-1 (HIF-1), erythropoietin, and lipopolysaccharides) are expressed and partake in neuroprotective signalling cascades within the penumbra (Figure 1.2) (Dirnagl et al. 2003, Mergenthaler et al. 2004). Within days to weeks from stroke onset, the ischemic brain attempts to self-repair, with initiation of endogenous regenerative mechanisms including plastic rewiring of neuronal circuitry, neurogenesis and angiogenesis. While the early days of stroke research focused exclusively on mechanisms of neuronal injury and therapeutic intervention, the ability of the brain to mount protective and regenerative responses to ischemic challenges provides new targets for consideration when investigating treatment options (Dirnagl et al. 2003, Dirnagl 2012).

Figure 1.1 The ischemic cascade of events triggered following stroke.
Energy failure leads to depolarisation of neurons and glutamate release which activates glutamate receptors (NMDA, AMPA, and Kainate receptors) and drastically increases intracellular calcium. Water enters the cell via osmotic gradients and the cell swells...
(oedema formation). Calcium over-activates numerous enzyme systems and results in mitochondrial disruption and ultimately free radical formation. Free radicals damage mitochondria, DNA, cell membranes (lipolysis), trigger caspase-mediated cell death (apoptosis), induce the formation of inflammatory mediators, and cause infiltration of blood-borne inflammatory cells. Figure adapted from Dirnagl et al. (1999).

Figure 1.2 The temporal profile of pathophysiological and repair mechanisms initiated following ischemic brain injury.

Focal cerebral ischemia results in initiation of complex pathological events that contribute to the spread of damage over time. The acute phase of stroke (within minutes from stroke onset) includes events such as energy failure and ionic homeostatic imbalances that lead to excitotoxicity, the subacute phase (minutes-hours from stroke onset) involves oxidative stress, BBB disruption, and peri-infarct depolarisation while subacute and chronic phases (days-weeks from stroke onset) encompass delayed mechanisms of inflammation and apoptosis. Initiation of concurrent protective mechanisms occurs as an attempt to restore function through neuroprotective processes during the acute-subacute phases and regenerative processes occurs during the recovery.
1.4.1 Energy failure and excitotoxicity

The brain is more vulnerable to ischemia than any other organ in the body with different regions of the brain varying in their susceptibility to ischemia (Rosner et al. 1986). This is due to its limited stores of glucose which is required for the generation of adenosine triphosphate (ATP). In particular, neurons rely exclusively on glucose for ATP as an energy substrate. Despite the small size of the brain which approximates to 2.5% of total body weight, it requires approximately 20% of the oxygen and 25% of the glucose consumed by the entire body to meet its high metabolic demands which are crucial to maintain normal function and viability (Zauner et al. 2002). Stroke deprives the brain of these essential nutrients and the death of neurons due to the depletion of ATP results in the formation of a lesion with ensuing neurological deficits and the incorporation of the penumbra into the infarcted core (Dirnagl et al. 1999, Lo et al. 2003).

More than 90% of oxygen consumed by the body is used by mitochondria to generate ATP. Within minutes of blood vessel occlusion, the dramatic decrease in oxygen and glucose delivered to mitochondria prevents the activity of the electron transport chain to maintain aerobic respiration for adequate ATP generation and energy production (Sciamanna and Lee 1993, Smith 2004). As a result anaerobic glycolysis takes over, causing rapid intracellular acidification, and lactate accumulation that contribute to the increased depletion of ATP and can enhance free radical generation and toxic conversion (LaManna et al. 1997). Additionally, severe lack of oxygen and diminished levels of glucose inhibit oxidative phosphorylation which then leads to ATP-synthase functioning backwards and consuming ATP further (Katsura et al. 1994). Progressive failure of ATP-dependent ion pumps, such as sodium (Na⁺) or potassium (K⁺) -ATPase results in loss of membrane potential causing neurons and glia to depolarise (Katsura et al. 1994). Uncontrolled cell membrane depolarisation and changes in concentration gradients of Na⁺ and K⁺ across the plasma membrane then causes activation of voltage-gated calcium (Ca²⁺) channels. Increased intracellular Ca²⁺ levels lead to the release of large and sustained amounts of excitatory amino acids (EAAs) such as glutamate, a major excitatory neurotransmitter in the brain, into the
extracellular compartment from presynaptic terminals (Lipton and Rosenberg 1994). Simultaneously energy dependent re-uptake transporters present on neurons and glia are impaired, which leads to further extracellular accumulation of glutamate.

The excitatory effects of glutamate are mediated through two types of receptors; ionotropic receptors, that are coupled directly to ion channels (Nakanishi 1992) and metabotropic receptors that are coupled to G-proteins and modulate intracellular second messengers (Schoepp and Conn 1993) found in the pre- and post-synaptic neuron membranes of the central nervous system (CNS). Over-activation of these glutamate ionotropic receptors; alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid (Kainate receptor) and N-methyl-D-aspartate (NMDA) receptors (Love 1999), results in excessive Ca$^{2+}$ entry and release and more Na$^+$ influx and K$^+$ efflux, that decrease ionic gradients, consume ATP, and promote even further glutamate release (Lee et al. 2000a). Excessive accumulation of glutamate leads to an over-activation of receptors that can mediate neuronal cell death through a process termed ‘excitotoxicity’ (Olney 1978). Virtually all glutamate receptor family members are believed to be involved in mediating excitotoxicity (Choi 1987), although NMDA receptor activation appears to be a key mediator of neuronal death through a whole host of Ca$^{2+}$ mediated or catalysed injuries (Choi 1988, Waxman and Lynch 2005). An additional consequence of glutamate induced over-activation of receptors is the extreme influx of Na$^+$ and Cl$^-$ into neurons through monovalent ion channels (Smith 2004). This influx of ions is larger than the efflux of K$^+$ resulting in the passive flow of water into neurons causing cellular swelling and oedema (O'brien 1979, Simard et al. 2007).

### 1.4.2 Calcium overload

After cerebral ischemia, a dramatic rise in intracellular calcium (Ca$^{2+}$) concentration occurs due to over-activation of glutamate receptors, NMDA and AMPA receptors operated channels, voltage-gated Ca$^{2+}$ channels, and reverse operation of Na$^+$/Ca$^{2+}$ exchanger. Further release from intracellular Ca$^{2+}$ stores within the endoplasmic reticulum, mitochondria, synaptic vesicles, and calcium binding proteins raises the Ca$^{2+}$ concentration (Pringle 2004). As a result calcium over-activates numerous enzyme systems including proteases, lipases, phospholipases, endonucleases, certain nitric oxide synthases and various others that can contribute to induction of genes or gene products which subsequently initiate degeneration of cellular and
subcellular structures (Choi 1988, Orrenius et al. 2003, Sattler and Tymianski 2000). Excessive Ca\textsuperscript{2+} levels trigger many downstream neurotoxic cascades including mitochondrial dysfunction, oxidative stress due to an increase free radicals, and initiation of molecular events that ultimately lead to apoptosis (Lai et al. 2014, Siesjö et al. 1995).

1.4.3 Reperfusion injury

In the treatment of ischemic stroke, re-establishment of blood flow through collateral blood supply, or spontaneous or thrombolytic recanalisation is believed to be beneficial in minimising neuronal damage and infarct extension by salvaging reversibly damaged penumbral tissue (Pan et al. 2007, Schaller and Graf 2004). Clinical trials have demonstrated functional improvements after reperfusion using thrombolysis in selected patients with acute stroke (NINDs rt-PA Study 1995). However, reperfusion can also carry certain risks. Some patients experience devastating side effects in the form of fatal oedema or intracranial hemorrhage after thrombolytic therapy (NINDs rt-PA Study 1997). Therefore, reperfusion may reduce infarct size and improve functional outcomes in some patients, while in others it can exacerbate brain damage leading to “reperfusion injury” (Aronowski et al. 1997, Dietrich 1994, Pan et al. 2007, Yang and Betz 1994). Multiple pathological processes are involved in reperfusion injury and these include leukocyte infiltration, platelet and complement activation, post-ischemic hyperperfusion, and BBB disruption (Pan et al. 2007).

1.4.4 Oxidative stress

A physiological imbalance between the cellular production of free radicals and the ability of cells to defend against them is referred to as oxidative stress (Gilgun-Sherki et al. 2002, Simonian and Coyle 1996). Free radicals are highly reactive products of cellular respiration (Simonian and Coyle 1996) and can be divided into two groups; reactive oxygen species (ROS) and reactive nitrogen species (RNS). Spontaneous or thrombolytic reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions turned on during ischemia, several of which involve the excessive generation of ROS/RNS (Nita et al. 2001, Schaller 2005). If not scavenged, ROS/RNS can modify macromolecules through breakdown of lipid membranes, oxidisation of DNA, denature enzyme proteins and interfere with signal transduction and inhibit their normal function to initiate cell death programs in the form of apoptosis or necrosis within hours.
1.4.5 The neurovascular unit

Ischemic stroke is a vascular disorder with neurological consequences that involves disruption of neurovascular communication and is required for the control and modulation of regional and local CBF (del Zoppo 2010, Koehler et al. 2009, Zonta et al. 2003). The study of blood vessels in cerebrovascular diseases has expanded from sole consideration of endothelial cells to include other cells within the practical framework described as the “neurovascular unit” (NVU) (Lo and Rosenberg 2009). The National Institute of Neurological Disorders and Stroke (NINDs 2002) progress review group identified the NVU as a conceptual model that emphasises the dynamic interactions between neurons, astrocytes, smooth muscle cells, endothelial cells, pericytes, basement membranes, extracellular matrix and supporting cells (microglia and oligodendroglia) necessary for normal function (Figure 1.3) (del Zoppo 2009, Dirnagl 2012, Lo and Rosenberg 2009, Moskowitz et al. 2010). Interaction between components of the ‘unit’ also partake in the pathobiology of stroke (del Zoppo 2010). The NVU provides a platform in understanding the evolution of stroke injury and in processes of brain repair for the success of appropriate clinical and experimental investigations.
The inter-relationship of microvessels and their dependent neurons via astrocytes and surrounding cells including microglia and oligodendrocytes where injury affects the function of the entire unit. Microvessels consisting of pericytes attached to the abluminal surface of the endothelial cells are surrounded by basement membrane and encompassed by astrocyte end-feet. Figure adapted from del Zoppo et al. (2010).

1.4.6 Blood brain barrier integrity

The CNS is considered to be immune privileged and is separated from the changeable milieu of the blood by the blood brain barrier (BBB) to allow conductivity of complex neural signalling without external interferences (Reese and Karnovsky 1967). The blood-cerebrospinal fluid barrier (BCSFB) established by the choroid plexus epithelial cells works in conjunction with the BBB to create a tight seal preventing passage of blood-borne molecules into the microenvironment of the brain (Engelhardt and Sorokin 2009). The BBB comprises of a complex system of highly specialised and unique endothelial cells, their underlying basement membranes where a large number of pericytes reside (Armulik et al. 2010), peri-vascular antigen-presenting cells, ensheathing astrocytic endfeet (Abbott et al. 2006a) and the associated parenchymal basement membrane (Engelhardt and Sorokin 2009). Specific interactions between brain endothelium and glia that are part of the NVU appear to be involved in regulating the BBB under physiological and pathological conditions (Abbott et al. 2006b). Under normal conditions, the tight junctions only allow the diffusion of very small water-soluble compounds via a par-cellular aqueous pathway, while the lipid membranes of the endothelium comprising of large surface areas offer effective diffusion of lipid-
soluble compounds via a trans-cellular lipophilic pathway (Rowland et al. 1992). Cerebral ischemia compromises the BBB integrity for days to even weeks and leads to permeability of previously excluded blood-borne molecules and inflammatory cells (Engelhardt and Sorokin 2009, Okada et al. 1994, Strbian et al. 2008). A cascade of microvascular events contribute to the breakdown of the BBB including fibrin accumulation, transmigration of leukocytes, generation of degrading enzymes, basal laminae breakdown with loss of astrocyte and endothelial cell contacts which causes vasogenic edema and potential hemorrhagic transformation (Engelhardt and Sorokin 2009, Okada et al. 1994, Petty and Wettstein 2001). The volumetric effect of edema formation cause local compression of microcirculation leading to further perfusion deficits, a rise in intracranial pressure, and dislocation of parts of the brain (Durukan and Tatlisumak 2007).

1.4.7 Inflammation

Cerebral ischemia and reperfusion elicits a strong inflammatory response that can exacerbate tissue damage and involves the rapid activation of resident cells (mainly microglia), infiltration inflammatory cells including T cells, neutrophils, monocytes/macrophages, activation of astrocytes, oxidative stress, increased production of inflammatory cytokines and chemokines, and increased expression and activation of adhesion molecules and matrix metalloproteinases (Jin et al. 2010, Stoll et al. 1998). Although, the inflammatory response has also been found to exert beneficial effects after stroke through generation of anti-inflammatory cytokines, rapid removal of debris, walling off the injured brain region to prevent the spread of damage, and influence repair and remodelling processes (Stoll et al. 1998). The detrimental or protective/restorative effects of inflammation may depend on the severity of stroke and the stages of ischemia in which inflammatory cells contribute (Wang et al. 2007). The majority of studies that have focused on inflammatory mechanisms have targeted acute neuronal injury, but emerging emphasis is now taking shape suggesting their role in promoting recovery after the acute phase (Gladstone et al. 2002, Ikonomidou and Turski 2002, Wahlgren and Ahmed 2003).

1.4.9.1 Leukocytes

Within the first few hours from stroke onset, initiation of an inflammatory reaction occurs, where cytokine signalling and subsequent expression of adhesion
molecules including intercellular adhesion molecule-1 (ICAM-1) on circulating leukocytes and at the vascular endothelium (Huang et al. 2006). Leukocytes or white blood cells including polymorphonuclear leukocytes (neutrophils) and mononuclear leukocytes (monocytes/macrophages) then adhere to the endothelium and transmigrate from the blood into the brain parenchyma. Neutrophils are generated in the bone marrow and are normally the first responders in the host defence mechanism to invading organisms and clearance of damaged tissue. The transmigration process of leukocytes is aided by but not dependent on BBB disruption, and is capable of causing additional injury to the blood vessels and surrounding brain parenchyma (Schaller and Graf 2004, Stoll et al. 1998). Once within the brain, leukocytes are capable of producing various proteases, lipid derived mediators and ROS that cause injury to potentially salvageable cells, and also contribute to ensuing oedema formation (Chou et al. 2004, Clark et al. 1993, Schaller and Graf 2004). Accumulation of neutrophils along with red blood cells and platelets can cause additional disruption to microvascular cerebral blood flow, referred to as the “no-flow” phenomenon (Wong and Crack 2008).

1.4.9.2 Microglia and macrophages

The CNS is composed of two cells types that are equally numerous within the brain; neurons and glial cells, where glial cells comprise of astrocytes, oligodendrocytes and microglia. Microglial cells, the resident macrophages of the CNS, act as sensors of neuronal pathology and are key elements in triggering the innate immune response (Stoll et al. 1998, Wang et al. 2007) (Kreutzberg 1996). When ischemia occurs, macrophages (microglia-derived and blood-derived) become visible within 24 hours (Kleinig and Vink 2009). Once activated, microglia morphologically transform from ramified resting state cells into activated amoeboid shaped phagocytes virtually indistinguishable from blood-borne macrophages and lack discriminating cellular markers (Jin et al. 2010, Schilling et al. 2003, Schilling et al. 2005). Direct evidence exists supporting the harmful effects after ischemia when microglia release cytotoxic molecules such as superoxide (Colton and Gilbert 1987), nitric oxide (Moss and Bates 2001), ROS, tumour necrosis factor α (TNFα) (Sawada et al. 1989), toxic prostanoids (Gibson et al. 2005), and neurotoxic pro-inflammatory cytokines (Block et al. 2007, Dirnagl et al. 1999, Hanisch 2002, Raivich et al. 1999, Yrjanheikki et al. 1999). However, some studies have suggested microglia/macrophages are also capable of protecting cells by phagocytosing invading neutrophils (Neumann et al. 2008), clearing
deleterious necrotic brain tissue (Anderson et al. 2003, Schroeter et al. 1997), regulating the inflammatory response by releasing anti-inflammatory cytokines (Battista et al. 2006) and growth factors (Lalancette-Hebert et al. 2007) (Lalancette-Hebert et al. 2007, Neumann et al. 2008, Thored et al. 2009). Following injury to the CNS, intermediately activated microglia have been found to promote beneficial effects (Gomes-Leal 2012, Thored et al. 2009), while over-activated microglia can be extremely destructive (Gomes-Leal 2012, Popovich et al. 1999). The dual detrimental and beneficial effects on ischemic injury and repair suggest a complex role of microglia in stroke (Nedergaard and Dirnagl 2005, Nguyen et al. 2002, Watanabe et al. 2000, Wyss-Coray and Mucke 2002).

1.4.9.3 Astrocytes

Astrocytes are recognised as plastic cells that play a number of roles in the CNS and exhibit various morphological and biochemical changes triggered by physiological and pathological events that alter their environmental milieu (Ridet et al. 1997, Sofroniew and Vinters 2010, Zhao and Rempe 2010). Astrocytes can change their physical and molecular phenotype and exist in pro-survival or “cytotrophic” and destructive “cytotoxic” distinctions (Maragakis and Rothstein 2006, Sofroniew and Vinters 2010). Numerous astrocytic mechanisms have evolved to protect neurons against ischemia, including glutamate uptake by astrocytes to prevent excitotoxic accumulation (Swanson et al. 2004). Metabolite transfer from astrocytes to neurons provides energy to neurons during stroke, where astrocyte glycogen stores serve as a carbon source and cells undergo anaerobic metabolism to support penumbral neurons (Potas et al. 2005, Swanson et al. 2004). Further support is provided through early detoxification of free radicals as astrocytes contain higher intracellular concentrations of antioxidants than oligodendrocytes and neurons, which contribute to their robust resistance to oxidative stress (Potas et al. 2005, Wilson 1997). Reactive astrocytes are known to up-regulate erythropoietin after ischemia, a paracrine messenger within the brain that can prevent apoptosis or excitotoxic stress (Bernaudin et al. 2000, Buemi et al. 2003, Nagai et al. 2001, Swanson et al. 2004). Reactive astrocytes also partake in vascular and neuronal regeneration after stroke by secreting several factors important for promoting angiogenesis, directing neural precursor migration, and glial plasticity (Acker et al. 2001, Mocchetti and Wrathall 1995, Seifert et al. 2006, Tokita et al. 2001).
In contrast to their potential support role, astrocytes actively participate in the demise of brain tissue after stroke if they become over-activated. Astrocyte over-activation results in the efflux of glutamate through reversal of ion channels and other routes can contribute to elevations in extracellular glutamate and exacerbate excitotoxicity (Sofroniew and Vinters 2010, Takano et al. 2005). Reactive astrocytes secrete ROS, pro-inflammatory cytokines and interleukins, matrix metalloproteinases, and other factors that contribute to delayed neuronal cell death, BBB disruption and facilitate oedema formation through aquaporin-4 channels abundantly expressed in astrocytic endfeet at the endothelial interface (Dong and Benveniste 2001, Endoh et al. 1993, Feuerstein et al. 1998, Swanson et al. 2004, Venero et al. 2001). Reactive astrocytes can also amplify ischemic injury though gap junctions that remain open, providing a conduit for the propagation of pro-apoptotic signals between dying and viable astrocytes (Cotrina et al. 1998, Swanson et al. 2004). Collectively, the role of astrocytes constitutes a finely gradated continuum of morphological changes from reversible pro-survival alterations to long-lasting scar formation around the lesion, a process referred to as reactive gliosis (Fawcett and Asher 1999, Sofroniew and Vinters 2010). Therefore it is an oversimplification to assign sole protective or destructive functions to astrocytes and rather highlights the intricacy of these mechanisms that collectively influence the brain microenvironment after ischemia.

1.4.9.4 Oligodendrocytes

Oligodendrocytes are abundant in both grey (comprising mainly of neuronal cell bodies) and white matter (primarily consisting of myelinated axonal bundles) and produce myelin sheaths for enhanced axonal transmission (Wood and Bunge 1984). Being the only cells capable of forming myelin, oligodendrocyte damage or death causes profound consequences on the function of myelinated CNS tracts, especially within the white matter (Arai and Lo 2009, Dewar et al. 2003, Potas et al. 2005). Compared to mechanisms of neuronal injury in grey matter, white matter injury and the role of oligodendrocytes during the early phase of cerebral ischemia is fairly understudied and thus poorly understood (Arai and Lo 2009). Oligodendrocytes are highly vulnerable to ischemic stress and excitotoxicity (Arai and Lo 2009, Lyons et al. 2000, McDonald et al. 1998, Tekkok and Goldberg 2001) with studies suggesting equivalent vulnerability as neurons to ischemia (Dewar et al. 2003). Oligodendrocytes and their precursor cells have also been found to remyelinate axons within penumbral
areas with up-regulated expression of major components of CNS myelin and increased precursor cell proliferation (Fitch and Silver 2008, Mandai et al. 1997, Nishiyama 2007). Remyelination of regenerating axons is essential for stroke recovery and fundamental research into these mechanisms is underway (Arai et al. 2011). However, like other pathophysiological mechanisms, oligodendrocytes and their precursors play dual roles in injury and repair processes, since regeneration and plasticity are halted by the production of myelin-associated neurite growth inhibitors within the penumbra (Bandtlow and Schwab 2000, Dewar et al. 2003).

1.5 Failed treatments for stroke

Numerous neuroprotective compounds have been tested in animal stroke models with drugs showing the most potential having moved into trials in human stroke patients. Despite promising results in preclinical experimental models, translation to clinical success has been a litany of disappointment (see Table 1.1). The failure of neuroprotective therapies to translate to clinical efficacy has been attributed to the following; therapeutic time window, age, history of disease, drug dose, duration of ischemic event, BBB permeability, gender, appropriate classification and stratification of stroke patients into treatment groups, and finally and most importantly the functional and morphological differences between the brains of humans and animals (Adams et al. 1995, del Zoppo 1995, del Zoppo 1998, Dirnagl et al. 1999). Even when the international recommendations for preclinical research were followed, based on the STAIR criteria, there was limited success in clinical translation (Fisher et al. 2009, Minnerup et al. 2012, STAIR 1999). Such examples include haematopoietic growth factors and free radical trapping agent, NXY-059, which fulfilled nearly all the STAIR criteria and yet failed to translate into the clinic (Diener et al. 2008, Sachs 1992, Shuaib et al. 2007). Dissection of molecular pathways and mechanisms should in theory bring major advances and success to neuroprotective strategies. However, these approaches are mainly neuronal focused. One of the major recommendations from the NINDS Stroke Progress Review Group was to shift focus away from chiefly neurocentric views and recognise that neurons are integrated into a complex network of multiple cell types that form the basis of the NVU and influence the ischemic microenvironment and therefore considers the brain from a functional perspective (Abbott et al. 2006b, Font et al. 2010, NINDs 2002). Furthermore, neuroprotective therapies have mainly focused on
preventing the spread of initial tissue damage, while advances in regenerative medicine, cell-based therapies, and tissue engineering focus on repair processes initiated after stroke and raise the possibility of coaxing rescue of neuronal circuits (Font et al. 2010).

Table 1.1 Neuroprotective compounds tested in clinical trials for stroke.
The failure of neuroprotective compounds to progress beyond Phase II/III clinical testing: drugs utilised target various sites of action highlighting the complex nature of events occurring in stroke and the difficulty translating findings in animal models to human stroke. Adapted from Green and Shuaib (2006).Abbreviations: BMS-204352, [5-chloro-2-methoxyphenyl]-1,3-dihydro-3-fluoro-6-[trifluoromethyl]-2H-indol-2-one; ICAM, intercellular adhesion molecule; NMDA, N-methyl-Daspartate; NOS, nitric oxide synthase. Lack of efficacy means that efficacy was not demonstrated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism of action</th>
<th>Outcome (Phase)</th>
<th>Reason</th>
</tr>
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<tbody>
<tr>
<td>Selfotel</td>
<td>NMDA receptor antagonist</td>
<td>Negative (III)</td>
<td>Adverse events</td>
</tr>
<tr>
<td>Aptiganel</td>
<td>NMDA receptor antagonist</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>Gavestinel</td>
<td>NMDA glycine-site antagonist</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>Eliprodil</td>
<td>NMDA, polyamine site blocker</td>
<td>Negative (II)</td>
<td>Adverse events</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>NMDA, channel blocker</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>Cervene</td>
<td>Kappa opioid receptor antagonist</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>Lubeluzole</td>
<td>NOS inhibitor and Na⁺ channel blocker</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
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<td>Sodium channel blocker</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>BMS-204352</td>
<td>K⁺ channel blocker</td>
<td>Negative (III)</td>
<td>Lack of efficacy</td>
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<tr>
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<td>Ca²⁺ channel antagonists</td>
<td>Negative (Meta-analysis)</td>
<td>Lack of efficacy</td>
</tr>
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<td>Anti-ICAM antibody</td>
<td>Negative (III)</td>
<td>Lack of efficacy and adverse events</td>
</tr>
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<td>Cell membrane stabilizer</td>
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<td>Lack of efficacy</td>
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<td>Negative (III) 6 trials</td>
<td>Lack of efficacy</td>
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<td>Lipid peroxidation inhibitor</td>
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<td>Lack of efficacy</td>
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<td>5-HT₁A receptor antagonist</td>
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<td>Lack of efficacy</td>
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<td>ONO-2506</td>
<td>Astrocyte modulating factor</td>
<td>Negative (II)</td>
<td>Lack of efficacy in futility analysis</td>
</tr>
<tr>
<td>Trafermin</td>
<td>Basic fibroblast growth factor</td>
<td>Negative (II/III)</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>UK-279,276</td>
<td>Neutrophil inhibitory factor</td>
<td>Negative (II)</td>
<td>Lack of efficacy in futility analysis</td>
</tr>
<tr>
<td>NXY-059 (Cerovive)</td>
<td>Free radical trapping agent</td>
<td>Positive - Saint 1 (III)</td>
<td>Improved primary outcome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative - Saint 2 (III)</td>
<td>Lack of efficacy</td>
</tr>
</tbody>
</table>

1.6 Current treatment for stroke
1.6.1 Recombinant tissue plasminogen activator (rt-PA)

Despite the high prevalence of stroke, unfortunately, thrombolytic or “Clot Buster” therapy with alteplase, is the only pharmacological intervention approved for
treatment of ischemic stroke rather than hemorrhagic stroke and is appropriate for less than 30% of ischemic patients (NINDs rt-PA Study 1995). Thrombolytic therapy focuses on recanalisation to either reverse ischemic damage or decrease the size of potential damage (Gilman 2006, Goldstein 2007). A computed tomography (CT) scan is performed to ensure the patient is not suffering from a hemorrhagic stroke, thus, rendering thrombolysis unsuitable for treating such cases. Intravenous treatment with thrombolytic drug, recombinant tissue-plasminogen activator (rt-PA) alteplase is capable of dissolving the clot, thus permitting reperfusion. Thrombolysis is approved for treating patients within 4.5 hours after ischemic onset (Donnan and Davis 2008) due to the risks associated with symptomatic intracerebral haemorrhage and neurotoxicity if administered outside this window (Wardlaw et al. 1997). The delay of a patient being presented to a hospital coupled with intrahospital delays such as that caused by a CT scan and the presence of any other contraindications means ~95% of patients do not receive this treatment. Therefore, only 5% of patients diagnosed ultimately receive the drug. Unfortunately, administration of the drug is not always successful as some patients still do not undergo reperfusion after being given the drug. In addition, the initiation of reperfusion after drug administration can still lead to hemorrhagic complications within the treatment window due to the severity of damage caused by the stroke insult (Wardlaw et al. 1997). Given the impoverished percentage of patients receiving thrombolysis, it is crucial to develop therapeutic treatments that target the majority of patients.

1.6.2 Mechanical recanalisation

Mechanical recanalisation is preferable in some cases where thrombolytic therapy is not suitable for treating stroke patients (Nakano et al. 2002, Nakano and Wakisaka 2004, Yoneyama et al. 2002). For large artery occlusions thrombolysis can result in the delivery of high concentrations of thrombolytic agents that result in failure of recanalisation in 25-40% of patients, and in some cases has also been associated with greater risks of hemorrhagic complications (del Zoppo et al. 1998, Furlan et al. 1999, Hacke et al. 1995, NINDs rt-PA Study 1995, Ringer et al. 2001). As a result, mechanical recanalisation is selected as an alternative first choice of treatment for such cases. Balloon catheters or clot extraction using retrieval devices are the only available and effective treatment methods for mechanical clot disruption (Nakano and Wakisaka 2004). However new mechanical devices are emerging including ultrasound, laser
energy, and suction-creating saline jets and are under investigation to determine safety and feasibility (Leary et al. 2003, Lutsep 2001, Nakano and Wakisaka 2004, Ringer and Tomsick 2002). Previous reports have suggested the combined use of both mechanical recanalisation and thrombolytic agents to result in improved clinical outcomes (Nakano et al. 1998, Nakano et al. 2002, Qureshi et al. 2002, Tsai et al. 1994, Ueda et al. 1998, Yoneyama et al. 2002). Mechanical recanalisation increases the surface area of the clot exposed to thrombolytic agents and potentially enhance the effectiveness of the drug, thereby improving recanalisation rates, reducing the concentration of thrombolytic agent administered and therefore decreasing potential serious hemorrhagic complications (Nakano and Wakisaka 2004). Mechanical recanalisation procedures also pose certain risks including arterial rupture, spasm, and distal embolism (Nakano et al. 1998, Nakano et al. 2002, Yoneyama et al. 2002). Further investigations into the safety of mechanical recanalisation have been recommended and as such have been limited to the treatment of patients that failed thrombolysis or were considered poor candidates for thrombolytic therapy.

1.7 The challenge of repairing the damaged brain after stroke

Stroke outcome is distinctly different from other neurological diseases as it involves the focal loss of many different cell types within the territory of arterial supply. As such therapies designed to recover the injured brain will require a more complex approach than simply targeting neuroprotection. The human brain is a complex unit of intricate circuitry, comprising of billions of cells, not just neurons, interconnecting to process multiple functional systems involved in sensory, motor, cognitive and emotional responses. Hence, several major challenges arise following brain injury including stroke where replacement of lost neurons is just one factor required for re-establishing function since a wide array of cell types are needed to arrange into complex networks that form the functional infrastructure of the nervous system (Chiu and Rao 2011).

The adult human CNS is composed of approximately $10^{12}$ cells, with the majority being fully differentiated and post-mitotic (Chiu and Rao 2011). Neurons comprise only approximately 10% of these cells with the remaining comprising of “support” cells: neighbouring populations of glia that play roles in neuronal
development, survival and/or function. Within the brain neurons can further be divided into different phenotypic sub-populations whose function varies depending on location. Excitatory pyramidal neurons constitute the majority of neocortical neurons (70-80%), with similar anatomical, physiological and molecular properties (DeFelipe and Farinas 1992) and vary in terms of their location within the cortical laminae and connection patterns (Markram et al. 2004). Pyramidal projection neurons can be classified as associative, commissural, or corticofugal, based on their principal output to other neocortical areas, opposite hemisphere, or subcortical regions respectively (Molyneaux et al. 2007). Each pyramidal neuron receives synaptic input from distinct cortical and subcortical areas (Aronoff et al. 2010, Miyashita et al. 1994, Porter and White 1983) and each input targets layer-specific dendritic domains on these neurons (Spruston 2008). The remaining non-pyramidal neurons are local interneurons (20-30%), which are mainly inhibitory and again consist of a diverse range of cells that differ based on their morphological, physiological, molecular and synaptic characteristics (DeFelipe 1993, Wonders and Anderson 2006).

In addition to cellular diversity the brain consists of complex axonal pathways that vary in the number of anatomical projections and distance covered. The majority of the cortical mantle project towards the basal ganglia in a topographical manner (Figure 1.4) (Bolam et al. 2000). The basal ganglia are a group of nuclei consisting of the striatum (caudate nucleus and putamen), globus pallidus (GPe: external segment, GPI: internal segment), the subthalamic nucleus (STN) and the substantia nigra (SN; SNc: dorsal pars compacta, SNr: ventral pars reticulata). These structures are associated with a variety of functions including motor, cognitive and associative control. In addition there is a ventral division of the basal ganglia (ventral striatum or nucleus accumbens; ventral pallidum and ventral tegmental area) that are associated with limbic functions. A major role of the basal ganglia system is to integrate sensorimotor, associative and limbic information and produce context-dependent behaviours (Bolam et al. 2000). The main entry point of cortical information to the basal ganglia is via corticostriatal pathways to the striatum and the subthalamic nuclei (Bolam et al. 2000). The heterogenous neuronal population of the striatum comprises of medium sized densely spiny projection neurons (90-95%) (Kemp and Powell 1971) and several populations of interneurons (Kawaguchi 1993, Kawaguchi et al. 1995). Classes of striatal interneurons
can be characterised based on chemical, physiological and morphological properties (Kawaguchi et al. 1995).

**Figure 1.4 The basal ganglia circuitry.**

Block diagram of the circuitry of the basal ganglia showing the “direct”, “indirect”, and “hyperdirect” pathways. Excitatory glutamatergic projections are illustrated in blue, while inhibitory GABAergic projections are illustrated in red. Projections from the SNC to the striatum use dopamine as a neurotransmitter and bind to dopamine receptors (D1 and D2 and is excitatory or inhibitory respectively). Adapted from Moroney et al. (2008). Abbreviations: STN, subthalamic nucleus; SNC, Substantia nigra pars compacta; SNr, Substantia nigra pars reticulata; GPe and GPi, external and internal segments of the globus pallidus respectively.

In the adult human brain, intricate neuronal pathways are assembled and refined over a long time period from development that extends into adolescence. These pathways are used throughout life and once established are rarely replaced, with the exception of certain regions of the brain including the olfactory bulb. The limited ability of the central nervous system to self-renew and regenerate as compared to other organs including the skin, blood, and bone creates immense difficulties in assigning treatment
options to promote normal repair processes. Unfortunately, majority of strokes affect the elderly population where neuronal circuits have had decades of refining, increasing the devastating impact of stroke and damage to these finely tuned pathways. Furthermore, the location and extent of neuroanatomical destruction following stroke varies from one individual to the next, increasing the difficulty in assigning treatments. The heterogeneity of impairments and specific brain regions affected relate to the anatomy of arterial blood supply and location of the occlusion. MCA obstruction results in invariable damage within the striatum and variable damage within the cortex due to the tortuous trajectory of artery and collateral blood supply within cortical tissue allowing perfusion to certain brain regions (Cheng et al. 2011, Smith et al. 2012). Stroke lesion topology is however important in assigning treatment strategies as the location of damage appears to be a key factor in recovery (Feys et al. 2000, Parkinson et al. 2009). Regenerative strategies should consider identification of the site and size of the lesion and the specific original neuronal phenotypes affected, including their functional properties and anatomical features to enhance understanding the requirements of promoting restoration and functionality of surviving networks (Dihne et al. 2011). Understanding the specific demands on the human nervous system and the extent of endogenous mechanisms activated after injury need to be fully addressed to anticipate challenges and improve quality and efficacy of therapeutic investigations.

1.8 Endogenous brain repair following stroke

Contrary to long held beliefs, it is now understood and accepted that the brain is highly malleable following injury. Some spontaneous recovery of function is usual although limited in its extent, but has been observed in humans and is known to involve synaptic plasticity, endogenous neurogenesis and angiogenesis. However, the intricate mechanisms of recovery are not completely understood. The microenvironment following stroke reflects a complex, dynamic and evolving molecular state, rather than a fixed cellular environment. Elucidating aspects of this environment and the contribution from endogenous repair mechanisms may reveal specific targets for a wide range of therapeutic strategies that augment brain repair. For this reason development of restorative therapies to treat stroke represent new hope to stroke survivors. Restorative therapeutic strategies currently under investigation include cell-based therapies, growth factor delivery, device-based strategies including electromagnetic stimulation, and non-
pharmacological methods comprising of task-oriented and repetitive training-based interventions, exercise/physical therapy (Cramer 2008a). Most importantly, the therapeutical treatment window for targeting neurorepair mechanisms during subacute and chronic phases after stroke is much wider than that for achieving neuroprotective during the acute phase (Zhang and Chopp 2009).

1.8.1 Spontaneous recovery

Early spontaneous recovery of neurological function after ischemic stroke is not uncommon in experimental models and human patients (Jones and Millikan 1976, Rothrock et al. 1995). Even with spontaneous recovery, more than 69% of patients experience functional motor impairments in the upper limbs and 56% experience hemiparesis up to 5 years post-stroke (Desrosiers et al. 2005, Gillot et al. 2003, Luke et al. 2004, Urton et al. 2007). Considerable variability across stroke victims and across neurological domains has long frustrated efforts to precisely determine the process of spontaneous recovery (Cramer 2008b, Luria 1963). To date, the main principles behind improvement appear to be that most spontaneous recovery occurs within the first three months from stroke onset; recovery of cognitive deficits appear to be more common than motor recovery; and patients with mild deficits recover faster than those with severe deficits with varying patterns of recovery existing across different neurological domains within the same individual (Cramer 2008b).

Animal studies have provided insight into a range of molecular and physiological events underlying recovery (Cramer 2008b). These events include increased expression of growth-associated proteins, cell-cycle proteins, and growth factors; structural changes in axons, dendrites, and synapses, increased activation and migration of endogenous neural stem cells; and alterations in extracellular matrix, glial cells, and angiogenesis (Carmichael 2006, Chopp et al. 2007, Cramer 2008b, Jones et al. 2003, Komitova et al. 2006, Nudo 2007). Observations in human patients have demonstrated brain mapping and reorganisation of surviving circuitry that support behavioural recovery, including increased activity within associated regions linked to injured zones, interhemispheric lateralisation, and a shift in representational maps within intact regions (Cramer 2008b). Since the first functional imaging study of brain reorganisation after stroke by Brion and colleagues (1989), many studies described the initial reaction to stroke as increased activity within cortical regions that previously

These two forms of post-stroke induced increased brain activity have been found to directly contribute to spontaneous improvements and are time dependent with increased activity observed early post-stroke and declining thereafter (Calautti et al. 2001, Cramer and Crafton 2006, Cramer 2008b, Small et al. 2002, Ward et al. 2004). The degree of persistently increased activity has been found greatest in those with poorest neurological outcomes (Cramer and Crafton 2006, Ward et al. 2003a, Ward et al. 2003b) however this is not always helpful as increased reorganisation can be associated with induction of epilepsy (Witte and Stoll 1996) or chronic pain (Chen et al. 2002).

The third compensatory response to brain damage appears to be reorganisation of somatotopic maps. Reassignment of function that occurs within peri-infarcted regions is similar to the map alterations described after peripheral nervous system perturbations (Cohen et al. 1993, Merzenich and Jenkins 1993). Few studies suggested the degree of somatotopic reorganisation is greatest in larger stroke injury (Cramer and Crafton 2006, Schaechter et al. 2008). Such maps are usually present in a numerous primary cortices such as motor, sensory, auditor, and visual cortex (Cramer 2008b). Somatotopic organisation also exists within the white matter, basal ganglia, secondary neocortex, and the hemisphere ipsilesional to movement (Alkadhi et al. 2002, Cramer 2008b, Fontaine et al. 2002, Godschalk et al. 1995, Maillard et al. 2000, Morecraft et al. 2002, Scholz et al. 2000). The same events that support spontaneous recovery are likely those that may assist studies aiming to therapeutically improve recovery (Cramer 2008b, Rocca and
Filippi 2007). However, multiple factors including demographics and socioeconomic factors, pre-stroke medical comorbidities and disability, age, genetics, and behavioural experience during the weeks to months after a stroke have been suggested to influence events that support stroke recovery and as such should be considered as covariates in restorative therapeutic trials (Baird 2007, Cramer 2008b, Dobkin 2003, Foley et al. 2003, Johansson 2000, Kwakkel 2006, Legg 2004, Sharp et al. 2007).

1.8.2 Synaptic plasticity after stroke

As suggested by Hebb more than half a century ago, neuronal cortical connections can be remodelled by our experience (Hebb 1949, Hebb 1947). Since then, chemical and anatomical plasticity within the cerebral cortex has been demonstrated in adult animals (Bennett et al. 1996, Holloway Jr 1966, Johansson 2000, Kleim et al. 1996, Olsson et al. 1994, Rosenzweig 1966, Torasdotter et al. 1998, Turner and Greenough 1985, Volkmar and Greenough 1972). Merzenich and co-workers extensively demonstrated first evidence of another aspect of brain plasticity where cortical representation areas and maps can be modified through sensory input, learning and experience, as well as in response to brain injury (Jenkins and Merzenich 1987, Jenkins et al. 1990, Merzenich et al. 1983, Merzenich et al. 1984, Nudo and Milliken 1996, Nudo et al. 1996, Pons et al. 1988, Xerri et al. 1998). The potential relevance of plasticity in stroke rehabilitation was proposed more than two decades ago (Jenkins and Merzenich 1987). Neuroplastic remodelling mechanisms can be enhanced by enriching the environment with complex tasks during rehabilitation and thus promoting further recovery (Gauthier et al. 2008, Johansson and Ohlsson 1996, Wolf et al. 2006).

Plasticity is defined as anatomical and functional alterations within the CNS that result in enhanced functional recovery (Johansson 2007, Lee and van Donkelaar 1995). The mechanisms involved include regulation of brain circuits and activation of parallel pathways that maintain functions lost, unmasking and activation of silent or dormant pathways, and/or long term plasticity through production of new dendritic spines and neurite outgrowth of surviving neurons as well as new synapse formation (Calabresi et al. 2003). Motor and sensory cortices are loosely organised into somatotopic functional maps that display high levels of use-dependent plasticity that can be modified based on activity (Murphy and Corbett 2009, Nudo et al. 1996). Spontaneous improvements in neurological outcomes observed in both animal stroke models and human patients most
likely occur through mechanisms involving remapping and reconnections as the number of newly generated neurons, which will be discussed later, are not sufficient to replace a substantial portion of lost cells to facilitate recovery (Brown 2006, Carmichael 2003, Fraser et al. 2002). Axonal sprouting and synaptogenesis has been observed after ischemia based on up-regulated expression of growth-promoting genes that mediate signalling within growth cone membranes, reorganisation of cytoskeletal elements and increased synaptophysin expression suggesting synapse formation within the penumbra (Carmichael 2003, Fitch and Silver 2008, Stroemer et al. 1995). It became clear in the last decade that the penumbra is not merely a region passively dying over time, but is actively recovering and as such is a principal target for therapies in neuroreparation (Font et al. 2010).

Acute brain injury including stroke can also trigger diaschisis phenomena, which involves a sudden loss of functions in regions remote to the injury site but possess anatomical connections to the lesioned area (Carmichael et al. 2004). Diaschisis resolution contributes to later functional recovery within the ipsilateral, contralateral hemispheres as well as the cerebellum and spinal cord (Font et al. 2010). The long-term changes and resolution of diaschisis are based on unmasking of silent synapses that can strengthen existing neural circuits, and axonal regeneration which creates and alters the shape, number and type of synapses (Calabresi et al. 2003). Dendritic spines, which are the major post-synaptic targets for glutamatergic transmission, are the focus of constant remodelling through the action of neurotransmitters, neurotrophic factors, newly synthesised synaptic proteins, and gene expression (Kawamata et al. 1999).

1.8.3 Neurogenesis within the adult brain

Brain plasticity is also supported by the generation of new cells, both neuronal and glial, through the process of neurogenesis that involves the generation of neural stem/progenitor cells traditionally thought to solely occur during the embryonic stages of development in the mammalian CNS (Cajal 1913). Until the mid 20th century, neurogenesis in the mammalian nervous system was believed to be restricted to fetal development and no regeneration occurred in the adult brain. In 1962 Joseph Altman discovered first evidence of neurogenesis in the brains of adult mammals (Altman 1962), and demonstrated adult neurogenesis in the dentate gyrus of the hippocampus (Altman 1963, Altman and Das 1965), olfactory bulb (Altman 1969) and neocortex.
Altman’s controversial discoveries crushed the century-old dogma deeply entrenched at the time that no new neurons are formed in the adult brain after birth and as such his discovery was essentially ignored until the 1980s. Regardless of this, Altman went on further to discover and name the rostral migratory stream using $[^{3}H]$-thymidine autoradiography to label proliferating cells in the rat brain (Altman 1969). Michael Kaplan re-examined the phenomenon of neurogenesis in the 1970-1980s (Kaplan and Hinds 1977) and demonstrated the presence of newborn cells in the adult brains of mice that had structural characteristics of neurons including dendrites and synapses. Meanwhile Fernando Nottebohm demonstrated neurogenesis in canaries and provided evidence for functional roles of adult neurogenesis in seasonal song learning (Goldman and Nottebohm 1983). It is now widely accepted that in most mammals neurogenesis occurs in three germinal regions in the mature brain; the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, the subventricular zone (SVZ) lining the lateral ventricles and the olfactory epithelium (Arnold et al. 1998, Kempermann et al. 2004, Ming and Song 2005, Taupin and Gage 2002). Neurogenesis has been found to occur throughout life in rodents (Altman and Das 1965, Altman 1969, Kaplan and Hinds 1977), non-human primates (Kornack and Rakic 2001) and humans (Eriksson et al. 1998, Gross 2000). In the intact adult mammalian CNS, neurogenesis outside these germinal zones appears to be particularly limited and under considerable debate (Ming and Song 2005). Currently neurogenesis in the adult brain is one of the hot topics in neuroscience, especially because of the new opportunities for treatment of brain damage and disease in either harnessing progenitors to regenerate lost tissue or by cell transplantation therapies.

### 1.8.3.1 Neurogenesis in the hippocampus

The hippocampus, located in the medial temporal lobe of the brain, is composed of multiple subfields; the dentate gyrus containing the fascia dentate and the hilus, and the cornus ammonis (CA) that is differentiated into fields termed CA1, CA2, CA3 and CA4 (O'Keefe and Nadel 1978). Neurogenesis that occurs in the hippocampus is believed to be involved in learning, memory and mood regulation (Abrous et al. 2005, Raber et al. 2004). Neural progenitor cells (NPCs) in the hippocampus are located in the subgranular zone (SGZ) at the border between the granule cell layer (GCL) and hilus of the dentate gyrus (DG) (Ming and Song 2005, Riddle and Lichtenwalner 2007). Studies have reported that the resident hippocampal precursor is a restricted progenitor cell capable
of proliferation and multipotential differentiation however cannot self-renew and thus proliferate indefinitely (Bull and Bartlett 2005, Seaberg and van der Kooy 2002). When these progenitors undergo division some daughter cells differentiate into neurons and develop the characteristic prominent apical dendrite of dentate granule neurons as they migrate into the GCL. Immature adult-born neurons extend axons into the hippocampal area CA3 as rapidly as 4-10 days after mitosis (Hastings and Gould 1999), and display passive membrane properties, action potentials and functional synaptic inputs similar to that found in mature dentate granule cells (van Praag et al. 2002). However, compared to the development of granule neurons born at the developmental peak of genesis, the structural and functional growth of adult born granule neurons is slower (van Praag et al. 2002, Zhao et al. 2006).

1.8.3.2 Neurogenesis in the subventricular zone and rostral migratory stream

The degree of adult neurogenesis within the hippocampus is only a fraction compared to that which occurs in the anterior portion of the adult subventricular zone (SVZ) (Riddle and Lichtenwalner 2007). The adult SVZ is a thin and persistent remnant of the secondary proliferative zone of the developing brain (Riddle and Lichtenwalner 2007). Olfactory bulb (OB) neurogenesis is reported to be involved in olfactory discrimination and olfactory memory (Abrous et al. 2005).

It has been reported that neural stem cells display similar characteristics to astrocytes, however not all astrocytes within the region are neural stem cells (Doetsch et al. 1997, Doetsch et al. 1999, Morshead et al. 1994). Extensive analysis of this region has revealed identification of three main cell types; (1) neuroblasts or Type A cells, (2) radial glial cells or SVZ astrocytes referred to as Type B cells, (3) undifferentiated cells also known as transit amplifying cells referred to as Type C cells, (4) a monolayer of ciliated ependymal cells that line the ventricle (see Figure 1.5) (Doetsch et al. 1997).

Neuroblasts born within the SVZ maintain their proliferative capacity as they migrate along a restricted pathway, called the rostral migratory stream (RMS) (Altman 1969), to the olfactory bulb, where they differentiate into periglomerular and granule neurons (Coskun and Luskin 2002, Doetsch et al. 1997, Luskin 1993, Luskin et al. 1997, Pencea et al. 2001). Throughout the migration, chains of neuroblasts are ensheathed by slowly proliferating astrocytes that potentially help maintain an
appropriate microenvironment for migration and division (Riddle and Lichtenwalner 2007). The division that occurs within the SVZ and RMS is far more extensive than that in the DG where division of progenitor cells is spatially restricted whereas the microenvironment that supports division within the RMS that is anatomically situated far from the SVZ. Division of progenitor cells within the DG is spatially restricted, whereas division of neuroblasts within the RMS, far from the SVZ, suggests the microenvironment that supports division is more extensive in the SVZ/RMS system. Furthermore, stem/progenitor cell populations between the SVZ and the SGZ differ (Ray and Gage 2006), where the adult hippocampus lacks true stem cells within the progenitor population and only contain more restricted progenitor cells that underlie hippocampal neurogenesis (Bull and Bartlett 2005, Seaberg and van der Kooy 2002). The postnatal SVZ is also a site for gliogenesis (Levison and Goldman 1993, Paterson et al. 1973), and in vitro experiments have demonstrated neural stem/progenitor cells can be isolated from adult or fetal SVZ and cultured, retaining self-renewing and multipotent properties capable of generating neurons and glia (McLeod et al. 2012).
Figure 1.5 Neurogenesis within SVZ and the DG of the normal adult rodent brain.
Sagittal view of the rat brain illustrating neurogenesis within the SVZ where neuroblasts migrate through the rostral migratory stream (RMS) into the olfactory bulb (OB) (A). Coronal view of adult rat brain showing the SVZ of the LV (B). Cell types and key components found within the adult subventricular zone (SVZ) niche of the lateral ventricle (LV) include; radial glial cells (b cells; blue) which are stem cells that produce migrating neuroblasts (a cells; red) via rapidly dividing transit-amplifying cells (c cells; green) that are destined for the olfactory bulb under normal conditions; multi-ciliated ependymal cells (e cells; grey) that line the lateral ventricle; and specialised basal lamina (BL; black), endothelial cells, and blood vessels (BV) (C). Adapted from Riquelme et al. (2008). Abbreviations: CC, corpus callosum; DG, dentate gyrus.

1.8.3.3 Neurogenesis in the olfactory epithelium

The birth of new specialised olfactory receptor neurons (ORNs) of the olfactory nerve within the adult olfactory epithelium (OE) has been previously described, (for review, see (Schwob 2002)). The receptor elements that subserve the olfactory sense
and transducer volatile chemical stimuli are set in the OE which lines a section of the nasal cavity and are in direct contact with the airborne environment (Farbman 1992, Schwob 2002). The relatively unprotected position within the nasal cavity means the OE is easily damaged by exposure to infectious agents, toxins, or mechanical trauma (Schwob 2002). Progenitor cells within the basal layer of the OE produce new receptor neurons that migrate superficially as they develop the characteristic apical dendrite and project an axon to the glomerular layer of the OB (Riddle and Lichtenwalner 2007). Indeed, the remarkable capacity of the olfactory system to recovery from injury allows maintenance of critical sensory function, despite the system’s vulnerability to damage (Schwob 2002). Olfactory loss has even been shown to be an early indicator of Alzheimer’s disease pathogenesis and age-related neural decline (Rawson and LaMantia 2007). Neurogenesis within the OE is less extensively studied than the SVZ/RMS and the DG and regulation mechanisms of neurogenesis within the olfactory system are only beginning to be elucidated (Schwob 2002).

1.8.3.4 Neurogenesis in the other regions

It is generally agreed that the three regions described above are the only sites for relatively large-scale and ongoing neuronal replacement in the normal mature brain, there is evidence supporting the potential for more widespread adult neurogenesis (Riddle and Lichtenwalner 2007). The properties of cells similar to stem cells or progenitor cells obtained from the hippocampus and anterior SVZ have been isolated from other regions of the brain including the cerebral cortex, striatum, septum, spinal cord, hypothalamus and even white matter (for review, see (Emsley et al. 2005)). However, it is debatable as to whether stem cells found within the cerebral cortex (Dayer et al. 2005, Gould et al. 1999b, Gould et al. 2001), amygdala (Bernier et al. 2002), spinal cord (Yamamoto et al. 2001) were produced by these regions and are evidence of widespread neurogenesis or represent cells that have originated from the DG and SVZ/RMS and have migrated. Evidence for constitutive neuronal replacement in regions other than the three main germinal zones remains controversial due to the methodological challenges of analysing cells that divide slowly or rarely as well as significant questions of what constitutes adequate proof a particular cell is newly born and that a newly born cell identified is a neuron (Nowakowski and Hayes 2000, Rakic 2002).
1.8.3.5  Aged-related decrease in neurogenesis

Earlier studies of adult neurogenesis using thymidine labelling and electron microscopy revealed neurogenesis is sustained in the senescent brain, albeit at a lower level compared to young adults (Kaplan 1985, Riddle and Lichtenwalner 2007). Experiments using BrdU labelling paradigms and immunolabelling of immature neuronal markers have demonstrated the extent and time course of aging related changes in the hippocampus of rats and mice (Kuhn et al. 1996, Lichtenwalner et al. 2001, Nacher et al. 2003, Riddle and Lichtenwalner 2007, Seki and Arai 1995). The profound reduction of 80% or more of new neurons occurs relatively early in the aging process with the greatest decline occurring by middle age and a modest decrease during later senescence (Kuhn et al. 1996, Lichtenwalner et al. 2001). Although detailed studies are limited, a similar decline has been observed in primates (Gould et al. 1999a, Riddle and Lichtenwalner 2007).

Aging within the SVZ has not been as extensively studied as that within the hippocampal region, however, a similar decline in proliferation has been observed. Greatest decline in cell genesis within the SVZ was observed between young adulthood and middle age with a moderate additional decline with further aging, similar to that in the hippocampus (Jin et al. 2003a, Luo et al. 2006). Aging-related decrease in progenitor proliferation within the SVZ appears to be further compounded by additional deficits in either the division of migrating neuroblasts within the RMS or a decrease in survival rates of these neuroblasts, which results in a greater decline in newborn neurons within the OB (Tropepe et al. 1997). Studies using electron and light microscopy suggest that SVZ neurogenesis becomes restricted to the dorsolateral aspect of the lateral ventricle and an increase in the number of astrocytes interposed among ependymal cells (Luo et al. 2006). As in comparison to the hippocampus, both the size of the SVZ and the number of migrating neuroblasts are reduced in aged macaques compared to young adults (Freundlieb et al. 2006).

Evidence of age-related changes in the genesis of ORNs is limited and further confounded by the virtue of its exposed location within the nasal cavity and the influence of external factors on their numbers (Riddle and Lichtenwalner 2007). Investigators have demonstrated numerous reports on decreased number of ORNs with
patchy replacement of sensory epithelium by respiratory epithelium, however the loss of ORNs cannot be assumed to be due to decreased genesis (Naessen 1971).

The mechanisms of aging-related influences on neurogenesis involves multiple points of regulation, including the size of progenitor cell populations, the rate of cell division, survival of daughter cells, the neuronal or glial commitment of daughter cells, and the rate of neuronal outgrowth and differentiation (for detailed review see, (Riddle and Lichtenwalner 2007)). Studies have demonstrated that BrdU labelling of proliferative cells supports the conclusion that aging does not diminish cell survival rates of newborn cells but rather it results in a decline in proliferation rates (Cameron and McKay 1999, Kempermann et al. 1998, Kuhn et al. 1996). Additionally, although survival rates appear unaffected by age, the percentage of newly generated progenitors that differentiate into neurons is much lower in middle-aged and old animals compared to young adult (Kempermann et al. 1998, Lichtenwalner et al. 2001, Rao et al. 2005, Seki and Arai 1995). Continued exploration of the mechanisms that control neurogenesis and mediate age-related decline in neurogenesis are important for the progress of harnessing the brain’s regenerative potential to restore function lost to stroke injury, which more often affects the elderly population.

1.8.3.6 Factors known to regulate neurogenesis in the adult brain

Intrinsic factors that influence neurogenesis are vast and evolving and these include transcription factors and cell cycle regulators, hormone and growth factors such as stress hormones, growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis, fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF) family, transforming growth factor-β family, retinoic acid and neurotransmitters (Abrous et al. 2005, Hagg 2005, Riddle and Lichtenwalner 2007). Recent studies report that changes in neurogenesis develop primarily due to alterations in the neurogenic microenvironment and factors that control cellular division, rather than progenitor cell loss or intrinsic cellular changes (Riddle and Lichtenwalner 2007). A striking advancement was understanding adult neurogenesis does not occur randomly or homogeneously throughout the neurogenic zones, instead dividing neural progenitor cells can be found in close association with microvasculature within a “neurovascular niche” with neurogenesis closely associated with active angiogenesis and remodelling.
(Alvarez-Buylla and Lim 2004, Doetsch 2003, Palmer et al. 2000, Ward and Lamanna 2004). It is understood that multiple steps of neurogenesis are controlled by glial, in particular astrocytes, which regulate proliferation, fate specification, migrating and integration of neuronal progeny into pre-existing circuits (Ma et al. 2005). The neurogenic zones are unique and highly specialised microenvironments (niches) that strictly regulate the development of adult neural stem cells.

Interestingly, several neurotransmitters have been implicated in adult neurogenesis and can act as trophic factors and synaptic messengers (Riddle and Lichtenwalner 2007). Loss of cholinergic projections from the basal forebrain to both the DG and OB have resulted in a decline in the number of newborn neurons within these regions (Cooper-Kuhn et al. 2004, Mohapel et al. 2005). Loss of dopaminergic projections from the substantia nigra to the SVZ in aged primates have resulted in decreases in the number of proliferating progenitor cells and developing neurons within the SVZ (Freundlieb et al. 2006). γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the adult brain, has been found to initially exert excitatory action on newborn neurons due to their high cytoplasmic chloride content during neuronal maturation of the fetal brain (Ben-Ari 2002, Delpire 2000, Owens and Kriegstein 2002, Payne et al. 2003). Ge et al. (2006) demonstrated that newborn granule cells in the dentate gyrus of the adult hippocampus were tonically activated by ambient GABA before the cells were sequentially innervated by GABAergic and glutamatergic synaptic inputs.

1.8.3.7   Neurogenesis after stroke

The brain attempts to self-repair after cerebral ischemia (Dancause et al. 2005, Zhang et al. 2008) and brain remodelling contributes to functional recovery in many patients (Weiller et al. 1992, Weiller et al. 1993). Under normal conditions, newly generated neuroblasts migrate to their appropriate destinations where SGZ neural progenitor cells differentiate into granule cells in the hippocampus (Cameron et al. 1993) or SVZ neural stem/progenitor cells differentiate into interneurons in the olfactory bulb; granule cells or periglomerular cells (Figure 1.5) (Altman 1969, Lois and Alvarez-Buylla 1993).
Under ischemic conditions, robust enhancement of neurogenesis has been observed in the SGZ/GCL in the hippocampus and the SVZ (Arvidsson et al. 2001, Arvidsson et al. 2002, Jin et al. 2003b, Parent et al. 2002, Takagi et al. 1999, Teramoto et al. 2003). There is an alteration in the pattern of adult neurogenesis following stroke whereby proliferation of cells within the SVZ increases and newly born, immature neurons migrate to areas of ischemic injury rather than the olfactory bulb (Figure 1.6) (Arvidsson et al. 2002, Ohab et al. 2006, Parent 2003, Zhang et al. 2001). A few studies suggest that following ischemic injury, particular brain functions revert to that seen during early developmental stages where the recovery process demonstrates similarities to ontogenesis (Cramer and Chopp 2000). Stroke can result in deficits in hippocampal-associated spatial memory (Yonemori et al. 1999), and enhanced neurogenesis observed within the DG has been suggested to be a compensatory adaptive response to ischemic injury that may contribute to recovery (Liu et al. 1998).

Stroke-induced neurogenesis has also recently been demonstrated in the adult human brain, even in patients of advanced age (Minger et al. 2007). In the adult brain newly born stem cells are capable of migrating to the damaged striatum (Arvidsson et al. 2002, Jin et al. 2003b) as well as the infarcted cortex (Jin et al. 2003b, Ohab et al. 2006). The ability of neural progenitor cells to migrate long distances is an impressive process due to the tissue boundaries of striatum, white matter and cortex they encounter while travelling through the brain from the SVZ (Ohab et al. 2006). Despite the robust response by NPCs more than 80% die within weeks after stroke. This low survival rate can be attributed to the inhospitable environment of the severely ischemic region with toxic signalling events, inflammation and reduced trophic support factors negatively influencing cell survival. Additionally, less than ~ 0.2% of NPCs become mature neurons with most surviving stem cells differentiating into astrocytes (Arvidsson et al. 2002, Zhang et al. 2001). Astrocytes are more robust and readily capable of surviving in hypoxic conditions (Gurer et al. 2009). None-the-less, of the cells that do differentiate into neurons has been shown to morphologically and electrophysiologically integrate into neural networks in the stroke damaged region (Hou et al. 2008). It remains to be established whether stroke-induced neurogenesis is sufficient to facilitate functional recovery, however, this response offers hope to translational and clinical possibilities (Emsley et al. 2005). Understanding active adult neurogenesis from neurogenic and
potential non-neurogenic regions is a step forward in generating treatment strategies that target repair processes for treatment of injuries to the CNS including stroke.

Figure 1.6 Neurogenesis following cerebral ischemia in the adult rodent brain. Coronal section of adult rat brain showing the SVZ of the LV (A). Schematic coronal view of neural precursor/progenitor cells and neuroblasts in the SVZ of non-ischemic (B) and ischemic (C) hemispheres where proliferation increases after stroke within the ischemic hemisphere and neuroblasts migrate towards infarcted brain regions. Adapted from Zhang et al. (2008). Abbreviations: CC, corpus callosum; LV, lateral ventricle.

1.8.4 Angiogenesis

In concert with neural plasticity and neurogenesis, brain repair processes after stroke also include complex patterns of vascular remodelling. The first primitive vascular plexus within the embryo is established through a process known as vasculogenesis where blood vessels form de novo from endothelial progenitor cells, while further expansion of the vascular plexus occurs through angiogenesis (Patan 2000). By definition, angiogenesis is the formation of new blood vessels from pre-existing vascular networks (Risau 1998). The history of vasculogenesis and vascular remodelling dates back 227 years to the work by John Hunter who first coined the term “angiogenesis” in 1787. John Hunter identified growing vessels in healing wounds, and embryos and carotid arteries in deer (Hunter 1870), while Meyer described sprouting of new blood vessels from pre-existing vessels from observations in tadpole tails (Meyer 1853, Skalak 2005). In the 20th century Judah Folkman pioneered scientific research
into the importance of angiogenesis mainly in tumour growth and cancer and is considered to be the “father of angiogenesis” (Folkman et al. 1962, Hall 2005, Ribatti 2008, Stephenson et al. 2013). Between 1975 and 1990 it became well accepted that microvessels can dramatically and rapidly adapt and their growth and regression processes have been of great interest as therapeutic targets (Skalak 2005).

Angiogenesis is a vital a process that occurs in growth and development and is strictly controlled in adults. Most blood vessels remain quiescent in the adult, however, endothelial cells retain the ability of rapid division based on physiological stimuli such as in response to ovulation (Karamysheva 2008). Many pathological states induce angiogenesis including wound healing, chronic inflammation, restenosis, atherosclerosis and tumours, as well as regenerative processes (Patan 2000). Angiogenesis occurs through two distinct processes, sprouting of endothelial cells and splitting of vessel lumens through intussusceptive angiogenesis (Patan 2000). Sprouting angiogenesis consists of several steps described by Ausprunk and Folkman (Ausprunk and Folkman 1977) along with many others (for review see (Burri and Tarek 1990, Folkman 1982, Folkman 1985, Folkman 1986)), including endothelial-cell proliferation, migration, tube formation, branching, and anastomosis (Carmeliet 2000, Risau 1997). Intussusceptive mode of angiogenesis consists of the formation of circular columns (pillars) of interstitial tissue that distinguish future vessels, pillar reshaping and fusions, delineation, segregation, growth, and extraction of newly formed vascular entities, and finally new branching generations and maturation of all components (for review see (Djonov et al. 2000, Patan 2000)). Endothelial cell sprouting occurs through migration and mitosis and results in new vessels composed of endothelial cells derived from parent vessels, while intussusceptive angiogenesis occurs by multiplication of existing vessels through lumen splitting (Patan 2000). It has been suggested that intussusceptive angiogenesis plays a role in early capillarisation, network remodelling as well as large vessel formation. Increased metabolic demand has been suggested to switch sprouting angiogenesis to intussusceptive angiogenesis for growth of capillary networks (Djonov et al. 2000). Angiogenesis and vascular remodelling are important processes that occur throughout the life of an organism, and are directed by genetic pre-determinants and environmental stimuli (Skalak 2005).
Over 20 endogenous regulators of angiogenesis have been described such as growth factors, matrix metalloproteinases, cytokines and integrins (Folkman and Klagsbrun 1987, Klagsbrun and D'Amore 1991). Important roles in the development, induction of endothelial cell division, and differentiation of vasculature occurs through many growth factors including vascular endothelial growth factor (VEGF), VEGF receptor 2, platelet-derived endothelial cell growth factor, fibroblast growth factors (FGFa and FGFb), epidermal growth factor (EGF), angiogenin, and ephrin receptors and ligands (Klagsbrun and D'Amore 1991, Plate 1999, Yancopoulos et al. 1998). Transforming growth factor-β and transforming growth factor-α inhibit endothelial cell proliferation but can induce three-dimensional tube formation and other aspects of angiogenesis (Klagsbrun and D'Amore 1991). Angiopoietins 1 and 2 and their tyrosine kinase receptor Tie2 are involved in maturation, stabilisation, and remodelling of vessels (Yancopoulos et al. 1998). These regulation factors represent potential mechanisms and molecular targets for angiogenic therapies after brain injury (Ergul et al. 2012, Xiong et al. 2010).

1.8.4.1 Angiogenic response to stroke

Angiogenesis following ischemic brain injury has been demonstrated in experimental models and in humans (Krupinski et al. 1994, Sbarbati et al. 1996, Taylor et al. 2013). During the early stages of angiogenesis, newly formed blood vessels are permeable and become less leaky as they mature (Plate 1999, Taylor et al. 2013, Zhang et al. 2000). Up-regulated expression of VEGF, VEGFR2, angiopoietins, and Tie2 was observed in rodent ischemic brain tissue for up to 28 days post-stroke (Hayashi et al. 2003, Taylor et al. 2013, Zhang and Chopp 2002). Stroke patients were observed to have high serum concentrations of VEGF between 7-14 days post-stroke (Slevin et al. 2000). These factors are known to mediate angiogenesis within the ischemic regions (Beck et al. 2000, Lin et al. 2000, Zhang et al. 2000, Zhang et al. 2002). Stroke-induced increase in VEGF and VEGF receptor 2 expression was found to promote cerebral vessel sprouting to form new permeable vessels while angiopoietin 1 and Tie2 up-regulation was found to lead to maturation of the newly formed vessels into functional structures (Beck et al. 2000, Lin et al. 2000, Zhang et al. 2000, Zhang et al. 2002).

Angiogenesis is essential for ischemic brain repair as it can improve blood flow perfusion to ischemic and surrounding tissues which then stimulates metabolism and in
turn may improve functional outcome following stroke (Sbarbati et al. 1996). A significant correlation was found between angiogenesis and survival times after stroke as patients with higher density of blood vessels were found to survive longer in comparison to patients with low vascular density (Krupinski et al. 1993, Krupinski et al. 1994, Slevin et al. 2000). Restorative therapies enhancing angiogenesis have been associated with improvements in functional outcome and suggest in addition to neurogenesis, angiogenesis should be further explored as a treatment modality (Wang et al. 2004, Wang et al. 2008, Zacharek et al. 2007, Zhang et al. 2003a). However, the benefits of angiogenesis can be negatively affected by premorbid disease, as observed in diabetes and/or hypertension, and should also be considered when investigating treatment options (Ergul et al. 2012, Navaratna et al. 2009).

1.8.5 Coupling of neurogenesis and angiogenesis

Indeed, it is well recognised that neurogenesis and angiogenesis are tightly co-regulated after stroke and brain injury (Leventhal et al. 1999, Moskowitz et al. 2010, Ohab et al. 2006, Palmer et al. 2000, Thored et al. 2007, Zhang et al. 2000). Neurogenesis and angiogenesis share similar molecular mechanisms that have been evolutionarily conserved so that similar pathways and mediators are involved in both processes (Carmeliet and Tessier-Lavigne 2005). Normally within the brain, complex mechanisms of cell to cell signalling occur between cerebral endothelium and neural stem/progenitor cells within the subventricular and subgranular zones of ongoing neurogenesis (Palmer et al. 2000). A significant question since the discovery of neural progenitors in the adult brain was how these precursors’ migrated long distances, through complex territories of the brain from the posterior to the anterior regions of the brain. A study by Snapyan et al. (2009) investigated the long-distance journey and demonstrated that blood vessels precisely outline the migratory stream to the olfactory bulb providing structural and molecular cues for neuroblast migration in the adult mammalian brain. These close relationships are maintained in the context of recovery after stroke (Thored et al. 2007). Ohab et al. 2006 have also shown proliferating endothelial cells form a continuous link along the entire migratory pathway of immature neurons from the SVZ to the infarcted region. Studies have shown migrating immature neurons closely associate with the newly formed microvessels within the peri-infarct cortex as early as 3 days following stroke (Ohab et al. 2006). Activated endothelial cells, injured and newly forming blood vessels release an array of trophic factors
including VEGF and BDNF which can evoke neurogenesis, are chemotactic for progenitor cells and may enhance the survival and integration of neuroblasts into the brain tissue (Leventhal et al. 1999, Minger et al. 2007, Shen et al. 2004). Enhancement of neural progenitor cell proliferation and differentiation has been found when co-cultured with endothelial cells (Leventhal et al. 1999, Shen et al. 2004). SVZ neural progenitor cells in return have been found to up-regulate their expression of angiogenic genes (Liu et al. 2007) and co-culturing experiments have shown neural progenitor cells promote angiogenesis by increasing capillary tube formation (Teng et al. 2008). Therefore there is a loop of interactions between stem cells and angiogenesis, and recovery of the brain after stroke comprises interdependent neurovascular plasticity and remodelling processes that employ multiple mediators and signals (Snapyan et al. 2009). Exploration of how these multi-cell and multi-signal processes are regulated and the extent in which they occur after stroke may represent a new frontier of neurovascular repair therapies (Moskowitz et al. 2010).

1.8.6 The limitations of angiogenesis and neurogenesis

Unfortunately, the timing of endogenous repair responses initiated after stroke that have the capacity to promote self-repair are askew. Although neuroblasts migrate to regions of damage, few cells differentiate to form functional neurons (Zhang et al. 2001). The potential for brain regeneration relies heavily on the surrounding microenvironment: in rats, stroke increases the percentage of proliferating SVZ cells that contribute to neurogenesis as soon as 2 days after stroke, peaking at 7 days, and returning to normal by 14 days (Zhang et al. 2001). New capillary buds appear 3 days after cerebral insult (Hayashi and Chan 2003) and although studies show that angiogenic factors are up-regulated between 3 and 7 days after stroke, new functional vascular networks do not appear until much later, around 14 days (Hayashi et al. 2003, Sbarbati et al. 1996, Taylor et al. 2013, Yu et al. 2007). Given that neurogenesis peaks at 7 days it may be that the developing vasculature at this time is not mature enough to support neuroblast differentiation into significant neuronal populations that result in functional integration. By the time new vascularisation is able to deliver oxygen and glucose for normal energy metabolism to sustain new circuitry, neurogenesis has subsided (Zhang et al. 2001). Conversely, newly formed blood vessels require signalling to remain viable and if the damaged brain is not replaced these vessels begin to regress around 90 days from stroke onset (Yu et al. 2007). Therefore, although a significant
signalling loop exists between neurogenesis and angiogenesis, new blood vessels capable of sustaining brain replacement are not extensively formed until weeks after neurogenesis has subsided.

1.9 Therapeutics to harness endogenous repair

Considering spontaneous plastic alterations and other neurorestorative processes induced by stroke have been found to diminish within months (Bolognini et al. 2009, Bütefisch et al. 2003, Delvaux et al. 2003, Shimizu et al. 2002), non-pharmacological treatments may help to prolong this therapeutic window of potential plastic changes for recovery of neurological functions. The uninjured brain tissue appears to be receptive to modulation by external non-pharmacological methods that include task-oriented strategies, repetitive training, physical activity, and neuromodulatory approaches such as non-invasive brain stimulation (Cramer 2008a). Extremity Constraint Induced Therapy Evaluation (EXCITE) trial reported significant improvements for arm motor function using repetitive constraint-induced therapy (Cramer 2008a, Wolf et al. 2008). Various types of physical exercise training studies have documented beneficial sensorimotor, strength, endurance, physiological, and psychological effects in stroke survivors (Brinkmann and Hoskins 1979, Duncan et al. 1998, Engardt et al. 1995, Gordon et al. 2004, Hesse et al. 1995, Silver et al. 2000, Weiss et al. 2000, Whitall et al. 2000). Non-invasive brain stimulation has been extensively used in rehabilitation research for the potential in promoting motor recovery after brain injury (Ergul et al. 2012, Ridding and Ziemann 2010). Such treatment modalities have been found to increase angiogenesis, neurogenesis and affect the CNS from the molecular to synaptic level of cortical maps and large scale neural networks to improve motor recovery (Baba et al. 2009, Forstreuter et al. 2002, Gertz et al. 2006, Hambrecht et al. 2003, Kojda et al. 2001, Nudo 2006, Ward and Cohen 2004, Xapelli et al. 2008).

Unfortunately, non-pharmaceutical approaches are mainly focused on improvement of motor function with only mild to moderate gains documented, and as such is often limited to a fraction of patients (Cramer 2008a, Hendricks et al. 2002, Schaechter 2004). Recent advancements have been made towards developing management of non-motor impairments through rehabilitation (Brewer et al. 2012, Carandang et al. 2006). Despite the progress in acute management of stroke using non-
pharmacological treatments, a large portion of stroke survivors are still left with significant impairments (Brewer et al. 2012). Combinatorial therapeutic approaches might prove to be more fruitful in promoting recovery of a range of functions. The developing understanding of brain repair processes and neural plasticity is of great interest, hence investigating alternate restorative therapeutic strategies require further focus. Such treatment modalities aimed towards endogenous neurorestorative processes include pharmacological and cell-based therapies, used in combination with rehabilitation or alone, can stimulate significant neurological recovery after stroke. Future studies may need to temporally and spatially coordinate restorative events initiated after stroke to either harness or enhance their regenerative capabilities.

1.10 The potential of cell-based therapies to repair the damaged brain

In addition to the aforementioned strategies, cell-based therapies may represent a new cutting edge treatment modality for stroke sufferers. The advent of stem cell therapies aimed at repairing the brain following stroke was developed in the mid-1990s following the discovery of endogenous neurogenesis (Bonn 1998). Stem cell therapy has the potential to rescue the stroke affected brain and restore neurological function while harnessing endogenous regenerative processes initiated weeks after stroke (Wang et al. 2004). One key difference separating pharmacological strategies and cell-based treatments is the ability of transplanted cells to actively interact with parenchymal cells depending on their microenvironment, while drugs interact with brain cells depending on their pharmacokinetic profiles (Zhang and Chopp 2009). Cell-based therapies shift the paradigm from symptomatic control and prevention of damage during the acute phase, to restoring neural networks lost following stroke injury (Urbaniak Hunter et al. 2010) and thus widening the therapeutic window. Stem cell treatments are also capable of potentiating the endogenous processes of brain plasticity, including neurogenesis and angiogenesis (Zhang et al. 2005). However, just as with endogenous repair processes’, understanding the microenvironment after stroke during the recovery phase is essential to certify the survival, functionality, and positive influence of transplanted stem cells.

Due to the complexities of brain composition (discussed earlier) it is the unique ability of stem cells to differentiate into cell types appropriate to the structure in which they are grafted that makes them excellent candidates for restorative therapy. A wide
repertoire of cell types from different sources have been tested in stroke models (summarised in Table 1.2) including human neural stem cells (Roitberg et al. 2006), restricted progenitors cells (neural or glial progenitors) (Goldman et al. 2012, Roy et al. 2004), immortalised cell lines such as human embryonal carcinoma-derived neuronal cells (hNT or NT2N) (Borlongan et al. 1998, Saporta et al. 1999), and embryonic murine immortalised neuroepithelial MHP36 cell line derived stem cells (Modo et al. 2002), trophic factor-secreting kidney cells (Johnston et al. 2001), endothelial progenitor cells (Moubarik et al. 2010), human bone marrow cells (Chen et al. 2001a), human umbilical cord blood cells (Chen et al. 2001b), peripheral blood cells (Shyu et al. 2006, Willing et al. 2003), and adipose tissue cells (Kang et al. 2003). In addition, combinatorial treatments have also been considered for neural repair including transplants containing scaffolds or extracellular matrix to provide support to exogenous cells (Arien-Zakay et al. 2009, Park et al. 2002). Transplant studies in most cases have shown successful survival of grafted cells and improved behavioural outcomes. Stem cells possess neuroprotective potential, immunomodulatory effects and deliver an array of growth and neurotrophic factors. This is based on the ability of engrafted stem cells to secrete and promote the endogenous release of factors including stromal derived factor-1 (SDF-1), BDNF, fibroblast growth factor (FGF), VEGF, granulocyte-monocyte colony-stimulating factor (GM-CSF), glial cell-line-derived factor (GDNF), and nerve growth factor (NGF) (Bersano et al. 2010, Llado et al. 2004). Exogenously grafted cells have the potential of protecting existing host neural circuitries, enhancing synaptic activity, and possibly providing a platform for host neural regeneration (Wechsler and Kondziolka 2003). Furthermore, it has been suggested that the improved neurological function observed may be due to the implanted cells stimulating the damaged brain and evoking remodelling and rewiring of neuronal circuitry, or awakening depressed neural circuits particularly within the border zone of the infarct (Takahashi et al. 2008). The proposed mechanism/s of action from exogenous cell engraftments are summarised in Table 1.3 with the most attractive method of approach focused on stimulating the normal host neurogenic regions to generate stem or progenitor cells that facilitate neuronal plasticity for self-repair (Llado et al. 2004). Benefits from exogenous transplants is dependent on the type and severity of stroke as well as adequate circulation within cerebral tissue which ultimately influence survival, integration, and efficacy of neural transplants in stroke patients (Savitz et al. 2002). For cell-based therapies to be of major clinical value, transplanted cells should be able to act through
several mechanisms including replacement of lost neurons, provide trophic or chemoattractive support to promote survival of affected neurons, recruit endogenous progenitors, modulate inflammation, remyelinate axons, and repair damaged neural circuitry (Lindvall et al. 2012). The choice of the most appropriate cell source will also be governed by accessibility, safety, ethical restrictions and most importantly, efficacy.

Table 1.2 Cell types and sources used for treatment of stroke.
Several cell types have been tested in experimental models where safety and efficacy have been demonstrated. Adapted from Chui and Rao (2011).

<table>
<thead>
<tr>
<th>Cell types and sources</th>
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<tbody>
<tr>
<td>1) Neural stem cells derived from pluripotent stem cells, fetal &amp; adult tissue</td>
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<tr>
<td>2) Restricted progenitor cells (neural or glial restricted progenitors, oligodendrocytic progenitors)</td>
</tr>
<tr>
<td>3) Immortalised cell lines (undifferentiated cells or post-mitotic immature neuronal cells)</td>
</tr>
<tr>
<td>4) Non-neural cells (mesenchymal stem cells, bone marrow stem cells, cord blood cells, kidney cells, adipose tissue cells)</td>
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<tr>
<td>5) Combinatory therapies (mixed populations of cells, cells plus scaffold or extracellular matrix, genetically engineered cells for gene therapy or for growth and other factors)</td>
</tr>
</tbody>
</table>
Table 1.3 Suggested mechanism/s of action of exogenously transplanted cells to the nervous system.

One or more mechanisms of exogenous cell engraftment potentially contribute to enhanced recovery from disorders of the nervous system. Adapted from Chui and Rao (2011).

<table>
<thead>
<tr>
<th>Modes of action</th>
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<tbody>
<tr>
<td>1) Support endogenous neurogenesis or neurite outgrowth</td>
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<tr>
<td>2) Stimulate remodelling and rewiring of host neural circuitry</td>
</tr>
<tr>
<td>3) Release of trophic factors</td>
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<tr>
<td>4) Promote or restore myelination</td>
</tr>
<tr>
<td>5) Provide missing molecules</td>
</tr>
<tr>
<td>6) Modulate scar formation and properties</td>
</tr>
<tr>
<td>7) Modulate the immune response</td>
</tr>
<tr>
<td>8) Re-establish the blood brain barrier</td>
</tr>
<tr>
<td>9) Enhance neuronal survival</td>
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Interestingly, Modo et al. (2002) highlighted an important consideration for future work with cell therapy, which was that stem cells do not usually remain at the site of injection. The implication of this migration pattern was investigated by Modo and colleagues (2002) where they identified differential effects of graft location on the type of functional recovery that ensued. Therefore with the development of cell therapy graft placement should also be considered to improve the benefit from such treatment. Parenchymal versus intraventricular graft locations have been implicated in recovery ranging from cognitive to sensorimotor deficits (Modo et al. 2002). Furthermore, multiple graft sites appear to be more beneficial as they provide varying microenvironments for grafted cells, which in turn influence graft connectivity and transmitter release and thus facilitate recovery of specific behaviours (Modo et al. 2002). Transplantation studies have focused on implanting cells within penumbral regions suggesting these areas would be less hostile and provide a supportive extracellular matrix for the grafts. However, the possibility of spreading waves of depression, excess amounts of excitatory neurotransmitters, and the inhibitory glial scar barrier are potential risk factors that could influence penumbral grafts (Smith et al. 2012). On the
other hand transplants directly into the core infarct could result in high cell death rates
due to the release of pro-inflammatory cytokines and various other toxic signalling
molecules. Several factors require consideration for the successful development of any
cell-based treatment for acute ischemic stroke including cell characterisation, delivery
route, transplant sites, in vivo biodistribution of exogenous cells, mechanism of action,
preclinical safety and efficacy, location of injury, timing of treatment after stroke, and
patient response. Recommendations identifying key translational barriers required for
facilitating successful development of cell based therapies for stroke have been
highlighted during the STEPS meetings (Stem Cell Therapy as an Emerging Paradigm
for Stroke) (Savitz et al. 2002, The-STEPS-Participants 2009) involving investigators
from academia, industry leaders, clinical and science researchers, and National
Institutes of Health representatives. The potential application of stem cell treatments is
vast and may emerge in routine practice in the future however development of its use for
improving recovery for stroke is still in its infancy.

1.11 Clinical trials of cell-based therapies after ischemic stroke

Following extensive meta-analysis of over 100 pre-clinical stem cell transplant
studies to treat stroke in various animal models (Lees et al. 2012) human clinical trials
have commenced. Human stem cell transplant therapies are well established for treating
patients suffering haematopoietic and lymphoid cancers, including other blood diseases
and autoimmune disorders (Bliss et al. 2007). Clinical cell-based trials to treat acute
ischemic stroke are currently underway and those that have completed Phase I or II, or
are ongoing can be found summarised in Table 1.4. The advancements in experimental
and clinical use of stem cells have highlighted the enormous potential of cell
transplantation for stroke therapy. However, a great majority of trials are safety and
tolerability studies, with small sample sizes and control groups that were unspecified.
The PISCES (Pilot Investigation of Stem Cells in Stroke) study was the first clinical
trial of a neural stem cell therapy for stroke patients that was initiated in 2010 (Mack
2011, ReNeuron Limited 2010). The PISCES study, which involved a clinical trial with
ReN001, a genetically engineered human neural stem cell line, recently reported a
progress update which revealed no cell-related or immunological adverse effects from
receiving treatment. In addition patients sustained reductions in neurological
impairments based on the National Institutes of Health Stroke Scale (NIHSS), Barthel
Index, and Modified Rankin Score and Summated Ashworth scores for affected limbs. A wide range of cell types from a variety of sources are under investigation and ongoing trials and follow up assessments will yield further data on the safety and efficacy of the specific stem cells that are currently being utilised. These processes are a necessary prelude to large-scale randomised efficacy trials with broad clinical endpoints to judge the balance of risks and benefits. Meetings from the series of STEPs proposals have previously and will continue to provide an excellent forum for methodological discussions in the cell therapy field that will enable future translation to stroke treatment (Savitz et al. 2014, The STEPS Participants 2009).
Table 1.4 Summary of completed and ongoing prospective observational and randomised control trials using stem cell therapy to treat ischemic stroke sufferers.

<table>
<thead>
<tr>
<th>Author (Year)</th>
<th>Cell Type</th>
<th>No. of patients (Active/Control)</th>
<th>Stroke Type</th>
<th>Duration*</th>
<th>Administration</th>
<th>Immuno-suppression</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Ongoing prospective clinical trials</td>
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<tr>
<td>Savitz et al. (2009)</td>
<td>Autologous BMMC’s</td>
<td>30</td>
<td>Ischemic cortical infarct</td>
<td>24-72 hours</td>
<td>Intravenous</td>
<td>None</td>
<td>Phase I, single arm, evaluating feasibility and safety</td>
</tr>
<tr>
<td>Detante et al. (2000)</td>
<td>Autologous mesenchymal stem cells</td>
<td>30</td>
<td>Carotid ischemic infarct</td>
<td>Within 6 weeks</td>
<td>Intravenous</td>
<td>None</td>
<td>Phase II, randomized, controlled, open, with 3 parallel groups, evaluating feasibility and safety</td>
</tr>
<tr>
<td>Hernandez et al. (2000)</td>
<td>Autologous CD34+ bone marrow cells</td>
<td>20</td>
<td>MCA infarct</td>
<td>5-9 days</td>
<td>Intra-arterial</td>
<td>None</td>
<td>Phase I/II, single arm, evaluating safety and efficiency</td>
</tr>
<tr>
<td>Andre et al. (2000)</td>
<td>Autologous BMMCs</td>
<td>15</td>
<td>MCA infarct</td>
<td>3 hours to 90 days</td>
<td>Intravenous/Intra-arterial</td>
<td>None</td>
<td>Phase I, two arms (nonrandomised), evaluating safety</td>
</tr>
<tr>
<td>Habib et al. (2007)</td>
<td>Autologous CD34- bone marrow cells</td>
<td>10</td>
<td>Total anterior circulation infarct</td>
<td>Within 7 days</td>
<td>Intra-arterial</td>
<td>None</td>
<td>Phase I/II, single arm, evaluating safety and tolerability</td>
</tr>
<tr>
<td>ReNeuron Limited, PISCES trial et al. (2010)</td>
<td>CTX0E03 neural stem cells</td>
<td>12 (male)</td>
<td>Subcortical white matter and/or basal ganglia infarct</td>
<td>6 months to 5 years</td>
<td>Stereotactic injection into putamen</td>
<td>None</td>
<td>Phase I, single arm, single administration, ascending dose, evaluating safety</td>
</tr>
<tr>
<td>Steinberg et al. (2011)</td>
<td>SB623 stem cells</td>
<td>18</td>
<td>Subcortical MCA or lenticulo-striate artery + cortical involvement</td>
<td>Between 6 to 24 months</td>
<td>Intracerebral implantation</td>
<td>None</td>
<td>Phase I/II, nonrandomised, evaluating safety</td>
</tr>
<tr>
<td>Levy et al. (2011)</td>
<td>Allogenic mesenchymal bone marrow cells</td>
<td>35</td>
<td>Ischemic stroke</td>
<td>Longer than 6 months</td>
<td>Intravenous</td>
<td>None</td>
<td>Phase I/II, nonrandomised, one label, single dose, evaluating safety</td>
</tr>
<tr>
<td>Hinson (2011)</td>
<td>ALD401 cells derived from autologous bone</td>
<td>100</td>
<td>MCA infarct</td>
<td>Within 2 weeks</td>
<td>Intra-arterial</td>
<td>None</td>
<td>Phase I/II, nonrandomised, double blind, evaluating safety</td>
</tr>
<tr>
<td>Shyu et al. (2011)</td>
<td>Olfactory ensheathing cells</td>
<td>6</td>
<td>Chronic infarct</td>
<td>6 to 60 months</td>
<td>Intracerebral transplant of cultured and expanded olfactory mucosal cells collected 1 to 2 months prior</td>
<td>None</td>
<td>Phase I, randomized, evaluating safety and efficacy</td>
</tr>
</tbody>
</table>
Table 1.4 (continued)

<table>
<thead>
<tr>
<th>Author (Year)</th>
<th>Cell Type</th>
<th>No. of patients (Active/Control)</th>
<th>Stroke Type</th>
<th>Duration*</th>
<th>Administration</th>
<th>Immunosuppression</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bang et al. (2005)</td>
<td>Autologous mesenchymal cells</td>
<td>5/25</td>
<td>MCA infarct with persistent deficits</td>
<td>Beyond 7</td>
<td>Intravenous</td>
<td>None</td>
<td>Phase I/II, questionable study quality</td>
</tr>
<tr>
<td>Savitz et al. (2005)</td>
<td>Fetal porcine</td>
<td>5/0</td>
<td>MCA infarct</td>
<td>Average 5 years</td>
<td>Stereotactic implantation</td>
<td>None (cells pre-treated with anti-MHC antibody)</td>
<td>Phase I, study stopped early after 2 SAEs</td>
</tr>
<tr>
<td>Kondziolka et al. (2005)</td>
<td>Immortalised neuronal cells</td>
<td>14/4</td>
<td>Basal ganglia infarct with stable deficits</td>
<td>1 to 6 years</td>
<td>Stereotactic implantation</td>
<td>Methylprednisolone during surgery, cyclosporine 1 week before surgery and continued for 6 months</td>
<td>Phase II, no significant effect on functional outcome</td>
</tr>
<tr>
<td>Kondziolka et al. (2000)</td>
<td>Immortalised neuronal cells</td>
<td>20/0</td>
<td>Basal ganglia infarct</td>
<td>6 months to 6 years</td>
<td>Stereotactic implantation</td>
<td>Methylprednisolone during surgery, cyclosporine 1 week before surgery and continued for 6 months</td>
<td>Phase I, improved functional outcome, PET showed increased metabolic activity</td>
</tr>
<tr>
<td>Lin et al. (2002)</td>
<td>Autologous peripheral blood CD34+</td>
<td>30</td>
<td>Chronic MCA infarct with stable deficits</td>
<td>6 months to 5 years</td>
<td>Intracerebral implantation</td>
<td>None</td>
<td>Phase II, randomised, evaluating safety and efficacy</td>
</tr>
</tbody>
</table>

Abbreviations: MCA, middle cerebral artery; SAE, serious adverse effects; BMMCs, bone marrow mononuclear cells; CTX0E03, neural stem cell line (ReNeuron); SB623, modified stromal cells; PET, positron emission tomography. *Duration since onset of stroke. Adapted from Sahota et al. (2011).
1.12 The importance of when to transplant

The optimal time point for transplantation of stem cells following stroke in the clinical setting is yet to be determined. Timing of stem cell transplants is crucial as factors that support neuronal reconstruction need to be identified and targeted so as to harness endogenous restorative processes initiated after stroke. The microenvironment of the brain dramatically changes over time after stroke and has become well recognised as a significant factor regulating the fate and function of exogenously transplanted cells (Chiu and Rao 2011). As such engraftment timing should coordinate with the critical pathophysiological and regenerative events. Neurologists appear to mainly favour delaying implantation of cells for months after stroke when behavioural deficits reach a plateau, and the microenvironment becomes less aggressive towards grafted cells. However, during this time scar tissue can form which may negatively interfere with transplanted grafts and newly formed vasculature may begin to regress (Yu et al. 2007). In addition to this, delaying transplantation risks the brain becoming irreversibly damaged with the loss of potentially salvageable penumbral tissue. One of the most important requirements for long term survival of transplanted neural progenitor cells into brain is adequate blood flow to support graft viability. For this reason, a great deal of attention has been paid towards enhancing the longevity of transplanted stem cells by providing support cells and enhancing vasculogenesis within the graft (Ishikawa et al. 2013, Moubarik et al. 2011, Nakagomi et al. 2009, Oyamada et al. 2008). However, the potential danger of uncontrolled proliferation of exogenous cells still remains. Even the formation of benign tumours poses a risk by affecting normal functioning of circuits through added pressure. Instead, rather than developing strategies to promote vasculogenesis to support exogenous stem cells, targeting endogenous angiogenesis may achieve a similar result and avoids added complications or variables. Recent studies in our laboratory show the presence of new blood vessels by 7 days after stroke with functional vascular networks evident by 14 days (Taylor et al. 2013). Cell-based therapies may benefit from an established microvascular bed to optimise survival and influence maturation of cells within stroke damaged tissue. No studies to our knowledge have targeted sites of new vascularisation during the natural course of recovery, which may be more effective in promoting graft longevity and functional recovery.
1.13 Differentiation of stem cells prior to transplant

Previous studies have mainly focused on the use of undifferentiated stem cells to treat the stroke affected brain (Jiang et al. 2005, Li et al. 2006, Zhang et al. 2003b), however assessment of the histopathological influences of these cells has not been well documented. Studies have reported undifferentiated stem cell transplants predominantly differentiated into astrocytes rather than mature neurons (Chen et al. 2001b, Takahashi et al. 2008). The ischemic damaged brain most likely evokes differentiation along the glial path due to the robust nature of astrocytes in comparison to neurons (Wieloch 1985). However, astrocytic differentiation in such cases could result in further contribution to glial scar formation that may inhibit neuronal regeneration and hinder functional recovery (Sofroniew and Vinters 2010). Another point of concern lies within the proliferative capacity of stem cells, where failure to exit the cell cycle could result in fatal neoplastic/tumour formation (Molcanyi et al. 2009, Riess et al. 2007). Hence, to avoid astrocytic differentiation of grafted cells and reduce the risk of tumorigenicity the use of cells pre-differentiated into specific neuronal phenotype/s prior to transplant may offer a better alternative. Studies supporting this concept first emerged when NPCs that were pre-programmed into a GABAergic phenotype and later transplanted into the damaged spinal cord promoted functional recovery and reduced neuropathic pain (Mukhida et al. 2007). Since then 3 other studies using similar approaches have reported functional improvements when transplanting GABAergic cells for the treatment of Parkinson’s and Huntington’s diseases (McLeod et al. 2012, Mukhida et al. 2008a, Mukhida et al. 2008b). Suggested mechanisms by which GABAergic transplants accelerated functional recovery were through potential restoration of information relays in cortico-basal ganglia circuits and no tumour formation was detected. Whether GABAergic cell transplants stimulate trophic, endogenous restoration or replacement of missing/altered inhibitory neurons, or awaken depressed neural circuits through neurotransmitter release in stroke injury models remains to be determined (Alvarez Dolado and Broccoli 2011). However the above studies suggest that pre-differentiating cells prior to transplant would greatly extend the utility of neural transplantation and offer an alternative approach for stroke treatment.
1.14 Animal models of ischemic stroke

The successful translation of positive outcomes in pre-clinical animal studies to the human condition depends heavily on the reliability and reproducibility of the experimental animal model used (Roulston et al. 2012). Animal models of stroke are an invaluable tool to recreate the human situation and help elucidate pathophysiological mechanisms involved after stroke to ultimately test new therapeutic strategies (Garcia 1984, Ginsberg 1990, McAuley 1995). Experimental models include both permanent and transient (involving reperfusion) ischemia, and since most human strokes involve a degree of reperfusion, animal models that incorporate this element are most relevant. The most widely used model of ischemic stroke involves occlusion of the middle cerebral artery (MCA), as it is the most commonly affected vessel in humans (Yaqub et al. 1991). Mice and rats are the most commonly used species, followed by the growing use of larger species including rabbits, cats, dogs, pigs, sheep and non-human primates (Casals et al. 2011). Multiple experimental MCAo models have been developed (Traystman 2003) including thromboembolic models using blood clot (Kaneko et al. 1985) or air (Kochanek et al. 1987) embolism, intraluminal filament (Matsuo et al. 1994), electrocoagulation (Barone et al. 1991), photothrombotic models (Markgraf et al. 1993), and the endothelin-1 stroke model (Sharkey et al. 1993). There is no ideal model of stroke with all models having advantages and drawbacks (Roulston et al. 2012). Certain disadvantages include exacerbation of ischemic damage; blood clot embolisation in rats can cause endothelial cell injury (Garcia et al. 1981) which results in increased BBB breakdown, inflammation and finally exacerbating cerebral injury (Nishimoto et al. 1978). The physical insertion of the intraluminal filament to create MCAo can lead to subarachnoid hemorrhage, damage to the external carotid artery as well as the endothelial lining potentially increasing adhesion molecule expression and promote neutrophil infiltration (Kanemitsu et al. 2002, Nishigaya et al. 1991, Schmid-Elsaesser et al. 1998). The electrocoagulation models requires invasive cranectomy to expose the MCA and causes permanent MCAo without reperfusion, thus resulting in a model less relevant to the clinical setting (McAuley 1994, Takamatsu et al. 1998). The photothrombosis stroke model has been associated with a relatively small ischemic penumbra (or at times, without a salvageable penumbra) with oxidative damage limited to the infarct core without progressive distribution through the peri-infarcted area as observed in other stroke models and in humans (Braeuninger and Kleinschnitz 2009,
Carmichael 2005, Kim et al. 2000, Nagayama et al. 2000). Also, simultaneous disruption of endothelial cell integrity and rapidly progressive infarction in a small cortical volume results in substantial local vasogenic oedema (Watson et al. 1985), a pattern which is very different to that observed in human stroke and more closely resembles traumatic brain injury (Albensi et al. 2000, Schneider et al. 2002). Alternatively, the endothelin-1 stroke model is less invasive than other models and is unlikely to result in damage to the endothelium or surrounding structures (Macrae 1992, Sharkey et al. 1993). Most importantly, the occurrence of stroke in humans is rarely under the influence of anaesthesia and the ET-1 model is the only model of stroke in experimental rodents that avoids complications associated with the use of general anaesthetics. A plethora of evidence exists to suggest anaesthetic agents can have neuroprotective properties that potentially confound experimental findings, based on their regulation of ROS production (Hagerdal et al. 1978), neurotransmitter release (Bhardwaj et al. 1990), and gene expression (Hamaya et al. 2000).

Occlusion of the MCA results in a reduction of CBF in both the striatum and cortex, however the degree and distribution of blood flow reduction and lesion volume depends on the duration of occlusion achieved, the occlusion site along the MCA, and the amount of collateral blood flow into the MCA territory (Traystman 2003). Rodents are the most frequently used models of focal brain ischemia caused by MCAo (Belayev et al. 1996, Boyko et al. 2010, Longa et al. 1989, Spratt et al. 2006, Yu et al. 2003). Their widespread availability and low cost of rodents as well as the ease associated with handling, minimal housing space requirements, and simplicity of conducting replicate studies make them ideally suited for experiments (Roulston et al. 2012). Despite their small size, rats share similarities with humans in their cranial vasculature and physiology (Yamori et al. 1976), and have a well documented behavioural profile which is vital when testing neurorestorative therapies (Hunter et al. 1998, Roulston et al. 2012).

1.15 Endothelin-1 induced stroke

Endothelin-1 (ET-1) is a potent and long-acting venous and arterial constrictor. Originally isolated by Yanagisawa et al. (1988) and generated by endothelial cells, ET-1 exerts its’ affects through two main receptors, endothelin-A receptor (ETA-R) and
endothelin-B receptor (ETB-R) (Haynes and Webb 1998). Sharkey *et al.* (1993) first described stereotaxic injection of ET-1 adjacent to the MCA in conscious rats that resulted in constriction of the MCA followed by gradual reperfusion, thus producing a viable model of focal cerebral ischemia.

Given the setbacks associated with neuroprotective drugs in clinical practice, it is clearly important to use animal models of stroke that mimic more closely the human condition. The vasoconstricting action of ET-1 on the MCA produces profound reductions in local cerebral blood flow that results in the development of cerebral infarctions equivalent to those observed in other models of MCAo (Fuxe *et al.* 1992, Sharkey *et al.* 1993). In humans, prognostic clinical approaches such as the use of the Scandinavian Stroke Scale allow prediction of functional outcome and survival of stroke sufferers in order to correctly stratify treatment groups in clinical trials (Counsell *et al.* 2002, Roulston *et al.* 2012). A similar approach was developed in rats by Roulston *et al.* (2008). Concurrent observation of behavioural changes that occur during ET-1 stroke induction including symptomatic counter-clockwise circling, clenching and dragging of the contralateral forepaw and spontaneous circling can be used to accurately predict stroke outcome that precisely correlate with histological damage and neurological deficits (Roulston *et al.* 2008). Predictive outcome models such as the ET-1 model enables stratification of rats into treatment groups ensuring that stroke severity is evenly represented across all groups (Roulston *et al.* 2012, Roulston *et al.* 2008). This holds particular importance and relevance to the clinical setting since humans are not pre-assigned to treatment groups before the onset of stroke, but rather are assessed after stroke using various neurological scales to then assign treatments (Counsell *et al.* 2002). Such post-stroke methods of stratification are rarely reported by other models of stroke for pre-clinical assessments as animals are randomised into treatment groups prior to stroke induction. Furthermore, the degree of variability in stroke damage observed between individuals can also be accounted for using the ET-1 model since all degrees of stroke severities can be assessed across the treatment groups. This enables complete characterisation of differential effects of the treatment on histological and functional outcome in rats with varying stroke severities (Roulston *et al.* 2012, Roulston *et al.* 2008). Moreover, ET-1 induced sustained reduction in CBF and gradual reperfusion resembles the majority of human stroke where thrombolytic therapy is not applied due to the narrow therapeutic window.
The stereotaxic application of ET-1 to the MCA to induce focal ischemic strokes has also been adapted for use in primates (Virley et al. 2004). Non-human primate models of stroke represent the most relevant platform for clinical translation to investigate ischemic stroke. In comparison to other species, the non-human primate brain is anatomically closer to that of humans, with analogous cerebrovasculature and more similar haemostatic components (Fukuda and del Zoppo 2003). Ultimately, a model of stroke induced in the absence of anaesthesia and incorporates gradual spontaneous reperfusion is most desirable for the translation of new treatments to promote brain repair in patients.

1.14.1 Angiogenesis in the ET-1 stroke model

More traditional rodent stroke models such as the thread occlusion model of MCAo have reported angiogenesis restricted to penumbral regions (Bosomtwi et al. 2008, Sbarbati et al. 1996). Using the ET-1 stroke model Taylor et al., (2013) have demonstrated development of new vasculature within the border region as well as across the infarcted core. Most importantly, this relates to human patients as significantly higher microvessel density was observed across the damaged region of the brain and correlated with a greater survival time following stroke (Krupinski et al. 1994). Therefore active angiogenesis appears to be beneficial for the stroke affected human brain and utilising the endothelin-1 stroke model permits investigation of an angiogenic microenvironment similar to that in humans. For the studies conducted in this thesis, the design of the ET-1 rat model of stroke appears to be the most suitable, reliable, and relevant model to be used.

1.16 Summary and Research Aims

Current treatment options for stroke are limited with no effective therapeutic treatment proven to improve neurological recovery in the post-ischemic phase (Molina 2011). Regenerative events initiated following brain damage offer a second window for treatment. As such promising therapeutic treatment strategies that target brain regeneration are currently under investigation. However there is still much to learn about the way in which the brain responds to stroke injury over different recovery phases. To this effect complete characterisation of long term histological changes that
occur in response to stroke recovery is yet to be explored and understanding endogenous remodelling mechanisms and their response to different grades of stroke severity is vital to establishing best treatment windows for restorative therapies. Cell-based therapies have the potential for positive therapeutic effects following stroke (Kondziolka and Wechsler 2008). However, complete understanding of the histopathological influences of exogenously transplanted stem cells has not been well documented. Furthermore, an alternative concept of transplanting pre-differentiated neurons to enhance neurological recovery after stroke is yet to be realised. Therefore, in this thesis we focus on changes within the brain microenvironment after stroke according to varying stroke severity, and subsequent use of stem cell transplants to support brain repair and functional recovery.

The first aim of this thesis was to establish and characterise endogenous repair mechanisms initiated after stroke using the ET-1-induced MCA vasoconstriction model and correlate these findings with infarct volume, inflammation, scar formation and functional recovery. The second aim of this thesis was to investigate the effect of undifferentiated NPC transplants versus pre-differentiated cell transplants into the rat brain 7 days post-stroke (during angiogenesis) on functional recovery. Non-autologous transplanted NPC survival requires the prolonged use of an immunosuppressive agent cyclosporine A (CsA). Due to previous reports of the potential neuroprotective effects of CsA (Butcher et al. 1997, Shiga et al. 1992, Uchino et al. 1998), it was imperative to investigate the effects of CsA on stroke outcome, in particular pathophysiological and regenerative processes, before exploring NPC transplant strategies. Overall, these studies examine injury and repair responses initiated following stroke and the potential for brain repair following human NPC transplantation in a clinically relevant model of stroke. Moreover, this thesis indicates the most suitable NPC phenotype for transplantation and optimal regenerative and phenotypic conditions to translate into significant functional recovery following cerebral ischemia.
Chapter 2: General Materials and Methods

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2.1 Introduction

The endothelin-1 model of middle cerebral artery constriction in rats was chosen for this thesis to address unanswered questions related to brain remodelling and repair processes initiated following stroke; and to investigate the potential use of human neural progenitor cell (NPC) transplants to promote functional recovery. Studies presented in Chapters 3, 4 and 5 implement identical experimental surgical techniques (to induce stroke) and neurological tests to assess functional outcomes, with the exception of Chapter 5 where the addition of a more comprehensive behavioural test was introduced to detect long term functional loss. Methods specific to individual studies can be found within each relevant chapter.

All experiments were performed in strict accordance with the guidelines of the National Health & Medical Research Council of Australia Code of Practice for the Care and use of Animals for Experimental Purposes in Australia. The protocol was approved by the St Vincent’s Hospital animal ethics committee (AEC009/09). To reliably acquire data with the use of animals, reducing stress to animals is crucial during the testing period. Each rat was acclimatized to their environment prior to all experimental procedures by gentle handling and taming of the animals in advance. Surgeries were performed under general anaesthesia, paracetamol (2mg/kg in drinking water) was provided 24 hours prior to and after surgery in order to minimize post-operative pain. Continuous monitoring was included for each rat throughout the length of the study to ensure their wellbeing. A daily checklist included weight gain, coat condition and physical response to handling during routine husbandry.

2.2 Endothelin-1 induced middle cerebral artery constriction in conscious rats

2.2.1 Animals

Male Hooded Wistar rats, aged 10-12 weeks (280-360g) were purchased from The University of Adelaide Laboratory Animal Services (SA, Australia). Rats were housed on a 12 hour day/night cycle with temperature maintained between 18°C and 22°C and maintained on a standard chow diet. Rats were dually housed prior to surgery.
2.2.2 Surgical preparation

Anaesthesia was administered intraperitoneally (i.p.) with a mixture of Ketamine/Xylazine (75mg/kg: 10mg/kg respectively) and maintained throughout surgery by inhalation of isoflurane (95% oxygen and 5% isoflurane). A heated pad was used to maintain normal rat body temperature throughout surgery. Based on the method first employed by Sharkey and colleagues (1993), rats were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and secured with a nose piece and ear bars. A midline scalp incision was made to retract the scalp bilaterally exposing the skull. The skull surface was cleaned and allowed to dry before bregma (the anatomical position on the surface of the skull demarcating the junction of the coronal and sagittal sutures) was located and its co-ordinates determined. To locate the middle cerebral artery, the stereotaxic co-ordinates first reported by Paxinos and Watson (1986) and modified by Callaway et al. (1999) for the Hooded Wistar strain of rats were utilised. These were 0.2 mm anterior, -5.9 mm lateral, and -5.2 mm ventral relative to Bregma. A burr hole was made over the MCA on the right side of the skull with a fine electric power drill. A 23-gauge stainless steel guide cannula (2cm) was stereotaxically implanted through the burr hole into the piriform cortex 2 mm dorsal to the right MCA. Bone wax was used to seal the hole around the guide cannula and this was secured to the skull by dental acrylic cement and one optical screw previously inserted into the skull to provide a suitable anchor for adherence of the cement. Once the cement was dry, the scalp was sutured closed around the cannula and rats were allowed to recover for at least 5 days prior to stroke induction. The guide cannula protruded 1cm from the surface of the skull, allowing for a microinjector to be inserted for stroke induction. Rats were housed individually on a 12 hour day/night cycle with room temperature (RT) maintained between 18°C and 22°C with ad libitum access to food and water.

2.2.3 Stroke induction

To induce stroke, the right MCA was constricted in conscious rats by perivascular administration of endothelin-1 (ET-1; American Peptide Company, Inc., Sunnyvale, CA, USA; 60μmol in 3μl of saline over 10 min). A 30-gauge microinjector was inserted into the previously implanted guide cannula, extending 2mm beyond the ventral end of the guide cannula. The microinjector was held in place by a poly-tubing cuff and attached to a length of PE tubing (~50cm) connected to a Hamilton syringe. The rat was placed in a plexiglass box for observation. An average of 3μl of ET-1 was
administered while the rat’s behaviour was continuously observed. During ET-1 infusion, characteristic behavioural changes indicating stroke induction included clenching and dragging of the contralateral forepaw and spasmodic or continuous circling in an anti-clockwise direction, validating the precise placement of the cannula. Stroke severity was rated 1 to 5 based on behavioural responses listed in Table 2.1, with a stroke rate of 5 being the most severe; with higher stroke ratings correlating with larger infarct volumes (Figure 2.1) (Roulston et al., 2008). Changes in behaviour occurred over 2 to 10 minutes from commencement of ET-1 infusion and these changes were observed and rated over 60 min. Rat behaviour returned to normal after this time, except where a severe stroke rating of 5 was recorded, and rats continued to circle for up to 2 hrs. Rats that did not display behavioural changes described in Table 2.1 indicative of stroke were considered not to have suffered one and were excluded from further experiments. This rating system enabled stroke severity to be predicted prior to treatment and thus enabled potential stroke outcome to be evenly represented across different groups (Roulston et al., 2008). Temperature was measured with a rectal probe prior to stroke and at 30 and 60 min intervals for 3 hrs after stroke. Sham-injected rats (n=4) underwent cannula implantation and saline infusion instead of ET-1 (with 14 day recovery), sham-operated rats (n=3) received cannula implant without infusion of ET-1 or saline (with 7 and 14 day recovery).
Table 2.1 Behavioural changes during stroke induction

Changes in behaviour upon ET-1 infusion in the conscious rat indicate stroke intensity that can be rated from 1 to 5, with 5 being the most severe. A score of 0 is assigned if none of the below responses are exhibited and the rate is excluded from further analysis (Roulston et al., 2008).

<table>
<thead>
<tr>
<th>Time</th>
<th>Observed behaviour</th>
<th>Stroke rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2 min</td>
<td>Systematic grooming, teeth chattering</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Tongue poking, licking bedding</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raised contralateral forepaw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raised and clenched contralateral forepaw</td>
<td></td>
</tr>
<tr>
<td>2-4 min</td>
<td>Biting cage and bedding</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Head turned to contralateral direction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Head bobbing in contralateral direction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spasmodic contralateral turns (not continuous)</td>
<td></td>
</tr>
<tr>
<td>5-10 min</td>
<td>Continuous contralateral turning</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Chin rubbing</td>
<td></td>
</tr>
<tr>
<td>10-30 min</td>
<td>Continuous ipsilateral circling</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Contralateral forepaw clenched</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chin rubbing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forepaw shuffling/digging while turning</td>
<td></td>
</tr>
<tr>
<td>&gt;30 min</td>
<td>Still circling after 60 minutes</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Loss of balance on rearing/walking</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Loss of righting reflex</td>
<td></td>
</tr>
</tbody>
</table>

2.2.4 Assessment of functional outcome

Behavioural tests were conducted on all rats prior to any surgical procedures (pre-surgery), immediately prior to ET-1-induced MCA constriction (pre-stroke), 24, 48, and 72 hrs post-stroke, 7 or 14 days post-stroke for Chapters 3 and 4 or up to 35 days post-stroke for Chapter 5, depending on experimental sequelae. Deficit scores from each rat post-stroke were compared to pre-stroke scores, thus each rat acted as its own control. Pre-surgical scores were compared to pre-stroke assessments to ensure no functional damage was caused by guide cannula implantation. All neurological observations compared deficits in the affected contralateral limbs (opposite side to the brain damage) to the unaffected ipsilateral limbs (same side as the brain damage).
2.2.4.1 Neurological deficit score

Rats were tested for neurological abnormalities using the neurological deficit scores based on visual detection of abnormal posture and hemiplegia previously described by De Ryck et al. (1989) and Yamamoto et al. (1988). Abnormal posture was assessed by suspending the rat by the tail ~10cm from the ground for ~5 seconds. Prior to stroke induction rats will reach for the ground with both front limbs outstretched. Thorax twisting and/or forelimb flexion were scored. Hemiplegia was evaluated by placing rats on a raised platform (3cm x 70cm) approximately 20cm from the bench. Limb dysfunction was deemed present when the contralateral forelimb and hind limb (opposite to the infarcted hemisphere) slipped off. All behaviours were scored based on the following scale: 0 = no deficits, 1 = slight deficit, 2 = moderate deficit, 3 = severe deficit. A maximum neurological deficit score of 12 would therefore, equate to all 4 limbs being severely affected. In the studies for this thesis, ipsilateral forelimb deficit was rarely detected therefore the maximum contralateral deficit was a score of 6. A score of 0 was considered normal and is thus represented as the baseline.
2.2.4.2 Tape test

Stroke patients can suffer from a neuropsychological condition known as sensory hemi-neglect, where the patient does not recognise, or attend to visual information on the side of the visual field contralateral to the brain damage. The tape test, developed to test this condition in rats, was first described by Schallert and Whishaw (1984). A circular adhesive label/tape, ~10mm in diameter, was attached to the distal-radial region of each forepaw. Placement of the first tape was randomised between contralateral and ipsilateral forepaws. The tapes were simultaneously touched by the investigator on both forepaws prior to placing the rats in a plexiglass box. The latency for the rat to then touch and remove the tape from the contralateral and ipsilateral forepaws was measured with a stopwatch and recorded. Rats that did not remove tapes within three minutes were given a maximum score of 180 sec.

2.2.4.3 Cylinder test

For studies involving cell-transplants, the cylinder test was performed using a modified version of the test (Lundblad et al., 2002) originally described by Schallert & Tillerson (2000). The cylinder test was developed to assess exploratory weight bearing motor movements to support the body of rats against the wall of a cylindrical enclosure. Rats will voluntarily rear and explore the wall using their forelimbs and while standing on their hindlimbs. The cylinder test enables comparisons between post-stroke preference and post-transplant correction of voluntary forelimb movements during vertical rearing movements per horizontal landing. Pre-lesion rats typically distribute the weight bearing movements equally on their ipsilateral and contralateral forelimbs during vertical rearing and both forelimbs simultaneously while landing. However, post-stroke rats no longer use their impaired contralateral forelimb while rearing. For accuracy in scoring, the test was videotaped and analysed in slow motion. A clear plexiglass cylinder with a height of 30cm, diameter of 20cm with an open top and closed bottom was used and a mirror was placed behind the cylinder to record movements when the rats turned away from the camera. The cylindrical shape encourages vertical exploration of the wall as well as landing activity. Ipsilateral, contralateral or simultaneous paw usage were calculated independently for vertical ‘rearing’ and horizontal ‘landing’. Vertical rearing wall touches were counted only when paw was flat on the wall with digits spread apart. Following a rearing movement of forelimb contacts on the wall, the first limb to contact the ground was scored as a
land for that limb and any movements along the ground following landing were excluded from analysis. Rats typically explore walls with three paw touches before landing, and therefore ten landings were quantified in order to obtain at least thirty vertical wall touches at each time point per rat. Inactive rats prior to surgery that fail to rear and complete 10 landings were excluded from the study.

2.3 Brain processing

For studies conducted in Chapters 3 and 4, rats were sacrificed at 7 or 14 days after stroke with a lethal overdose of Lethabarb (0.1ml; Virbac Australia Pty Ltd, Peakhurst, NSW, Australia) and decapitated. The brains were quickly removed and frozen over liquid nitrogen and stored at -80°C. Prior to processing brains were thawed to -18°C and coronal cryostat sections (16 µm thick) were cut in serial sections, using a Leica cryostat, at eight pre-determined coronal planes throughout the brain from -3.2 to 6.8 mm relative to Bregma to encompass the frontal and parietal cortex and dorsal and ventral striatum, in accordance with the method used by Callaway et al. (1999). Sections were then stored at -80°C before processing.

For studies conducted in Chapter 5, rats were harvested 28 days after transplantation (35 days post-stroke). Prior to harvest, anaesthesia was administered using Ketamine/Xylazine (75mg/kg; 10mg/kg respectively, i.p.). All rats were transcardially perfused with 300mL of cold phosphate buffered saline (PBS; 0.1M, pH 7.4) followed by 300mL of cold 4% paraformaldehyde (PFA; in PBS, 0.1M pH 7.4) for 15 minutes. The brains were quickly removed and post-fixed overnight in 4% PFA in 0.1M PBS at 4°C followed by cryoprotection overnight in 30% sucrose in 0.1M PBS at 4°C. Brains were then cut in 40µm coronal sections on a Leica cryostat at -25°C from -3.2 to 6.8 mm relative to Bregma. Sections were stored at -80°C before processing.

2.4 Quantification of ischemic damage

2.4.1 MCID image analysis

The extent of brain injury following stroke was determined in triplicate unstained slide mounted sections for Chapters 3 and 4. For Chapters 3 and 4 infarct area was measured across eight predetermined planes throughout the brain; +3.2, +1.2, +0.2, -0.8, -1.8, -2.8, -4.8 and -6.8 mm relative to bregma using the ballistic light method
described by Callaway et al. (2000). Infarct area was measured using a computerised image analysis system (MCID/M4: Micro Computer Imaging Device, Imaging Research Inc, St Catherines, Ontario, Canada). This method relied on basic principles of light propagation through unstained sections such that light passing directly through transparent and undamaged tissue enter the camera lens. Damaged brain tissue deviate the light and therefore is not captured by the small camera aperture. The resultant image appears light grey in undamaged regions while damaged regions appear distinguishably darker (ref Figure 2.1). The image analysis system is then used to easily outline and select the ischemia area and record the results. Total infarct volume was determined by integrating the cross-sectional area of damage at each stereotaxic level with the distances between levels (Osborne et al., 1987). The influence of oedema was corrected for by applying the following formula: (area of normal hemisphere/area of infarcted hemisphere) x area of infarct (Leach et al., 1993).

2.4.2 NeuN fluorescence staining

The use of the ballistic light method to accurately measure damage beyond 7 days after stroke has not been established. Therefore brain injury following stroke in Chapter 5 was determined in triplicate neuronal nuclear antigen (NeuN) stained sections. For Chapter 5, brain tissue sections were collected and stained with NeuN to highlight areas devoid of neurons and thus infarcted regions of the brain. NeuN labelled endogenous rat neurons allowed visualisation of healthy brain tissue while lack of staining highlighted regions of ischemic damage. Triplicate sections from each level were visualised with an Olympus microscope (Albertslund, Denmark) and analysed using National Institute of Health ImageJ software (USA). Total infarct volume was determined by integrating the cross-sectional area of damage at each stereotaxic level with the distances between levels (Osborne et al., 1987). The influence of oedema was corrected for by applying the following formula: (area of normal hemisphere/area of infarcted hemisphere) x area of infarct (Leach et al., 1993).

2.5 Immunohistochemistry and immunofluorescent techniques

2.5.1 Slide mounted tissue sections

The immunohistochemical procedures used for studies conducted in Chapters 3 and 4 involved incubation of slide mounted 16µm fresh frozen coronal tissue sections in
the presence of commercially purchased antibodies raised against specific antigens. The primary antibodies listed in Table 2.2 were employed to investigate endogenous remodelling processes of angiogenesis, SVZ cell proliferation, migration and neuronal differentiation, activation of microglia/macrophages, astrogliosis and scar formation. First, slide mounted tissue sections were fixed with methanol (-20°C for 15 min) (for sections to be incubated in vWF); in 4% PFA (in 0.1M PBS, pH 7.4) for 15 min RT (for sections to be incubated in Ki67 or GFAP); in 4% PFA (in 0.1M PBS, pH 7.4 at RT) for 15 min and then washed in PBS (0.1M; 3 x 5 min) prior to antigen retrieval with 10% proteinase K in 0.1M PBS for 3 min at RT followed by incubation in 30% fetal calf serum to stop proteinase K activity for 30 min (for sections to be incubated in DCX); or in serial acetone dilutions (50:100 x 2 min each at RT) (for sections to be incubated in OX42). Sections were washed in PBS (0.1M; pH 7.4) containing 0.05% Tween 20 detergent (PBT; 3 x 5 min) followed by a 30 min block in PBT containing 5% normal goat serum (NGS) (for sections to be incubated in vWF); or in PBS (0.1M; 3 x 5 min) followed by incubation in blocking solution containing 5% NGS and 0.3% Triton X-100 in 0.1M PBS for 30 min (for sections to be incubated in Ki67, DCX, OX42, or GFAP). Sections were then incubated with the appropriate primary antibody listed in Table 2.2 in a mixture of 5% NGS in PBT (vWF); or 5% NGS and 0.3% Triton X-100 in PBS (0.1M, pH 7.4) (Ki67, DCX, OX42, or GFAP) for 1 hour at RT.
Table 2.2 List of primary antibodies used

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit anti-von willebrand factor (vWF)</td>
<td>1:200</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Monoclonal mouse anti-neuron specific nuclear protein (NeuN)</td>
<td>1:400</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Monoclonal rabbit anti-Ki67</td>
<td>1:1000</td>
<td>Labvision Thermo Scientific, Fremont, CA, USA</td>
</tr>
<tr>
<td>Polyclonal guinea-pig anti-doublecortin (DCX)</td>
<td>1:500</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Monoclonal mouse anti-OX42/CD11b (OX42)</td>
<td>1:100</td>
<td>Serotec, Raleigh, NC, USA</td>
</tr>
<tr>
<td>Monoclonal mouse anti-glial fibrillary acidic protein (GFAP)</td>
<td>1:400</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-glial fibrillary acidic protein (GFAP)</td>
<td>1:400</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
<tr>
<td>Monoclonal mouse anti-Nestin</td>
<td>1:400</td>
<td>Cell Signalling Technology, Danvers, MA, USA</td>
</tr>
</tbody>
</table>

Following incubation with the primary antibodies, tissue sections were then washed in PBT (vWF) or PBS (0.1M, pH 7.4, 3 x 5 min) (Ki67, DCX, OX42, or GFAP) and transferred for incubation with the biotinylated secondary antibodies (Table 2.3) (biotinylated goat anti-rabbit for vWF or Ki67 staining; biotinylated goat anti-guinea-pig for DCX staining; biotinylated goat anti-mouse for OX42 or GFAP staining) in PBT (vWF) containing 5% NGS; or in a mixture of PBS (0.1M, pH 7.4) containing 5% NGS and 0.3% Triton-X100 (GFAP, OX42, Ki67, DCX) for 30 min at RT. All sections were analysed with a standard ABC detection kit (Vector Labs) for 30 mins followed by washes in PBS (0.1M pH 7.4; 3 x 5min) and 5 min incubation with diaminobenzidine (DAB). Sections were then washed and dehydrated in serial alcohol dilutions (1 x 70%, 1 x 100%, and 1 x 100%), cleared by standing in histolene, drained and coverslipped. The resultant colour reaction was visualised with an Olympus BH-2 bright field microscope (Albertslund, Denmark).
Table 2.3 List of secondary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated goat anti-rabbit</td>
<td>1:200</td>
<td>Vector Labs, Burlingame, CA, USA</td>
</tr>
<tr>
<td>Biotinylated goat anti-guinea-pig</td>
<td>1:200</td>
<td>Vector Labs, Burlingame, CA, USA</td>
</tr>
<tr>
<td>Biotinylated goat anti-mouse</td>
<td>1:200</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

For immunofluorescent staining of endogenous remodelling processes of angiogenesis, SVZ cell proliferation, migration and neuronal differentiation, activation of microglia/macrophages, astrogliosis and scar formation, tissue sections adjacent to those used for immunohistochemistry were fixed in 4% PFA for 10 min and washed in PBS (0.1M, pH 7.4, 3 x 5 min). All sections were then subjected to block with DAKO serum-free protein block (Glostrup, Denmark) for 30 min. Dual immunofluorescent techniques were employed where the primary antibodies listed in Table 2.2 were used and sections were incubated overnight at 4°C in a mixture of PBS (0.1M, pH 7.4) containing 2% normal donkey serum (NDS), 2% NGS, 0.3% Triton X-100 (staining for vWF and either NeuN, OX42 or GFAP); or in a mixture of PBS (0.1M, pH 7.4) containing 2% NGS and 0.3% Triton X-100 (staining for Ki67 and either GFAP, or DCX; or Nestin and GFAP).

Tissue sections were then washed in PBS (0.1M, pH 7.4, 3 x 5 min) and transferred for incubation with an appropriate secondary antibody (1:500 for all) for 2 hr at RT (Table 2.4); Alexa-568 goat anti-rabbit and Alexa-488 donkey anti-mouse for the detection of vWF with either OX42 or GFAP; or Alexa-568 goat anti-mouse and Alexa-488 donkey anti-rabbit for detection of vWF with NeuN; or Alexa-568 goat anti-rabbit and Alexa-488 goat anti-mouse for detection of GFAP with either Nestin or Ki67; or Alexa-568 goat anti-rabbit and Alexa-488 goat anti-guinea pig for detection of Ki67 with DCX. Tissue sections were then washed in PBS (0.1M, pH 7.4, 3 x 5 min) and cover-slipped with DAKO fluorescent mounting medium. Resulting sections were examined with a Zeiss fluorescence microscope equipped with a 578-603nm filter set for detection of red fluorescence and 495-519nm filter set for the detection of green fluorescence (ZeissAxioskop2, North Ryde, Australia), or a laser scanning inverted confocal imaging system (Nikon Instruments Inc., Melville, NY, USA).
In control experiments for immunohistochemistry and immunofluorescent techniques, primary antibodies were omitted or the appropriate IgG control (Table 2.7) was included to verify the specificity of each antibody.

**Table 2.4 List of secondary fluorophore-conjugated antibodies used for immunofluorescence**

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa-568 goat anti-rabbit</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-568 goat anti-mouse</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-488 donkey anti-mouse</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-488 donkey anti-rabbit</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
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<tr>
<td>Alexa-488 goat anti-mouse</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-488 goat anti-guinea pig</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
</tbody>
</table>

**2.5.2 Free floating tissue sections**

The immunofluorescent techniques used for studies conducted in Chapter 5 involved incubation of free floating tissue sections of 40µm thick in the presence of commercially purchased antibodies raised against specific antigens. Free floating sections were placed in 24 well tissue culture plates (11cm diameter) in 500ml of 0.1M PBS (pH 7.4), one section per well. The primary antibodies listed in Table 2.5 were employed to investigate SVZ-derived human neural progenitor cell (hNPC) cell survival and phenotypes *in vivo*, and visualise endogenous blood vessels, SVZ proliferation, migration, and neuronal differentiation.

For dual immunofluorescent staining with HuNu and either GABA, Tuj1, Nestin, GFAP, Ki67, GAD, or Casp3, free floating serial sections were blocked with 5% NGS in 0.3% Triton X-100 in PBS (0.1M, pH 7.4) for 30 min followed by washes in PBS (0.1M, pH 7.4, 3 x 5 min), prior to incubation with the primary antibody overnight at 4°C. For dual staining with HuNu and either CB, CR, or vWF; or triple staining with HuNu and either SYN and Tuj1; or GABA and PV, free floating sections were
incubated in 40% proteinase K in 0.1M PBS for 4 min at RT for antigen retrieval followed by incubation in 30% fetal calf serum for 30 min. Sections were washed in PBS (0.1M, pH 7.4; 3 x 5 min) followed by incubation in blocking solution containing 5% NGS (or 1% bovine serum albumin (BSA) for all staining with goat anti-PV) and 0.3% Triton X-100 (and 3% skim milk for sections to be incubated with vWF) in PBS (0.1M, pH 7.4) for 30 min prior to incubation with the primary antibodies overnight at 4°C in a mixture of PBS (0.1M, pH 7.4), 2% NGS, and 0.3% Triton X-100.
<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal mouse anti-human specific nuclear antigen (HuNu)</td>
<td>1:1000</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Monoclonal rabbit anti-neuronal class III β-tubulin (Tuj1)</td>
<td>1:1000</td>
<td>Covance Inc., Sydney, NSW, Australia</td>
</tr>
<tr>
<td>Monoclonal chicken anti-neuronal class III β-tubulin (Tuj1)</td>
<td>1:200</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-gamma-aminobutyric acid (GABA)</td>
<td>1:1000</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Monoclonal mouse anti-neuron specific nuclear protein (NeuN)</td>
<td>1:400</td>
<td>Millipore, Billerica, MA, USA</td>
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<tr>
<td>Polyclonal rabbit anti-Nestin</td>
<td>1:1000</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Monoclonal rabbit anti-SOX2</td>
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<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-glial fibrillary acidic protein (GFAP)</td>
<td>1:1000</td>
<td>DAKO, Glostrup, Denmark</td>
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<tr>
<td>Monoclonal rabbit anti-Ki67</td>
<td>1:500</td>
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</tr>
<tr>
<td>Polyclonal guinea-pig doublecortin (DCX)</td>
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<td>Millipore, Billerica, MA, USA</td>
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<tr>
<td>Monoclonal rabbit anti-glutamate decarboxylase (GAD) 65&amp;67</td>
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<td>Millipore, Billerica, MA, USA</td>
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<tr>
<td>Polyclonal rabbit anti-synaptophysin (SYN)</td>
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<td>Sigma, St. Louis, MO, USA</td>
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<tr>
<td>Monoclonal mouse anti-calbindin-D28k (CB)</td>
<td>1:1000</td>
<td>Swant, Marly, Switzerland</td>
</tr>
<tr>
<td>Monoclonal goat anti-parvalbumin (PV)</td>
<td>1:1000</td>
<td>Swant, Marly, Switzerland</td>
</tr>
<tr>
<td>Monoclonal rabbit anti-calretinin (CR)</td>
<td>1:1000</td>
<td>Swant, Marly, Switzerland</td>
</tr>
<tr>
<td>Monoclonal rabbit anti-cleaved caspase-3 (Casp3)</td>
<td>1:500</td>
<td>Cell Signalling Technology, Boston, MA, USA</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-von willebrand factor (vWF)</td>
<td>1:200</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
</tbody>
</table>

All sections were then washed in PBS (0.1M, pH 7.4, 3 x 5 min) and staining was visualised by incubation for 2 hr at RT with a combination of 2 or 3 of the following fluorophore-conjugated secondary antibodies (1:500 for all) (Table 2.6);
Alexa-568 goat anti-mouse and Alexa-488 goat anti-rabbit for the detection of HuNu and either Tuj1, GABA, Nestin, GFAP, Ki67, NeuN, vWF, CR, CB, or Casp3; or Alexa-350 goat anti-mouse, Alexa-488 goat anti-rabbit, and Alexa-555 goat anti-chicken for detection of HuNu co-labelled with Tuj1 and SYN; or Alexa-488 goat anti-mouse, Alexa-647 goat anti-rabbit, and Alexa-568 donkey anti-goat for detection of HuNu with GABA and PV, in a mixture of PBS (0.1M, pH 7.4), 2% NGS, and 0.3% Triton X-100.

For double-staining with HuNu and terminal transferase-mediated dUTP nick end-labelling (TUNEL), a TUNEL staining kit was used according to manufacturer’s instructions (Promega). Free floating sections were incubated in 20µg/ml Proteinase K for antigen retrieval for 2 min at RT followed by washes in PBS (0.1M, pH 7.4) and incubation in 10% NGS for 5 min. Sections were then incubated in equilibration buffer for 10 min at RT, followed by incubation in rTdT reaction mix for 60 min at 37°C. Reaction was then terminated with 2x SSC (1:10 in dH2O) for 15 min at RT. Endogenous peroxidises were quenched by incubating sections in 0.3% H2O2 in PBS (0.1M, pH 7.4) for 5 min at RT, followed by using standard ABC methodology to process cells for DAB, and washed in dH2O (2 x 2min). Sections were then incubated in blocking solution containing 5% NGS and 0.3% Triton X-100 for 30 min prior to washes in PBS (0.1M, pH 7.4) and incubation in primary antibody mixture containing HuNu, 2% NGS, and 0.3% Triton X-100 in PBS (0.1M, pH 7.4) overnight at 4°C. Sections were then washed in PBS (0.1M, pH 7.4) prior to incubation in secondary antibody solution containing Alexa-568 goat anti-mouse in PBS (0.1M, pH 7.4) and 2% NGS and 0.3% Triton X-100 for 2 hr at RT.
Table 2.6 List of secondary fluorophore-conjugated antibodies used

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa-647 goat anti-chicken</td>
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<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-568 goat anti-rabbit</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-568 goat anti-mouse</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-568 donkey anti-goat</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Alexa-555 goat anti-chicken</td>
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<td>Alexa-488 goat anti-rabbit</td>
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<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
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<tr>
<td>Alexa-488 goat anti-guinea pig</td>
<td>1:500</td>
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</tr>
<tr>
<td>Alexa-350 goat anti-mouse</td>
<td>1:500</td>
<td>Invitrogen Life Technologies, Grand Island, NY, USA</td>
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</tbody>
</table>

In all experiments, DNA counterstain DAPI (Molecular Probes) was applied before coverslipping with ProLong gold anti-fade reagent (Invitrogen). Control experiments included either omission of each primary antibody from the protocol or the inclusion of the appropriate IgG control (Table 2.7) to verify the specificity of each antibody. Positive controls for TUNEL staining included treating sections with DNase I (1:100) in DNase buffer (1:10 in dH₂O) (TUNEL kit) to cause DNA fragmentation for 10 min followed by washes in dH₂O (2 x 2min). Resulting sections were examined with a Nikon confocal laser scanning microscope (Nikon). Images obtained using a 40 and 60 objectives were presented as collapsed reconstructions (z stacks) of 17 optical sections captured every 0.2 µm on the z-axis, at a scan speed of 0.5 frame/sec, a resolution of 1024 x 1024 pixels with the line acquisition mode, with orthogonal imaging.

2.6 Stereology

For Chapters 3 and 4, resulting tissue sections were viewed using an Olympus BH-2 microscope under x20 magnification with at least three sections per region used for assessment. Four common regions of interest where damage was routinely observed...
were identified in each section for assessment: the damaged cortex, damaged striatum, and the border region surrounding the damage for both the striatum and cortex. Each area used for analysis was then compared with the appropriate corresponding mirror region in the contralateral hemisphere, as well as to sham-operated controls, using exactly the same sample area. vWF labelled blood vessels, OX42 labelled microglia/macrophages, and GFAP labelled astrocytes were quantified in the same brain regions of interest using digital video imaging (TK C 1480E; JVC, Wayne, NJ, USA), an automated systematic random sampling point-counting system was applied with a Computer Assisted Stereological Toolbox (CAST System; Olympus). The number of vWF-labelled blood vessels detected in the ipsilateral sample region was then compared to the contralateral region and expressed as 100% control, while OX42 and GFAP positive cells were expressed as a percentage of the total number of counts (cross hairs) within the defined sample area. Within the SVZ Ki67-positive proliferating cells, Ki67/GFAP-positive proliferating radial glial cells, and DCX-positive immature migrating neurons were quantified using the cell counter plugin for National Institute of Health ImageJ software (USA). Due to a small counting frame within the SVZ total cell numbers were quantified using standard stereological techniques and ImageJ. The total number of proliferating cells, proliferating radial glial cells, and immature neurons in the ipsilateral SVZ was then compared to the corresponding contralateral SVZ and expressed as 100% control. Images of the SVZ were obtained using a laser scanning confocal microscope (Nikon) under x40 magnification and presented as collapsed reconstructions of 10 optical sections captured every 0.5µm on the z-axis, at a scan speed of 0.5 frame/sec, a resolution of 1024 x 1024 pixels with the line acquisition mode.

For Chapter 5, cell counts were performed using the optical fractionator stereological method to obtain unbiased estimates that was applied with Stereo Investigator software (MBF Bioscience, Williston, VT, USA). Sections were analysed using a fluorescence microscope (Olympus BH-2, Tokyo, Japan) equipped with a QIClick scientific camera (Surrey, BC, Canada) at a magnification of x60, where a three-dimensional optical dissector counting probe (x, y, z dimension of 30µm x 30µm x 10µm respectively) was applied to a systematic random sample of sites within the cell graft. The number of HuNu-positive cells that expressed Tuj1, GABA, Nestin, GFAP, and Ki67 was manually quantified. Estimates of the total number of cells co-localising with markers of interest were obtained using the following formula: \( E = k \Sigma N \), where
E is the estimate of the total number of stained cells in each case, $\sum N$ is the sum of n values in the 3 sections analysed, and k indicates that every $k^{th}$ section was considered (k=8). N was corrected according to Abercrombie’s formula: $N = nt/(t + D)$, where $n$ is the number of cells counted in each section, $t$ is the section thickness, and D is the mean diameter of the cells (Abercrombie and Johnson, 1946). Co-localised cell counts were expressed as the percentage of the total number of HuNu-positive cells.

2.7 Buffers

A variety of buffers and fixatives were used routinely during experimental procedures carried out in this thesis. Buffers were made up either on the day or the day before. Chemical fixatives were prepared either the day before or a week in advance and stored at either RT, 4°C or -20°C.

- **Phosphate Buffered Saline (PBS) (0.1M, pH 7.4):** NaCl (137mM), Na$_2$HPO$_4$ (10mM), NaH$_2$PO$_4$.2H$_2$O (10mM)
- **PBT Buffer:** 0.05% Tween 20 detergent diluted in 0.1M PBS
- **Antigen Retrieval Buffer Solution:** 10-40% Proteinase K solution diluted in 0.1M PBS
- **Fixative:** 4% Paraformaldehyde (PFA) (HO(CH$_2$O)$_n$H) dissolved in 0.1M PBS
- **Fixative:** 100% Methanol (CH$_3$OH)
- **Fixative:** 50% Acetone (CH$_3$COCH$_3$) diluted in 0.1M PBS and 100% Acetone
### 2.8 Drugs and chemicals

**Table 2.7 List of drugs and chemicals used**

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC detection kit</td>
<td>Vector Labs, Burlingame, CA, USA</td>
</tr>
<tr>
<td>Acetone (CH$_3$COCH$_3$)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Chromium(III) potassium sulfate dodecahydrate (Chrom Alum: CrK(SO$_4$)$_2$.12$H_2$O)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Cremophor EL (Castor oil)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>Sandimmune, Novartis Pharmaceuticals Corporation, NJ, USA</td>
</tr>
<tr>
<td>DAPI (4’, 6-Diamidino-2-Phenylindole, Dihydrochloride)</td>
<td>Molecular Probes, Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Di-sodium hydrogen orthophosphate (Na$_2$HPO$_4$)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Endothelin-1 (ET-1)</td>
<td>American Peptide Company, Inc. CA, USA</td>
</tr>
<tr>
<td>Ethanol (CH$_3$CH$_2$OH)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td>Gibco, Life Technologies, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Histolene (C$<em>{10}$H$</em>{16}$)</td>
<td>Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>Isopentane (2-Methylbutane: C$<em>3$H$</em>{12}$)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Laminin</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Liquid diaminobenzidine (DAB) kit</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
<tr>
<td>Methanol (CH$_3$OH)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Normal chicken IgY</td>
<td>Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>Normal donkey serum (NDS)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Normal goat serum (NGS)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Normal goat IgG (H+L)</td>
<td>PeproTech, Rocky Hill, NJ, USA</td>
</tr>
<tr>
<td>Normal mouse IgG (H+L)</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier and Location</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Normal rabbit IgG (H+L)</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA) (HO(CH₂O)ₙH)</td>
<td>ProSciTech Pty Ltd, Thuringowa Central, QLD, Australia</td>
</tr>
<tr>
<td>Poly-L lysine</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Skim milk</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Transferase-mediated dUTP nick end-labelling (TUNEL) kit</td>
<td>Promega DeadEnd Colorimetric TUNEL system, Madison, WI, USA</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Tween 20 detergent</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
</tbody>
</table>

### 2.9 Statistical analyses

All statistical analysis was conducted in consultation with a statistical consultant, Rachel Sore of the Statistical Consulting Centre with the University of Melbourne, Victoria, Australia. Statistical analysis was carried out on raw data, except where the difference in ratios (expressed as a percentage) of the contralateral to ipsilateral hemispheres or of the total number of cells quantified within a defined region. Neurological deficit scores were analysed by Kruskal-Wallis non-parametric analysis of variance (ANOVA) followed by Dunn’s post-test. Adhesive tape test data was analysed by a two-way repeated-measures (RM) ANOVA (side x hr/days after stroke) to compare latencies in the ipsilateral and contralateral forepaws over time followed by a Bonferroni post-hoc test to compare between sides at different time points. A RM two-way ANOVA, followed by Bonferroni post-hoc test was used to analyse cylinder test behaviour data comparing treatment groups at different time points.

Infarct volumes were analysed by ANOVA followed by a Bonferroni post-hoc test for comparing treatment groups (Chapter 4 & 5). Infarct area was analysed by a two-way ANOVA followed by a Bonferroni post-hoc test to compare treatment groups.
at each stereotaxic position relative to bregma (Chapter 4 & 5). To explore correlations between infarct volume and either: angiogenesis, SVZ cell proliferation, migrating SVZ neuroblasts or inflammatory cell activation, the Pearson product-moment coefficient, r, for ordinal values was determined (Chapter 3 & 4).

For analysis of endogenous remodelling responses, the number of angiogenic blood vessels, SVZ cell proliferation, migrating SVZ neuroblasts, and or inflammatory cell activation were each analysed by one-way ANOVAs followed by Bonferroni post-hoc test to compare 7 and 14 day recovery groups as well as shams for Chapter 3, or to compared between vehicle-treated, CsA-treated and shams for Chapter 4. A Mann Whitney test was used to compare between sham and 7 day recovery groups for Chapter 3.

Total number and percentage of HuNu-positive cells remaining within grafted regions and co-localised cell counts in vivo (HuNu-positive cells co-localised with Tuj1, GABA, Nestin, GFAP, or Ki67) were analysed by two-way ANOVA followed by Bonferroni post-hoc test to compare treatment groups and graft location (Chapter 5). Co-localised cell counts in vivo (HuNu-positive cells co-localised with Tuj1, GABA, Nestin, GFAP, or Ki67) were analysed by two-way ANOVA followed by Bonferroni post-hoc test to compare treatment groups and cellular markers (Chapter 5).

Data presented as boxplots include hinges extending from the 25th to 75th percentiles, the median line within the box and whiskers extending to the minimum and maximum values of the dataset. All other data are tabulated as mean ± SEM, unless otherwise stated. The assumptions of Normality and constant variance of residuals for all ANOVAs performed were examined and were deemed satisfactory. All statistical analyses were performed using GraphPad Prism, version 6 (GraphPad Software Inc., San Diego CA). P-values less than 0.05 were considered significant.
Chapter 3: Brain Remodelling Following Endothelin-1 Induced Stroke in Conscious Rats

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3.1. Introduction

Despite attempts to prevent brain injury following ischemic stroke, it is often too late for reperfusion or neuroprotective therapies. Most sufferers end up with long term deficits. Therapeutic strategies that target brain repair represent new hope for stroke survivors with long term functional deficits. Prior to developing such options, it is imperative to first establish and characterise endogenous pathophysiological and regenerative mechanisms that are activated in response to various stroke severities, since not all stroke outcomes are the same. For this reason establishing a reliable animal model for which to investigate new approaches to accelerate/promote brain repair is vital for successful clinical translation.

The injured brain is primed with processes that have the potential to facilitate functional recovery and these include plasticity, neurogenesis and angiogenesis (Carmichael and Chesselet, 2002; Cramer, 2008; Jacobs and Donoghue, 1991). Full characterisation of the stroke affected microenvironment will assist in the development of better treatment strategies and help with the correct assignment of patients to clinical trial. Therefore in the current chapter we used the endothelin-1 model of stroke in conscious rats to characterise the cellular response to ischemic reperfusion injury and repair. This model has several advantages as stroke is induced without anaesthesia and rats can be assigned a predictive outcomes score, similar to that which occurs with humans upon presentation to a Stroke Response Unit.

3.2. Hypothesis and Aims:

Hypothesis: Following ischemic stroke with reperfusion, injury and repair mechanisms are simultaneously activated which influence recovery and correlate with the severity of stroke damage.

Specific Aims: To investigate:

1. The correlation between stroke severity and repair processes including new blood vessel formation, cell proliferation, migration and neural differentiation within the subventricular zone (SVZ) that influence functional recovery 7 and 14 days after ischemic stroke and reperfusion in the rat brain.
2. The correlation between stroke severity, functional recovery, and injury mechanisms including microglial activation and macrophage infiltration, reactive astrogliosis, and scar formation 14 days after stroke.

3.3. **Experimental design**

In order to determine the effect of ET-1 stroke on brain remodelling and stroke severity, we chose two recovery times: 7 and 14 days (Figure 3.1). Neurogenesis in rats has been found to peak 7 days post-stroke and return to normal levels by 14 days (Zhang et al., 2001), while new blood vessels are present at 7 days but don’t appear to from functional networks until 14 days (Hayashi et al., 2003; Sbarbati et al., 1996; Taylor et al., 2013; Yu et al., 2007). As we were interested in investigating cell proliferation, migration, and neural differentiation within the subventricular zone (SVZ) we chose a 7 day recovery period to investigate these effects (Zhang et al., 2001). To investigate angiogenesis, inflammation, and scar formation, we chose a 14 day recovery point since reports suggest these events occurred or were sustained at this time (Taylor et al., 2013).

Angiogenesis, SVZ cell proliferation, migration and neuronal differentiation, activation of microglia/macrophages, astrogliosis and scar formation were identified using immunohistochemistry and immunofluorescent techniques and quantified using the Computer Assisted Stereological Toolbox (CAST) system and ImageJ. Findings were then correlated to infarct volume within each rat. To ensure that stroke outcome was evenly represented across the two recovery groups, rats were assigned to a recovery group after stroke induction and once they had been given a predictive outcome score.
Figure 3.1 Experimental timeline

Animals were assigned to either a 7 or 14 day recovery group after stroke induction to ensure that stroke severity was evenly represented across groups. All behaviour tests (neurological deficit scores and adhesive tape test) were performed as single trials pre-surgery, pre-stroke (day 0), and at 24 hr, 48 hr, 72 hr, 7 and 14 days post-stroke as highlighted in blue. All rats underwent surgery to stereotaxically implant a guide cannula and were allowed to recover for 5 days prior to stroke induction. Animals were euthanised and brains processed for histological analysis at the conclusion of the study.
3.4. Publication

Data generated for this thesis chapter has been published as an original research article in the open access journal Public Library of Science: PLoS One. The article PDF has been inserted here as a thesis chapter in accordance with the University of Melbourne guidelines.

The article can be found online at:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4029108/
Brain Remodelling following Endothelin-1 Induced Stroke in Conscious Rats

Hima C. S. Abeysinghe1*, Laita Bokhari1,3, Gregory J. Dusting4,5, Carli L. Roulston1

1 Neurotrauma Research team, Department of Medicine, St Vincent's Campus, University of Melbourne, Victoria, Australia, 2 Department of Surgery, St Vincent’s Campus, University of Melbourne, Victoria, Australia, 3 Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Victoria, Australia, 4 Cytoprotection Pharmacology Program, Centre for Eye Research, The Royal Eye and Ear Hospital Melbourne, Victoria, Australia, 5 Department of Opthamology, Faculty of Medicine, University of Melbourne, Victoria, Australia

Abstract

The extent of stroke damage in patients affects the range of subsequent pathophysiological responses that influence recovery. Here we investigate the effect of lesion size on development of new blood vessels as well as inflammation and scar formation and cellular responses within the subventricular zone (SVZ) following transient focal ischemia in rats (n=34). Endothelin-1-induced stroke resulted in neurological deficits detected between 1 and 7 days (P<0.001), but significant recovery was observed beyond this time. MCID image analysis revealed varying degrees of damage in the ipsilateral cortex and striatum with infarct volumes ranging from 0.76-77 mm³ after 14 days, where larger infarct volumes correlated with greater functional deficits up to 7 days (r=0.53, P<0.05). Point counting of blood vessels within consistent sample regions revealed that increased vessel numbers correlated significantly with larger infarct volumes 14 days post-stroke in the core cortical infarct (r=0.81, P<0.0001), core striatal infarct (r=0.91, P<0.005) and surrounding border zones (r=0.66, P<0.005; and r=0.73, P<0.05). Cell proliferation within the SVZ also increased with infarct size (P<0.01) with a greater number of Nestin/GFAP positive cells observed extending towards the border zone in rats with larger infarcts. Lesion size correlated with both increased microglia and astrocyte activation, with severely diffuse astrocyte transition, the formation of the glial scar being more pronounced in rats with larger infarcts. Thus stroke severity affects cell proliferation within the SVZ in response to injury, which may ultimately make a further contribution to glial scar formation, an important factor to consider when developing treatment strategies that promote neurogenesis.

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Competing Interests: The authors have declared that no competing interests exist.

* E-mail: himaa@student.unimelb.edu.au

Introduction

The degree of brain injury varies between each stroke victim and infarct volume has been shown to directly correlate with functional improvements 90 days post-stroke [1]. A number of events including excitotoxicity, oxidative stress, inflammation and apoptosis are initiated during and following stroke that contribute to the evolution of brain injury beyond the initial insult (see for reviews: [2,3]). Evolution of infarcts tends to differ according to the underlying vascular lesion and cerebral territories involved [4]. Non-malignant infarcts evolve approximately over 3-7 days post-stroke in rodents and become stable thereafter [5], while in humans infarct evolution takes days to weeks [6,7] with most stabilising between 1-3 months from stroke onset [8].

The importance of correctly diagnosing initial stroke severity and progression of injury relates to correct assignment of treatment options in stroke management since the initial size of a stroke lesion affects subsequent pathophysiological responses [9,10]. To this end, prognostic clinical approaches such as the use of the NIHSS (National Institutes of Health Stroke Scale) allow prediction of functional outcome and survival in stroke patients in order to support clinical management and to correctly stratify treatment groups in clinical trials aimed at achieving neuroprotection [11-13]. However, with the development of treatment strategies that promote brain repair, a similar comprehensive evaluation of patients following stroke will be required to assist in defining patients more likely to respond to
brain restoration treatments and rule out those that lack the biological factors required to achieve improved functional outcomes [14,15]. This highlights the need to further understand endogenous repair mechanisms and their response to different grades of stroke severity and subsequent damage.

Ischemic insults have now been shown to trigger neural progenitor cell proliferation and stem cell migration from the subventricular zone (SVZ) of the lateral ventricle to damaged regions of the brain [16,17] even in patients of advanced age [18]. Additionally, angiogenesis is one of the pivotal restorative mechanisms initiated after ischemic injury and involves the formation of new blood vessels from the damaged vascular networks [19,20]. Neurogenesis and angiogenesis are tightly coupled to one another and may influence brain remodelling and subsequent functional recovery in many patients following stroke [21-24]. The potential affect the size of the infarct has on the degree of angiogenesis has not been previously reported despite many studies focusing on the pathways involved in the induction of angiogenesis after ischemic brain injury. Likewise factors that influence the rate of neurogenesis remain to be defined. Here we investigate the effect of stroke severity on angiogenesis and SVZ cell proliferation and migration after cerebral ischemia using the endothelin-1 (ET-1) rat model of stroke, and correlate these findings with infarct volume, inflammation, scar formation and functional recovery.

Materials and Methods

Ethics statement

All experiments were performed in strict accordance with the guidelines of the National Health & Medical Research Council of Australia Code of Practice for the Care and use of Animals for Experimental Purposes in Australia. The protocol was approved by the St Vincent’s Hospital animal ethics committee (AEC009/09). All surgery was performed under general anaesthesia, and paracetamol (2mg/kg in drinking water) was provided for 24 hr prior to and after surgery in order to minimize suffering and included monitoring each rat throughout the length of the study to ensure their wellbeing.

Surgical preparation

A total of 37 adult male Hooded Wistar rats weighing 300-360g (Laboratory Animal Services, University of Adelaide, Australia) were included in this study. Rats were group-housed (4 rats to a cage), until endothelin-1 induced middle cerebral artery constriction, whereupon they were housed in separate cages under diurnal lighting with ambient temperature maintained between 20 and 22°C. Rats were given free access to food and water. Rats were anaesthetised with a mixture of Ketamine/Xylazine (75mg/kg:10mg/kg respectively i.p.) and maintained throughout surgery by inhalation of isoflurane (95% oxygen and 5% isoflurane). A 23-gauge stainless steel guide cannula was stereotaxically implanted into the cortex 2 mm dorsal to the right middle cerebral artery (MCA) (0.2 mm anterior, -5.9 mm lateral, -5.2 mm ventral) as in previous studies [5,14]. Rats were allowed to recover for 5 days prior to stroke induction.

Stroke induction

Focal cerebral ischemia was induced in conscious rats by constriction of the right middle cerebral artery with perivascular administration of endothelin-1 (ET-1; American Peptide Company, Inc. CA, USA; 40pmol in 2μl of saline over 10 min) to rats (n=34) similar to previous studies [14]. Characteristic behavioural changes indicating stroke included clenching and dragging of the contralateral forepaw and continuous circling in an anti-clockwise direction. These behavioural changes observed during stroke induction were scored 1 to 5 for stroke severity (according to our previously established protocol) with 5 being the most severe [14]. Changes in behaviour occurred routinely over 2 to 60 minutes from initiation of ET-1 infusion and rats that did not display any behavioural change were considered not to have suffered a stroke and were excluded from further study. Sham-operated rats (n=3) underwent cannula implantation without ET-1 delivery.

Assessment of functional outcome

Neurological assessments were conducted on all rats prior to any surgical procedures (pre-surgery), immediately prior to ET-1-induced stroke (pre-stroke) and 24, 48, 72hrs, 7 and 14 days post-stroke. Animals were tested and scored for neurological abnormalities based on abnormal posture and hemiplegia as previously described [25,26]. Forelimb asymmetry between the contralateral and ipsilateral forepaws was evaluated using the sticky label test by measuring latency to touch and remove attached stimuli [27]. Neurological scores from each rat post-stroke were compared to pre-stroke scores, thus each rat acted as its own control.

Quantification of ischemic damage

Rats were decapitated 7 (n=15) or 14 days (n=19) after stroke, forebrains removed, frozen over liquid nitrogen, and stored at -80°C prior to processing. Coronal sections (16 μm thick) were prepared using a Leica cryostat (Leica Microsystems, Wetzlar, Germany) at eight pre-determined coronal planes throughout the brain from -3.2 to 6.8 mm relative to Bregma. The extent of brain injury following stroke was determined in triplicate unstained slide mounted sections using a micro computer imaging device (MCID M4 image analyser, Imaging Research Inc., St. Catharines, ON, Canada) according to the methods of Callaway and colleagues [28]. Total infarct volume was determined by integrating the cross-sectional area of damage at each stereotaxic level with the distances between levels [29]. The influence of oedema was corrected for by applying the following formula: (area of normal hemisphere/area of infarcted hemisphere) x area of infarct [30].

Immunohistochemistry

Immunohistochemical staining was performed in adjacent sections to those used to quantify damage in order to identify blood vessels, proliferating SVZ cells, migrating SVZ neuroblasts, radial glial cells, as well as inflammatory cells after stroke. Control experiments included either omission of each primary antibody from the protocol or the inclusion of the appropriate IgG control to verify the specificity of each antibody. All sections were analysed with an ABC detection kit (Elite Vectastain, Vector Labs, Burlingame, CA, USA) for 30 mins followed by washes in phosphate buffered saline (PBS 0.1M pH 7.4; 3 x 5min) and 5 min incubation with
diaminobenzidine (DAB; Sigma, St. Louis, MO, USA). The resultant colour reaction was visualised with an Olympus microscope (Albertslund, Denmark).

**Blood vessel detection.** For detection of blood vessels, sections were first fixed with methanol (-20°C for 15 minutes) and then washed in PBS (0.1M; pH 7.4) containing 0.05% Tween 20 detergent (PBT) (3 x 5 min) followed by a 30 min block in 10% normal goat serum in PBT prior to washes in PBS (0.1M; 3 x 5 min). Sections were then incubated for 1 hour with rabbit anti-von Willebrand factor (vWF; 1:200, Millipore, Billerica, MA, USA) in PBT containing 5% NGS (0.1M, pH 7.4) for 10 min and washed in PBS (0.1M; 3 x 5 min) and 30 min incubation with secondary antibody biotinylated goat anti-rabbit (1:200, Vector Labs, Burlingame, CA, USA). Sections were further analysed as described above.

**SVZ cell proliferation.** For the detection of proliferating cells within the SVZ sections were fixed in 4% paraformaldehyde (PFA in 0.1M PBS, pH 7.4, for 15 min RT) and then washed in PBS (0.1M; 3 x 5 min) prior to incubation in blocking solution containing 5% NGS and 0.3% Triton X-100. Sections were then incubated with rabbit anti-Ki67 (Ki67; 1:1000, Labvision Thermo Scientific, Fremont, CA, USA) in PBT containing 5% NGS and 0.05% Tween 20 detergent (PBT) (3 x 5 min) and incubating for 30 min with secondary antibody biotinylated goat anti-rabbit (1:200, Vector Labs, Burlingame, CA, USA) for 30 min. Sections were further analysed as described above.

**Immature migrating neurons within the SVZ.** For the detection of migrating immature neurons or neuroblasts within the SVZ sections were fixed in 4% paraformaldehyde (PFA in 0.1M PBS, pH 7.4, for 15 min RT) and then washed in PBS (0.1M; 3 x 5 min) prior to antigen retrieval with 10% proteinase K (DAKO, Glostrup, Denmark) in 0.1M PBS for 3min following by incubation in 30% fetal calf serum to stop proteinase K activity for 30 min. Sections were washed in PBS (0.1M; 3 x 5 min) followed by incubation in blocking solution containing 5% NGS in 0.1M PBS for 30 min. Sections were then incubated with guinea-pig anti-doublecortin (DCX; 1:3000, Millipore, Billerica, MA, USA) for 1 hour in a mixture of PBS (0.1M; pH 7.4) containing 5% NGS prior to PBS washes (0.1M; 3 x 5 min) and incubation with secondary antibody biotinylated goat anti-rabbit (1:200, Vector Labs, Burlingame, CA, USA) for 30 min. Sections were further analysed as described above.

**Astrocytes.** For detection of astrocytes sections were fixed in 4% paraformaldehyde (PFA in 0.1M PBS, pH 7.4, for 15 min RT) and then washed in PBS (0.1M; 3 x 5 min) prior to incubation in block solution containing 5% NGS and 0.3% Triton X-100 in PBS (0.1M). Tissue sections were then incubated with mouse anti-glial fibrillary acidic protein (GFAP; 1:400, Millipore, Billerica, MA, USA) before washing in PBS (0.1M; 3 x 5 min) and incubating for 30 min with secondary antibody biotinylated goat anti-mouse (1:200, DAKO, Glostrup, Denmark) and analysed as above.

**Immunofluorescence.** Adjacent sections to those used for immunohistochemical staining, were fixed in 4% PFA for 10 min and washed in PBS (0.1M, pH 7.4, 3 x 5 min). Sections were then subjected to block with DAKO serum-free protein block (Glostrup, Denmark) for 30 min. Dual immunofluorescent techniques were employed where the primary antibody for vascular endothelial cells (vWF, 1:200) was incubated overnight at 4°C in a mixture of PBS (0.1M, pH 7.4) containing 2% NDS, 2% NGS, 0.3% Triton X-100, together with either: mouse anti-OX42/CD11b (1:100) to detect microglia/macrophages; mouse anti-neuron specific nuclear protein (NeuN; 1:400, Millipore, Billerica, MA, USA) to identify neurons; or mouse anti-GFAP (1:400, Millipore, Billerica, MA, USA) to detect astrocytes.

For characterisation of cells within the SVZ, rabbit anti-Ki67 (Ki67; 1:2000, Labvision Thermo Scientific, Fremont, CA, USA) was co-incubated with either mouse anti-GFAP (1:400, Millipore, Billerica, MA, USA) or guinea-pig anti-doublecortin (DCX; 1:500, Millipore, Billerica, MA, USA); and mouse anti-Nestin (1:400, Cell Signalling Technology, Danvers, MA, USA) was co-incubated with and rabbit anti-GFAP (1:400, DAKO, Glostrup, Denmark) overnight at 4°C in a mixture of PBS (0.1M, pH 7.4) containing 2% NGS and 0.3% Triton X-100.

**Microglia/macrophages.** Microglia/macrophages, sections were fixed by serial acetone dilutions (50:100 x 2 min each at RT) and then washed in PBS (0.1M; 3 x 5 min) prior to 30 min incubation in DAKO serum-free protein block (DAKO, Glostrup, Denmark). Sections were then incubated for 1 hour with mouse anti-OX42/CD11b (OX42; 1:100, Serotec, Raleigh, NC, USA) prior to washes in PBS (0.1M; 3 x 5 min) and 30 min incubation with secondary antibody biotinylated goat anti-mouse (1:200, DAKO, Glostrup, Denmark). Sections were further analysed as described above.

**Quantification of Immunohistochemistry.** Resulting tissue sections were viewed using an Olympus BH-2 microscope (Albertslund, Denmark) under x20 magnification with at least three sections per
region used for assessment. MCID generated images that were used to assess infarct were used as a reference and four common regions of interest where damage was routinely observed were identified in each section for assessment: the damaged cortex, damaged striatum, and the border region surrounding the damage for both the striatum and cortex. Each area used for analysis was then compared with the appropriate corresponding mirror region in the contralateral hemisphere, as well as to sham-operated controls, using exactly the same sample area (see figure 1A-L).

Blood vessels. Blood vessel quantification was assessed 14 days post-stroke in vWF-labelled sections at two anatomical levels (+0.2 mm and -0.84 mm relative to bregma) shown in previous studies to routinely result in damage. Using digital video imaging (TK C 1480E; JVC, Wayne, NJ, USA), an automated systematic random sampling point-counting system was applied with a Computer Assisted Stereological Toolbox (CAST System; Olympus) [31]. Sampling commenced randomly within each defined field and the number of points that overlay either blood vessels or where appropriate immuno-labelled cells, was scored and then divided by the total number of points counted in each field. The number of blood vessels detected in the ipsilateral sample region was then compared to the contralateral region and expressed as 100% control. For quantification of blood vessels a common area of interest within the cortex and striatum across all stroke rats was identified based on the appearance of both small and large vWF labelled microvessel and compared directly with the contralateral mirror image.

SVZ cell proliferation. Ki67 positive cells within the SVZ were quantified at both 7 and 14 days post-stroke in triplicate consecutive sections (+1.2 mm bregma) to ensure the same location within the SVZ was analysed for all animals. Cells within the ipsilateral SVZ and contralateral SVZ were quantified using the cell counter plugin for National Institute of Health ImageJ software (USA). Images were obtained using an Olympus microscope (Albertslund, Denmark) under x20 magnification (the same magnification utilised with CAST counts). Qualitative images of proliferating immature neurons (Ki67/DCX positive) observed within the SVZ were obtained using a laser scanning inverted confocal imaging system (Nikon Instruments Inc., Melville, NY, USA) under x40 magnification (a higher magnification was required to achieve greater image resolution for cell counts). Images were presented as collapsed reconstructions of 10 optical sections captured every 0.5 µm on the z-axis, at a scan speed of 0.5 frame/sec, a resolution of 1024 x 1024 pixels with the line acquisition mode. Due to a small counting frame within the SVZ cell numbers were quantified using standard stereological techniques and ImageJ. The total number of proliferating radial glial cells detected in the ipsilateral SVZ was then compared to the corresponding contralateral SVZ and expressed as 100% control.

SVZ neuronal cell migration. Migrating immature neurons or neuroblasts (DCX positive) within the ipsilateral SVZ and contralateral SVZ were quantified using the cell counter plugin for National Institute of Health ImageJ software (USA). DCX positive cells within the ipsilateral SVZ and contralateral SVZ were quantified using the cell counter plugin for National Institute of Health ImageJ software (USA). Images were obtained using an Olympus microscope (Albertslund, Denmark) under x20 magnification (the same magnification utilised with CAST counts). Qualitative images of proliferating immature neurons (Ki67/DCX positive) observed within the SVZ were obtained using a laser scanning inverted confocal imaging system (Nikon Instruments Inc., Melville, NY, USA) under x40 magnification (a higher magnification was required to achieve greater image resolution for cell counts). Images were presented as collapsed reconstructions of 10 optical sections captured every 0.5 µm on the z-axis, at a scan speed of 0.5 frame/sec, a resolution of 1024 x 1024 pixels with the line acquisition mode. Due to a small counting frame within the SVZ cell numbers were quantified using standard stereological techniques and ImageJ. The total number of immature neurons in the ipsilateral SVZ was then compared to the corresponding contralateral SVZ and expressed as 100% control.

Activated microglia/macrophages. Quantification of inflammatory cells was assessed within the same brain regions of interest as assessed for blood vessel quantification using the automated systematic random sampling point-counting system. The number of OX42 positive cells that displayed an amoeboid morphology with reduced processes (indicative of activation) were counted and expressed as a percentage of the total number of counts (cross hairs) within the defined sample area within the ipsilateral or contralateral hemispheres. Resting microglia displaying morphologies of small cell bodies with highly ramified processes were excluded from counts.

Astrocytes. GFAP labelled reactive astrogliosis was assessed and quantified based on morphological stage of activation [32]. Different gradations of reactive astrogliosis and glial scar formation were identified based on cellular hypertrophy, and length and thickness of processes and classified as either activated astrocytes or diffuse astrocytes. Quantification of activated or diffuse astrocytes within the cortical and striatal core damage and border regions was also counted using the automated systematic random sampling point-counting system and expressed as a percentage of the total number of counts (cross hairs) within the defined sample area.

Statistical analyses

All statistical analysis was conducted in consultation with a statistical consultant, Rachel Sore of the Statistical Consulting Centre with the University of Melbourne, Victoria, Australia. Neurological outcome
data was analysed by Kruskal-Wallis non-parametric ANOVA followed by Dunn’s post-test. Hemineglect data were analysed by repeated measures for two-way ANOVA (side x hr/days after stroke) to compare latencies in the ipsilateral and contralateral forepaws over time. Individual comparisons were made using Bonferroni multiple comparisons test for all analyses where ANOVA yielded a significant result or Mann Whitney test for comparisons between two data-sets. Values are presented as mean ± SEM unless stated otherwise. To test for correlation between infarct volume and either: angiogenesis, SVZ cell proliferation, migrating SVZ neuroblasts or inflammatory cell activation, the Pearson product-moment coefficient, r, for ordinal values was determined using GraphPad Prism, version 6 (GraphPad Software Inc., San Diego CA). P-values less than 0.05 were considered significant.

Results

Functional outcome

Significant neurological deficits were observed 24 hrs to 7 days post-stroke when compared to pre-stroke scores (P<0.0001 n=19, non-parametric ANOVA; Figure 1A) but not beyond 7 days. Latency to touch and remove sticky labels from the stroke affected contralateral forepaw was also significantly increased when compared with the ipsilateral forelimb between 24 and 72 hrs after stroke (P<0.05, Two-way ANOVA; Figures 1B, C), but no significant deficits were detected after this time.

Infarct analysis

Histopathology 14 days after stroke revealed damage in the frontal, parietal and insular cortex and the striatum. Rats assigned lower stroke ratings during induction of stroke (ET-1 infusion) displayed damage within the cortex whereas those assigned larger stroke ratings demonstrated damage within both the cortex and striatum (Figure 1J-L) as in previous studies [14]. Rats with higher stroke severity scores exhibited significantly greater infarct volumes in both 7 and 14 day recovery groups (P<0.05, P<0.0001, Figure 1D). A positive correlation between total infarct volume and neurological deficit score was also found 24hr (r=0.52, P<0.05; Figure 1E), 48hr (r=0.70, P<0.001; Figure 1F), 72hr (r=0.60, P<0.05; Figure 1G) and 7 days (r=0.54, P<0.05; Figure 1H), but not at 14 days post-stroke (r=0.16; Figure 1I).

Angiogenesis and infarct correlation

vWF labelled blood vessels revealed regions of angiogenesis both within and around the core infarct of the cortex and where relevant in the damaged striatum. Both large and thin walled microvessels were point counted within a defined sample area (800µm²) from the same anatomical region for all rats and compared to the contralateral mirror image (undamaged tissue). Blood vessel counts were then correlated with the quantified infarct volume for each rat. Positive correlations between infarct size and blood vessel numbers were detected in the ipsilateral core cortex (r=0.81, P<0.0001; Figure 2A), surrounding border cortex (r=0.66, P<0.005; Figure 2B), core striatum (r=0.91, P<0.005; Figure 2C) and border striatum (r=0.73, P<0.05; Figure 2D), with larger infarcts correlating with increased vascular density. The number of blood vessels (vWF+) significantly increased within the ipsilateral core cortex (P<0.0001), border cortex (P<0.01), core striatum (P<0.01) and border striatum (P=0.05, Scatter plots, non-parametric ANOVA; Figure 2E-H) 14 days post-stroke when compared to the corresponding contralateral hemisphere or when compared to either hemisphere of sham-operated animals. No significant differences were observed between the contralateral hemisphere of ET-1 stroke affected animals and either hemisphere of sham-operated animals (Figure 2E-H). To confirm an association between damage after stroke and angiogenesis, immunofluorescent double labelling for neurons (NeuN) and blood vessels (vWF) confirmed that greater levels of angiogenesis occurred in regions where there was greatest neuronal loss (Figure 2I-K).

SVZ cell proliferation, migration, neural differentiation and infarct correlation

In order to determine the effects of stroke on the neurogenic niche we first characterised cell proliferation within the SVZ. Ki67 positive cells were counted in both the ipsilateral and contralateral SVZ region from both 7 and 14 day recovery groups. The total number of proliferating cells within the SVZ significantly increased 7 days post-stroke compared to sham-operated animals (P<0.05, Scatter plots, non-parametric ANOVA; Figure 3A). This increase was no-longer detected 14 days post-stroke. At 7 days post-stroke a positive correlation was found between infarct volume and Ki67 labelled SVZ cells (r=0.73, P=0.005, n=15; Figure 3B), with larger infarcts associated with increased cell proliferation. No correlation between infarct volume and SVZ proliferation was found 14 days post-stroke (r=0.07, n=19; Figure 3C).

We then examined the effects of stroke on stem cell differentiation within the SVZ. We detected both Ki67/GFAP/Nestin positive radial glial cells as well as immature migrating neuronal cells identified as DCX staining. We therefore quantified Ki67/GFAP+ cells and DCX+ cells separately along the lateral ventricle in both the ipsilateral and contralateral SVZ 7 days post-stroke at the same anatomical level for all sections (+1.2mm relative to bregma). The number of proliferating radial glial cells (Ki67+/GFAP+) increased 7 days post-stroke compared to sham-operated animals (P<0.05, Scatter plots, Mann Whitney test; Figure 3D). There was also a significant correlation between infarct volume and the number of Ki67/GFAP co-labelled cells within SVZ (r=0.73, P<0.005, n=15; Figure 3E) 7 days post-stroke. The number of DCX+ cells was also increased within and surrounding the SVZ 7 days post-stroke compared to sham-operated animals (P<0.05, Scatter plots; Mann Whitney test; Figure 3F). These DCX+ cells, did not stain with either Ki67 or GFAP and a significant correlation was also found between infarct volume and the number of DCX positive cells within the SVZ (r=0.77, P<0.001, n=15; Figure 3G) 7 days post-stroke. Immunofluorescence staining revealed a greater number of Ki67 positive (Figure 4A-C) and DCX positive (Figure 4D-F) cells within the SVZ of animals with larger infarct volumes. Nestin/GFAP positive immunostaining revealed a large number of radial glial cells extending from the SVZ towards the cortical and striatal penumbral regions 7 days post-stroke, with many new activated Nestin+ astrocytes detected within the
Lesion Size Affects Brain Remodelling Post-Stroke

Figure 1. Neurological outcome 14 days after ET-1 induced stroke. Combined neurological deficit scores (A). Data presented as box plots include hinges extending from the 25th to 75th percentiles, the median line within the box and whiskers extending to the minimum and maximum values of the dataset. ***P<0.0001 vs 0 hr pre-stroke score (n=19, non-parametric ANOVA). Latency to touch (B) and remove an adhesive (C) on the contralateral (stroke affected) forelimb compared with the ipsilateral forelimb. Data presented as mean ± SEM, *P<0.05 compared with the ipsilateral forelimb at the same time measurement (ANOVA). Scatter plots depicting correlation between infarct volumes and stroke severity rating (r=0.88, P<0.0001; D) (Pearson product moment correlation coefficients). A significant correlation was found between total infarct volume and neurological deficit score at 24 (r=0.52, P<0.05; E), 48 (r=0.70, P<0.001; F), 72hr (r=0.60, P<0.01; G), and 7 day (r=0.54, P<0.05; H) post-stroke but not thereafter (r=0.16; I) (Pearson product moment correlation coefficients). MCID images of coronal sections taken from three animals with stroke ratings ranging from low to high; stroke rating #2 (J), #3 (K), and #4 (L) displaying damage to varying degrees within the cortex and striatum marked by the black dotted line. Consistent regions used for quantification within the infarcted cortex and striatum can be visualised by the red boxes (J-L). doi:10.1371/journal.pone.0097007.g001

Inflammatory response and infarct correlation

Activated Microglia/Macrophages. OX42 labelling revealed both resting and activated microglia/macrophages in the contralateral and ipsilateral hemisphere respectively. Resting microglia were observed to have a ramified morphology with small cell bodies and long fine branched processes (Figure 5A) [33]. Activated microglia appeared more amoeboïd in morphology with large cell bodies with retracted processes, similar in appearance to blood derived macrophages [34,35] (Figure 5B, C). Since reactive microglia can develop into a phagocytic phenotype that is indistinguishable from infiltrating blood borne macrophages and lack discriminating cellular markers [35-37] both activated microglia and blood borne macrophages were quantified together. A significant increase in the number of activated microglia/macrophages was detected in the ipsilateral hemisphere up to 14 days after stroke. A positive correlation was found between the number of activated microglia/macrophages and infarct volume within the ipsilateral core cortex (r=0.88, P<0.0001, n=19; Figure 5D), border cortex (r=0.70, P<0.001, n=19; Figure 5E), core striatum (r=0.86, P<0.01, n=8; Figure 5F) and border striatum (r=0.77, P<0.05, n=8; Figure 5G) with larger infarcts containing more OX-42 positive cells within a consistent sample area. Contrastingly, within the contralateral hemisphere in regions that mirrored areas defined in the ipsilateral hemisphere, the number
Lesion Size Affects Brain Remodelling Post-Stroke

of activated microglia/macrophages negatively correlated with the size of infarct. This was observed within the core cortical \((r=0.81, P<0.0001, n=19; \text{Figure } 5\text{H})\), border cortex \((r=0.75, P<0.0005, n=19; \text{Figure } 5\text{I})\), core striatum \((r=0.75, P<0.05, n=8; \text{Figure } 5\text{J})\) and border striatum \((r=0.75, P<0.05, n=8; \text{Figure } 5\text{K})\). Activated microglia within the contralateral hemisphere were observed through the corpus callosum \((\text{Figure } 5\text{L-O})\) with increased state of activation observed closer to the site of damage.

Astrogliosis. Astrocytes were quantified based on morphological distinctions. Astrocytes of normal quiescent appearance were found in regions remote from the lesion site or within the contralateral hemisphere (Figure 6A) and were identified by low GFAP expression with long slender processes that did not overlap. Activated astrocytes were defined by an up-regulation of GFAP expression with cellular hypertrophy without pronounced overlap of processes (Figure 6B). Finally, severely diffuse astrocytes were identified based on pronounced up-regulation of GFAP expression, prominent cell body and process

Figure 2. Angiogenesis and infarct volume 14 days post-stroke.
Scatter plots depict a significant correlation found between degree of angiogenesis and infarct volume within cortex core \((r=0.81, P<0.0001, n=19; \text{A})\), cortex border \((r=0.66, P<0.005, n=19; \text{B})\), striatum core \((r=0.91, P<0.005, n=8; \text{C})\) and striatum border \((r=0.73, P<0.05, n=8; \text{D})\) (Pearson product moment correlation coefficients). Scatter plots of blood vessels \((\text{vWF}^+)\) from sham-operated \((n=3)\) and 14 day post-stroke animals \((n=19)\) revealed a significant increase in the number of blood vessels within the ipsilateral cortical and striatal core and border regions only \((\text{non-parametric ANOVA}; E-H)\). Data presented as mean ± SEM. *\(P<0.05\), **\(P<0.01\), ****\(P<0.0001\) compared with the contralateral hemisphere of ET-1 stroke affected animals and either hemisphere of sham-operated animals. No significant differences were observed between the contralateral hemisphere of ET-1 stroke affected animals and either hemisphere of sham-operated animals. Angiogenesis and neuronal loss within the stroke damaged brain \((\text{I-K})\). Immunohistochemical localisation of neurons (red) and blood vessels (green) in the cortex of stroke affected rat brains with total infarct volumes ranging from 0.76-77mm\(^3\). Increased angiogenesis is observed in regions with greatest neuronal loss (ipsilateral columns I, J and K). Scale bar: I-K 100 µm. doi:10.1371/journal.pone.0097007.g002
hypothesis, with distinct process overlap and dense packing of cells to form a glial scar (Figure 6C). Within the core cortex, little or no activated astrocytes were observed 14 days post-stroke (Figure 6D) and diffuse astrocytes were detected in very low numbers in this region (Figure 6E). Increased GFAP immunoreactivity was predominantly observed within the border zones of the infarct. In cortical regions surrounding the infarct (border zone), infarct volume negatively correlated with the number of moderately activated astrocytes (r=0.87, P<0.0001, n=19; Figure 6F) but positively correlated with diffuse astrocytes (r=0.96, P<0.0001, n=19; Figure 6G). Little or no activated astrocytes were observed within the core striatum 14 days post-stroke (Figure 6H). A modest correlation was observed between infarct size and diffuse astrocytes within the core striatum (r=0.93, P<0.001, n=8; Figure 6I). Within the border zone surrounding the striatal infarct, lesion volume negatively correlated with activated astrocytes (r=0.85, P<0.01, n=8; Figure 6J) but positively correlated with diffuse astrocytes (r=0.89, P<0.005, n=8; Figure 6K). Larger infarct volumes were associated with greater glial scar formation encircling the damaged striatum and cortex.

Discussion

In the present study we confirm that the ET-1 model of stroke is a valuable tool for studying brain remodelling and long term recovery, specifically angiogenesis, microglia/macrophage activation, astrocytic morphological transition and SVZ cell proliferation, differentiation and migration. There is a positive correlation between lesion size and proliferation and differentiation of cells within the SVZ 7 days post-stroke, as well as between lesion size and the degree of angiogenesis, microglia activation and astrogliosis 14 days after stroke. This confirms and extends the initial report of Nagai et al. (2010) who showed pathological responses initiated by stroke affects the magnitude of the inflammatory response, and identifies the effect of stroke severity on subsequent repair mechanisms, important factors to consider when exploring treatment strategies that promote recovery of the injured brain.

Predictive outcome and long term recovery

Neurological deficit scores after stroke have commonly been used as a predictor of long term outcomes [38,39]. The current scales however, do not take into account spontaneous recovery of functions that
occur weeks to months after stroke without change to the resulting brain infarct. None-the-less use of such scales has proven beneficial when assigning patients to treatment. We have previously reported the benefits of predicting stroke outcome using the ET-1 model of stroke in conscious rats, where variability in stroke severity reliably correlates with infarct volumes up to 3 day post-stroke [14]. To this end stroke outcomes using the ET-1 model allow clinically relevant assessments of stroke pathology since stroke in humans is highly variable: a large infarct can result following occlusion of a major artery such as the MCA or a small infarct can result due to occlusion of a single penetrating artery [40]. Predicting stroke outcome using rodent models has also been described where neurological deficits are tested upon recovery from anaesthesia [41]. In the present study we demonstrate that whilst a neurological scale at the time of stroke can be reliably used to predict histological outcome up to 14 days, it is not a reliable long-term predictor of neurological outcome, since recovery of deficits observed 14 days after stroke did not correlate with reduced lesion size.

Angiogenesis and infarct size
The potential for brain regeneration relies heavily on the surrounding microenvironment and for neuronal replacement to occur it requires supporting vasculature. Angiogenesis is a fundamental process occurring during...
development and in wound healing in adults, and previous studies using the MCA thread occlusion model have reported new vasculature develops mostly within the peri-infarct zone [19,42]. In contrast, we showed evidence of angiogenesis across the entire infarct in addition to the border zone [5], similar to that which occurs in humans where a significantly higher microvessel density is observed across the damaged region of the brain [20]. We now demonstrate that larger infarct volumes result in increased microvessel density within the core infarct as well as the peri-infarct zone, suggesting that even the most severely damaged brain might be capable of supporting neuronal replacement.

Figure 5. Immunohistochemical analysis of microglia and macrophages 14 days post-stroke. Immunohistochemical images of OX42 labelled microglia/macrophages within the contralateral undamaged hemisphere (A), ipsilateral core cortex of a small infarct (B) versus a large infarct (C). Within the ipsilateral hemisphere, a positive correlation was observed between infarct volume and the number of microglia and macrophages in the core cortex (r=0.88, P<0.0001, n=19; D), surrounding cortex (r=0.70, P<0.001, n=19; E), core striatum (r=0.86, P<0.01, n=8; F), and border striatum (r=0.77, P<0.05, n=8; G). In contrast, the number of microglia and macrophages in the mirror image areas on the contralateral side negatively correlated with infarct volumes within the core cortex (r=0.81, P<0.0001, n=19; H), border cortex (r=0.75, P<0.0005, n=19; I), core striatum (r=0.75, P<0.05, n=8; J), and border striatum (r=0.75, P<0.05, n=8; K) (Pearson product moment correlation coefficients). Activated microglia can be observed within the corpus callosum, and potentially represent microglia migrating from the contralateral undamaged hemisphere to sites of damage within the ipsilateral hemisphere (L). Magnified immunohistochemical images that correspond to boxes labelled (M to O) in (L) illustrate the possible migration pathway of microglia along the corpus callosum as indicated by the arrows. Scale bar: (A-C) 100µm, (L) 2500µm, (M-O) 50µm.

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Previously the degree of angiogenesis, measured by increased cerebral blood flow and microvessel density, has been correlated with longer survival in stroke affected patients [20] suggesting that active angiogenesis may be beneficial to the damaged brain. Indeed we have previously reported that with spontaneous functional recovery newly formed vascular networks are detected within damaged regions [5]. One possible mechanism may be associated with the damage sustained to cerebral blood vessels themselves, such that a greater degree of vascular sprouting may result from an increased number of damaged vessels. Cerebral ischemia initially compromises blood vessels and may result in extensive damage to the vessel wall. In the present study we confirmed that a greater degree of angiogenesis occurs in regions associated with extensive neuronal loss and increased stroke severity. Thus increased vascular damage is associated with increased neuronal loss, resulting in increased angiogenesis.

The angiogenic process can improve the perfusion of oxygen and nutrients to ischemic and surrounding tissues where partially affected neurons that have the capability of surviving reside, which in turn may improve functional outcome following stroke [19]. Proliferation of endothelial cells within damaged brain regions are initiated within days after ischemic events [43,44] and the process of active angiogenesis may be beneficial for the ischemic brain. Interestingly, the
correlation between angiogenesis and improved neurological outcome following stroke has been observed in both animal stroke models and human stroke patients [20,45,46]. Angiogenesis has also been linked directly with other endogenous recovery mechanisms including neurogenesis, synaptogenesis and neuronal and synaptic plasticity which are all events involved in the long-term repair and restoration process of the brain after stroke. If these repair processes are not achieved then vessel density may later regress. Clearly angiogenesis is a potential target for improved regenerative recovery after ischemic stroke.

Neurogenesis and infarct size

Ischemic insults to the brain have now been shown to trigger progenitor cell proliferation and stem cell migration from the SVZ of the lateral ventricle to damaged regions of the brain. We saw increased SVZ cell proliferation in the ipsilateral hemisphere 7 days post-stroke. Importantly, rats with larger infarcts were shown to have a greater degree of SVZ cell proliferation. Previous studies have shown proliferating SVZ stem cells increase as early as 2 days post-stroke, an effect that peaks by 7 days and returns to normal by 14 days [47,48]. Ours is the first study to show that this effect is initially amplified with increasing stroke severity, but is not sustained beyond this time.

The adult SVZ contains a heterogeneous population of cells including SVZ radial glial cells (type B cells), rapidly dividing transient amplifying cells (type C cells) and migratory neuroblasts (type A cells) [49,50]. Normally within the adult SVZ, newly generated stem cells migrate through the rostral migratory stream (RMS) into the olfactory bulb and differentiate into interneurons [51]. Cerebral ischemia alters the migration pathway and neuroblasts generated from the SVZ migrate to the injured brain regions instead, where only a few differentiate into new neurons (<0.2%) [48]. In the present study rats with larger infarcts showed evidence of increased SVZ proliferation with a greater number of cells generated following a neuronal differentiation path as evidenced by increased DCX staining. DCX positive neuroblasts were not however evident within penumbral regions (Figure S1D-G), possibly suggesting a low survival rate of these new neurons with greater stroke severity. Arvidsson et al. [48] previously described the unfavourable hostile environment of severely ischemic tissue for newly formed neurons.

A notable loop of interactions between newly formed stem cells and blood vessels has been reported [24] and this interaction may be magnified with larger infarct volumes. Activated endothelial cells, injured and newly forming blood vessels release an array of trophic factors including vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) which can evoke neurogenesis, are chemotactic for progenitor cells and may enhance the survival and integration of neuroblasts into the brain tissue [18,22,52].

In the present study larger infarct volumes also correlated with an increase in the number of radial glial cells generated within the SVZ 7 days post-stroke. Radial glial cells retain neural stem cell-like properties and are capable of migrating from the SVZ towards the damaged brain where they can differentiate into either neurons or astrocytes based on the microenvironment [50,53,54]. Our results demonstrate that an increase in the number of SVZ derived radial glial cells 7 days post-stroke appear to be extending towards cortical and striatal penumbral regions of animals with larger infarcts (Figure S1A-C). By 14 days Nestin expressing reactive astrocytes are detected within the glial scar and possibly indicate a new cell population of reactive astrocytes that have migrated from the SVZ to the site of injury in response to stroke. These results indicate that without intervention, newly generated stem cells extend towards the damaged brain but have a greater propensity to become astrocytes that in turn contribute to glial scar formation that poses a major obstacle to brain repair.

Microglial response to lesion size

Previous studies have reported a positive correlation between infarct size and microglia activation up to 7 days following discrete photochemically induced thrombotic lesions of the cerebral cortex [9]. We now show that this effect is also true for brain injury following focal ischemia using the ET-1 model with a 14 day recovery. Furthermore, we observed within the contralateral hemisphere a decrease in the percentage of activated microglia/macrophages in rats with larger infarcts. Microglia can become motile and actively move to the site of injury following chemotactic signals [55,56] and have also been found to migrate through the corpus callosum to arrive to their desired destination [57]. Indeed in the present study we observed in rats a reduction in the number of microglia in the contralateral hemisphere as well as graded changes in morphological activation throughout the corpus callosum, suggesting movement of microglia from uninjured brain regions to the infarcted hemisphere as described by others [55,56]. Therefore, the accumulation of microglia within the infarcted region at 14 days is likely to comprise of proliferating resident microglia as well as migrating microglia [58].

The accumulation of activated microglia/macrophages within ischemic regions occurs early after stroke with a peak response occurring at 7 days [59] when the inflammatory response peaks [5]. Our data now indicate a significant inflammatory response persists 14 days post-stroke where the size of the lesion still dictates the level of inflammatory cell activation. Persistent microglial activation can become maladaptive with the release of cytotoxic molecules [60,61] and pro-inflammatory cytokines that have been found to be neurotoxic [2,62,63]. In contrast, recent reports suggest that activated microglia may also play a role in brain repair. For it has been implicated in increasing neurogenesis in vitro [64] and in vivo [65] following stroke, and free radicals derived from activated microglia might also have a role in promoting angiogenesis [5]. Microglia may also exert neuroprotection by producing neurotrophic molecules such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), and several other growth factors [66]. However the degree of injury may be an important stimulus for microglia, determining whether they play a detrimental or beneficial role. Establishing the microglia/macroage response after an ischemic insult will assist in the understanding of endogenous processes occurring to highlight the positive and negative influences these inflammatory cells may have on endogenous repair mechanisms that support neuronal differentiation and survival of migrating neural progenitor cells.

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Astrocytic response to varying infarct volumes

Positive correlations between lesion size and astrocytic activation has also been reported by Nagai et al. (2010). Quantification of activated astrocytes according to morphological transition in correlation with infarct volume has not been previously reported. Astrogliosis following injury to the brain is not a simple all-or-none phenomenon, but a finely graded continuum of morphological change that can either influence brain preservation or result in long-lasting scar formation and reorganisation of tissue structure [32,67]. We now confirm that larger infarct volumes contain an increased percentage of severely diffuse reactive astrocytes, and a decrease in the percentage of moderately activated astrocytes within border regions of the infarct.

Astrocytes with moderately activated morphologies have been thought to initially provide trophic and protective support to neurons [68] that may promote neuroprotection and could possibly create an environment conducive to facilitate neurite extension and axonal outgrowth to aid in regeneration [69]. If immediate restoration of blood flow after initial stroke insult is initiated, astrocytes of mild or moderately active morphologies have the potential to resolve and return to their appearance in healthy tissue once the triggering mechanism is resolved [70]. On the other hand if the insult continues to persist without resolution, astrocytes transition into severely diffuse reactive distinctions and may not revert to their previous morphological states [70]. These severely diffuse reactive astrocytes may no longer be beneficial to neurons and can instead majorly impede axonal regrowth through formation of the glial scar and secretion of extracellular matrix molecules that prevent axonal regeneration [67,71-73].

Based on our findings larger infarcts induce astrocytic transition to severely diffuse morphologies that could possibly contribute to the spread of damage as well as combining to form a physical barrier to brain remodelling and repair. Importantly we now show that increased cell proliferation within the SVZ of rats with larger infarcts contribute to increased gial scar formation since we observed a greater proportion of Nestin+/GFAP positive cells extending from the SVZ as well as within the border zone. Much focus in developing treatment strategies that encourage brain repair has been to target increased proliferation and migration of neural progenitor cells from the SVZ after brain injury [74-78]. Treatments aimed towards up-regulating endogenous proliferation from either the SVZ or dentate gyrus of the hippocampus should also confirm the fate of the newly generated cells to appropriately assess the therapeutic benefit [79]. Given the results presented in the current study it is now clearly important to characterise the long term impact of these treatments across differing stroke severities. Particularly since increasing stem cell proliferation and migration may be counterproductive if the microenvironment supports conversion to astrocytes that accentuate the glial scar rather than conversion to new neurons.

Conclusion

The potential for brain regeneration relies heavily on the surrounding microenvironment and this includes the pathogenic contribution from inflammatory cells, angiogenesis and neural stem cells. Ours is the first study to demonstrate a positive correlation between stroke severity and the effects on pathological responses to brain recovery using a focal model of cerebral ischemia. In addition, our results have highlighted the importance of taking into consideration infarct size when developing therapies that are targeted towards promoting neurogenesis. Therapeutic strategies aimed at increasing cell proliferation and migration from the SVZ need to pay particular attention to the phenotypic transformation of neural progenitor cells following treatments to avoid unwanted additional scar formation. Understanding the endogenous pathways initiated after ischemic insult will enable us to design better therapies to augment the restorative power of the cerebral vasculature as well as improve brain remodelling for improved functional outcomes.

Supporting Information

Figure S1. SVZ cell extension towards infarct and neural differentiation.

Photomicrographs of the contralateral (A) and ipsilateral (B) hemispheres depicting radial glial cells mainly within the ipsilateral hemisphere extending from the SVZ towards the cortical and striatal penumbral regions (Nestin+/GFAP+; green/red respectively with co-expression giving a yellow appearance). Core infarct regions are marked by a white dotted line. Merged immunofluorescent image of radial glial cells extending towards the penumbra from the SVZ as indicated by the arrows (Nestin/GFAP/DAPI; green/red/blue respectively with Nestin/GFAP co-expression giving a yellow appearance). C. Immunofluorescent images depicting immature neuronal cells (DAPI/DCX+; blue/green respectively; D-G) within the penumbra cortex (D) and SVZ of the ipsilateral hemisphere (E) and mirror images of the contralateral hemisphere (F, G). All images were taken from animals with large infarcts. LV: Lateral ventricle, Ctx: Cortex, Stm: Striatum. Scale bar: A,B 400µm, C 40µm, D, G 100µm, E, F 50µm.

Author Contributions

Conceived and designed the experiments: CLR GJD. Performed the experiments: HCSA LB CLR. Analysed the data: HCSA. Contributed reagents/materials/analysis tools: CLR. Wrote the paper: HCSA CLR.

References


Supporting Information

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Chapter 4: Treatment with cyclosporine A reduces overactivation of astrocytes and averts deficits following transient focal ischemia in rats

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4.1 Introduction

Studies now show that the injured brain is capable of some repair with spontaneous functional recovery observed in a cross section of stroke patients during rehabilitation without pharmaceutical intervention (Gauthier et al. 2008). Subsequently exercise and environmental enrichment strategies have shown some success in improving functional outcomes (Minger et al. 2007). To this end, ischemic insults have now been shown to trigger brain remodelling and stem cell migration from the subventricular zone (SVZ) of the lateral ventricle to damaged regions of the brain, even in patients of advanced age (Garzon-Muvdi and Quinones-Hinojosa 2009). Despite these attempts at repair complete restoration of function is often not achieved (Muntner et al. 2002). For this reason exogenous cell-based therapies to complement endogenous repair mechanisms are currently being trialed following extensive meta-analysis of over 40 studies reporting significant improvements in function when transplanted after stroke in animal models (Lees et al. 2012). Early reports suggest that transplanted embryonic stem cells, fetal stem cells, and immortalised cell lines can survive the grafting process and improve functional outcome (Borlongan and Sanberg 2002, Wechsler and Kondziolka 2003). However, the survival and success of transplanting a non-autologous stem cell relies heavily upon preventing host rejection of the graft with immunosuppression routinely used in conjunction with cell-based therapies (Hicks et al. 2009, McLeod et al. 2012, Mukhida et al. 2007, Mukhida et al. 2008).

CsA is the most widely used immunosuppressive agent in allograft transplant procedures and treatment of autoimmune disorders (Borel et al. 1976). Previously, CsA has been shown to be neuroprotective in experimental animal models of stroke (Butcher et al. 1997, Shiga et al. 1992, Uchino et al. 1998) which has prompted further research into its use as a treatment agent (Osman et al. 2011). However, experimental data investigating the effects of CsA in focal models of stroke remain limited with conflicting reports of the efficacy of CsA treatment (Borlongan et al. 2005, Kuroda et al. 1999, Leger et al. 2011, Puka-Sundvall et al. 2001, Shiga et al. 1992, Yu et al. 2004). Furthermore, previous preclinical studies have not focused on the effect of CsA on critical cellular events associated with brain repair in response to injury which is necessary to evaluate the impact of CsA on overall stroke outcome. Therefore, prior to commencing stem cell transplant studies targeting the regenerative process of
angiogenesis, we now focus on the effect of CsA treatment on stroke outcome. We first examined the cellular effects of CsA on microglia activation, astrogliosis, angiogenesis, stem cell proliferation and migration as well as neural differentiation within the neurogenic niche of the subventricular zone (SVZ), and correlated findings to infarct volume and functional recovery 7 days after transient focal cerebral ischemia in rats.

4.2 Hypothesis and Aims

Hypothesis: The use of immunosuppressive agent, CsA, affects responses to stroke injury and attempts at brain repair that influence functional recovery.

Specific Aims: To investigate:

1. The effects of CsA on inflammatory processes including microglia/macrophage activation, astrogliosis, functional recovery and infarct volume 7 days after transient focal ischemia in rats.

2. The effects of CsA treatment on repair mechanisms such as angiogenesis and stem cell responses within the neurogenic niche of the subventricular zone (SVZ), 7 days after ischemia in rats.

4.3 Experimental Design

In order to determine potential neuroprotective effects of CsA on the brain after stroke, two treatment groups were used with the first group receiving CsA and the second group receiving vehicle, each allowed to recover for 7 days post-stroke (Figure 4.1). All outcomes were compared to sham-operated control animals with the same recovery period. To investigate the effects of CsA on endogenous remodelling processes, angiogenesis, stem cell responses, activation of microglia/macrophages, astrogliosis and scar formation were analysed using immunohistochemistry and immunofluorescent techniques and quantified using the Computer Assisted Stereological Toolbox (CAST) system and ImageJ. All rats were assigned to a treatment group before initial surgery, and stroke was induced in conscious rats by ET-1 infusion. Each rat was rated for stroke severity to ensure that each group was not unevenly represented for predictive stroke outcome. All experiments performed were conducted in adherence to current RIGOR guidelines (Lapchak PA 2013, Landis SC et al. 2012) and included randomisation of treatments, blinding during assessment, inclusion of
appropriate control groups, and full statistical analysis involving power calculations in consultation with the Statistical Consulting Centre, University of Melbourne, Victoria, Australia.

Figure 4.1 Treatment protocol
All animals received treatment 2 days prior to stroke (CsA: \(n=8\); vehicle: \(n=8\)) and everyday thereafter. All behaviour tests (neurological deficit scores and adhesive tape test) were performed as single trial pre-surgery, pre-stroke (day 0), and at 24 hr, 48 hr, 72hr and 7 days post-stroke as highlighted in blue. All rats underwent surgery to stereotaxically implant a guide cannula and allowed to recover for 5 days prior to stroke induction with ET-1. Animals were euthanised and brains processed for histological analysis at the conclusion of the study.

4.4 Methods
The ET-1 model of MCA vasoconstriction was used to induce stroke in all rats and the associated surgical procedures and behavioural tests (neurological deficit score and adhesive tape test) were carried out, as described in Chapter 2.2. Infarct volume was determined using MCID analysis of unstained sections (Section 2.4.1). Animal details and specific methods used in this study are presented below.

4.4.1 Animals
Adult male Hooded Wistar rats (300-360g) purchased from Laboratory Animal Services, University of Adelaide (SA, Australia) were utilised for experiments. All rats were maintained on a 12 hr light/dark cycle at temperatures between 20 and 22°C, with \textit{ad libitum} access to food and water. Rats were dually housed prior to surgery and
individually housed following surgery. Sham control operations (n=3) were performed where animals underwent cannula implantation without stroke with a 7 day recovery period. A total of 25 rats were used for this study.

4.4.2 Cyclosporine administration

CsA (10mg/kg, i.p; n=8, Sandimmune, Novartis Pharmaceuticals Corporation, NJ, USA) or vehicle control (2.6% ethanol; 1% castor oil in saline; n=8) was administered 2 days prior to stroke induction and then again everyday thereafter. The dosing regime was chosen based on previous studies demonstrating 10mg/kg is sufficient to prevent host rejection of stem cell transplant in rats (Mukhida et al. 2007). Pre-treatment of rats with CsA or vehicle did not affect behavioural signs indicative of stroke induction and therefore allowed rats to be assigned a stroke severity score during stroke induction to ensure that within each treatment group stroke severity was equally matched as in previous studies (Roulston et al. 2008).

4.4.3 Immunohistochemistry

Astrocytes, microglia/macrophages, proliferating SVZ cells, migrating SVZ neuroblasts, radial glial cells, and blood vessels were identified in rat brain sections adjacent to those used to quantify damage by utilising immunohistochemical staining techniques described in Section 2.5.1. Primary antibodies used included the following: mouse anti-glial fibrillary acidic protein (GFAP; 1:400, Millipore, Billerica, MA, USA), mouse anti-OX42/CD11b (OX42; 1:100, Serotec, Raleigh, NC, USA), rabbit anti-Ki67 (Ki67; 1:1000, Labvision Thermo Scientific, Fremont, CA, USA), guinea-pig anti-doublecortin (DCX; 1:3000, Millipore, Billerica, MA, USA), rabbit anti-von Willebrand factor (vWF; 1:200, Millipore, Billerica, MA, USA). Biotinylated secondary antibodies used included goat anti-mouse (1:200, DAKO, Glostrup, Denmark), goat anti-rabbit (1:200, Vector Labs, Burlingame, CA, USA), goat anti-guinea-pig (1:200, Vector Labs, Burlingame, CA, USA), goat anti-rabbit (1:200, Vector Labs, Burlingame, CA, USA). Standard ABC methodology was used to process cells for diaminobenzidine (Sigma, St. Louis, MO, USA) immunohistochemistry and resultant colour reaction was visualized with an Olympus microscope (Albertslund, Denmark).
4.4.4 Immunofluorescence

Adjacent sections to those used for immunohistochemistry, were used for the detection of cells within the SVZ with dual immunofluorescent techniques described in Section 2.5.1. Primary antibodies used included the following: rabbit anti-Ki67 (Ki67; 1:2000, Labvision Thermo Scientific, Fremont, CA, USA), mouse anti-GFAP (1:400, Millipore, Billerica, MA, USA), guinea-pig anti-doublecortin (DCX; 1:500, Millipore, Billerica, MA, USA), mouse anti-Nestin (1:400, Cell Signalling Technology, Danvers, MA, USA), and rabbit anti-GFAP (1:400, DAKO, Glostrup, Denmark). Secondary antibodies (1:500 for all) used included; Alexa-568 goat anti-rabbit, Alexa-488 goat anti-mouse, Alexa-488 goat anti-guinea pig. Resulting sections were examined with a laser scanning confocal microscope (Nikon Instruments Inc., Melville, NY, USA) using a x40 magnification (a higher magnification was required to achieve greater image resolution for cell counts).

4.4.5 Quantification of immunohistochemistry

Cells of interest were quantified according to methods described in Chapter 2.6. Four common regions of interest where damage was routinely observed were identified in each section for assessment: the damaged cortex, damaged striatum, and the border region surrounding the infarct for both the cortex and striatum. Each analysis area was then compared with the appropriate corresponding mirror region within the contralateral hemisphere (undamaged tissue), as well as to sham-operated controls, using the same sample area for all regions assessed.

4.4.5.1 Astrocytes

GFAP labelled reactive astrogliosis were quantified based on phenotypic distinctions (Lau et al. 2012, Lau et al. 2014, Sofroniew and Vinters 2010) 7 days post-stroke. An automated systematic random sampling point-counting system was applied with a CAST System (Olympus) (Lokmic and Mitchell 2011). Different gradations of reactive astrogliosis were identified based on cellular hypertrophy, length and thickness of processes, and categorised as either activated astrocytes or diffuse astrocytes. Activated or diffuse astrocytes were quantified within the cortical and striatal core damage and border regions and expressed as a percentage of the total number of counts within the defined sample area. The number of resting/quiescent astrocytes was also
quantified in the contralateral hemisphere for comparison across treatment groups and to sham-operated controls.

4.4.5.2 **Activated microglia/macrophages**

Quantification of OX42 positive cells using the point-counting system was assessed within the same regions of interest as assessed for astrocyte cell counts. OX42 positive cells that displayed activated amoeboid morphology with reduced processes were counted and expressed as a percentage of the total number of counts within the defined sample area within either hemisphere for comparison across treatment groups and to sham-operated controls. Resting microglia displaying small cell bodies with highly ramified processes were excluded from counts (rarely identified within the damaged zone).

4.4.5.3 **SVZ cell proliferation and neural differentiation**

Ki67 positive proliferative cells, proliferating radial glial cells (Ki67/GFAP positive), or migrating immature neurons (DCX positive) within the SVZ were quantified separately at 7 days post-stroke. Cells within the ipsilateral SVZ and contralateral SVZ were quantified using the cell counter plug-in for National Institute of Health ImageJ software (USA). Ki67 positive images were obtained using an Olympus microscope (Albertslund) under x20 magnification, while Ki67/GFAP positive and Ki67/DCX positive images were obtained using a laser scanning confocal microscope (Nikon) under x40 magnification. The small counting frame within the SVZ required quantification of cell numbers using standard stereological techniques and ImageJ. The total number of proliferating cells, proliferating radial glial cells, or immature neurons detected in the ipsilateral SVZ was then compared to the corresponding contralateral SVZ and expressed as 100% control. Qualitative images of Nestin/GFAP positive radial glial cells observed migrating from the SVZ toward border regions was obtained using a confocal microscope (Nikon) under x40 magnification.

4.4.5.4 **Blood vessels**

Blood vessel quantification was assessed 7 days post-stroke in vWF-labelled sections within the same brain regions of interest used for quantification of inflammatory cells using the CAST point-counting system. The number of both small
and large blood vessels (vWF positive) detected in the ipsilateral sample region was then compared to the contralateral region and expressed as 100% control.

4.4.6 Statistical analyses

Data were analysed as described in Chapter 2.9. Briefly, neurological outcome data was analysed by Kruskal-Wallis non-parametric ANOVA followed by Dunn’s post-test. Forelimb asymmetry data was analysed by a two-way repeated measures (RM) ANOVA followed by Bonferroni test. Infarct area was analysed using two-way ANOVA followed by Bonferroni test. Infarct volume and cell counts were analysed by one-way ANOVA followed by Bonferroni test. To test for correlation between infarct volume and cell types of interest the Pearson product-moment coefficient, r, for ordinal values was determined. A value of $P<0.05$ was considered significant.

4.5 Results

4.5.1 Functional outcome

A total of 19 rats were included in this study. Rats that were excluded from the study included two that did not show signs of having a stroke during ET-1 infusion and one that died as a result of a severe stroke. ET-1 induced stroke resulted in significantly higher neurological deficits in vehicle treated animals at 24 ($P<0.01$), 48 ($P<0.001$), 72 hrs ($P<0.001$) and 7 days ($P<0.05$, $n=8$, Kruskal-Wallis ANOVA followed by Dunn’s post-test; Figure 4.2 A) post-stroke when compared to pre-stroke scores. Rats receiving CsA also displayed deficits between 24hrs to 7 days post-stroke ($P<0.05$, $n=8$, ANOVA; Figure 4.2 A) when compared to pre-stroke ratings. However deficits in CsA treated rats were significantly less at 48 hrs when compared to vehicle treated controls ($P<0.05$, ANOVA; Figure 4.2 A).

Latency to touch and remove adhesive labels from the stroke affected contralateral forepaw was significantly increased when compared with the ipsilateral forelimb at 24 (vehicle: $P<0.0001$, CsA: $P<0.001$), 48 (vehicle: $P<0.0001$, CsA: $P<0.05$), and 72 hrs (vehicle: $P<0.01$, CsA: $P<0.05$, Two-way ANOVA) after stroke, but not after this time (Figure 4.2 B-E) No significant difference existed in latency to touch or remove adhesive labels between vehicle and CsA treated rats at any time.
Sham-operated animals showed no evidence of neurological deficits displayed after cannula-implant surgery.

### 4.5.2 Infarct outcome

Histological analysis 7 days post-stroke from both treatment groups revealed damage in the parietal, insular, and frontal cortex, and the striatum consistent with previous studies (Abeyesinghe et al. 2014, McCann et al. 2008, Roulston et al. 2008, Taylor et al. 2013) (Figure 4.2 I, J). Treatment with CsA had no significant effect on infarct area at any anatomical level relative to bregma (Figure 4.2 F, G) or overall volume of infarct (Figure 4.2 H) in the cortex and striatum when compared to vehicle treated control rats. Sham-operated animals showed no evidence of quantifiable ischemic damage in the region of the MCA and were therefore not included in infarct correlation analysis.

![Figure 4.2 Effects of CsA and vehicle treatments on functional outcome and infarct volume 7 days post-stroke.](image)

Neurological deficit scores from both treatment groups (A). Data presented as box plots include hinges extending from the 25th to 75th percentiles, the median line within the box and whiskers extending to the minimum and maximum values of the dataset
(\(n=8\)/group). *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\) relative to 0 hr scores, #\(P<0.05\) compared with vehicle treated (Kruskal-Wallis non-parametric ANOVA followed by Dunn’s post-test). Latency to touch (B, C) and remove adhesive (D, E) for both treatment groups. Data presented as mean ± SEM, *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), ****\(P<0.0001\) compared with 0hr score in the same forelimb, #\(P<0.05\), ##\(P<0.01\), ###\(P<0.001\), ####\(P<0.0001\) compared with ipsilateral forelimb at the same time measurement (two-way ANOVA with Bonferroni post-hoc test). CsA treatment had no significant effect on infarct area at any of the stereotaxic positions relative to bregma (F, G) or infarct volume (H) within the cortex and striatum post-stroke when compared to vehicle treated rats (two-way ANOVA). Data presented as mean ± SEM. Core infarct areas of the cortex and striatum were identified using MCID images generated from unstained sections in vehicle treated (I) and CsA treated (J) rats where white dotted lines mark damage within the cortex and striatum. CsA: Cyclosporine A; MCID: micro computer imaging device.

4.5.3 Histopathology outcome

4.5.3.1 Astrogliosis

Astrogliosis was assessed by quantifying astrocytes based on three morphological distinctions. First, astrocytes of normal quiescent appearance were identified by low GFAP expression with long slender processes that did not overlap and were normally found in both hemispheres of sham-operated controls (Figure 4.3 A, D) or within regions remote to the infarct site or the contralateral hemisphere of stroke affected rats. Second, severely diffuse astrocytes were identified based on pronounced up-regulation of GFAP expression, hypertrophy of the cell body and processes with distinct process overlap, and dense packing of cells as seen in vehicle treated controls (Figure 4.3 B, E). Third, activated astrocytes were defined by an up-regulation of GFAP expression with cellular hypertrophy without pronounced overlap of processes as observed in CsA treated animals (Figure 4.3 C, F). Activated and diffuse astrocytes were counted and compared to sham control rats. No astrocytes were present within core infarcted regions analysed and as such only cell counts within the border cortical and striatal regions were presented. The number of activated and diffuse astrocytes within the ipsilateral hemisphere of vehicle and CsA treated rats were significantly greater than sham-operated controls (\(P<0.05\) compared to sham-operated animals,
scatter plot, ANOVA, Figure 4.3 G-J). Rats that received CsA treatment showed a significant increase in the number of astrocytes with activated morphologies along with significantly reduced numbers of astrocytes with severely diffuse morphologies in the surrounding cortical (Activated; \(P<0.05\), Diffuse; \(P<0.01\), Figure 4.3 G, H respectively) and striatal (Activated & Diffuse; \(P<0.05\), Figure 4.3 I, J respectively) border regions in comparison to vehicle treated animals. The effects of CsA treatment on quiescent resting astrocytes was also examined. Quantification of astrocytes with a resting morphology within the contralateral hemisphere of sham-operated, vehicle and CsA treated animals revealed no significant difference in appearance or number of astrocytes between groups (ANOVA, Figure 4.3 K, M). No activated or diffuse astrocytes were observed within the contralateral hemisphere of either treatment group or sham-operated controls (ANOVA, Figure 4.3 L, N).

Differences between astrocyte morphological activation across treatment groups was further investigated in relation to stroke severity by comparing the number of activated and diffuse astrocytes across infarct volumes for each treatment group. No significant correlation was observed between activated astrocytes and lesion size in either treatment group within the border regions of the cortex and striatum (Figure 4.3 O, Q respectively). In contrast, greater lesion size was found to correlate significantly with a higher number of severely diffuse astrocytes within the border cortex (\(r=0.85, P<0.01, n=8\), Figure 4.3 P) and striatum (\(r=0.86, P<0.05\), Figure 4.3 R) of vehicle treated animals, an effect that was attenuated with CsA treatment despite similar stroke outcomes.
Figure 4.3 Effects of CsA treatment on activated versus diffuse astrocytes and correlation with infarct volume.

Bright-field immunohistochemical photomicrographs depicting GFAP staining of resting astrocytes within the ipsilateral hemisphere of sham-operated controls (A, D), diffuse astrocytes (B, E) and activated astrocytes (C, F) within the border cortex and striatum of the ipsilateral hemisphere from vehicle and CsA treated animals using consistent sample areas. Short arrows represent cells in resting states with long slender processes and low level of GFAP expression, open arrow heads indicate activated
astrocytes with cellular hypertrophy up-regulation of GFAP without process overlap, and filled arrow heads represent diffuse astrocytes with cellular hypertrophy and pronounced process overlap. Scatter plots demonstrate CsA significantly increased the number of activated and decreased the number of diffuse astrocytes within the border cortex and striatum (G-J, ANOVA). Data are presented as mean ± SEM, *P<0.05 compared to sham-operated animals, #P<0.05, ##P<0.005 compared to vehicle treated animals. No difference in the number of resting, activated/diffuse astrocytes in the contralateral hemisphere across all groups analysed (L-N) (ANOVA). No correlation between infarct volume and the number of activated astrocytes in either treatment groups within the cortex border (O). A significant correlation was found between infarct volume and the number of severely diffuse astrocytes within the border cortex for both treatment groups (vehicle: r=0.85, P<0.01, n=8, CsA: r=0.81, P<0.05, n=8; P). No correlation was observed between infarct volume and the number of activated astrocytes in either treatment groups within the striatum border (Q). A positive correlation was found between volume of infarct and diffuse astrocyte numbers within the border striatum of vehicle treated animals only (vehicle: r=0.86, P<0.05, n=5; R) (Pearson product moment correlation coefficients). Scale bars A-F = 100µm. CsA: Cyclosporine A; GFAP: glial fibrillary acidic protein.

4.5.3.2 Microglia/macrophage activation

Microglial/macrophages labelled with OX42 were observed in resting states within the contralateral hemisphere of stroke affected rats and in both hemispheres of sham-operated controls. Resting microglia displayed ramified morphology with small cell bodies and long fine branched processes within the ipsilateral cortex and striatum of sham-operated controls (Figure 4.4 A, D). Activated microglia/macrophages were observed in the ipsilateral hemisphere of stroke affected rats and appeared amoeboid with large cell bodies and retracted processes similar in appearance to blood derived macrophages. Both activated microglia and blood borne macrophages were quantified together and were mainly observed within the core damaged regions of the cortex (Figure 4.4 B, C) and striatum (Figure 4.4 E, F) as well as within surrounding border zones in both vehicle and CsA treated rats. Following stroke there was a significant increase in the number of activated microglia/macrophages detected across the infarct at 7 days in both treatment groups compared to the ipsilateral hemisphere of sham-operated controls (P<0.05). There was no difference in the number of activated
microglia between CsA treated rats and vehicle treated controls (Scatter plots; Figure 4.4 G-J). Correlation analysis between lesion volume and microglia activation revealed larger infarct volumes correlated with increased numbers of activated microglia/macrophages in both treatment groups in the core cortical (vehicle: $r=0.96$, $P<0.0005$, $n=8$, CsA: $r=0.97$, $P<0.0001$, $n=8$; Figure 4.44 K), cortical border (vehicle: $r=0.97$, $P<0.0001$, CsA: $r=0.78$, $P<0.05$; Figure 4.4 L), striatal core of the vehicle treated animals only (vehicle: $r=0.90$, $P<0.05$, $n=5$; Figure 4.4 M), and striatal border (vehicle: $r=0.91$, $P<0.05$, $n=5$, CsA: $r=0.92$, $P<0.05$, $n=5$; Figure 4.4 N).

Figure 4.4 Effect of CsA on activated microglia and macrophages 7 days post-stroke and correlation with infarct volume.

Immunohistochemical photomicrographs of OX42 labelled microglia/macrophages in consistent sample areas within the cortex (A-C) and striatum (D-F) representing resting
states within the ipsilateral hemisphere of sham-operated controls and activated states within the core damaged regions of the ipsilateral hemisphere from both vehicle and CsA treated animals 7 days post-stroke. Short arrows represent cells in resting states with highly ramified processes, while arrow heads indicate activated microglia and blood borne macrophages with amoeboid morphology, large cell bodies and retracted processes. Scatter plots demonstrate no significant differences in the number of microglia/macrophages between treatment groups in all regions analysed (G-J) (ANOVA). Data are presented as mean ± SEM, *P<0.05 compared to sham-operated animals. A positive correlation was observed between infarct volumes and activation of microglia/macrophages in the core cortex (vehicle: r=0.96, P<0.0005, n=8, CsA: r=0.97, P<0.0001, n=8; K) and border cortex (vehicle: r=0.97, P<0.0001, CsA: r=0.78, P<0.05; L) of both treatment groups, core striatum (vehicle: r=0.90, P<0.05, n=5, CsA: r=0.74, n=5; M) of the vehicle treated rats only, and striatal border of both treatment groups (vehicle: r=0.91, P<0.05, n=5, CsA: r=0.92, P<0.05, n=5; N) (Pearson product moment correlation coefficients). Scale bars A-F = 100µm. CsA: Cyclosporine A; OX42: CD11b antigen on microglia/macrophages.

**4.5.3.3 SVZ cell proliferation, migration and neural differentiation**

In order to determine the effects of CsA treatment on the neurogenic niche we examined cell proliferation, migration and differentiation within the SVZ (Figure 4.5 A-D). Stroke resulted in an increase in the number of cells positive for Ki67, Ki67/GFAP, and DCX in the ipsilateral SVZ of both CsA treated and vehicle treated rats when compared to the SVZ of the non-ischemic hemisphere, and sham-operated controls (Ki67+ cells and Ki67/GFAP+ cells: P<0.05 for both treatment groups; DCX+ cells: P<0.05, for vehicle treated only, Scatter plots, ANOVA, Figure 4.5 I-K). Treatment with CsA had no effect on the overall number of Ki67+ proliferating cells, Ki67/GFAP+ proliferating radial glial cells, or the generation of migratory DCX+ neuronal cells within the SVZ when compared to vehicle treated rats (Figure 4.5 I-K). GFAP+ radial glial cells with stellated phenotypes were quantified within the SVZ. Comparisons between lesion size and cell numbers within the SVZ revealed larger infarct volumes significantly correlated with an increased number of Ki67+ proliferating cells within the ipsilateral SVZ 7 days from stroke onset for both treatment groups (vehicle: r=0.76, P<0.05, n=8, CsA: r=0.78, P<0.05, n=8; Figure 4.5 L). The same was observed for Ki67/GFAP+ proliferating radial glial cells (vehicle: r=0.77,
Similarly, a positive correlation was found between infarct volume and the number of DCX+ immature neuronal cells generated from the SVZ (vehicle: $r=0.77$, $P<0.05$, $n=8$, CsA: $r=0.91$, $P<0.005$, $n=8$; Figure 4.5 N).

Further staining with nestin revealed GFAP+ radial glial cells extending from the SVZ towards injured regions of the brain with no visible differences between treatment groups (Figure 4.5 E, F). A larger proportion of new Nestin/GFAP+ astrocytes was observed within the border regions of vehicle treated rats compared to CsA treated rats (Figure 4.5 G, H).

Figure 4.5 Effect of CsA on SVZ cell proliferation/migration, and neural differentiation and infarct correlation 7 days post-stroke.

Merged immunofluorescent images of Ki67+ proliferating cells (red) with either GFAP+ stellated astrocytes (green; A, B); or immature migrating neuroblasts (DCX; green; C, D) within the SVZ of the lateral ventricle (LV). Co-localisation with Ki67 can be observed within the SVZ of both treatment groups highlighting an apparent increase in the number of proliferating radial glial cells (Ki67/GFAP+) and little co-localisation with immature neuroblasts (Ki67/DCX+). Merged immunofluorescent images of neural
progenitor cell marker Nestin (green) and astrocytic marker GFAP (red) double labelling immature radial glial cells (yellow) within the SVZ can be seen migrating towards the infarct with little difference between vehicle (E) and CsA (F) treated groups. Greater co-localisation of Nestin/GFAP+ (yellow) immature astrocytes was apparent within cortical border regions of vehicle treated animals (G) than compared to CsA treated animals (H). Scatter plots confirm no significant differences in the number of Ki67+, Ki67/GFAP+, and DCX+ cells within the SVZ between vehicle and CsA treated groups (I-K respectively, ANOVA). Data are presented as mean ± SEM. *P<0.05 compared to sham-operated animals. Positive correlations were observed between infarct volume and the number of Ki67+ (vehicle: r=0.76, P<0.05, n=8, CsA: r=0.78, P<0.05, n=8; L), Ki67/GFAP+ (vehicle: r=0.75, P<0.05, CsA: r=0.94, P<0.001; M), and DCX+ cells (vehicle: r=0.76, P<0.05, CsA: r=0.90, P<0.005; N) within the lateral ventricle of the SVZ in comparison to the respective contralateral SVZ (Pearson product moment correlation coefficients). Scale bars A-H = 50µm. CsA: Cyclosporine A; GFAP: glial fibrillary acidic protein; DCX: doublecortin; SVZ: subventricular zone.
4.5.3.4 Angiogenesis

vWF immuno-stained blood vessels revealed non-angiogenic regions with organised blood vessel staining within the contralateral hemisphere of stroke affected rats and in both hemispheres of sham-operated controls (Figure 4.6 A, D). vWF-stained blood vessels also revealed areas of blood vessel hyperdensity consistent with angiogenesis within and around the core cortical (Figure 4.6 B, C) and striatal infarcts (Figure 4.6 E, F) in stroke affected rats. Both large and thin walled microvessels were point counted within a defined sample area (800µm²) from the same anatomical region for all rats and compared to the contralateral mirror image within treatment groups, and to sham-operated controls.

Stroke resulted in a significant increase in the number of blood vessels counted in all regions analysed when compared to the corresponding non-ischemic hemisphere and when compared to sham-operated controls \((P<0.05)\) (Scatter plots; Figure 4.6 G-J). The number of vessels detected in the stroke affected brain was not significantly different between treatments. Blood vessel counts were correlated with quantified infarct volumes for each rat and revealed a positive correlation existed between the infarct volume and the number of blood vessels in both treatment groups within the core cortex (vehicle: \(r=0.94, P<0.001, n=8\), CsA: \(r=0.96, P<0.0005, n=8\); Figure 4.6 K), border cortex (vehicle: \(r=0.79, P<0.05\), CsA: \(r=0.78, P<0.05\); Figure 4.6 L), core striatum (vehicle: \(r=0.93, P<0.01, n=5\), CsA: \(r=0.97, P<0.01, n=5\); Figure 4.6 M) and border striatum regions (vehicle: \(r=0.88, P<0.05\), CsA: \(r=0.96, P<0.01\); Figure 4.6 N).
Figure 4.6 Effect of CsA on angiogenesis and correlation with infarct volume 7 days following ET-1 induced stroke.

Immunohistochemical photomicrographs of vWF staining in consistent sample areas from the ipsilateral hemisphere of sham-operated controls demonstrating normal vascularisation (A, D) and the ipsilateral core damaged regions of the cortex (B, C) and striatum (E, F) demonstrating blood vessel hyperdensity consequent to angiogenesis from both treatment groups at 7 days post-stroke. No difference was observed between vehicle and CsA treated animals in the number of blood vessels detected in all regions used for analysis (G-J, ANOVA). Data are presented as mean ± SEM, *P<0.05 compared to sham-operated animals. A positive correlation was observed between infarct volume and core cortical (vehicle: r=0.94, P<0.001, n=8, CsA: r=0.95, P<0.0005, n=8; K), bordering cortical (vehicle: r=0.79, P<0.05, CsA: r=0.78, P<0.05; L), core
striatal (vehicle: r=0.93, P<0.01, CsA: r=0.97, P<0.01; M) and bordering striatal areas (vehicle: r=0.88, P<0.05, CsA: r=0.96, P<0.01; N) for both treatment groups (Pearson product moment correlation coefficients). Scale bars A-F = 200µm. vWF: von willebrand factor; CsA: Cyclosporine A.

4.6 Discussion

This is the first report to show that pre-treatment with CsA reduced over activation of astrocytes in the stroke affected brain and retained their pro-survival morphology following acute cerebral ischemia. We also show that CsA treatment reduced initial deficits that occur in the days after stroke. In contrast to previous reports, CsA treatment did not affect infarct size or overall functional outcome. Additionally we observed an apparent reduction in GFAP staining in new radial glial cells in the surrounding border regions in rats treated with CsA. These findings suggest a targeted effect of CsA on astrocytes since infarct volume, activation of microglia/macrophages, and other critical endogenous repair mechanisms remain unaffected by treatment. Reduced astrogliosis with retained astroglia support may therefore account for reduced neurological deficit detected early after stroke with CsA treatment.

4.6.1 Functional outcome and infarct volume

Treatment with CsA prior to ET-1 induced stroke did not significantly affect overall functional outcomes 7 days post stroke, nor did it attenuate infarct volume. Despite this, CsA treatment did significantly improve initial neurological deficits detected post-stroke indicating an early protective effect of CsA on the development of deficits. Spontaneous recovery observed within the vehicle control group by 7 days may also account for a lack of effect between treatment groups at this time. None-the-less, CsA treatment did not improve hemineglect deficits with the adhesive label test at any time after stroke, which highlights the need to incorporate multiple behavioural measures since it is difficult to relate such diffuse damage that results from MCA stroke to a single behavioural measure (Windle and Corbett 2005).

Few studies have evaluated pre-treatment of CsA in models of focal ischemia with assessments made 24 hrs – 3 days post-stroke (Cho et al. 2012, Kuroda et al. 1999, Leger et al. 2011, Shiga et al. 1992) and only one study assessed the influence of CsA
on functional outcome up to 24 hours with no benefits reported (Cho et al. 2012). Behavioural analysis as early as 24 hrs post-stroke may lack sensitivity to detect neurological deficits since maximal infarct volume is not reached until at least 3 days following stroke (Lansberg et al. 2001). Our study expands on previous data as it is the first to evaluate CsA pre-treatment on both neurological outcome and infarct size beyond 3 days when the infarct has completely matured in the ET-1 model (Taylor et al. 2013). It is important to understand that improvements in neurological function after stroke can occur without change to overall stroke volume, as shown with other treatments. Previous studies using Rho-associated kinase (ROCK) inhibitors, Y-27632 and Fasudil, report functional recovery 40 days post-stroke in the absence of neuroprotection (Lemmens et al. 2013). Studies now suggest improved functional outcomes can be achieved without reduction in infarct volume through neural rescue, enhanced plasticity and brain remodelling (Lemmens et al. 2013).

Despite the above, studies assessing the effects of CsA on stroke volume in other models of stroke have in contrast reported positive effects when compared to vehicle control animals (Matsumoto et al. 1999). These discrepancies may relate to the dose of CsA used. Two high doses of CsA at 20mg/kg each delivered immediately after reperfusion and 24 hrs post-reperfusion successfully reduced infarct size (Matsumoto et al. 2002). However, in the same study a low dose of 10mg/kg resulted in no change to total infarct volumes and higher doses of CsA ranging from 30-50mg/kg were associated with high mortality rates, suggesting CsA-induced toxicity. Additionally Cho et al, (2012) recently reported reduced infarct volume 24 hours after stroke following pre-stroke treatment with CsA (10mg/kg), but they did not investigate effects of CsA beyond this recovery time. Furthermore, Yu et al. (2004) reported CsA administered at an even lower dose of 1mg/kg and a second dose of 10mg/kg demonstrated both non-neuroprotective and neuroprotective effects respectively 3 days post-stroke in a rat model of MCA occlusion. Previous reports mainly utilised the intraluminal filament model of MCA occlusion and we now report that a low dose of CsA (10mg/kg/day) commencing prior to stroke induction does not reduce overall damage in the endothelin-1 model of stroke. Importantly, CsA does appear to prevent the development of initial neurological deficits early after stroke compared to vehicle controls. Our results highlight the importance of testing neuroprotective or restorative drug treatments in a variety of experimental stroke models ranging from small to large animals and the
incorporation of dose-response curves for appropriate evaluation of preclinical data as outlined from previous STAIR (Stroke Therapy Academic Industry Roundtable) meeting proposals (STAIR 1999).

4.6.2 Effects of CsA on astrocytes

Treatment with CsA significantly reduced the number of diffuse astrocytes known to contribute to extensive glial scar formation surrounding cortical and striatal infarcts (Abeysinghe et al. 2014, Sofroniew and Vinters 2010). Astrocytes are known to exist in a variety of phenotypes that have pro-survival (cytотrophic) and destructive (cytotoxic) components that arbitrate brain integrity, neuronal death, and subsequent rescue and repair after brain injury (Lau et al. 2014, McMillian et al. 1994, Panickar and Norenberg 2005, Sofroniew and Vinters 2010). The degree of astrocyte activation is a gradated continuum of morphological change that influences brain preservation to long-lasting glial scar formation and is considered manageable by pharmacological intervention (Mueller et al. 2009, Ridet et al. 1997, Sofroniew and Vinters 2010). Strategies aimed at targeting astrocyte activation must be capable of reducing their involvement with glial scarring; however it is equally imperative to retain their pro-survival morphology. Pro-survival astrocytic phenotypes have previously been associated with up-regulation of brain-derived neurotrophic factor (BDNF), glutamate transporters, anti-oxidant enzymes (Lau et al. 2012) and improved glycogen metabolism (Hossain et al. 2014). The findings from this study demonstrate that treatment with CsA attenuates the development of neurological deficits possibly by reducing over-activation of astrocytes, with retained pro-survival morphological characteristics (as described by Lau et al., 2014 and Sofroniew et al. 2010). Pro-survival astrocytes have previously been shown to be important for neuronal rescue and restoration of neurotransmission, brain plasticity, synaptogenesis and maintenance of the blood brain barrier (Gee and Keller 2005, Sofroniew and Vinters 2010).

We previously report that the majority of cells within the neurogenic niche after ET-1 stroke differentiate into astrocytes and extend towards the glial scar, where they are found as diffuse astrocytes incorporated into the glial scar by 14 days (Abeysinghe et al. 2014). We now report that CsA treatment appears to reduce the transition of radial glial cells into diffuse astrocytes, since many nestin positive astrocytes bordering the infarct retained pro-survival profiles without evidence of overlapping processes typical
of cells within the glial scar. We therefore suggest that CsA treatment may improve the impact of newly generated astrocytes from the SVZ by reducing their contribution to scar formation whilst generating astrocytes for trophic support. Recent reports describe key positive functions of astrogliosis for neuronal support (Maragakis and Rothstein 2006, Sofroniew and Vinters 2010), thus highlighting the importance of maintaining beneficial effects of reactive astrogliosis with pharmacological strategies.

Initial motor deficits detected after stroke are often due to depression or ‘silencing’ of surviving pathways rather than lost neurons (Font et al. 2010, Netz et al. 1997). Spontaneous recovery after stroke is thought to be due to unmasking or activation of these silent pathways with restoration of nerve transmission between surviving cells, which could account for spontaneous recovery observed in vehicle-treated controls. In this study we have shown the treatment with CsA retains pro-survival astrocyte phenotypes which is very important for neuronal support, in particular glutamate re-uptake (Anderson and Swanson 2000, O'Shea et al. 2006, Sofroniew and Vinters 2010). Maintenance of synaptic function by astrocytes is becoming increasingly recognized with astrocytic dysfunction linked to increased synaptic glutamate accumulation and excitotoxicity-mediated neuronal cell death (Maragakis and Rothstein 2004). CsA treatment may prevent silencing of pathways within the border regions by permitting glutamate turnover and restoring neurotransmission through retained trophic astrocytic support and thus account for reduced deficits observed at 48 hrs post-stroke. Brunkhorst et al. (2013) recently reported functional recovery from stroke following treatment with neuroprotective compound FTY720, a sphingosine-1-phosphate antagonist, with improvements observed attributed to reduced astrogliosis and glial scarring, modulation of synaptic morphology, and increased expression of neurotrophic factors including vascular endothelial growth factor (VEGF) within the border regions of damage. In addition, Taylor et al. (2013) previously report that recovery of function after stroke plateaus as scar formation increases within the border regions of the infarct, indicating an association between glial scarring and functional outcome.

Although this is not the first report to show that CsA treatment reduces reactive gliosis after stroke, we now provide quantitative analysis of this effect and distinguish astrocytes based on morphological transition beyond the initial reports conducted 48
hours post-stroke (Leger et al. 2011). Extending this time window is important since astrocytes transition occurs over days to weeks in response to stroke and dynamically change from supportive to major contributors to glial scar formation (Abeysinghe et al. 2014, Taylor et al. 2013). By extending recovery to 7 days we have shown that we can quantify astrocyte transition.

4.6.3 Correlation of treatment with infarct volume

Previous studies report that stroke severity can also affect the degree of pathological response (Abeysinghe et al. 2014, Nagai et al. 2010). Given this correlation, it was essential to determine if the reduction in astrogliosis after CsA administration was attributed to direct effects of treatment rather than variations in the size of the lesion between treatment groups. An attenuated correlation was observed between diffuse astrocyte numbers and infarct size in CsA treated animals within the cortical border but overall number of diffuse astrocytes was significantly less than that of vehicle treated animals. No correlation was observed between infarct volume and diffuse astrocytic numbers within the striatal border of CsA treated animals. Therefore the increase in the number of activated astrocytes and decrease in the number of diffuse astrocytes in CsA treated groups was directly related to treatment rather than variation in infarct size across groups.

4.6.4 Impact of CsA on microglia/macrophage activation

The brain is thought to be a site that is “immunoprivileged” (Mrass and Weninger 2006) and reports on effects of CsA on brain inflammation are limited. Microglia are the immune cells of the central nervous system and have been studied in great detail regarding their over activation following injury to the brain (Nedergaard and Dirnagl 2005, Nguyen et al. 2002, Wyss-Coray and Mucke 2002). Surprisingly we found that CsA had no effect on activated microglia/macrophages 7 days post-stroke suggesting CsA mediated effects appear to be specific to astrocyte transition. This is in direct contrast to recent work where pre-treatment with CsA resulted in reduced microglia/macrophage numbers 24 hours post-stroke (Cho et al. 2012). This again highlights the need to incorporate longer recovery periods to enable full maturation of the infarct to occur as well as testing of pharmacological treatments in a variety of stroke models.
Given the initial improvements in functional deficits observed with CsA treatment where changes in microglia responses are not observed, the question arises as to how important is inflammation to stroke outcome (Iadecola and Anrather 2012, Jin et al. 2013, Wang et al. 2007)? The inflammatory contribution from microglia and macrophages following stroke is thought to create a toxic environment that contributes to the spread of damage in the days after a stroke event (McCann et al. 2008). Indeed in the present study where CsA has no effect on microglia/macrophage activation we observed no differences in overall infarct in comparison to vehicle control. Any toxic effects on functionally depressed neurons in the border however, may in this case be counteracted due to the positive trophic effects of activated astrocytes, thus resulting in brain rescue. Similar to previous reports, reduced astrogliosis and functional recovery following treatment with neuroprotective compound FTY720 was observed in the absence of attenuation of microglia/macrophages activation (Brunkhorst et al. 2013). These results suggest that whilst CsA does not affect microglia/macrophage activation, reduction in over-activation of astrocytes and astrogliosis alone may be physiologically beneficial.

4.6.5 CsA effects on SVZ cell proliferation/migration and neural differentiation

When assessing treatments that alter the post-stroke environment it is important to understand the effects on mechanisms associated with neurogenesis. In the present study we demonstrate that treatment with CsA does not alter cell proliferation/migration or neural differentiation within the SVZ. Normally, the adult SVZ consists of a subpopulation of cells including radial glial cells (type B cells), rapidly dividing transient amplifying cells (type C cells) and migratory neuroblasts (type A cells) (Doetsch et al. 1999, Gregg and Weiss 2003). In the present study, the total number of Ki67+ proliferating cells, Ki67/GFAP+ proliferating radial glial cells and DCX+ immature migratory neuroblasts generated within the SVZ increased significantly 7 days post-stroke in both treatment groups. CsA administration did not affect overall cell proliferation within the SVZ, identified by Ki67 staining, in comparison to vehicle treated controls. Furthermore, DCX staining, a marker for immature migrating neuroblasts, revealed no significant impairment in neural differentiation within the SVZ following CsA administration relative to vehicle treated control animals.
Stroke increases proliferation and migration of neural progenitor cells within the SVZ towards injured brain regions (Zhang et al. 2001, Zhang et al. 2005), where they have the ability to differentiate into either neurons or astrocytes based on the local microenvironment (Gregg and Weiss 2003, Merkle et al. 2004, Zhang et al. 2007). Treatments that aim to improve brain regeneration must therefore avoid negatively influencing this process (Abeysinghe et al. 2014). In the current study we observed no effect on the number of Ki67/GFAP+ proliferating radial glial cells within the SVZ with CsA treatment. However, in comparison to vehicle treated controls, CsA treatment did appear to reduce the amount of Nestin/GFAP+ staining in the ischemic border zone. This may be due to reduced activation of new astrocytes in this region. Therefore attenuation of astrogliosis with CsA may be due to a collective contribution from reduced differentiation of new progenitor cells into astrocytes, as well as reduced over activation of pre-existing astrocytes. How CsA exerts this effect over astrocyte transition remains to be determined. However the potential of CsA treatment to reduce astrogliosis while releasing trophic factors that support survival and neural differentiation of newly generated progenitor cells is quite promising and represents a desirable treatment outcome.

4.6.6 CsA effect on angiogenesis

Although mature functional blood vessels are not evident until 14 days post-stroke, activated, injured and newly forming blood vessels at 7 days are known to release an array of trophic factors including VEGF and brain-derived neurotrophic factor. In addition to being angiogenic, these trophic factors can evoke neurogenesis, are chemotactic for progenitor cells and may enhance the survival and integration of newly generated neuroblasts in the injured brain tissue (Leventhal et al. 1999, Minger et al. 2007, Shen et al. 2004). Interestingly VEGF has also been demonstrated to enhance synaptic plasticity (Licht et al. 2011) and can be beneficial in experimental stroke (Hermann and Zechariah 2009). Brunkhorst et al. (2013) demonstrated up-regulated expression of VEGF within border regions of the infarct following FTY720 treatment associated with reduced astrogliosis, however angiogenesis was not evaluated in their study. In the present study CsA had no affect on the angiogenic response 7 days post-stroke. Combined trophic signalling from angiogenic vessels with reduced astrogliosis may work in concert to support neuronal rescue for functional return.
4.7 Conclusion

Pharmacological manipulation of reactive astrogliosis and maintenance of pro-survival astrocytic characteristics has great potential for rescue of neurological deficits that occur due to functional depression in pathways that lay outside of the damaged brain. It has been suggested that reactive astrogliosis and scar formation seen in animal models is reflective of that which occurs in the human brain following stroke (Huang et al. 2014). This further highlights the importance of gaining a better understanding of glial cells and the influence on brain recovery to facilitate the development of therapeutic strategies for future clinical use. Here we provide evidence for the use of CsA for reducing early functional deficits after stroke, even in the absence of neuroprotection. Restorative strategies aimed towards creating a more supportive microenvironment devoid of scar tissue with retained astrocytic support has great potential for early rescue of depressed pathways. Treatments aimed towards overcoming astrogliosis and scar formation could also be used to complement other brain restoration treatments such as cell-based therapies to facilitate their integration and maturation. Given the similarities in glial scar formation between animal models and the human brain after injury, further investigation into novel methods for attenuating astrogliosis may be of great benefit for restoring function in stroke sufferers.
Chapter 5: Pre-differentiation of human neural progenitor cells into GABAergic neurons prior to transplant results in greater repopulation of the damaged brain and accelerates functional recovery after transient ischemic stroke

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5.1 Introduction

Regenerative events initiated following brain injury are active for weeks following stroke (Dancause et al., 2005; Zhang et al., 2008) that potentially provide a second window for treatment. Promising treatment strategies that target brain regeneration are currently under investigation. Consequently there is now strong evidence indicating extensive practice, exercise and physical therapy are required to substantially improve motor recovery after stroke through brain plasticity (Nudo and Friel, 1998; Nelles, 2004; Pascual-Leone et al., 2005). However many stroke survivors are often not able to participate in meaningful physiotherapy until many weeks after a stroke event and delayed rehabilitation results in worse outcomes (Gauthier et al., 2008).

For reasons above exogenous cell-based therapies to complement endogenous repair mechanisms are currently being trialled in humans following extensive meta-analysis of over 40 studies reporting significant improvements in function when transplanted after ischemia in animal models (Kondziolka et al., 2000; Sahota and Savitz, 2011; Lees et al., 2012). Despite early reports of functional benefits in humans (Wechsler, 2004; Bliss et al., 2007), a cellular basis for neurological improvement still remains elusive. Whilst neural cell replacement may be achieved, new research shows that neural stem cells can exert trophic function through secretion of protein factors that induce change in the host tissue to promote functional improvements. In addition to identifying how these cells work, it is equally important to isolate factors that influence stem cell survival and long-term integration within host tissue.

To date, all pre-clinical and clinical stem cell transplant studies for the treatment of stroke have been conducted using undifferentiated stem cells (Chen et al., 2001b; Takahashi et al., 2008; Sahota and Savitz, 2011). Survival rate with these cells ranges between 0.5-30% (Saporta et al., 1999; Chen et al., 2001a; Hicks et al., 2007; Takahashi et al., 2008; Hicks et al., 2009; Leong et al., 2012; Smith et al., 2012), and although capable of forming neuronal populations after transplant in animal models, most predominantly differentiate into astrocytes (Chen et al., 2001b; Hicks et al., 2007; Takahashi et al., 2008). Functional recovery in these studies has therefore been
suggested to be due to trophic support (Chiu and Rao, 2011; Smith et al., 2012) but characterisation of this effect has not been well documented.

Recent reports using other models of neurological disease suggest that pre-differentiating stem cells into a neuronal phenotype prior to transplant maybe a better approach (Mukhida et al., 2007; Mukhida et al., 2008; Alvarez Dolado and Broccoli, 2011; McLeod et al., 2012). Indeed differentiated human neural progenitor cell-derived GABAergic neurons injected into the spinal cord following spinal cord injury results in long term survival of GABAergic cells that generate glutamic acid decarboxylase, GABA, and β-III tubulin resulting in functional improvement (Mukhida et al., 2007). We therefore see value in using a similar approach for promoting recovery after stroke. To this end, we have isolated and characterized human neural progenitor cells (hNPCs) from the subventricular zone (SVZ) and directed their differentiation into GABAergic neuronal cells. In the present study we investigated the effect of transplanting undifferentiated SVZ-hNPCs versus pre-differentiated SVZ-hNPCs into the rat brain 7 days after stroke. As such this study aims to determine optimal phenotypic conditions that translate into best outcomes in terms of cell survival, histopathology and functional recovery.

5.2 Hypothesis and Aims

Hypothesis: Pre-differentiating stem cells prior to transplant is a favourable alternative for promoting graft survival, neural rescue and structural reorganisation of the damaged brain to facilitate functional recovery after stroke.

Specific Aims: To investigate:

1. The effect of transplanting undifferentiated SVZ-hNPCs versus pre-differentiated SVZ-hNPCs into stroke affected regions of the rat brain on cell survival and influence on functional recovery 28 days post-transplant.
2. The phenotypic profile of transplanted human NPCs versus pre-differentiated hNPCs 28 days post-transplant.
3. The effects of stem cell transplants on endogenous recovery processes.
5.3 Experimental Design

Experimental findings in Chapter 3 suggested that an optimal time to transplant stem cells was 7 days after stroke. Significant factors that lead to this decision included vascular recovery with increased activity within the neurogenic niche at this time, and importantly the absence of an established glial scar. In order to investigate the effects of stem cell transplants on stroke outcomes, three treatment groups were established 7 days after stroke: Group 1 received media infusion without cells (control); Group 2 received undifferentiated SVZ-hNPCs transplants; and Group 3 received pre-differentiated SVZ-hNPCs transplants. All treatment groups were recovered to 28 day post-treatment (day 35 post-stroke) (Figure 5.1). Cell survival, phenotypic differentiation and maturation profiles of hNPCs, endogenous SVZ proliferation, migration and neural differentiation, as well as neuronal replacement was investigated using immunofluorescence and quantified using ImageJ and Stereo Investigator software. Functional assessments were also carried out to determine the effects of stem cell transplant on recovery of functions. All rats were assigned to a treatment group before initial surgery, and stroke was induced using the conscious ET-1 model ensuring stroke severities were evenly represented across groups. All experiments performed were conducted in adherence to current RIGOR guidelines (Lapchak PA 2013, Landis SC et al. 2012) and included randomisation of treatments, blinding during assessment, inclusion of appropriate control groups, and full statistical analysis involving power calculations in consultation with the Statistical Consulting Centre, University of Melbourne, Victoria, Australia.

Figure 5.1 Treatment protocol
Animals were assigned to treatment groups after stroke induction ensuring stroke severity was evenly represented across groups. All animals received transplants 7 days after stroke (undifferentiated SVZ-hNPCs: \( n=7 \); vehicle: \( n=7 \); and pre-differentiated
SVZ-hNPCs: n=7). All behaviour tests were performed as single trials with pre-surgery, pre-stroke (day 0), and pre-transplant scores (7 days after stroke) obtained prior to each procedure. Stereotaxic guide cannula implant was conducted 5 days prior to stroke induction with ET-1. The forelimb asymmetry test (cylinder test), neurological deficit scores and adhesive tape test were used to assess functional outcome at time points highlighted in blue. All animals received daily injections of cyclosporine A (i.p. 10mg/kg) throughout the duration of the experiment. Animals were euthanised and brains processed for histological analysis at the conclusion of the study.

5.4 Methods

The ET-1 MCA constriction model was used to induce stroke in all rats with behaviour assessments (neurological deficit score and cylinder test) carried out as described in Chapter 2.2. Infarct volume was determined using NeuN staining according to methods described in Section 2.4.2. Animal details and specific methods utilised in this study are presented below.

5.4.1 Animals

A total of 30 adult male Hooded Wistar rats weighing 300-360g purchased from Laboratory Animal Services, University of Adelaide (SA, Australia) were used for experiments. Rats were dually-housed, until ET-1 induced stroke, whereupon they were individually housed on a 12 hr light/dark cycle with ad libitum access to food and water.

5.4.2 In Vitro Stem Cell Preparation

5.4.2.1 Cell expansion, medium preparation and handling

Experiments were conducted using hNPCs derived from the subventricular zone (SVZ) of human fetal brain tissues (14-21 weeks) that were obtained following donor consent under the strict guidelines approved by the Singapore National Healthcare Group Domain Specific Review Board. The SVZ was dissected, processed and cultured as free floating neurospheres using protocols previously described (Kobayashi et al. 2010) and stored as cryopreserved aliquots until use.

Prior to transplant procedures, cryopreserved SVZ-hNPC spheres were quickly thawed in a 37°C water bath followed by the addition of pre-warmed complete media
(Neurocult NS-A human neural stem cell proliferation media) and supplement (Stem Cell Technologies Inc., VIC, Australia), human Epidermal Growth Factor (EGF, 20ng/ml; Peprotech, Rocky Hill, NJ, USA), human basic Fibroblast Growth Factor (bFGF, 20ng/ml; Peprotech), heparin (2µg/ml; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Gibco BRL, Life Technologies, Grand Island, NY, USA). Cells were seeded onto 6 well plates and passaged 48 hrs later, as follows. Media containing neurospheres were collected into fresh falcon tubes and briefly left to stand at room temperature (RT) to allow cells to settle prior to centrifugation at 190xg for 5 minutes. The supernatant was collected into fresh 50mL falcon tubes to be used as conditioning media at time of plating. The pellet was then resuspended in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) and re-pelleted by centrifugation at 190xg for 4 minutes. Pre-warmed TrypLE (0.6ml; Gibco BRL) was then added to each pellet, the spheres gently resuspended and incubated for 4 mins at 37ºC in a water bath. After incubation, further DMEM/F12 was added and the cells gently triturred. The dissociated neurospheres were then spun at 190xg for 5 minutes and the supernatant discarded. Fresh pre-warmed DMEM/F12 was added and pellet resuspended. SVZ-hNPC density and viability (90%) were determined with a haemocytometer using the standard 0.1% trypan blue (Sigma-Aldrich) exclusion test. Approximately 0.7 x 10⁶ cells/well were plated into low adherence 6-well plates (Corning Costar, Sigma-Aldrich). Conditioned and complete media were added to the cell suspension during plating at a ratio of 1:2, to a final volume of 3ml/well. For expansion, cultures were maintained in complete media at 37ºC in a humidified incubator (5% CO₂ atmosphere) for a minimum of 14 days to fully recover and reach appropriate numbers required for differentiation and transplantation procedures. A 50% media change was performed every second day and plates were gently shaken daily to prevent spheres from fusing together. Cell density and viability (≥90%) was determined using the standard 0.1% trypan blue exclusion test.

5.4.2.2 In vitro differentiation of hNPCs

For differentiation of SVZ-hNPCs, first hNPC neurospheres were dissociated into single cell suspensions with TrypLE, and then cultured in differentiation media consisting of DMEM/F12 (Invitrogen, Life Technologies, Grand Island, NY, USA) and neurobasal media (1:1) supplemented with 1% StemPro neural (Invitrogen), 0.5% N2 (Gibco) and 50 ng/ml brain-derived neurotrophic factor (BDNF; Peprotech) to induce
differentiation as described previously (Kobayashi et al., 2010). All cultures were maintained at 37°C in a humidified culture incubator (5% CO₂ atmosphere) for 7 days, with a 50% differentiation media change every second day.

5.4.2.3 In vitro characterisation of hNPCs

To determine the phenotype of undifferentiated (day 0) cells, SVZ-hNPC neurospheres were seeded onto poly-L lysine (Sigma) and laminin (Sigma) coated 8 well chamber slides (Nunc Lab-Tek, Thermo Fisher Scientific, Waltham, MA, USA) at a density of approximately 200,000 cells/mL. Coated chamber slides were first prepared by incubation with 5µg/ml laminin (Sigma) and poly-L-lysine (0.1% solution) (Sigma) for 2 hours at 37°C. Slides were washed twice with DMEM/F12 before use. After plating, for day 0 analysis, neurospheres were allowed to attach for 2 hours at 37°C under 5% CO₂ before removal of media and fixation with 4% paraformaldehyde (PFA) in 0.1M PBS for 15 minutes for immunocytochemical analysis. For analysis of differentiated SVZ-hNPCs (day 7), hNPCs were seeded onto poly-L lysine (Sigma) and laminin (Sigma) coated 8 well chamber slides as described above. hNPCs were placed in differentiation media as described earlier and after 7 days, the cells were fixed with 4% PFA for 10 min at room temperature, washed (1 x 5min in 0.1M PBS), and processed for immunocytochemistry.

Immunofluorescence was performed on in vitro cell culture chamber slides to analyse phenotypic profiles as previously described (Mukhida et al., 2007). Cells were blocked with 5% normal goat serum (NGS) (or 1% bovine serum albumin (BSA, Sigma) for all staining with goat anti-parvalbumin antibody) in 0.1% Triton X-100 in 0.1M PBS for 20 mins before SVZ-hNPCs were identified using primary antibodies including mouse anti-human specific nuclear antigen (HuNu; 1:1000, Millipore), rabbit anti-neuronal class III β-tubulin (Tuj1; 1:1000, Covance Inc., Sydney, NSW, Australia), rabbit anti-gamma-aminobutyric acid (GABA; 1:1000, Sigma, St. Louis, MO, USA), rabbit anti-Nestin (1:1000, Millipore), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000, DAKO), guinea-pig anti-doublecortin (DCX; 1:500, Millipore, Billerica, MA, USA), rabbit anti-neuronal nuclear antigen (NeuN, 1:1000, Millipore), rabbit anti-Ki67 (1:500, Thermo Scientific), rabbit anti-SOX2 (1:400, Millipore), rabbit anti-glutamate decarboxylase 65&67 (GAD; 1:1000, Millipore), rabbit anti-synaptophysin (SYN; 1:200, Sigma), chicken anti-neuronal class III β-tubulin (Tuj1; 1:200, Millipore), mouse anti-calbindin-
D28k (CB; 1:1000, Swant, Marly, Switzerland), goat anti-parvalbumin (PV; 1:1000, Swant), rabbit anti-calretinin (CR; 1:1000, Swant). Primary antibodies were diluted in 5% NGS in 0.1M PBS and cells were incubated for 2 hrs at RT followed by washes in 0.1M PBS. Secondary fluorophore-conjugated antibodies (1:500 for all) included Alexa-568 goat anti-mouse, Alexa-488 goat anti-mouse, Alexa-568 goat anti-rabbit, Alexa-488 goat anti-rabbit, Alexa-488 goat anti-guinea pig, Alexa-555 goat anti-chicken, Alexa-647 goat anti-chicken, Alexa-568 donkey anti-goat (Invitrogen). Antibodies were diluted in 5% NGS in 0.1M PBS and cells were incubated for 1 hr followed by washes in 0.1M PBS. In all experiments, DNA counterstain DAPI (Molecular Probes) was applied before coverslipping with ProLong gold anti-fade reagent (Invitrogen). Resulting sections were examined with a Nikon confocal laser scanning microscope (Nikon Instruments Inc., Melville, NY, USA).

Stereology for cultured cells was assessed by manually quantifying immunolabeled cells at a magnification of x60 using the confocal microscope. Cells in two wells were analysed for each immunocytochemical stain. Cell counts were performed at 6 randomly selected sites within each culture well. The total number of cells immunoreactive for Tuj1, GABA, Nestin, GFAP, SOX2, and Ki67 was determined and expressed as a percentage of the total number of HuNu-positive cells.

5.4.2.4 Preparation of hNPCs for transplantation

For preparation of undifferentiated human SVZ-NPCs for transplant, the neurospheres were collected and treated with 0.01% DNase/DMEM for 20 mins at 37°C. The neurospheres were rinsed in 0.01% DNase/DMEM and centrifuged at 190\(\times\)g for 5 min, re-suspended in 0.01% DNase/DMEM and gently trituated into a single-cell suspension.

For pre-differentiated SVZ-hNPC cell preparation for transplantation, neurospheres were collected and then cultured in the presence of BDNF and neural supplements to induce differentiation as described in Section 5.4.2.2. After 7 days, differentiated SVZ-hNPCs were collected and centrifuged at 190\(\times\)g for 5 min, and a single cell suspension was made in preparation for transplantation in vivo.
Undifferentiated SVZ-hNPCs or pre-differentiated cell suspensions with a final concentration of 400,000 cells/µl were prepared, and a viability of ≥90% for transplantation.

5.4.3 *In Vivo Transplant Studies*

5.4.3.1 *Surgical Transplantation*

7 days after ET-1 induced stroke rats were re-anaesthetized as described above. A total of 800,000 cells in 2µl (400,000 cells/µl) were seeded to each graft site via a Hamilton syringe attached to a glass capillary (open diameter 50-70µm). The glass capillary was left in place for a further 5 min after each dose before retrieval. A total of 8 graft sites relative to bregma were chosen across the striatum and cortex as follows: Site 1 1.4 mm anterior, -4.9 mm lateral, -3 & -5 mm ventral; Site 2 1.4 mm anterior, -2.8 mm lateral, -3.6 & -5.6 mm ventral; Site 3 0.2mm anterior, -3.8 mm lateral, -4.8 & -5.8 mm ventral; and Site 4 -2.16 mm anterior, -6 mm lateral, -3.3 & -4.3 mm ventral. All rats were immunosuppressed by daily i.p. injections of cyclosporine (10mg/kg i.p. daily; Sandimmune, Novartis Pharmaceuticals, NJ, USA) commencing 2 days prior to cell transplantation.

5.4.3.2 *Immunofluorescence*

Immunofluorescence staining was performed on tissue sections to analyse and quantify graft survival, maturation and differentiation profile as described in Section 2.5.2. Primary antibodies used included mouse anti-HuNu (1:1000, Millipore), rabbit anti-Tuj1 (1:1000, Covance), rabbit anti-GABA (1:1000, Sigma), rabbit anti-Nestin (1:1000, Millipore), rabbit anti-GFAP (1:1000, DAKO), rabbit anti-NeuN (1:1000, Millipore), rabbit anti-Ki67 (1:500, Thermo Scientific), rabbit anti-SOX2 (1:400, Millipore), rabbit anti-GAD (1:1000, Millipore), rabbit anti-SYN (1:200, Sigma), chicken anti-Tuj1 (1:200, Millipore), mouse anti-CB (1:1000, Swant), goat anti-PV (1:1000, Swant), rabbit anti-CR (1:1000, Swant), rabbit anti-cleaved caspase-3 (Casp3; 1:500, Cell Signalling Technology, Boston, MA, USA), rabbit anti-Von Willebrand Factor (vWF; 1:200, Millipore). Secondary fluorophore-conjugated antibodies (1:500 for all) included Alexa-568 goat anti-mouse, Alexa-488 goat anti-mouse, Alexa-350 goat anti-mouse, Alexa-488 goat anti-rabbit, Alexa-647 goat anti-rabbit, Alexa-555 goat anti-chicken, Alexa-647 goat anti-chicken, Alexa-568 donkey anti-goat (Invitrogen). Staining for terminal transferase-mediated dUTP nick end-labelling (TUNEL), required
a TUNEL staining kit and was followed according to manufacturer’s instructions (Promega DeadEnd Colorimetric TUNEL system, Madison, WI, USA). Resulting sections were examined with a Nikon confocal microscope (Nikon). Orthogonal projections were performed using National Institute of Health (NIH) ImageJ software.

5.4.4 Quantification and Stereology

5.4.4.1 Quantification and characterisation of transplanted cells

5.4.4.1.1 Stereological analysis

The optical fractionator stereological method was used to obtain unbiased estimates of the total number of HuNu-positive nuclei within undifferentiated and pre-differentiated cell graft sites \((n=7/\text{group})\) using Stereo Investigator software (MBF Bioscience, Williston, VT, USA) (Chapter 2.6) (Hicks et al. 2007, Mukhida et al. 2008, West et al. 1991). Briefly, every third section was analysed using a fluorescence microscope (Olympus BH-2, Tokyo, Japan) equipped with a QIClick scientific camera (Surrey, BC, Canada) at a magnification of x60, where a three-dimensional optical dissector counting probe \((x, y, z \text{ dimension of } 30\mu m \times 30\mu m \times 10\mu m \text{ respectively})\) was applied to a systematic random sample of sites within the cell graft (Mukhida et al. 2008). Only HuNu-positive cells with clearly visible fluorescence was quantified.

5.4.4.1.2 Phenotypic analysis

The number of HuNu-positive cells that expressed Tuj1, GABA, Nestin, GFAP, and Ki67 was manually counted for phenotypic analysis of cells in vivo within transplant sites (Chapter 2.6). Cells were considered co-localised if labelling of markers was seen either throughout the extent of the HuNu-positive nucleus, or if a cytoskeletal/cytoplasmic marker surrounded the HuNu-positive nuclear marker. Quantitative analysis was performed in 3 tissue sections 280 µm apart per graft site (4 graft sites/rat) using z-scan confocal microscopy at x40 magnification, for animals that received undifferentiated SVZ-hNPCs or pre-differentiated cells \((n=7/\text{group})\) as previously described (Corvino et al. 2005, Corvino et al. 2014, Geloso et al. 1996, Geloso et al. 1997, Geloso et al. 2007). Briefly, estimates of the total number of cells co-localising with markers of interest were obtained using the following formula:

\[
E = k \sum N,
\]

where \(E\) is the estimate of the total number of stained cells in each case, \(\sum N\) is the sum of \(n\) values in the 3 sections analysed, and \(k\) indicates that every \(k^{th}\) section was considered \((k=8)\). N was corrected according to Abercrombie’s formula:
\[ N = \frac{nt}{(t + D)} \], where \( n \) is the number of cells counted in each section, \( t \) is the section thickness, and \( D \) is the mean diameter of the cells (Abercrombie and Johnson 1946). Co-localised cell counts were expressed as the percentage of the total number of HuNu-positive cells.

### 5.4.5 Statistical Analysis

Data were analysed as described in Chapter 2.9. Briefly, cylinder test behaviour data was analysed by a two-way repeated-measures (RM) ANOVA followed by Bonferroni test. Infarct area and volume was analysed using two-way ANOVA followed by Bonferroni test. Neurological deficit scores were analysed by Kruskal-Wallis non-parametric ANOVA followed by Dunn’s post-test. All cell counts were analysed by two-way ANOVA followed by Bonferroni test. Data were presented as mean ± SEM. Statistical significance was defined as \( P < 0.05 \).

### 5.5 Results

#### 5.5.1 In vitro characterisation of SVZ-derived hNPCs

Confocal analyses of immunolabeled undifferentiated SVZ-hNPCs in vitro revealed formation of neurospheres that were co-localised with human-specific nuclei antigen (HuNu) and the undifferentiated cell marker SOX2 (98.9 ± 0.7%) (Figure 5.2 A & N). These cells also co-localised with proliferation marker Ki67 (59.5 ± 3.1%) and NPC marker Nestin (95.1 ± 0.7%) (Figure 5.2 B) but did not express neuronal markers Tuj1, GABA, GAD or DCX (Figure 5.2 C-F). In contrast, pre-differentiated SVZ-hNPCs labelled with HuNu were positive for neuronal marker Tuj1 (92.3 ± 1.4%) (Figure 5.2 G & N) and inhibitory neurotransmitter GABA (90.1 ± 2.7%) (Figure 5.2 H), as well as the GABA-producing enzyme GAD (Figure 5.2 I) and immature neuronal marker DCX (Figure 5.2 J). Very few pre-differentiated cells expressed Ki67 (9.6 ± 2.5%) (Figure 5.2 K) or the NPC markers Nestin (28.5 ± 2.6%) (Figure 5.2 L) and SOX2 (12.1 ± 1.7%) (Figure 5.2 M). No expression for GFAP, synaptophysin or intracellular calcium binding proteins (CBPs) calbindin-D28k, calretinin, or parvalbumin was detected in any cell in vitro.
Figure 5.2 Undifferentiated hNPC and pre-differentiated cell phenotypes in vitro

Immunofluorescent confocal images of undifferentiated SVZ-hNPC in culture expressed HuNu (red) and SOX2 (green; panel A); and Ki67 (red) and Nestin (green; panel B) with co-localisation giving a yellow appearance. Undifferentiated SVZ-hNPC immunopositive for HuNu (red; C-F) in culture were not observed to express Tuj1 (green; panel C), GABA (green; panel D), GAD (green; panel E), or DCX (green; panel F). Pre-differentiated cells cultured for 7 days demonstrated immunoreactivity for HuNu
(red; G-M) and co-labelled with Tuj1 (green; panel G), GABA (green; panel H), GAD (green; panel I) and DCX (green; panel J). Pre-differentiated cells HuNu (red) cultured for 7 days demonstrated little immunoreactivity for Ki67 (green; panel K), Nestin (green; panel L) or SOX2 (green; panel M). Numbers of HuNu-positive cells co-expressing SOX2, Ki67, Nestin, Tuj1, and GABA from day 0 undifferentiated hNPCs and day 7 pre-differentiated cells in vitro (N). The numbers of cells are presented as a percentage of the total number of HuNu-positive cells counted in culture. Data are mean ± SEM. ****P<0.0001 compared with undifferentiated SVZ-hNPC cell counts (two-way ANOVA with Bonferroni post-test). Scale bar: (A-F) 100µm, (G-M) 50µm. Abbreviations: HuNu, human nuclei antigen; Tuj1, neuronal class III β-tubulin; GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase 65&67; DCX, doublecortin.

5.5.2 Transplant studies

5.5.2.1 Treatment groups

A total of 21 rats were included in this study. 7 rats were excluded prior to stem cell transplant based on low stroke severity scores and minimal functional deficits detected at 7 days (Roulston et al., 2008; Abeysinghe et al., 2014). Only rats with a stroke rating of 4 and a clear functional deficit of ≥3 were randomly assigned to three experimental groups (n=7/group): Group 1 received media without any cells (vehicle control); Group 2 received undifferentiated SVZ-hNPC transplants; and Group 3 received pre-differentiated cells.

5.5.2.2 Functional Outcomes

5.5.2.2.1 Cylinder test

No significant bias in forelimb use upon rearing was detected using the cylinder test prior to stroke induction in any treatment group. After stroke, all rats exhibited asymmetrical limb use indicative of stroke damage with preferential ipsilateral (unimpaired) forelimb use during rearing (Figure 5.3 A). Transplantation of either undifferentiated SVZ-derived hNPCs and pre-differentiated SVZ-derived hNPCs appeared to decrease forelimb asymmetry after treatment, however this effect was only statistically significant in rats receiving pre-differentiated hNPCs at 7 and 28 days post-transplant in comparison to vehicle control rats receiving media alone (Figure 5.3 A) (P<0.05, two-way ANOVA).
5.5.2.2 Neurological deficit score

No neurological deficits were observed prior to ET-1 induced stroke, however, after stroke significant deficits were observed in all treatment groups between 24 hours and 7 days after stroke (day 0 post-transplant) \((P<0.001\), non-parametric ANOVA, Figure 5.3 B). By 28 days rats receiving vehicle control or undifferentiated SVZ-hNPCs showed significant recovery in their deficits when compared to pre-transplant scores \((P<0.01)\). Rats that received pre-differentiated SVZ-hNPCs demonstrated earlier recovery with significant improvements observed 7 days post-transplant \((P<0.001)\) when compared to pre-transplant scores, with no significant deficits detected 28 days post-transplant when compared to pre-stroke scores \((P>0.05)\).

5.5.2.3 Infarct assessment

Absence of NeuN immunoreactivity revealed stroke induced damage to the parietal, insular, and frontal cortex, as well as the striatum as reported previously (Roulston et al. 2008). Both infarct area (Figure 5.3 C, D) and infarct volume (Figure 5.3 E) within the cortex and striatum were consistent across treatment groups with no significant differences detected between groups \((P>0.05\), two-way ANOVA).

Figure 5.3 Functional outcome following transplantation.
Effects of transplantation on contralateral limb use when rearing (A) in the cylinder test after ET-1 induced stroke. Data are mean ± SEM expressed as a percentage of contralateral (impaired) forelimb use. Each rat acted as its own control, results following stroke were compared to 0hr pre-stroke scores. \( \phi P<0.05 \) relative to 0hr post-stroke baseline scores for vehicle treated rats \((n=7)\); **\( P<0.01 \), ***\( P<0.001 \), ****\( P<0.0001 \) compared to 0hr post-stroke baseline scores for undifferentiated treated rats \((n=7)\); #\( P<0.05 \), ##\( P<0.01 \) relative to 0hr post-stroke baseline scores for pre-differentiated treated rats \((n=7)\); δ\( P<0.05 \), δδ\( P<0.01 \) vehicle treated rats compared to pre-differentiated treated rats (two-way ANOVA followed by Bonferroni post-test). Combined neurological deficit scores (B). Data presented as box plots include hinges extending from the 25\(^{th}\) to 75\(^{th}\) percentiles, the median line within the box and whiskers extending to the minimum and maximum values of the dataset \((n=7/group)\). *\( P<0.05 \), ***\( P<0.001 \), ****\( P<0.0001 \) relative to 0hr post-stroke baseline scores \((n=7/group)\); #\( P<0.05 \), ##\( P<0.01 \), ###\( P<0.001 \), ####\( P<0.0001 \) relative to pre-transplant scores (Kruskal-Wallis ANOVA followed by Dunn’s test). The effect of transplanting vehicle, undifferentiated SVZ-hNPCs, or pre-differentiated cells on infarct area (C, D) and total infarct volume (E) within the cortex and striatum. Data presented as mean ± SEM of infarct area measured at 8 pre-determined coronal planes through the brain (two-way ANOVA followed by Bonferroni post-test).

5.5.2.4 **Characterisation of cells within grafts**

5.5.2.4.1 **Cell counts**

HuNu immunostaining revealed large numbers of SVZ-hNPCs within each graft site situated both within and outside the damaged regions 28 days post-transplant. Rats receiving undifferentiated SVZ-hNPCs displayed evidence of cells within each transplant site, however many HuNu positive SVZ-hNPCs were also detected beyond the transplant regions and were also visible within the infarct border zone. Rats that received pre-differentiated SVZ-hNPCs showed dense clustering of cells within each site. Stereological cell counting revealed that the number of undifferentiated SVZ-hNPCs \((64,712 ± 16,866 \text{ cells and } 260,278 ± 14,112 \text{ cells})\) within the cortex and striatum respectively was significantly lower than that of pre-differentiated HuNu-positive cells remaining within cortical \((444,852 ± 22,181 \text{ cells})\) and striatal graft regions \((508,098 ± 10,031 \text{ cells})\) \((P<0.0001; \text{ Figure 5.4 A})\). A greater number of cells, both pre-differentiated \((P<0.05)\) and undifferentiated \((P<0.0001)\) SVZ-hNPCs remained
within striatal grafts compared with cortical graft sites (Figure 5.4 A). However the overall percentage of transplanted pre-differentiated cells remaining within cortical (27.8 ± 1.3 %) and striatal graft sites (31.8 ± 0.5 %) was significantly more than that of undifferentiated SVZ-hNPCs within the cortex (4.0 ± 1.0 %) and striatum (16.3 ± 0.9 %) (\(P<0.0001\), Figure 5.4 B).

Survival of transplanted cells within graft sites was confirmed by immunohistochemical analysis for apoptotic marker cleaved caspase-3 (Casp3) and TUNEL stain. Neither Casp3 immunostaining (Figure 5.4 C-E) nor TUNEL (Figure 5.4 F, G) staining was observed within cells 28 days post-transplant. The low level of TUNEL staining within the graft was similar to that observed within the contralateral hemisphere (Figure 5.4 H). Many transplanted undifferentiated SVZ-hNPCs were associated within the glial scar and were GFAP-positive (Figure 5.4 I, J), whilst transplanted pre-differentiated cells were clearly associated with blood vessels within the infarcted regions (Figure 5.4 K).
Figure 5.4 Transplanted cells survive within the stroke affected brain.

The total number (A) and percentage (B) of HuNu-positive cells remaining within cortical and striatal grafts from undifferentiated \((n=7)\) and pre-differentiated \((n=7)\) treatment groups. Data presented as mean ± SEM, ****\(P<0.0001\) relative to undifferentiated treated animals in the same region; ####\(P<0.0001\) relative to undifferentiated grafts within the cortex; \(\phi P<0.05\) relative to pre-differentiated grafts within the striatum (two-way ANOVA followed by Bonferroni post-test). Transplanted cells immunopositive for HuNu (red; C, F) did not express apoptotic markers including cleaved caspase-3 (Casp3, green; D), merged image (E); or TUNEL (G), with the level
of TUNEL staining similar to the contralateral mirror image (H). Transplanted HuNu-positive (red) pre-differentiated cells were associated with vWF stained blood vessels (green) within infarcted brain regions (I). Many HuNu positive undifferentiated SVZ-hNPCs (red) were found within border regions consisting of GFAP-positive astrocytes (GFAP; green; J). Magnified immunofluorescent image (K) that corresponds to box highlighted in (J) illustrating incorporation of undifferentiated SVZ-hNPCs into the glial scar bordering the infarct. Scale bar: C-E 20µm, F-H 100µm, I, K 200µm, J 100µm. Abbreviations: vWF, Von Willebrand Factor; TUNEL, terminal transferase-mediated dUTP nick end-labelling; I, ipsilateral hemisphere; C, contralateral hemisphere.

5.5.2.4.2 Phenotypic analysis

Confocal image analysis 28 days post-transplant revealed undifferentiated SVZ-hNPCs mainly expressed markers for GFAP and Nestin (Figure 5.5 A, B) with many cells also positive for Ki67 (Figure 5.5 C). Some undifferentiated SVZ-hNPCs were also found to express GABA and Tuj1 (Figure 5.5 D, E). In contrast, pre-differentiated cells mainly expressed Tuj1 and GABA (Figure 5.6 A, B) with little co-labelling with Nestin and Ki67 (Figure 5.6 C, D) and a distinct lack of GFAP expression (Figure 5.6 E).

Immunofluorescent NeuN staining was used to confirm positioning of graft sites within the infarcted region for stereological analysis across all groups receiving SVZ-hNPC transplants (Figure 5.7 A-H). Cell counts within each site revealed that there were significantly less undifferentiated SVZ-hNPCs that expressed Tuj1 (6.6 ± 1.3%; P<0.0001) and GABA (5.4 ± 1.4%; P<0.0001) in comparison to the number of pre-differentiated cells across all transplant sites (Tuj1: 89.6 ± 1.9%; GABA: 94.3 ± 1.6%) (Figure 5.7 I-L, ANOVA). Conversely, there was a significantly greater proportion of undifferentiated SVZ-hNPCs that expressed markers for GFAP (93.0 ± 1.9%; P<0.0001), Nestin (88.1 ± 2.8%; P<0.0001) and Ki67 (11.9 ± 1.6%; P<0.05) compared to transplanted pre-differentiated cells (GFAP: 4.7 ± 1.3%; Nestin: 11.6 ± 3.3%; Ki67: 4.6 ± 1.1%) (Figure 5.7 I-L).

Further confocal analysis revealed pre-differentiated SVZ-hNPC grafts expressed GAD65/67 (Figure 5.8 A), as well as intracellular CBPs, calbindin-D28k
(Figure 5.8 B), calretinin (Figure 5.8 C), and parvalbumin (Figure 5.8 D). In addition, pre-differentiated SVZ-hNPCs were observed to express synaptophysin (SYN) that was mostly localised to the cell body of cells transplanted into the core infarct region (Figure 5.8 E), but was also additionally observed along the cytoskeleton of cells that were transplanted into sites outside of the infarct within the peri-infarct territory (Figure 5.8 F). Pre-differentiated SVZ-hNPC grafts located within the peri-infarct territory appeared to extend long neurites revealed by HuNu and Tuj1 immunostaining (Figure 5.8 G). Grafts located within the core infarct were surrounded by the glial scar consisting of densely packed GFAP-positive astrocytes, with limited dispersion of SVZ-hNPCs in closest proximity to the scar (Figure 5.9 A-C). Parallel negative control experiments omitting primary antibodies depict a low level of autofluorescence within the core infarct (Figure 5.9 D-F).
Figure 5.5 Undifferentiated hNPC cell phenotypes in vivo.

Confocal immunofluorescent photomicrographs of undifferentiated SVZ-hNPCs at 28 days post-transplant within cortical border regions with orthogonal reconstructions. Undifferentiated SVZ-hNPCs maintained expression of HuNu (red) and co-labelled mainly with GFAP (panel A), Nestin (panel B), and Ki67 (panel C), with little expression of GABA (panel D), and Tuj1 (panel E) (green). Orthogonal reconstructions from confocal z-series are presented as viewed in x-z (top) and y-z (right) planes. HuNu
immunoreactivity of undifferentiated SVZ-hNPCs was observed completely surrounded by GFAP and Nestin. Scale bar: A-E 50µm, orthogonal images 5µm. Abbreviations: HuNu, human nuclear antigen; GFAP, glial fibrillary acidic protein; GABA, gamma-aminobutyric acid; Tuj1, neuronal class III β-tubulin.

Figure 5.6 Pre-differentiated cell phenotypes in vivo.
Immunofluorescent confocal images of pre-differentiated cells 28 days post-transplant within the stroke damaged cortex maintained expression of HuNu (red) and co-labelled
with Tuj1 (panel A), and GABA (panel B), Nestin (panel C), Ki67 (panel D) (green), with lack of co-localisation with GFAP (green; panel E). Orthogonal reconstructions from confocal z-series are presented as viewed in x-z (top) and y-z (right) planes. HuNu immunoreactivity of pre-differentiated cells was observed completely surrounded by Tuj1 and GABA. Scale bar: A-E 20µm, orthogonal images: 5µm. Abbreviations: HuNu, human nuclear antigen; GFAP, glial fibrillary acidic protein; GABA, gamma-aminobutyric acid; Tuj1, neuronal class III β-tubulin.
Figure 5.7 Phenotypic profile of transplanted undifferentiated hNPCs versus pre-differentiated cells.

Coronal sections immunostained with NeuN (green) highlight undamaged brain regions with lack of staining within stroke affected areas (white dotted line) at cortical graft sites +1.4mm and -2.16mm relative to bregma (A, C) and striatal graft sites +1.4mm and +0.2mm relative to bregma (E, G), where white boxes depict graft location. Representative images of graft sites stained with HuNu (red) and NeuN (green; B, D, F, H) from regions highlighted by white boxes in (A, C, E, G). Numbers of HuNu-positive
cells co-expressing TuJ1, GABA, Nestin, GFAP and Ki67 from undifferentiated SVZ-hNPC \( n=7 \) and pre-differentiated cell \( n=7 \) treated animals from cortical graft sites; +1.4mm (I) and -2.16mm (J) relative to bregma; and striatal graft sites +1.4mm (K) and +0.2mm (L) relative to bregma. The numbers of cells are presented as a percentage of the total number of HuNu-positive cells counted. Data are mean ± SEM. ***\( P<0.001, ****P<0.0001 \) compared with undifferentiated SVZ-hNPC cell counts (two-way ANOVA with Bonferroni post-test). Scale bar: A, C, E, G 2000µm, B, D, F, H 200µm. Abbreviations: HuNu, human specific nuclear antigen; NeuN, neuron specific nuclear antigen.
Figure 5.8 Further maturation of pre-differentiated cells 28 days post-transplant. Confocal photomicrographs of pre-differentiated cells 28 days post-transplant within the stroke damaged brain expressed HuNu (red) double labelled with either GAD65/67 (green; panel A); or calbindin-D28k (CB; green; panel B); or calretinin (CR, green; panel C). Pre-differentiated cells expressing HuNu (blue) tripled labelled with
parvalbumin (PV, green) and GABA (red; panel D); or tripled labelled with Tuj1 (red) and pre-synaptic vesicle protein synaptophysin (SYN, green) within cortical core (panel E) and border (panel F) regions. Some pre-differentiated cells HuNu (red) grafted to border regions appeared to extend long neurites Tuj1 (green, G). Orthogonal reconstructions from confocal z-series are presented as viewed in x-z (top) and y-z (right) planes. Scale bar: panels A, B 10µm, orthogonal image 5µm; panels C, D 40µm, orthogonal image 5µm; panels E, F 10µm, orthogonal image 5µm; panel G 20µm, orthogonal image 5µm.

Figure 5.9 Pre-differentiated cells grafted to the core infarct in vivo.

Pre-differentiated cell graft (HuNu, red; A) located within the infarcted core region was surrounded by GFAP-positive astrocytes on one side (green; B) that form the glial scar border (arrow), merge (C), with dispersion of HuNu-positive cells observed in the core furthest away from the scar (arrow heads). Negative control; primary antibodies were absent (D-F). Scale bar: A-F 100µm.
5.5.2.4.3  Effect of stem cell transplant on the neurogenic niche

To investigate the effects of SVZ-hNPC transplant on endogenous recovery mechanisms we examined changes within the neurogenic niche in response to stem cell transplant. Confocal analysis revealed an apparent increase in the number of Ki67-positive proliferating cells within the ipsilateral SVZ of rats that received pre-differentiated SVZ-hNPCs in comparison to rats that received undifferentiated SVZ-hNPCs or vehicle controls (Figure 5.10 A-F). In particular, immunofluorescent labelling of cells within the SVZ revealed an apparent increase in the number of newly generated DCX-positive neurons in rats with pre-differentiated SVZ-hNPC transplants in comparison to the contralateral SVZ, and rats with undifferentiated SVZ-hNPC transplants or vehicle controls (Figure 5.10 B, D, F). Further analysis of immunolabeled cells within the SVZ revealed an apparent increase in the number of GFAP-positive radial glial cells with long processes directed towards the infarct that did not co-localise with Nestin in animals that received pre-differentiated SVZ-hNPC transplants compared to the contralateral SVZ, and compared to the other treatment groups (Figure 5.10 G-L).
Figure 5.10 Effect of treatment on endogenous neurogenesis and radial glial cell populations within the SVZ.

Immunofluorescent images of proliferating cells (Ki67; red) and migrating immature neuroblasts (DCX; green) within the contralateral and ipsilateral SVZ of vehicle treated (A, B), undifferentiated hNPC treated (C, D) and pre-differentiated cell treated (E, F) animals. Nestin (green; arrow heads) and GFAP (red) immunopositive cells (co-expression giving a yellow appearance; arrows) within the contralateral and ipsilateral SVZ of vehicle treated (G, H), undifferentiated treated (I, J), and pre-differentiated treated (K, L) animals. All images were taken at the same anatomical location from animals with similar infarct volumes. Scale bar: A-F 500µm, G-L 50µm. Abbreviations: Undiff, undifferentiated SVZ-hNPC treated; Prediff, pre-differentiated treated; LV, lateral ventricle; CC, corpus callosum; Stm, Striatum.
5.6 Discussion

The use of undifferentiated stem cells has attracted special interest for treatment of neurological diseases including stroke and as such have undergone intense investigation in preclinical models (Svendsen et al., 1997; Bjorklund and Lindvall, 2000; Martinez-Serrano et al., 2001). Undifferentiated stem cells are favoured due to their ability to provide trophic factor support to injured or “depressed” neural circuitry and respond to host microenvironmental cues by migrating and differentiating into various cell types to influence functional recovery. An alternative approach to cell-based therapies has evolved which includes pre-differentiating hNPCs prior to transplant to improve functional outcomes in animal models of Huntington’s disease, Parkinson’s disease and neuropathic pain models (Mukhida et al., 2007; Mukhida et al., 2008; McLeod et al., 2012).

The present study shows first evidence in a model of stroke for accelerated improvements in motor functions following transplantation of human derived pre-differentiated GABAergic cells compared to undifferentiated hNPC transplants. We demonstrate that GABAergic neuronal transplants survive within the stroke-affected rat brain, mature and extend processes with evidence of synaptogenesis and calcium signalling events associated with neurotransmission. Moreover, rats receiving pre-differentiated cells showed evidence of increased endogenous neurogenesis with immature neurons seen extending from the SVZ towards the damaged striatum. Contrastingly, undifferentiated SVZ-hNPC transplants predominantly differentiated into astrocytes and appeared to contribute to further scar formation within the peri-infarct border zone. The results from the current study suggest that differentiation of hNPCs into a neuronal phenotype prior to transplant may represent a favourable alternative for cell restoration strategies after stroke.

5.6.1 Phenotypic characterisation of hNPCs in vitro

In vitro differentiation of hNPCs prior to transplantation at day 7 revealed a highly enriched population of GABAergic neurons that expressed Tuj1, GABA and GAD65&67. The expression of both GAD 65&67 isoforms confirmed functional differentiation of these cells with an ability for neurons to produce and release GABA (Soghomonian and Martin, 1998; Le Magueresse and Monyer, 2013) and excluded the
possibility of GABA immunoreactivity resulting from *in vitro* uptake (McLeod et al., 2012). The decreased expression of markers Nestin, Ki67 and SOX2 with *in vitro* differentiation also confirmed phenotypic GABAergic conversion. Few pre-differentiated cells were positive for Ki67 and those that were, were likely not to have undergone differentiation. In contrast examination of undifferentiated hNPCs revealed a larger population of Nestin, Ki67 and SOX2 immunopositive cells confirming the uncommitted early phase of these NPCs. Taken together, differentiation *in vitro* results in down regulation of naive precursor cell markers and an up-regulation of neuronal markers where the majority of pre-differentiated cells displayed GABAergic neuronal phenotypes.

5.6.2 Effect of hNPCs on functional recovery

Treatment with GABAergic hNPCs accelerates functional return when compared to rats treated with undifferentiated hNPCs. In particular, neurological deficit scores were improved at earlier times after stroke in comparison to undifferentiated hNPC treated rats. Recovery observed in GABAergic hNPC treated rats 28 days post-transplant was consistent with pre-stroke neurological scores. Forelimb asymmetry was also improved back to pre-stroke scores with GABAergic hNPC treatment and was the only group that showed statistical improvement with this assessment despite an observed positive effect in rats treated with undifferentiated cells compared to the media alone treatment. Results obtained using the cylinder test should be viewed with caution since the number of rats in each group may have been under-powered and increasing the sample size could yield a different result. Data interpretation is also made difficult by spontaneous improvements in media alone treated rats by 28 days. Unfortunately, marked spontaneous recovery is common in rodent models of stroke and most likely explains behavioural improvements observed in vehicle controls (Schaar et al., 2010). The inclusion of more comprehensive behavioural tests like the cylinder test (Schallert and Woodlee, 2005) are therefore required in future studies (Schaar et al., 2010). Never-the-less these results show that differentiation into a GABAergic cell lineage prior to transplant has beneficial effects on recovery to both the cortical and subcortical motor systems.
5.6.3 Transplant survival and migration

Only rats with the same stroke severity scores were included in these experiments which resulted in little variation detected between infarct volumes across treatments. As such we were able to assess cell responses to the host microenvironment without having to take into account variations in brain injury between groups (Abeysinghe et al., 2014).

Despite the above, comparison of hNPC survival 28 days post-transplant between treatment groups was difficult due the apparent migration of undifferentiated cells away from the graft site. In our study only 16% of undifferentiated hNPCs were detected within striatal grafts and only 4% within cortical grafts 28 days post-transplant. Undifferentiated hNPCs favour less hostile environments and have previously been reported to migrate towards border regions (Kelly et al., 2004; Okada et al., 2005), which could account for the low cell numbers detected in the original grafts. It is for this reason that the peri-infarct territory is currently the target area for transplantation as it is thought to be less hostile and may provide a more supportive extra-cellular matrix for hNPC grafts to anchor (Saporta et al., 1999; Roitberg et al., 2006; Mine et al., 2013). In contrast, we now show for the first time that a greater percentage of cells can be retained in the target region if cells are pre-differentiated first. Approximately 30% of GABAergic hNPC transplants were still detected in cortical and striatal graft sites even within the severely damaged brain 28 days post-transplant. These GABAergic cells were not in a state of decay since cells did not stain positive for apoptotic markers Casp3 or TUNEL stain.

Previous reports suggest that between 0.5-15% of exogenously grafted cells survive in the core infarct and only 20-30% survive in locations distant to the infarct (Saporta et al., 1999; Hicks et al., 2007; Hicks et al., 2009; Leong et al., 2012; Smith et al., 2012). We now show that greater cell retention following transplant can be improved through pre-differentiation. Despite this positive step, we still observed significant cell loss when compared to the original number transplanted. This process may actually be necessary since pruning and refinement of cell numbers to facilitate development of efficient networks is an important process during development (Iglesias et al., 2005; Craik and Bialystok, 2006). It may also suggest that not as many cells need to be transplanted in order to achieve significant outcomes.
The question remains, how do GABAergic cells survive within the severely damaged brain? The answer may relate to trophic factor support provided from blood vessels detected within and surrounding grafts (Leventhal et al., 1999; Shen et al., 2004; Minger et al., 2007). Angiogenesis occurs as early as 3 days after ET-1 stroke (Taylor et al., 2013) and is thought to provide a platform for brain repair. Cells grafted to sites of re-vascularisation after stroke might be expected to do better (Chopp and Li, 2008; Zhang and Chopp, 2009; Taylor et al., 2013). Current clinical trials favour delaying cell-based treatments until behavioural deficits reach a steady plateau and the microenvironment less aggressive (Kondziolka et al., 2000; Rabinovich et al., 2005). Unfortunately, delaying treatment also allows time for newly formed vasculature to regress (Yu et al., 2007). Our results suggest that cell-based therapies may benefit from a developed microvascular bed for optimising survival and influence of exogenous grafts on functional recovery. Timing cell-based therapy within weeks after stroke, when standard therapeutic procedures have been exhausted and relative stabilisation of the infarct has occurred, may be a more suitable option for intervention.

5.6.4 Post-transplant histology

Immunohistochemical analysis of hNPCs that were not pre-differentiated prior to transplant revealed predominant differentiation into GFAP-positive astrocytes 28 days post-transplant in all brain regions targeted. Astrocytes are becoming recognized as a restorative therapeutic target for brain injury, including stroke, as their phenotype can mediate aspects of brain integrity, neuronal cell death as well as repair (Sofroniew and Vinters, 2010). Astrocyte activation and reactive astrogliosis can result in both beneficial and deleterious responses, and is dependent on the intensity and hostility of the stroke damaged environment (Sofroniew and Vinters, 2010; Abeysinghe et al., 2014; Lau et al., 2014). In the ET-1 stroke model, reactive astrogliosis and glial scarring is established by 14 days (Taylor et al., 2013) and is a major obstacle to brain repair and functional recovery (McGraw et al., 2001). For this reason we chose not to transplant cells into an established glial scar but rather targeted transplant prior to its formation. Like in many previous studies, hNPCs that were not pre-differentiated prior to transplant were found to be localised within the glial scar by 28 days and were intensely stained with GFAP indicating post-transplant differentiation. Undifferentiated hNPCs also displayed a significantly greater proliferative capacity as indicated by Ki67
immunoreactivity compared to pre-differentiated hNPCs confirming their capacity for self-renewal and expansion after transplant. Interestingly, a small subpopulation of undifferentiated hNPCs spontaneously differentiated into Tuj1 and GABA expressing neurons by 28 days demonstrating their ability to enter a neural lineage despite a greater propensity towards a glial lineage (Kelly et al., 2004; Roitberg et al., 2006).

In contrast to the above, immunohistochemical analysis of GABAergic cell transplants revealed robust expression of Tuj1, GABA, and GAD within the cell bodies confirming retention of their GABAergic neuronal phenotype 28 days post-transplant, even in the severely damaged brain. Pre-differentiated cells displayed low levels of proliferative potential, without aberrant trans-differentiation. Nor did they revert back to an undifferentiated state, suggesting these cells could be a safe source for transplantation. As such pre-differentiating cells into neurons prior to transplant may provide an edge in their therapeutic potential by reducing the risk of contributing further to glial scar formation and low risk for tumorigenicity.

5.6.5 Neuronal repopulation and trophic support for functional recovery

How GABAergic transplants improve the recovering brain is yet to be fully characterised. However possible mechanisms include neuronal repopulation to bridge the gap between damaged circuits, rescue or re-activation of dormant but surviving circuitry, or influences on neuronal reconstruction. In particular GABAergic cells transplanted into the striatum resulted in >30% new neurons expressing immunohistochemical markers that are typically expressed by striatal GABAergic medium sized spiny projection neurons and aspiny interneurons, being positive for GABA, GAD, Tuj1, and calcium binding proteins that are essential components for restoring neurotransmission (Gerfen et al., 1985; Gerfen, 1992; Bennett and Bolam, 1993; Cicchetti et al., 1998; Riedel et al., 2002). Whilst grafts to the core infarct were clearly isolated by the glial scar barrier by 28 days, grafts within the border zone appeared to be better positioned to influence host circuitry and promote recovery. Future studies using anterograde and retrograde labelling would be required to substantiate connectivity of graft cells to host neural systems (Lindvall et al., 2012).

GABAergic cell transplants also up-regulated markers that were absent at the time of transplant, indicating further maturation in vivo to potentially aid recovery. In
our study, only pre-differentiated cells expressed synaptophysin, a pre-synaptic vesicle protein, suggesting increased synaptic vesicle formation with potential roles in neurotransmission and plasticity (Ujike et al., 2002; Weimer and Jorgensen, 2003; Hajjar et al., 2013). The localisation of synaptophysin to the cellular cytoskeleton of grafts within the peri-infarct territory could indicate a more advanced state of maturation compared to grafts within the core infarct. Furthermore, only pre-differentiated cells were observed to express intracellular CBPs, calbindin-D28k, calretinin and parvalbumin, potentially indicating calcium signalling events associated with neurotransmission, transmitter release, and plasticity (Felmy and Schneggenburger, 2004; Yanez et al., 2012). Overall, paracrine delivery of GABA, up-regulated synaptophysin and CBP expression may restore neurotransmission and promote plasticity to improve behavioural outcomes.

Alternatively, GABAergic transplants may function by means of awakening depressed pathways known to lie dormant following stroke (Dancause, 2006; Cramer, 2008; Benowitz and Carmichael, 2010), either through restoration of neurotransmission or graft-induced enhancement of neurogenesis to stimulate pathways and influence repair. Trophic and chemoattractive functions exerted by interneurons during development through the release of depolarising GABA may be recapitulated by GABAergic transplants in our study to promote proliferation/migration of NPCs, provide positional cues to migrating NPCs and immature neurons, and influence synapse maturation (Lopez-Bendito et al., 2003; Represa and Ben-Ari, 2005; Le Magueresse and Monyer, 2013). Such reasoning is based on the likelihood that the human GABAergic transplants in our study are immature, excitatory and release depolarising GABA as the switch to an inhibitory phenotype only occurs late in the first postnatal year in humans (Xu et al., 2011). Furthermore, within the ipsilateral SVZ, only pre-differentiated cell treated animals displayed GFAP-positive radial glial cells with long processes known to function in guiding immature neurons during neurogenesis (Gubert et al., 2009). Usually, the endogenous neurogenic response peaks 7 days post-stroke and returns to normal by 14 days (Arvidsson et al., 2002; Zhang et al., 2006), therefore the apparent increase in activity within the neurogenic niche in our study 35 days post-stroke suggests a trophic influence from transplanted GABAergic cells. Trophic factors secreted from migrating endogenous NPCs and immature neurons, or GABAergic transplants themselves could act to stimulate rewiring or awakening of
host circuits enabling remaining healthy tissue to restore function of lost connections (Wright et al., 2003; Dancause, 2006; Andres et al., 2011).

5.7 Conclusion

Findings from this study demonstrate pre-differentiating SVZ-hNPCs prior to transplantation as a novel strategy to accelerate functional recovery after cerebral ischemia. In contrast, undifferentiated hNPC transplants have the potential to delay or hinder functional improvements through contributions to further scar formation. Neuronal repopulation through GABAergic neuronal transplants may achieve greater viability in a clinical setting by targeting a smaller ischemic region, as seen in lacunar strokes (Carmichael, 2005; Arboix and Marti-Vilalta, 2009). However, GABAergic transplants also show promise for targeting severe stroke through production of GABA, synaptogenesis and calcium signalling events associated with neurotransmission, to bridge the gap between damaged pathways, enhance plasticity, or rescue depressed circuitry to re-establish function. Alternatively, known trophic effects of depolarising GABA during development may be recapitulated in our study to enhance endogenous neurogenesis, re-activate circuitry and promote plasticity for functional recovery in severe strokes with widespread damage. The principles behind differentiation of hNPCs into a desired neuronal cell type may be a favourable alternative for treating stroke.
Chapter 6: Summary and Conclusions

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6.1 Summary

Ischemia is a highly complex disorder, leading to “silencing” or death of diverse cell types with intricate connectivity patterns that result in significant functional impairments. There is no proven therapeutic treatment aside from thrombolytic therapy or intense physiotherapy to treat acute stroke sufferers. In the case of intense rehabilitation, most stroke survivors are unable to participate in meaningful treatment until weeks after a stroke event resulting in suboptimal outcomes (Gauthier et al., 2008). In addition, the characteristics and complexity of the human brain adds numerous challenges in considering treatment strategies for stroke. However, the adult brain responds to ischemia by triggering processes in an attempt to self-repair, which are active for days to weeks after stroke onset. As a result, treatment strategies that compliment endogenous brain repair through cell-based or pharmacological methods provide a unique approach for promoting regeneration. The stroke-damaged milieu influences the potential for endogenous brain repair. Hence, careful planning and attention to specific demands of the brain and use of appropriate preclinical models are required to improve the quality and efficacy of restorative treatments.

Restoring functional outcome is the ultimate goal for any stroke therapy. However restorative strategies that target brain regeneration need to comprehensively take into account the influence of stroke severity and, in regards to investigating cell-based therapies, factors that influence exogenous stem cell survival and long-term integration within host tissue to improve overall therapeutic efficacy. Therefore the studies conducted in this thesis were designed to initially characterise brain remodelling events taking into account stroke severity, and subsequently investigate the use of human neural progenitor cell (hNPC) transplants for promoting brain recovery.

Endogenous neurogenesis, angiogenesis, and axonal plasticity are interlinked remodelling events that potentially underpin improvements in neurological function after stroke (Zhang and Chopp, 2009). Here we investigated the correlation between stroke severity and effects on pathological responses to brain recovery after brain ischemia and reperfusion in the rat. As such we have now, for the first time, fully characterised long term remodelling events in a relevant rat model of stroke and highlight the importance of taking into consideration infarct size when developing
therapies and assessing outcomes. In addition, we also recommend that treatments aimed towards increasing endogenous neurogenesis also target neuronal differentiation so as to avoid further contribution to the glial scar through increased stem cell generation.

A major component of this thesis was to develop an exogenous cell-based therapy to complement endogenous repair mechanisms that are activated after stroke. Since many xenotransplants require combined immunosuppression, we first investigated the effects of Cyclosporine A (CsA) use alone. Neuroprotective effects of CsA treatment have been observed in experimental animal models of stroke (Butcher et al., 1997; Shiga et al., 1992; Uchino et al., 1998). However the effect of CsA on brain remodelling and repair mechanisms activated after stroke had not been reported. Therefore, prior to commencing stem cell transplant studies, determining potential CsA-mediated effects on brain remodelling and events within the neurogenic niche were conducted. As such we determined that treatment with CsA did not affect critical endogenous repair mechanisms including angiogenesis and neurogenesis. However, we found that CsA treatment did reduce over activation of astrocytes in response to stroke, and retained their pro-survival morphology that could aid repair. Astrocytes, in particular, have been targeted for regenerative neurobiology as their dynamically changing phenotypes arbitrates brain integrity, neuronal cell death, and subsequent repair (McMillian et al., 1994; Sofroniew and Vinters, 2010). While these novel finding are promising, studies to confirm that retained astroglial support promotes neural rescue are required and this warrants future investigation.

A cellular basis for functional recovery following cell-based treatment remains unclear. Given the elusive nature of functional improvements after such treatment, it was important to delineate how the stroke-affected environment influences stem cell survival as well as their ability to integrate within host tissue to help reveal how these cells work. Hence, we targeted stem cell transplants to the period of angiogenesis after stroke so that exogenous grafts would benefit from a redeveloped microvascular bed. Additionally, we also investigated the effects of transplanting undifferentiated hNPCs versus pre-differentiated hNPCs into the rat brain after stroke. One of the major findings of our investigation was that undifferentiated hNPC transplants contributed further to glial scar formation since the majority of these cells were positive for GFAP and located
within the established host scar. Contrasting, pre-differentiated GABAergic cells transplants accelerated functional return and grafts displayed evidence of GABA production, synaptogenesis and calcium signalling events associated with neurotransmission. In addition, rats receiving pre-differentiated cell transplants appeared to have increased neurogenic activity with greater neural differentiation suggesting a trophic role by GABAergic transplants. GABAergic transplants may also promote neurite outgrowth, influence plasticity, re-activate silent pathways or bridge the gap between affected pathways, all of which require further investigation. This preclinical study now has important implications for transplantation of differentiated cells as a favourable and feasible alternative for cell-based strategies to promote behavioural recovery in stroke.

6.2 Limitations and future directions

6.2.1 Control data

For studies presented in Chapters 3 and 4, we used the contralateral (non-ischemic) hemisphere as the control for stroke-induced changes detected within the ipsilateral (ischemic) hemisphere. This was advantageous as we allowed each animal to act as its own control, thereby avoiding the influence of natural inter-animal variations on comparisons. However, to avoid potential alterations in various parameters within the contralateral hemisphere as a result of stroke, or confirm lack thereof, sham-operated controls were used to compare to stroke-affected animals. Sham-operated controls were harvested at 7 and 14 days after stroke in accordance to experimental time-course requirements for studies presented in Chapters 3 and 4. These animals showed no track mark from cannula implantation, or quantifiable ischemic damage in the region of the MCA with no neurological deficits displayed after surgery. In Chapter 5, we used vehicle treated ET-1 stroke induced animals as a control for stroke-induced hNPC treated groups. The same medium used to suspend pre-differentiated or undifferentiated hNPCs was used for vehicle transplantation to ensure the cell phenotype was the only variable between treatment groups. Animals in all treatment groups received stroke ratings of 4 to ensure stroke severity in each group was evenly represented. For future safety and efficacy studies, the influence of either pre-differentiated or undifferentiated hNPC transplants on endogenous processes in naïve
controls (non-stroke) may be required to fully address the influence of these cells within a non-hostile environment.

6.2.2 Administration of CsA post-stroke

In Chapter 4, the influence of CsA on neurological and histopathological outcomes after stroke was assessed when administered prior to stroke induction. Testing of neuroprotective compounds often commence with a pre-stroke treatment approach to determine efficacy, and as such we used a similar approach (Danton and Dietrich, 2004). Future experiments testing CsA at different intervals following stroke onset may be required to investigate CsA effects on astrogliosis and glial scaring and thus determine its potential use for supporting brain repair mechanisms.

6.2.3 Functional outcome and spontaneous recovery

Spontaneous recovery was observed in all behavioural tests employed for studies conducted in this thesis. Animals, like humans, show a degree of spontaneous recovery, and as a result it can be difficult to evaluate and pinpoint treatment effects. Unfortunately, there lies significant difficulty in relating widespread stroke damage to a single behavioural measure and so a multitude of high power behaviour tests are required to assess functional outcomes. Therapeutic strategies aimed towards enhancing plasticity after stroke require assessment of long-term functional recovery, as such preclinical tests demonstrating long-lasting deficits are more relevant for the application of treatment in clinical situations (Bouët et al., 2007; Freret et al., 2006; Schaar et al., 2010; Schallert et al., 2000). Additionally, selecting the most appropriate frequency of assessments is important as repeated behavioural testing can confound restoration of function, because plasticity is influenced by experience (Borlongan, 2000; Schallert et al., 2000; Will and Kelche, 1992). The selection of behaviour tests continues to be a subject of considerable debate and requires careful consideration and evaluation. Overall, preclinical tests should be sensitive to the particular injury location, extent of damage, and treatment benefit for successful functional evaluation after stroke (Schaar et al., 2010).

6.2.4 Further characterisation of pre-differentiated cell grafts in vivo

All tissue sections generated from rat brains treated with stem cell transplants were exhausted by the end of this study. As such many markers to characterise cell
maturation and fully characterise effects of transplants on endogenous responses were not achieved. Future experiments should include investigating reconstruction or reorganisation of neuronal circuits, and defining potential synaptic host-graft connections. To further confirm maturation of pre-differentiated cells, staining with microtubule-associated protein 2 (MAP2) could be used as it is an abundant neuronal cytoskeletal protein that is present in the later phases of maturation. TuJ1 (class III β-tubulin) is a neuronal marker that is expressed early after leaving the cell cycle and so may represent both immature and mature neuronal populations. In addition, investigating the expression of various other proteins including SNARE or SNAP proteins would assist in confirming calcium signalling, synapse docking and neurotransmission events involving exogenous cell grafts within the stroke damaged brain (Hanson et al., 1997). Further functionality studies could include investigating the expression of glutamate receptor (GluR) 2/3 or D2 dopamine receptors within graft sites, thus indicating either re-establishment of cortico-striatal pathways or nigrostriatal dopaminergic pathways respectively for cognitive and motor functional recovery (Chen et al., 2002; Dunnett et al., 1988; Dunnett and Rosser, 2007; Helm et al., 1991; Lovinger, 2010; Mukhida et al., 2008). Ultimately, the use of anterograde and retrograde labelling would substantiate connectivity of graft-host neural systems (Lindvall et al., 2012). Unfortunately due to the limitations and practicality of our study, these experiments could not be performed.

Calcium binding proteins (CBPs) are expressed by both glutamatergic and GABAergic neurons (DeFelipe, 1993). Confirmation of CBP expression by grafted GABAergic cells in Chapter 5 required triple immunofluorescent staining with markers for CBP, human-specific HuNu, and GABA. Unfortunately, due to limitations in the species our antibodies were raised in, triple staining could only be performed with parvalbumin, and not calbindin-D28k or calretinin. It is important to note that we utilised adjacent sections for staining with GABA, calbindin-D28k, calretinin or parvalbumin from the same graft site and animal. Furthermore, transplanted human GABAergic neurons morphologically possess smaller nuclei that are easily distinguishable to human glutamatergic neurons. SVZ-derived hNPCs have a greater propensity to become GABAergic rather than glutamatergic neurons (Kobayashi et al., 2010) and this was confirmed by stereological cell counts of immunolabelled cells, where 90-93% hNPCs were positive for GABA within the pre-differentiated hNPC.
cohort. Therefore it is highly unlikely transplanted cells converted to glutamatergic and we believe expression of calbindin-D28k and calretinin was indeed from human GABAergic cell transplants in our study.

6.2.5 Cell-based therapy for lacunar stroke sufferers

The new advances described in this thesis may open the door to techniques applicable to a variety of cell sources for cell-based treatment strategies for stroke. However, tissue replacement as a therapeutic goal for severe stroke is almost certainly beyond the scope of current therapeutic approaches. The aftermath of severe strokes results in widespread damage of functionally diverse brain regions and loss of specific neuronal subtypes and highly complex circuits involved in a multitude of functions (Carmichael, 2005). The lack of appropriate signals for axonal regeneration and the hostile environment of the damaged brain significantly hamper the ability of exogenously transplanted cells to replace the full complement of neuronal subtypes affected and re-construct functional circuitry. Given this immense challenge with widespread damage, lacunar strokes may be more suitable for the application of cell replacement based strategies. Lacunar strokes are small subcortical infarcts that result in damage to deep brain structures including the basal ganglia, thalamus, internal capsule, corona radiate and brainstem that is caused by occlusion of a single penetrating artery (Fisher, 1982; Lammie, 2000; Norrving, 2008). The benefits from exogenous stem cell transplants independent of promoting neuronal differentiation or integration into host circuitry and rather mediating trophic effects and promoting plasticity may suffice to treat stroke patients suffering large damage (Takahashi et al., 2008). However, as highlighted from the STEPS meeting in 2014, application of stem cells to replace lost brain tissue and re-establish lost connections is still an important goal for future studies and requires continued investigation (Dihne et al., 2011; Savitz et al., 2014). Therefore, the use of pre-differentiated cell transplants may be more suitable for treatment of lacunar stroke sufferers with small subcortical infarcts as a cell replacement strategy (Arboix and Marti-Vilalta, 2009). Nonetheless, extensive advances in preclinical testing of pre-differentiated cells for stroke treatment are required before implementation in clinical studies (Dihne et al., 2011).
6.3 Key Themes and Conclusions

Key elements that have emerged across all studies conducted in this thesis include harnessing endogenous recovery events such as angiogenesis and identifying reactive gliosis as a barrier to brain remodelling and neurological recovery. Experimental evidence provided throughout this thesis highlight the important influence of the surrounding microenvironment on brain regeneration after cerebral ischemia, which is critical in developing restorative therapies. Restorative treatments possess the potential to treat the majority of patients without being hampered by reduced tissue perfusion, which is known to restrict the delivery of neuroprotective agents, nor do they require rapid intervention immediately after stroke. In view of the complexity of the mechanisms involved, current therapies that stimulate and amplify endogenous restorative responses may also provoke unwanted side-effects such as additional glial scar formation, a major barrier for brain repair. Hence steps are required to ensure adverse side-effects do not outweigh the benefits of treatment. In this thesis we investigated both inflammatory and regenerative responses to stroke severity in hopes to better understand mechanisms that lead to functional recovery as well as the histopathological hurdles that delay or impede recovery, so as to highlight the need to augment naturally occurring positive capabilities of the brain. We have emphasised varying stroke severities are an important factor to consider when investigating therapeutic interventions to ensure subsequent results are due to treatment and not varied lesion size. More interesting however is the potential for treatments aimed towards enhancing endogenous SVZ cell proliferation and migration in contributing further to astrogliosis and scar formation and thus may require additional efforts to promote neuronal differentiation to avoid such a result.

A number of considerations have emerged in moving forward with cell-based therapies in preclinical studies described in this thesis. First we confirmed CsA did not mediate neuroprotective effects or alter repair processes after stroke and therefore would not confound experimental findings in cell-based treatment studies. However CsA pre-treatment was observed to attenuate over activation of astrocytes and retain pro-survival morphologies supportive of brain repair with reduced neurological deficits early after stroke that warrant further investigation. Next we identified potential risks with the use of undifferentiated hNPC transplants as they predominantly differentiated
into astrocytes and contributed further to scar formation. This again supports the concept of promoting neural differentiation of undifferentiated stem cells/progenitor cells prior to transplant to avoid further complications. As such, a major novel strategy we have highlighted is the use of pre-differentiated human neuronal transplants to accelerate and improve functional recovery and potentially optimise the prescription of restorative cell-based treatments. The influence of pre-differentiated cell transplants on neurogenesis and subsequent functional improvements possibly suggest regenerative events are enhanced by the use of neural lineage cells for transplant.

Potential applications for stem cell therapies are vast with a plethora of experimental and clinical trials assessing their use in stroke. Development of its use and understanding of the mechanisms of stem cell repair are still in its infancy. Many challenges remain; however, the principles behind differentiating cells prior to transplant to re-establish function after stroke may serve as a favourable alternative in cell-based therapies to be considered in future efforts. It is with hope that the work presented in this thesis has expanded our understanding for endogenous remodelling mechanisms after ischemic stroke and has identified potential treatment strategies to reduce the devastating impact that stroke has on human life.
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