



The adult neural stem cell niche in ischaemic stroke

A thesis submitted to the Department of Physiology, Anatomy and Genetics,
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Doctor of Philosophy

by

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For my parents

ABSTRACT

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Ischaemic stroke is a major cause of mortality and chronic disability for which there is no effective treatment. The subventricular zone (SVZ) is an adult neurogenic niche which mediates limited endogenous repair following stroke. To harness this phenomenon for therapy, it is important to understand how the SVZ niche is altered in stroke, and the processes that recruit neural precursors to the site of injury, which becomes a *de facto* neurogenic niche. Galectin-3 (Gal-3) is a β -galactoside binding protein involved in cellular adhesion, inflammation and tumour metastasis. Gal-3 is specifically expressed in the SVZ and maintains neuroblast migration to the olfactory bulb, although its role in post-stroke neurogenesis is not well-understood. Therefore, this project aimed to (1) characterise the cytoarchitecture of the SVZ in response to stroke, and (2) examine the role of Gal-3 in stroke outcome and tissue remodelling, and test the hypothesis that Gal-3 is required for neuroblast ectopic migration into the ischaemic striatum.

Using the intraluminal filament model of middle cerebral artery occlusion (MCAO) in mice, and whole mounts of the lateral ventricular wall, significant SVZ reactive astrogliosis and increased vascular branching were observed, thereby disrupting the neuroblast migratory scaffold. Stroke increased SVZ cell proliferation without increase in cell death. Post-stroke ependymal cells were enlarged and non-proliferative, and assumed a reactive astroglial phenotype, expressing *de novo* high levels of glial fibrillary acidic protein. This was associated with focal planar cell polarity misalignment, and turbulent and decreased rate of cerebrospinal fluid flow. These findings demonstrate significant changes in multiple SVZ cell types which are positioned to influence post-stroke neurogenesis and regulation of the neural stem cell niche

Gal-3 was up-regulated in the ischaemic brain and ipsilateral SVZ. To elucidate the role of Gal-3 after stroke, MCAO was performed in wildtype and Gal-3 null (Gal-3^{-/-}) mice, and parameters of stroke outcome and post-stroke neurogenesis compared. The deletion of Gal-3 did not affect infarct volumes or neurological outcomes, although neuroblast migration into the ischaemic striatum was increased in Gal-3^{-/-} brains. Gal-3^{-/-} mice failed to mount an angiogenic response in the ischaemic striatum, and this was associated with lower levels of vascular endothelial growth factor (VEGF) and increased anti-angiogenic protein levels. Loss of Gal-3 further disrupted the pro-proliferative neurovascular interaction at the basement membrane. The current data indicate that Gal-3 is a pleiotropic molecule which has distinct roles in both the SVZ and the post-stroke striatum as niches of adult neurogenesis.

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AUTHORSHIP STATEMENT

I conceptualised and designed the experiments in this thesis with inputs from my supervisor, Dr Francis Szele.

For characterisation of the post-stroke subventricular zone, Ms Judith van der Harg assisted in parts with tissue dissection, immunohistochemistry and quantification under my supervision.

For the galectin-3 study, the 2-photon neuroblast migration experiment was carried out in collaboration with Dr Isabelle Comte and Dr Yongsoo Kim. Dr Comte performed stereotactic injections, Dr Kim performed time-lapse imaging and I performed quantification and analysis. I trained Ms Micaela Vidal Pascual, who perform blinded neurological status assessment in my presence. Ms Nicola Lewis helped run immunohistochemistry, genotyping and contributed to blinded quantification.

Parts of Chapter 1 have been published in (Young et al., 2011; Chen et al., 2011). Parts of Chapter 5 have been published in (Comte et al., 2011). Parts of Chapter 3 have been submitted as a manuscript for peer-review.

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Movie 5.1. Neuroblast migration in the RMS in wildtype

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ABBREVIATIONS

ace tub	acetylated tubulin
ADAMTS1	a disintegrin and metalloproteinase with thrombospondin motifs 1
ANOVA	analysis of variance
β -cat	beta-catenin
BrdU	5-bromo-2'-deoxyuridine
CBF	cerebral blood flow
CCA	common carotid artery
CSF	cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
Dcx	doublecortin
ECA	external carotid artery
ECL	enhanced chemiluminescence
FGF	fibroblast growth factor
GABA	gamma-aminobutyric acid
γ -tub	gamma-tubulin
Gal-1	galectin-1
Gal-3	galectin-3
GFAP	glial fibrillary acidic protein
HRP	horseradish peroxidase
ICA	internal carotid artery
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein

IL-1 β	interleukin 1 β
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
NeuN	Neuronal Nuclei
NSC	neural stem cell
NSPC	neural stem/progenitor cell
OB	olfactory bulb
OPC	oligodendrocyte precursor cell
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween
PCP	planar cell polarity
PCR	polymerase chain reaction
PECAM	platelet endothelial cell adhesion molecule-1
PHi3	phosphorylated histone H3
PSA-NCAM	polysialylated neural cell adhesion molecule
RMS	rostral migratory stream
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SGZ	subgranular zone
Shh	sonic hedgehog
siRNA	small interfering RNA
SVZ	subventricular zone
TNF α	tumour necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

“Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centres, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.”

- Ramón y Cajal, 1928

Chapter 1

Introduction

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1.1. Stroke: The global burden of disease

Stroke is a neurological condition caused by the interruption of the blood supply to the brain, when a blood vessel is narrowed or blocked by a clot (ischaemic stroke), or if a vessel ruptures and bleeds (haemorrhagic stroke; Mackay and Mensah, 2004). The acute cessation of oxygen and nutrient supply causes damage to brain tissue and results in brain infarction. The effects of stroke depend on which part of the brain is injured and how severely it is affected, and are characterised by the rapid onset of focal neurological signs and symptoms (Longmore et al., 2002). The most common symptom of stroke is sudden motor weakness or numbness of the face, arm or leg, usually on one side of the body. Other symptoms include: confusion; difficulty speaking or understanding speech; visual disturbances; difficulty walking, with loss of balance or coordination; severe headache; and altered level of consciousness. A very severe stroke can cause sudden death.

Stroke is the third leading cause of death in the world, accounting for approximately 10% of all deaths (5.5 million), behind coronary heart disease and all forms of cancer combined (World Health Organisation, 2002; Fig 1.1A). Annually, 15 million people worldwide are affected by stroke, with ischaemic stroke constituting ~90% of cases, while haemorrhagic stroke accounts for ~10% (Mackay and Mensah, 2004). Successful management of cerebrovascular risk factors such as the treatment of hypertension and reduced prevalence of cigarette smoking has contributed to a decreased incidence of stroke in the last 20 years in several developed countries including the UK (Rothwell et al., 2004). However, stroke is predominantly a disease of the elderly (Fig 1.1B) and therefore remains a major public health concern in our ageing society (Feigin et al., 2003). A recent US study further reports an alarming 50% increase in stroke incidence in men between the ages of 35 – 45 from 1995 to 2008 (George et al., 2011).

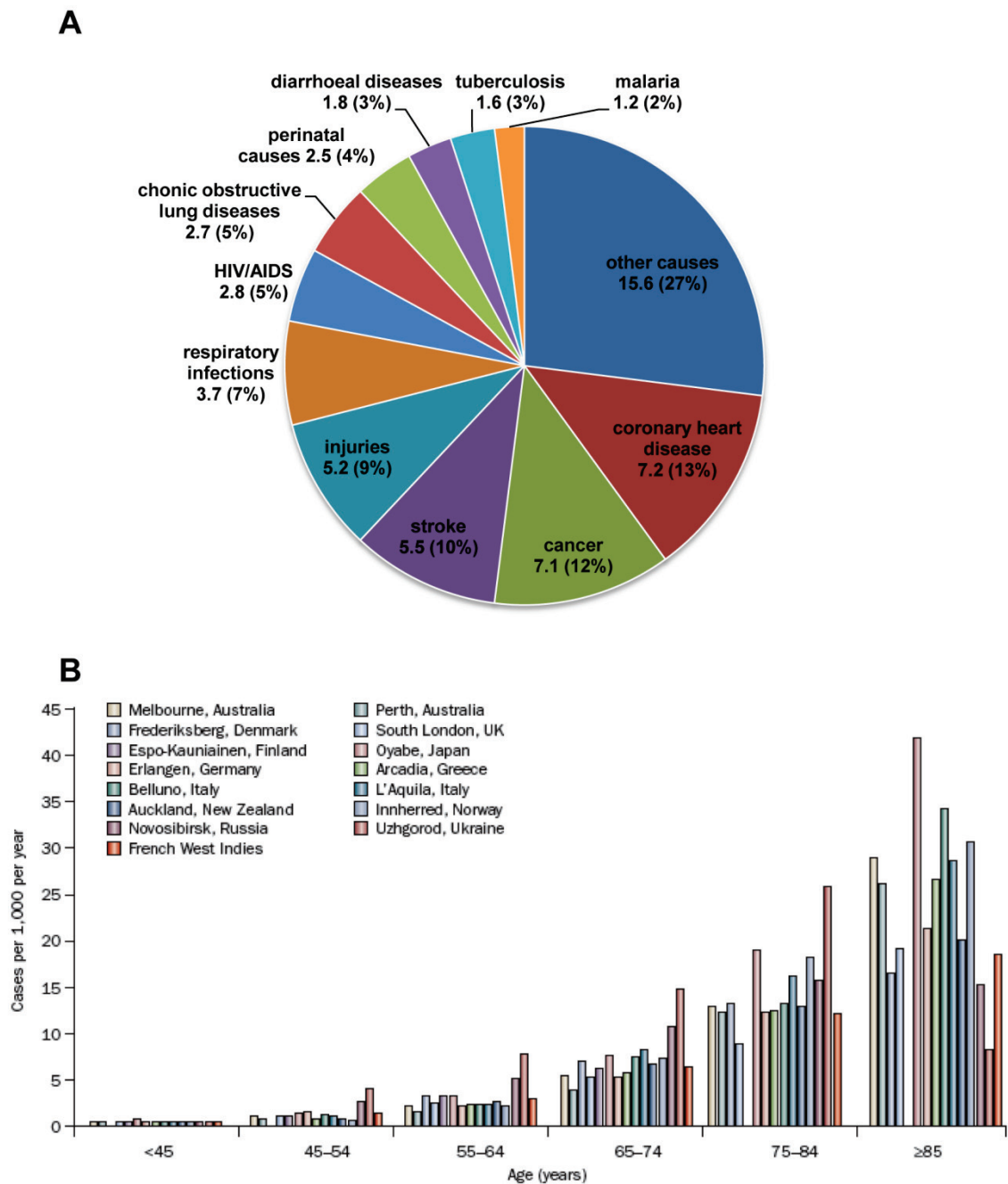


Figure 1.1. Stroke: The burden of disease

- (A) Causes of mortality world-wide (World Health Organisation, 2002).
- (B) Incidence of ischaemic stroke in different ages groups (Chen et al., 2010).

Stroke is a leading cause of chronic disability and results in permanent disability in a third of the 15 million stroke patients each year. In the UK there are over 350,000 people living with moderate to severe disability due to stroke, with an estimated annual cost of over £5-billion to the economy (National Audit Office, 2005). At an individual level, stroke can be a devastating entrapment and place huge financial and emotional pressures on one's family and carers.

In developing countries, communicable diseases account for ~75% of deaths. However, as developing communities adopt “Western” lifestyles without access to adequate healthcare, the incidence of stroke is often higher and the outcomes worse than in developed countries (Fig 1.2). This is evidenced by the higher loss of disability-adjusted life years (DALY) due to stroke in developing countries compared to developed countries (Fig 1.2B). For instance, the loss of DALY due to stroke in South Africa (12.84 DALYs lost /1000 population) is nearly 4 times that of the UK (3.48 DALYs). The global burden of disease is highlighted by the high rates of mortality and DALYs lost in emerging BRICS nations (Brazil, Russia, India, China and South Africa) which constitute close to 50% of the world's population (Fig 1.2A).

1.2. Treatments for ischaemic stroke

Ischaemic stroke¹ occurs when cerebral blood flow is physically obstructed due to atherosclerosis and thrombus formation or thromboembolic event. Atherosclerosis results in build up of atherosclerotic plaques on the vessel wall which hardens and narrows the vessel. These changes, together with long-standing hypertension, damage the arterial wall and lead to platelet aggregation and thrombus formation with varying degree of flow

¹ “Stroke” refers to ischaemic stroke in this thesis unless otherwise specified.

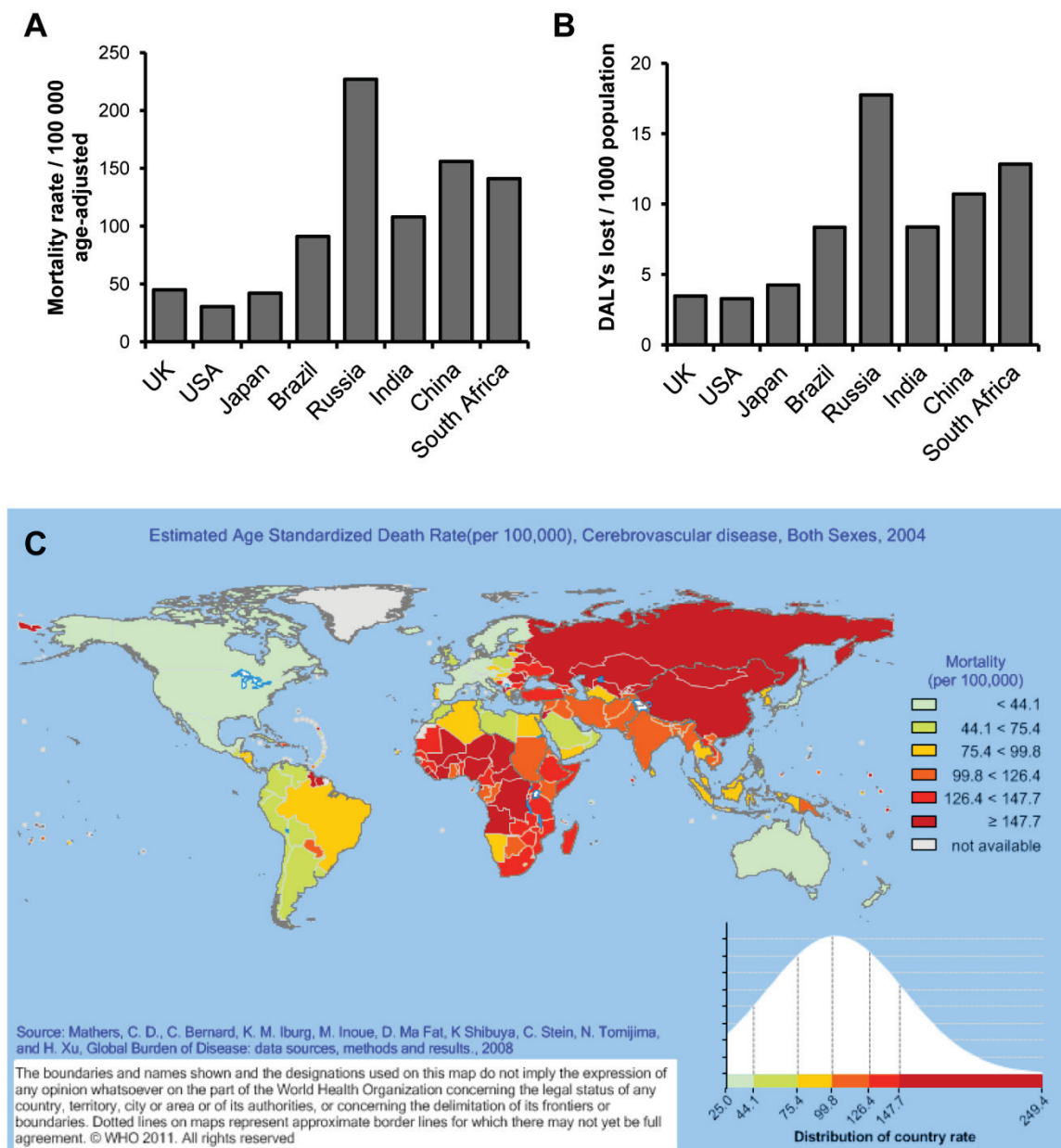


Figure 1.2. The stroke epidemic in developing countries

- (A) Age-adjusted mortality rate due to stroke.
- (B) Disability-adjusted life years lost due to stroke.
- (C) World map showing mortality rate due to stroke. Darker colour represents higher mortality.

Data from WHO Online Database <https://apps.who.int/infobase/Comparisons.aspx>

obstruction. If this process occurs in small cerebral vessels, such as the lenticulostriate arteries, local thrombotic occlusion may cut off blood flow to brain tissue distal to the thrombus and in the absence of collateral blood supply, result in stroke. Alternatively, fragments of atherosclerotic plaques and thrombi in a large artery outside the brain, e.g. the carotid artery, may become dislodged and carried by the circulation into the cerebral vasculature, eventually occluding smaller downstream vessels, and resulting in thromboembolic stroke. Atrial fibrillation, myocardial infarction and prosthetic cardiac valves are other common cardiogenic causes of thromboembolic stroke.

The most effective strategy against stroke has therefore been one of prevention, targeting risk factors associated with atherosclerosis, such as hypertension, hypercholesterolaemia, type 2 diabetes and cigarette smoking (Chen et al., 2010). Patients and their physicians have become knowledgeable about lifestyle choices and have at their disposal a wide selection of effective pharmacological agents to reduce these risk factors (Rothwell et al., 2004).

The emphasis on stroke prevention partially reflects the limited therapeutic options available once stroke has occurred. Limiting the extension of acute stroke and establishing reperfusion of the ischaemic brain are the cornerstones of current treatment. Anti-platelet and anti-coagulant therapies are the first-line treatments to prevent the progression of stroke. For patients with no contraindications, and where expertise is present, systemic intravenous thrombolysis with recombinant tissue plasminogen activator (rt-PA) is associated with significantly improved neurological outcome (Sutherland et al., 2011). In a large non-randomised and single-blinded trial, stroke patients treated within the first 3 hours with intravenous rt-PA were 30% more likely to have a favourable outcome compared to the placebo group when followed up at 1 year (Kwiatkowski et al., 1999). For patients in whom intravenous thrombolysis is contraindicated, intra-arterial

thrombolysis, endovascular embolectomy and percutaneous transluminal angioplasty are some of the highly advanced surgical techniques used to restore blood flow. However, the safety and benefits of these procedures remain to be confirmed (Brinjikji et al., 2011; Fiorella et al., 2011).

Currently, neuronal loss is permanent and there is no effective neurorestorative therapy which can repair or replace damaged neuronal circuits. Physiotherapy and rehabilitation can lead to some degree of neural plasticity, adaptation and functional recovery (Bosnell et al., 2011), although for most patients, the improvements are inadequate and unsatisfactory.

1.3. Stem cells for the treatment of stroke

Unlike traditional preventative and neuroprotective strategies, stem cells offer the possibility of repairing damaged neural tissue at a delayed time-point following stroke. Therefore, over the last twenty years, there has been great impetus to determine whether stem cells from a variety of sources may be utilised for functional brain repair following ischaemic stroke. “Stem cell” is a term used to broadly define cell populations characterised by self-renewal and multipotency (Hanna et al., 2010). Self-renewal enables the stem cell population to replenish itself in long-term maintenance through a balance of symmetric and asymmetric cell divisions (Spradling et al., 2001). Multipotent cells give rise to daughter cells which differentiate into multiple distinct cell types. The extent to which self-renewal and multipotency have to be satisfied for inclusion as stem cells is debated. Therefore, stem cells encompass numerous cell types from various stages of development in different organ systems. Stem cells can be classified into three types according to their abilities to differentiate (Jaenisch and Young, 2008). The first type is

totipotent cells which can generate all cell types including germ cells and extra-embryonic cells, and give rise to the entire organism. The only totipotent cells are fertilised eggs and their cleavage product at a very early stage of development. The second type is pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). They give rise to derivatives of all three embryonic germ layers including ectoderm, mesoderm and endoderm. The third type is multipotent stem cells which are reservoirs of undifferentiated cells that divide to generate some or all of the major cell types of the tissue or organ, and can be found among differentiated cells during various stages of development and adulthood. The subventricular zone (SVZ) in the adult brain is a specialised niche where multipotent stem cells reside.

An increasing number of clinical trials and preclinical studies have provided evidence that neurorestorative stem cell-based therapies can lead to functional recovery in stroke patients (Burns et al., 2009). Clinical approaches to stem cell therapy in stroke can be broadly divided into 2 categories: (1) the stimulation of *endogenous* multipotent stem cells, e.g. adult neural stem and progenitor cells (NSPCs), and bone marrow haematopoietic and mesenchymal stem cells, and (2) the transplantation of diverse populations of *exogenous* stem cells derived from embryonic tissue (ES), foetal and adult brain, other organs (e.g. bone marrow) and immortalised or iPS cell lines (Hess and Borlongan, 2008). The enormous therapeutic potential of stem-cell therapy for stroke is emphasised by a number of completed clinical trials which demonstrate at least safety and feasibility of delayed intracerebral stem cell delivery. In the first clinical trial using an immortalised human neural stem cell line for stroke therapy, functional recovery in long-term follow-up was limited (Kondziolka et al., 2000). In a subsequent Phase II trial, 6 of the 14 patients who received cell therapy showed improvements as measured by a standardised stroke scale, although this was not statistically significant compared to

control subjects (Kondziolka et al., 2005). However, post-mortem analysis in one patient, who died from a myocardial infarction 2 years following cell transplant, showed that the transplanted cells were present and retained neuronal phenotype, did not form tumours, and did not elicit injurious tissue inflammation (Nelson et al., 2002). In 2010, the first UK-based stem cell clinical trial for stroke was commenced to assess the safety and feasibility of a proprietary foetal-derived neural stem cell line for the treatment of chronic ischaemic stroke (Stroemer et al., 2009; ReNeuron, 2010). There are also multiple Phase I/II trials underway to test the efficacy of autologous haematopoietic stem cells and mesenchymal stem cell transplantation for chronic stroke (Table 1.1). The autologous approaches, like the stimulation of endogenous NSPCs in the SVZ, are clinically attractive as they bypass ethical concerns and nullify the risk of immune-rejection. The systemic administration of granulocyte-colony stimulating factor (G-CSF) also shows tremendous promise in mobilising endogenous haematopoietic stem cell pools in pre-clinical studies (Piao et al., 2009) and a randomised trial using subcutaneous G-CSF has yielded promising results (Shyu et al., 2006), although the mechanism of improvement is not clear.

This thesis concentrates on neural stem cells that are found endogenously in the adult SVZ, with a particular focus on the mechanisms which permit neural precursors to divert from their normal migratory pathway, and relocate towards the ischaemic brain tissue where they mediate endogenous repair. The following sections review current understanding of the endogenous neural stem cell population and its response to ischaemic stroke, as well as its therapeutic potential.

Table 1.1. Clinical trials of stem cell therapy for stroke

Cells type	Study design	Stroke type & location	No. of patients	Latency to delivery	Route of delivery	Endpoint / outcome	Status	Ref / NLM ID
NT2 teratoma-derived neurons	Phase I NR-SB	Ischaemic Striatum	12	6 months – 6 years	IC	Safe and feasible. Some functional improvement	Complete	Kondziolka et al., 2000
NT2 teratoma-derived neurons	Phase II R-SB	Ischaemic / Haemorrhagic Striatum	18	1 – 6 years	IC	Safe and feasible. No significant functional improvement	Complete	Kondziolka et al., 2005
NT2 teratoma-derived neurons	Phase II R-OB	Ischaemic Basal ganglia	9	29 – 70 months	IC	Safe and feasible. Some functional and cognitive improvements	Complete	Stilley et al., 2004
Autologous MSC	Phase I/II R-OL	Ischaemic Striatum / cortex	30	4 – 9 weeks	IV	Safe and feasible. No significant functional improvement	Complete	Bang et al., 2005
Autologous BMMNC	Phase I NR-OL	Ischaemic Striatum / cortex	6	3 – 90 days	IA	Safe and feasible	Complete	Battistella et al., 2011
Proprietary foetal neural stem cell line	Phase I NR-OL	Ischaemic Striatum / subcortical	12	6 months – 5 years	IC	Safety and feasibility	Ongoing	NCT01151124
Autologous BMMNC	Phase I NR-OL	Ischaemic Not specified	10	24 – 72 hours	IV	Safety and feasibility	Ongoing	NCT00859014

Autologous MSC	Phase II R-OL	Ischaemic carotid embolic	30	< 6 weeks	IV	Safety, feasibility and efficacy	Ongoing	NCT00875654
Autologous BMMNC	Phase I/II NR-OL	Ischaemic carotid embolic	12	7 – 10 days	IV	Safety, feasibility and efficacy	Ongoing	NCT01028794
Autologous CD34+	Phase II R-OL	Ischaemic MCA-T	30	6 – 60 months	IC	Efficacy	Ongoing	NCT00950521
Autologous CD34+	Phase I/II R-OL	Ischaemic MCA-T	10	7 days	IA	Safety, tolerability and efficacy	Ongoing	NCT00535197
Autologous CD34+	Phase I/II	Ischaemic MCA-T	20	5 – 9 days	IA	Safety and efficacy	Ongoing	NCT00761982
Autologous BMMNC / stromal cells	Phase I	Ischaemic MCA-T	33	< 72 hours	IV	Safety of 2 autologous cell types	Recruiting	NCT00908856
Allogenic MSC	Phase I/II NR-OL	Ischaemic not specified	35	> 6 months	IV	Safety and efficacy	Recruiting	NCT01297413

NLM ID, National Library of Medicine Identifier (<http://www.clinicaltrials.gov>); NR, Non-randomised; R, Randomised; OL, Open labelled; SB, Single blinded, BMMNC, bone marrow mononuclear cells; MSC, mesenchymal stem cells; MCA-T, Middle cerebral artery territory; IC, Intracerebral; IV, Intravenous; IA, Intra-arterial.

Modified from joint publication in (Chen et al., 2011).

1.4. Adult neurogenesis

A central dogma of neurobiology was that new neurons were not added to the adult mammalian brain (Gross, 2000). It was thought that neurogenesis was confined to embryonic development and a short postnatal period (Cajal, 1991) and the assumption was never seriously challenged until the mid 20th century. Smart and Altman began to erode the concept of an immutable adult mammalian brain by demonstrating cell division in the subependymal (Messier et al., 1958) and hippocampal (Altman and Das, 1965) regions with tritiated thymidine autoradiography, and evidence of newborn cells with neuronal morphology. Using electron microscopy, (Kaplan and Hinds, 1977) demonstrated that the newborns cells in the olfactory bulb possessed ultrastructural features of neurons. However, it was in the 1990s that the concept of adult neurogenesis gained widespread acceptance, particularly following generation of self-renewing and multipotent neurospheres from the adult mammalian brain (Reynolds and Weiss, 1992) and the use of thymidine analogues and retrovirus lineage tracing to label newborn cells combined with their colocalisation with neurone-specific molecular markers (Luskin, 1993; Kuhn et al., 1996).

Two distinct populations of endogenous neural stem cells reside in the mammalian brain and continue to generate neurons throughout adult life. This process, known as adult neurogenesis, occurs in the SVZ which lines the lateral ventricles (Fig 1.3), and the subgranular zone (SGZ) of the hippocampal dentate gyrus. Adult neurogenesis has been documented in all mammalian species examined, including rodents, dogs, cats, non-human primates and humans (Lois and Alvarez-Buylla, 1993; Gould et al., 1999; Eriksson et al., 1998; Sanai et al., 2004). In the SVZ, astrocyte-like neural stem cells and progenitor cells reside in a specialised niche, and give rise to neuroblasts which migrate

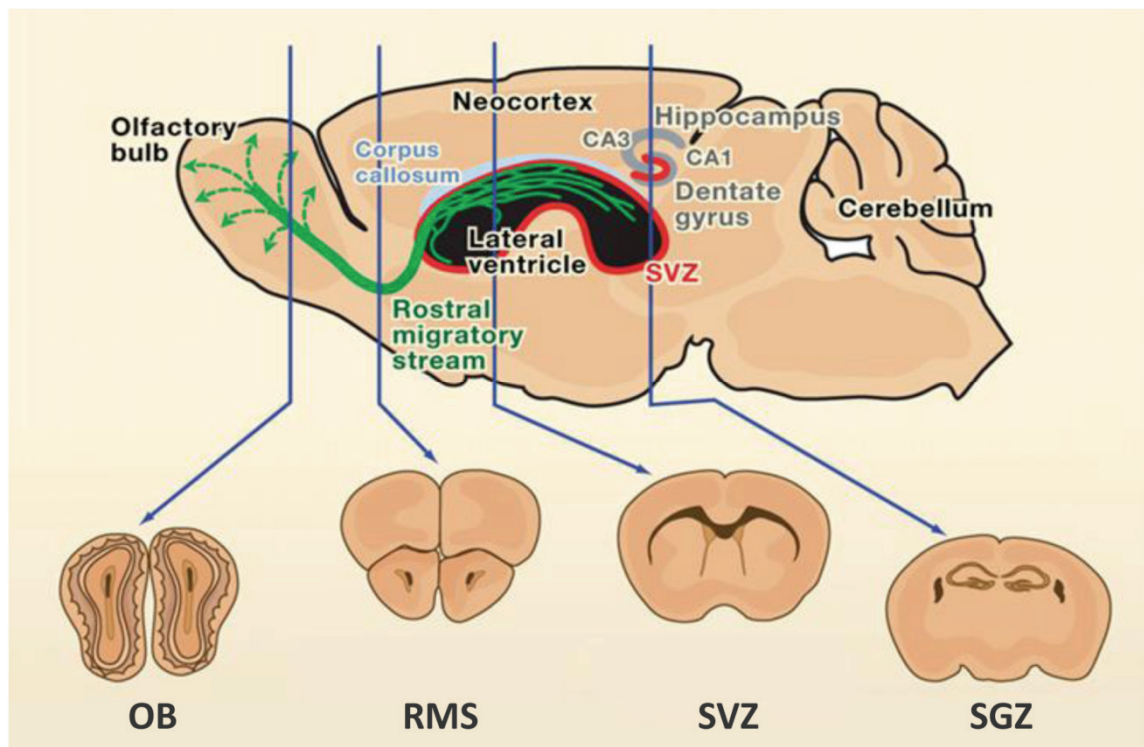


Figure 1.3. Adult neurogenesis in the mammalian brain

Sagittal section through the mouse brain showing sites of adult neurogenesis. New neurons are generated in the subventricular (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (*red*). In the SVZ, newborn neural precursors migrate long-distance to the olfactory bulb and differentiate into mature neurons (*green*). *Blue* lines indicate plane of sectioning and corresponding appearance in coronal sections. Modified from (Zhao et al., 2008).

OB, olfactory bulb. RMS, rostral migratory stream.

long distances through the rostral migratory stream (RMS) to the olfactory bulb (OB), where the majority differentiate into GABAergic interneurons (Lois and Alvarez-Buylla, 1994), and some into glutamatergic interneurons (Brill et al., 2009). In the SGZ, neurogenesis gives rise to granule interneurons that provide excitatory inputs to area CA3 (Kempermann, 2002). It is estimated that ~30,000 neurons are generated each day in the murine SVZ (Lois and Alvarez-Buylla, 1993). Although their functions are incompletely understood, evidence suggests that the interneuron populations confer a high degree of plasticity to the neural circuits in the olfactory bulb and dentate gyrus (Lledo et al., 2006). SVZ neurogenesis is involved in olfactory discrimination, while SGZ neurogenesis plays a role in learning and memory formation (Zhao et al., 2008). Defective or insufficient adult neurogenesis has been associated with a wide spectrum of neurodevelopmental and neurodegenerative diseases ranging from schizophrenia to Alzheimer's disease (Steiner et al., 2006). Tantalisingly, adult neurogenesis contributes to the endogenous repair mechanism after different types of brain injury such as traumatic brain injury, multiple sclerosis, intracranial haemorrhage and stroke (Szele and Chesselet, 1996; Calzà et al., 1998; Jin et al., 2001; Masuda et al., 2007). In addition, recent data further suggest that stem/progenitors cells may in fact be widely present in dormant states in various brain regions, and may be activated / re-programmed following injury to give rise to neurons and glia (Robel et al., 2011).

1.4.1. The subventricular zone neural stem cell niche

The adult neural stem cell niche is classically defined as a unique cell tissue and its non-cellular substrates which retain stem cells and control their self-renewal and progeny production, *in vivo*, in the postnatal brain (Spradling et al., 2001; Conover and Notti, 2007). The SVZ is an adult neural stem cell niche and is composed of several distinct cell

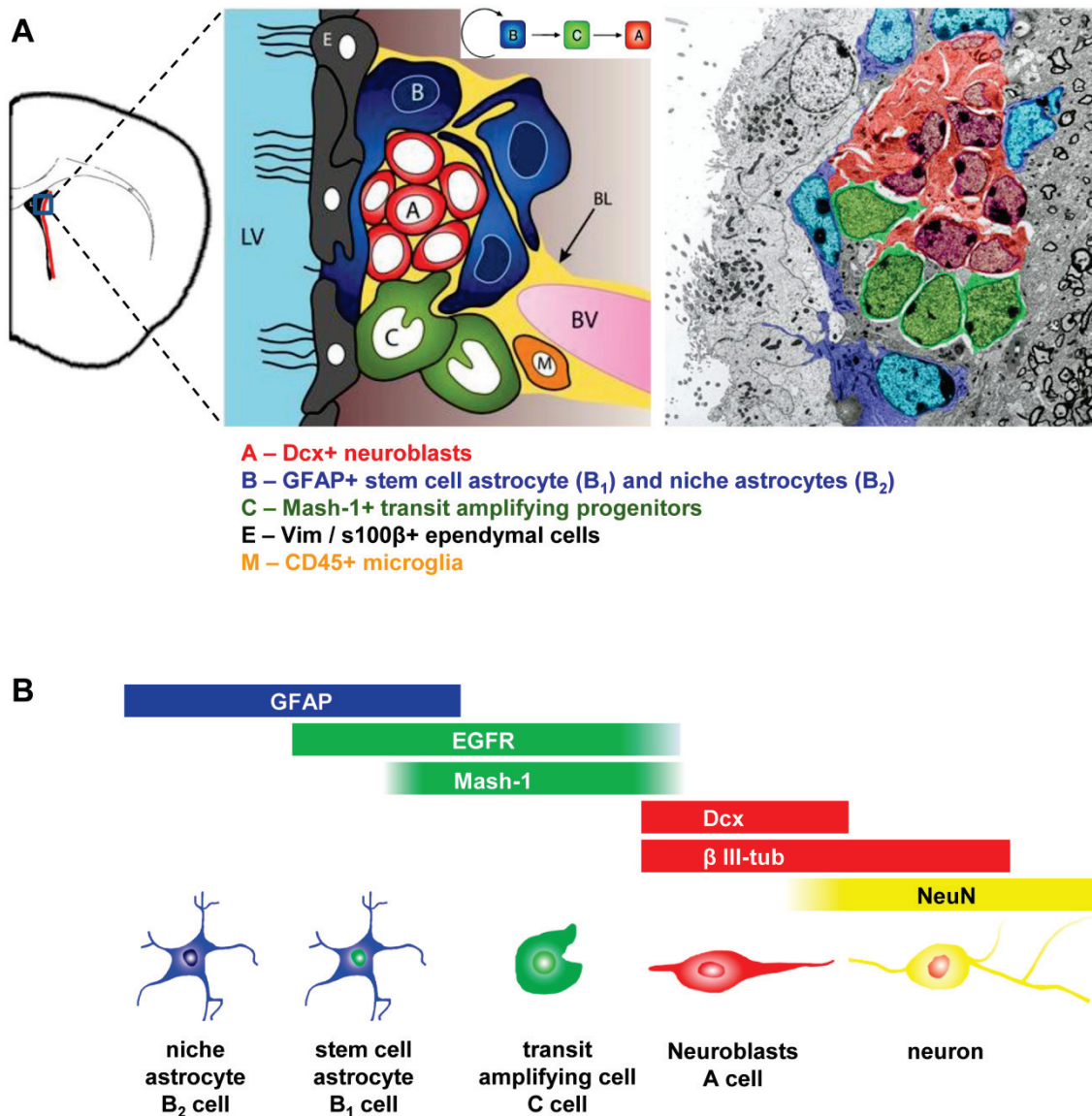


Figure 1.4. Cell types of the SVZ and their molecular expression profile

- (A) Schematic and electron-micrograph of the SVZ showing the different cell types and their close anatomical relationship with one another, as well as non-cellular components including the cerebrospinal fluid, extracellular matrix and vasculature. Modified from (Doetsch et al., 1997)
- (B) Common molecular markers used to label SVZ cell types. Note the overlapping expression of several markers such as EGFR, Mash-1 and NeuN, in different cell types.

types (Fig 1.4A) involved in the generation of new neurons and the regulation of the niche (Doetsch et al., 1997, 1999). Astrocyte-like stem cells (B_1 cells) which express glial fibrillary acidic protein (GFAP; Fig 1.4B) are derived from radial glia (Tramontin et al., 2003; Spassky et al., 2005; Garcia et al., 2004; Merkle et al., 2004) and give rise to transit amplifying progenitors (C cells). These Mash-1- and epidermal growth factor- (EGFR) expressing progenitors divide rapidly to generate doublecortin- (Dcx) expressing neural precursors (neuroblasts) which migrate through the RMS to the OB and differentiate into interneurons. In addition, astrocyte-like stem cells also give rise to a small population of NG2⁺ oligodendrocyte precursor cells (OPCs) which generate myelinating oligodendrocytes (Aguirre et al., 2004; Menn et al., 2006). The processes of quiescence, proliferation, migration, differentiation and integration are regulated by a host of different cellular and molecular signalling pathways, and closely involve other cells types in the SVZ including GFAP⁺ niche astrocytes, CD45⁺ microglia, vimentin / α 100 β expressing ependymal cells and the vasculature (Conover and Notti, 2007). The niche is also regulated by extracellular matrix (ECM) interactions (Kazanietz and French-Constant, 2011a), as well as molecules in the cerebral spinal fluid and blood (Ihrie and Alvarez-Buylla, 2011).

A remarkable feature of newborn neuroblasts in the SVZ is their ability to migrate long-distance from the SVZ, through the RMS to the olfactory bulb (SVZ-RMS-OB). This intrinsic migratory property underpins their ability to migrate into the ischaemic striatum after stroke, and makes these cells ideal therapeutic targets as they potentially solve the difficult question of cell delivery in stem cell therapy. To understand the mechanisms which govern the migration of SVZ neuroblasts into the ischaemic striatum, it is important to appreciate the tight regulation of constitutive tangential neuroblast

migration to the OB. Niche mechanisms operating at cellular and molecular levels tightly regulate this long-distance migration.

Neuroblasts form chains and migrate along one another (Wichterle et al., 1997), under the influence of cell-surface molecules such as polysialylated neural cell adhesion molecule (PSA-NCAM), and $\alpha 6 \beta 1$ integrin and its ECM protein interactions (Whitman and Greer, 2009). PSA-NCAM is a glycoprotein expressed by neuroblasts and facilitates tangential migration from the SVZ to the OB. It is a member of the NCAM surface immunoglobulin family, and the addition of PSA is believed to facilitate de-adhesion during homotypic cell migration (Cayre et al., 2009). Genetic ablation of NCAM results in small olfactory bulbs with accumulation of neural precursors in the SVZ (Tomasiewicz et al., 1993; Cremer et al., 1994). Targeted cleavage of PSA by neuraminidase acutely disrupts neuroblast migration by interfering with homotypic cell interactions and neuroblast chain formation (Hu et al., 1996). Integrins are heterodimeric cell-surface receptors that bind ECM proteins including laminin (Cayre et al., 2009), and their interactions play important roles in SVZ regulation. Functional blocking antibody against integrin $\alpha 6$ inhibits neural precursor migration and chain formation on an $\alpha 6 \beta 1$ -binding substrate *in vitro* (Jacques et al., 1998) while blocking either $\alpha 6$ or $\beta 1$ integrin subunits *in vivo* disrupts RMS neuroblast chain structures (Emsley and Hagg, 2003). The binding of $\alpha 6 \beta 1$ integrin to laminin is likely important, as loss of laminin resulted in similar phenotype (Belvindrah et al., 2007).

In addition to homotypic chain migration, neuroblasts are closely associated with blood vessels which form a migratory scaffold and mediate migration through brain-derived neurotrophic factor- (BDNF) signalling (Bovetti et al., 2007; Snapyan et al., 2009; Whitman et al., 2009). Niche astrocyte processes loosely ensheath neuroblast chains to form “glial tubes” and regulate cell movements via GABA-signalling (Bolteus and

Bordey, 2004). Niche astrocyte and neuroblast interactions through neuregulin/ErbB4 (Ghashghaei et al., 2006) and Slit1/Robo (Kaneko et al., 2010) pathways maintain astrocytic cytoarchitecture and normal neuroblast migration. Chemokines such as stromal-derived factor 1 alpha (SDF-1 α / CXCL12) promote neuroblast motility *in vitro* (Kokovay et al., 2010). Although its role in normal SVZ neuroblast migration *in vivo* is yet to be determined, SDF-1 α plays important roles in neuronal migration in the cerebellum during development (Ma et al., 1998). SVZ microglia are semi-activated and neuroblasts express several chemokine receptors including CXCR4 and CCR2, the cognate receptor for SDF-1 α and monocyte chemoattractant protein 1 (MCP-1/CCL2) respectively (Goings et al., 2006; Tran et al., 2007). The chemorepulsive Slit2-Robo signalling pathway appears to play an important role in directing neuroblasts away from the SVZ. Slit2 is expressed in the SVZ, septum and choroid plexus, and its receptors, Robo-2 and Robo-3 are expressed by neuroblasts (Nguyen Ba-Charvet et al., 1999; Wu et al., 1999). Cerebral spinal fluid (CSF) flow, which is propelled by the beating cilia of ependymal cells, establishes a chemorepulsive Slit2 gradient to direct neuroblast migration rostrally towards the olfactory bulb (Wu et al., 1999; Sawamoto et al., 2006). In addition, there is evidence that the OB may be the source of chemoattractants such as Prokineticin-2, Netrin-1, glial-derived neurotrophic factor and BDNF which direct neuroblast migration towards the olfactory bulb (Ng et al., 2005; Murase and Horwitz, 2002; Paratcha et al., 2006; Chiaramello et al., 2007). In support of the presence of chemoattractants, although removal of the olfactory bulb does not completely stop migration of neuroblasts, nor disturb the directionality of migration, there is evidence that loss of the OB results in decreased numbers of SVZ neuroblasts migrating into the RMS (Jankovski et al., 1998; Kirschenbaum et al., 1999; Liu and Rao, 2003).

1.5. The subventricular zone in post-stroke neurogenesis

After focal ischaemic stroke, there is increased SVZ cell proliferation, with accompanying ectopic migration of many neuroblasts out of their normal SVZ-RMS-OB migratory pathway, towards the injured brain regions (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002; Parent et al., 2002). Neuroblast migration towards the injury occurred in relatively large numbers, although as many as 80% of these neuroblasts died within the first 2 weeks (Arvidsson et al., 2002). Nevertheless, some of these ectopic neuroblasts would survive and differentiate into mature neurons with evidence of synaptic formation and electrophysiological activity. Using genetic Cre-loxP labelling, Yamashita and colleagues demonstrated that SVZ-derived neuroblasts migrated into the ischaemic striatal penumbra and gave rise to Neuronal Nuclei-positive (NeuN+) neurons (Yamashita et al., 2006). Newborn neurons expressed protein markers characteristic of striatal medium spiny neurons such as DARPP-32 (Arvidsson et al., 2002; Parent et al., 2002). Hou and colleagues used electron microscopy to show that SVZ derived neurons formed synapses, and electrophysiological recordings demonstrated action potentials (Hou et al., 2008). Nascent neurons also sent out axonal projections to distant structures (Magavi et al., 2000), although it is not known to what extent these newborn neurons become functionally integrated into the local neuronal circuit. The type of neuron that the SVZ can generate after stroke is also controversial. There are inconsistencies in the literature in rat and murine cerebral ischaemia models which alternatively suggest that post-injury neurogenesis in the striatum gives rise to striatum-appropriate DARPP-32+ medium spiny neurons and parvalbumin+ interneurons (Parent et al., 2002; Teramoto et al., 2003; Yoshikawa et al., 2010; Zhang et al., 2010), while others report restricted generation of calretinin+ interneurons (Liu et al., 2009b; Wei et al., 2011; Yang et al., 2008). The emerging understanding is that SVZ stem cells are heterogeneous and fate-restricted, and

unable to generate diverse neurons as previously believed (Merkle et al., 2007; Young et al., 2007). In addition there is evidence that the diversity of OB interneurons generated by the SVZ diminishes with age; the majority of interneurons made by the adult SVZ are calretinin+ (Batista-Brito et al., 2008), indicating that age-dependent restriction of cell fate choices (multipotency) may limit the SVZ's reparative potential. However, it is possible that SVZ cells may revert to a more immature state after injury.

SGZ neurogenesis increases after global and focal ischaemic stroke (Lichtenwalner and Parent, 2006). However, unlike the SVZ, newborn NPCs from the SGZ do not migrate ectopically after injury to generate functional neurons in the CA1 region where pyramidal cells are lost (Lichtenwalner and Parent, 2006).

In addition to the SVZ and SGZ where adult neurogenesis normally occurs, evidence is emerging that quiescent or potential neural stem/progenitor cells (NSPCs) may reside in normally non-neurogenic regions. These cells can be activated, and/or reprogrammed by injury to generate new cells, and participate in tissue repair. Following focal cortical injury, reactive astrocytes in the cortex and striatum generate neurons and glia. Using genetic fate mapping, Buffo and colleagues demonstrated that reactive astrocytes were able to proliferate and produced neurons *in vitro*, although *in vivo*, their progenies were restricted to glial lineage (Buffo et al., 2008). The presence of quiescent NSPCs was recently identified in neocortical layer 1 in rats. After mild ischaemic injury, these cells expressed molecular markers of embryonic NPCs such as Nkx2.1 and MafB, underwent cell division, and gave rise to functional GABAergic neurons in the cortex (Ohira et al., 2010). NSPCs have been isolated from the post-stroke cortex in mice, and gave rise to neurons and glia *in vitro* (Nakagomi et al., 2009). Cortical progenitors have been reported in Macaque monkeys following global cerebral ischaemia which were capable of dividing and generating mature neurons (Tonchev et al., 2005). Together, these

studies suggest that neurogenesis may be induced in astrocytes found outside of the SVZ and SGZ in the injured brain environment.

1.5.1. Clinical evidence of increased post-stroke neurogenesis

Although the majority of studies examining post-stroke neurogenesis have been carried out in small rodents, experimental stroke in macaque monkeys shows increased proliferation in the SVZ and SGZ, and 15 days after stroke, 1% of newborn cells express the mature neuronal marker NeuN (Tonchev et al., 2005). Important from a translational perspective, adult neurogenesis also occurs in the human brain and has been documented in the human SVZ and SGZ in post-mortem tissues (Eriksson et al., 1998; Bernier et al., 2000; Sanai et al., 2004; Curtis et al., 2007). Proliferative cells in human neurogenic compartments express similar molecular markers as those described in rodents, such as nestin, PSA-NCAM and GFAP. After stroke, increased numbers of proliferating cells and PSA-NCAM⁺ neuroblasts are observed in the ipsilateral SVZ and adjacent striatum respectively (Macas et al., 2006; Jin et al., 2006; Marti-Fabregas et al., 2010). Importantly, although neurogenesis decreases with age (Lazarov et al., 2010), aged brains retained the capacity to respond to ischaemia by increasing cell proliferation and generating PSA-NCAM⁺ neuroblasts (Macas et al., 2006). Nevertheless, the number of PSA-NCAM⁺ neuroblasts that is at the ischaemic penumbra is relatively low, and it is not known how many of them survived long term or differentiated into functional neurons. In rodent models, post-stroke neurogenesis continues for at least a year (Kokaia et al., 2006), therefore, cumulative addition of new cells, even in low numbers, could make a considerable contribution over time.

1.5.2. Mechanism of clinical improvement

Strategies which augment post-stroke neurogenesis and neuroblast migration to the injured brain regions have been associated with improved functional recovery and reduced infarct size in preclinical stroke models (Sun et al., 2003; Teramoto et al., 2003; Sugiura et al., 2005; Thored et al., 2006). The positive contribution of SVZ-mediated cellular repair is highlighted by recent works in which the selective genetic ablation of SVZ neuroblasts in a mouse model of stroke worsens functional outcome and increases stroke size (Jin et al., 2010). However, the mechanism of improvement in SVZ-mediated endogenous repair after stroke is unclear, and there is currently no direct evidence that post-stroke neurogenesis improves stroke outcome by the replacement of lost neurons or damaged neural circuits *per se*; the time frame of improvement within days, often precedes robust neuroblast migration to the stroke. Furthermore, Arvidsson and colleagues estimated that endogenous neurogenesis replaces less than 1% of neurons lost in MCAO stroke (Arvidsson et al., 2002), and although some of these newborn neurons exhibit evidence of synapse formation and fire action potentials (Hou et al., 2008), their functional significance have not been directly examined. Therefore, it is thought that neuroprotective mechanisms, including the secretion of growth factors, promotion of angiogenesis and inflammatory modulation, are responsible for the therapeutic effect of SVZ-mediated cellular repair (Ohtaki et al., 2008; Bacigaluppi et al., 2009; Li et al., 2010a; Daadi et al., 2010). It is important to note that many of the strategies which increase neurogenesis by administration or genetic over-expression of growth factors are confounded by effects other than neurogenesis. For example, in addition to its effect on increasing SVZ proliferation and recruitment of neuroblasts towards ischaemic injury, vascular endothelial growth factor (VEGF) is also a potent activator of angiogenesis and has been shown to improve cerebral blood flow in the ischaemic penumbra after stroke. Nevertheless,

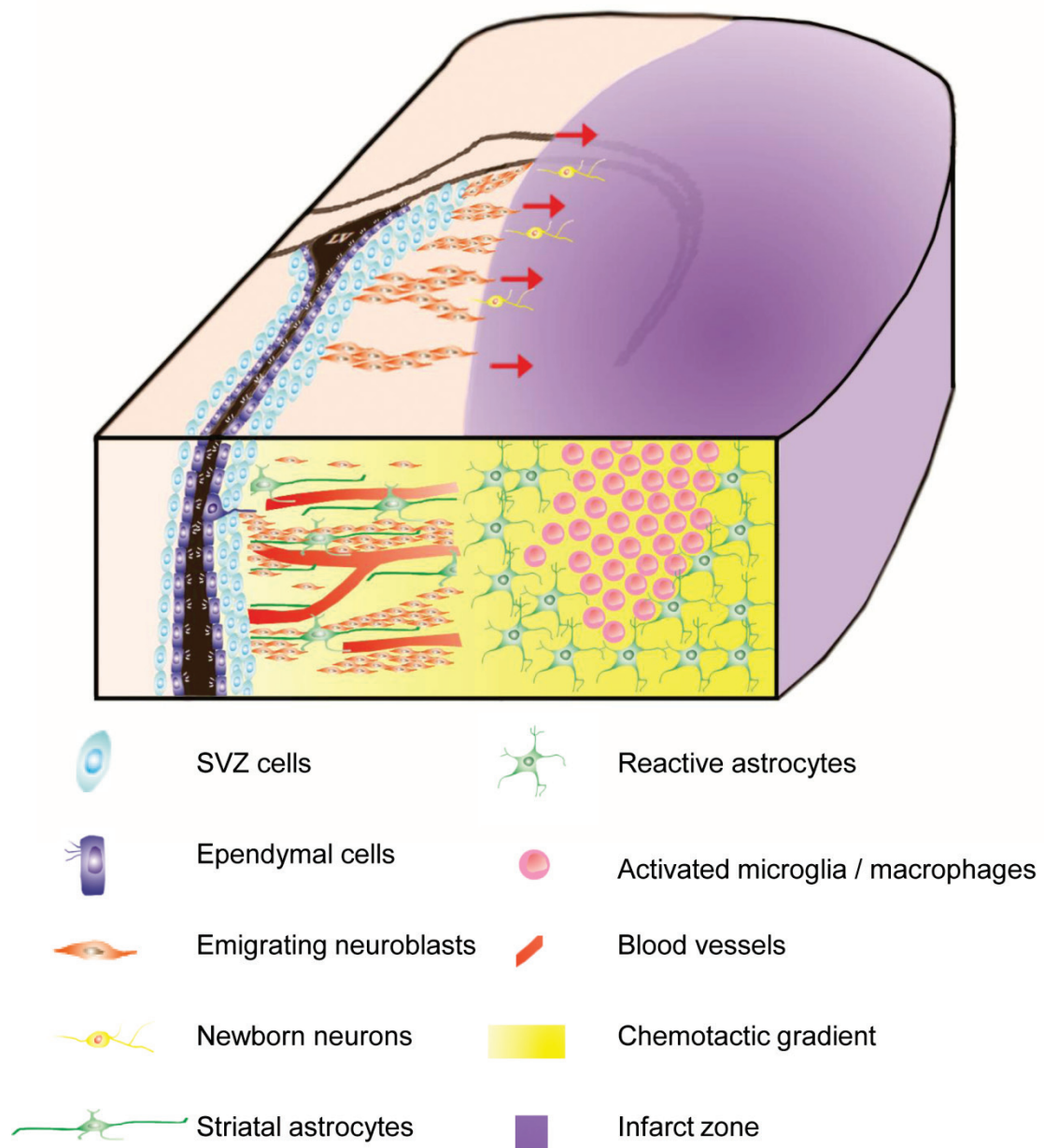


Figure 1.5. Neural precursors migrate into the ischaemic striatum after stroke

Ischemic stroke leads to a robust inflammatory response in the infarct area, with accumulation of large numbers of reactive astrocytes and activated microglia / macrophages. Inflammatory cells release various chemokines and chemoattractants such as stromal-derived factor 1 alpha and monocyte chemoattractant factor 1. Neuroblasts from the SVZ migrate up chemotactic gradients along blood vessels and astrocytic processes towards the injury. Relatively little is known about the changes in the SVZ neurogenic niche which may facilitate the exit of neuroblasts out of their normal migratory pathway into the striatum. Published in (Young et al., 2011)

despite possible confounding effects, the emerging picture is an intertwined relationship between neurogenesis and angiogenesis which may be mutually supportive (Shen et al., 2004; Roitbak et al., 2008).

1.5.3. Mechanisms of SVZ neuroblast emigration

Following animal models of stroke, large numbers of SVZ neuroblasts are able to overcome the tight regulation described above to exit their normal migratory pathway and migrate towards the injured brain regions (Parent et al., 2002; Jin et al., 2003; Zhang et al., 2001; Arvidsson et al., 2002). Neuroblast emigration commences 3 - 4 days following stroke, remains robust for several weeks thereafter, and has been observed to continue for as long as 1 year after stroke (Kokaia et al., 2006). Several molecular signals and cellular interactions which direct neuroblasts towards the ischaemic brain regions have been described. The role of reactive astrocytes and activated microglia/macrophages at the site of injury is important, as are blood vessels and astrocytic processes which form migratory scaffolds, recapitulating the route of constitutive SVZ neuroblast migration along the SVZ-RMS-OB axis (Fig 1.5).

Role of reactive astrocytes and activated microglia / macrophages

With the onset of ischaemic stroke, delivery of oxygenated blood to the brain is drastically decreased. Neurons have high metabolic demands and come under increasing oxidative stress (Lakhan et al., 2009). This results in the accumulation of reactive oxygen species and ischaemic cell death. Neuronal cell death leads to activation of the complement cascade and the initiation of the inflammatory response (Kim et al., 2008). In the ischaemic brain regions, reactive astrocytes and activated microglia / macrophages secrete

a variety of substances, including a number of chemokines and chemo-attractants which recruit SVZ neuroblasts towards the ischaemic striatum. Reactive astrocytes secrete SDF-1 α and neuroblasts express the corresponding CXCR4 receptor (Imitola et al., 2004; Ji et al., 2004; Tran et al., 2007). Over-expression of SDF-1 α results in increased neuroblast recruitment, while functional blocking antibodies or small interfering RNA (siRNA) to the CXCR4 receptor have the opposite effect (Robin et al., 2005; Thored et al., 2006; Liu et al., 2008). ERK signalling may mediate the effect of SDF-1 α on neuroblasts migration (Luo et al., 2005). Interestingly, a recent report suggests that complement derived anaphylatoxin C3a potentiates neuroblast migration at low SDF-1 α concentration, while inhibiting neuroblast migration at high SDF-1 α concentration in an ERK1/2 phosphorylation-dependent process (Shinjyo et al., 2009). This implies that neuroblasts can respond dynamically to the concentration of this chemokine. MCP-1 also induces post-stroke neuroblast emigration into the striatum. MCP-1 is highly up-regulated by reactive astrocytes and activated microglia / macrophages in the ischaemic striatum and cortex, while emigrating neuroblasts express its cognate receptor CCR2 (Yan et al., 2006b; Liu et al., 2007b; Tran et al., 2007). Accordingly, MCP-1^{-/-} and CCR2^{-/-} mice have significantly decreased post-stroke neuroblast emigration compared to wildtype controls (Yan et al., 2006b). However, in a separate study, CCR2^{-/-} decreases post-stroke neutrophil recruitment and reduces expression of pro-inflammatory cytokines such as TNF α , resulting in reduced infarct size compared to wildtype mice (Dimitrijevic et al., 2007).

In addition to classic chemokines, activated microglia/macrophages secrete VEGF which has chemo-attractant, angiogenic and neurogenic properties (Plate et al., 1999; Sun et al., 2003). *In vitro*, VEGF is a chemo-attractant and promotes neuroblast migration through VEGF receptor 2 (VEGFR-2; Zhang et al., 2003; Barkho et al., 2008). Transgenic

over-expression of VEGF increases SVZ cell proliferation and the number of neuroblasts in the ischaemic brain regions after ischaemic stroke (Wang et al., 2007). However, the authors of the study point out that *in vivo* it is difficult to distinguish whether the increased number of neuroblasts observed is due to a specific effect on ectopic migration, or merely an indirect result of increased proliferation. Activated microglia/macrophages in the ischaemic brain also express high levels of osteopontin, an acidic glycoprotein, which has chemo-attractive properties, as well as cell adhesion and extracellular matrix signalling functions via activation of integrin receptors (Meller et al., 2005; Doyle et al., 2008). After ischaemic stroke, osteopontin mediates neuroblast emigration in a β 1-integrin-dependent manner (Yan et al., 2009b). Osmotic pump infusion of a functional blocking antibody to osteopontin decreases neuroblast emigration into the striatum without affecting SVZ proliferation, compared to infusion of IgG controls. *In vitro*, blockade of β 1-integrin which is expressed by neurosphere-derived migrating neural precursor cells, decreases cell migration on laminin substrate. Reactive astrocytes and activated microglia/macrophages are also involved in tissue remodelling of the ischaemic striatum, and express matrix metalloproteinases (MMPs) which cleave and degrade extracellular-matrix components to facilitate neuroblast migration through the striatum (Rosenberg et al., 2001; Lee et al., 2006). Thus, a number of different ligands are secreted by glial cells in response to stroke, and these molecules diffuse from the injury to the SVZ to recruit neuroblasts via different mechanisms.

Role of angiogenic vasculature as substrates for migration

Following ischaemic stroke, there is increased endothelial proliferation and formation of new blood vessels (angiogenesis), in the peri-infarct region (Liu, 1988; Kovacs et al., 1996; Beck et al., 2000; Marti et al., 2000; Zhang et al., 2000). Endothelial proliferation

in the peri-infarct region is seen as early as 12-24-hours post-stroke, resulting in significantly increased blood vessel density by 3-days (Beck and Plate, 2009). Peri-infarct angiogenesis is increased for more than 3 weeks following cerebral ischaemia (Hayashi et al., 2003). Angiogenesis functions to increase blood supply to vulnerable brain regions, and to provide trophic support which plays an important role to reduce further neuronal death in the days and weeks following stroke (Leventhal et al., 1999; Plane et al., 2010). Endothelial cells promote NSC proliferation and neuronal differentiation (Shen et al., 2004), and they secrete growth factors such as BDNF and angiopoietin (Ang-1) to promote neuroblast recruitment towards the ischaemic brain tissue. BDNF is known to promote proliferation, migration, survival and differentiation of SVZ neural precursor cells (Leventhal et al., 1999; Benraiss et al., 2001). After focal stroke, endothelial cells express Ang-1, which act on Tie-2 receptors on neuroblasts to promote emigration (Ohab et al., 2006). A recent study found that Ang-1 also promotes SVZ proliferation constitutively (Rosa et al., 2010). In addition, finger-like laminin structures contiguous with the vascular basement membrane, known as fractones, may play important roles in neural precursor cell maintenance by regulating the bioavailability of growth factors (Mercier et al., 2002; Colognato et al., 2005; Kerever et al., 2007; Shen et al., 2008). As discussed above, SVZ neuroblast migration to the olfactory bulb is partly mediated by the vascular scaffold that is present in the SVZ-RMS-OB migratory pathway. Several studies using different models of cerebral ischaemia have shown that neuroblast migration in the striatum is also associated with the vasculature. In static brain sections, many neuroblasts in the ischaemic striatum are in close proximity to blood vessels (Ohab et al., 2006; Thored et al., 2007; Yamashita et al., 2006). Time-lapse microscopy of acute slices after focal ischaemia suggest that some of the Dcx⁺ neuroblasts made contacts with blood vessels during active migration, both in the early phase as the neuroblasts exit the SVZ, as

well as in the striatum (Zhang et al., 2009; Kojima et al., 2010). These experiments suggest that blood vessels serve not only to provide a physical substrate for migration, but form dynamic molecular interactions with neuroblasts to provide directionality and trophic support in a hostile environment, in a recapitulation of the normal migratory pathway.

Molecular and migratory properties of SVZ neuroblasts are altered after stroke.

SVZ neuroblasts undergo intrinsic changes after focal stroke. Neuroblasts express MMPs which can cleave and degrade ECM components. MMP-3 and MMP-9 are up-regulated by neuroblasts in response to chemokine stimulation and focal ischaemia (Lee et al., 2006; Barkho et al., 2008). Neuroblast emigration into the ischaemic striatum is significantly reduced by administration of the broad-spectrum MMP-inhibitor GM6001, while MMP-3 and MMP-9 blockade with specific siRNA decreased neuroblast migration *in vitro* (Lee et al., 2006; Barkho et al., 2008). However, it is yet unclear whether MMPs are required to merely “digest” a pathway through the extracellular matrix to facilitate neuroblast emigration, or whether MMPs directly modulate ECM interactions with proteins such as integrin and laminin, and thus affect intracellular signalling. Nevertheless, these studies suggest a model wherein the striatum presents a molecular/mechanical barrier through which neuroblasts have to bore to reach the lesion.

Attempts have been made to characterise changes in gene expression in the SVZ following ischaemic stroke. Using a combination of gene microarray and real time RT-PCR, Liu and colleagues showed the up-regulation of several gene products including hypoxia inducible factor alpha (HIF-1 α), Notch4 and Ephrin-B2 in both dissected SVZ tissue and cultured SVZ neurospheres after MCAO (Liu et al., 2007a). Their functional implication in the post-stroke SVZ niche and their effects on neuroblast emigration remains to be elucidated.

Time-lapse microscopy demonstrates that neuroblasts actively exiting the SVZ migrate significantly faster than neuroblasts inside the SVZ (Zhang et al., 2009). This is surprising since 2-photon time-lapse data from the Szele Laboratory show that SVZ neuroblasts slow down significantly when emigrating from the RMS (Nam et al. 2007), suggesting that mechanisms such as GABAergic signalling which regulate speed may be significantly altered after stroke. *In vitro*, SVZ cells derived from post-stroke animals spend less time in cytokinesis, and migrate further when plated on Matrigel substrate (Zhang et al., 2007a). Using 2-photon time-lapse imaging, neuroblast chains remain very stable during normal migration (Nam et al., 2007). After ischaemic stroke, there is evidence in static sections that neuroblast chains may be dispersed as neuroblasts exit their normal migratory pathway (Jin et al., 2003). It will be important to detail the extent to which neuroblast chain migration is disturbed, and the molecular and cellular signals within the SVZ which facilitate neuroblast emigration.

1.6. Galectin-3 as a regulator of the post-stroke SVZ

Galectins (Gal) are a family of carbohydrate binding proteins (Gal-1 to Gal-15). They contain evolutionarily conserved carbohydrate recognition domains (CRD) of approximately 130 amino acids on the carboxyl terminus which bind to β -galactoside residues and facilitate cell to cell and cell to extracellular matrix interactions (Liu and Rabinovich, 2005). There are three different types of galectins: (1) the proto-galectins which contain a single CRD and form homodimers, (2) a chimeric galectin, characterized by a single CRD and a large amino terminal domain that contributes to self-aggregation, and (3) the tandem-repeat galectins in which at least two CRDs occur within a single polypeptide and are bridged by a small peptide domain (Barondes et al., 1994). Galectins regulate various biological processes including cell migration, cell-ECM adhesion and cell

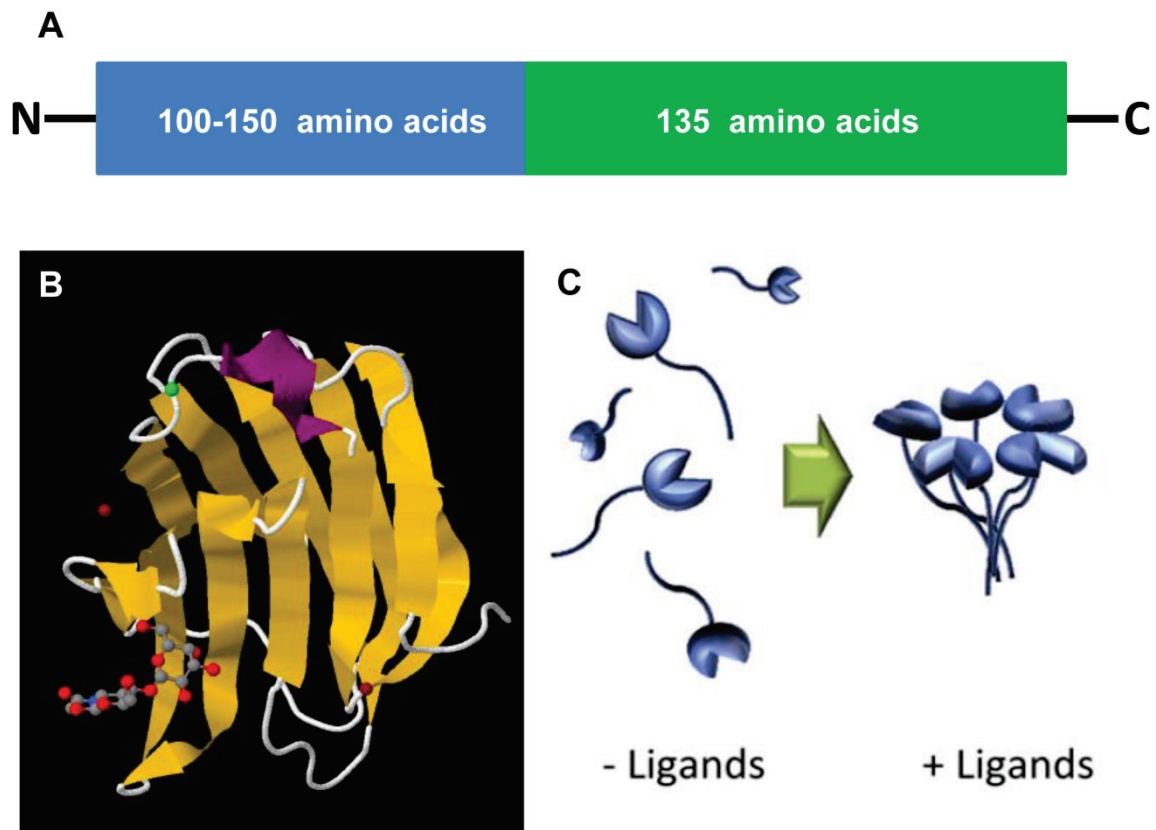


Figure 1.6. Galectin-3 structure

- (A) Schematic of the Galectin-3 (Gal-3) protein showing the N-terminal domain (*blue*) which varies in length depending on the species and the C-terminal (green) carbohydrate recognition domain (CRD).
- (B) Crystal structure of human Gal-3 CRD bound to the disaccharide N-acetyllactosamine (*grey* [Carbon], *red* [Oxygen] and *blue* [Nitrogen]). β -strands *yellow*, α -helix *purple* and loops *white*. Structure From Protein Data Bank (ID: 1KJL) and viewed on Jmol Chemical Structure Viewer.
- (C) Pentamerisation of Galectin-3 in presence of ligands via its N-terminal domain, the head representing C-terminal carbohydrate recognition domain and the tail representing the N-terminal domain.(Argüeso and Panjwani, 2011).

survival, depending on their intra- or extracellular localization (Elola et al., 2007). They have important actions in inflammation and cancer, but their potential functions in brain development and pathology are only now being revealed. It was recently discovered that galectin-1 increased SVZ neurogenesis and improved recovery after stroke (Sakaguchi et al., 2006; Ishibashi et al., 2007), but the expression and function of other galectins in the SVZ were not known.

Galectin-3 (Gal-3; also known as Mac-2, CB 35, Lgals3, L-34) is unique in the galectin family as it is the only chimeric member, and has an extended amino-terminal region composed of tandem repeats of proline, glycine and tyrosine residues (Fig 1.6A; (Cooper, 2002). This chimeric configuration permits the formation of Gal-3 oligomers, which can simultaneously bind multivalently to glycoconjugates on multiple binding partners (Fig 1.6C; Ahmad et al., 2004). It is also cleaved by MMPs such as MMP-9, which increases its binding affinity to glycoconjugates (Ochieng et al., 1998). Gal-3 ligands include laminin, collagen, fibronectin, elastin, NG2 proteoglycans, integrins, NCAM, tenascin, advanced glycosylation end products and many others. As discussed above, several of these ligands are involved in the regulation of the neural stem cell niche. Gal-3 is abundant in a variety of tissues and its intracellular and extracellular function has been implicated in many biological processes (Table 1-2).

Gal-3 has been shown to be anti-apoptotic, particularly in tumour cells. It has been suggested that Gal-3 prevents apoptosis by blocking changes in membrane potential in the mitochondria, possibly interacting with other apoptotic regulators at the membrane (Fukumori et al., 2006). Gal-3 also has significant sequence similarity with the apoptotic regulators of the B-cell lymphoma 2 (Bcl-2) family and shares with them the highly conserved and functionally important motif; Asp-Trp-Gly-Arg, known as the anti-death

motif (Nangia-Makker et al., 2008). Whether Gal-3 works in a similar way to Bcl-2 proteins is yet to be determined.

The binding of Gal-3 to integrins may be central to its role in cell migration. In corneal wound healing, the interaction of Gal-3 with glycans on $\alpha3\beta1$ -integrin is required for corneal epithelial cell migration, and its multivalent properties facilitate the clustering of $\alpha3\beta1$ -integrin and downstream signalling activation (Saravanan et al., 2009). Interestingly, in the thymus, Gal-3 inhibits the interaction between immature thymocytes and thymic epithelial cells, and is thought to act as a de-adhesion signal to facilitate migration (Villa-Verde et al., 2002).

Gal-3 has several roles in the immune response. One of these roles includes the negative regulation of T-cell receptor- (TCR) mediated CD4⁺ T-cell activation (Chen et al., 2009). Gal-3 has also been implicated in chemoattraction of monocytes and macrophages, recruiting them to sites of inflammation (Papaspnyridonou et al., 2008; Nieminen et al., 2008)

Gal-3 promotes metastatic angiogenesis and endothelial cell morphogenesis into capillary tubes (Nangia-Makker et al., 2000b). A recent study suggests that Gal-3 may mediate angiogenesis via the stabilisation of the VEGFR-2 receptor (Markowska et al., 2011).

In spite of the myriad functions described above, the function of Gal-3 in the normal brain, if any, was unknown at the commencement of the current project. In collaboration with Isabelle Comte, Gal-3 was found to be specifically expressed in the adult SVZ and maintained normal neuroblast migration to the OB (Comte et al., 2011). A recent study suggested that Gal-3 may be enriched in astrocyte-like stem cells in the SVZ (Beckervordersandforth et al., 2010), while another group reported that Gal-3 binding may

be necessary for oligodendrocyte differentiation *in vitro*, and myelin integrity *in vivo* (Pasquini et al., 2011). In addition, Gal-3 is frequently associated with pathological conditions in the central nervous system (CNS), especially in diseases where there is a strong inflammatory component. In an environmental gene microarray study of injured brain tissue after an aspiration model of traumatic brain injury, Gal-3 was upregulated 5-fold (unpublished data E. Kang and F. Szele). Gal-3 has also been implicated in an experimental autoimmune encephalomyelitis model of multiple sclerosis, where it is believed to be required for the damaging autoimmune inflammatory process (Jiang et al., 2009). Through its interaction with extracellular-matrix proteins, Gal-3 mediates corneal epithelial repair in the eyes (Saravanan et al., 2009). Gal-3 is also up-regulated following models of ischaemic stroke and hypoxia/ischaemia, and 2 studies have started to address the role Gal-3 may play following ischaemic brain injury (Yan et al., 2009a; Doverhag et al., 2010). These studies will be discussed in detail in Chapter 5.

Table 1.2. Functions of galectin-3

1	Cell migration Thymocyte release from the thymic microenvironment (Villa-Verde et al., 2002). Epithelial cell lamellipodia formation during corneal wound healing (Saravanan et al., 2009). SVZ neuroblasts migration to the olfactory bulb (Comte et al., 2011). $\alpha 6$ integrin / laminin mediated keratinocyte motility (Kariya et al., 2010).
2	Cell adhesion Homotypic and heterotypic aggregation of breast carcinoma cells during vascular metastasis (Nangia-Makker et al., 2008). Tumour cell adhesion to laminin, collagen 4 and fibronectin (Takenaka et al., 2004).
3	Cell survival Breast (Akahani et al., 1997) and prostate (Wang et al., 2010) carcinoma cell resistance to cisplatin-induced apoptosis.
4	Inflammation Macrophage activation and amplification of monocyte chemotaxis during atherosclerosis (Papaspnyridonos et al., 2008). Neutrophil recruitment following <i>Streptococcus pneumonia</i> lung infection (Nieminen et al., 2008). Negative regulation of peritoneal B1-cell differentiation into plasma cells (Oliveira et al., 2009).
5	Angiogenesis Stimulates endothelial morphogenesis and neovascularisation (Fukushi et al., 2004).
6	Membrane receptor expression Increased $\alpha 6 \beta 1$ expression in breast carcinoma cells (Warfield et al., 1997). Stabilisation of cell surface EGFR, TGF-βR (Partridge et al., 2004) and VEGFR-2 (Markowska et al., 2011).

1.7. Investigating the adult neural stem cell niche in ischaemic stroke

From the review of the literature, it was apparent that despite significant advances over the last decade in understanding the recruitment of SVZ neural precursors towards the ischaemic injury, there was insufficient information on how the SVZ as a specialised adult neural stem niche responds to ischaemic stroke. The experiments described above point to a model whereby release of soluble factors recruits neuroblasts towards ischaemic brain regions with guidance from glial processes and blood vessels. The overwhelming focus has been on factors from the site of stroke which recruit neural precursors by chemotaxis. This thesis proposes that *de novo* cellular or molecular processes localised within the SVZ facilitate early events in post-stroke neuroblast emigration i.e. exit from the SVZ-RMS-OB migratory pathway. What are the changes within the SVZ which facilitate neural precursor emigration? Are SVZ components such as neuroblast chains, astrocytic “glial tubes”, vascular scaffold and extracellular matrix proteins, which regulate normal SVZ migration, altered after ischaemic stroke? In our attempts to explore the therapeutic potential of adult neurogenesis, detailed knowledge and mechanistic understanding of the SVZ response to stroke are important, as is the recognition that the adult neural stem cell population exist in a tightly regulated environment, and any changes are likely to affect the intricate system in complex ways. The central theme of this thesis is the changes to the SVZ niche in response to ischaemic stroke. In addition, given the unique expression of Gal-3 in the normal SVZ, its role in constitutive neuroblast migration, as well as its up-regulation following brain injury, Gal-3 is well-positioned to regulate the post-stroke SVZ.

For this thesis, a reproducible model of experimental ischaemic stroke in mice was set up to document changes in the adult neural stem cell niche in response to stroke, and to investigate the role of Gal-3 in post-stroke SVZ-mediated endogenous repair. In Chapter

3, the intraluminal filament MCAO in mice was identified as a suitable stroke model for this purpose, and it contains the experiments that were performed to validate the model. In Chapter 4, the cytoarchitectural reorganisation of the SVZ after MCAO stroke was examined in detail. Using whole mounts of the lateral ventricular wall in immunohistochemical and functional studies, I found significant structural changes to cellular components of the SVZ, with implication for their functions. In Chapter 5 and 6, Gal-3 null mice were used to investigate whether this carbohydrate-binding lectin, specific to SVZ in the normal brain and highly up-regulated after stroke, may be involved in the post-stroke SVZ reorganisation and neuroblast migration towards the ischaemic striatum. I found that Gal-3 was essential for post-stroke angiogenesis in the ischaemic striatum, and modulated the pro-proliferative interaction between ectopic neuroblasts and the vasculature, while negatively regulating the neuroblast emigration. Lastly, in Chapter 7, I discuss the significance of the current finding and what they inform us about the neural stem cell niche in ischaemic stroke. I also critically assess the limitations of my studies, and propose future work to address unanswered questions that have arisen from this thesis.

Chapter 2

Material and Methods

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2.1. Animals

Male 129sv mice aged 8-12 weeks, weighing 23-28g were obtained from University of Oxford Biomedical Services Specific Pathogens Free Breeding Unit (Oxford, UK). Congenic 129sv Gal-3 +/- mice were obtained from the Poirier Laboratory (Colnot et al., 1998). Ten Gal-3^{+/-} breeding pairs were set up to generate male Gal-3^{+/+} and Gal-3^{-/-} littermate mice used in this thesis. Animals were maintained in individually ventilated cages on 12-hour light / dark cycles, with free access to food and water. Procedures were carried out with University of Oxford Research Ethics Committee approval, in accordance with the Animals (Scientific Procedures) Act of 1986 (UK). All efforts were made to minimise animal suffering and distress.

2.2. Galectin-3 null mice

Gal-3 null mice were generated by targeted mutation and homologous recombination by the Poirier Laboratory (Colnot et al., 1998). A targeting vector (*tmlPoi*) containing neomycin resistance and herpes simplex virus thymidine kinase genes was used to disrupt 3.7kb of sequence that includes exons 2, 3 and 4. The vector was electroporated into WW6 embryonic stem cells (derived from 129/sv, C57BL/6, SJL mixed background mice) and enriched with G418 and gancyclovir. Correctly targeted ES cells were injected into MF1 blastocysts and implanted into pseudo-pregnant dames. Germ-line chimeric male offsprings were crossed with female 129sv mice to produce heterozygotes for the recombined allele. Heterozygotes were crossed to generate wildtype (+/+), heterozygote recombinants (+/-) and homozygote recombinants (-/-).

2.3. Genotyping

2.3.1. DNA Extraction

The genotype of Gal-3 wildtype, heterozygote and null mice from Gal-3 heterozygote breeding pairs was determined by analysis of earclip DNA. Earclip samples were used to extract DNA for genotyping polymerase chain reaction (PCR). A Proteinase K-based spin column system was used to extract DNA (E.Z.N.A Tissue DNA Isolation Kit, Omega Bio-tek).

2.3.2. Polymerase chain reaction and gel electrophoresis

Ten microlitre of the extracted DNA was added to a master mix for a reaction solution (50 μ L final volume) consisting of 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl; Sigma-Aldrich, Dorset, UK); 0.2 mM dNTP; 0.6 μ M *common*, *Gal-3*^{+/+} and *Gal-3*^{-/-} primers; 2.5 mM MgCl₂; 2.5 μ L dimethyl sulfoxide (DMSO); 2.5 μ L Red Taq polymerase (1 unit/ μ L; #D4309, Sigma-Aldrich). The PCR programme consisted of 94° C for 3 min, then 34 cycles with denaturation at 94° C for 45 sec, annealing at 55° C for 30 sec, and elongation at 72° C for 2 min, followed by final elongation at 72° C for 5 min. The customised primers (Eurofins) were as follows: *common* 5'-CAC GAA CGT CTT TTG CTC TCT GG-3'), *Ga*;-3^{+/+} 5'-TGA AAT ACT TAC CGA AAA GCT GTC TGC-3' (single band of ~300 bp) and *Gal-3*^{-/-} 5'- GCT TTT CTG GAT TCA TCG ACT GTG G-3' (single band of ~500 bp).

PCR products were stored at 4° C until separation by agarose gel electrophoresis (1X TAE buffer, 2% agarose, 5 μ g ethidium bromide/100 mL agarose) at 100 V for 60 min. DNA bands were visualised under ultraviolet light and captured by a charge-coupled device digital camera.

2.4. Visualisation of cerebrovascular anatomy

Adult male mice were overdosed with intraperitoneal pentobarbitone (1 g/kg body weight), and transcardiacally perfused with (1) 15 mL of cold normal saline via a syringe, (2) 15 mL of 4% paraformaldehyde (PFA) and (3) 5 mL of warm 3.75% gelatin carbon black solution (15 mL of 10% gelatin in H₂O, 10 mL India ink, 15 mL H₂O). The specimens were kept at 4 °C overnight to allow gelatin to set. Each brain was then carefully dissected out by carefully nudging the brain away from the base of the skull, cutting the trigeminal nerve tracts and optic chiasma which tethered the brain. The brains were post-fixed in 4% PFA overnight before imaging.

2.5. Middle cerebral artery occlusion

Thirty, forty or fifty minutes (min) of left-sided intraluminal filament MCAO surgery were carried out as a model of transient focal cerebral ischaemia. All surgeries in this study were performed between 10h00 – 18h00.

2.5.1. Anaesthesia

The mouse was placed in an anaesthetic induction chamber and anaesthesia was induced with 2% isoflurane in 30% oxygen (0.3 L/min) and 70% nitric oxide (0.7 L/min). The mouse was then transferred onto the operating table (Fig 2.1A), and anaesthesia was maintained with 1.5% isoflurane in 30% oxygen (0.3 L/min) and 70% nitric oxide (0.7 L/min) delivered through a nosepiece. During surgery, the animal's core body temperature was monitored by continuous rectal temperature monitoring, and regulated at 36.5°C using a feedback heating pad (PhysiTemp TCAT-2LV; Linton Instruments). The eyes and cornea were protected by application of Lacri-Lube ocular lubricants (Allergan, Marlow, UK).

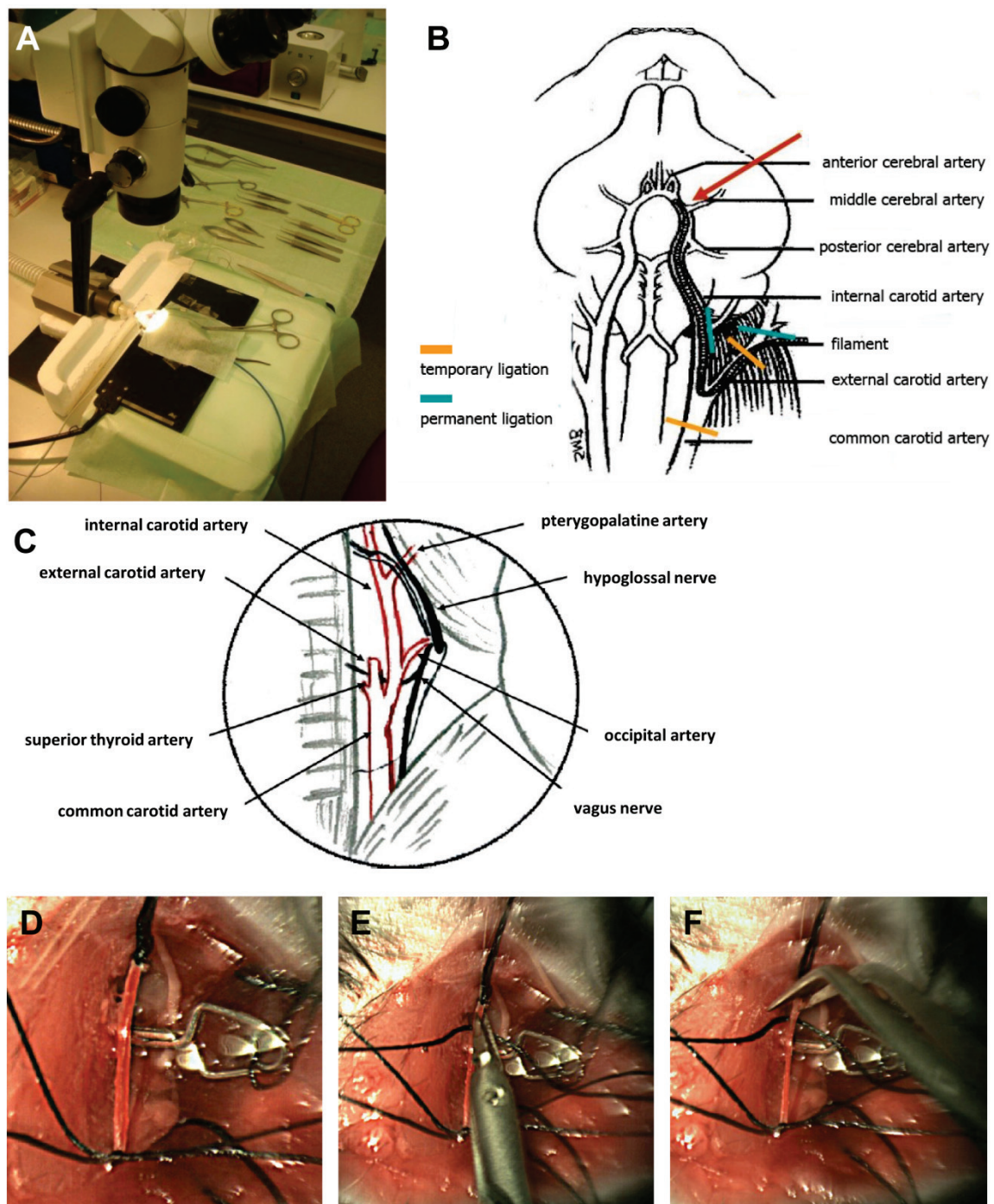


Figure 2.1. MCAO surgery

- (A) Aseptic surgical setup for MCAO surgery.
- (B) Schematic representation of intraluminal filament MCAO, with filament occlusion of the origin of the MCA. Image courtesy of Dr Joe Casto.
- (C) Ventral neck dissection to show anatomy of carotid arteries (Chen et al., 2008).
- (D) ECA and CCA ligated and positioned for arteriotomy. The aneurysm clip temporarily clamps the ICA. The laterally displaced vagus nerve (*white*) can be seen in the background.
- (E) Arteriotomy with 2 mm iris scissors.
- (F) Arteriotomy at the tip of the forceps is ready for filament insertion.

2.5.2. Laser Doppler Cerebral Blood Flow

During MCAO surgery, cerebral blood flow (CBF) in the MCA territory was monitored using a laser Doppler flowmetre (LDF; Oxford Optronix, Oxford, UK). With the animal lying prone, the head was shaved and cleaned with 10% povidone-iodine in sterile water. Observing aseptic surgical techniques, a 1cm midline incision was made in the scalp to expose the skull. The laser Doppler probe was affixed to the skull over the MCA vascular territory (3 mm lateral and 1 mm posterior of Bregma) with cyanoacrylate adhesive.

2.5.3. MCAO Surgery

After obtaining stable laser Doppler CBF reading, the mouse was turned supine and positioned with the neck slightly extended. The ventral neck was shaved and cleaned with 10% povidone-iodine in sterile water. Observing aseptic surgical techniques, a 1.5 cm skin incision was made in the midline neck from the sternal notch towards the top of the trachea. Under an operating microscope, the salivary glands and connective tissues were displaced laterally to visualise the anterior triangle of the neck. The left sternocleidomastoid and omohyoid muscles were gently retracted to expose the carotid vessels (Fig 2.1C). The common carotid artery (CCA), the left external carotid artery (ECA), and the left internal carotid artery (ICA) were isolated (Fig 2.1B). The left CCA and ICA were temporarily occluded with a loose suture and aneurysm clip (Codman, Raynham, MA, USA) respectively to create a bloodless field, and a small arteriotomy was made with fine iris scissors (Fig 2.1D-F). A silicon-coated nylon suture measuring 200 μm in diameter (Docol, Redlands, CA, USA) was introduced into the ECA and advanced 9-10 mm up the ICA from the carotid bifurcation to block the origin of the MCA. Upon successful MCAO, CBF decreased to less than 30% of baseline values. The filament was held in place with a security tie during MCAO. After the desired ischaemic time (30, 40

or 50 min), the filament was removed and the ECA permanently tied. The MCA was reperfused as evidenced by rapid rise of CBF towards baseline values. The surgical field was rinsed with sterile saline and checked for haemorrhage. The overlying salivary glands were repositioned, followed by skin closure with 5/0 silk suture, and final cleaning of surgical wound with 10% povidone-iodine in sterile water. Laser Doppler CBF was monitored for 15 min after reperfusion, then removed for scalp wound closure with 5/0 silk suture.

2.5.4. Sham surgery

Sham surgeries were carried out to minimise the likelihood that the observed effects were directly attributable to factors such as surgical stress, anaesthesia, or the presence of a large surgical neck/scalp wound. For MCAO, sham surgery included identical preparatory steps and surgical steps except for the introduction of the silicone filament into the ECA and subsequent blocking of the origin of the MCA. Sham-operated animals were kept anaesthetised on the surgical table with laser Doppler CBF monitoring and temperature regulation for a total duration of approximately 120 min. The length of time was selected based on the mean total MCAO surgical time in our pilot study. Littermates were randomly assigned to undergo MCAO or sham surgery.

2.5.5. Recovery and Post-Operative Care

At the end of surgery, each animal received an intraperitoneal injection of buprenorphine (0.3 µg/kg body weight) diluted in 0.5 mL saline for analgesia and fluid replacement. Animals were recovered in heated incubators set at 28° C (Wolfensohn and Lloyd, 2003) for 2 or 24 hours to maintain body temperature at 35 - 36° C before being returned to their home cages (~20° C). Body temperatures were measured daily at between 11h00 and

13h00 using a digital rectal thermometer. Fluid balance was maintained by daily intraperitoneal injections of 5% normal saline (0.4 mL daily). Animals supplemented with glucose received daily intraperitoneal injections of 5% glucose in normal saline (0.4 mL daily) in place of normal saline injection. Transwean soft feed (Lillico, Redhill, Surrey, UK) was provided during the survival period, and animals were weighed daily.

2.5.6. Bromodeoxyuridine Labelling

Bromodeoxyuridine (BrdU), a thymidine analogue, was used to document significant DNA replication and label proliferating cells. BrdU (Sigma-Aldrich) was reconstituted in normal saline 10 mg/mL. Animals which were survived for 7 days post-MCAO received a single intraperitoneal injection of BrdU (100 mg/kg body weight) 2 hours before sacrifice. Animals which were survived for 14 days post-MCAO were cumulatively labelled and received intraperitoneal injections of BrdU (100 mg/kg body weight) for five days from Day-4 to Day-8 post-MCAO.

2.6. Evaluation of neurological status

2.6.1. Modified Neurological Deficit Score

Twenty-four hours after MCAO, assessment of neurological status was carried out. Animals were coded so that I was blinded to the animals' experimental groups. A modified neurological deficit score (mNDS) based on Bederson's system was used to quantify gross motor neurological deficit (Table 2.1; Bederson et al., 1986b; Barber et al., 2004).

The mouse was held by the mid-tail by the standing assessor with an outstretched arm. Neurologically intact mice had symmetrical extension of their limbs, while hemiparetic mice showed flexion limbs contralateral to the MCAO. Posture varied from mild wrist flexion and shoulder adduction with extension at the elbow to severe posturing with full flexion of wrist, elbow, and adduction with internal rotation of the shoulder (Bederson et al., 1986b). Axial flexion towards the contralateral side may also be present. Animals which displayed these deficits and no other abnormalities were given a score of 1. The mouse was then placed prone on table counter and held by the tail. The assessor exerted lateral pressure with his index finger on the animal's left, then right shoulder. In normal animals, pressures from both sides were countered equally and while the animal may slide on the table, it remained standing. In neurologically affected animals, asymmetry of motor strength was evident, and when pressure was exerted on the left shoulder towards the right, the weakened contralateral limbs provided less resistance and the animal often fell onto its right side. The presence of this deficit together with contralateral limb flexion when held by the tail scored 2. For the final part of this assessment, the mouse was placed in open-field and allowed to explore. The gait of the animal was observed. Asymmetry with gait deviating from a straight line towards the contralateral side, or walking in large circles > 50 cm in diameter was scored 3. Motor asymmetry with circling gait in small circles (10 – 50 cm) was scored 4. Motor asymmetry with circling about the same spot was scored 5.

2.6.2. Modified Neurological Severity Score

The modified Neurological Severity Score (mNSS) was adapted and modified from (Chen et al., 2001, 2005). Commencing 24 hours after MCAO, neurological status was assessed and scored by a blinded investigator.

Table 2.1. Modified Neurological Deficit Score (mNDS)

Neurological deficit	Score
Normal	0
Consistent forelimb and axial flexion towards the contralateral side when lifted by the tail	1
Score 1 PLUS consistently reduced resistance to lateral push	2
Score 2 PLUS gait toward the paretic side or large weak circling (> 50 cm diameter) toward the paretic side	3
Score 2 PLUS small circling (10-50 cm diameter) toward the paretic side	4
Score 2 PLUS tight circling (<10 cm diameter) toward the paretic side	5

The mouse was placed in an openfield and observed for spontaneous motor activity for 2 min. Motor hemiparesis and asymmetry were assessed as for mNDS. For visual and tactile placing of forepaw, the mouse was lifted by its tails and brought to approach a table edge. A normal mouse rapidly extended both forelimbs and symmetrically planted paws onto the table surface, while a neurologically affected mouse reached out towards the table slowly and/or did not plant paw(s). For the test of proprioception, the mouse was placed prone on the edge of the table and the hindlimb was laterally displaced over the edge of the table. A normal mouse resisted lateral displacement and immediately retracted the limb back onto the table surface, while a neurologically affected mouse had decreased resistance to lateral displacement and slowed/absent limb retraction reflex. The corneal reflex was elicited by touching the cornea with a fine cotton strand which normally caused blinking. The startle reflex was elicited by a sudden high pitched sound generated through pursed lips from behind the

Table 2.2. Modified Neurological Severity Score (mNSS)

Openfield activity (select appropriate score out of 3)	3
<i>Place the mouse in openfield grid for 2 min</i>	
Moves and approaches at least 3 sides of cage	0
Moves but does not approach at least 3 sides of cage	1
Reduced and slow movements	2
Minimal movement	3
Motor hemiparesis (score 1 for each feature present)	3
<i>Lifting the mouse by the tail</i>	
Flexion of forelimb	1
Flexion of hindlimb	1
Trunk curl / Head deviation	1
Symmetry of movement (score 1 for each feature present)	3
<i>Placing the mouse on the floor</i>	
Inability to walk straight	1
Circling	1
Falling down on the paretic side	1
Sensory test (score 1 for each feature present)	2
Visual and tactile placing of forepaw	1
Proprioceptive test	1
Absent reflexes / abnormal movement	3
Loss of corneal reflex	1
Loss of startle reflex (response to loud high pitch sound)	1
Tremours, shakes, pilo-erection, staring, loss of grooming	1
Maximum score (higher score means greater disability)	14

mouse and normally resulted in flinching movements best visualised by observing the pinnae of the ears.. The general well-being of the mouse was assessed by observing for negative features including tremors, pilo-erection, loss of grooming, and staring immobility.

2.7. Measurement of infarct volume

Twenty-four hours after MCAO, brains were sectioned in a mouse brain matrix into four 1.5 mm thick coronal brain sections (Bregma +1.5 mm, 0 mm, -1.5 mm and -3.0 mm; Paxinos and Franklin, 2001) and incubated in 2% triphenyl-tetrazolium (Sigma-Aldrich) for 30 min at 36.5° C in the dark. After TTC-staining, the sections were imaged with a digital camera (Sony DC-T100) on millimetre graph paper. The infarct volume was reconstructed according to the Cavalieri principle (Swanson et al., 1990). The areas of the infarct on the brain sections were measured using Volocity 4 software (PerkinElmer, Cambridge, UK) and the volume of the infarct in the section was calculated as follows:

$$V^1 = \frac{1.5 (Area^1 + Area^2)}{2}$$

Where V^1 = infarct volume of first brain section, $Area^1$ = infarct area of the first section, $Area^2$ = infarct area of the second section, 1.5 = thickness of section in millimetre. The equation was repeated for all 4 sections to calculate the total infarct volume. To compensate for brain swelling and tissue loss, the infarct area of each section was measured using the indirect method (infarct area = total contralateral hemisphere area – ipsilateral non-stroke area; Swanson et al., 1990; Lin et al., 1993), and the infarct volume was expressed as a percentage of the contralateral hemisphere. In a separate group of animals, 7-days after MCAO, fluorescence immunohistochemistry for NeuN and GFAP

was carried out on 5 brain sections (Bregma +1.5 mm, 0.5 mm, -0.5 mm, -1.5 mm and -2.5 mm) to similarly reconstruct infarct volume (Liu et al., 2009a).

2.8 Histology and immunohistochemistry

2.8.1. Perfusion and tissue preparation

At the end of the survival period, animals were overdosed with intraperitoneal pentobarbitone, and transcardiacally perfused with 25 mL of cold normal saline via a syringe to flush out blood, followed by 25 mL of 4% PFA. Brains were carefully dissected out of the skull, post-fixed overnight in 4% PFA in 4° C, cryoprotected for 48 hours in 30% sucrose in 0.1M phosphate buffer (PB), and frozen at -80 °C.

2.8.2. Microtome Sectioning

Frozen brains were microtomed (Leica SM2000R; Leica, Milton Keynes, UK) on a frozen stage into 30 µm serial coronal sections from the olfactory bulbs to the caudal hippocampus. A 25G syringe needle was used to mark the cortex contralateral to stroke lesion to aid identification of the ipsilateral and contralateral hemispheres. Brain sections were stored as free-floating sections on 48-well plates in cryoprotectant (Appendix 1) at -20 °C.

2.8.3. Fluorescent immunohistochemistry

Free-floating fluorescent immunohistochemistry was performed over 2 days at room temperature unless otherwise stated. Incubation steps were carried out on 12-well plates with 2 mL of solution in each well. A maximum of 6 free-floating brain sections were

placed on porous well inserts (CLS3477, Sigma-Aldrich) and used to transfer sections between wells.

Sections were washed in 0.1 M phosphate buffered saline (PBS) 3 times 10 min, treated with 50 mM glycine in PBS to reduce tissue autofluorescence, washed in PBS a further 3 times, and blocked for 1 hour in 10% donkey serum in PBS with 0.5% Triton X-100. Double and triple immuno-staining were carried out by co-incubating primary antibodies produced in different host species at 4° C overnight. On Day-2, sections were washed in PBS 3 times 10 min and incubated for 1 hour with appropriate fluorophore-conjugated secondary antibodies. After washing in PBS 3 times 10 min, sections were incubated for 10 min in 4',6-diamidino-2-phenylindole (DAPI, 10 µg/mL, Sigma-Aldrich) to counterstain cell nuclei, and rinsed 3 times 10 min in 0.1M phosphate buffer (PB). Sections were mounted on Superfrost Plus slides (VWR, Lutterworth, Leicestershire, UK) and excess fluids blotted with filter paper. The slides were air-dried for 45 min, and coverslipped (#1.5 thickness) with Fluorsave mounting media (Calbiochem-Merck, Nottingham, UK). Slides were dried in a dark drawer overnight and stored in slide boxes at 4 °C.

Table 2.3 lists the primary antibodies used in the thesis. Alexa Fluor 488, 568 and 647 (Invitrogen Paisley, Renfrewshire, UK), and Cy3 (JacksonImmunoResearch, West Grove, PA, USA) conjugated secondary antibodies raised against the host species of the primary antibodies were used.

2.8.4. Bromodeoxyuridine immunohistochemistry

For brain sections undergoing immunohistochemistry for BrdU, the above fluorescent immunohistochemical procedure was preceded by 20 min incubation in deoxyribonuclease

(DNase) buffer (Appendix 1), followed by 30 min incubation in DNase (2 mg/mL in DNase buffer , 569 Kunitz Units/mg, Sigma-Aldrich) at 36.5 °C.

Table 2.3. Antibodies for immunohistochemistry and immunoblotting

Antigen	Dilution	Host	Manufacturer Cat. #
Acetylated tubulin	1:1000	mouse	Sigma-Aldrich T6793
β -actin	1:1000	mouse	Sigma-Aldrich A4700d
β -catenin	1:1000	rabbit	Sigma-Aldrich C2206
β -tubulin	1:12000	mouse	Abcam Ab7287
BrdU	1:200	mouse	Dako M0744
BrdU	1:500	sheep	Abcam Ab1893
Calretinin	1:2000	rabbit	Millipore AB5054
CD45	1:200	rat	Millipore CBL1326
Dcx	1:100	goat	Santa Cruz SC 8066
γ -tubulin	1:200	goat	Santa Cruz SC 7396
Galectin-3	1:100	rabbit	Santa Cruz SC 20157
Galectin-3	1:100	rat mono	Santa Cruz SC 23938
GFAP	1:500	mouse	Millipore 557355
GFAP	1:200	rabbit	Sigma-Aldrich G9269
GFAP	1:400	rat mono	Invitrogen 13-0300
Mash1	1:100	mouse	BD 556604
NeuN	1:400	mouse	Millipore MAB377
PECAM (CD31)	1:200	rat	BD 557355
PHi3	1:400	rabbit	Millipore 06-570
S100b	1:100	mouse	Sigma-Aldrich S2657
VEGF	1:100	rabbit	Santa Cruz SC 507
Vimentin	1:200	goat	Santa Cruz SC 7558

2.8.5. Controls

Signals from fluorescence immunohistochemistry were compared to manufacturers' product datasheets, published works, and unpublished data from the Szele and Buchan laboratories. The Allen Brain Atlas (Lein et al., 2007) *in-situ* hybridisation data was consulted for general expression of gene products of interest. Specificity of anti-gal-3 and anti-VEGF antibodies were further tested by Western blot experiments which identified protein bands at the predicted molecular weights.

Negative controls omitting primary antibodies were carried out for all experiments. In double and triple label experiments, I verified the specificity of fluorescent signals and systematically ruled out cross-labelling by applying each primary antibody with inappropriate secondary antibody combinations, which showed lack of fluorescence.

2.8.6. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

Brain sections were mounted on Superfrost slide. Sections were rinsed 2 times 10 min in Tris buffered saline (TBS), dehydrated and rehydrated in graded isopropanol (70, 90 and 100%), rinsed 2 times 10 min in TBS, and pre-incubated with terminal deoxynucleotidyl transferase (TdT) buffer (#11684795910, Roche Diagnostics, Burgess Hill, West Sussex, UK) for 15 min at 37° C. Brain sections were incubated with TdT and fluorescein-conjugated dUTP mixture (5µL TdT and 45 µL fluorescein-conjugated dUTP per brain section) for 30 min at 37° C. Positive control sections were pre-treated with 2 mg/mL DNase (Sigma-Aldrich) for 15 min, and negative controls did not receive TdT. Sections were rinsed 3 times 10 min in TBS, counterstained with DAPI and coverslipped with Fluorsave media.

2.9. Whole mount dissection and immunohistochemistry

2.9.1 Whole mount dissection

Following deep anaesthesia with intraperitoneal pentobarbitone, 25 mL of normal saline was transcardiacally perfused to flush out blood. The brain was extracted, cut in the midline, and the overlying cerebral cortex, medial ventricular wall and hippocampus were dissected away to reveal the lateral ventricular wall (Mirzadeh et al., 2010a). Under a dissecting microscope, using the tips of 25G syringe needles and fine forceps, a coronal incision was made through the hippocampus (Bregma -2.5 mm). Here in the posterior lateral ventricles, the hippocampus forms the medial wall and the cortex forms the dorsal (roof) and lateral walls. An incision was made to separate the medial hippocampus from the dorsal cortex. The cortex was gently peeled away from the hippocampus, all the way around towards the ventral hippocampus. Here a second incision through the piriform cortex was made to free the hippocampus. The hippocampus, freed of its dorsal and ventral cortical connections, was gently peeled anteriorly. As the medial wall (containing the hippocampus) was peeled away, the lateral ventricular wall is visualised from the posterior towards the anterior, and the glistening ventricular surface and choroid plexus sheets could be seen. Excess dorsal cortical tissues were cut away, leaving the lateral ventricular wall intact. The medial wall remained connected to the lateral wall anteriorly. Two more incisions were made, one dorsal through the anterior corpus callosum and dorsal cortex, and one ventral through the anterior nucleus accumbens to allow further retraction to open the anterior-most extent of the lateral ventricles. One last cut was then made to completely separate the medial and lateral walls.

2.9.2. Whole mount immunohistochemistry

Whole mounts were carefully placed ventricle-side up on 24-well plates and post-fixed in 4% PFA with 0.1% Triton-X 100 at 4°C overnight with the constant rocker on *slow* setting. This prevents the whole mounts from being turned up-side-down and potentially damaging the ventricular wall. Between each immunohistochemistry incubation step, the whole mount was carefully transferred between wells for the same reason. On Day-2, they were washed 3 times 10 min in PBS with 0.1% Triton-X 100 (PBS*), blocked in 10% donkey serum in PBS* for 1 hour, and incubated at 4° C for 48 hours in primary antibodies in PBS containing 10% donkey serum and 2% Triton X-100 to aid antibody penetration. Antibodies used are listed in Table 2.2. On Day-4, after rinsing in PBS* 2 times 8 min and 3 times 20 min, whole mounts were incubated for a further 48 hours with appropriate fluorophore-conjugated secondary antibodies at 4° C. On day-6, whole mounts were washed in PBS* 3 time 10 min, counterstained with DAPI for 10 min before final PB wash 3 time 10 min.

2.9.3. Final dissection and mounting

Whole mounts were trimmed under the stereotactic microscope to 200 - 300 µm sections. Taking care not to touch the ventricular wall, the whole mount was transferred back into a dissecting Petri dish. The dorsal cortex and the corpus callosum were trimmed away, leaving the lateral ventricular wall and underlying striatum. A long horizontal cut was made along the ventral aspect of the whole mount. This cut would allow the whole mount to be positioned anatomically, with the ventricular wall now upright, perpendicular to the surgery table. With the cortex and corpus callosum having been removed, the SVZ could be visualised dorsally and fine iris scissors was used to cut away the striatum adjacent to the SVZ. Lastly, the excess ventral tissues were trimmed, leaving a thin and flat whole

mount of the lateral ventricular wall. This was mounted on Superfrost slides, and coverslipped (#1.5 thickness) with adhesive spacers and Flurosave mounting media. Mounted slides were dried facing down in a dark drawer for 48 hours before storage in slide book at 4° C.

2.9.4. Controls

Negative controls omitting primary antibodies were performed for each of the antibodies used, and absence of fluorescence was documented. I examined astrocytes, ependymal cells, neuroblasts and vasculature in whole mounts of the contralateral hemispheres post-MCAO and sham-operated controls ($n = 5$), and I did not find obvious differences. Thereafter, contralateral hemispheres post-MCAO were used as controls, unless otherwise stated.

2.10. Cerebrospinal fluid flow assay

For dynamic CSF flow studies, the animals were coded by a colleague and I did not know the experimental group of the animal until the final analysis had been performed. Seven-days post-MCAO, the animal was overdosed with intraperitoneal pentobarbitone and the brain was quickly extracted omitting the perfusion step. The lateral ventricular wall was dissected in 36.5° C Leibovitz's (L-15) media (Invitrogen). The whole mount was pinned to a Petri dish coated with Sylgard 184 elastic polymer, and filled with warmed L-15 media. The whole mount was positioned under the Zeiss StereoLumar fluorescence microscope and brought into focus. The needle bevel of the Hamilton syringe which contained the fluorescent beads was carefully advanced by adjusting the stereotactic frame so that the flat bevel broke the fluid surface above the lateral ventricular wall, but did not

contact brain tissue. Five nanolitres of two μm -diameter green fluorescent microsphere beads (F8827, Invitrogen), reconstituted 45% bead, 45% water and 10% glycerol, were released over the anterior dorsal ventricular wall with a 0.5 μL Hamilton syringe connected to a stereotactic injector (Stoelting, Illinois, USA) at 120 nanolitres/min. Bead movements were visualised using Zeiss AxioCam camera and imaged for 60 seconds at 3-frames-per-second. Three to five rounds of bead release and imaging were performed for each whole mount to obtain optimal time-lapse images of bead flow. Fluorescent beads were cleared between each round by addition of fresh warm L-15 media. A maximum of 30 min elapsed between death and completion of imaging. For negative controls, absence of flow was confirmed when beads were released over the striatum, as well as on the ventricular surface following 5 min incubation with 70% ethanol. *Post-hoc* whole mount immunohistochemistry was performed after completion of CSF flow study and analysed under confocal microscope to exclude damage to the ventricular wall from dissection and to document features of post-stroke changes.

2.10.1. Analysis and quantification

Time-lapse images were analyzed using Volocity 4 software. For each whole mount, 20 beads (dorsal and ventral streams, 10 beads each) were manually tracked by x:y coordinates for at least 10 frames to calculate flow velocity. The paths of flowing beads were plotted on 2-dimensional Cartesian plane with Graphis software (Kylebank, <http://www.kylebank.com>). I used the Pythagorean equation to determine distance travelled between 2 frames: $d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$ where d = distance travelled between frame 1 and 2, x_2 = x-coordinate in frame 2, x_1 = x-coordinate in frame 1, y_2 = y-coordinate in frame 2, y_1 = y-coordinate in frame 1. By calculating the distance travelled

by a single bead over the 10 frames, and dividing that distance by the time lapsed in 10 frames, the velocity of the bead flow could be calculated.

2.11. Two-photon time-lapse microscopy

Cell tracker green 5-chloromethylfluorescein diacetate (CTG; 10 mM, 0.6 μ L; Invitrogen) in DMSO was injected into lateral ventricle using a stereotactic frame and microlitre syringe injector (stereotactic coordinates A-P 0.1, M-L 0.70, D-V 2.5 from Bregma for Gal-3^{+/+}; A-P 0.15, M-L 0.70, D-V 2.5 for Gal-3^{-/-}). Migration was imaged two days after injection. After deep anaesthesia, mice were decapitated and the brain quickly dissected out. Brains were submerged in ice-cold artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 2.5 mM KCl, 2 mM, CaCl₂, 1 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM H₂PO₄, and 25 mM glucose at pH 7.4) and 300 μ m sagittal slices cut on a vibratome (Campden Instrument, Loughborough, Leicester, UK). Brain slices were incubated in oxygenated aCSF at 36.5° C for 45 min to permit acclimatisation to *ex-vivo* environment. The slice was then placed in the imaging chamber which was regulated at 36.5° C with continuous circulation of oxygenated aCSF at 0.5 – 1.0 mL/min. A Mira 900 two-photon laser (Coherent, Santa Clara, CA, USA) was used to acquire optical z-stacks (51 sections spaced 1 μ m apart) every 3 min over the elbow of the RMS for 2 hours.

Time-lapse z-stacks were processed in Volocity 4 for 3-dimensional cell tracking. In each slice, all CTG+ migrating neuroblasts that were traceable for at least 1 hour of the imaging period were included in analysis. To obtain migration speed, x:y:z coordinates over the first hour for each cell were recorded. Total speed was the sum of migratory distance between each frame over time, while net speed was the distance between the initial position and the final position over time. For a visual representation of cell

migration, the paths of 10 randomly selected migrating neuroblasts were plotted on 3-dimensional Cartesian plane with Graphis software.

2.12. Microscopy and quantifications

Experimental groups were coded, and images were acquired and quantified by a blinded investigator.

2.12.1 Epifluorescence microscopy

Epifluorescence images were acquired with Openlab software (Improvision) on a Leica DMIRB microscope with a digital camera (Hamamatsu C4742-95) and analysed with Volocity 4 and ImageJ (NIH, Bethesda, MD, USA) softwares.

2.12.2. Confocal microscopy

High-magnification optical sections of coronal sections and whole mounts were obtained with a Zeiss LSM 710 laser scanning confocal microscope using 40X oil immersion (Zeiss Plan-Neofluar 40X/1.3 Oil DIC) and 20X (Zeiss Plan-Neofluar 20X/0.50) objectives. Four laser excitation lines (405, 488, 561 and 633 nm) were used to excite DAPI, Alexa Fluor 488, Alexa Fluor 567 / Cy3 and Alexa Fluor 647 fluorphores. Emission spectra were set as per Zeiss Zen image acquisition software. Where images were intended for comparison of fluorescence intensity, images were acquired at the same settings across individuals and the experimental groups. Confocal images were analysed in LSM Image Browser 4.2 (Zeiss), Volocity and ImageJ softwares.

2.12.3. Whole mounts

Whole mounts were examined globally and images were acquired from 4 specific regions of the lateral ventricular wall. The adhesion zone was not included in the analysis. To measure average ependymal cell surface area, the area of each 40X confocal field was divided by the number of cells occupying each field. Planar cell polarity of each ependymal cell was measured by drawing a vector between the centres of the cell and basal body cluster. The vectors were expressed as an angle away from the median value measured, and represented using a histogram (Mirzadeh et al., 2010a). Vascular density and angiogenesis were measured by counting the number of blood vessel branches in projected 40X 10 μ m confocal *z*-stacks from 4 locations on each whole mount. Vessel diameter was measured using Volocity 4.

2.12.4. Digital threshold area measurement

In Chapter 4, vascular density and size of neural precursor pool were assessed by digital threshold area measurement. Confocal images were imported into ImageJ, transformed into greyscale and the threshold was set manually. The area which contained pixels above the set threshold was then measured and expressed as a percentage of the total surface area.

2.12.5. Proliferating cells and co-localisation studies

Quantification of PHi3⁺ and BrdU⁺ cells, and co-localisation studies (Dcx, Mash-1, s100 β , PECAM, GFAP and CD45) were performed in 40X orthogonal projections of 10 μ m confocal *z*-stacks (dorsal, middle and ventral SVZ or striatum/ischaemic penumbra) in 5 evenly spaced sections from Bregma +1.5mm to -0.5mm.

All co-localisation studies were performed in 40X confocal z-stacks and shown in figures as single confocal optical sections or orthogonal projections.

2.12.6. TUNEL+ apoptotic cells

TUNEL+ cells in the entire SVZ was counted under 20X epifluorescence microscope in brain sections at Bregma +1 mm, +0.2 mm and -0.5 mm. TUNEL+ cells in the striatum were counted in a representative 20X epifluorescence image.

2.13. Western blot

At the end of the survival period, animals were killed with intraperitoneal injection of pentobarbitone. The brain was quickly dissected and the ipsilateral and contralateral hemispheres from Bregma +2 mm to -3 mm were placed in sterile 2 mL PCR tubes, frozen in liquid nitrogen, and stored at -80° C.

2.13.1. Brain homogenisation and protein quantification

Brain tissues were mechanically homogenised (5 seconds bursts repeated 3 times; Omni Tissue Homogenizer 115, Omni International, Kennesaw, Georgia, USA) in 50 mL Falcon tubes (BD, Oxford, UK) in 4° C cold-room in homogenisation buffer (1 mL/100 mg brain tissue; Appendix 1) supplemented with phenylmethanesulphonylfluoride (1 mM; Sigma-Aldrich) and protease inhibitor cocktail (#11873580001, Roche Diagnostic). A clean homogeniser blade was used for each sample. Brain homogenates were kept on a constant rocker in 4° C for 30 min, and centrifuged at 10 G at 4° C for 10 min. The supernatant was transferred and aliquoted into labelled 0.5 mL PCR tubes. A modified Lowry colorimetric protein assay (Bio-Rad DC Protein Assay; Bio-Rad, Hemel Hempstead,

Hertfordshire, UK) was used to calculate protein concentrations against a bovine serum albumin (BSA) standard. The Lowry assay is based on the reduction of Cu^{2+} into Cu^{+} in alkali solution, which is affected by the concentration of amino acid residues in solution, and the subsequent reduction product of Folin reagent which is quantified. Five-microlitre of BSA protein standards and sample proteins, diluted 1:10 in homogenisation buffer, were loaded on 96-well plates (BD) in triplicates. Twenty-five microlitre of Reagent A (alkaline copper tartrate solution) and 200 μL of Reagent B (Folin reagent) were added to each well and allowed to incubate on a constant rocker for 15 min. The absorbance was read by a microplate absorbance reader at 750 nm (Bio-Rad). The 3 absorbance readings for each sample were averaged and calculated against the BSA standard curve to obtain protein concentrations.

2.13.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and membrane transfer

To prepare the sample for SDS-PAGE, 32.5 μg (1.25 mg / mL) of sample protein were mixed with 10 μL 2X Laemmli sample buffer (Bio-Rad) and 4 μL dithiothreitol (0.1 M final concentration; Sigma-Aldrich), for a final volume of 40 μL , and denatured on a Eppendorf Thermomixer (Eppendorf, Cambridge, UK) set at 95°C and 300 rpm for 3 min. The samples were centrifuged to 10G for 3 seconds.

Molecular weight standards (10 μL ; Dual-Colour Precision Plus Protein Standards, Bio-Rad), positive control sample (30 μL ; HeLa cell lysate, Santa Cruz) and negative control sample (30 μL ; distilled water), and protein samples (30 μL) were loaded into pre-cast SDS-PAGE gels (Criterion Tris-HCl gel 12.5% or Criterion TGX 4 – 20%; Bio-Rad) for vertical 2D electrophoresis at 100V (Criterion Cell, Bio Rad) in 1X Running Buffer

(Appendix 1), until the line of bromophenol blue reached the end of the gel. The gel was carefully removed and rinsed with Transfer Buffer (Appendix 1).

After 30-second activation of the polyvinylidene fluoride membrane in methanol, a wet transfer sandwich was prepared with the membrane positioned on the anode side of the gel. The sandwich was inserted into the transfer box (Criterion Blotter, Bio Rad) and filled with cold Transfer Buffer, together with cooling unit and magnetic stir bar. Proteins were transferred from the gel to the membrane at 100V for 45 min on a magnetic stirrer. At the end of the transfer, the sandwich was opened and the membrane rinsed in 0.1M phosphate buffered saline with 0.1% Tween 20 (PBST). Complete transfer of the visible molecular weight standards from the gel onto the membrane was accepted as evidence of successful protein transfer.

2.13.3. Immunoblotting

Membrane was blocked overnight at 4° C in 5% w/v skim milk in PBST. All incubations steps were performed on a constant rocker. The membrane was incubated for 1 hour in primary antibody diluted in PBST. After 3 times 10 min PBST wash, the membrane was incubated for 1 hour with appropriate horseradish peroxidase-conjugated secondary antibody, and final 3 times 10 min PBS wash. Immunoreactive bands were revealed by luminol enhanced chemiluminescence (ECL) reaction for 5 min (PS-3, Lumigen, Southfield, Michigan, USA). The membrane was drained of excess luminol solution, placed between transparency films, and developed using the Biospectrum AC imaging system (UVP, Cambridge, UK). Each membrane was sequentially exposed for 1 min, 3 min and 10 min to ensure optimal and consistent chemiluminescence signal collection.

2.13.4. Densitometry

Protein band densitometry was made using Visual Works software (UVP) and normalised against β -tubulin and/or β -actin loading control bands. Normalised band densities between protein samples from individual animals in the groups were averaged and expressed as the mean $\pm$ standard error of the mean.

2.13.5. Membrane Restoration and Re-probing

A membrane restoration kit (Blot-Restore, Merck) was used to wash off chemiluminescent reagents and remove primary and secondary antibodies bound to the membrane. To validate the removal of bound primary and secondary antibodies, a restored membrane was re-probed with the same secondary antibody and developed with luminol ECL, while omitting the primary antibody. This membrane did not reveal detectable bands after 30 min of exposure time. Restored membranes were re-probed by immunoblotting for different proteins of interest for maximum of 3 times.

Antibodies used for immunoblotting are listed in Table 2.3. See Appendix 1 for constituents of solutions used.

2.14. Proteome array

Proteome Profiler Mouse Angiogenesis Array (#ARY015, R&D Systems, Abingdon, Oxfordshire, UK) was used. Brain homogenates were prepared and quantified as for Western blot to obtain protein samples. Protein samples from individual animals in each group were pooled ($n = 3$). Samples were incubated for 1 hour with biotinylated anti-mouse antibodies (R&D) to permit subsequent detection by Streptavidin detection antibodies. Each sample was incubated with nitrocellulose membrane containing 53

different capture antibodies spotted in duplicates (Appendix 2), in an individual tray at 4° C overnight on constant rocker. On Day-2, membranes were washed 3 times 10 min in 1X Wash Buffer (R&D) to remove unbound proteins. The membranes were then incubated with HRP-conjugated Streptavidin detection antibodies (R&D) for 30 min. Following 3 times 10 min wash in 1X Wash Buffer, the membranes were placed on transparency films and incubated with ECL as for Western blots. Membranes were simultaneously exposed for 15 min with Biospectrum AC imaging system to collect ECL signal.

Relative protein expressions were quantified by measuring spot densities in Image J software and subtracting background spot densities of negative control spots. The 2 duplicate spots were averaged and expressed as a percentage of the positive control spots and/or percentage of wildtype naïve brain protein levels. See Appendix 2 for full list of spotted capture antibodies and coordinates.

2.15. Statistics

Continuous numerical data were presented as mean $\pm$ standard error of the mean. Differences between two groups were assessed using Student's t-test and amongst three or more groups with analysis of variance (ANOVA) with Bonferroni post-hoc test. Groups were considered significantly different when at least a 95% confidence level ($P < 0.05$) was obtained. To analyse ordinal data (e.g. neurological deficit score), the Mann-Whitney non-parametric test was used. To assess the distribution of PCP vector angles in ependymal cells, Kolmogorov-Smirnov test was performed. Analyses and graphical representations were performed in Microsoft Excel 2007 with XL Toolbox 2.80 (<http://xltoolbox.sourceforge.net>) and GraphPad Prism 5 (GraphPad, La Jolla, CA, USA).

Chapter 3

Middle cerebral artery occlusion model of stroke in mice

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3.1 Introduction

For nearly 200 years, investigators have devised ingenious methods to model clinical stroke. In 1836, the renowned vascular surgeon, Sir Astley Cooper of Guy's Hospital in London, made the first physiological observations on the effects of carotid and vertebral arteries ligations in dogs (Cooper, 1836). Stroke modelling has evolved significantly with better understanding of its pathophysiology and the advent of modern vascular and neurosurgical techniques. Experimental stroke models have enabled deep understanding of the pathogenesis and pathophysiology of stroke at molecular and cellular levels. In combination with modern scientific tools and advancements in the fields of molecular medicine, transgenic animals and stem cell biology, experimental stroke models are central to the efforts to devise novel therapeutic strategies for the treatment of stroke. Key areas of scientific investigation using experimental stroke have included: (1) understanding the pathogenesis and pathophysiology of stroke, (2) discovery of therapeutic targets and drug discovery, and (3) development of efficacious and safe therapeutic strategies, with potential for translation into the clinics. Investigators have relied on experimental stroke models in laboratory animals to study adult neurogenesis and its response to stroke.

3.1.1. Challenges to stroke modelling

A variety of mammalian species have been used to surgically model stroke, including rodents, rabbits, pigs, cats, dogs, and non-human primates. Today, the majority of preclinical studies are carried out in mice and rats (Liu and McCullough, 2011), although there is much contention in the scientific community regarding whether they serve as adequate model animals for humans.

The human brain accounts for only 2% of total body weight, however it receives approximately 15% of cardiac output and consumes nearly 20% of oxygen used in the entire body (Kandel et al., 2000). Neural tissue has a high metabolic demand, and loss of oxygenated blood supply for even a few minutes can lead to permanent neuronal damage (Kandel et al., 2000). The brain has physiological and anatomical features which prevent circulatory compromise. In a process known as auto-regulation, cerebral blood vessels can detect changes in systemic blood pressure and brain oxygenation, and dilate or constrict to maintain homeostasis (Ganong, 2005). Anatomically, the mammalian brain is protected from circulatory compromise by an interconnected ring of arteries at the base of the brain known as the circle of Willis (Fig 3.1). The cerebral arteries, which carry oxygenated blood to the brain, form an anastomosis through a series of communicating arteries at the base of the brain, thus connecting the anterior (internal carotid arteries) and posterior (vertebral arteries) cerebral circulations, and the left and right hemispheric circulations. In effect, should the blood flow in any of the 4 main brain-supplying arteries become obstructed proximal to the circle of Willis, the anastomosis ensures collateral blood supply, and prevent brain ischaemia. As a result, a near complete obstruction of a carotid vessel (> 90%) may be entirely asymptomatic (ACAS, 1995). Ironically, this neuroprotective mechanism presents a hurdle to investigators, since occlusion of a single, or even both carotid arteries is ineffective to cause stroke. The focal nature of clinical stroke and the circle of Willis anastomosis have meant invasive techniques are required to block cerebral blood vessels at least at the level of the circle of Willis, in order to model stroke.

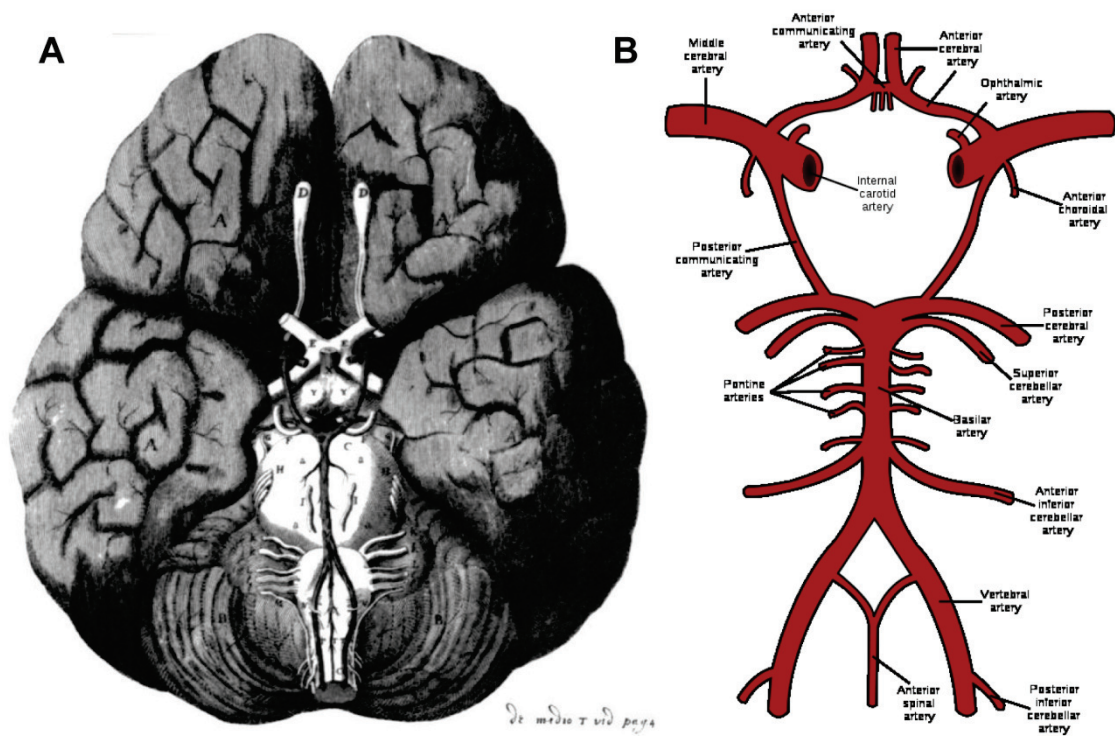


Figure 3.1. Circle of Willis in stroke anatomy

- (A) Christopher Wren's engraving of the human brain viewed ventrally published in *Cerebri Anatome* by Thomas Willis (O'Connor, 2003).
- (B) Circle of Willis vascular anastomosis at the base of the brain.

3.1.2. Animal models of stroke

Techniques used to model stroke can broadly be divided into 2 categories: focal cerebral ischaemia and global cerebral ischaemia. Focal ischaemia in the MCA territory seeks to model the 70% of clinical stroke that affects the MCA territory (Fig 3.2). On the other hand, global ischaemia mimics acute arterial insufficiency during cardiopulmonary arrest. However, from a stroke perspective, global ischaemia models permit the study of general principles of neuronal response to ischaemia, as well as investigations of ischaemia in regions unaffected by focal models (e.g. CA3 of the hippocampus; Hoyte et al., 2004).

Focal MCA stroke models

Focal stroke models involve the obstruction of cerebral blood flow to a specific region of the brain, resulting in localised ischaemia and infarction. This is usually achieved with surgical manipulation of the MCA. Early attempts in Macaque monkeys and dogs (Harvey and Rasmussen, 1951) involved transcranial surgical approaches through the temporal skull and clipping or ligation of isolated MCA at different distances from its origin (distal MCAO). These methods have been modified and successful attempts have been made to approach the MCA using a trans-orbital approach whereby invasive craniotomy is avoided, although significant surgical skills are required (Howells et al., 2010). Novel methods of occluding the MCA have been developed, including electrocautery and endothelin injection, in attempts to lessen mechanical perturbation of brain tissue during surgical ligation. However, there is concern over electrocautery and the effect of thermal current on surrounding brain tissue (Chauveau et al., 2010). Doubts have also been raised over the use of endothelin, a potent vasoconstrictor, which may potentiate NMDA-mediated neurotoxicity (Yamashita et al., 2000).

Variations of distal MCAO methods have been transferred to rodents, and are commonly used today. Distal MCAO produces a precise, reproducible, and relative small infarct lesion, largely confined to the cortex. This method has been used to demonstrate the feature of SVZ-mediated endogenous repair following stroke, and that cortical infarct distant from the SVZ is able to increase SVZ proliferation and recruit newborn neuroblasts to migrate towards the injury (Wang et al., 2007; Ohab et al., 2006). However, transcranial distal MCAO involves significant surgical trauma with craniotomy and breach of the dura and the CSF compartment, which potentially confound the effects of brain ischaemia (Howells et al., 2010)

Intraluminal filament MCAO

The intraluminal filament MCAO with reperfusion in mice is widely used by investigators. In the hands of a well-trained investigator, it is a reliable and reproducible model, producing consistent infarcts affecting mainly the striatum and the cortex to a lesser degree, depending on the strain of mice used (Carmichael, 2005). The intraluminal filament MCAO was first developed in rats (Koizumi et al., 1986) whereby a ventral cervical approach was used to access the carotid vessels. After temporarily occluding the carotid arteries to create a bloodless field, a silicone-coated nylon suture was introduced through an arteriotomy into the CCA and advanced up the ICA to block the origin of the MCA at the circle of Willis. The initial technique did not allowed for cerebral reperfusion at the end of the ischaemia time, now considered a major contributor of stroke pathophysiology (Bulkley, 1987; Leff and Repine, 1990) and modifications were later made to introduce the filament through the ECA which was permanently tied upon withdrawal of the filament at end of the ischaemic time and permitted reperfusion of the

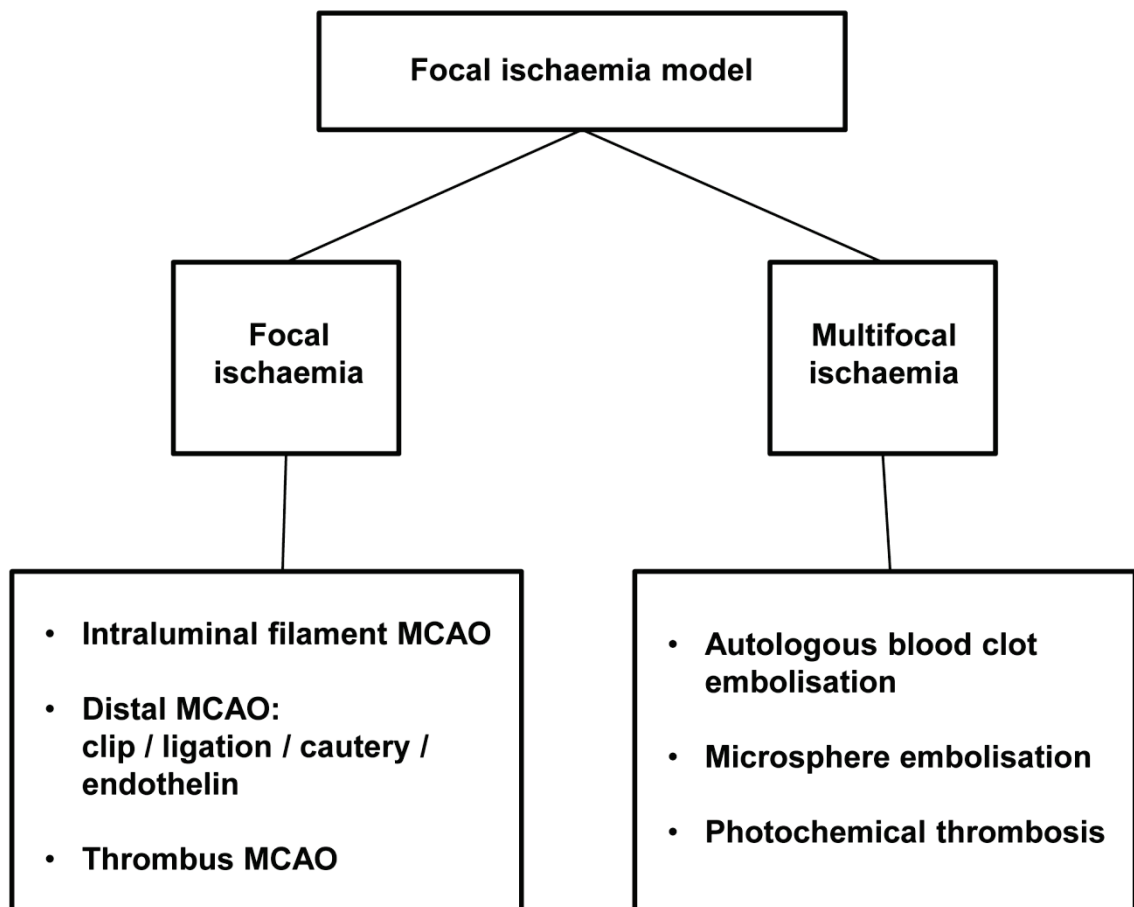


Figure 3.2. Common rodent models of ischaemic stroke

Modified from (Liu and McCullough, 2011)

ipsilateral CCA and ICA, with the ECA supply territory compensated by adequate collaterals. The intraluminal MCAO with reperfusion was adapted for mice to make use of transgenic mice lines (Yang et al., 1994).

The Szele Laboratory had not previously examined the SVZ after ischaemic brain injury. Therefore, based on the wide use of the intraluminal MCAO in mice, and experience with the model in the collaborating Buchan Laboratory, this model was adopted for the investigation of the murine SVZ after stroke. However, there were considerable technical challenges with the model, as well significant variation depending on surgical technique, strain of mouse used and the length of MCA occlusion time. In addition, neither laboratories had carried out MCAO in the 129sv mouse strain, the background strain of the transgenic mouse line which will be used in the Gal-3 study. In this chapter, I used the intraluminal filament MCAO on 129sv mice, and documented the extent of infarct volume after 30, 40 and 50 min of MCAO and assessed neurological outcomes. The consistency of the MCAO model with regards to infarct volumes and neurological outcomes was examined, and effects of post-operative temperature regulation and glucose supplementation were assessed. In doing so, a 50-min MCAO protocol was established for the studies in this thesis.

3.2 Results

3.2.1. MCAO surgery

Eleven out of eighteen mice successfully underwent MCAO. MCAO surgery was completed in 13 out of 18 mice (72%), with MCAO times of 30, 40 and 50 min. In the remaining 5 animals (28%), MCAO surgery was incomplete and they were excluded from the experiment. Of the 5 failed attempts, 3 were excluded due to excessive intra-operative haemorrhage and the remaining 2 due to insufficient CBF suppression after filament insertion. Two of the thirteen mice which completed surgery died in the post-operative period within 24 hours. They had undergone 30- and 40-min MCAO respectively. Post-mortem was carried out to determine the cause of death. Subarachnoid haemorrhage is a common cause of mortality after experimental MCAO (Tsuchiya et al., 2003). However, this was not evident in either animal. In both animals, large haematomas were found subcutaneously in the neck and thoracic regions. The cause of death in both animals was likely massive haemorrhage from the surgical site.

The average time from the start of the anaesthesia to the point of filament insertion to occlude the MCA was 80 ± 5 min. The total duration of anaesthesia was 145 ± 4 min. During the course of these MCAO surgeries, their surgery time shortened as surgical competency improved (Fig 3.3A).

3.2.2. Changes in cerebral blood flow

During MCAO surgery, the CBF to the MCA vascular territory was monitored by a laser Doppler flowmetre. MCAO decreased CBF to less than 30% of the pre-ischaemic baseline (Buchan et al., 1992). In all cases, occlusion of the ipsilateral CCA and ICA (which creates a bloodless field necessary for filament insertion) was associated with a considerable drop in CBF (*CCA clamp*, Fig 3.3B). Filament insertion and advancement up the ICA to occlude the origin of MCA produced further drop in CBF to less than 30% of baseline (*MCAO*, Fig 3.3B). This was maintained whilst the filament remained *in-situ*. After 30, 40 or 50 min of MCAO, the filament was slowly withdrawn, and CBF monitoring continued for a further 15 min. The MCA territory was reperfused, initially via the circle of Willis, followed by re-established ipsilateral internal carotid supply. Reperfusion corresponded with return of CBF to at least 50% of pre-ischaemic baseline maintained for 15 min. Sham surgery animals, which did not have filament insertion, did not drop their CBF to < 30 % of baseline, and maintained a steady baseline CBF for the duration of anaesthesia.

3.2.3. Neurological deficit

Twenty-four hours following recovery from MCAO surgery, the animals were assessed for neurological deficit using the modified Neurological Deficit Score (mNDS; Barber et al., 2004). The gross assessment system documents evidences of motor asymmetry and hemiparesis. Thirty-minutes MCAO caused minor flexion of the contralateral trunk and limbs in all 3 mice (mNDS 1). Forty-minutes MCAO resulted in different grades of neurological deficit. The first (40-min MCAO #1) displayed limb flexion and lateral

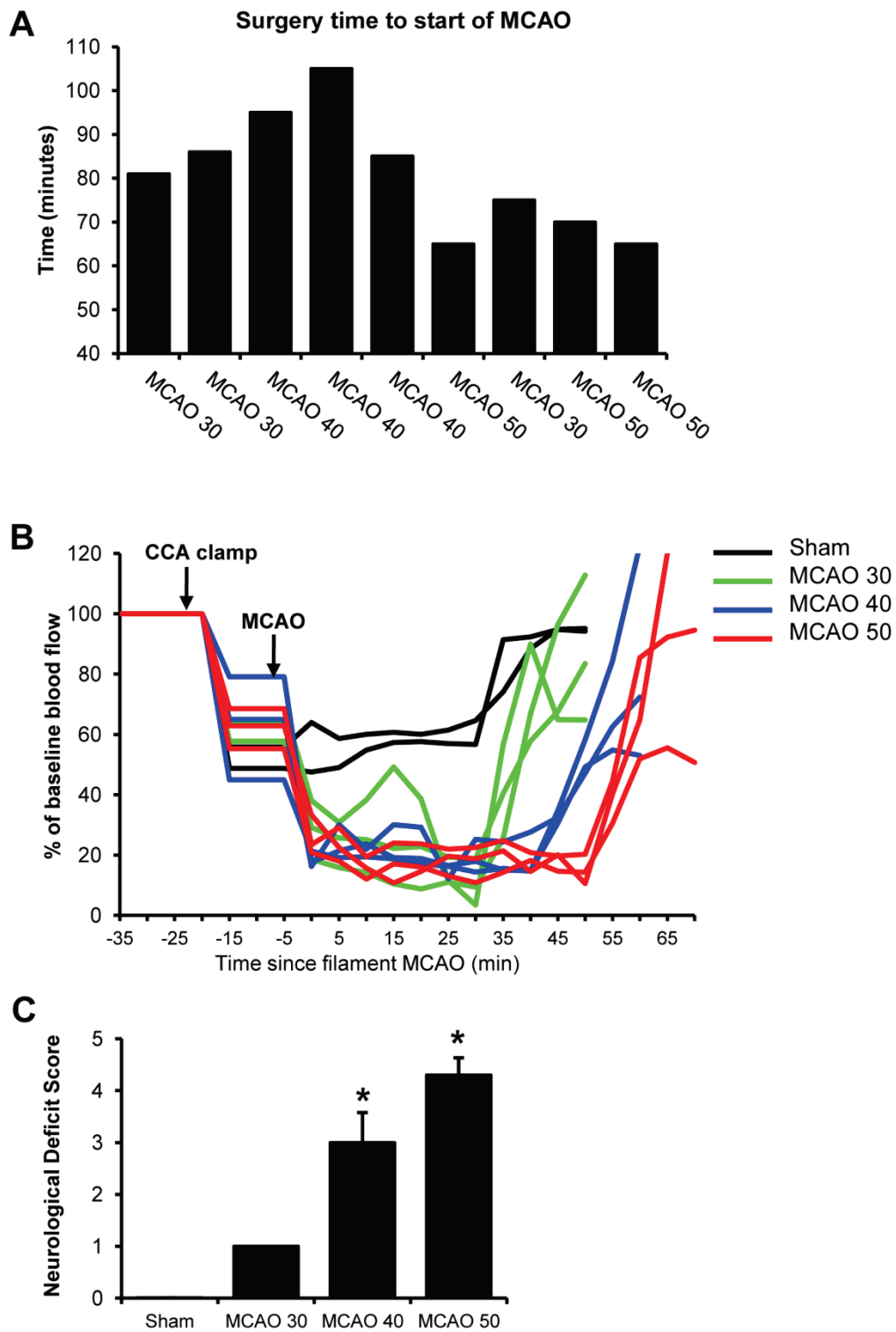


Figure 3.3. Cerebral blood flow and neurological status assessment

- (A) Surgery time to commencement of MCAO. Presented in the order the surgeries were carried out.
- (B) Laser doppler measurement of cerebral blood flow.
- (C) Neurological status measured after MCAO.

instability (mNDS 2). The second (40-min MCAO #2) displayed limb flexion, lateral instability, as well as tight circling gait (< 50cm diameter) towards the paretic side (mNDS 4). The third (40-min MCAO #3) displayed limb flexion, lateral instability, and minor circling gait (> 50cm diameter; mNDS 3). Fifty-min MCAO produced the most obvious and severe neurological deficit, consistently with limb flexion, lateral instability and tight circling gait (mNDS 4 and 5). Sham-operated animals did not exhibit observable neurological deficits (mNDS 0).

3.2.4. Infarct volume

Twenty-four hours after MCAO surgery, after assessment of neurological status, the mice were sacrificed. TTC-staining was used to assess infarct volumes. Unstained brain areas demarcated infarcted brain tissue. Sham-operated brain sections were uniformly stained deep red, except for white matter tracts such as the corpus callosum and anterior commissure (Fig 3.4A). Thirty minutes of MCAO resulted in small unstained infarct lesion in the striatum in one animal, but no visible infarct in the others. Forty minutes of MCAO resulted in infarct of the striatum. Fifty minutes of MCAO produced the largest infarct, involving mainly the striatum and the cortex to a lesser extent (Fig 3.4B). Portion of the thalamus and piriform cortex were also lesioned. Importantly, in all three 50-min MCAO animals, the medial border of the infarct did not appear to involve the periventricular zone (*green line* Fig 3.4B). The infarct volume after 50-min MCAO (17.39 ± 2.56 % of the contralateral hemisphere) was significantly greater than both 30- (2.75 ± 2.76 %) and 40-min (8.46 ± 0.29 %) MCAO (Fig 3.4C; $P = 0.02$; ANOVA).

To better ascertain the extent of the brain infarct, and its evolution over time, a separate cohort of animals were sacrificed 7-days following 50-min MCAO. Loss of

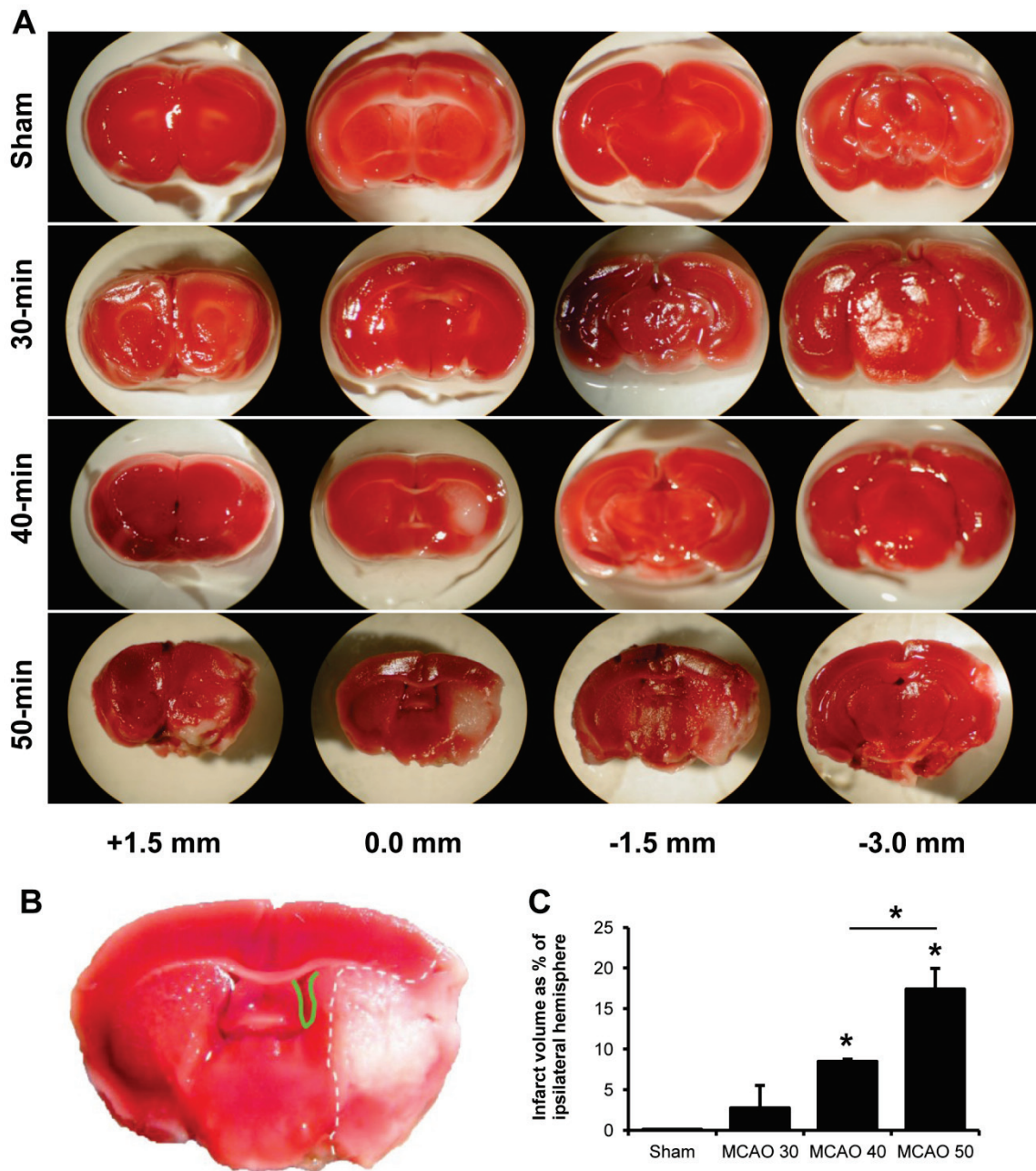


Figure 3.4. Infarct volumes with different MCAO time

- (A) TTC-stained brain slices depicting area of infarct (white unstained) after different MCAO time.
- (B) Infarct of the ipsilateral striatum and cortex (*white dotted line*) sparing the SVZ (*green*).
- (C) Infarct volume reconstruction using TTC-stained slices.

NeuN and initiation of GFAP+ glial scar formation were independently used to assess infarct volume in coronal brain sections (Fig 3.5A-C). The infarct volumes measured by NeuN (21.1 ± 1.5 % of the hemisphere) and GFAP (25.1 ± 2.8 %) did not differ significantly ($n = 6$; Fig 3.5D). The measurements were not statistically different than those obtained with TTC-staining 24 hours post-stroke. The ischaemic lesion extended from the medial striatum to the lower cortical layers, consistent with published works on MCAO in the 129sv strain (Endres et al., 1998; Pham et al 2010). The SVZ can be seen as a cell nuclei-dense region between the striatum and the septum, and is well visualised in GFAP- and DAPI-stained sections (Fig 3.5E). Critically, in agreement with the previous TTC-staining results, 7-days post-stroke the SVZ does not constitute part of the ischaemic lesion, and it is separated from the border of the lesion by a rim of healthy NeuN+ cells. I never observed ischaemic lesion in shams or contralateral hemispheres.

3.2.5. Temperature regulation in MCAO

In the experiment to determine an optimal MCAO time, 24 hours post-MCAO surgery, at the time of sacrifice, I noticed that the animals' bodies were cool, and they were not moving freely in their cages. The rectal temperature of one such animal was measured at 28.0°C . Hypothermia is neuroprotective, and could have been a significant confounder in my studies. I therefore compared 2 strategies aimed to keep body temperatures within the physiological range.

Two groups of mice ($n = 5$) underwent 50-min MCAO. Post-operatively, one group was kept in a warmed incubator for 2 hours at 28°C (2-hour group), and the second group kept in the same incubator for 24 hours (24-hour group). Rectal temperature was measured immediately after removal from the incubator and then daily in the middle of

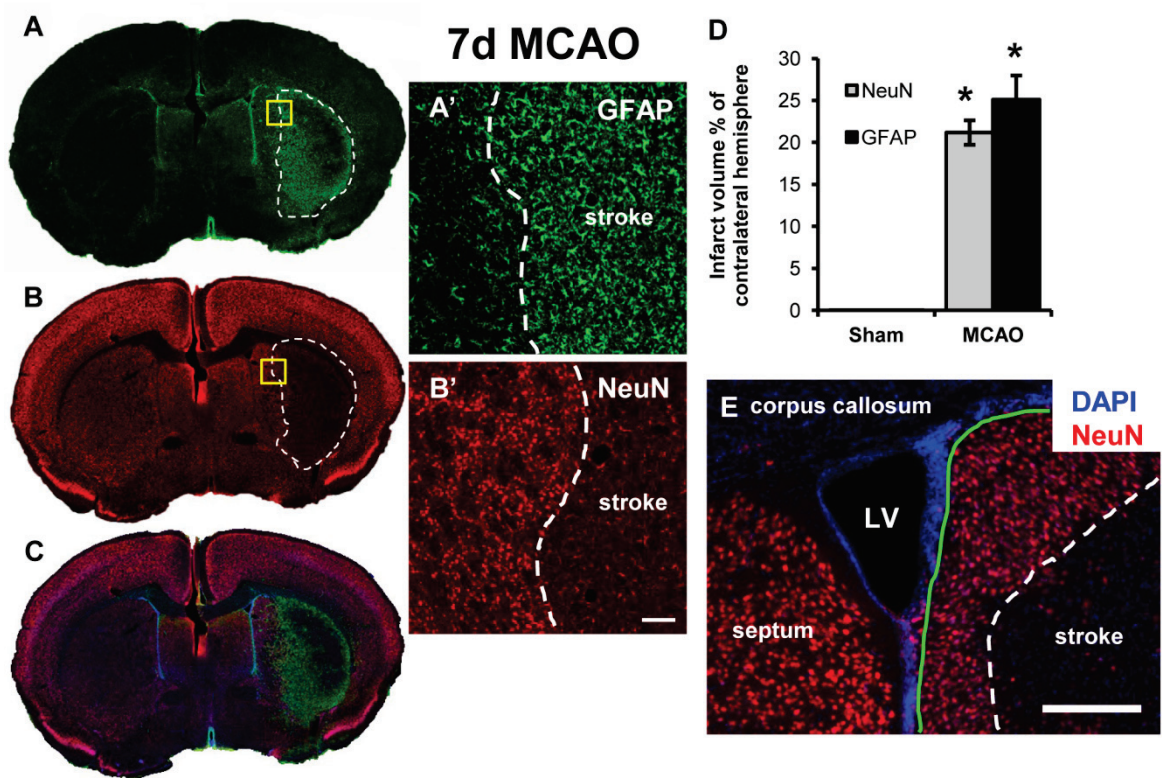


Figure 3.5. Histological assessment of infarct volume 7-days post-MCAO

- (A-C) Representative coronal sections showing area of infarct (*white dotted line*). (A') and (B') *yellow boxed areas* in (A) and (B): The medial infarct edge approaches but does not abut the SVZ.
- (D) Infarct volume reconstruction using NeuN and GFAP immunohistochemistry showing significant brain infarct.
- (E) NeuN immunofluorescence merged with DAPI to show relative anatomical position of lateral ventricle (LV), subventricular zone (*green line*), and infarct.

Scale bar 100 μ m.

the daylight cycle (11h00 - 14h00) for 3 consecutive days. The incubator maintained body temperature at $35 \pm 1^\circ \text{C}$ in both groups. However, 1 day after MCAO, the 2-hour group recorded significantly lower body temperatures ($29.3 \pm 0.9^\circ \text{C}$) compared to the 24-hour group ($35.0 \pm 0.2^\circ \text{C}$; $P < 0.001$, *Day 1*, Fig 3.6A). Two days after MCAO, the 24-hour group had physiological temperatures ($36.2 \pm 0.3^\circ \text{C}$), compared to significantly lower temperatures in the 2-hour group ($28.8 \pm 1.0^\circ \text{C}$; $P < 0.001$). Three days after MCAO, the abnormally low body temperatures persisted in the 2-hour group ($P < 0.001$).

3.2.6. Glucose saline supplementation

Post-MCAO, mice received daily intraperitoneal injections of normal saline to help maintain hydration. However, in the 3-day survival temperature regulation experiment, significant weight loss in post-MCAO mice was noticed. By Day-3 post-MCAO, some had lost almost 20% of their baseline body weight. The effect of daily intraperitoneal injection of 5% glucose in normal saline (glucose saline group) was compared to normal saline (saline only group). Glucose administration was started 24 hours post-MCAO. In both groups, the body weight had decreased significantly by Day-1. However, by Day-3, although the body weights of the groups were not significantly different, I observed increased activity in the glucose saline group compare to the saline only group. By Day-7, there was significant difference in body weight ($P = 0.02$; Fig 3.6B). The body weight of the glucose saline group stabilised and continued to decrease in the saline only group.

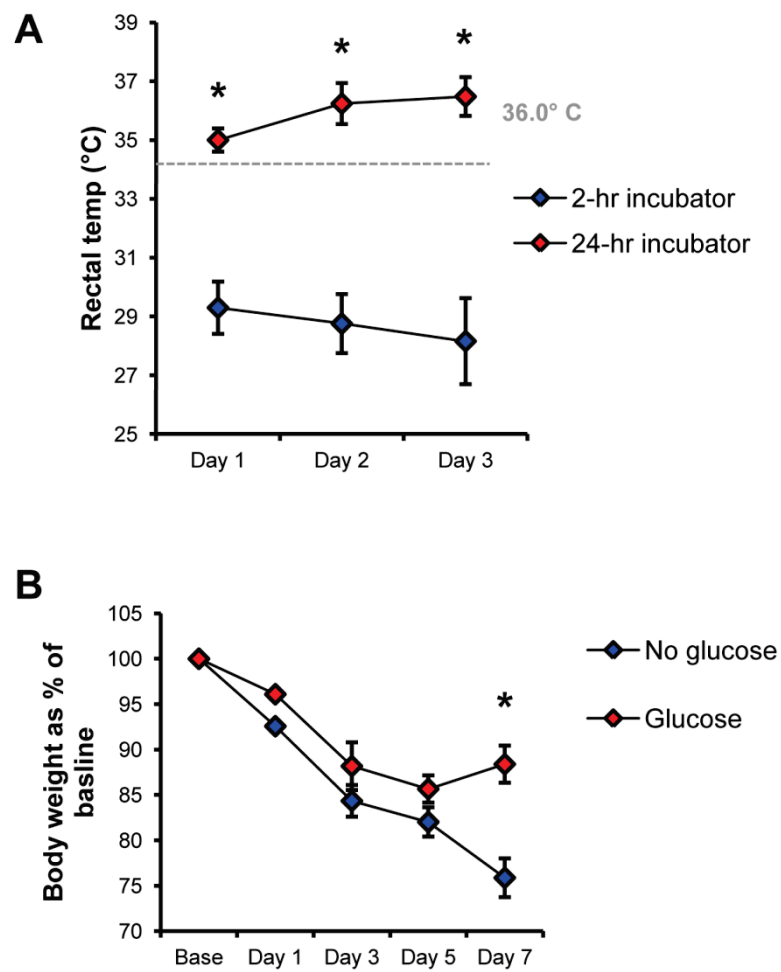


Figure 3.6. Physiological parameters post-MCAO

(A) Rectal temperature post-MCAO measured during middle of daylight cycle.

(B) Body weight post-MCAO.

3.3. Discussion

Intraluminal filament MCAO is a feasible method to model ischaemic stroke in mice with a high degree of reproducibility. Here I confirmed that this method resulted in significant cerebral ischaemia and produced brain infarct, as demonstrated by (1) decrease of cerebral blood flow to less than 30% of baseline, (2) functional neurological deficit, and (3) histological evidence of tissue infarction. Post-MCAO mice were frequently hypothermic, as previously reported (Barber et al., 2004) and this was rectified by extended recovery time of 24 hours in warmed incubator set at 28° C. Post-MCAO mice were prone to metabolic imbalances and showed significant weight loss. Daily glucose supplementation gradually began to restore body weight over the 7-day survival period.

3.3.1. Reliability and reproducibility of the experimental stroke model

A reproducible and predictable stroke model is important to allow meaningful scientific interpretation of results. For example, a comparable baseline infarct size or neurological deficits are necessary to assess the effects of genetic manipulations or pharmacological treatments. The pursuit of reliability and reproducibility occasionally conflict with validity. For instance, although the thrombus model of MCAO may perhaps better resemble the pathogenesis and pathophysiology of clinical stroke, its unpredictability and difficulty in determining the baseline threshold makes it a less-favoured model in the research community.

Many factors (Table 3.1) affect the reliability and reproducibility of intraluminal filament MCAO. The size and location of infarcts can vary significantly between mice strains due to differences in the patency of the posterior communicating arteries and the extent

Table 3.1. Factors which affect MCAO stroke outcome

Factor	Remarks
Animal strain	Difference in circle of Willis anatomy and collateral blood supply.
Age	Aged animals sustain larger infarct volume and more prone to complications. Surgery more difficult in aged.
Sex	Oestrogen-mediated protection in females. Differences in inflammatory response and cell death pathways.
Body weight	Smaller animals sustain larger infarct volume. Post-MCAO body weight correlates with functional recovery.
Body temperature	Hypothermia results in reduced infarct volume and weight loss.
Diameter and stiffness of occluding filament	Larger diameter results in larger infarct volume. Stiff filament increases risk of intracranial trauma.
Ischaemic time	Needs to be optimised for each laboratory and surgeon. No reperfusion injury component in permanent occlusion
Surgical expertise	Consistent surgical approach with minimal blood loss.

of cortical collateral between the anterior and middle cerebral artery territories (Connolly et al., 1996). 129sv mice have well-developed posterior communicating arteries (Fujii et al., 1997) and cortical collateral vascular supply (Maeda et al., 1998) compared to C57Bl/6 mice. The body weight is also an important factor which impacts the infarct size. Using filaments of the same diameter, differences in body weight by more than 5 g significantly changed the infarct size, with the larger animal sustaining a smaller infarct (Connolly et al., 1996; Takaba et al., 2004). Similarly, a difference of 50 μm in the filament diameter can affect the tightness of the occlusion at the MCA origin and affect infarct size (Connolly et al., 1996). Temperature regulation is also very important to achieve consistent results (Barber et al., 2004). Mice have a large skin surface area to body weight ratio, and become hypothermic in the post-operative period.

Fifty minutes of MCAO is a reproducible model of stroke

In the above experiments, I showed that the length of time that the MCA is occluded by the intraluminal filament (MCAO time) determines infarct size. MCAO times ranging from 30 to 60 min have been used for model stroke in 129sv mice (Carmichael, 2005). The Buchan Laboratory has used 45 min of MCAO time in C57Bl/6 mice to obtain reproducible infarcts of the striatum and cortex (Barber et al., 2004). Fifty minutes of MCAO time resulted in functional neurological deficits and brain infarct volume with greater consistency compared to thirty and forty minutes of MCAO. Therefore, 50-min MCAO were used for all MCAO experiments in the rest of this thesis.

Functional neurological deficit after MCAO was assessed using a modified version of the NDS originally designed for assessing deficits in post-MCAO rats (Bederson et al., 1986b). In the original study, the score correlated well with TTC-stained infarct areas, although a small infarct area (< 10%) resulted in a negative score (mNDS 0). Motor

disturbances including hemiparesis and motor asymmetry are well known sequelae of lesions of frontal cortex and striatum in rodents, and the severity of neurological deficit has been correlated with an increase in the size of the lesion (Bederson et al., 1986a). NDS was used to document improved functional recovery after MCAO in mice deficient in neuronal nitric oxide synthase (Huang et al., 1994) and those over-expressing human superoxide dismutase (Yang et al., 1994). However, in addition to gross motor deficits, a variety of sensory-motor, learning and species-specific behaviour (Deacon, 2006) may be affected, and more sensitive testing batteries will be necessary to document these and other more subtle motor deficits. For instance, in this study, there were occasions when post-stroke mice scored similarly on the mNDS, although one may be alert and active, while the other remained immobile unless physically stimulated. Clearly, the mNDS does not account for such differences in behaviour. Furthermore, the deficits measured by mNDS seem to resolve fairly rapidly following MCAO. Therefore it may be less useful for measuring stroke impairments in the medium or longer term. Indeed, more detailed composite neurological deficit scoring systems have been used which incorporate components of motor, sensory and reflex testing (Li et al., 2000; Hunter et al., 2000; Rogers et al., 1997). Functional assessment in mice is not as well as established compared to testing batteries designed for rats, and training mice for motor tasks may be more time-consuming requiring greater behavioural expertise (Hoyte et al., 2004).

The SVZ is spared from the MCAO infarct

TTC is reduced by intact mitochondrial dehydrogenase to form formazan, a deep red and fat-soluble compound (Bederson et al., 1986a). It is routinely used to allow rapid analysis of the extent of irreversible ischaemic damage in the brain and heart, and has been validated following both rat and mouse intraluminal filament MCAO (Türeyen et al.,

2004). A high degree of correlation has been reported between TTC-staining and traditional histopathological methods such as haematoxylin and eosin, and cresyl violet stains, which allow direct visualisation of vacuolated cytoplasm and early stages of nuclear disintegration (Park et al., 1988).

The infarct affects the ipsilateral brain hemisphere, involving the striatum and cortex. However, despite the extensive striatal involvement, there was no obvious direct extension of the infarct to the SVZ. This was an important consideration in order to distinguish the effects of a large but distant focal brain infarct on the SVZ, as opposed to the direct effects of ischaemic injury to the SVZ. For example, following controlled cortical impact injury, lesions which directly involved the SVZ resulted in SVZ microglial activation, while more superficial lesions did not (Goings et al., 2006). However, despite the absence of macroscopic SVZ infarct/ischaemia based on TTC-staining, minor degrees of SVZ hypoxia can affect the neurogenic niche (Thored et al., 2007). Furthermore, ischaemic stroke and reperfusion is an evolving injury (Lin et al., 1993; Liu and McCullough, 2011). In the ischaemic striatum, there is a balance between neuronal survival and continued neuronal deaths which will affect final infarct volume. Acute tissue oedema which affects cerebral-vascular perfusion pressure is also important. However, 7-days post-MCAO when the infarct lesion would have fully evolved (Du et al., 1996; Garcia et al., 1993), immunohistochemical examination of the infarct confirmed that the stroke model used in this study indeed did not injure the SVZ directly. Notably, the infarct volumes measured by GFAP and NeuN immunolabelling at 7-days post-MCAO, although statistically non-significant due to the small sample size, appeared larger than those obtained by TTC-staining at 1-day, suggesting either an evolution in the size of the stroke volume over 7 days or differences between the methods of measuring infarct volume.

3.3.2. Improving post-surgical morbidity and mortality

Even after 3 months of training, the rate of failed MCAO surgery was 28%. This was accompanied by 15% mortality rate. However, surgical refinements have been introduced which minimise intra-operative haemorrhage and unnecessary tissue trauma, with the overall aim of reduced surgical complications and mortality. Despite improved surgical experience, each animal undergoing MCAO is expected to remain anaesthetised for approximately 2 hours. Prolonged anaesthesia disturbs the normal physiology and in particular difficulty arises in maintaining body temperature and body weight in the post-operative period. Careful post-operative care is essential to ensure adequate physiological recovery. I expect that incorporating the surgical refinements, temperature regulation and nutritional supplementation, post-MCAO mortality rates would decrease significantly. The post-MCAO mortality rate is a good indication of the standard of surgery and post-operative care. Mortality rate less than 10% is quoted in the literature as a benchmark (Dirnagl, 2010).

Mice are physically more delicate and less able to tolerate surgical stress than the more robust rats (Hoyte et al., 2004). The small size of mice also renders the surgical procedures technically challenging. In my experiments, post-MCAO mice are prone to hypothermia. Their large skin surface area to body weight ratio makes them sensitive to temperature fluctuations during surgery and the post-operative period of decreased motor activity. Post-MCAO mice are also affected by significant weight loss. Nevertheless, strategies have been devised to overcome these limitations.

Extended recovery in incubator regulates body temperature post-MCAO

Changes in body temperature have been clinically demonstrated to affect stroke outcome (Zgavc et al., 2011). In rat MCAO models, the hypothalamus may be involved, resulting in post-stroke hyperthermia (Hoyte et al., 2004). In contrast, post-MCAO mice were profoundly hypothermic, despite intra-operative temperature regulation and 2 hours in warmed incubator. This length of time was selected based on stroke literature which reports that animals were kept in an artificially warmed environment until they have recovered from anaesthesia and become ambulatory (Dirnagl et al., 2010). The Buchan Laboratory previously reported that temperature regulation with feedback heating lamp and intra-abdominal temperature telemetry probes regulated post-MCAO within the physiological range (Barber et al., 2004). In this study, I demonstrate that extended recovery time in an incubator set at 28°C for 24 hours post-MCAO was sufficient to maintain normal body temperature in the post-operative period. Importantly, extended incubation also did not cause hyperthermia which could exacerbate ischaemia brain injury and introduce experimental inconsistency.

Nutrient supplementation is necessary post-MCAO

Oral dietary supplementation with Transwean, which was provided freely in cages, and daily intraperitoneal normal saline injections were insufficient to prevent weight loss post-MCAO. Daily injection of 5% glucose in normal saline did not prevent post-MCAO weight loss altogether, although the animals appeared more alert and mobile, and weighed significantly more than the animals which received only normal saline 7-days post-MCAO. Clinically, acute hyperglycaemia at the time of presentation, or persistent hyperglycaemia over days, are associated with worse outcome after stroke (Baird et al.,

2003; Sykora et al., 2011). Animal studies also suggest that hyperglycaemia at the time of acute brain ischaemia results in superoxide-mediated neuronal death (Suh et al., 2008). Nevertheless, the dose of glucose infused in my study equates to ~0.8 g/kg body weight; a dose lower than the 1 - 2.5 g /kg routinely used of intraperitoneal glucose tolerance test in mice (IPGTT; Andrikopoulos et al., 2008). Low dose IPGTT (1 – 1.5 g/kg) results in transient increase in blood glucose levels by approximately 2-3 mM, which returns to normal within 60 min (Gunton et al., 2005). There is no evidence that such transient increase in blood glucose levels affects stroke outcome in healthy normoglycaemic animals. Furthermore, the majority of animal studies linking hyperglycaemia with adverse stroke outcomes either models Type 1 diabetes, or has prolonged blood glucose levels in excess of 20 mM (MacDougall and Muir, 2011), more than twice the concentration transiently reached with my post-stroke intraperitoneal treatment. Therefore, I believe that the current regimen of glucose supplementation contributes to animal well-being post-MCAO (and post-sham surgery), without adversely impacting stroke outcome or confounding experimental results.

3.3.3. Validity of the experimental stroke model

A major challenge facing stroke researchers is the choice of the appropriate experimental stroke model. There are many factors to consider when choosing a model. The single most important consideration is undoubtedly the extent to which the model corresponds with the human disease, and thus critically the degree to which findings can be generalised from the experimental laboratory animal to human physiology.

A major criticism of the intraluminal filament MCAO is that it is not a physiological model of ischaemic stroke. The acute occlusion of the MCA, although

focal, does not take into account the variable nature of the thromboembolic stroke which may lodge at various sites of the vascular bed, and interact with diseased, atherosclerotic vessels (Millikan, 1992). Although MCAO allows reperfusion following ischaemia, it does not accurately represent the process of gradual recanalisation over days and weeks after thromboembolic stroke.

The thrombus model of MCAO was developed to address these questions, and significantly led to the development of rt-PA which remains the major pharmacological success in stroke research in the past 50 years. Thromboembolic stroke in rabbits was modelled by autologous infusion of 15 - 150 mg of thrombus clots into the CCA and lodged at varying locations of the cerebral arterial bed (Zivin et al., 1985). This method resulted in neurological abnormality including behavioural disturbances, hemi-paresis and reduced activity, and not uncommonly, death. Histological examination of brain tissue revealed embolic infarcts at random locations in the cortical grey matter. Administration of intravenous rt-PA through the ear vein at the time of the thrombus infusion resulted in significantly decreased neurological deficit and mortality, without evidence of intracerebral haemorrhage (Zivin et al., 1985). This proof of principle study has led to the development of thrombolysis as a routine therapy available in much of the UK and the developed world, with up to 25% of patients presenting with stroke suitable for thrombolysis (Rudd et al., 2011). An additional advantage from the stroke modelling perspective is that the carotid catheters can be implanted several days prior to the thrombus infusion, obviating the need of anaesthesia at the time of cerebral ischaemia. Common anaesthetic agents such as isoflurane may have neuroprotective effects during brain ischaemia (Stratmann et al., 2009).

Variations of the thrombus model have been used, including the placement of a large thrombus directly into the MCA in mice and rats (Zhang et al., 1997a, 1997b) which

resulted in the infarct location and size similar to the intraluminal filament method with good reproducibility. There is also evidence that the clot is subjected to degradation and recanalisation with time or therapeutic intervention (Asahi et al., 2005).

Ninety-five percent of ischaemic stroke occurs in people over the age of 45 (Feigin et al., 2003), it is therefore surprising that the majority of investigators (including myself) model stroke in young adult animals. This mismatch may well contribute significantly to the failure to translate therapeutic strategies which appear promising in preclinical testing. This short-coming is likely largely due to the exorbitant cost of aged laboratory animals. In addition, clinical stroke occurs in a complex milieu of co-morbid diseases including ischaemic heart disease, hypertension, type 2 diabetes mellitus and atherosclerosis, interacting with various pharmacological regimens (Wiebers et al., 1990). The current stroke models have not begun to look into the significance of such interactions.

3.3.4. The mice model of intraluminal filament MCAO for stroke research

Despite its short-comings, intraluminal filament MCAO in mice has led to the development of promising therapeutic strategies and pharmacological agents which are now in advanced clinical trials, including hypothermia (Lyden et al., 2010), granulocyte-colony stimulating factor (Belkin, 2009), and mesenchymal stem cells (Detante, 2009).

Mice models further benefit from the availability of a wide selection of transgenic mice which are invaluable research tools that can be used to test various hypotheses. “Knockout mice” in which a specific gene is rendered non-functional have allowed researchers to ascertain the role of specific molecules and signalling pathways in stroke pathophysiology. In Chapter 4, mice homologous for a targeted deletion in the Gal-3 gene are used to examine the role of Gal-3 in neuroblast ectopic migration and stroke outcome.

Conditional knockout mice have also been developed, whereby the researcher has temporal and cellular control to determine precisely when and in which cell type(s) the gene of interest is inactivated (Beckervordersandforth et al., 2010). Bacterial artificial chromosome transgenics are used to create reporter mouse lines which allow endogenous stem cell lineage tracing (Kim et al., 2009). It also provides a tool to study gene mutations identified in patients that are associated with either increased risk or protection from cerebrovascular disease. For example, 11 β hydroxysteroid dehydrogenase type 1 (11 β HSD-1) is an enzyme which regenerates active cortisol from its inactive form, cortisone, and was found at higher levels in obese men (Gura, 2001). Researchers generated transgenic mice with targeted overexpression 11 β HSD-1 in adipose tissue, which resulted in increased visceral fat deposition and truncal obesity, proving for the first time that abnormality of steroid conversion may underlie obesity syndromes (Masuzaki et al., 2001).

3.4. Conclusion

In this chapter, I have identified 50-min intraluminal filament MCAO in mice as a reliable and reproducible model of transient focal stroke. The ischaemic lesions resulted in predictable neurological deficits and infarcts which impacted mainly the striatum while sparing the neurogenic niche. Temperature regulation and metabolic support are likely important factors in this injury model. Despite its shortcomings as a model of clinical stroke, in terms of the species differences between mice and men, and the pathogenesis of ischaemia, it remains a valuable tool for investigators to probe the mechanisms of ischaemic damage and endogenous repair at the cellular and molecular level.

Chapter 4

Cytoarchitectural reorganisation in the subventricular zone after ischaemic stroke

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4.1. Introduction

4.1.1. Change to the adult neural stem cell niche after stroke

A unique feature of the SVZ adult neural stem niche is its participation in endogenous repair following different types of brain injury. After focal ischaemic stroke, SVZ cell proliferation increases, and neuroblasts leave their normal SVZ-RMS-OB migratory pathway to migrate towards injured brain regions (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002., Parent et al., 2002). These phenomena have been observed using different experimental stroke models in all mammalian species examined, including humans. In neurosphere assays, post-stroke neural stem and progenitors cells display increased self-renewal and preferential neuronal and oligodendrocyte differentiation (Zhang et al., 2004; Deierborg et al., 2010). In addition to changes in cell proliferation and migratory destination, other cellular components of the SVZ are altered. A transient increase in endothelial cell proliferation was observed with a paradoxical decrease in vascular density after MCAO in rats (Thored et al., 2007). The same study also reported increased microglial proliferation in the SVZ, and microglia which adopted pro-neurogenic phenotype.

Post-stroke, SVZ neuroblasts begin to express markers of mature neurons such as calretinin and tyrosine hydroxylase, indicative that stroke is driving them towards neuronal differentiation (Liu et al., 2009c). Neuroblasts in the post-stroke SVZ also express MMPs which appear to digest pathways for their ectopic migration (Lee et al., 2006; Barkho et al., 2008).

4.1.2 Ependymal cells

Ependymal cells are post-mitotic and multi-ciliated cells derived from radial glia during the first post-natal week, and they have important physiological functions in SVZ niche maintenance. Ependymal cells regulate the neurogenic niche via Noggin/BMP (Lim et al., 2000) and Foxj1/Ank3 pathways (Paez-Gonzalez et al., 2011), establish CSF flow over the ventricular surface (Sawamoto et al., 2006), and form a neuro-protective metabolic barrier at the brain-CSF interface (Del Bigio, 2010). A cluster of 20 - 50 motile cilia are found on an ependymal cell and their coordinated beating mediates CSF flow on the ventricular surface (Sawamoto et al., 2006). Each motile cilium is a thin protrusion of cell membrane which surrounds a ciliary axoneme connected to a basal body. The axoneme consists of a central microtubule pair linked by radial spokes to the 9 outer microtubules pairs that are connected to a dynein motor to facilitate mechanical movement (Louvi and Grove, 2011). In ependymal cells, the basal bodies and clusters of motile cilia are oriented in the apical plane towards the downstream position with reference to the direction of CSF flow (Mirzadeh et al., 2008). This orientation of basal bodies and cilia in the apical plane confers planar cell polarity (PCP) to ependymal cells, and is critical for the maintenance of normal CSF flow (Mirzadeh et al., 2010b). Genetic or acquired conditions which disrupt ependymal ciliary beating result in aberrant CSF circulation and hydrocephalus (Banizs et al., 2005; Sawamoto et al., 2006; Monkkenen et al., 2007; Tissir et al., 2010). However, the effect of ischaemic brain injury on ependymal motile ciliary function has not been investigated.

The identity of neural stem cells in the SVZ has remained elusive, and ependymal cells are controversial contenders (Chojnacki et al., 2009). Although both ependymal cells and astrocyte-like stem cells share a common radial glial origin (Tramontin et al., 2003; Spassky et al., 2005; Jacquet et al., 2009), ependymal cells do not contribute adult

neurogenesis in the normal brain (Chiasson et al., 1999; Capela and Temple, 2002; Laywell et al., 2000; Gregg and Weiss, 2003; Spassky et al., 2005). After stroke, ependymal cells express markers of radial glia such as Ser-55 phosphorylated-vimentin and brain-lipid-binding protein (Zhang et al., 2007b), and may be a source of new neuroblasts and astrocytes after ischaemic injury (Carlen et al., 2009). This raises the possibility that the post-stroke environment may revert ependymal cells to an immature radial glial-like state. The question also arises whether astrocyte-like stem cells and niche astrocytes are similarly reprogrammed or de-differentiated?

4.1.3. Whole mounts to study the SVZ

Whole mounts of the lateral wall of the lateral ventricles have been used to reveal novel features of the SVZ. These functionally significant discoveries have played major roles in defining our understanding of adult neurogenesis and the neural stem cell niche. Whole mounts have tremendous advantage over traditional coronal or sagittal sections as it provides an un-obstructed *en-face* view of the neural stem cell niche in relatively uninterrupted three-dimensional brain-tissue. The technique was initially developed in the Alvarez-Buylla Laboratory, and involved careful dissection of the brain hemisphere, removal of overlying cortex, medial septum and hippocampus to reveal the intact underlying lateral ventricular wall (Mirzadeh et al., 2010a). The tissue could then be processed for immunohistochemistry or assessment of ventricular-surface CSF flow. The technique was used to describe the network of tangential neuroblast chains which migrate to the olfactory bulb (Doetsch and Alvarez-Buylla, 1996). The Szele Laboratory used whole mounts to detail the distribution of Dcx⁺ neuroblasts in the SVZ (Yang et al., 2004). The whole mount preparation was also instrumental in demonstrating the role of

ependymal motile cilia in maintaining neuroblast migration to the olfactory bulb. In a set of elegant experiments, (Sawamoto et al., 2006) showed that ventricular-surface CSF flow directed neuroblast migration. Small volumes of India ink released onto freshly-dissected whole mounts revealed a pattern of CSF flow highly reminiscent of the network of neuroblast chains. In contrast, misdirected neuroblasts were associated with absence of the stereotyped CSF flow in *Tg737^{orp}* mutant mice which have defective ciliogenesis (Banizs et al., 2005; Sawamoto et al., 2006). Importantly, without whole mounts, it would have been difficult to make this observation in histological sections as the neuroblasts still formed chains; the anatomical defect only became apparent when a global view of the neuroblast chain population was obtained.

(Mirzadeh et al., 2008) used whole mounts to reveal the specialised epithelial organisation of the neural stem cell niche and the close interaction between astrocyte-like stem cells and blood vessels. Astrocyte-like stem cells in the SVZ have apical processes with primary cilia that contact the lateral ventricle (Doetsch, 2003). These apical primary cilia protrude between adjacent ependymal cells to form rosettes, or pinwheel-like structures (Mirzadeh et al., 2008). Although the significance of this visually striking cellular arrangement remains unknown, the absence of this feature in non-neurogenic areas (e.g. the third ventricle) and the presence of adherens and gap junctions between stem and ependymal cells suggest functional importance. Furthermore, an extensive blood vessel network spans the entire ventricular wall beneath the ependymal layer (Shen et al., 2008; Tavazoie et al., 2008). The majority of proliferating astrocyte-like stem and progenitors cells are located in close proximity to the SVZ vasculature (Shen et al., 2008). Each apical-contacting astrocyte-like stem cell also has a basal process which terminated on a blood vessel, while transit amplifying progenitors frequently contact endothelial cells, without intervening aquaporin-4⁺ astroglial endfeet or NG2⁺ pericytes (Tavazoie et al.,

2008). Prompted by this observation, the investigators further found a leaky blood brain barrier in the SVZ and intravenous infusion of small molecules entered the SVZ with relative ease. Taken together, the whole mounts studies have provided detailed insights into the unique cytoarchitecture of the SVZ.

4.1.4. Research questions

To understand the changes that occur in the SVZ after an external insult such as an adjacent stroke lesion, it is essential to consider both the molecular signalling pathways and the observable anatomical correlates, as they provide complementary information and answers. Therefore, given the observed increase in SVZ cell proliferation and ectopic migration of neuroblasts, as well as molecular changes, the time is ripe for a detailed examination of the SVZ using the whole mount technique. In particular, I am interested in SVZ components that regulate neuroblast migration. My hypothesis is that in order for neuroblasts to exit the tightly regulated migratory pathway and migrate into the ischaemic striatum, significant cytoarchitectural changes are necessary, in addition to the described chemokine signalling mechanisms. Specifically, in pilot studies using coronal sections from post-MCAO brains, features of reactive astrogliosis in the SVZ were observed. Therefore, a potential mechanism that alters neuroblast migration after stroke is the disruption of the astrocyte glial tubes. The unresolved issue of the stem cell identity of the SVZ is also intriguing. Could ependymal cells act as neural progenitors and become depleted during post-stroke endogenous repair? I hypothesised that if such drastic changes occurred in ependymal cell fate, associated cellular and molecular changes would lead to significant ependymal dysfunction. Ependymal cells abut the ventricular surface; whole mounts would provide a unique view on their response to stroke.

In this chapter, I used a combination of the whole mount preparations of the lateral ventricular wall and coronal brain sections, to provide a detailed examination of post-stroke neurogenesis and SVZ cytoarchitecture. There was significant cellular reorganisation after MCAO stroke in mice in niche astrocytes and the vasculature. Ependymal cells also assumed a non-proliferative “reactive” ependymal cell phenotype with altered ciliary function in response to distant ischaemic injury.

4.2. Results

4.2.1. Middle cerebral artery occlusion model of stroke

Fifty minutes of MCAO caused reproducible cerebral ischaemia and neurological deficits. MCAO decreased CBF in the left MCA territory by >70% from baseline values, followed by reperfusion after filament removal (Fig 4.1A). Twenty-four hours after recovery from 50-min MCAO, neurological assessment showed consistent contralateral motor hemiparesis and asymmetry of movement. There was gradual recovery of neurological function as measured by the mNDS over the survival period (Fig 4.1B).

4.2.2. Reactive astrocytosis in the SVZ after stroke

Seven days post-MCAO, coronal sections through the ipsilateral SVZ showed increased GFAP immuno-reactivity compared to sham-operated controls (Fig 4.2A-B). In controls, astroglial processes were radially-orientated and a few GFAP+ processes made contact with the lateral ventricular surface (Fig 4.2A'). In contrast, post-MCAO, astrocytes displayed features of reactive astrocytosis and GFAP processes appeared tortuous and hypertrophic (Fig 4.2B'). There were also significantly more ventricular-contacting GFAP+ processes compared to controls (Fig 4.2E; $n = 4$; $P = 0.03$).

To better assess this phenotype post-stroke, I prepared whole mounts of the lateral ventricular wall which permitted unobstructed *en-face* views of the SVZ and overlying ependyma (Fig 4.3A-B). At the ventricular surface, ependymal cells are clearly visualised by the distinct cobblestone cellular arrangement when immunolabelled with β -catenin (Fig 4.3C). The demarcated adhesion zone (AZ Fig 4.3C) is a small area of the ventricular wall

where the lateral ventricles are non-patent and voided of ependymal cells during the whole mount preparation (Fig 4.9M; Sawamoto et al., 2006).

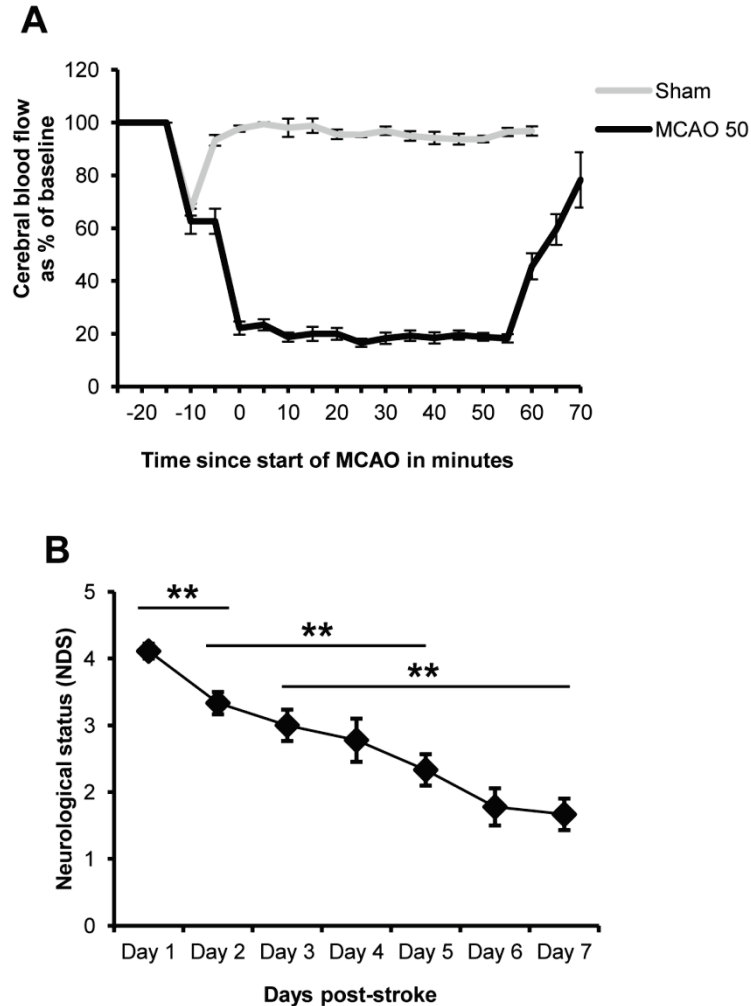


Figure 4.1. Cerebral blood flow and neurological status assessment

- (A) Laser Doppler measurements of cerebral blood flow in shams and during MCAO.
- (B) Neurological assessment using the modified Neurological Deficit Score. Significant recovery over the survival period (Mann Whitney non-parametric test; ** $P < 0.01$; $n = 10$)

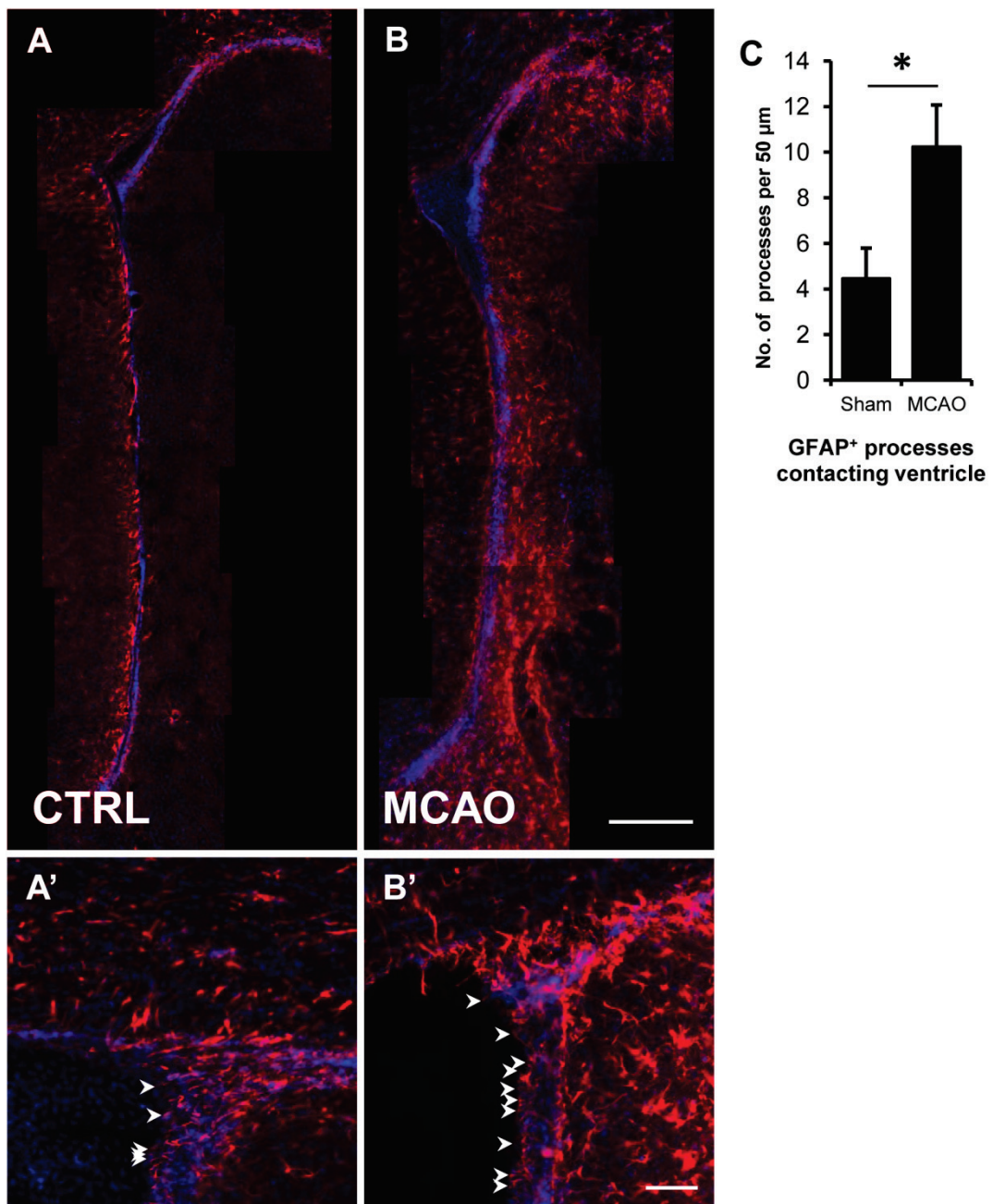


Figure 4.2. Reactive SVZ astrocytosis after stroke: Coronal sections

- (A) GFAP is expressed in the SVZ. (A') is an enlarged view of the dorsal SVZ with aligned glial processes.
- (B) Reactive astrocytosis in the ipsilateral SVZ after stroke. (B') *arrow-heads* show increased GFAP elements which are in contact with the ventricular surface.
- (C) Number of GFAP+ processes contacting the ventricular surface. * $P = 0.03$.

Scale bar 200 μm and 50 μm

GFAP+ astrocytes in the SVZ extended longitudinal processes anteroposteriorly to form glial tubes which ensheath neuroblast chains (Fig 4.3D, F, H). Although the ischaemic lesion did not reach the SVZ, there was significant reactive astrocytosis in the ventricular wall ($n = 8$; Fig 4.3D-E). Reactive astrocytosis was uniform on the lateral ventricular wall and it was not confined to specific regions. At low magnification, reactive astrocytosis was especially evident in the intraventricular adhesion zone devoid of ependyma (*arrow* Fig 4.3E). This gives the impression that the adhesion zone was more affected. However, examination at high magnification showed that this was not the case. Seven days post-stroke, astrocytes at different locations on the ventricular wall were hypertrophic, and glial processes became tortuous, disrupting the migratory scaffold (Fig 4.3G, J). Some post-stroke astrocytes also expressed vimentin, another marker of reactive astrocytosis (Fig 4.13B; Robel et al., 2011). In controls, protoplasmic astrocytes in the SVZ were located adjacent to blood vessels, and frequently made contact with them, although they typically did not wrap processes around multiple vessels (Fig 4.3I). Interestingly, post-stroke, numerous large protoplasmic astrocytes extended multiple processes to several blood vessels (*arrows* Fig 4.3J).

4.2.3. Neuroblast chain structure is maintained despite glial tube disruption

Newborn neuroblasts in the SVZ expressed doublecortin (Dcx), a marker of immature migratory cells with neuronal specification (Gleeson et al., 1999). On whole mounts, Dcx+ neuroblast chains were organised in a tangential network on the lateral ventricular wall (Fig 4.4A). Bundles of neuroblast chains spanned the entire extent of the lateral ventricular wall and were oriented along the rostrocaudal migratory axis. Tangential neuroblast chains were concentrated in the dorsal and ventral ventricular wall, while chains in the adhesion zone appeared shorter and were less aligned to the migratory axis.

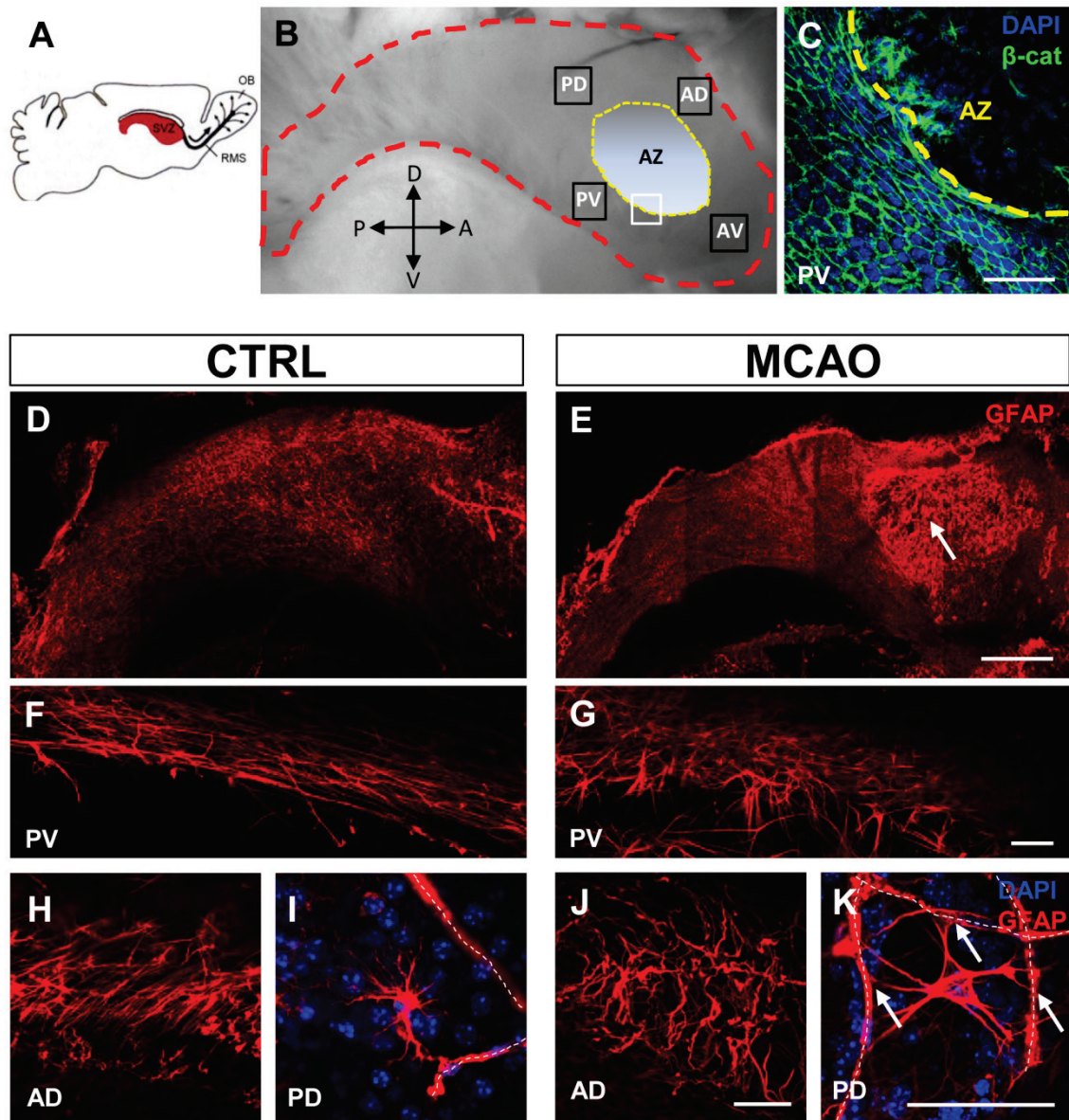


Figure 4.3. Reactive SVZ astrocytosis after stroke: Whole mounts

- (A) Schematic showing the lateral ventricular wall and SVZ (red). OB olfactory bulb, RMS rostral migratory stream. Adapted from (Doetsch et al., 1997).
- (B) Dissected whole mount; lateral ventricular wall outlined in red. The orientation as in (A). Analyses were performed in 4 boxed areas AD, anterior dorsal; AV, anterior ventral; PD, posterior dorsal; and PV, posterior ventral. AZ is the intraventricular adhesion zone where the striatum and septum are joined and the ventricle is non-patent. D, dorsal V, ventral; A, anterior; P, posterior.
- (C) White boxed area in (B): Junction between normal endymal cell layer and adhesion zone devoid of endymal cells.

(D-K) Whole mount GFAP immunofluorescence. Niche astrocytes in the SVZ have long processes aligned in the direction of neuroblast chain migration, in the posterior ventral (F) and anterior dorsal (H) SVZ. Post-stroke, reactive astrocytosis with hypertrophy and tortuous glial processes appear across the SVZ (G,J). Some astrocytes in the SVZ are located near blood vessels (I *dotted lines*) make contact with them. After stroke, reactive astrocytes near blood vessels (*dotted lines*) make multiple contacts (*arrows* K).

Scale bar 50 μm , (C, E) 500 μm .

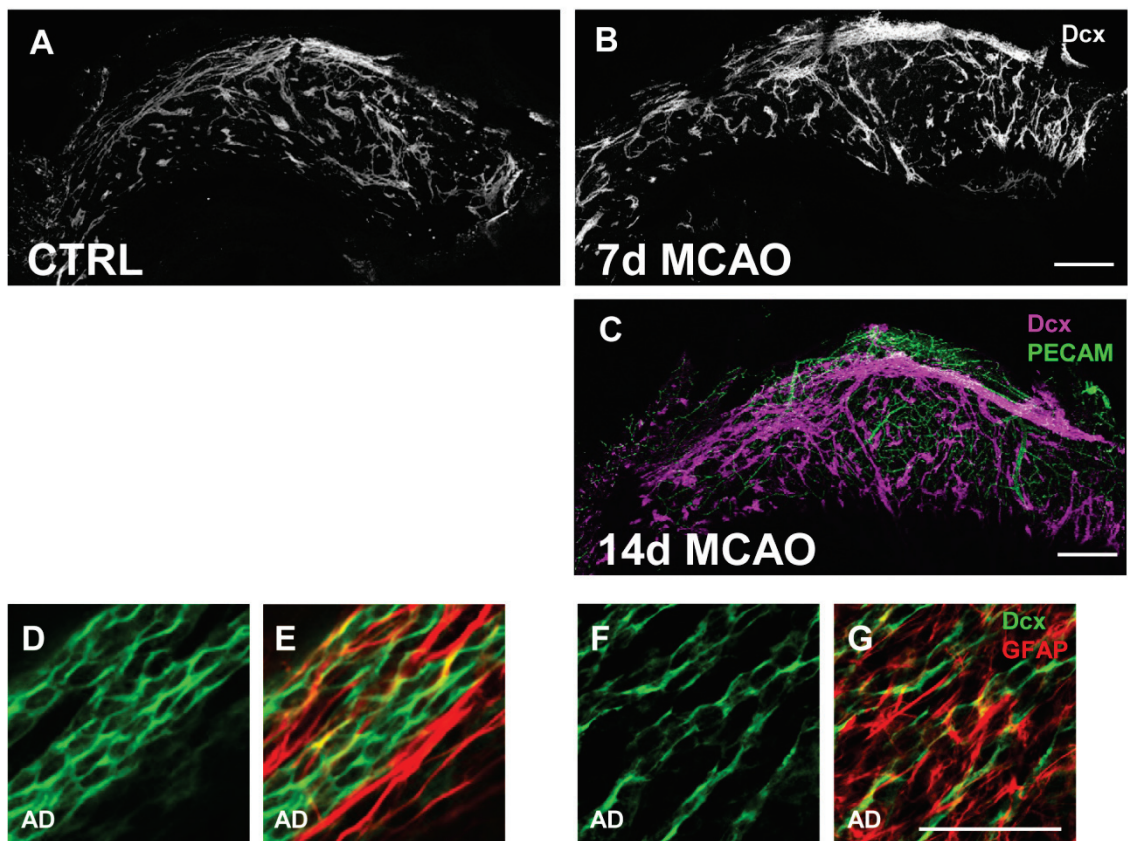


Figure 4.4. Disrupted glial tube migratory scaffold around neuroblast chains

(A-C) The tangential networks of neuroblast chains on the lateral ventricular wall remain intact 7- and 14-days post-MCAO.

(D-G) Niche astrocytes form glial tubes which ensheath neuroblast chains (D-E). Reactive astrocytes have disrupted glial tube structure after stroke (F-G).

Scale bar (A-C) 500 μm (D-G) 50 μm .

Surprisingly, the gross anatomical organisation of neuroblasts did not change 7- and 14-days after stroke (Fig 4.4B-C). The integrity of the homotypic interaction between neuroblasts was also maintained (Fig 4.4F). However, reactive astrogliosis post-stroke disrupted the tangential orientation of the glial processes with respect to neuroblast chains (Fig 4.4G).

4.2.4. Vascular engorgement and increased vascular density in the post-stroke SVZ

The altered glial-vascular interaction after stroke (Fig 4.3K) suggested the vascular niche may be disrupted. Post-stroke vascular engorgement was visible in freshly dissected and unperfused whole mounts (*arrow* Fig 4.5D). Whole mounts immunostained for platelet endothelial cell adhesion molecule 1 (PECAM) showed increased vascular density in the SVZ 7- and 14-days post-stroke (Fig 4.5B-C, E-F'; $n = 4$). The number of PECAM+ vessel branches increased at both time points (Fig 4.5G; $P = 0.03$, ANOVA), while vessel diameter was larger at 7-days (Fig 4.5H; $P = 0.02$, ANOVA).

4.2.5. Increased neural-lineage cell proliferation after stroke

The number of SVZ cells expressing phosphorylated histone H3 (PHi3), which is specifically phosphorylated during mitosis, increased 7-days post-stroke ($n = 4$; $P = 0.02$; Fig 4.6A-C). Proliferating cells in the SVZ include astrocyte-like stem cells, transit amplifying progenitors and neuroblasts, as well as a small percentage of microglia, niche astrocytes and endothelial cells. To determine which cells increased proliferation after stroke, PHi3 was co-labelled with Dcx (neuroblasts) and Mash1 (transit amplifying

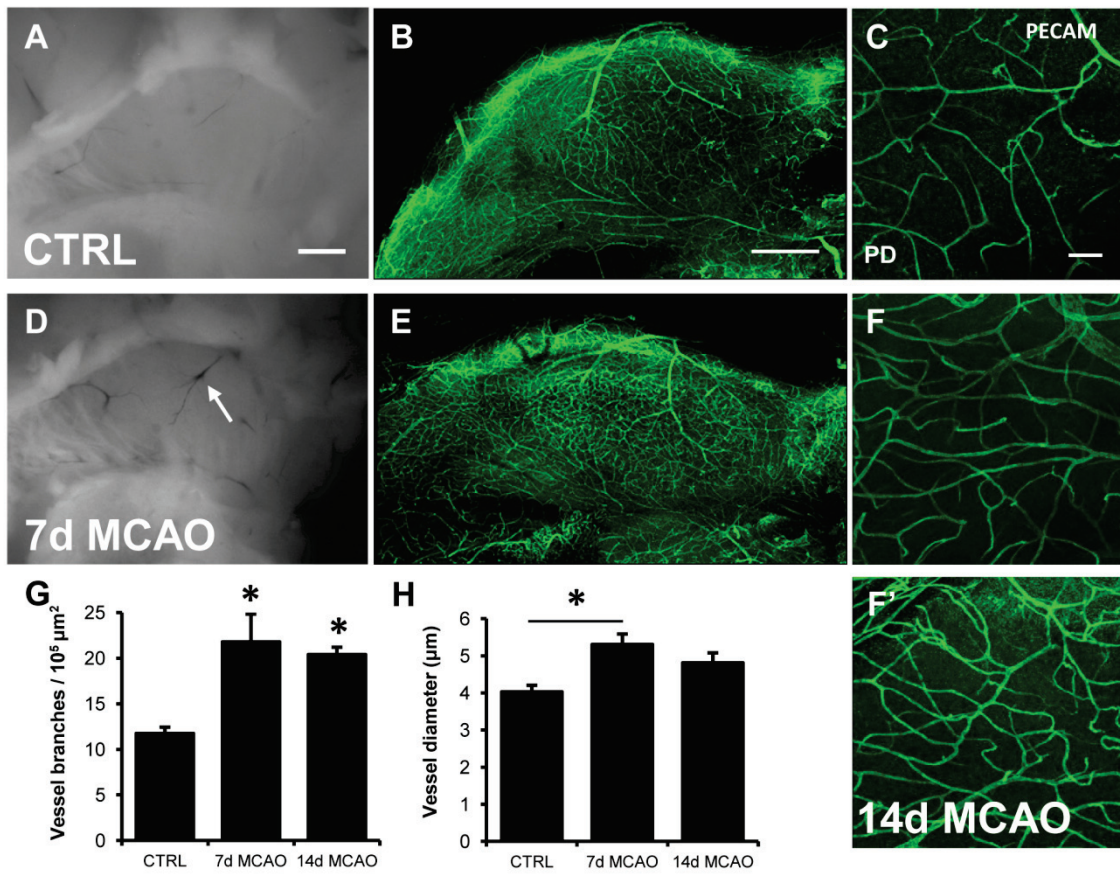


Figure 4.5. Stroke increases vascular density and blood vessel diameter

- (A-C) PECAM immunofluorescence of control whole mount SVZ vasculature.
 (D-F) After stroke, engorged blood vessels become visible in the ventricular wall (*arrow* D). (E-F') Representative images of increased vascular density 7- and 14-days after stroke.
 (G) Number of blood vessel branches increased 7- and 14-days after stroke.
 (H) Increased blood vessel diameter 7-days after stroke.

Scale bar (A, B, D, E) 500 μm (C, F') 50 μm.

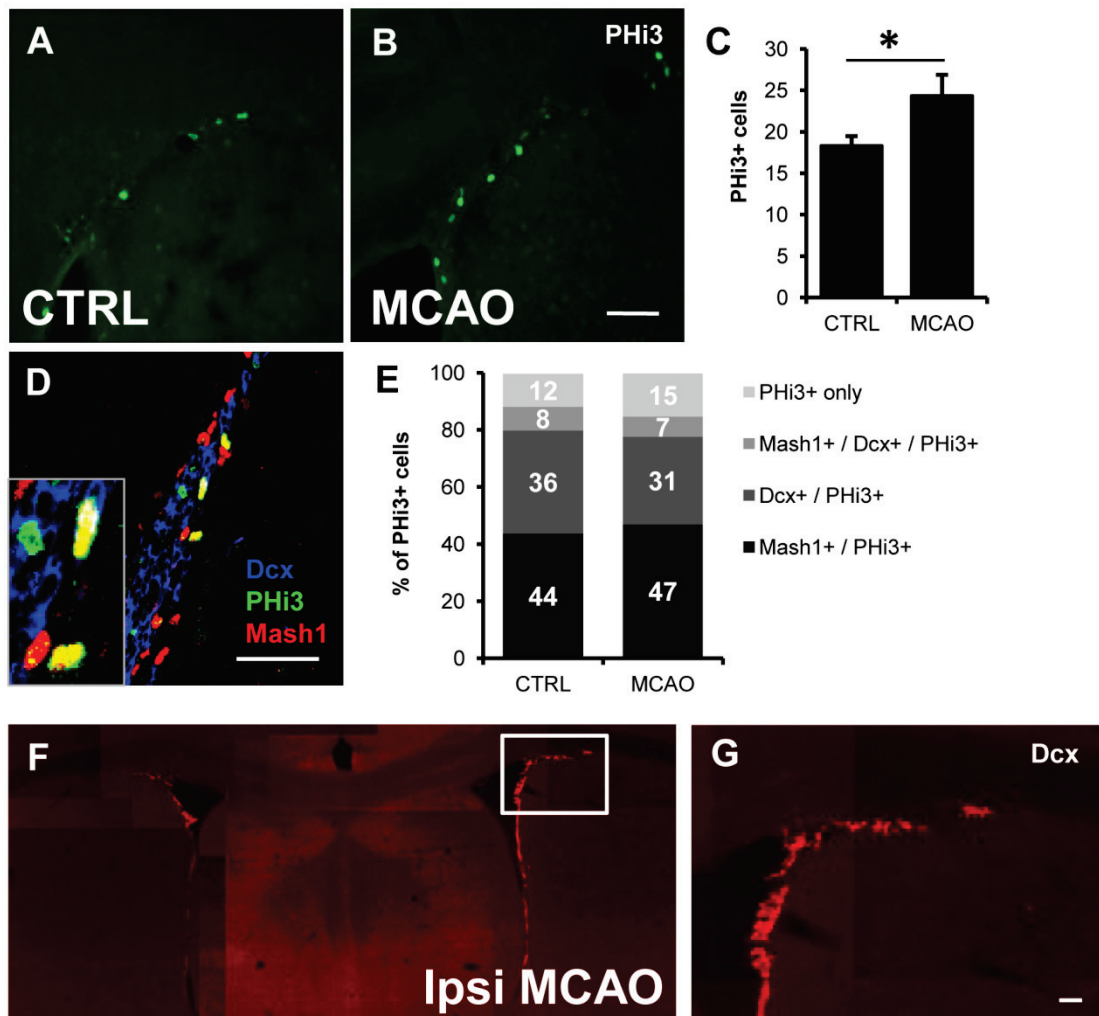


Figure 4.6. Increased neural cell proliferation in the SVZ after stroke

- (A-B) Representative PHI3+ cells in the dorsolateral SVZ in control and 7-days after stroke.
- (C) Number of PHI3+ cells increased after stroke. * $P = 0.02$.
- (D) Triple immunofluorescence for PHI3, Dcx and Mash1 to determine ratio of proliferating cell types.
- (E) Percentage of PHI3+ cells in the SVZ which co-localised with Mash1 and/or Dcx.
- (F-G) Dcx+ neuroblast pool increased in the ipsilateral SVZ after stroke.

Scale bar 50 μm .

progenitors; Fig 4.6D-E; 499 cells examined). In controls, 44 ± 5.6 % of proliferating cells were transit amplifying progenitors (PHi3+ / Mash1+); 36 ± 2.0 % were neuroblasts (PHi3+ / Dcx+); and 8 ± 3.3 % co-localized with both Mash1 and Dcx (PHi3+ / Mash1+ / Dcx+), which were likely young neuroblasts (*inset* Fig D). After stroke, 47 ± 4.3 % of proliferating cells were transit amplifying progenitors; 31 ± 8.8 % were neuroblasts; 7 ± 3.7 % were young neuroblasts. Thus, neural lineage cells (Dcx and/or Mash1-positive cells) constituted the majority of proliferating cells in controls ($88 \pm 4\%$) and did not change significantly after stroke ($85 \pm 9\%$). PHi3+/Dcx-/Mash1- cells are predominantly astrocytes and microglia (Goings et al., 2006); and they accounted for only 12 ± 4.6 % and 15 ± 9.1 % of mitotic cells in controls and after stroke, respectively. Increased SVZ proliferation led to increased neuroblast pool in the ipsilateral SVZ, as seen by the increased Dcx immunolabelling post-MCAO (Fig 4.6F-G).

4.2.6. Ependymal cells are non-proliferative

Ependymal cells have been reported to proliferate after injury. However, 7- and 14-days post-stroke, proliferating ependymal cells were not observed. Following a pulse injection of BrdU 2 hours prior to sacrifice, many BrdU+ cells were found in the SVZ 7-days after stroke (Fig 4.7A). Several BrdU+ cells were positioned in close proximity to the ependymal layer (*arrows*, Fig 4.7B). S100 β + ependymal cells and BrdU+ proliferating cells were analysed in orthogonal projection of confocal z-stack, especially BrdU+ cells that on epifluorescence microscopy appeared as if they could be in the ependymal layer. Not a single s100 β + / BrdU+ double-labelled cell was found (Fig 4.7C; $n = 5$ mice). If ependymal cells could divide after stroke, they may do so slowly, and fail to label with a single pulse of BrdU. However, 5 days of cumulative BrdU-labelling in the 14-day group

also did not identify BrdU+ / S100 β + double-labelled ependymal cells ($n = 6$; Fig 4.7D-F). Using different markers for ependymal cells and proliferating cells, PHi-3+ / Vim+ double-labelling cells in contact with the ventricle at both time-points were not detected (data not shown).

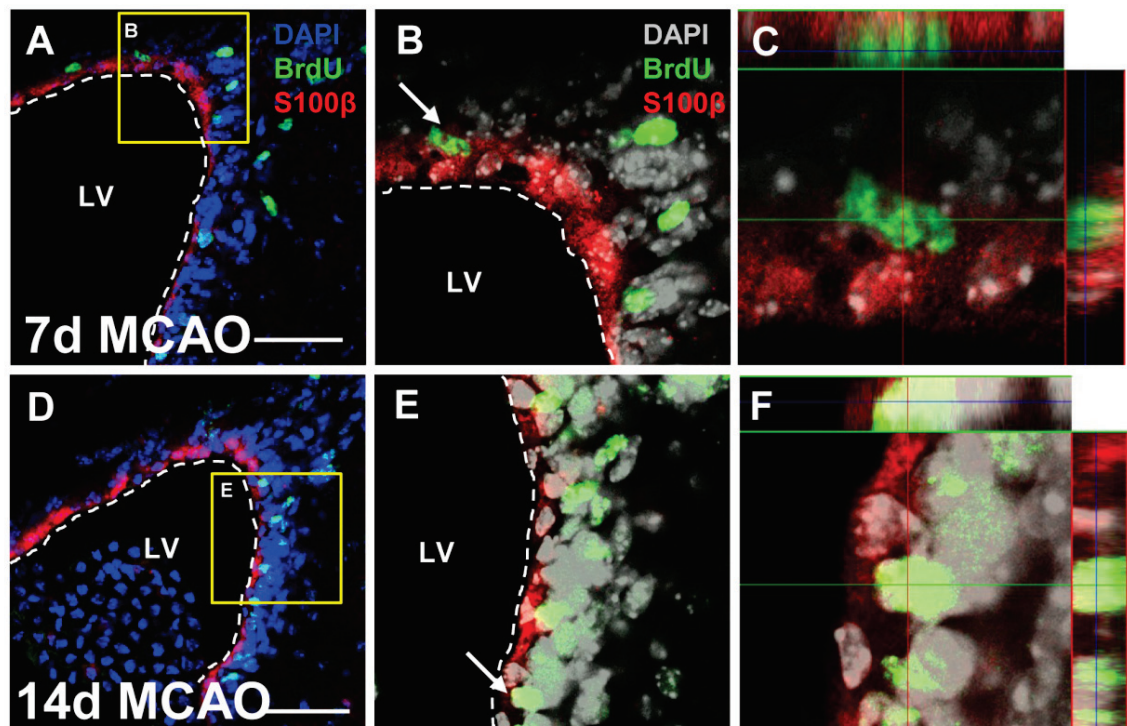


Figure 4.7. Ependymal cells are non-proliferative after stroke

BrdU+ mitotic cells around the lateral ventricle 7-days post-stroke (A; single BrdU pulse labelling) and 14-days post-stroke (D; cumulative BrdU labelling). (B, E) *Boxed areas* in (A) and (D), respectively: BrdU+ cells were closely associated with, but distinct from, s100 β + ependymal cells. (C, F) Arrowed cell (B) and (E) respectively: Orthogonal projections of high-powered confocal z-stack. BrdU+ cells did not co-localise with the ependymal marker s100 β

Scale bar 50 μ m.

4.2.7. Stroke does not increase apoptosis in the SVZ

Apoptosis can cause reactive astrocytosis, and vice versa, but the number of TUNEL+ cells in the control and 7-days post-stroke SVZ remained similar ($n = 4$; $P = 0.3$; Fig 4.8A-C). The absolute numbers of apoptotic cells in the SVZ were very low after stroke (2.5 ± 0.9 cells per section in the SVZ) and similar to the number in controls (1.4 ± 0.6 cells per section in the SVZ) which contrasts with the ischaemic striatum where large numbers of TUNEL+ cells were seen (135 ± 24 cells per 20X microscope field; Fig 4.8D-E).

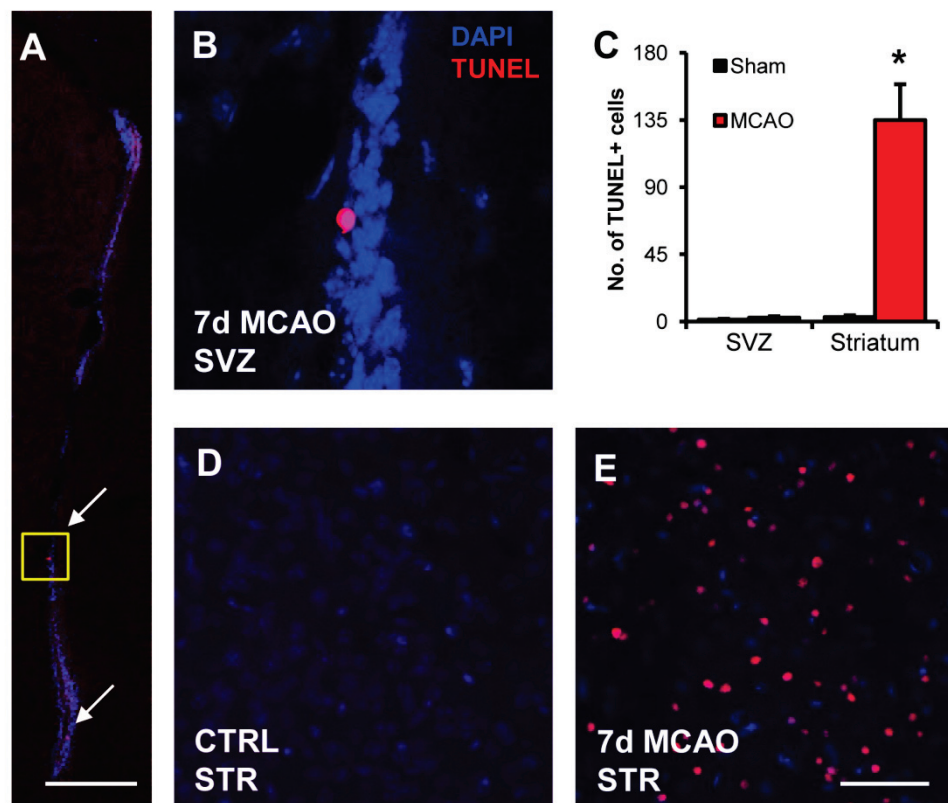


Figure 4.8. Low levels of apoptosis in the post-stroke SVZ

- (A) TUNEL+ apoptotic cells in the SVZ 7-days after stroke. There were very few TUNEL+ apoptotic cells (*white arrows*) even in the post-stroke SVZ.
 - (B) *Boxed area* (A).
 - (C) Number of TUNEL+ apoptotic cells in the entire SVZ per brain section and per 20X microscope field in the striatum. ($n = 5$; $* P < 0.001$).
 - (D-E) TUNEL labelling in the striatum.
- Scale bar (A) 100 μ m (B-E) 50 μ m.

4.2.8. Ependymal cells remain intact, but have enlarged cell surface area post-stroke

Ependymal cells can be damaged due to neurodegeneration and with age (Luo et al., 2008); and may be lost after stroke (Carlen et al., 2009). Seven- and fourteen-days after stroke, the lateral ventricular ependymal cell layer remained intact when visualised *en-face* on whole mounts with β -catenin immunofluorescence which labels ependymal plasma membranes (Fig 4.9D, I). Ependymal cells displayed a typical cobblestone appearance with tufts of dense motile cilia at the ventricular surface (Fig 4.9A-E). Using various labelling strategies to visualise ependymal cells and their cellular components, ependymal denudation and tears at either time point post-stroke were not observed. The preservation of the ependymal layer was evidenced by the retention of the cobblestone appearance (β -catenin; Fig 4.9F,G, 3.11D-F, 3.15E-H; $n = 20$), preservation of motile cilia (acetylated tubulin; Fig 4.9H, J; $n = 10$), and γ -tubulin basal body clusters which are unique to cells possessing multiple motile cilia (Fig 4.11J, 3.14B; $n = 10$). Furthermore, increased apoptosis was not seen in the SVZ and ependymal layer after stroke (Fig 4.8). In coronal sections, s100 β -labeling showed preserved ependymal cell layer around the lateral ventricles (Fig 4.9L-M; $n = 5$).

In examining the ependyma for evidence of post-stroke damage, I noticed enlarged *en-face* ependymal cell surface area, while individual cilia tufts seemed more disperse (Fig 4.9I, J). Seven-days post-stroke, ependymal cells ipsilateral to stroke had increased cross-sectional surface area ($146 \pm 10 \mu\text{m}^2$) compared to contralateral controls ($111 \pm 6 \mu\text{m}^2$; $P = 0.02$; $n = 6$; 2630 cells; Fig 4.9K). The effect persisted and ependymal cells remained enlarged at 14-days post-stroke ($170 \pm 9 \mu\text{m}^2$; $P = 0.01$; $n = 3$; 1149 cells). Motile cilia numbers and length was unchanged after stroke (Fig 4.9J).

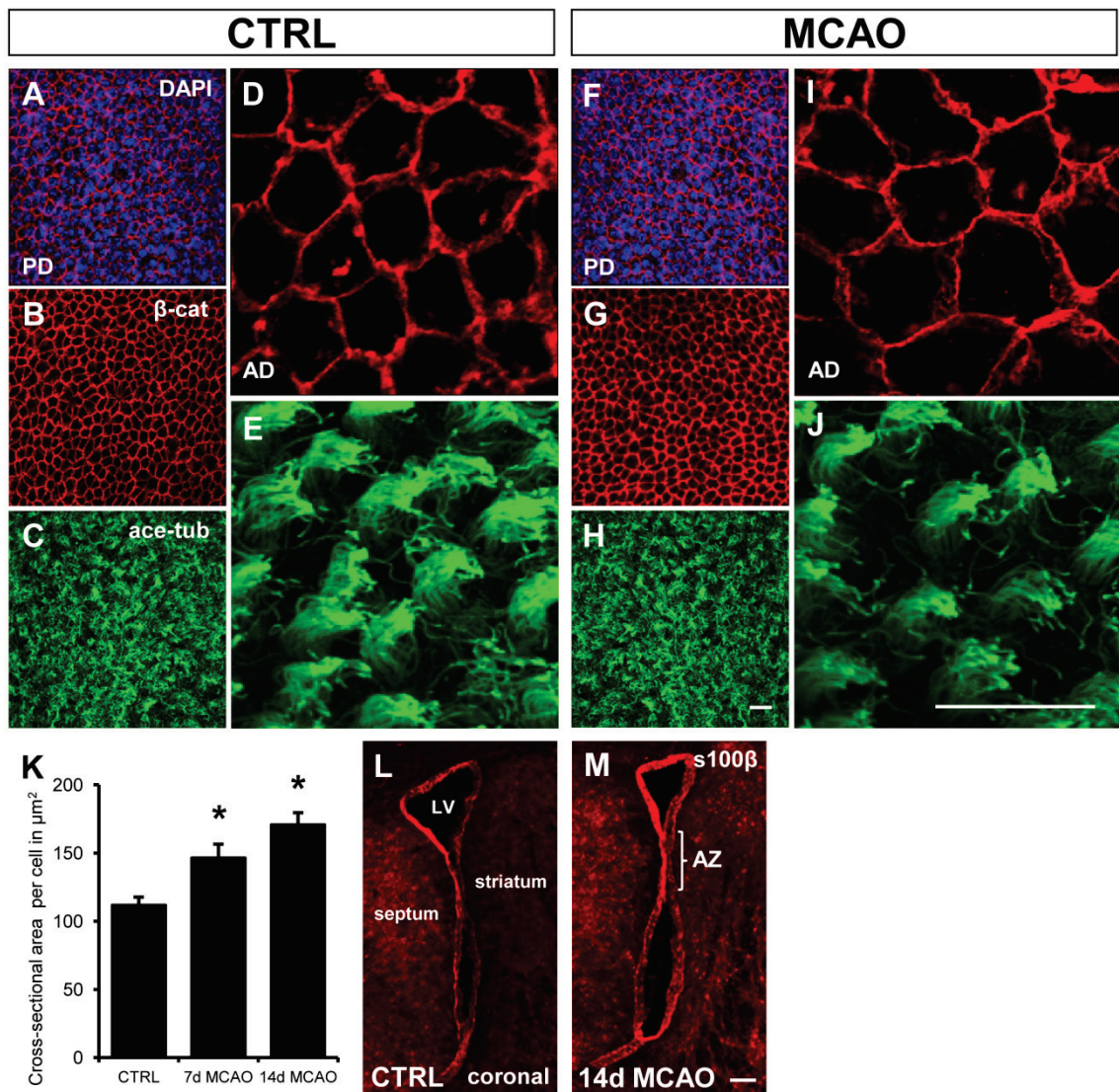


Figure 4.9. Preserved ependymal cells have enlarged surface area

- (A-J) En-face whole mount immunohistochemistry for ependymal cell membranes β -catenin *red*), and motile cilia (acetylated tubulin, *green*). Cell nuclei counterstained with DAPI (blue). (A-B) Ependymal cells lining the lateral ventricles form a cobblestone cell layer. (C,E) Tufts of motile cilia visualised on ependymal cell surface. (F,G) After stroke ependymal cell loss/denudation is not seen, and (H, J) motile cilia morphology is preserved.
- (K) Quantification of ependymal cell cross-sectional surface area.
- (L-M) Coronal section: s100 β immunofluorescence to show integrity of ependymal cell layer. AZ adhesion zone.

Scale bar (A-J) 20 μ m (L-M) 100 μ m.

4.2.9. Ependymal cells expressed glial fibrillary acidic protein after stroke

Astrocyte-like stem cells have small apical processes that are intercalated between ependymal cells at ventricular surface (Fig 4.10A). The single primary cilium of each apical process can be visualised with γ -tubulin immunohistochemistry which labels the basal bodies of primary and motile cilia (*arrow* Fig 4.10A). These apical processes were GFAP⁺ and gave rise to the visually striking pinwheel architecture of the ependymal layer (Fig 4.10B). Ventricle-contacting astrocyte-like stem cells sent a long basal process to reach a blood vessel deep in the SVZ (Fig 4.10C).

GFAP expression at the apical surface in the ependymal layer was largely restricted to ventricle-contacting astrocyte-like stem cells. Very few ependymal cells (<5%) contralateral to MCAO and in shams expressed GFAP (Fig 7A-C; $n = 8$). Unexpectedly, 7- and 14-days after stroke >85% of ependymal cells expressed high levels of GFAP ipsilateral to the lesion (Fig 4.11; $n = 8$). This *de novo* GFAP expression was apparent in high-powered single confocal optical sections through the ependyma, and associated with the cytoplasmic aspect of ependymal cell (*arrow*, Fig 4.11M). Confocal z-stack analysis demonstrated that GFAP filaments were aligned *within* ependymal cells at the ventricular surface (Fig 4.11M'), and not only *between* them. I considered the possibility that these apical GFAP⁺ cells may have been SVZ astrocytes that relocated to the ependymal layer and taken on ependymal morphology after stroke. However, GFAP filaments were found on the cytoplasmic aspect of cells with tufts of motile cilia and dense γ -tubulin⁺ basal bodies consistent with mature ependymal cell phenotype (Fig 4.11J-K). GFAP immuno-labelling was validated using 3 primary antibodies and 3 fluorophore-conjugated secondary antibodies (Table 2.3.). Negative controls omitting the primary antibody did not fluoresce.

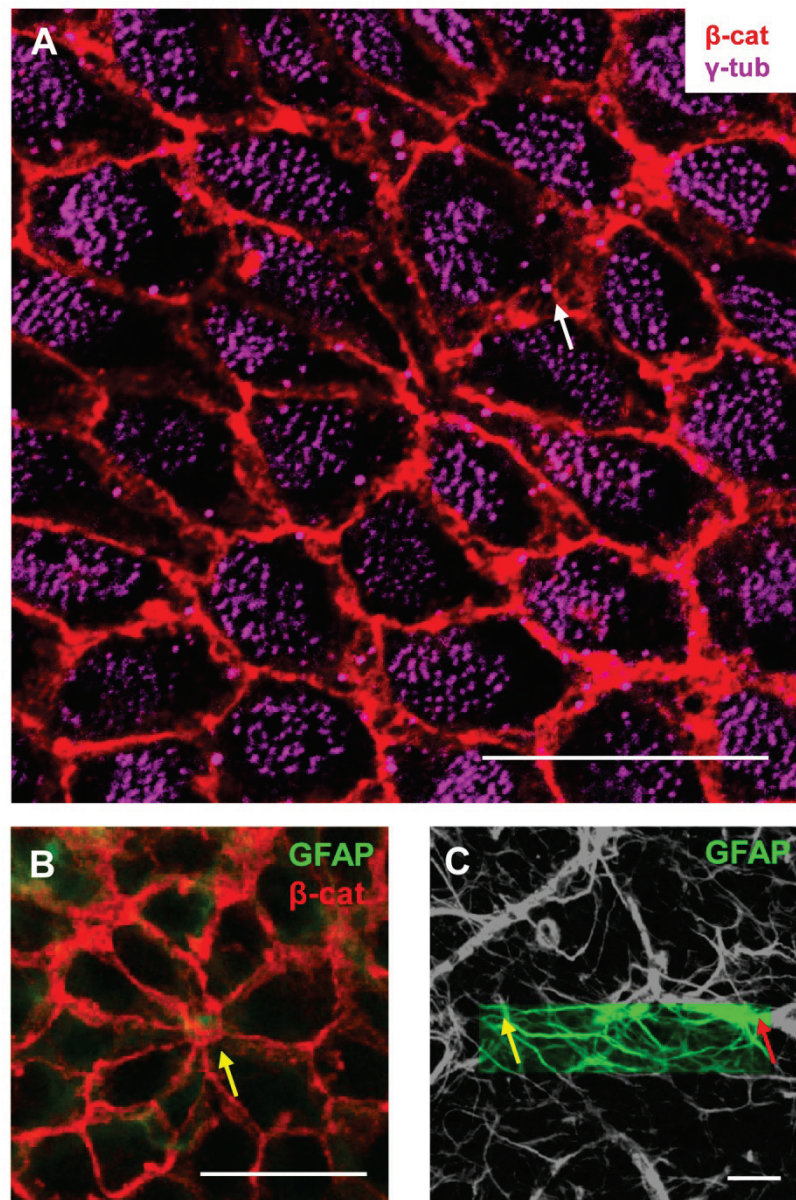


Figure 4.10. Pinwheel neuroepithelial architecture of the neurogenic niche

- (A) At the ventricular surface, multi-ciliated ependymal cells surround the apical process of astrocyte-like stem cells (B1 cell). Each B1 cell has a single primary cilium (*arrow*). The basal bodies of motile and primary cilia are labelled by γ -tubulin
- (B) GFAP+ B₁ cells (*yellow arrow*) and the surrounding ependymal cells form pinwheel-like cell arrangement.
- (C) The same cell in (B) is traced into the basal SVZ in a projected confocal z-stack, and terminates on a blood vessel (*red arrow*).

Scale bar 20 μ m.

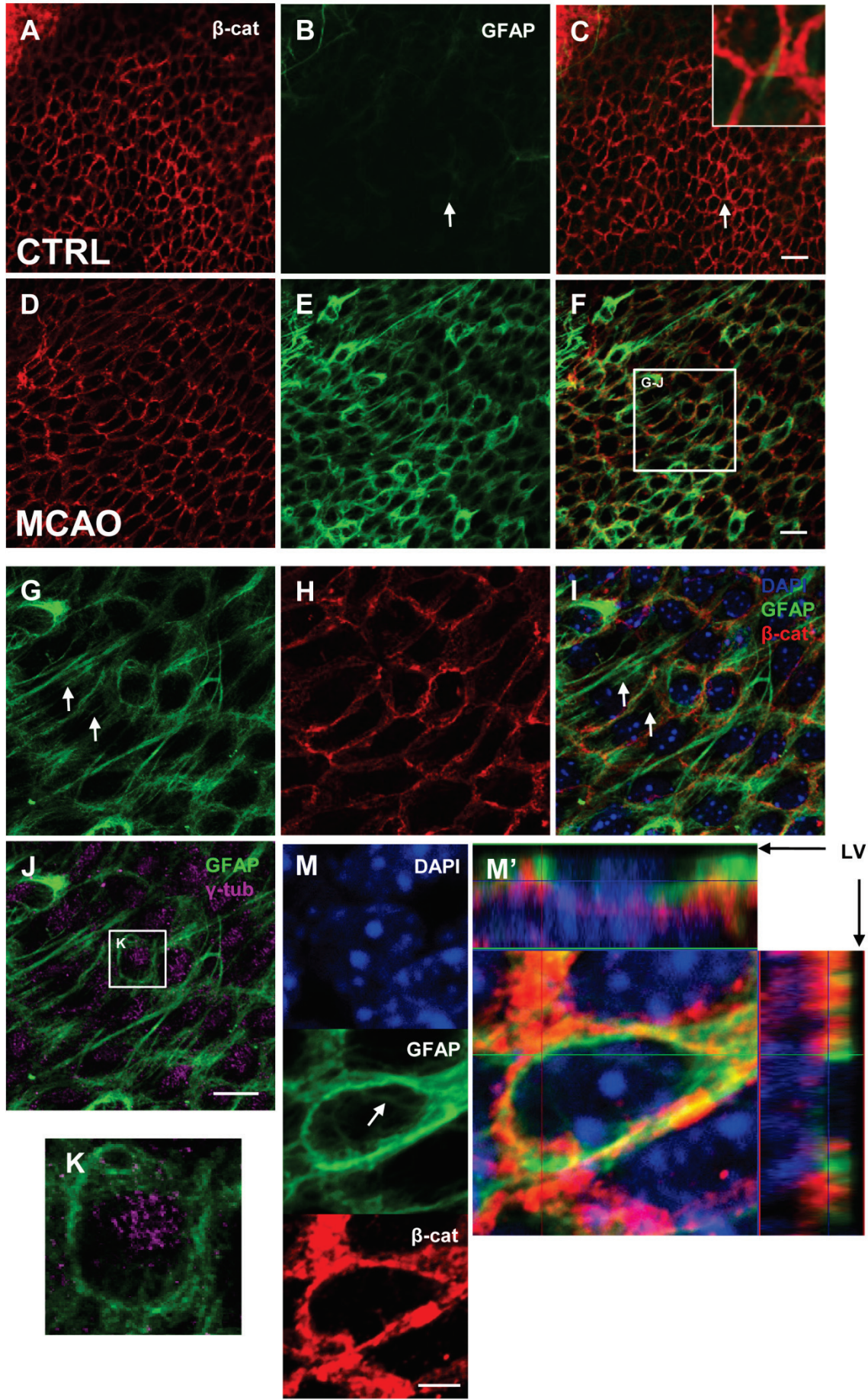


Figure 4.11. Reactive astroglial ependymal cells after stroke

Single confocal optical sections of whole mounts at the ventricular surface.

- (A-C) Most resting ependymal cells do not express detectable GFAP. In contrast, GFAP+ NSCs have apical processes which contact the ventricles (*white arrows B-C; inset*)
- (D-I) After stroke, ependymal cells express high levels of GFAP. (G-I) *Boxed area* (F). GFAP+ ependymal cells extended long processes parallel to the ventricular surface (*white arrows I*).
- (J-K) Motile cilia basal bodies immunolabelled with γ -tubulin (γ -tub) in GFAP+ ependymal cells. (K) *Boxed area* (J): Cytoplasmic GFAP in close association with ciliary basal bodies.
- (M) High-power single optical sections showing cytoplasmic (*arrow*) GFAP intermediate filament. (M') Orthogonal projection of confocal z-stack of ependymal cell at ventricular surface. LV lateral ventricle.

Scale bar 20 μ m (M) 5 μ m.

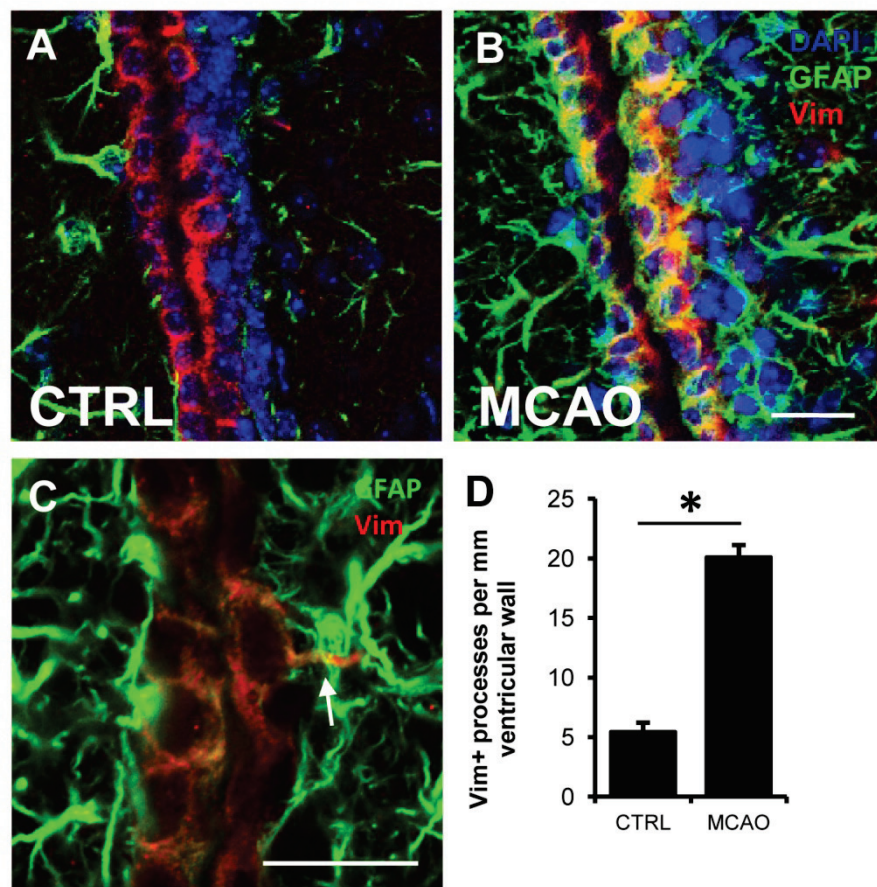


Figure 4.12. Ependymal cells extend basal processes after stroke

- (A-C) Coronal sections 7-day post-stroke. Immunofluorescence for GFAP (*green*) and vimentin (*red*). Ventricle-contacting Vim⁺ ependymal cells co-localise with GFAP. (C) Example of ependymal process extending into SVZ.
- (D) Quantification of Vim⁺ basal processes per mm of SVZ.

Scale bars 20 μ m

To confirm ependymal GFAP expression, vimentin was used as a marker of ependymal cells in coronal sections. Seven-days post-stroke, vimentin+ ependymal cells expressed GFAP (Fig 4.12), and some extended basal processes into the SVZ (*arrow* Fig 4.12C; (Zhang et al., 2007b)). The number of vimentin+ ependymal processes which extend into the SVZ increased post-stroke (20.1 ± 1.8) compared to controls (5.6 ± 1.0 ; $P < 0.0001$; Fig 4.12D).

4.2.10. Disrupted ependymal planar cell polarity and changed CSF flow

PCP in ependymal cells is distinguished by the cellular position of motile cilia, their orientations and beat direction, and hence the direction of CSF flow (Mirzadeh et al., 2010b). As expected, γ -tubulin+ ciliary basal bodies were regular and located downstream of CSF flow direction in controls (Fig d.13A; Mirzadeh et al., 2010b). Seven-days post-stroke, in the anterior dorsal lateral ventricular wall (Fig 4.3B location *AD*), the polarised cellular position of basal bodies was disrupted; many basal bodies were displaced and found upstream of CSF flow (*yellow arrows*, Fig 4.13B). Quantification of normal PCP orientation vectors (Fig 4.13C) showed a tight distribution around the median angle (*green bars* Fig 4.13D). In contrast, there was significant increase in PCP dispersion after stroke (*red bars* Fig 4.14D; $n = 3$, 302 cells; Two-sample Kolmogorov-Smirnov Test, $D = 0.32$, $P < 0.0001$). In other regions of the lateral ventricular wall, ependymal PCP was preserved.

I hypothesised that the ependymal changes after stroke may disrupt cilia function and normal CSF flow. To assess CSF flow, fluorescent bead movement over the anterior lateral ventricular wall was measured in freshly dissected whole mounts 7-days post-stroke ($n = 4$). Beads flowed anteriorly in dorsal and ventral streams around the intraventricular adhesion zone (Fig 4.14A-B). After stroke, the general direction of CSF

flow was unchanged. However, tracking the xy-coordinates of individual bead movement in time-lapse frames (Fig 4.14G-H) showed that post-stroke, CSF flow rate over the entire ventricular surface decreased by 26% (Fig 4.14I; Movies 4.1 and Movie 4.2; $n = 4$; $P = 0.01$). Furthermore, in the region of PCP disruption, there were focal turbulent pockets wherein fluorescent beads moved in a whirlpool-like pattern (*inset* Fig 4.14B; Movie 4.1). Post-hoc immunohistochemistry showed intact ependymal layer over the turbulent region, thus making it unlikely that the turbulence observed was due to mechanical tissue damage (Fig 4.14C-F). Motile cilia dysfunction and CSF flow disruption can cause ventricular dilation and hydrocephalus. Seven-days post-stroke, ipsilateral ventricular dilation (>50%) relative to the contralateral ventricle was observed in 4 out of 10 animals (40%; Fig 4.14J); and 14-days post-stroke, in 7 out of 13 animals (54%; Fig 4.14K). Relative ventricular dilation in shams ($n = 7$) or in any of the contralateral hemispheres was never observed.

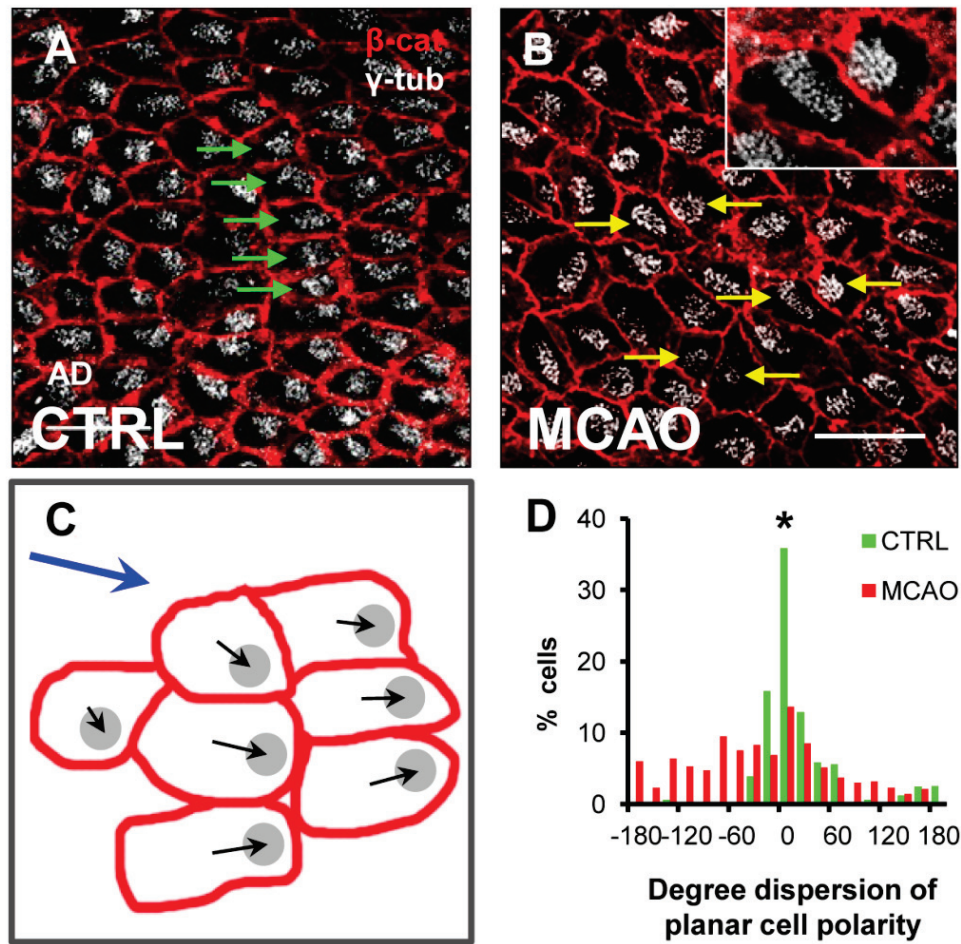


Figure 4.13. Misaligned ependymal planar cell polarity after stroke

- (A-B) Whole mount immunofluorescence for β -catenin (red) and γ -tubulin (white) shows ependymal planar cell polarity. (A) γ -tub+ cilia basal bodies are patterned to one side of the cells (green arrows), downstream of CSF flow direction. (B) After stroke, in the anterior dorsal lateral ventricular wall, ciliary basal bodies are randomly positioned within ependymal cells (yellow arrows).
- (C) Schematic to show the direction of CSF flow (blue arrow) in relation to vectors of ependymal PCP (black arrows)
- (D) Histogram of PCP orientation vectors in controls and 7-days after stroke. $P < 0.0001$; Two-sample Kolmogorov-Smirnov Test.

Scale bars 20 μ m

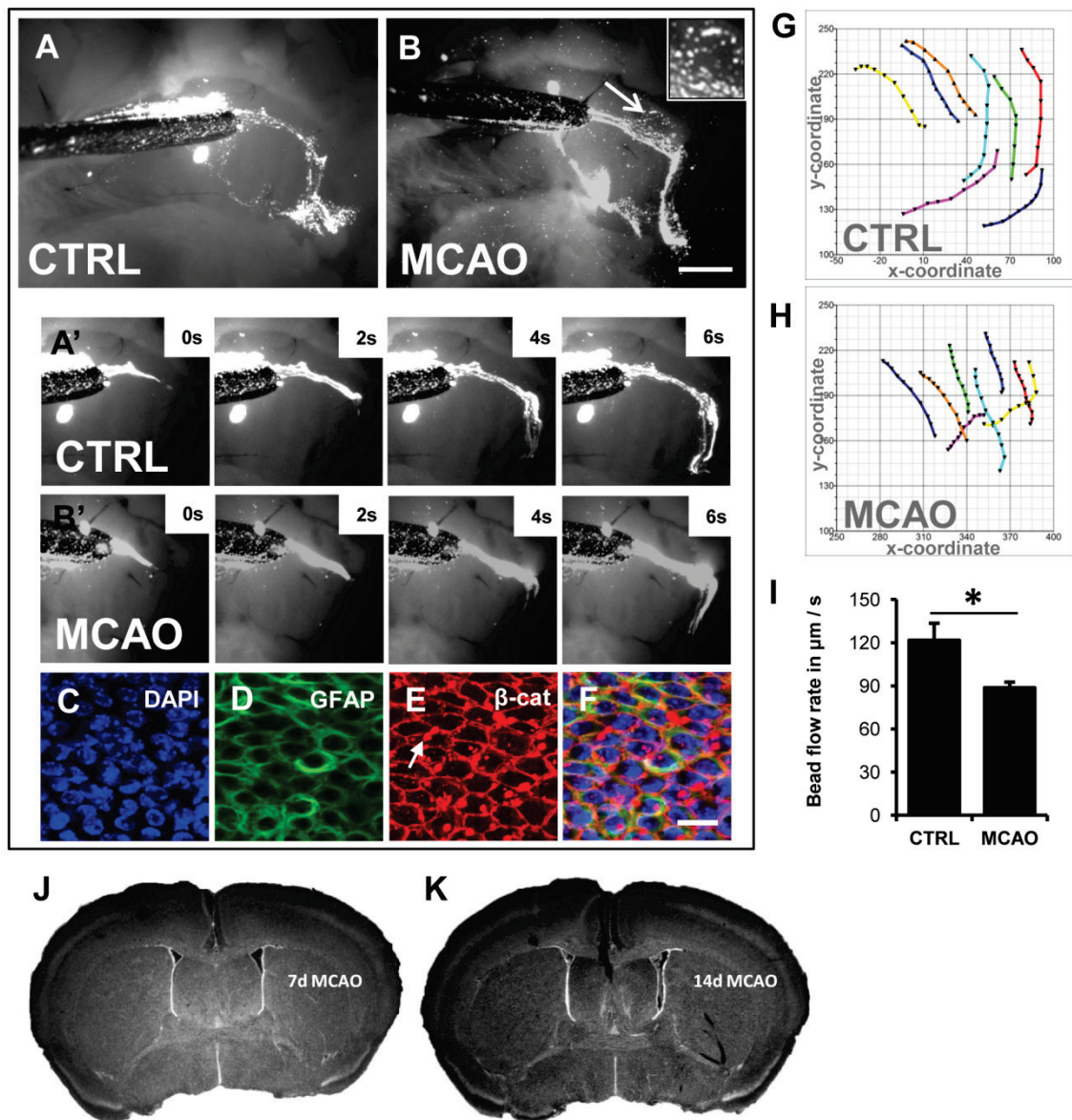


Figure 4.14. Turbulent and decreased cerebrospinal fluid flow after stroke

(A-B) Dynamic CSF flow study. Please see Movie 4.1. Fluorescent beads were deposited on lateral ventricular walls and time-lapse imaged. (A) Still-frame from Movie 4.1 showing CSF flow in control. (B) Still-frame from Movie 4.1 showing CSF flow 7-days post-stroke. *Inset*: Stroke increases flow turbulence over the anterior dorsal SVZ. Magnified view of the anterior dorsal lateral wall (arrow E, Movie 4.1). (A', B') Frames from time-lapse imaging of fluorescent bead movement over the anterior lateral ventricular wall.

(C-F) Post-hoc whole mount immunohistochemistry over area of flow turbulence in (B). Some fluorescent beads adhere to ventricular surface (arrow K).

- (G-H) Cartesian plots of bead movement over the anterior ventricular wall. Each trace represents one bead followed over 10 time-lapse frames.
- (I) Speed of fluorescent bead movement as a read-out for CSF flow and motile ciliary function. * $P = 0.02$.
- (J-K) Coronal sections showing example of ipsilateral ventricular dilation 7- and 14-days post-MCAO.
- Scale bar (B) 500 μm , (F) 50 μm .

Movie 4.1. CSF flow on the ventricular wall surface

CSF flow in normal lateral ventricle

Fluorescent beads were deposited over the anterior lateral ventricular wall in a control whole mount. Time-lapse imaged at 3 frames per second and playback at 2X real-time. The Hamilton syringe is visible in the field. Note the smooth anterior direction of bead flow, and the dorsal and ventral streams by-passing the intraventricular adhesion zone.

Stroke decreases CSF flow speed

CSF flow on whole mount 7-days post-MCAO. Some fluorescent beads from a preceding trial which have settled onto the ventricular surface are visible. Beads moved slowly and not in the free steady stream observed in the control. Turbulent flow is visible in the anterior dorsal region where PCP disruption was observed.

Stroke increases CSF flow turbulence

Enlarged view of inset in Fig 4.14B / anterior dorsal region. Bead flow is bi-directional and whirlpool-like. Mechanical damage to the ependyma with post-hoc immunohistochemistry (Fig 4.14C-F).

Timestamp in minutes: milliseconds

Please see accompanying DVD for *Movie 4.1 CSF flow – CY Thesis.mp4*

Movie 4.2 Negative controls

Lack of CSF flow on non-ependymal striatal surface

Negative control. Fluorescent beads deposited on brain tissue devoid of ependymal cells do not exhibit flow.

Lack of CSF flow following 70% ethanol incubation

Negative control. Following whole mount incubation in 70% ethanol, fluorescent beads deposited on the ventricular surface do not exhibit flow.

Please see accompanying DVD for *Movie 4.2 CSF NEG CTR – CY Thesis.mp4*

4.3. Discussion

This study shows that stroke alters the cellular components of the neurogenic niche; astrocytes, blood vessels and ependymal cells. Post-stroke reactive astrocytosis in the SVZ and a disrupted neuroblast migratory scaffold are novel changes which have not been previously described. Blood vessels, which aid neuroblast migration and contribute to the stem cell niche, increased in size and density after stroke. SVZ neuronal proliferation increased without changes in apoptosis. In contrast to what others have found, ependymal cell division after stroke was not detected and the ependymal layer remained intact. Unexpectedly, enlarged ependymal cells expressed robust levels of GFAP. While ependymal motile cilia appeared normal, ependymal cell PCP was misaligned in the anterior dorsal SVZ. Functional studies demonstrated turbulent CSF flow and decreased flow rates after stroke.

Whole mounts provide a unique opportunity to examine the SVZ, and previous studies have meticulously detailed the lateral ventricular wall in healthy mice (Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). In this chapter, whole mounts were used in histological and functional studies to better understand the cellular and molecular responses in this neurogenic compartment following stroke.

4.3.1 SVZ reactive astrocytosis

MCAO lesions encompassed most of the striatum but the SVZ did not form part of the stroke lesion. Although the SVZ is transiently hypoxic during MCAO (Thored et al., 2007), in the present stroke model, SVZ neurogenesis was robust and the number of apoptotic cells remained negligibly low. NeuN⁺ striatal neurons adjacent to the SVZ also

appeared healthy. However, SVZ astrocytes assumed a reactive phenotype, suggesting that SVZ astrocytes responded to the distant injury via diffusible, vascular-borne, and/or CSF-borne signals. SVZ astrocytes normally form glial tubes and provide a physical scaffold for neuroblast migration (Thomas et al., 1996). Disruption of SVZ astrocytes leads to abnormal neurogenesis and migration (Anton et al., 2004; Kaneko et al., 2010). For example, Galectin-3 gene deletion caused thickened SVZ glial processes and defective neuroblast migration (Comte et al., 2011). These observations point towards a model whereby multiple mechanisms maintain glial tube and neurogenic niche integrity.

After stroke, neuroblast migration to the olfactory bulb decreases (Ohab et al., 2006), although neuroblasts increase migration speed as they exit the SVZ and enter the striatum (Zhang et al., 2009). Such dynamic changes during neuroblast emigration may be orchestrated in part by SVZ reactive astrocytosis and glial tube dysmorphology. GABA tonically decreases neuroblast migration speed (Bolteus and Bordey, 2004), and reactive astrocytes are deficient in GABA inhibition (Ortinski et al., 2010), suggesting that after stroke reactive astrocytes may alter neuroblast migration speed via a GABA-mediated mechanism. Astrocytes are stationary in the unperturbed SVZ (Nam et al., 2007). After stroke however, SVZ-derived astrocytes migrate into the ischaemic striatum (Li et al., 2010c), and it is tempting to associate this capacity with stroke-induced reactive astrocytosis. Another intriguing question is whether stroke drives SVZ niche astrocytes towards a stem-like phenotype, as has been observed in cortical reactive astrocytes following injury, and whether they have the capacity to function as NSCs in the ischaemic striatum (Buffo et al., 2008).

4.3.2. Stroke increases proliferation in neural-lineage cells

Stroke increases SVZ neural precursor proliferation (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003). I found similar increase in proliferation and showed the percentages of mitotic SVZ neural progenitors and neuroblasts were unchanged. The stable ratio of proliferating glia (PHi3+/Dcx-/Mash1-) further suggested a proportional increase of GFAP+ astrocyte proliferation, a subset of which are astrocyte-like stem cells. Even though the SVZ does not normally generate significant numbers of astrocytes and oligodendrocytes (Menn et al., 2006), brain injury can lead to shift in cell fate decisions *in vivo*. Recent *in vivo* lineage tracing studies provide evidence that neural stem and progenitor cells in the SVZ generate astrocytes and oligodendrocytes after stroke (Li et al., 2010c; Zhang et al., 2010). In a model of demyelination, Dcx+ neuroblasts migrated into the corpus callosum and differentiated into oligodendrocytes (Jablonska et al., 2010).

In vitro, stroke and hypoxic/ischaemic injury lead to increased self-renewal and multipotency as reflected by increased number of neurospheres obtained per number of starting SVZ cells, and increased percentage of neurospheres which gave rise to neurons, astrocytes and oligodendrocytes (Zhang et al., 2004; Deierborg et al., 2010). My studies were not designed to detect quiescent NSCs and it is not known if self-renewal changed after stroke. In coronal sections, the number of GFAP+ processes contacting the ventricle was increased. Initially, this observation was thought to correspond with the concept of NSC activation, i.e. apical ventricular contact was necessary for quiescent stem cells to divide, or that niche astrocytes respond to environmental signals and reprogramme into a stem-like state. This concept had previously been described in the SVZ following intraventricular infusion of EGF, which doubled the number of astroglial processes in contact with the ventricular surface (Doetsch et al., 2002). In hindsight, the majority of GFAP+ processes thought to be contacting the ventricle in coronal sections probably

reflected GFAP expression in ependymal cells, and not the apical processes associated with stem-ness. The whole mount would have been an obvious way to assess if the number of apical processes contacting the ventricle increased, and indeed whether there was expansion of the B₁ NSC population after stroke. However, the robust expression of GFAP in the ependymal cells precluded such quantifications.

4.3.3. Vascular changes in the post-stroke SVZ

The importance of the vascular niche for SVZ neurogenesis and migration is well-established (Shen et al., 2008; Tavazoie et al., 2008; Kokovay et al., 2010). Ectopic migration into the ischaemic striatum occur along blood vessels (Yamashita et al., 2006; Ohab et al., 2006; Thored et al., 2007; Kojima et al., 2010). In the SVZ and RMS, endothelial cell derived BDNF and SDF-1 α regulate neuroblast migration (Snayyan et al., 2009; Kokovay et al., 2010). In the post-stroke striatum, the roles of these molecular pathways as the basis for the cellular affinity remain to be confirmed. Engorged SVZ vasculature and increased angiogenesis were reported following thermocoagulatory cortical lesions (Gotts and Chesselet, 2005). However, in a rat model of MCAO, (Thored et al., 2007) reported the paradoxical observations of transiently increased endothelial proliferation and decreased vascular density in the post-stroke SVZ. Inter-species differences could explain the differences in these results. I have also found it difficult to assess vascular density in the narrow SVZ in coronal sections, and the measurement is variable depending on the plane in which a blood vessel has been cut. Therefore, whole mount assessment of the SVZ vasculature is more precise and accurate. The observation that astrocytes increased contacts with blood vessels post-stroke raises the possibility that they actively modulate blood flow to the SVZ (Attwell et al., 2010). Since post-stroke

neurogenesis and angiogenesis are coupled in the ischaemic striatum (Ohab et al., 2006), the present data suggest this may also be the case in the post-stroke SVZ. In addition, changes to glial-vascular interactions at the vascular basement membrane could have implication for the integrity of the blood brain barrier.

4.3.4. Ependymal cells resemble radial glia and reactive astrocytes post-stroke

A subset of GFAP+ SVZ astrocytes are NSCs but whether ependymal cells may be neural precursors remains elusive (Spassky et al., 2005; Johansson et al., 1999; Gregg and Weiss, 2003; Gleason et al., 2008). I did not find proliferating ependymal cells 7- and 14-days after stroke, using pulse and long-term BrdU labelling, respectively. In contrast, post-stroke rat ependymal cells incorporates BrdU and expresses the mitotic radial glia marker phosphorylated vimentin (Zhang et al., 2007b). There may be inter-species differences, alternatively ependymal cells divided so rarely that the cumulative labelling strategy was insufficient. Similar cumulative labelling also did not detect BrdU+ ependymal cells (Carlen et al., 2009). Using a *Foxj1-cre* expressing lentivirus which labels ependymal cells and a subset of SVZ astrocyte-like stem cells (Jacquet et al., 2009), it was suggested that murine cells did not divide in the ependymal layer but delaminated into the SVZ and divided to generate neuroblasts and astrocytes (Carlen et al., 2009). However, the lack of ependymal specificity of the *Foxj1* promoter makes the result difficult to interpret. The authors also claimed delamination led to significant ependymal cell loss (time-point not specified). While I did not fate-map ependymal cells, detailed examination of whole mounts and coronal sections showed preserved ependymal cell layer, without obvious cell loss or denudation up to 14-days post-stroke.

GFAP is a major structural intermediate filament protein of SVZ astrocytes, reactive astrocytes and radial glia. SVZ astrocytes and ependymal cells derive from GFAP⁺ radial glia, but postnatal ependymal cells lose GFAP and develop motile cilia (Spassky et al., 2005). Surprisingly, ependymal cells expressed GFAP after stroke. Cytoplasmic GFAP was found at the ventricular surface in ependymal cells, identified in single confocal optical section by ciliary basal body clusters and β -catenin expression in the cell membrane. β -catenin can be expressed by activated SVZ cells (Cooper and Isacson, 2004), reactive astrocytes (White et al., 2009) and SVZ B₁ stem cells (Mirzadeh et al., 2008), thus it is possible that some of its expression was not ependymal. However, ependymal cells are easily distinguished from other SVZ cell types by their apical location abutting the lateral ventricles and β -catenin expression which confers the typical cobblestone arrangement of the ependymal cell layer. Nevertheless, immuno-electron microscopy would provide useful confirmation of GFAP expression in ependymal cells.

The GFAP detected in ependymal cells post-stroke may have been *de novo* expression or expression which increased to the point of immunohistochemical detectability. Adult ependymal cells do not usually express GFAP (Pastrana et al., 2009), although they readily do so *in vitro* (Weibel et al., 1986; Chiasson et al., 1999). A previous study made the distinction that mouse monoclonal antibodies against GFAP did not label ependymal cells, while polyclonal antibodies did (Doetsch et al., 2002). In this study, spurious labelling was excluded by using 3 different antibodies (Table 2.3) and with each antibody, the post-stroke ependyma was strongly GFAP⁺, while the ependyma were GFAP⁻ in sham operated controls and the contralateral hemisphere.

GFAP⁺ cells have also been reported in the ependyma of aged mice, and following neuraminidase denudation (Luo et al., 2008) and genetic niche disruption (Kuo et al., 2006). SVZ astrocytes replaces lost ependymal cells and assume ependymal features such

as adherens junctions, basal bodies and motile cilia (Luo et al., 2008). Postnatal deletion of Numb/Numbl like in the SVZ damages the ependymal cell layer, and endogenous repair results in a multi-layered stratified epithelium which co-express GFAP and α 100 β (Kuo et al., 2006). Under EphB2 regulation, the opposite also occurs and low numbers of ependymal cells delaminate into the SVZ, lost ependymal phenotype, and express GFAP (Nomura et al., 2010). After stroke, it was more likely that ependymal cells acquired features of radial glia and reactive astrocytes. Firstly, the ependymal layer was intact after stroke; even if delamination occurred, astrocytes were unlikely to have replaced >85% of ependymal cells within a week. Indeed, genetic disruption of the ependymal cell layer resulted in incomplete repair over a 6 week period (Kuo et al., 2006). Secondly, ependymal GFAP⁺ cells had cilia and basal bodies typical of mature ependymal cells. It was improbable that astrocytes could form dense basal body clusters and grow motile cilia tufts in only 7-days. Lastly, ependymal cell was shown to switch phenotype after neonatal hypoxia and stroke in rats (Zhang et al., 2007b; Ganat et al., 2002).

Vimentin⁺ ependymal cells that co-expressed GFAP in coronal histological sections provided further proof of the unexpected result. GFAP co-expression with vimentin is required for reactive astrogliosis and scar formation. Loss of either intermediate filament alone is insufficient to abolish these functions (Pekny et al., 1999; Wilhelmsson et al., 2004). It may be that after stroke, GFAP and vimentin co-expression in ependymal cells allow them to serve a barrier function similar to glial scars to limit damage (Li et al., 2007). Similarly, GFAP and vimentin co-expression increases the physical stiffness of astrocytes (Lu et al., 2011) suggesting GFAP may alter the mechanical properties of ependymal cells.

4.3.5. Planar cell polarity, motile cilia function and CSF flow

Ependymal PCP in the epithelial plane orients the cilia beating direction and hence CSF flow. Also referred to as translational polarity, ependymal PCP can be represented by the anatomical position of the basal body cluster in relation to the centre of the cell (Mirzadeh et al., 2010b). PCP in ependymal cells is established in the first post-natal week, through an interplay between hydrodynamic forces in the ventricle, and the asymmetric cellular distribution of the core PCP protein, Van Gogh-like 2 (Vangl2; Guirao et al., 2010).

After stroke, enlarged GFAP⁺ ependymal cells in the anterior dorsal ventricular wall lost PCP orientation. Whereas ciliary basal bodies were polarised in controls located downstream of the direction of flow, their location shifted dramatically and became randomly distributed post-stroke. To my knowledge, this is the first report of acute injury in a non-proliferating tissue disrupting PCP, after PCP has been successfully established during development. A possible mechanism may be that as ependymal cells acquire an astroglial phenotype, GFAP intermediate filaments cause relative shifts of basal body locations. Notably, the kaolin-infusion model of neonatal hydrocephalus caused ependymal cell stretching and qualitative impression of motile cilia displacement (Weller et al., 1978). It is not known if these changes were due to the induced hydrocephalus, or ependymal reaction to kaolin.

Lateral membrane contacts between ependymal cells via N-cadherins may be critical for SVZ proliferation and neuroblast chain formation (Paez-Gonzalez et al., 2011). The anterior dorsal ventricular wall where PCP was disrupted after stroke is the so-called neurogenic “hot-spot” with a high density of B₁ stem cells (Mirzadeh et al., 2008). Therefore, it will be interesting to examine whether PCP misalignment / intermediate

filament reorganisation would disrupt adherens junctions between adjacent ependymal cells.

The ependymal cell response to stroke was functionally significant, decreasing CSF flow speed and increasing turbulence 7-days post-stroke. Disruption of CSF fluid dynamics potentially disturb the molecular equilibrium between CSF and brain parenchyma (Johanson et al., 2011), such as the Slit2 chemorepellent gradient which mediates SVZ neuroblast migration (Sawamoto., et al 2006). The regulation of other CSF-borne molecules such as insulin-like growth factor (IGF), fibroblast growth factor (FGF) and Sonic hedgehog (Shh) (Lehtinen et al., 2011), may also to be affected. These molecules contribute to neurogenic niche maintenance and the SVZ's proximity to the ventricles makes it vulnerable to such imbalances.

The mechanism of unilateral ventricular dilation as a late consequence of stroke is unclear. Fourteen-days post-stroke, brain oedema would have largely resolved (Lin et al., 1993), reducing the likelihood of CSF outflow obstruction. Clinically, hydrocephalus *ex-vacuo* is recognised as ventricular dilation due to brain volume loss or tissue atrophy, and is likely to be a factor. However, congenital and acquired ciliary dysfunction results in hydrocephalus (Banizs et al., 2005; Tissir et al., 2010; Sarnat, 1995; Monkkonen et al., 2007), and CSF flow disturbances post-stroke may contribute to post-stroke ventricular dilation. While hydrocephalus is not a common acute complication after clinical stroke, a history of ischaemic stroke has been identified as a possible risk factor for idiopathic normal pressure hydrocephalus (Krauss et al., 1996; Boon et al., 1999). Moreover, post-stroke ventricular dilation may account for the increased ependymal cell surface area as the cells are mechanically stretched. This kind of mechanical force may provide a non-molecular signal which contributes to the changes observed in the post-stroke SVZ. In a comparative study of the rat and murine SVZ, the number of astrocyte-like stem cells

closely correlated with the number of ependymal cells (Kazanis and French-Constant, 2011b). The number of ependymal cells is directly proportional to the lateral ventricular surface area. Therefore a corollary of this observation may be that the number of NSCs correlates with the surface area of the lateral ventricles. It will be interesting to determine whether ventricular dilation alone would increase the NSC population, and thus potentially serve as a novel mechanism that increases post-stroke SVZ cell proliferation.

4.4. Conclusion

The roles of SVZ niche astrocytes, the vasculature and ependyma in the regulation of SVZ neurogenesis are well-known, and disruption of their function, such as abnormal glial tube formation and disrupted CSF flow are recognised features which impacts neuroblast migration. SVZ-mediated endogenous repair post-stroke is likely an intricate and complex process, as highlighted by the multiple cellular changes documented in this study. These functional changes are potential mechanisms which contribute to the endogenous repair process, and future work will need to elucidate to what extent these changes are parts of the stroke pathology, or whether they serve to facilitate aspects of SVZ-mediated repair.

Chapter 5

Galectin-3 is a regulator of SVZ neuroblast migration which is up-regulated after ischaemic stroke

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5.1. Introduction

A feature of adult neurogenesis in the SVZ is its capacity to respond to injury and mediate attempted endogenous repair. As discussed in Chapter 1 and demonstrated in Chapter 4, the SVZ is regulated by an intricate and complex system of molecular pathways and cellular interactions which regulate the neural stem cell niche in health and disease. The Szele Laboratory has been interested in the regulation of neuroblast emigration towards injured brain regions, in different models of brain injury (Goings et al., 2004; Ramaswamy et al., 2005; Kim and Szele, 2008; Dizon et al., 2010; Young et al., 2011). Therefore, to identify molecular interactions which facilitate recruitment of SVZ neuroblasts towards injury, a gene expression array was carried out on cortical and striatal tissue following an aspiration model of traumatic brain injury. Focusing on genes which mediate cell-cell and cell-extracellular matrix interactions, the array identified 3 members of the galectin family of carbohydrate-binding proteins which were significantly up-regulated: Gal-1 (11 fold), Gal-3 (5 fold) and Gal-9 (9 fold) (E. Kang and F. Szele, unpublished data).

Gal-3 was originally identified as the Mac-2 antigen, specific to a sub-population of macrophages (Ho and Springer, 1982). It is up-regulated in activated microglia/macrophages in the brain following ischaemic stroke (Walther et al., 2000) and experimental autoimmune encephalitis (Reichert and Rotshenker, 1999). *In-situ* hybridisation data from the Allen Brain Atlas (Lein et al., 2007) suggests that Gal-3 mRNA is restricted to the SVZ in the adult brain, although its cellular expression and function in the healthy CNS is hitherto unknown. The biological action of Gal-3 in other organs has been attributed to its carbohydrate binding properties, and Gal-3 is known to bind ECM proteins such as laminin, collagen, tenascin and fibronectin, as well as cell

surface molecules such as integrins, NG2 proteoglycans and NCAMs (Hughes, 2001). Critically, the interaction between these cell surface receptors and their ECM ligands (e.g. $\beta 1$ integrin and laminin) contributes to the regulation of the neural stem cell niche (Kazaniet al. and French-Constant, 2011a), and Gal-3 binding has been shown to modulate such ligand/receptor interactions (Kariya et al., 2010; Saravanan et al., 2009). The identification of Gal-3 as a molecule of interest coincided with the publication of a study in which Gal-1, expressed by dividing SVZ astrocytes, promoted cell proliferation (Sakaguchi et al., 2006). In a gerbil model of stroke, the same group demonstrated that infusion of recombinant Gal-1 increased post-stroke neurogenesis in the ischaemic striatum in a carbohydrate-binding dependent manner (Ishibashi et al., 2007). Therefore, given its specific expression in the SVZ and up-regulation after brain injury, Gal-3 was an ideal candidate for further investigation.

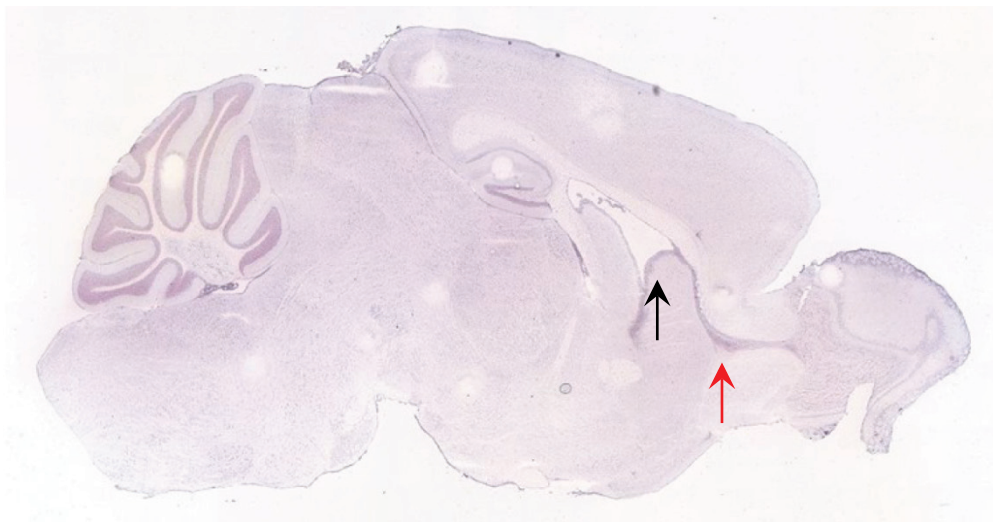


Figure 5.1. Gal-3 *in-situ* hybridisation in the adult mouse brain (Allen Brain Atlas; Lein et al., 2007)

The SVZ is indicated by *black arrow*, and the RMS by *red arrow*.

Dr Isabelle Comte, a post-doctoral researcher in the Szele Laboratory, confirmed the specific expression of Gal-3 in the SVZ, and in a series of elegant experiments (Comte et al., 2011), demonstrated that loss of Gal-3 resulted in delayed neuroblast migration to the OB *in vivo*, without affecting cell proliferation or cell death. *In vitro*, addition of functional blocking antibodies significantly inhibited neuroblast migration in RMS explant cultures, while addition of recombinant Gal-3 promoted migration. The *in vitro* experiments further suggested that the effect was mediated via extracellular interactions.

5.1.1. Gal-3 null mice

Mice which have a targeted deletion of the Gal-3 gene locus by homologous recombination are used in this study to perform loss-of-function studies to investigate the role of Gal-3 in neuroblast migration. Gal-3^{-/-} mice bred to congenic 129sv strain were generated by the Poirier Laboratory, and they were reported to be viable and fertile, without gross developmental abnormalities. (Colnot et al., 1998a) Indeed, anatomical defect in brain structures is not detected (Comte et al., 2011). However, deficiencies of various inflammatory processes have been reported in this mutant, including defective polymorphonuclear leukocyte recruitment following experimental peritonitis (Colnot et al., 1998b), decreased numbers of periaortic mononuclear cells in a model of atherosclerosis (Nachtigal et al., 2008), and reduced leukocyte infiltration into the CNS in experimental autoimmune encephalitis (Jiang et al., 2009).

5.1.2. Two-photon time-lapse microscopy to study cell migration

Cell migration can be imaged by time-lapse microscopy to provide information about the dynamic features of migration that is not available from static tissue studies. Static studies using cell tracers such as BrdU incorporation allows net result of migration to be inferred, and provide information regarding the number of cells that had migrated and their destination (Comte et al., 2007). However, they do not inform the investigator of the manner in which migration occurred. Novel aspects of neuroblast migration in the RMS have been demonstrated using 2-photon time lapse microscopy in acute brain slices, such as: the bidirectional nature of neuroblast migration, the stability of the neuroblast chains, periods of rest versus motility, tangential versus local exploratory movements, and migratory response to exogenous signals (Nam et al., 2007; Kim et al., 2009; James et al., 2011). Two-photon lasers are suitable for *ex vivo* time-lapse imaging in acute brain slices as they induce significantly less photobleaching and phototoxicity due to the focal nature of the incident beams, allowing for prolonged imaging with minimal cell death (James et al., 2011). This contrasts with confocal and epifluorescence microscopy where the entire tissue under the objective is targeted by the incident light throughout the imaging process (Zipfel et al., 2003). The long excitation waves also facilitates deeper tissue penetration, up to 500 μm into the brain slice, as well as reduced energy absorbance by the brain tissue compared to conventional imaging modalities (Zipfel et al., 2003).

5.1.3. Research questions

I hypothesised that Gal-3 is expressed by specific cell type(s) in the SVZ, and that Gal-3 is necessary to maintain the normal dynamic features of neuroblast migration. In addition, it is postulated that Gal-3 may be up-regulated in the ischaemic brain, in temporal and

cellular expression profiles that would be compatible with a role in regulating the SVZ response to stroke. The aims of this chapter are therefore to: (1) determine the cellular localisation of Gal-3 in the SVZ, (2) determine the temporal profile of Gal-3 expression after MCAO, and its post-stroke cellular localisation, and (3) determine if loss of Gal-3 results in abnormal dynamic features of neuroblast migration.

5.2. Results

5.2.1. Gal-3 is expressed by a subset of SVZ cells

Immunohistochemistry was carried out on coronal and sagittal brain sections to determine the cellular localisation of Gal-3 expression. In agreement with *in-situ* hybridisation data from the Allen Brain Atlas (Fig 5.1), Gal-3 was selectively expressed in the adult mouse brain in the SVZ, RMS and accessory OB (Fig 5.2A). In the SVZ and RMS, Gal-3 immunoreactivity was punctate and diffuse (Fig 5.2A'), and its expression pattern was reminiscent of the glial tubes surrounding neuroblasts. Indeed, GFAP+ SVZ astrocytes (Fig. 5.2D–F) and ependymal cells lining the lateral ventricles (Fig. 5.2F) expressed Gal-3. Gal-3 formed a dense network surrounding PSA-NCAM+ neuroblast chains, but Gal-3 was not expressed by neuroblasts (Fig 5.2C). A subset of Mash1+ transit-amplifying progenitor cells also co-localised with Gal-3, primarily in the cytoplasm (Fig 5.2C). CD45+ microglia, which are constitutively semi-activated (Goings et al., 2006), did not express Gal-3. Gal-3 immunoreactivity was absent in the main OB, cortex and the striatum. These findings demonstrated the selective expression of Gal-3 by specific cell types of the adult neurogenic niche.

5.2.2. Genotyping and phenotyping of Gal-3 deficient mice

To examine the function of Gal-3 in the regulation of adult neurogenesis and its response to stroke, I used transgenic 129sv mice which have homozygous genetic deletion of exons II – IV of the Gal-3 gene locus (Fig 5.3A; Colnot et al., 1998a). Genotyping PCR was performed to determine the genotype of offsprings from Gal-3 heterozygote null (Gal-3^{+/-})

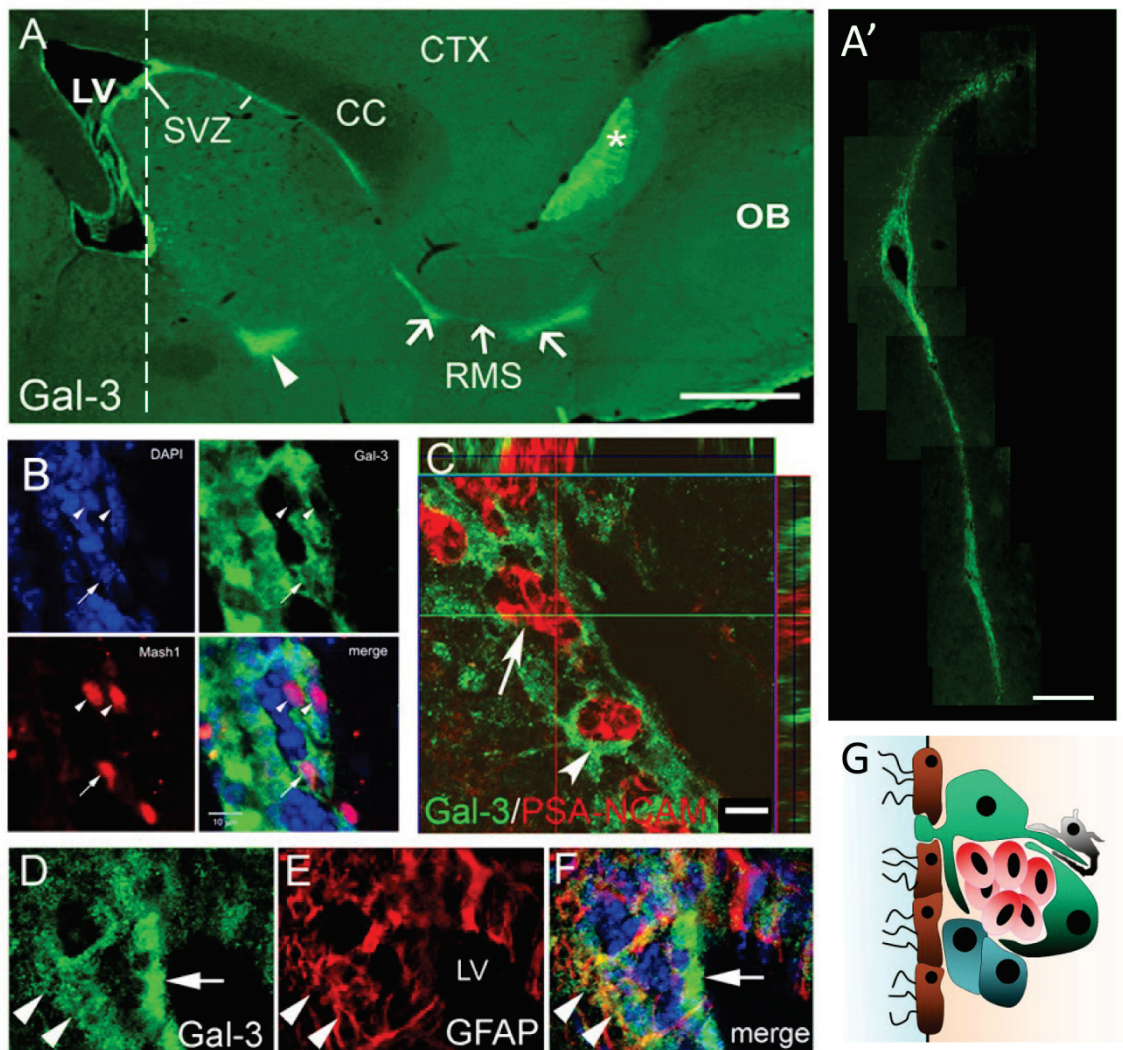


Figure 5.2. Gal-3 expression on astrocyte lineage cells surrounds neuroblast chains

- (A) Gal-3 immunohistochemistry in an adult sagittal brain section. Gal-3 is specifically expressed in the SVZ, RMS (*arrows*) and accessory olfactory bulb (*asterisk*). CC, corpus callosum; CTX, cerebral cortex; LV, lateral ventricle; OB, olfactory bulb. (A') Coronal montage through the *dotted line* in (A).
- (B) Gal-3 is expressed by subset of Mash-1+ transit amplifying progenitors. Confocal single optical section.
- (C) Gal-3 expression envelopes (*arrowhead*) neuroblast chains, although PSA-NCAM+ neuroblasts (*arrow*) do not express Gal-3.
- (D-F) Gal-3 immunofluorescence is diffuse and is associated with GFAP+ cells (*arrowheads*). Many ependymal cells also express Gal-3 (*arrow*).
- (G) Cellular schematic of subventricular zone.

Scale bar (A-A') 250 μ m, (C) 10 μ m.

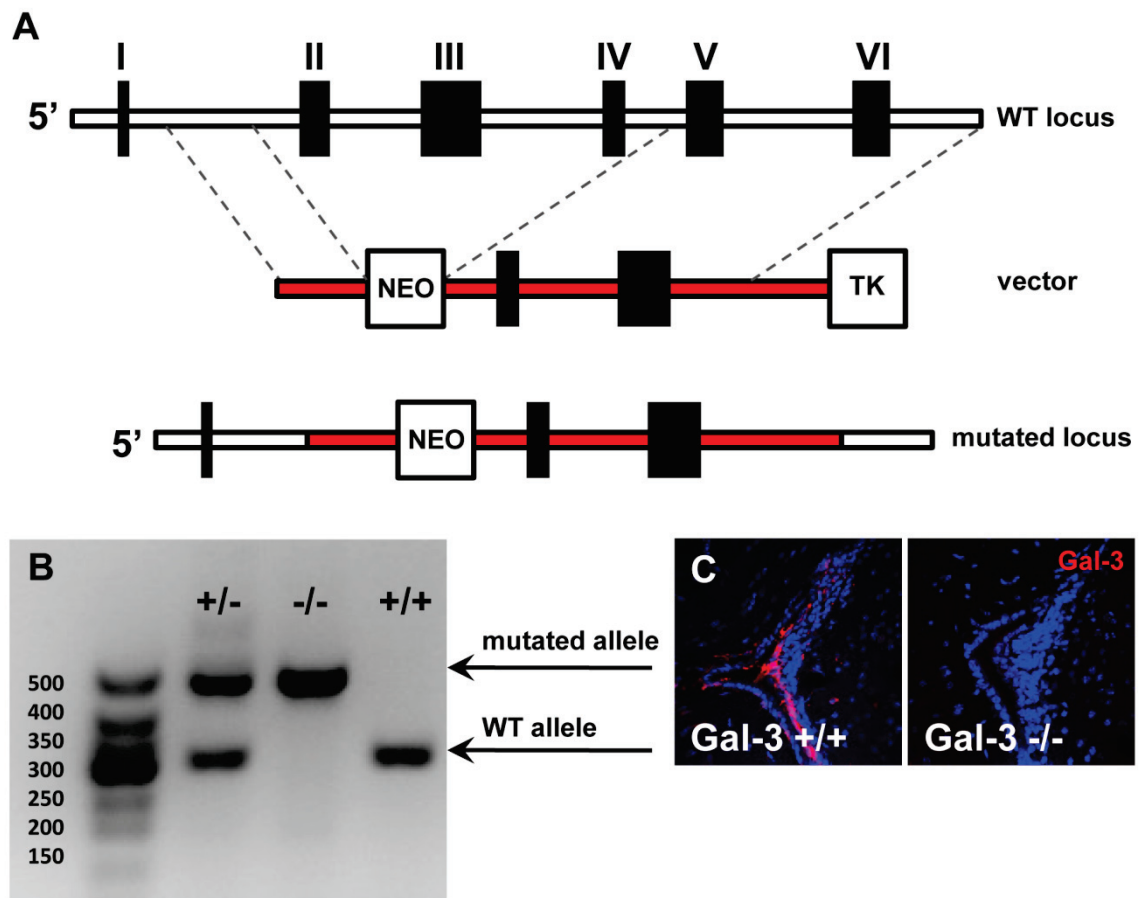


Figure 5.3. Galectin-3 homozygous null mutant mice

- (A) Targeted deletion of exons II – IV of the *Lgals3* gene locus by homologous recombination. Modified from (Colnot et al., 1998).
- (B) Gel electrophoresis of genotyping PCR products.
- (C) Immunohistochemistry for Gal-3 confirms gene deletion and absence of post-translational product.

breeding pairs. Wildtype mice (Gal-3^{+/+}) homozygous for the wildtype allele produced single DNA products ~300 bp while Gal-3 homozygous null (Gal-3^{-/-}) mice for the mutant allele produced single DNA products ~500 bp (Doverhag et al., 2010). Gal-3^{+/-} heterozygotes produced both ~300 bp and ~500 bp DNA products of reduced intensity (Fig 5.3B). The absence of functional Gal-3 gene was confirmed with immunohistochemistry with 2 different anti-Gal-3 antibodies, which showed loss of immunodetectable Gal-3 protein in the SVZ (Fig 5.3C). Rat monoclonal and rabbit polyclonal antibodies were raised against the full-length mouse Gal-3 protein and amino acids 1 - 160 on the N-terminus of recombinant human Gal-3, respectively.

Gal-3^{+/-} mice were fertile and mated normally, producing litters of ~6 - 8 pups. Gal-3^{-/-} mice were viable and found in appropriate numbers (~25%) as per Mendelian inheritance. Body weights were virtually identical for Gal-3^{+/+} (24.9 ± 0.4 g) and Gal-3^{-/-} (24.8 ± 0.5 g) aged 8 - 12 weeks ($n = 20$).

5.2.3. Gal-3 maintains neuroblast motility in the SVZ without affecting cell proliferation

In vitro and *in vivo* experiments from the Szele Laboratory suggested that neuroblasts migration in the SVZ-RMS-OB pathway was defective in Gal-3^{-/-} mutants (Comte et al., 2011). To understand the effects of Gal-3 deletion on dynamic features of neuroblast motility, I undertook *ex vivo* two-photon time-lapse microscopy of migrating neuroblast (Nam et al., 2007) in collaboration with Drs Isabelle Comte and Yongsoo Kim. SVZ cells were labelled with intraventricular injections of cell tracker green (CTG; De Marchis et al., 2001) and migration was studied 2 days later (Fig 5.4A–B; Movies 3 and 4).

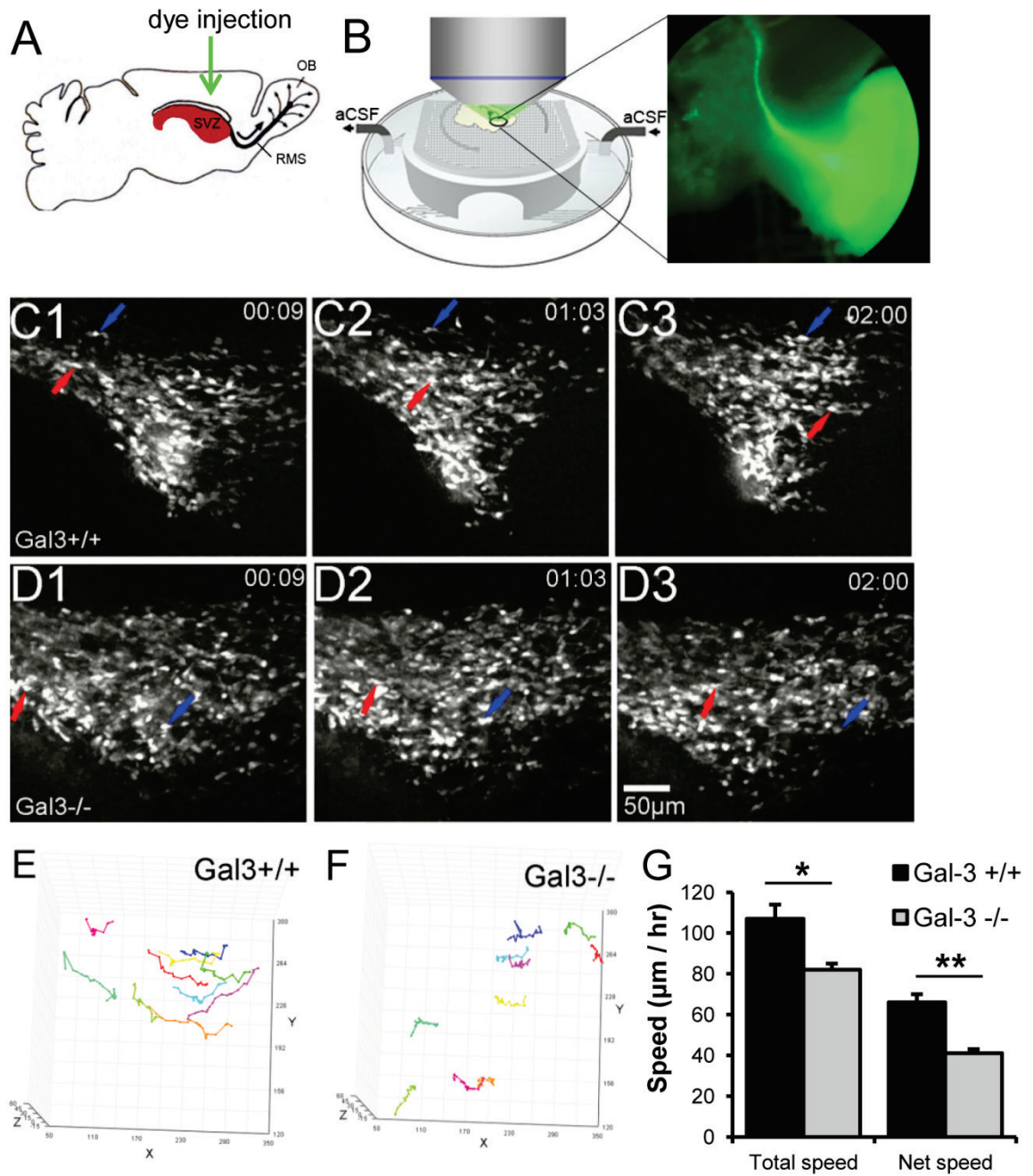


Figure 5.4. Two-photon time-lapse microscopy reveals slower and more complex motility in *Gal-3*^{-/-} mice

Figure 5.4. Two-photon time-lapse microscopy reveals slower and more complex motility in Gal-3 null mice

- (A-B) Cell tracker green was injected into the lateral ventricle and 2 days later neuroblast migration was analyzed at the RMS elbow in slices. (B) Low-magnification photomicrograph on right shows CTG-labelled cells migrating in the RMS.
- (C-D) Still images from a two-photon time-lapse movie from WT (Movie 5.1) and Gal-3^{-/-} brains (Movie 5.2). Coloured arrows show the positions of two CTG-labelled neuroblasts. Time stamps are in hours:minutes.
- (E-F) Migratory paths of neuroblasts over time. Each colour trace represents an individual neuroblast. Gal-3^{-/-} neuroblasts exhibit decreased motility and were more exploratory compared to Gal-3^{+/+}.
- (G) Quantification of total speed (total distance travelled over time) and net speed (net distance travelled over time). * $P < 0.05$, ** $P < 0.01$.

Scale bar 50 μ m

Movie 5.1. Two-photon time lapse microscopy of wildtype neuroblast migration

Wildtype neuroblasts, tracked by blue and red arrows. Migration is fast and straight.
Timestamp in hours:minutes

Please see accompanying DVD for *Movie 5.1 Gal-3 WT – CY Thesis.mp4*

Movie 5.2. Two-photon time lapse microscopy of Gal-3^{-/-} neuroblast migration

Gal-3^{-/-} neuroblasts, tracked by blue and red arrows. Migration is slow and exploratory.
Timestamp in hours: minutes

Please see accompanying DVD for *Movie 5.2 Gal-3 KO – CY Thesis.mp4*

Using this strategy, we examined cells that had migrated long distances from the SVZ to the RMS. Three-dimensional tracings of the migratory pathways of individual neuroblasts revealed that Gal-3^{-/-} neuroblasts moved in a more complex local manner and travelled less distance compared to Gal-3^{+/+} wildtypes (Fig 5.4E-F). Gal-3^{+/+} neuroblasts migrated at an average speed of $106 \pm 7 \mu\text{m} / \text{hour}$, which was similar to results from previous studies (Nam et al., 2007; Kim et al., 2009). Neuroblasts moved significantly slower in Gal-3^{-/-} ($84 \pm 3 \mu\text{m} / \text{hour}$; $P < 0.05$; 10 cells per brain slice; $n = 4$; Fig. 5.4G).

Pulse labelling of proliferating cells with BrdU (100 mg/kg) 2 hours before sacrifice showed comparable number of BrdU+ cells in the SVZ between Gal-3^{+/+} wildtypes (79.0 ± 16.8 cells) and Gal-3^{-/-} mutants (74.4 ± 7.6 cells), suggesting that Gal-3 did not regulate SVZ cell proliferation under normal conditions.

5.2.4. Gal-3 is up-regulated in the ischaemic brain

To assess the expression profile of Gal-3 after stroke, Western blots were performed to measure Gal-3 protein levels in the ischaemic brain 3- and 7- days after MCAO, in comparison to the contralateral hemisphere and naïve controls (Fig 5.5A). HeLa cell lysate which contains high levels of Gal-3 was used as positive control (Dagher et al., 1995), and revealed a strong immunoreactive protein band ~ 30 kDa, while a distilled water negative control showed no protein band. Gal-3 was barely detectable in naïve brain homogenates. Three-days post-MCAO, Gal-3 protein level in the ipsilateral hemisphere was significantly increased (12 ± 1.8 fold) compare to controls (Fig 5.5B). Seven-days post-MCAO, Gal-3 expression increased further to 40 ± 8.9 fold of naïve levels ($n = 3$; $P < 0.001$. One-way ANOVA with Bonferroni post-hoc test). In contrast, Gal-3 levels in the contralateral hemispheres 3- and 7-days after MCAO were not significantly different from controls. Equivalent protein loading was confirmed by

immunoblotting for β -tubulin, a ubiquitous microtubule structural protein (Liu and Xu, 2006).

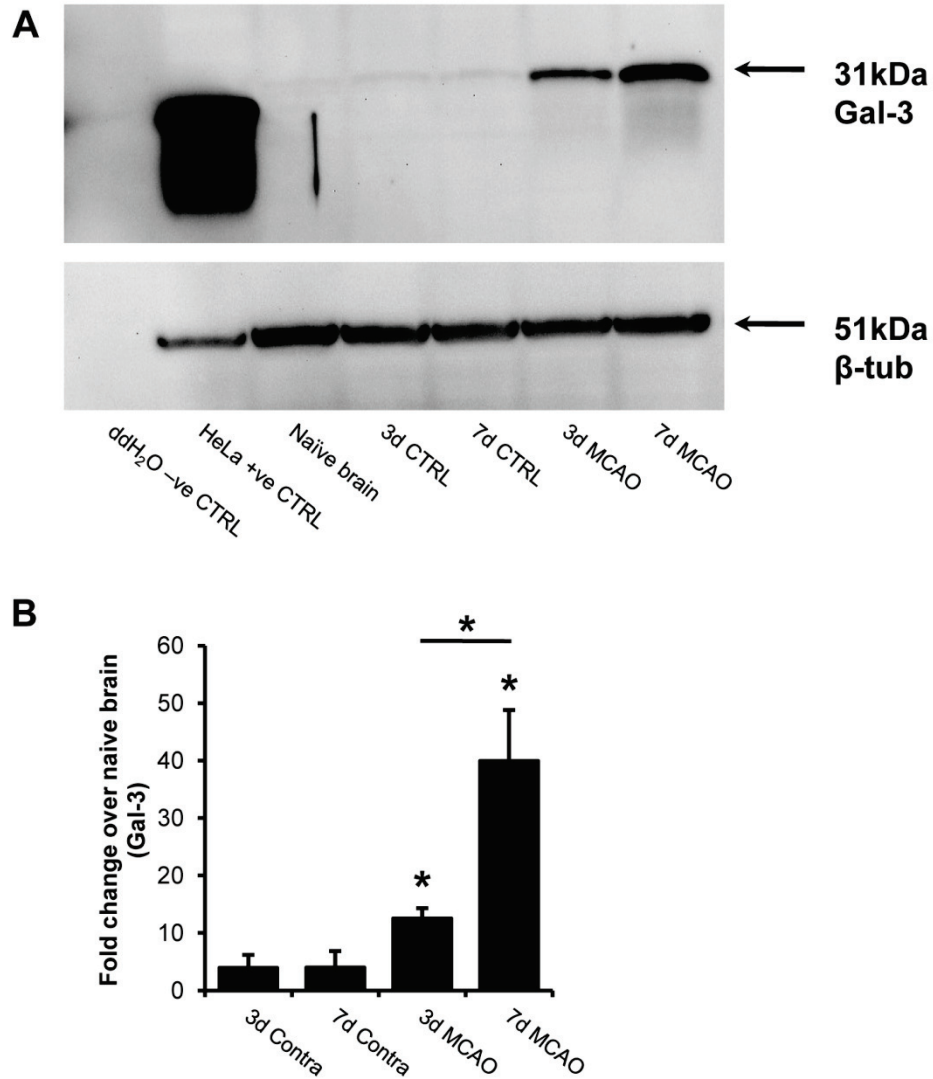


Figure 5.5. Gal-3 is up-regulated in the ischaemic brain

- (A) Western blot was performed on brain homogenates 3- and 7-days after 50-min MCAO.
- (B) Gal-3 was increased in the ipsilateral brain hemispheres at 3-days (12-fold increase) and 7-days (40-fold increase) after MCAO compared to naïve brains. $n = 3$ per group. * $P < 0.05$

5.2.5. Galectin-3 expression is increased in the corridor of neuroblasts emigration and the ischaemic striatum

In confirmation of the Western blot results, 7-days post-stroke there was marked increase in Gal-3 immunofluorescence in the brain hemisphere ipsilateral to MCAO (Fig 5.6A). In the post-MCAO SVZ, the cellular localisation of Gal-3 remained stable. Unchanged from naïve animals, Gal-3 was expressed by astrocytes, ependymal cells (Fig 5.6B), and a subset of transit amplifying progenitors cells (Fig 5.6D), but not by Dcx+ neuroblasts (Fig 5.6C). However, in the dorsal SVZ, GFAP and Gal-3 expression was increased compared to controls (Fig 5.6E-H). Increased Gal-3 was largely localised to GFAP+ astrocytes and ependymal cells post-MCAO. Interestingly, the increase in Gal-3 appears to be restricted to the SVZ and GFAP+ elements lateral to the ventricle. GFAP+ astrocytes in the corpus callosum medial to the SVZ weakly expressed Gal-3 (*arrow* Fig 5.6F). In the dorsolateral SVZ, a corridor of early neuroblast emigration after stroke, Gal-3 expression was increased compared to controls (Fig 5.6H).

The striatum and cortex did not normally express Gal-3. Stroke induced expression of Gal-3 in the ischaemic striatum and cortex, in GFAP+ reactive astrocytes concentrated around the infarct border, and CD45+ activated microglia / macrophages in the infarct core (Fig 5.7A-B). Gal-3 was not expressed by blood vessels and did not co-localise with NeuN+ neurons (Fig 5.7C-D).

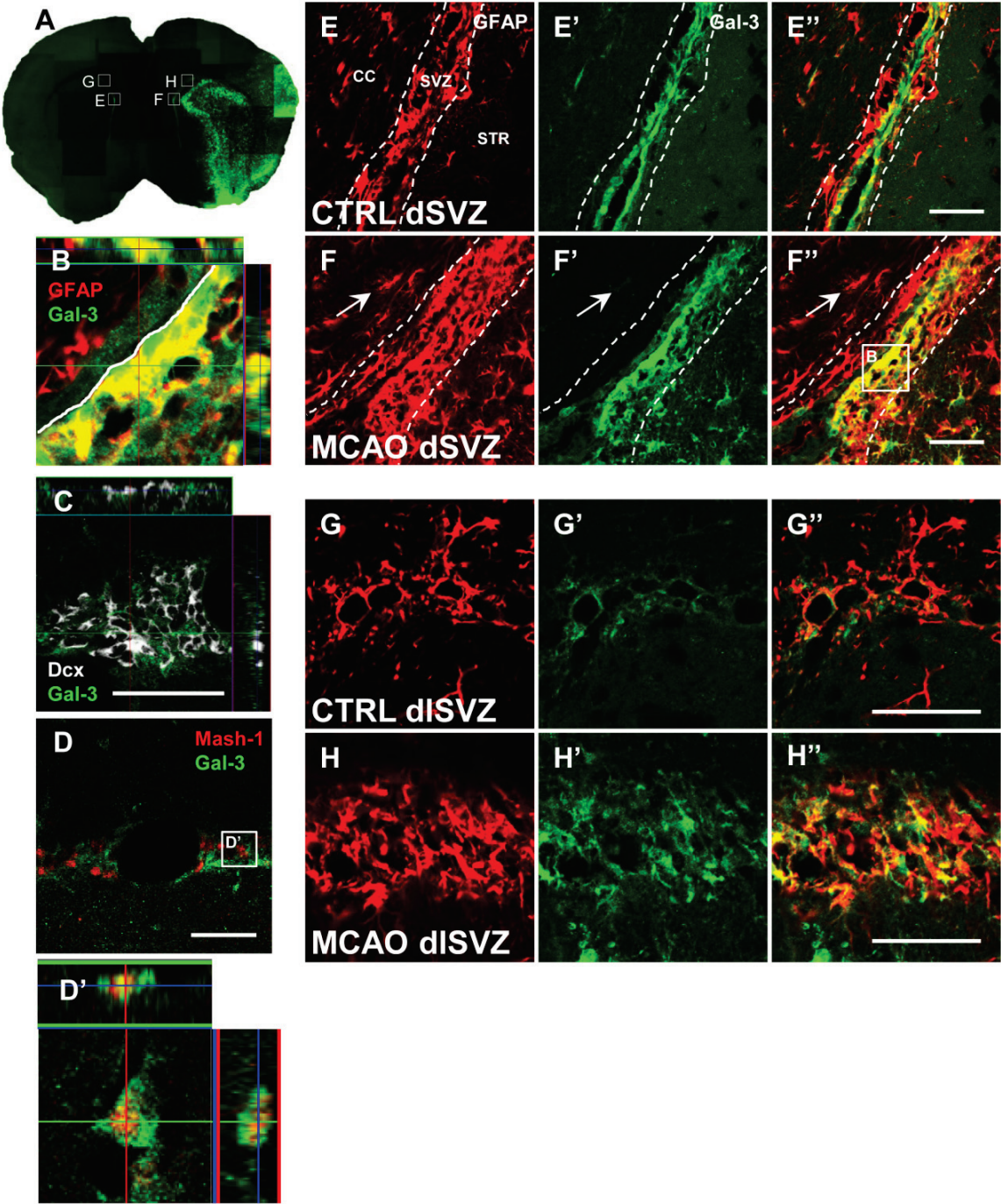


Figure 5.6. Gal-3 expression in the SVZ 7-days after MCAO

Figure 5.6. Gal-3 expression in the SVZ 7-days after MCAO

- (A) Induction of Gal-3 expression in the ischaemic striatum and cortex after MCAO.
- (B-D) Localisation of Gal-3 by immunohistochemistry. In the post-stroke SVZ, cellular localisation of Gal-3 expression remains stable, and is expressed in astrocytes, ependymal cells (B), and a subset of Mash-1+ transit amplifying progenitors (D-D'). Dcx+ neuroblasts do not express Gal-3 (C).
- (E-F) Gal-3 is normally expressed in the SVZ in the non-stroke contralateral SVZ (E). 7-days after MCAO, there is increased GFAP expression in the dorsal SVZ compared to the non-stroke control SVZ. This is accompanied by a marked increase in Gal-3 immunoreactivity, which co-localises with GFAP+ cellular elements (F). Interestingly, GFAP+ astrocytes in the corpus callosum, medial to the lateral ventricle, expressed lower Gal-3 (arrow F). CC, corpus callosum.
- (G-H) Gal-3 and GFAP expression in the dorsolateral SVZ is similarly increased post-MCAO.

Scale bar 50 μ m.

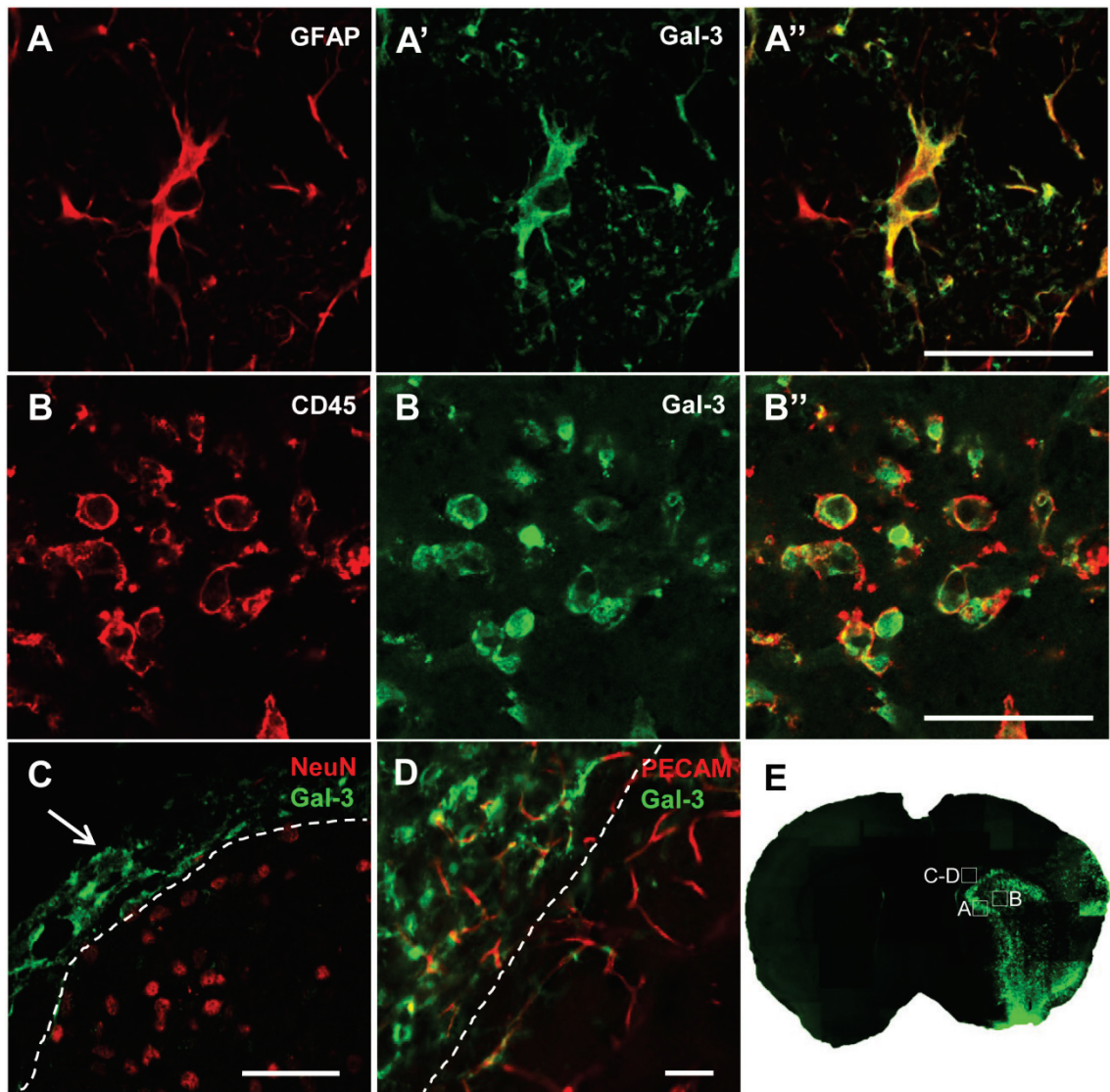


Figure 5.7. Gal-3 expression in the ischaemic striatum

- (A-B) Gal-3 is localised to GFAP+ reactive astrocytes and CD45+ activated microglia / macrophages in the ischaemic striatum.
- (C-D) In the contralateral non-stroke hemisphere, Gal-3 was expressed in the SVZ only. Gal-3 did not co-localise with the neurons (NeuN) or blood vessels (PECAM). Gal-3+ elements, presumably astroglial endfeet, are closely associated to PECAM+ blood vessels. *Dotted lines* demarcate lateral border of SVZ.
- (E) Induction of Gal-3 expression in the ischaemic striatum and cortex after MCAO. Same figure as Fig 5.6A.

Confocal single optical sections. Scale bar 50 μ m

5.3. Discussion

Gal-3 is a pleiotropic carbohydrate binding protein which is involved in inflammation and cancer biology. However, its function in the brain is not well-understood. In this study, Gal-3 was found to be constitutively expressed in the SVZ and along the RMS migratory pathway. Although Gal-3 did not regulate cell proliferation in the SVZ, Gal-3 maintained dynamic features of neuroblast migration in the RMS. Neuroblast migration in Gal-3^{-/-} mutants was slower and more exploratory compared to Gal-3^{+/+} wildtypes. After stroke, Gal-3 was up-regulated in the SVZ, while in the ischaemic brain regions, Gal-3 expression was induced in reactive astrocytes and activated microglia / macrophages.

5.3.1. Gal-3 expression in the SVZ neurogenic niche

The expression of Gal-3 in the CNS has been reported in reactive astrocytes and microglia in pathological conditions (Walther et al., 2000; Yan et al., 2009a; Doverhag et al., 2010). In the healthy brain, Gal-3 is specifically expressed by ependymal cells, astrocytes (including astrocyte-like stem cells) and a subset of progenitor cells in the neural stem cell niche. However, neuroblast and differentiated interneuron progenies do not express Gal-3. Interestingly, striatal and cortical astrocytes outside the SVZ, including the minority that are GFAP⁺, also do not express Gal-3. A recent study used fluorescence-activated cell sorting to isolate GFAP⁺/CD133⁺ adult NSCs in the SVZ, and suggested that Gal-3 was highly enriched in the adult NSC population compared to diencephalic astrocytes (Beckervordersandforth et al., 2010). However, it is imperative to point out that although Gal-3 is expressed by astrocyte-like stem cells, it is not an exclusive marker for adult NSCs, and is also robustly expressed by other SVZ cell types. Immuno-electron microscopy has shown that a subset of SVZ astrocytes contained nuclear Gal-3

immunoprecipitates, while in another, Gal-3 was nuclear and cytoplasmic (Comte et al., 2011). Nuclear Gal-3 participates in splicing of pre-mRNA (Dagher et al., 1995), and it remains to be seen whether the differential cell compartment expression in the SVZ may distinguish astrocyte-like stem cells from niche astrocytes in the SVZ.

5.3.2. Gal-3 increases exploratory neuroblast movement and decreases speed of migration

Static tracing studies *in vivo* with BrdU showed that generation of newborn neurons in the olfactory bulb was reduced in Gal-3^{-/-} mutants (Comte et al., 2011). From this, it was inferred that Gal-3 maintained neuroblast migration to the OB. Two-photon time-lapse microscopy studies previously demonstrated that only a subset of neuroblasts was migratory at any time and that they migrated in multiple directions, at different speeds and in long distance tangential and local exploratory patterns (Nam et al., 2007). Net neuroblast migration observed in static studies at a population level was the summation of these dynamic cellular migratory features. For example, Dcx was a known regulator of neuronal migration in the SVZ (Ocbina et al., 2006) and embryonic cortex (Feng and Walsh, 2001). Time-lapse imaging studies of dynamic features of migration demonstrated that Dcx was required to maintain nuclear translocation during migration, and it interacted with dynein to mediate nucleus-centrosome coupling (Koizumi et al., 2006). The findings that Gal-3^{-/-} neuroblasts were more exploratory and migrated slower were in agreement with static tracing studies which showed delayed neuroblast arrival the OB, with partial arrest in the RMS (Comte et al., 2011). Furthermore, they provided evidence that Gal-3 was required to maintain specific dynamic features of neuroblast migration, and maintained the pro-migratory environment in the SVZ-RMS-OB axis.

Gal-3 may regulate neuroblast migration through its interaction with ECM proteins. Gal-3 binds β -galactoside residues on laminin, integrin, NCAM and tenascin, all of which influence neuroblast migration (Comte et al., 2011; Cremer et al., 1994; Thomas et al., 1996; Jacques et al., 1998; Mercier et al., 2002; Emsley and Hagg, 2003). *In vitro*, inhibition of secreted Gal-3 with a functional blocking antibody significantly reduces neuroblast migration from RMS explants, while addition of recombinant Gal-3 increases migration (Comte et al., 2011). Gal-3 also mediates glioblastoma cell migration via an $\alpha 6 \beta 1$ -dependent mechanism, although there are conflicting results whether Gal-3 is in fact a *positive*, or *negative* regulator of migration and integrin expression (Camby et al., 2001; Debray et al., 2004). This may be indicative of Gal-3's pleiotropic functions under different biological conditions. On the other hand, Gal-3 may regulate neuroblast migration via maintenance of glial tube scaffold. Gal-3 is expressed by niche astrocyte which formed the glial tubes that envelope migrating neuroblast chains. Deletion of Gal-3 results in hypertrophied astrocyte processes and glial tube dysmorphology (Comte et al., 2011), similar to the phenotype that is associated with abnormal neuroblast migration (Ghashghaei et al., 2006; Kaneko et al., 2010).

5.3.3. Gal-3 expression after stroke: implications for neuroblast emigration

Gal-3 protein was virtually undetectable by Western blot in naïve brains. After stroke, Gal-3 was significantly up-regulated in the ipsilateral cerebral hemisphere both 3- and 7-days after MCAO. This was in agreement with previous study in rats demonstrating absence of Gal-3 in control brains, and significant increase from 1 day up to 2 months after MCAO in rats (Walther et al., 2000; Yan et al., 2009a). In a hypoxia/ischaemia brain injury model in mice, Gal-3 mRNA was detected in the ipsilateral cerebral hemisphere as early as 8 hours after hypoxic brain injury (Doverhag et al., 2010).

Seven days post-MCAO, a time of significant post-stroke neuroblast emigration (Ohab and Carmichael, 2008), Gal-3 expression in the SVZ and the ischaemic striatum was up-regulated. In the SVZ, the increased expression of Gal-3 was localised to astrocytes and ependymal cells. Interestingly, the pattern of Gal-3 up-regulation was congruent with the increased GFAP expression in the post-stroke SVZ. One can therefore hypothesise that Gal-3 may be involved in the post-stroke cytoarchitectural reorganisation detailed in Chapter 4, particularly the features of reactive astrogliosis in SVZ astrocytes and ependymal cells.

After stroke, in static brain section, a significant proportion of the ectopic neuroblast population migrate into the striatum from the dorsolateral SVZ (Lichtenwalner and Parent, 2006). Gal-3 was up-regulated in this corridor of neuroblast emigration. Therefore, given its function to maintain neuroblast migration and its robust expression in the corridor of neuroblast emigration, Gal-3 was well-positioned to influence post-stroke neuroblast migration/emigration.

5.3.4. Gal-3 expression in the ischaemic striatum

Consistent with previous reports (Walther et al., 2000; Yan et al., 2009a; Doverhag et al., 2010), Gal-3 was localised to GFAP⁺ reactive astrocytes and CD45⁺ activated microglia / macrophages in the ischaemic brain. Gal-3 expression in reactive astrocytes and activated microglia / macrophages, but not in resting astrocytes and microglia, suggested that Gal-3 may have a role in the neuro-inflammatory response after stroke. In a mouse model of neonatal hypoxia/ischaemia, the deletion of Gal-3 decreased the expression of MMP-9 and nitrotyrosine (a product of reactive nitrogen species) by ~2 fold, in the ischaemic brain, while the level of interleukin-1 β (IL-1 β), a key mediator of the inflammatory cascade, was unchanged (Doverhag et al., 2010). In another study, Gal-3 was associated with IGF-1

expressing microglia after MCAO stroke, and selective ablation of this cell population was associated with an increase (~2 fold) in IL-1 β , interleukin-6 and tumour necrosis factor α (TNF α ; Lalancette-Hébert et al., 2007). If Gal-3 regulated immune cell activation or secretion of cytokines post-stroke, it could contribute significantly to the SVZ endogenous repair response and stroke outcomes.

Since Gal-3 is normally a feature of SVZ astrocytes, Gal-3 expression in reactive astrocytes suggests that they convert / revert to a phenotype similar to SVZ astrocytes. This agrees with the emerging concept that reactive astrocytes are reprogrammed and acquire stem cell properties after brain injury (Buffo et al., 2008; Kronenberg et al., 2010; Heinrich et al., 2010). Reactive astrocytes assume a dedifferentiated molecular phenotype and express GFAP, vimentin, nestin and brain lipid binding protein (Robel et al., 2011), which are associated with either radial glia during development, or adult NSCs in the SVZ. Gal-3 is therefore an additional molecule whose expression is shared by SVZ astrocytes and reactive astrocytes in a classically non-neurogenic region after injury. Interestingly, Gal-3 expression in ependymal cells was up-regulated following stroke as they assumed the astroglial phenotype described in the previous chapter.

There is evidence that β 1 integrin signalling at the astrocyte basement membrane may be important in regulating astrocyte quiescence. Conditional deletion of β 1 integrin in GFAP⁺ astrocytes displayed features of mild reactive astrocytosis, namely cellular hypertrophy and up-regulation of GFAP (Robel et al., 2009). These astrocytes displayed abnormality at the interface between astroglial endfeet and the vascular basement membrane, with loss of aquaporin 4 and dystrophin-associated glycoproteins polarisation towards the endfeet (Robel et al., 2009). SVZ astrocytes express multiple integrin receptors including β 1 integrin (Kazanis et al., 2010), and their glial processes are hypertrophic in Gal-3^{-/-} mutants (Comte et al., 2011). Could the astrocyte dysmorphology

in Gal-3^{-/-} be the result of β 1 integrin dysregulation? In support, Gal-3 regulates integrin-dependent epithelial lamellipodia formation and cell migration by binding to N-glycan residues on α 3 β 1 and α 6 β 4 integrins and facilitated downstream tyrosine kinase receptor activation (Saravanan et al., 2009; Kariya et al., 2010). In breast carcinoma cells, α 6 β 1 integrin expression increased in the presence of Gal-3 *in vitro* (Warfield et al., 1997). It will be intriguing to test the hypothesis that Gal-3 mediates integrin signalling in the brain, both in the SVZ, where it regulates multiple facets of adult neurogenesis (Jacques et al., 1998; Emsley and Hagg, 2003; Mobley et al., 2009; Mercier et al., 2002; Leone et al., 2005;), and the ischaemic striatum where it may be positioned to contribute to reactive astrocyte reprogramming.

5.4. Conclusion

In this chapter, the specific expression of Gal-3 in the SVZ and its cellular expression were demonstrated. In confirmation of static tracing studies, Gal-3 promoted neuroblast migration and was required to maintain dynamic features of cell migration in the RMS. After stroke, Gal-3 was up-regulated in the SVZ, while its expression was induced in the ischaemic brain regions. The temporal profile and cellular location of Gal-3 expression in the post-stroke brain suggest that it is well-positioned to influence post-stroke SVZ neuroblast emigration and tissue remodelling in the ischaemic striatum. Control of post-stroke SVZ cytoarchitectural reorganisation and inflammatory response in the ischaemic brain tissue are potential mechanisms to be investigated.

Chapter 6

The role of galectin-3 in subventricular zone-mediated post-stroke neurogenesis

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6.1. Introduction

Post-stroke neurogenesis and ectopic migration of neuroblasts into the ischaemic striatum are regulated by a number of cellular interactions and molecular signalling pathways (Young et al., 2011; Ohab and Carmichael, 2008; Kernie and Parent, 2010). Changes occur in the ischaemic striatum, within the neuroblast population, and as demonstrated in Chapter 4, within the SVZ neurogenic niche, to facilitate post-stroke neurogenesis. Neuroblast chemotaxis is mediated by chemokines such as SDF-1 α (Ohab et al., 2006) and MCP-1 (Yan et al., 2006b) which are released by reactive astrocytes and activated microglia / macrophages, while emigrating neuroblasts secrete MMPs which are thought to digest ECM proteins and facilitate migration in the ischaemic tissue (Lee et al., 2006). Ectopic neuroblasts migrate along blood vessels (Yamashita et al., 2006) and astrocyte processes (Parent et al., 2002) in the ischaemic striatum, reminiscent of migratory mechanisms within the SVZ-RMS-OB pathway. In addition, SVZ components which regulate the neural stem cell niche such as the glial tube migratory scaffold, vasculature and ependyma-mediated CSF flow are disrupted after stroke.

In Chapter 5, Gal-3 was found to be specifically expressed in the SVZ and maintained dynamic features of neuroblast migration. After stroke, Gal-3 was up-regulated in the SVZ, and its expression was induced in reactive astrocytes and activated microglia / macrophages in the ischaemic brain tissue. Corresponding to its expression in these neuro-inflammatory cells, there is evidence that Gal-3 mediates the post-stroke inflammatory response (Doverhag et al., 2010; Lalancette-Hébert et al., 2007). These findings suggest that Gal-3 may have significant roles in the 2 domains, the SVZ and

ischaemic striatum, which remodel in response to stroke and facilitate post-stroke neurogenesis and ectopic neuroblast migration.

Gal-3 up-regulation has previously been reported, although it is unclear whether Gal-3 has a deleterious or neuroprotective role following ischaemic brain injury (Yan et al., 2009a; Doverhag et al., 2010). After MCAO in rat, intracerebral infusion of recombinant Gal-3 stimulated proliferation of SVZ neural progenitors and striatal endothelial cells without affecting infarct volume (Yan et al., 2009a). In contrast, in a model of neonatal hypoxia/ischaemia in mice, Gal-3^{-/-} mutants developed smaller ischaemic lesions (Doverhag et al., 2010).

For this chapter, I hypothesised that Gal-3 regulates the ectopic migration of neuroblasts by (1) facilitating aspects of cellular reorganisation in the post-stroke neural stem cell niche, and (2) remodelling of the ischaemic striatum. To this end, in a series of loss-of-function studies, Gal-3 null mice (Colnot et al., 1998a) were used to examine the effects of Gal-3 gene deletion on (1) neurological and histological stroke outcomes, (2) post-stroke SVZ neurogenesis, (3) post-stroke ectopic neuroblast migration, (4) post-stroke SVZ cytoarchitectural reorganisation, (5) the angiogenic and inflammatory cellular response in the ischaemic striatum, and (6) the proteome expression profile in naïve and post-stroke brains.

6.2. Results

6.2.1. Gal-3 deletion does not cause gross cerebrovascular abnormality

As discussed earlier in the thesis, strain-related differences in stroke outcome is a well-known confounder of the MCAO model, and can be attributed to differences in the cerebral vascular anatomy. Differences in the anatomy of the circle of Willis (Fujii et al., 1997) and pial anastomosis (Maeda et al., 1998) significantly influence stroke outcome. To interpret the effect of Gal-3 deletion, if any, it is essential that differences of vascular anatomy be excluded. The cerebral vasculature was visualised following transcardiac perfusion with India ink gelatin solution ($n = 6$). I did not detect gross differences in the pial anastomosis at the watershed between the anterior carotid artery and MCA vascular supply territories (*dotted line* Fig 6.1A-B). The size and extent of the MCA as it looped around the lateral forebrain were similar (*arrows* Fig 6.1A'-B'). There was variability in the arrangement of the posterior parts of the circle of Willis from animal to animal, irrespective of their genotype. However, both Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants on the 129sv strain had well-developed and patent *pars basilaris* posterior communicating arteries (Fig 6.1C-D).

6.2.2. Gal-3 deletion does not affect neurological outcome and infarct volume after stroke

Fifty minutes of MCAO were carried out on Gal-3^{+/+} and Gal-3^{-/-} young adult male mice (8-12 weeks old) weighing between 23 to 28 g from Gal-3^{+/+} breeding pairs (Fig 6.2). There was no difference in CBF in the MCA vascular territory during MCAO surgery between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants (Fig 6.3A). In both groups, laser Doppler

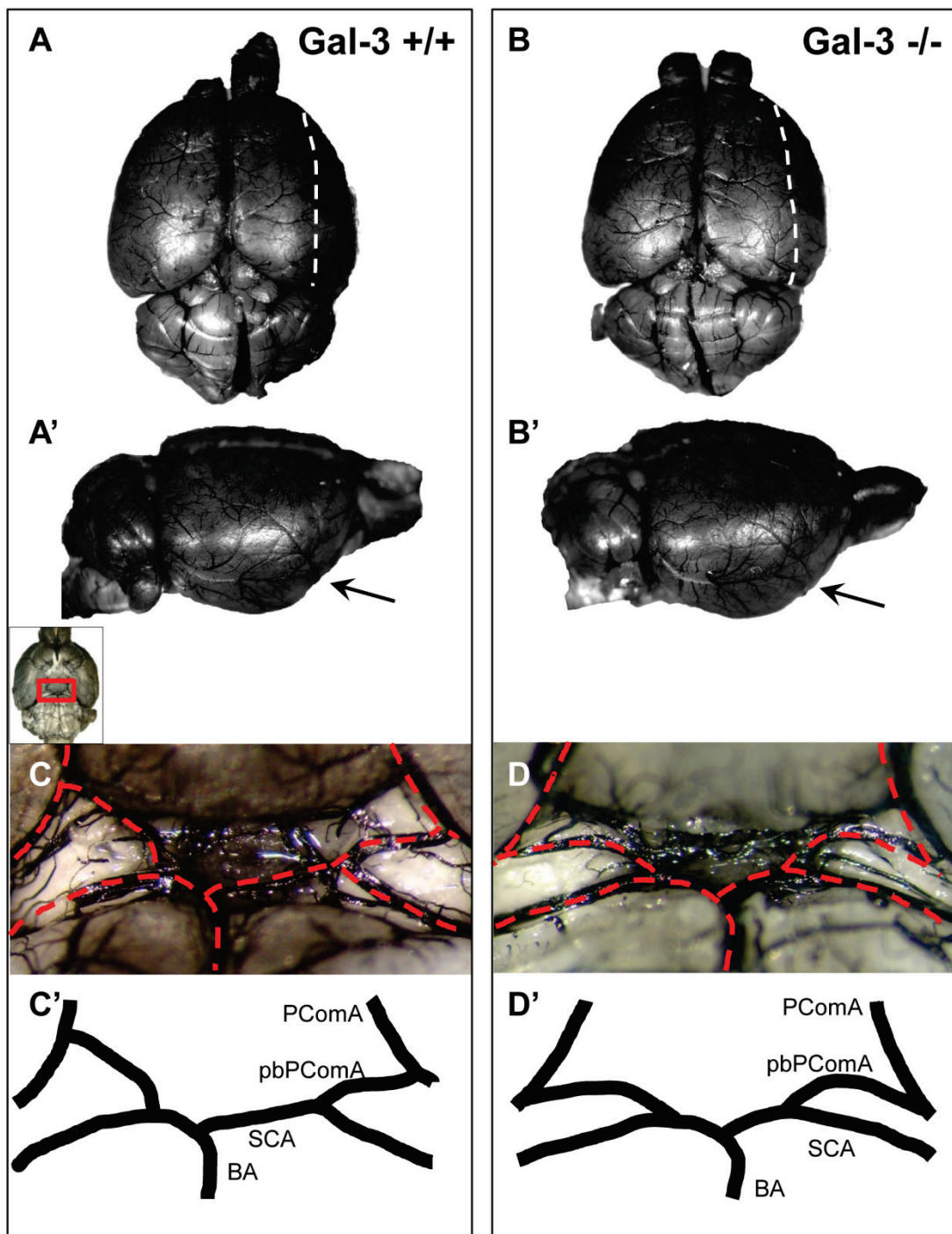
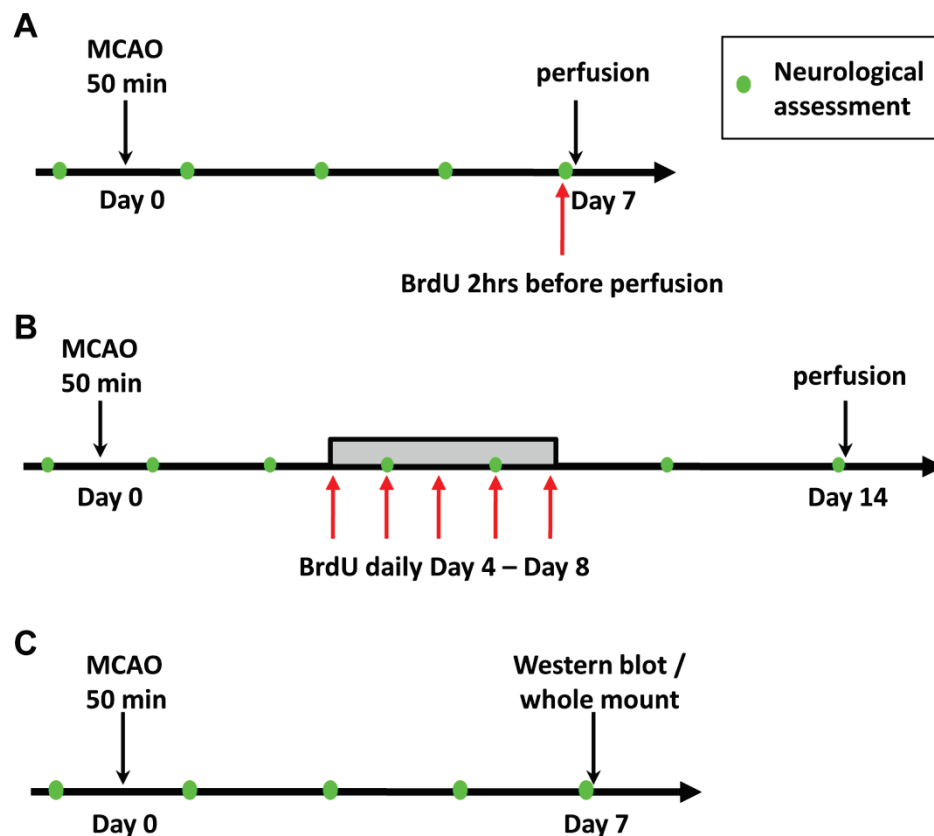


Figure 6.1. Cerebrovascular anatomy

Figure 6.1. Cerebrovascular anatomy

Animals were transcardiacally perfused with warmed India ink / gelatin solution to allow visualisation of the cerebral vasculature anatomy.

- (A-B) Assessment of pial anastomosis in the watershed zone (*dotted line*) between middle cerebral artery and anterior cerebral artery supply territories did not reveal gross differences. The distribution of the middle cerebral artery and its main branches were similar between Gal-3^{+/+} and Gal-3^{-/-} brain (*arrows A'-B'*).
- (C) Magnified view of the posterior aspect of circle of Willis (*red dotted lines*). Well-formed and patent *pars basilaris* posterior communicating arteries are observed in Gal-3^{+/+} and Gal-3^{-/-}. *PComA*, posterior communicating artery; *pbPComA*, pars basilaris posterior communicating artery; *SCA*, superior cerebellar artery; *BA*, basilar artery.

**Figure 6.2. MCAO experimental paradigms**

Animals underwent 50-min MCAO and were sacrificed 7 or 14 days later. Evaluation of neurological status (*green circles*) was carried out before MCAO to determine baseline, and on alternative days in the first post-stroke week, Day 10 and Day 14. In groups for coronal section immunohistochemistry (A-B), BrdU was administered as indicated.

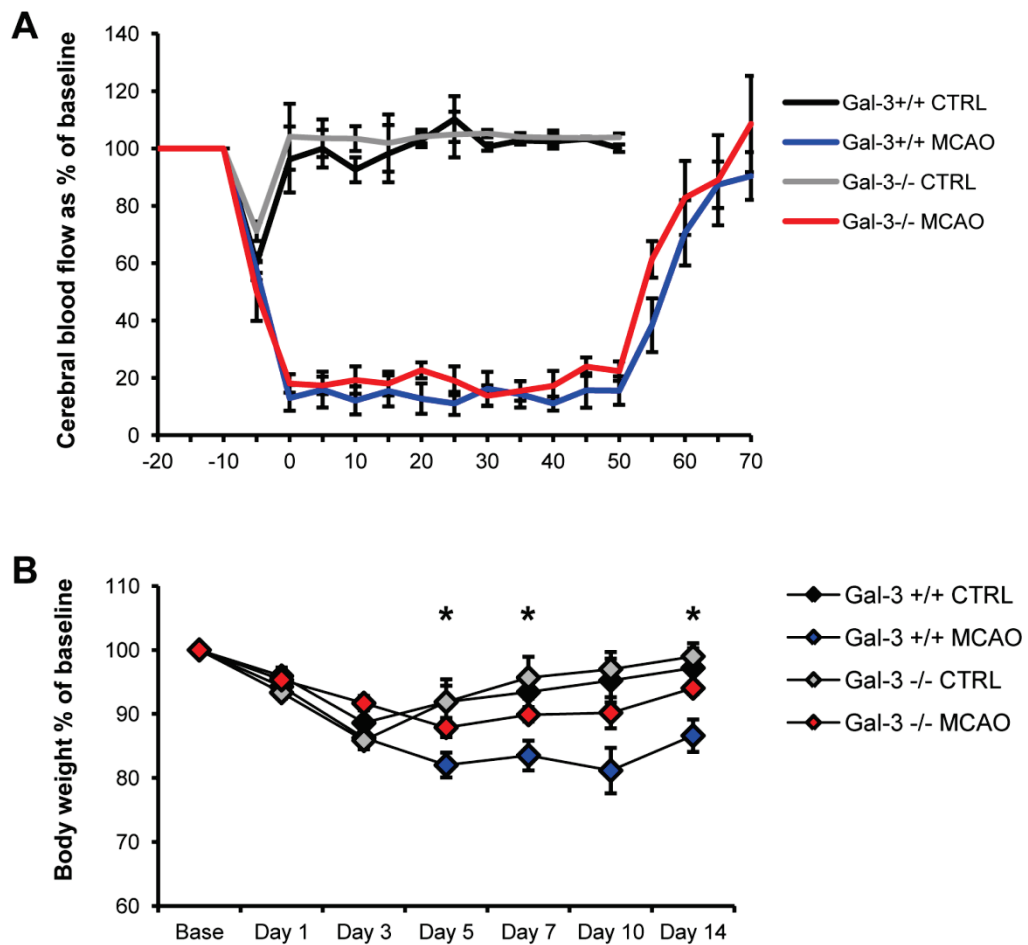


Figure 6.3. Cerebral blood flow in MCAO and post-stroke body weight

- (A) Laser Doppler flow measurements of cerebral blood flow during MCAO surgery.
- (B) Body weight measurements as a percentage of pre-stroke weight. * $P < 0.05$.

measurement of CBF showed decreased CBF to less than 30% of baseline levels during MCAO, and good reperfusion following removal of filament at the end of MCAO.

Neurological assessment

Assessment of neurological status was carried out using the modified Neurological Severity Score (mNSS) and openfield quantification of movement over 7 or 14-days survival post-MCAO (Fig 6.2). Mice were assessed 1 day before surgery to obtain baseline performance, and then on alternative days commencing 24 hours post-MCAO.

One day before MCAO surgery, animals did not display neurological abnormality and there was no difference between Gal-3^{+/+} and Gal-3^{-/-} mice. Animals explored openfield freely, with symmetrical limb movements, and normal sensory-motor reflexes. Twenty-four hours post-stroke, there was obvious deficit in neurological function, typified by reduced general mobility, limb asymmetry and hemiparesis, as well as loss of sensory motor reflexes (Fig 6.4A). The most frequently affected were the startle reflex and hind-

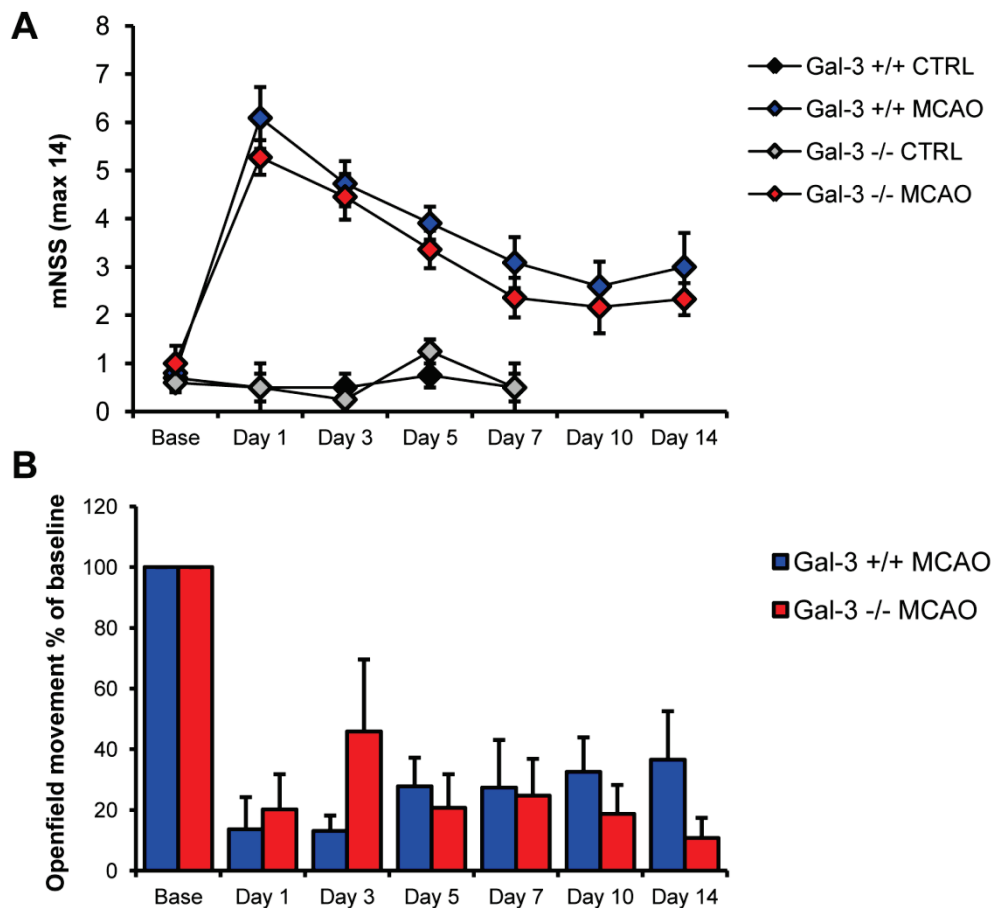


Figure 6.4. Evaluation of neurological status after MCAO

- (A) Assessment of neurological status with modified Neurological Severity Score (mNSS). Higher score = greater disability.
- (B) Openfield assessment of motor activity in a 2-min period as a percentage of pre-stroke distance run.

limb righting reflex. No difference in mNSS was observed between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants ($P = 0.158$; Mann Whitney non-parametric test), while sham-operated controls showed no deficit. The neurological status of both Gal-3^{+/+} and Gal-3^{-/-} mice improved during the first post-MCAO week. During the second post-MCAO week, there were remnant but persistent neurological signs consisting mostly of decreased general mobility and motor asymmetry. Difference in neurological status between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants was not observed at any point during the 2 weeks survival.

To quantify the reduction of motor activity, I analysed the total amount of spontaneous movement in openfield as a percentage of baseline. Throughout the post-stroke survival period, the distance travelled remained at ~20 - 30 % of baseline, with no sign of significant recovery. Consistent with assessment using the mNSS, there was no difference between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants (Fig 6.4B).

Weight loss after MCAO

As reported in Chapter 2, mice undergoing MCAO were prone to weight loss. Mice lost ~10 % body weight post-MCAO (Fig 6.3B). Strikingly, even sham-operated animals lost weight, although they regained normal weight more rapidly than those that underwent MCAO. This suggested that prolonged anaesthesia was likely an important contributing factor to weight loss. Importantly, at Day 5, 7 and 14 after MCAO, there were small but significant differences in bodyweight between Gal-3^{+/+} wildtype and Gal-3^{-/-} mutants. Day 5 post-MCAO, when maximal weight loss was observed, Gal-3^{+/+} wildtypes had lost significantly more weight compared to Gal-3^{-/-} mutants ($P = 0.03$). This was followed by similar rates of gradual weight recovery.

Infarct volume

Seven-days post-MCAO, loss of NeuN and initiation of GFAP+ glial scar formation were independently used to assess infarct volume in 5 evenly spaced coronal brain sections. Gal-3^{+/+} and Gal-3^{-/-} brains showed similar pattern of neuronal loss and reactive astrocytosis, affecting mainly the striatum and the cortex to a limited extent (Fig 6.5A-B). No difference in the infarct volume was found between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants when the infarct was delineated either by GFAP (Gal-3^{+/+} 25.1 ± 2.8%; Gal-3^{-/-} 24.2 ± 5.1%; *n* = 6) or NeuN (Gal-3^{+/+} 21.2 ± 1.5%; Gal-3^{-/-} 23.0 ± 3.9%; Fig 6.5C; *n* = 6).

6.2.3. Gal-3 is not required for increased SVZ cell proliferation post-MCAO

Stroke increased proliferation in the SVZ (Fig 6.6A-D). Seven-days post-MCAO, the number of proliferating PHi3+ cells in the SVZ was increased in both Gal-3^{+/+} (66.4 ± 6.3 cells) and Gal-3^{-/-} (61.5 ± 7.9 cells) compared to Gal-3^{+/+} non-stroke controls (38.6 ± 6.4 cells; *P* = 0.02) and Gal-3^{-/-} non-stroke controls (30.7 ± 2.7 cells; *P* = 0.02). There was no difference in the number of PHi3+ cells in the SVZ between Gal-3^{+/+} and Gal-3^{-/-} sham-operated controls, and 7-days post-MCAO. The preserved post-stroke increase in SVZ proliferation in Gal-3^{-/-} mutants indicates that Gal-3 is not a key regulator of this process. The effect of increased SVZ proliferation was confirmed by increased neuroblast population in the ipsilateral SVZ 7-days post-MCAO in both Gal-3^{+/+} and Gal-3^{-/-} brains (Fig 6.7). In addition, differences in the number of cells undergoing apoptosis between Gal-3^{+/+} and Gal-3^{-/-} brains were not detected (Fig 6.6G).

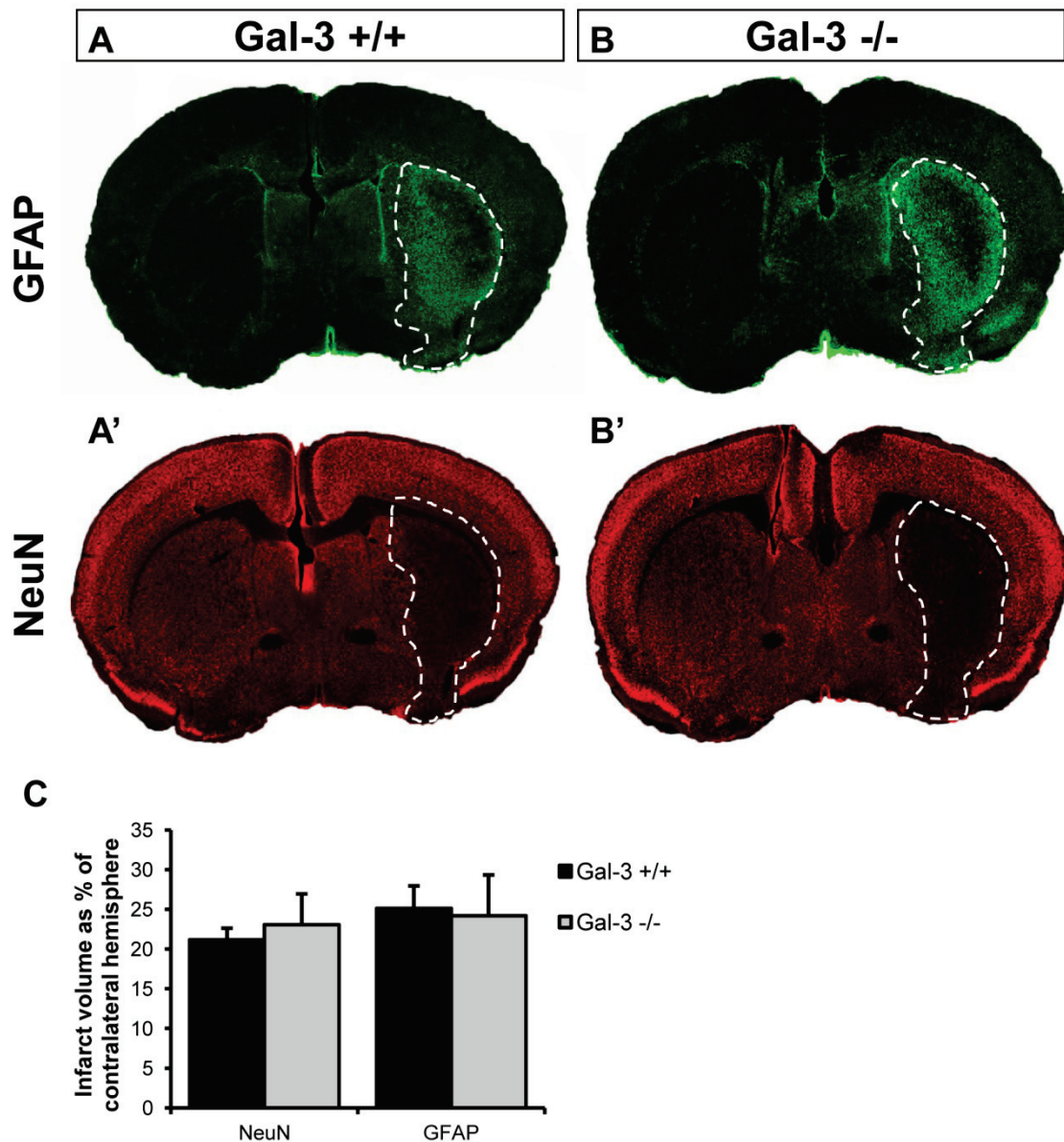


Figure 6.5. Infarct volumes

- (A-B) Double-immunohistochemistry for GFAP and NeuN shows similar infarcts largely confined to the striatum in Gal-3^{+/+} and Gal-3^{-/-} mice 7-days after 50-min MCAO.
- (C) Volume reconstruction by the indirect method in 5 evenly-spaced coronal sections shows no difference in infarct volume between Gal-3^{+/+} and Gal-3^{-/-} brains. Quantification of infarct by GFAP reactive astrocytosis or loss of NeuN⁺ neurons give similar results. Same sections shown here for each animal.

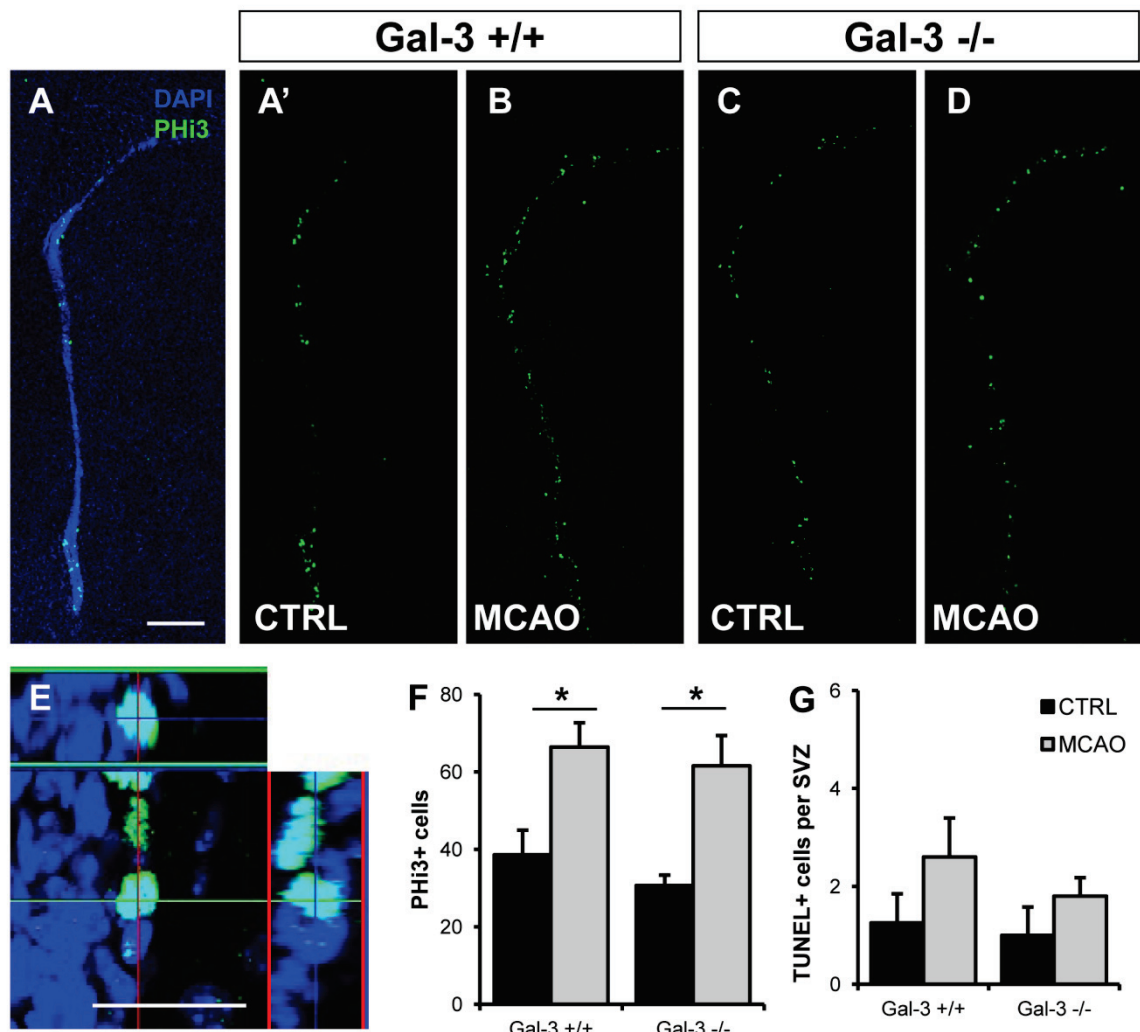


Figure 6.6. Increase in SVZ cell proliferation after stroke

- (A-D) Immunohistochemistry for phosphorylated histone H3 (PHi3) to label mitotic cells in the ipsilateral SVZ.
- (E) Orthogonal projection of PHi3+ nucleus in the SVZ.
- (F) Gal-3^{+/+} and Gal-3^{-/-} SVZ both demonstrate robust increase in cell proliferation 7-days post-MCAO. * $P = 0.02$.
- (G) The absolute number of TUNEL+ apoptotic cells in the SVZ was low, and did not increase significantly after stroke in Gal-3^{+/+} ($P = 0.25$) or Gal-3^{-/-} ($P = 0.23$).

Scale bar 200 μ m, (E) 20 μ m.

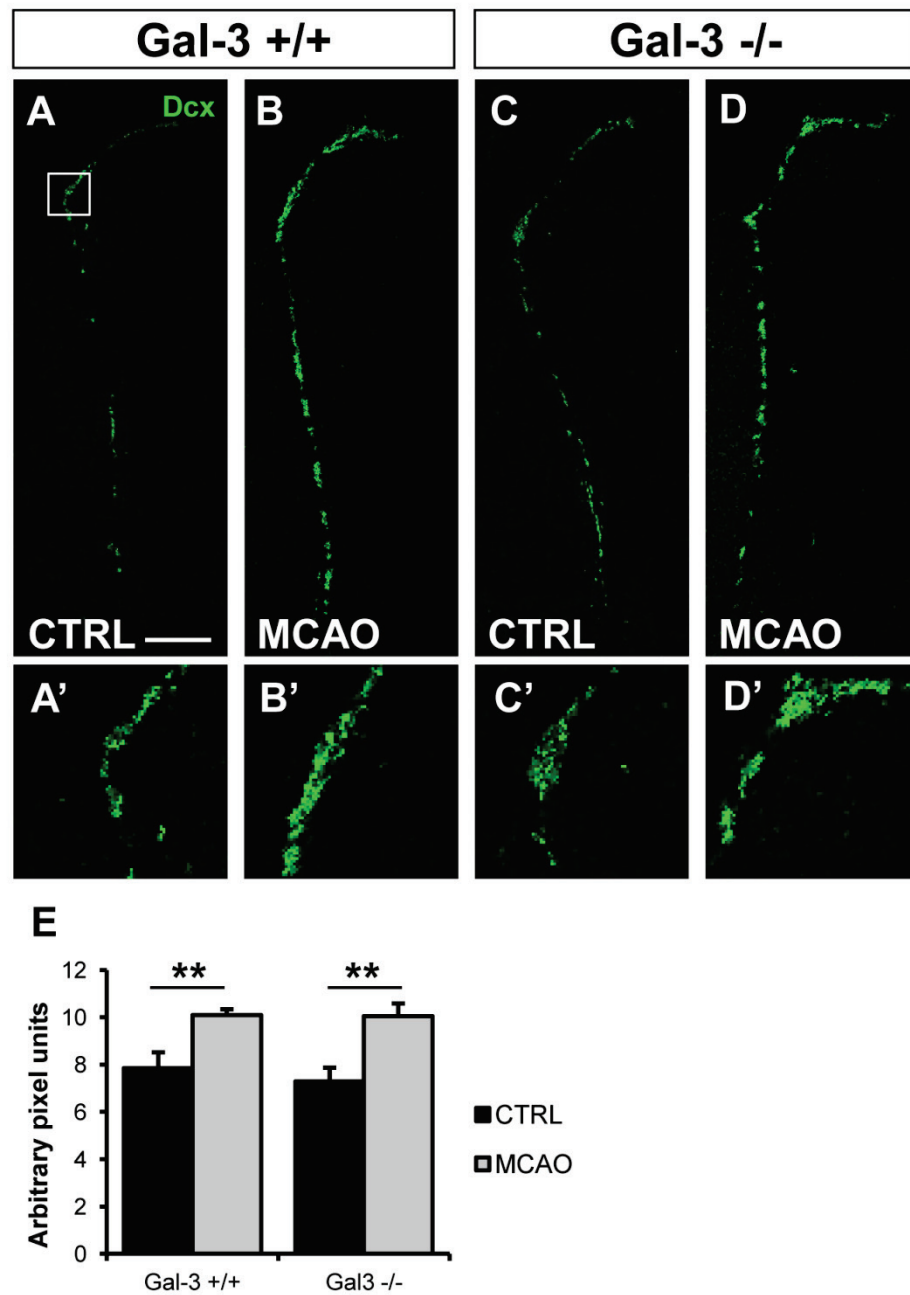


Figure 6.7. Increase neural precursor pool in the SVZ after stroke

- (A-D) Immunohistochemistry for doublecortin (Dcx), a marker of neuroblasts, in the ipsilateral SVZ. (A'-D') *Boxed area* in (A) and same dorsal position in (B-D). Confocal images acquired at the same settings.
- (E) Quantification of neural precursor pool by measurement of Dcx+ areas in the SVZ. The neural precursor pool increased significantly after stroke in both Gal-3^{+/+} (** $P = 0.006$) and Gal-3^{-/-} (** $P = 0.004$).

Scale bar 200 μ m.

6.2.4. Gal-3 deletion promotes ectopic migration of SVZ neuroblasts into the ischaemic striatum

In sham-operated controls, neuroblasts processes occasionally protruded into the surrounding striatum and septum (*arrow* Fig 6.8B) and occasional neuroblasts were observed outside the SVZ (Yang et al., 2004). In contrast, 14-days post-MCAO, frequent Dcx⁺ neuroblasts were observed in the ischaemic striatum and corpus callosum (*arrows* Fig 6.8D-F). Ectopic migration of neuroblasts was robust in the dorsolateral SVZ (Fig 6.8D). Regional quantification of emigrating neuroblasts showed that in the striatum adjacent to the dorsolateral SVZ, there were significantly more emigrating neuroblasts in Gal-3^{-/-} mutants ($53,823 \pm 4,160$ cells / mm³) compared to Gal-3^{+/+} wildtypes ($39,474 \pm 4,563$ cell / mm³; $P = 0.03$). A similar trend towards increased emigrating neuroblasts in Gal-3^{-/-} mutants was also observed in the striatum adjacent to the mid-SVZ, although this was not statistically significant ($P = 0.24$).

Emigrating neuroblasts did not form obvious chain-structures extending out of the SVZ. Neuroblasts were frequently found in clusters (*yellow arrow* Fig 6.8D), and ~30 % of neuroblasts > 50 µm away from the SVZ contacted blood vessels (Fig 6.9A; Table 6.1). Fourteen-days post stroke, $28.7 \pm 3.6\%$ of emigrating neuroblasts made contact with blood vessels in Gal-3^{+/+} wildtypes, compared with $28.0 \pm 3.1\%$ in Gal-3^{-/-} mutants. Interestingly, emigrating neuroblasts retained the ability to proliferate outside the neurogenic niche. Similar proportion of emigrating neuroblasts underwent mitosis in Gal-3^{+/+} wildtypes ($5.9 \pm 2.0\%$) and Gal-3^{-/-} mutants ($3.9 \pm 1.2\%$; $P = 0.38$; Fig 6.9B). In Gal-3^{+/+} wildtype brains, PHi3⁺ / Dcx⁺ proliferating neuroblast were frequently associated with PECAM⁺ blood vessels (Fig 6.9B). Significantly higher percentage of the mitotic neuroblasts in the striatum were in contact with blood vessels in Gal-3^{+/+} wildtypes (68.3 ± 8.4 % of dividing neuroblasts), compared to only $26.8 \pm 9.1\%$ in Gal-3^{-/-} mutants ($P =$

0.005; *black bars* Fig 6.9B). In the ischaemic striatum near the infarct, a small percentage of Dcx⁺ neuroblast expressed the mature neuronal marker NeuN (Fig 6.9C) and the GABAergic interneuron marker Calretinin (Fig 6.9D).

After stroke, in both Gal-3^{+/+} and Gal-3^{-/-} SVZ, neuroblasts assumed features of neuronal differentiation, as evidenced by the formation of multiple cell processes which extended into the striatum and frequently contacted blood vessels (*arrow* Fig 6.9E). Cells with such morphology also expressed the mature neuronal marker NeuN (Fig 6.9E').

Table 6.1. Ectopic neuroblasts in the ischaemic striatum

	Ectopic neuroblasts	
	Gal-3 ^{+/+}	Gal-3 ^{-/-}
In contact with blood vessel	28.7 ± 3.6%	28.0 ± 3.1%
Proliferating (PHi3+)	5.9 ± 2.0%	3.9 ± 1.2%
Proliferating on blood vessel	4.0 ± 0.5%	1.1 ± 0.4%*

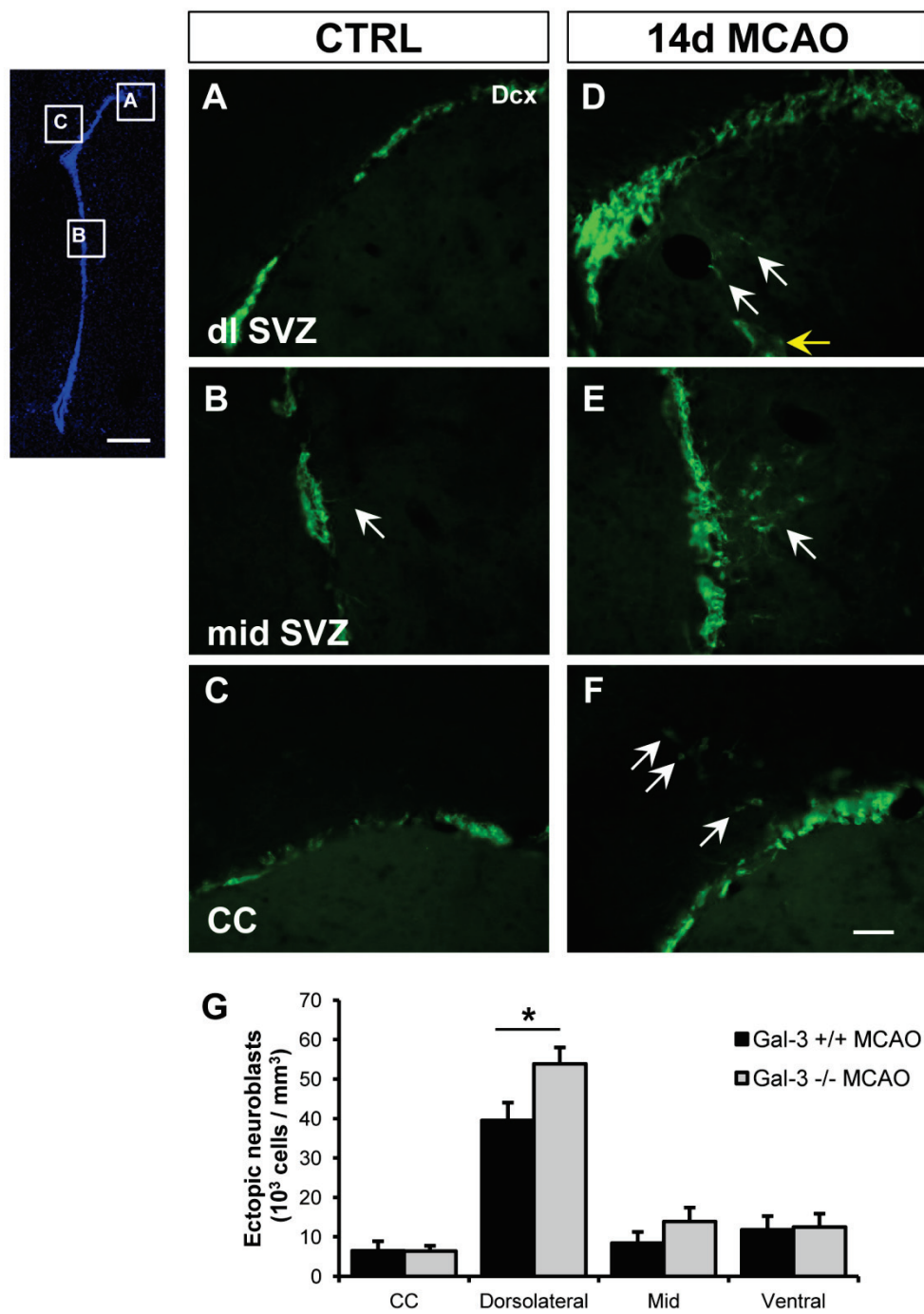


Figure 6.8. Increased ectopic neuroblast migration in *Gal-3*^{-/-} after stroke

Figure 6.8. Increased ectopic neuroblast migration in Gal-3^{-/-} after stroke

- (A-F) Immunohistochemistry for doublecortin (Dcx), 14-days after MCAO. In controls, occasional neuroblasts extends process into the striatum (*arrow B*). After stroke, neuroblasts emigrate into the striatum from the dorsal, dorsolateral, mid and ventral SVZ (*arrows D-F*). Ectopic neuroblasts are not found in chains but frequently form clusters (*yellow arrow D*).
- (G) Quantification of ectopic neuroblasts more than 50 μm from the edge of the SVZ in the corpus callosum (CC), dorsolateral, mid and ventral SVZ. The number of ectopic neuroblasts is increased in the dorsolateral SVZ in Gal-3^{-/-} (* $P = 0.03$).

Scale bar 50 μm .

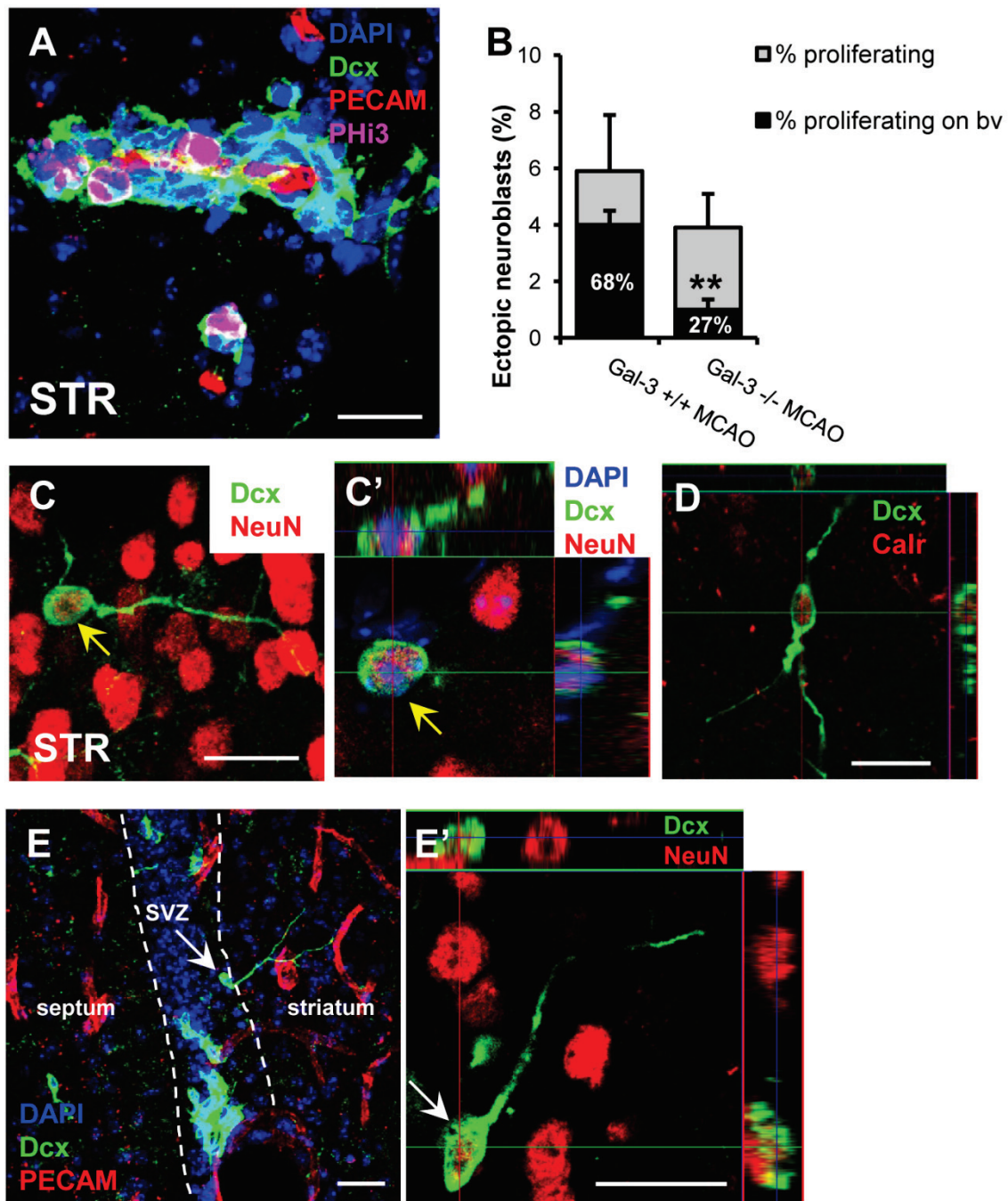


Figure 6.9. Ectopic neuroblasts in the ischaemic striatum

Figure 6.9. Ectopic neuroblasts in the ischaemic striatum

- (A) Ectopic neuroblasts in the striatum, are frequently associated with blood vessels and able to undergo proliferation. *STR*, striatum.
- (B) Percentage of ectopic neuroblasts undergoing proliferation (*grey bars*; PHi3+ / Dcx+). *Black bars* represent the proportion of the proliferating neuroblasts that are found on blood vessels. Although the overall rate of proliferation was not different between Gal-3^{+/+} and Gal-3^{-/-} ($P = 0.38$), a significantly lower percentage underwent proliferation in contact with blood vessels in Gal-3^{-/-} (** $P = 0.004$).
- (C-D) Ectopic neuroblasts began to express mature neuronal markers such as NeuN and calretinin 14-days post-MCAO in the striatum.
- (E) 14-days post-MCAO, neuroblasts with differentiated phenotype are found in the ipsilateral SVZ. A neuroblast in (*arrow E*) extended branching processes into the striatum and made contact with a blood vessel. This neuroblast also expressed NeuN (*E'*).

Scale bar (A, E) 50 μm , (C-D, E') 20 μm

6.2.5. Gal-3 deletion does not affect stroke-associated cytoarchitectural reorganisation in the SVZ

In Chapter 4, I demonstrated cytoarchitectural reorganisation of the subventricular zone following ischaemic stroke, including reactive astrogliosis and glial tube disruption, as well as a reactive astroglial ependymal phenotype with localised loss of PCP and altered CSF flow. Gal-3 is constitutively expressed by SVZ astrocytes and ependymal cells, and is up-regulated after stroke. I therefore considered the possibility that Gal-3 deletion might influence the orchestration of stroke-induced changes in the SVZ. Post-stroke whole mounts from Gal-3^{-/-} mutants were examined globally and confocal microscopy images were sampled in 4 different locations (Fig 6.10A). In Gal-3^{-/-} whole mounts 7-days post-MCAO, SVZ astrocytes demonstrated features of reactive astrocytes, including hypertrophied and tortuous GFAP+ processes (Fig 6.10B-C; *n* = 3). The post-stroke ependymal cell layer was similarly preserved after stroke in Gal-3^{-/-} mutants (Fig 6.10D; *n* = 5), while post-stroke ependymal cells similarly expressed GFAP (Fig 6.10E; *n* = 5). As previously observed 7-days post-MCAO in wildtype mice, PCP was disrupted in the anterior dorsal SVZ (Fig 6.10F-G; *n* = 3). These data suggest that cytoarchitectural reorganisation in the post-stroke SVZ is independent of Gal-3 function.

6.2.6. Gal-3 deletion does not affect the level of reactive astrogliosis and microglial activation in the ischaemic striatum

Seven-days following MCAO, there was an intense reactive astrogliosis in the infarcted striatum, with the formation of a GFAP+ astroglial scar which surrounded the lesion (Fig 6.11A-B). CD45+ microglia in the striatum became activated and assumed intermediate and amoeboid morphologies (Fig 6.11A'-B'). Qualitatively, I could not detect a difference in the morphology and density of reactive astrocytes and activated microglia

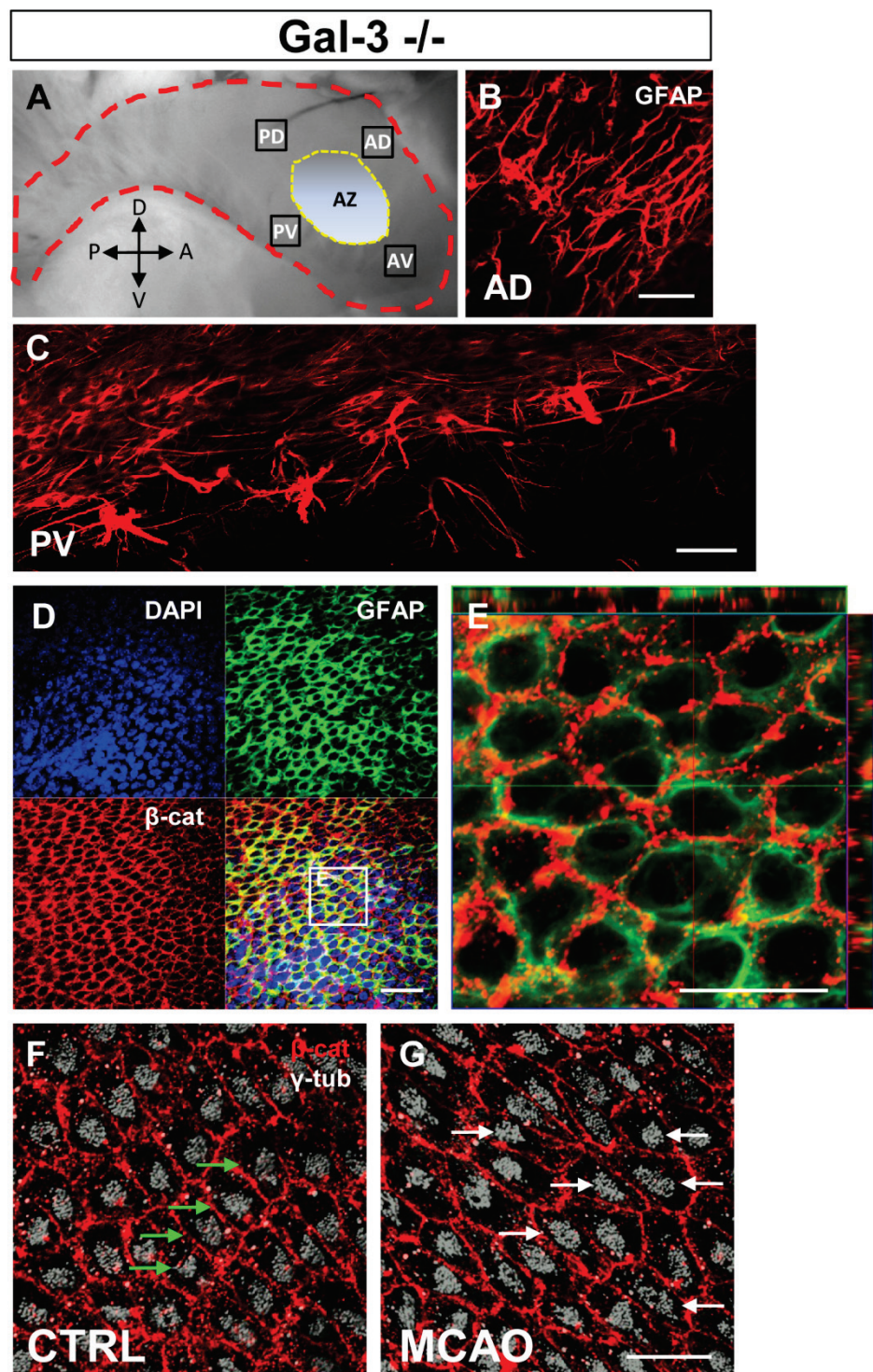


Figure 6.10. Post-stroke cellular reorganisation in the Gal-3^{-/-} SVZ

Figure 6.10. Post-stroke cellular reorganisation in the Gal-3^{-/-} SVZ

- (A) Dissected whole mount; lateral ventricular wall outlined in red. Analyses were performed in 4 boxed areas *AD*, anterior dorsal; *AV*, anterior ventral; *PD*, posterior dorsal and *PV*, posterior ventral. *AZ* is the intraventricular adhesion zone. *D*, dorsal; *V*, ventral; *A*, anterior; *P*, posterior.
- (B-C) Whole mount immunohistochemistry for GFAP showing hypertrophic and tortuous glial processes.
- (D-E) Confocal single optical sections at ventricular surface showing reactive astroglial ependymal phenotype with *de novo* GFAP expression (D). Orthogonal projection of confocal z-stack showing cytoplasmic GFAP intermediate filaments in ependymal cells (E).
- (F-G) Assessment of planar cell polarity in ependymal cells by position of ciliary basal body clusters (grey). *Green arrows* in (F) show normal PCP orientation and similar alignment of basal body clusters in Gal-3^{-/-} pre-stroke controls. *White arrows* in (G) show loss of basal body cluster alignment in the anterior dorsal SVZ 7-days post-MCAO in Gal-3^{-/-}.

Scale bar (B-C) 50 µm, (D-G) 20 µm

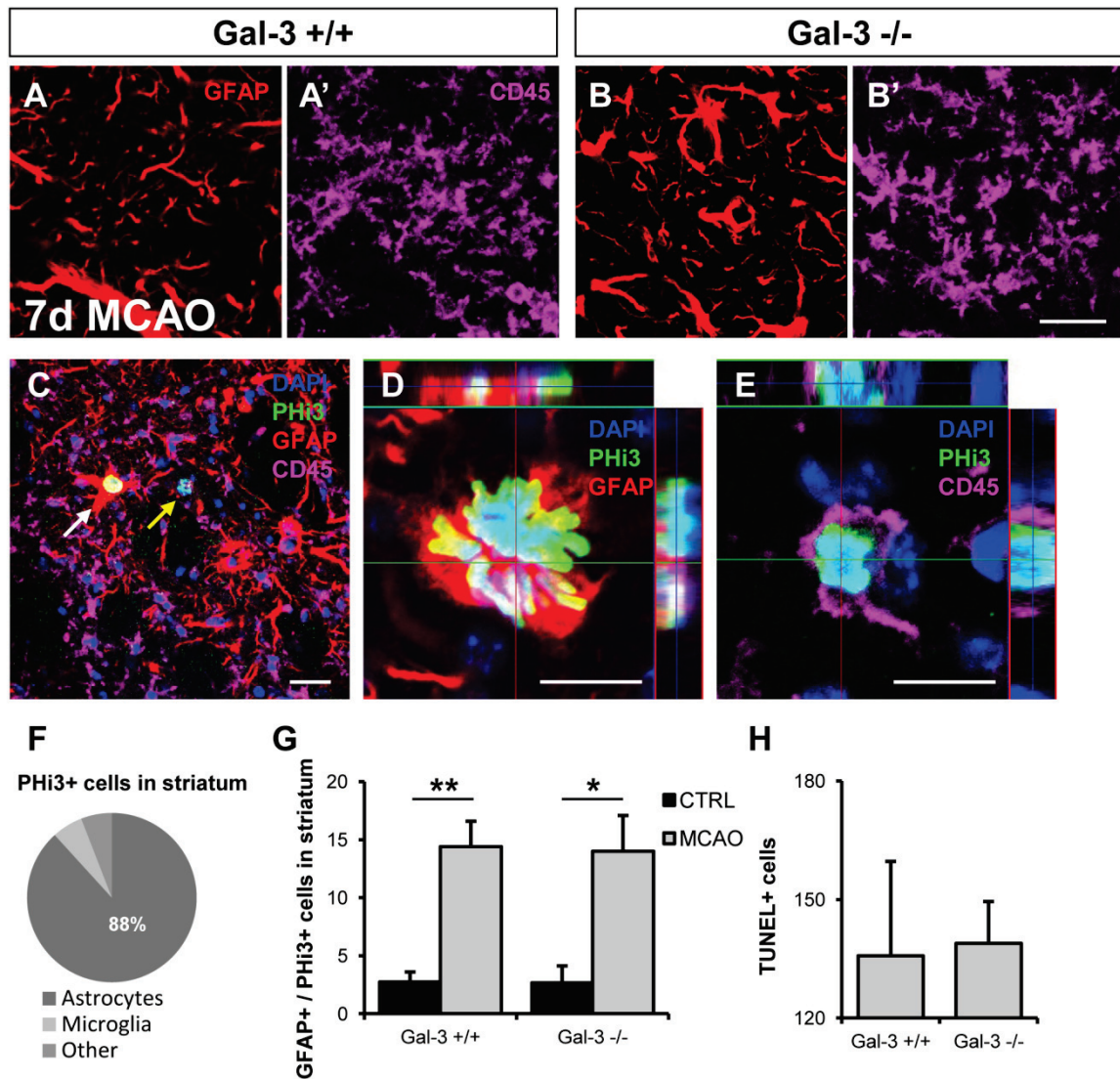


Figure 6.11. Reactive astrogliosis & microglial activation in the ischaemic striatum

(A-B) Immunohistochemistry for GFAP and CD45 in Gal-3^{+/+} and Gal-3^{-/-} brains 7-days post-MCAO.

(C-E) Reactive astrocytes and a small number of activated microglia co-localised with the mitotic marker PHi3.

(F) Quantification of proliferating cell types in the ischaemic striatum. Reactive astrocytes constituted the major proliferating cell population (88%).

(G) Quantification of proliferating reactive astrocytes 7-days post-MCAO showed similar levels of proliferating astrocytes between Gal-3^{+/+} (** $P = 0.009$) and Gal-3^{-/-} (* $P = 0.02$) brains compared to controls.

(H) Quantification of TUNEL+ apoptotic cells in the striatum 7-days post-MCAO.

Scale bar (A-C) 50 μ m, (D-E) 20 μ m

between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants. Interestingly, there were numerous PHi3⁺ mitotic cells in the striatum (Fig 6.11C), of which $88 \pm 4\%$ co-localised with GFAP (Fig 6.11D) and $6 \pm 2\%$ with CD45 (Fig 6.11E). This suggested that reactive astrocytes constituted the major proliferating population in the ischaemic striatum. The number of GFAP⁺ / PHi3⁺ proliferating astrocytes in the Gal-3^{+/+} striatum increased significantly 7-days post-MCAO (14.4 ± 2.2 cells) compared to controls (2.8 ± 0.9 cells; $P = 0.009$), and this increase in astrocyte proliferation was unaffected by the deletion of Gal-3 (Fig 6.11G). There were also large numbers of TUNEL⁺ apoptotic cells in the striatum, as previously documented in Chapter 4. However, the loss of Gal-3 did not alter programmed cell death in the post-stroke striatum (Fig 6.11H).

6.2.7. Gal-3 mediates post-stroke angiogenesis in the ischaemic penumbra

The different relationship between Gal-3^{+/+} wildtype and Gal-3^{-/-} mutant emigrating neuroblasts with regards to their proliferation on blood vessels called for an examination of the vasculature in the ischaemic striatum. In controls, the density of PECAM⁺ blood vessels in the medial striatum was not different between Gal-3^{+/+} and Gal-3^{-/-} mice (Fig 6.12C-D). Seven-days post-MCAO, in Gal-3^{+/+} wildtypes, the vascular density in the striatum was increased (Fig 6.12E). Surprisingly, post-MCAO increase in the vascular density was not observed in the Gal-3^{-/-} striatum (Fig 6.12F). Quantification of vascular density by measuring the percentage area covered by PECAM⁺ elements in projected confocal z-stacks (Fig 6.12B) confirmed robust angiogenesis in Gal-3^{+/+} striatum post-MCAO ($P < 0.001$), while in Gal-3^{-/-}, the vascular density remained at the pre-stroke baseline (Fig 6.12G).

Post-stroke angiogenesis was further assessed by quantification of proliferating endothelial cells. Seven-days post-MCAO, PHi3⁺ / PECAM⁺ mitotic endothelial cells

were exceedingly rare in the striatum and I did not detect more than 1 double-labelling cell in any of the brain sections examined. Therefore, brain sections from the 14-days survival group, which had received cumulative intraperitoneal injection of BrdU from Day 4 - Day 8, were examined. In sham-operated Gal-3^{+/+} and Gal-3^{-/-} brains, BrdU+ / PECAM+ cells were not detected. In contrast, post-MCAO, in Gal-3^{+/+} wildtypes, cumulative labelling showed robust endothelial cell proliferation ($22,123 \pm 3,128$ cells / mm³), which was significantly decreased in Gal-3^{-/-} ($6,637 \pm 1,277$ cells / mm³; $P = 0.003$).

Given the observation in Chapter 3 that stroke led to vascular engorgement and increased vascular density in the SVZ, would Gal-3 deletion dampen this angiogenic response in the SVZ similar to its effect in the ischaemic striatum? I assessed PECAM+ blood vessels in Gal-3^{+/+} and Gal-3^{-/-} whole mounts 7-days post-MCAO. Confocal images from 4 different locations of the lateral ventricular wall (Fig 6.10A) showed extensive network of blood vessels in the SVZ, which was not different between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants (Fig 6.13A-B). However, in contrast to the striatum, 7-days post-MCAO, in both Gal-3^{+/+} and Gal-3^{-/-} SVZ, there was equivalent increase in the vascular density in the lateral ventricular wall (Fig 6.13C-D). “Loops” of blood vessels, suggestive of intussusceptive “splitting” angiogenesis (Carmeliet and Jain, 2000), were observed after stroke (*arrows* Fig 6.13C-D). Quantification confirmed similar vascular density in sham-operated Gal-3^{+/+} wildtype ($15.1 \pm 1.5\%$) and Gal-3^{-/-} mutant SVZ ($14.2 \pm 0.6\%$). Seven-days post-stroke, there was significant increase in SVZ vascular density in both Gal-3^{+/+} ($23.7 \pm 1.3\%$; $P = 0.002$) and Gal-3^{-/-} brains ($20.3 \pm 1.9\%$; $P = 0.03$). This is a surprising result and suggests a specific role for Gal-3 in post-stroke angiogenesis in the ischaemic striatum, but not in the SVZ.

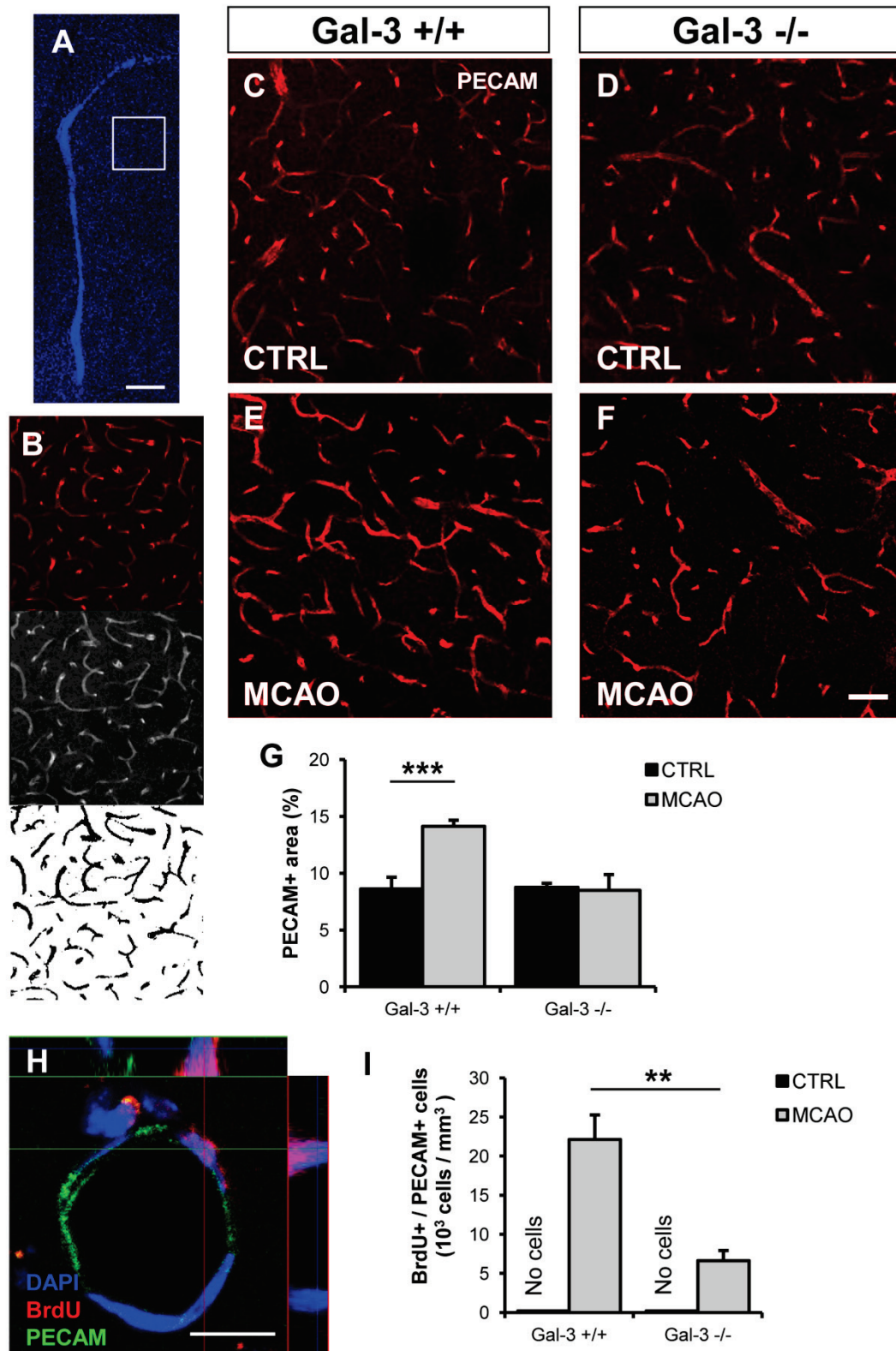


Figure 6.12. Gal-3 is required for angiogenesis in the ischaemic striatum

Figure 6.12. Gal-3 is required for angiogenesis in the ischaemic striatum

- (A) Low magnification image showing sampled area in the ischaemic striatum.
- (B) Digital threshold quantification of PECAM+ surface area in projected confocal z-stacks in the sampled area as a measure of vascular density.
- (C-F) Representative confocal images to show normal level of vascular density in the wildtype and Gal-3^{-/-} brains (C-D) and 7-days post-MCAO. Blood vessels labelled by PECAM immunohistochemistry.
- (G) Quantification of PECAM+ area shows significant increase in vascular density in the ischaemic penumbra 7-day post-MCAO in wildtype mice (***) $P < 0.001$. In Gal-3^{-/-}, vascular density remained at baseline levels.
- (H) Orthogonal projection of confocal z-stack showing BrdU+ / PECAM+ endothelial cell.
- (I) Quantification of BrdU+ / PECAM+ cell following cumulative BrdU labelling to assess endothelial proliferation in the ischaemic striatum. No cells were detected in non-stroke controls. 7-days post-MCAO, there were significantly more BrdU+ / PECAM+ cells in Gal-3^{+/+} compared to Gal-3^{-/-} brains (** $P < 0.003$).

Scale bar (A) 200 μm , (F) 50 μm , (H) 10 μm .

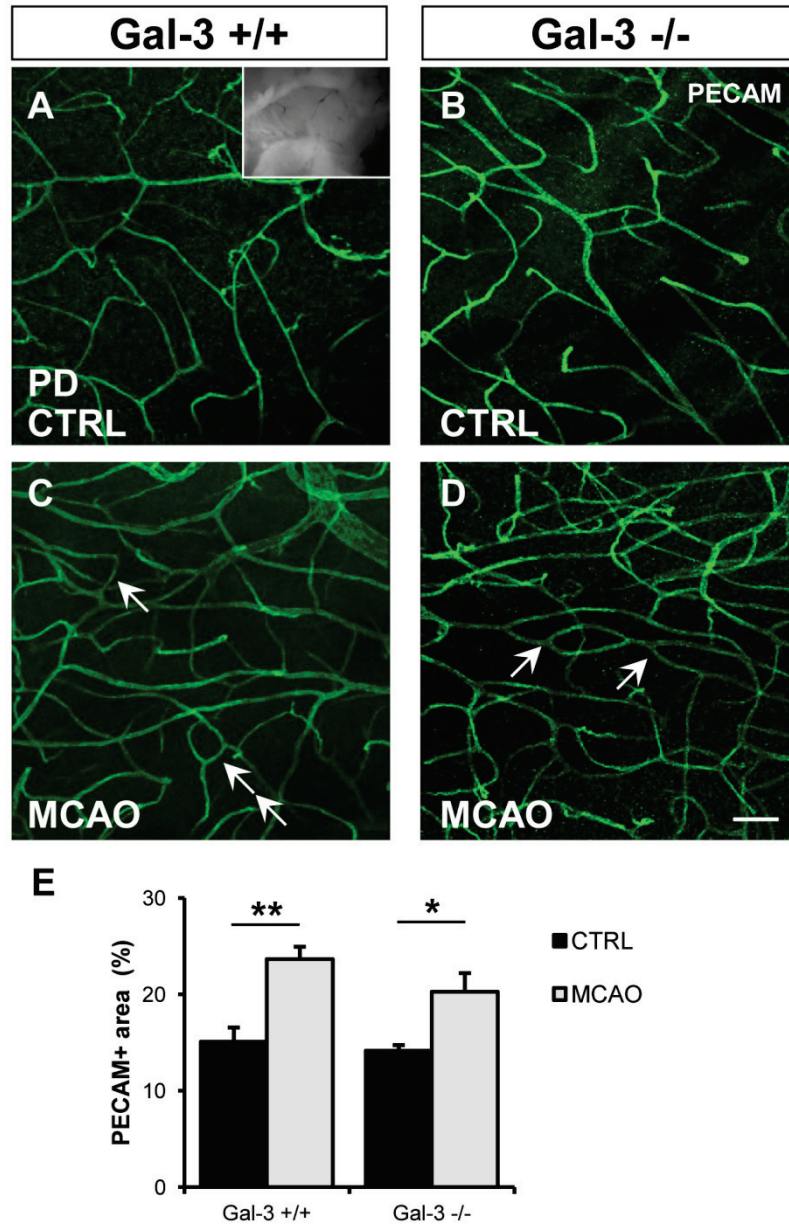


Figure 6.13. Preserved post-stroke angiogenesis in the SVZ in Gal-3^{-/-} mice

- (A-B) PECAM immunohistochemistry in whole mounts shows planar orientation of blood vessels in the SVZ parallel to the ventricular wall.
- (C-D) 7-days post-MCAO, increased vascular density in the SVZ with evidence of “splitting” angiogenesis (arrows) in wildtype and Gal-3^{-/-} brain.
- (E) Digital threshold quantification of PECAM+ surface area shows robust post-stroke angiogenesis in the SVZ in wildtype (** $P = 0.001$) and Gal-3^{-/-} (* $P = 0.03$) mice.

Scale bar 50 μ m.

6.2.8. Decreased VEGF levels in Gal-3^{-/-} brains after stroke

VEGF and its tyrosine kinase receptors are key regulators of post-stroke angiogenesis (Beck and Plate, 2009) and Gal-3 was previously shown to modulate VEGF signalling and its downstream pathways (Markowska et al., 2011). To determine whether the loss of post-stroke angiogenesis in Gal-3^{-/-} mice is mediated via VEGF signalling, Western blots were used to quantify VEGF protein levels in brain homogenates from Gal-3^{+/+} and Gal-3^{-/-} mice. The polyclonal antibody detected VEGF₁₈₈, VEGF₁₆₄ and VEGF₁₂₀ splice isoforms of murine VEGF, and the isoforms are seen as distinct protein bands when resolved on 4 - 20% gradient gel for SDS-PAGE. In sham-operated controls, immunoblotting for VEGF showed 2 distinct protein bands of ~27 kDa and ~22 kDa which corresponded with VEGF₁₈₉ and VEGF₁₆₅, respectively (Fig 6.14A; Chen et al., 2002). VEGF protein levels were not different between Gal-3^{+/+} wildtype and Gal-3^{-/-} mutant naïve brains ($n = 3$). However, in Gal-3^{+/+} brains 7-days post-stroke, VEGF₁₈₉ and VEGF₁₆₅ proteins were significantly increased compared to controls (Fig 6.14B-C; VEGF₁₈₉ $P = 0.02$; VEGF₁₆₅ $P = 0.016$). A faint ~17 kDa band also became visible which corresponded with VEGF₁₂₀. In contrast, in Gal-3^{-/-} brains 7-days post-stroke, VEGF isoform levels remained similar to control levels.

6.2.9. Gal-3^{-/-} mice express anti-angiogenic proteomes post-stroke

Numerous molecules and signalling pathways are implicated in post-stroke angiogenesis and neuroblast ectopic migration. Therefore, I used a proteomic approach to identify molecules and signalling pathways which interact with Gal-3. The semi-quantitative proteome array is based on principles of immunoblotting and enzyme-linked immunosorbent assay. A series of control and specific capture antibodies were spotted in duplicate on nitrocellulose membranes (see Appendix 2 for full list of capture antibodies).

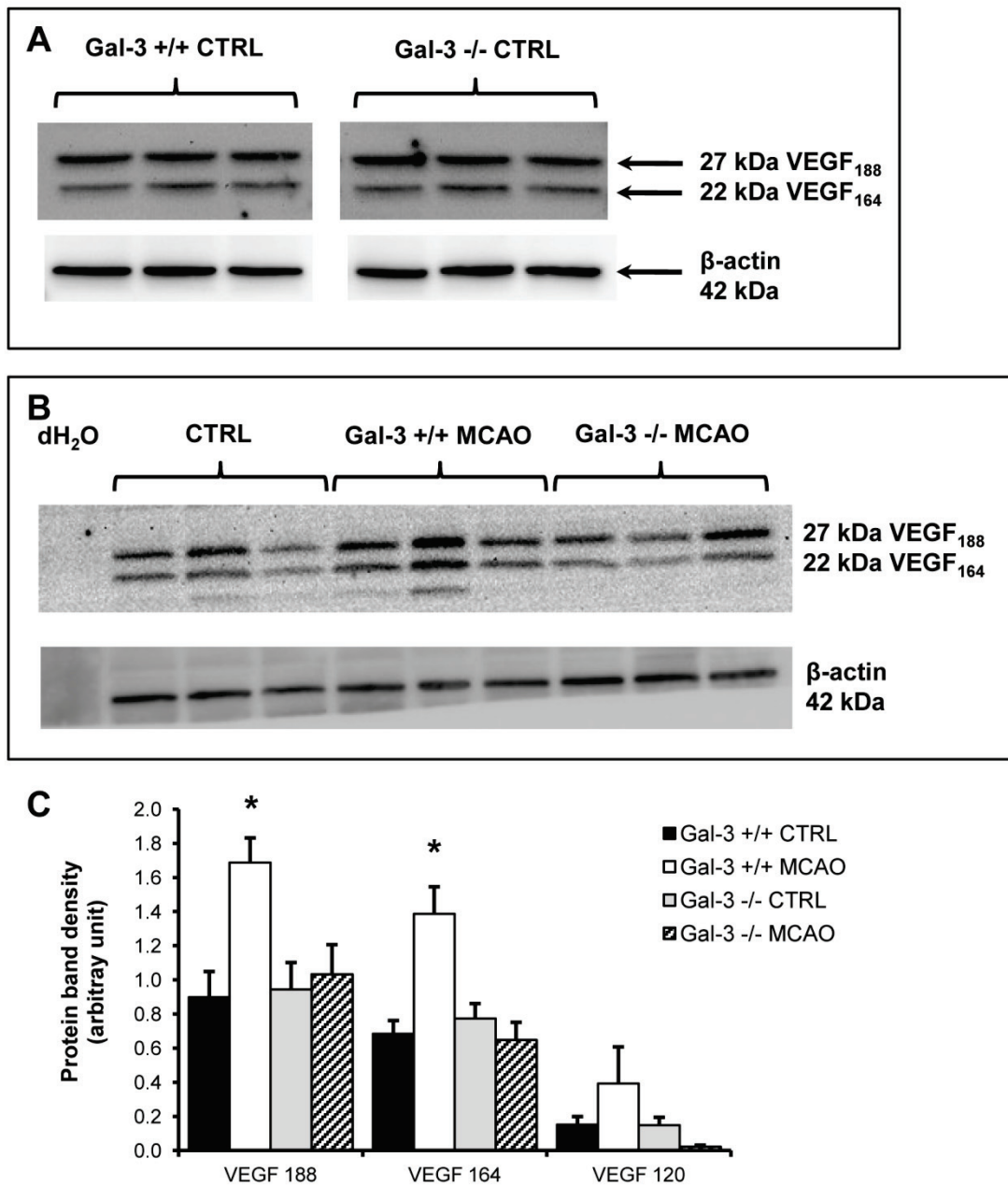


Figure 6.14. VEGF is decreased in the Gal-3^{-/-} ischaemic striatum

- (A) Immunoblotting for VEGF reveals protein bands corresponding to VEGF₁₈₈ (27 kDa) and VEGF₁₆₄ (22 kDa). Each lane represents an individual animal.
- (B) 7-days post-MCAO, VEGF isoforms were increased in Gal-3^{+/+}, but not in Gal-3^{-/-} brain samples.
- (C) Protein band densitometry quantification. VEGF188 (**P* = 0.019) and VEGF164 (**P* = 0.016) isoforms were significantly increased in the Gal-3^{+/+} brain 7-days post-MCAO compared to non-stroke controls. VEGF levels in Gal^{-/-} brains did not increase after MCAO (*n* = 3).

Each membrane was incubated separately with brain homogenate protein samples. Proteins captured by the spotted antibodies were detected with an HRP-conjugated secondary antibody and developed with ECL to determine the relative protein expression levels.

(Fig 6.15A-B) shows exposed nitrocellulose membranes following incubation with brain homogenates from naïve and post-stroke Gal-3^{+/+} and Gal-3^{-/-} mice. The positive control spots (*green box*) were strongly detected on all 4 membranes, while there was no signal in the negative control spots (*red box*). Proteins which showed relative expression of at least 20% of the positive control and/or showed at least 3-fold differences between any of the 4 groups were selected for further analysis (Table 6.2). A number of interesting molecules were identified, including the anti-angiogenic ADAMTS1 (A disintegrin and metalloproteinase with thrombospondin motifs 1) and thrombospondin-2; the hormone prolactin; the cytokines fibroblast growth factor-1 (FGF-1), platelet factor 4 (CXCL4) and osteopontin; and the family of insulin-like growth factor binding proteins (IGFBP).

ADAMTS1 was constitutively up-regulated in Gal-3^{-/-} mutants compared to Gal-3^{+/+} wildtypes (3.08 fold), and is further up-regulated in Gal-3^{-/-} mutants after stroke (5.23 fold). Thrombospondin-2 expression demonstrated a similar profile, and was expressed 3.08 fold higher in Gal-3^{-/-} mutant compared to Gal-3^{+/+} wildtype control brains. Post-MCAO, thrombospondin-2 expression in Gal-3^{-/-} mutants was 5.23 fold that of Gal-3^{+/+} wildtype controls. The hormone prolactin was expressed in naïve Gal-3^{+/+} wildtype brains, and was down-regulated by stroke. Surprisingly, prolactin was virtually absent in Gal-3^{-/-} brains both in controls and post-MCAO. Osteopontin, a phosphorylated glycoprotein which mediates cell migration, was robustly up-regulated post-MCAO in both Gal-3^{+/+} and Gal-3^{-/-} brains. CXCL4 protein was similarly increased in Gal-3^{+/+} (2.47

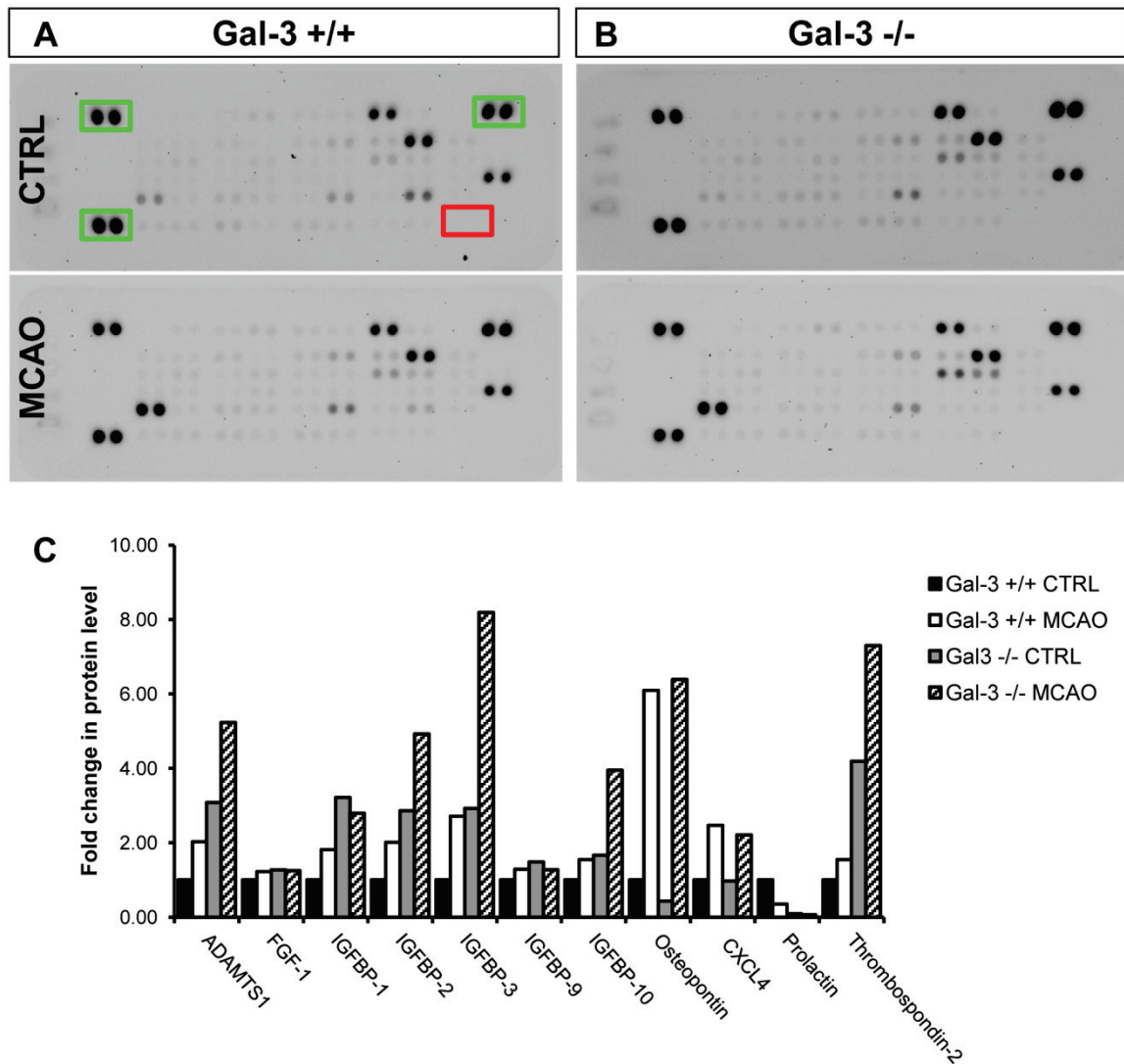


Figure 6.15. Proteome profile analysis

- (A-B) Nitrocellulose membranes were spotted with positive control antibodies and series of capture antibodies in duplicate (Appendix). The membranes were independently incubated with brain homogenates (pre-treated with biotinylated detection antibodies) from each group to detect proteins of interest. After wash, bound proteins were detected with Streptavidin-HRP and chemiluminescence to show relative expression level. *Green boxes* are positive control spots. *Red box* is the negative control spot.
- (C) Densitometry quantification of captured protein spots expressed as fold change compared to wildtype control brains. Proteins which showed more than 3-fold change between any of the experimental groups were selected for analysis ($n = 3$).

fold) and Gal-3^{-/-} (2.21 fold) brains post-MCAO. FGF-1 was present at high levels in Gal-3^{+/+} brains, and was moderately increased post-MCAO (1.22 fold), while in Gal-3^{-/-} mutants, FGF-1 was highly expressed and did not increase following MCAO. IGFBP-1, -3, -9 and -10 were constitutively expressed in Gal-3^{+/+} brains, and up-regulated moderately post-MCAO (1.29 – 2.71 fold). IGFBPs levels were higher in Gal-3^{-/-} mutants, with further increases post-MCAO.

These results showed that the deletion of Gal-3 was associated with considerable changes to the proteome profile in the mouse brain, both in health and after stroke (Figure 6.15C).

Table 6.2. Comparison of proteome profile

	Gal-3 ^{+/+}		Gal-3 ^{-/-}	
	CTRL	MCAO	CTRL	MCAO
ADAMTS1	0.41	0.82	1.25	2.12
	1.00	2.03	3.08	5.23
FGF-1	63.74	77.93	81.12	79.66
	1.00	1.22	1.27	1.22
IGFBP-1	0.50	0.91	1.60	1.39
	1.00	1.82	3.21	2.79
IGFBP-2	6.88	13.80	19.69	33.83
	1.00	2.01	2.86	4.92
IGFBP-3	2.81	7.62	8.21	23.00
	1.00	2.71	2.92	8.19
IGFBP-9	49.44	63.59	73.38	62.93
	1.00	1.29	1.48	1.27
IGFBP-10	0.90	1.40	1.50	3.57
	1.00	1.55	1.66	3.94
Osteopontin	14.81	90.27	6.38	94.61
	1.00	6.09	0.43	6.39
Platelet Factor 4 / CXCL4	9.82	24.26	9.54	21.68
	1.00	2.47	0.97	2.21
Prolactin	24.51	8.54	2.14	1.58
	1.00	0.35	0.09	0.06
Thrombospondin-2	0.60	0.93	2.51	4.38
	1.00	1.55	4.19	7.30

Protein expression as percentage of positive control spot

Protein expression as percentage of naïve Gal-3^{+/+} wildtype control

6.3. Discussion

In the current studies, the roles of Gal-3 in stroke outcomes and tissue remodelling in the ischaemic striatum, as well as in SVZ-mediated endogenous repair were investigated. Loss of Gal-3 did not change neurological outcomes and infarct volumes after stroke. The cellular inflammatory response and rate of apoptotic cell deaths in the ischaemic cerebral hemisphere were also unaffected by loss of Gal-3. However, deletion of Gal-3 was found to attenuate post-stroke angiogenesis in the ischaemic striatum, and this was associated with decreased VEGF protein levels and an anti-angiogenic protein profile.

The SVZ neural stem cell niche was significantly changed after stroke. Increased SVZ cell proliferation and features of SVZ cytoarchitectural reorganisation were observed, and were unaffected by the loss of Gal-3. Within the SVZ, some neuroblasts acquired a differentiated phenotype. Neuroblasts migrated ectopically after stroke, and numerous neuroblasts were found in the ischaemic striatum. Ectopic neuroblasts were associated with blood vessels, and retained the capacity to undergo cell division. Unexpectedly, the deletion of Gal-3 increased the number of ectopic neuroblasts in the ischaemic striatum. However, the pro-proliferative interaction between neuroblasts and blood vessels was diminished in Gal-3^{-/-} mutants. This finding, in combination with the loss of post-stroke angiogenesis, suggests a role for Gal-3 in the formation of the neurovascular niche ischaemic striatum, as it becomes a target site of adult neurogenesis in the post-stroke brain.

6.3.1 Gal-3^{-/-} mice for loss-of-function studies in MCAO

The “knockout mouse” technology was used to perform Gal-3 loss-of-function studies in the intraluminal filament model of MCAO. In Gal-3^{-/-} mutant mice, the functional Gal-3 gene was replaced with a non-functional allele by homologous recombination. This is an example of the versatility of the murine MCAO model for stroke research. In other species, similar loss-of-function study would have required the introduction of a pharmacological or biological agent into the brain, usually via intracerebral or intraventricular injections.¹ These invasive procedures increase animal morbidity and mortality, and confound experimental data.

129sv Gal-3^{+/-} mice were fertile and there was no obvious phenotypic difference in offsprings of different genotypes. Gal-3^{-/-} mutants were viable and there was no evidence of increased spontaneous abortion. Age, strain and body weight can all affect stroke outcome (Connolly et al., 1996) while oestrogen may mediate neuroprotection after ischaemic stroke (Li et al., 2010b). Therefore, the present experiments were carried out exclusively in male mice obtained from 129sv Gal-3^{+/-} breeding pairs, aged between 8 - 12 weeks and weighing between 23 - 28 g. Animals were genotyped by PCR as demonstrated in Chapter 5 from ear clip samples obtained at weaning, and confirmed using immunohistochemistry for Gal-3. Animals were coded and the investigator was blinded to their genotypes until analysis of results was performed.

MCAO resulted in similar degrees of neurological deficits in wildtype and mutant mice, which peaked 24 hours post-stroke, and recovered during the first post-operative week. In the second post-operative week, there were residual neurological deficits which persisted, demonstrated by both the mNSS and openfield assessment. In addition to gross

¹ Transgenic rats, cats and marmosets have been engineered (Sasaki et al., 2009), although the low rate of successful germ-line transmission currently restricts their utility.

motor deficits measured by the mNDS used in Chapter 3, the mNSS incorporated evaluation of other neurological modalities to provide a more detailed assessment of functional status. The mNSS took into account the animal's mobility, motor function, and sensory-motor reflex actions, as well as general behaviour (Li et al., 2000).

Consistent with the similar degrees of neurological deficits, the loss of Gal-3 did not affect stroke volume measured 7-days post-MCAO. Stroke is an evolving process, and there is a balance of neuronal survival and death in the ischaemic penumbra in the hours and days after the initial infarction (Liu et al., 2009a). However, using various strategies to measure the infarct volume, including staining with TTC, Fluoro-Jade B and haematoxylin and eosin, as well as immunolabelling for NeuN, the maximum infarct volume measure was obtained 1 - 3 days post-MCAO (Liu et al., 2009a; Lin et al., 1993). Therefore, 7-days after MCAO, the stroke lesions measured in this study were likely to have fully-evolved. Loss of NeuN and GFAP immunolabelling were independently used to measure infarct size by 2 blinded investigators, and no statistical difference was found between the 2 strategies.

6.3.2. Neuroblast maturation in the ischaemic SVZ

In chapter 4, increased proliferation of neural lineage cell types was documented in the post-stroke SVZ. In the present study, robust increase in SVZ cell proliferation led to neuroblast hyperplasia (Parent et al., 2002; Zhang et al., 2004). Neuroblasts in the post-stroke SVZ also appeared to acquire a mature / differentiated phenotype, as evidenced by advanced branching morphology and expression of the mature neuronal marker, NeuN. Similarly, SVZ neuroblasts following murine MCAO expressed neuronal specific markers such as tyrosine hydroxylase and displayed sodium currents (Liu et al., 2009c). These findings are remarkable as a primary feature of the SVZ neurogenic niche is its ability to

maintain neural precursors in a de-differentiated and migratory state until their arrival at their destination in the olfactory bulb, where their terminal differentiation is regulated by the basic-helix-loop-helix (bHLH) transcription factor NeuroD1 (Boutin et al., 2010). The authors showed that artificial over-expression of NeuroD1 in the SVZ induced neuroblasts to assume a complex branching phenotype and expressed NeuN. Many of these NeuroD1-induced neuroblasts also migrated ectopically out of the SVZ into the adjacent striatum. The uncanny similarities between these observations in the post-stroke SVZ and following the over-expression of NeuroD1 begged the question whether NeuroD1 was up-regulated by stroke. In addition, would it then be feasible to consider the process of ectopic neuroblast migration into the striatum as a recapitulation of radial migration in the olfactory bulb? If this were the case, it would suggest that stroke altered the molecular milieu of the niche significantly to resemble the olfactory bulb, thus triggering the neural differentiation programme. A provocative suggestion might be that the post-stroke SVZ recapitulated the embryonic VZ and ectopic migration into the striatum was analogous to cortical migration. The parallels are striking. During development, newborn neurons migrated along radial glia processes to reach their cortical targets (Rakic, 1972; Noctor et al., 2001). Post-stroke, neuroblast migrated in association with astrocyte processes (Parent et al., 2002) and recent genetic lineage tracing study using a nestin-CreER^{T2}:R26R-YFP reporter line indicated that some of these striatal astrocytes may be derived from the SVZ (Li et al., 2010c)

6.3.3. Deletion of Gal-3 increases ectopic neuroblast migration after stroke

Given that Gal-3 promoted neuroblast migration and its inhibition reduced migration, and that Gal-3 was highly up-regulated in both the post-stroke SVZ and the ischaemic striatum, it was reasonable to hypothesise that loss of Gal-3 would interfere with

neuroblast emigration after stroke. Unexpectedly, Gal-3 deletion increased the number of ectopic neuroblasts in the ischaemic striatum. In the dorsolateral SVZ where the most robust emigration occurred, the number of neuroblasts in the striatum was significantly increased in Gal-3^{-/-} mutants compared to Gal-3^{+/+} wildtypes 14-days post-stroke. The similar rates of SVZ cell proliferation and apoptosis in the ischaemic striatum further suggested a difference in the capacity to emigrate between Gal-3^{+/+} and Gal-3^{-/-} neuroblasts. Increased exploratory migratory patterns in Gal-3^{-/-} neuroblasts described in Chapter 5 may underlie the increased neuroblast emigration. In the only study ever carried out to assess dynamic features of SVZ neuroblast migration after stroke, neuroblasts were found to exit the SVZ in an exploratory manner (Zhang et al., 2009). Post-stroke time-lapse imaging in Dcx-eGFP brain slices showed that neuroblast on the border of the SVZ extended and retracted cell processes in multiple directions, and after stroke, some extended processes laterally and emigrated into the striatum. Emigration was also not uni-directional out of the SVZ; some ectopic neuroblasts were able to re-enter the SVZ. It is plausible that the slower and exploratory neuroblasts in Gal-3^{-/-} are better positioned to respond to the environmental cues which induce ectopic migration into the ischaemic striatum. This is supported by the absence of ectopic neuroblast migration in naïve or sham-operated Gal-3^{-/-} brain; ectopic migration only occurred when appropriate cell emigration signals are present, and not merely due to the loss of Gal-3. Furthermore, infusion of recombinant Gal-3 above the RMS did not elicit ectopic neuroblast migration (I. Comte and F. Szele unpublished data), and Gal-3^{-/-} mice had increased post-stroke neuroblast emigration, thus abrogating Gal-3 as a key chemotactic molecule in the ischaemic striatum.

6.3.4. An adult neurogenic niche in the ischaemic striatum

Neuroblasts' ability to proliferate during ectopic migration was previously documented (Zhang et al., 2007a), although the extent of this phenomena was not known. Using PHi-3 which only labels cells actively undergoing mitosis, I found that ~5% of neuroblasts in the striatum were proliferating. If this degree of proliferation persisted, it could have significant therapeutic implications, as the small number of ectopic neuroblasts could potentially generate large numbers of daughter cells. It was also not improbable that transit amplifying progenitors which have much greater proliferative potential may be able to migrate into the ischaemic striatum (Aguirre et al., 2004; Gonzalez-Perez et al., 2009). This is a paradigm shift as strategies to augment post-stroke neurogenesis would no longer be confined to recruiting the maximum number of neuroblasts possible. Instead, a novel approach may focus on inducing cells with proliferative capacity to emigrate, and creating a local environment that would be permissive for their division at the site of brain injury.

After stroke, ~30% of ectopic neuroblasts in the striatum made contact with blood vessels, very similar to the ~35% of ectopic neuroblasts which were found within 5 μ m of a blood vessel 2 weeks after MCAO in rats (Thored et al., 2007). However, nearly 70% neuroblasts which divided in the striatum were localised to blood vessels. This is reminiscent of proliferative NSPCs in the SVZ and stresses the importance of vascular basement membrane signalling mediated via SDF-1 α /CXCR4 and α 6 β 1 integrins. SDF-1 α mediates chemotaxis of NSPCs towards blood vessels and up-regulates α 6 integrin expression which is necessary for NSPC adherence to endothelial cells (Kokovay et al., 2010). In OPCs, α 6 integrin signalling potentiates the effects of growth factors to enhance survival (Colognato et al., 2002). Such interaction in the ischaemic striatum may also help explain the so-called coupling between post-stroke angiogenesis and neurogenesis. In Gal-3^{-/-} mutants, the affinity of ectopic neuroblasts for blood vessels and their capacity to

divide in the ischaemic striatum were not affected. However, whereas the majority of proliferating ectopic neuroblasts were found on blood vessels in Gal-3^{+/+} wildtypes, the proportion was significantly reduced in Gal-3^{-/-}. The fact that Gal-3^{-/-} ectopic neuroblasts were able to “home” in on blood vessel but then did not appear to receive (or respond to) the proliferative signal suggests impaired basement membrane signalling between neuroblasts and endothelial cells.

6.3.5. Gal-3 is not required for post-stroke reactive astrocytosis and microglia activation

Gal-3 expression coincided with the cellular inflammatory response after stroke. Gal-3 immunolabelling was also positively correlated with the degree of post-stroke microglial activation (Inacio et al., 2010). Therefore, Gal-3 was well-positioned to regulate reactive astrocytosis and microglial activation. After stroke, exposure to purines / pyrimidines from dying cells and plasma extravasations triggers the initial events of reactive astrocytosis (Buffo et al., 2010). This is followed by the release of cytokines such as TNF α and IL-1 β which further enhance reactive astrocytosis and microglia recruitment and activation, in complex feed-forward and feed-back loops (Lakhan et al., 2009). Reactive astrocytosis is characterised by intermediate filament expression (GFAP and vimentin), hypertrophy of intermediate filament processes, astrocyte hyperplasia, changes in secretory activity, and glial scar formation (Buffo et al., 2010). Seven-days post-stroke, there was intense reactive astrocytosis with the initiation of glial scar formation around the infarct core. I did not detect qualitative differences in the degree of reactive astrocytes between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants. Reactive astrocyte proliferation was also unaffected by the deletion of Gal-3. There may be redundant pathways in operation

involving the members of the galectin family, such as Gal-1 and Gal-9. As an example of such redundancy, deletion of both GFAP and vimentin was necessary to attenuate reactive astrogliosis (Wilhelmsson et al., 2004). Loss of either GFAP or vimentin alone had no obvious effect (Li et al., 2007). The issue of redundancy will need to be considered in future experimental strategies which will be discussed in the next chapter.

Microglia are distributed throughout the brain parenchyma and activated upon brain injury. Microglia activation states are revealed by their morphology ranging from (1) ramified (resting), (2) intermediate, (3) amoeboid, to (4) round (macrophage-like) (Goings et al., 2006; Thored et al., 2009). Seven-days post-stroke, in the ischaemic striatum, the majority of microglia were found in the intermediate or amoeboid state, with no observable difference between Gal-3^{+/+} wildtypes and Gal-3^{-/-} mutants. Contrary to previous reports (Lalancette-Hébert et al., 2007), microglia proliferation after stroke was detected at low levels in both Gal-3^{+/+} or Gal-3^{-/-} brains. Furthermore, similar numbers of cells underwent delayed programmed cell death in Gal-3^{+/+} and Gal-3^{-/-} post-stroke brains.

6.3.6. Gal-3 may mediate post-stroke angiogenesis via VEGF

Angiogenesis, the sprouting of new blood vessels from existing vessels (Carmeliet and Jain, 2000), is an endogenous repair mechanism in the ischaemic penumbra following stroke (Beck and Plate, 2009). In this study, 2 independent strategies were used to assess post-stroke angiogenesis in the ischaemic striatum; by measuring the vascular density in the striatum, followed by quantification of proliferating endothelial cells. The deletion of Gal-3 resulted in a failure to mount an angiogenic response in the ischaemic striatum following stroke. Gal-3 is known to promote vasculogenesis and angiogenesis. Gal-3 promoted recruitment of human umbilical vein endothelial cells and increased capillary tube formation (Nangia-Makker et al., 2000a). After rat MCAO, infusion of functional

blocking anti-Gal-3 antibody attenuated post-stroke increase in vascular density in the striatum, while recombinant Gal-3 increased endothelial proliferation *in vitro* (Yan et al., 2009). In another example of Gal-3/integrin interaction, NG2 proteoglycan binding to $\alpha 3 \beta 1$ integrin on endothelial cell surface was stabilised by Gal-3, and the transmembrane signalling induced cell motility *in vitro* and corneal angiogenesis after injury *in vivo* (Fukushi et al., 2004).

VEGF is a cytokine which binds to the tyrosine kinase receptor VEGFR-2 to stimulate endothelial proliferation and migration (Holmes et al., 2007). Gal-3 regulates the cell surface expression of VEGFR-2, and enhances its phosphorylation (Markowska et al., 2011). In Gal-3^{-/-} brains after stroke, levels of VEGF₁₈₈ and VEGF₁₆₄ isoforms were decreased compared to Gal-3^{+/+} wildtypes. VEGF levels in control Gal-3^{+/+} and Gal-3^{-/-} brains were not different, suggesting that the ability to up-regulate VEGF was impaired in Gal-3^{-/-} mutants after stroke.

Alternative splicing of VEGF mRNA at exon 6 and 7 results in 6 isoforms, with VEGF₁₂₀, VEGF₁₆₄ and VEGF₁₈₈ (human isoforms have 1 additional amino acid) being the most abundant (Robinson and Stringer, 2001). The isoforms differ in their interaction with VEGFR co-receptors heparin sulphate proteoglycans and neuropilin, which alter their binding to and activation of VEGFR (Holmes et al., 2007). VEGF₁₂₀ lacks the heparin binding domain and is freely secreted as a diffusible molecule. VEGF₁₆₄ has moderate affinity for heparin and ~50 - 70% of secreted molecules remain associated with the cell and ECM, while VEGF₁₈₈ strongly binds heparin and is almost completely sequestered within the ECM (Robinson and Stringer, 2001). Secreted VEGF₁₂₁ and VEGF₁₆₅ promote endothelial cell proliferation, although the mitogenic activity of VEGF₁₆₅ is ~50 fold greater compared to VEGF₁₂₁ (Neufeld et al., 1999). In contrast, recombinant VEGF₁₈₉ is not mitogenic to endothelial cells (Houck et al., 1991). However, ECM sequestered

VEGF₁₈₈ is likely a reservoir of the growth factor which is mobilised by proteolytic enzymes such as urokinase-type plasminogen activator (uPA; Neufeld et al., 1999). After cleavage, uPA-VEGF₁₈₉ displays endothelial mitogenic activity equivalent to VEGF₁₆₅, and uPA is significantly increased in the ischaemic brain tissue (Hosomi et al., 2001). Therefore, in the post-stroke brain, VEGF₁₆₄ and VEGF₁₈₈ may help to facilitate the temporal profile of angiogenesis in the ischaemic penumbra.

How does Gal-3 contribute to the up-regulation of VEGF after stroke? After stroke, hypoxia is central to the induction of angiogenesis (Pugh and Ratcliffe, 2003). VEGF transcription is up-regulated by HIF-1 α , a bHLH transcription factor which binds to hypoxia responsive elements in the VEGF promoter region. In the ischaemic brain, decreased degradation of HIF-1 α by HIF prolyl hydroxylases leads to increased HIF-1 α levels, resulting in VEGF up-regulation (Liu et al., 1995). Hypoxia also stabilises VEGF mRNA by RNA-binding proteins and prolongs its half-life (Levy et al., 1998). Furthermore, VEGF mRNA translation is preserved in unfavourable hypoxic conditions due to the presence of an internal ribosomal entry site (Stein et al., 1998). After stroke, VEGF is expressed by reactive astrocytes (Kalaria et al., 1998) which express cytoplasmic and nuclear Gal-3. HIF-1 α promotes Gal-3 expression under hypoxic conditions (Zeng et al., 2007). Therefore, Gal-3 is well positioned to be an upstream regulator of VEGF production. In thyroid tumour cells, Gal-3 up-regulates gene transcription by binding to a transcription factor homeodomain (Paron et al., 2003), and it will be interesting to test whether Gal-3 regulates VEGF at a transcription level in a similar manner. In a model of diabetic retinopathy, retina from Gal-3^{-/-} mutants expressed significantly lower levels of VEGF compared to Gal-3^{+/+} wildtypes following pancreatic β -islet cell depletion, while naïve animals had similar protein levels (Canning et al., 2007).

The finding of preserved post-stroke angiogenesis in the SVZ in Gal-3^{-/-} mutants was unexpected. This suggests that either (1) angiogenesis in the post-stroke SVZ did not depend on VEGF stimulation, or (2) VEGF up-regulation in the SVZ was preserved in Gal-3^{-/-} mutants. The mitogenic effects of VEGF on SVZ NSPCs *in vivo* and *in vitro* are well documented in healthy and injured brains (Jin et al., 2002; Sun et al., 2003). The response of SVZ endothelial cells to VEGF has not been specifically examined, and it is possible that analogous to SVZ astrocytes and microglia, they have unique properties which distinguish them from endothelial cells from other regions. Anatomically, SVZ vasculature have distinct anatomical features such as its planar architecture parallel to the ventricular wall and its direct basement membrane contact with NSPCs without intervening astroglial endfeet or pericytes (Shen et al., 2008; Tavazoie et al., 2008). It will be informative to measure VEGF levels in the post-stroke SVZ to determine its role in SVZ angiogenesis and to shed light on the difference in angiogenesis between the post-stroke Gal-3^{-/-} mutant SVZ and ischaemic striatum.

6.3.7. An anti-angiogenic proteome profile in Gal-3^{-/-} after stroke

To understand the attenuated post-stroke angiogenesis in Gal-3^{-/-} mutants, and the disparity between the ischaemic striatum and SVZ, a proteome array was performed to compare the protein expression profile between naïve and post-stroke brains in Gal-3^{+/+} and Gal-3^{-/-} mice. This technique has the advantage of allowing semi-quantitative measurement of actual protein levels as opposed to transcription products measured by PCR-based approaches. The increase of osteopontin in both Gal-3^{+/+} and Gal-3^{-/-} samples after stroke served as a useful positive control, as osteopontin, a phosphorylated acidic glycoprotein, was known to be up-regulated after stroke (Yan et al., 2006a, 2009b).

Platelet factor 4 / CXCL4

Platelet factor 4 / CXCL4 was up-regulated in both Gal-3^{+/+} and Gal-3^{-/-} brains after stroke. It is released by activated platelets and mediates key steps of haematopoiesis and the coagulation cascade (Kowalska et al., 2010). It was the first chemokine shown to inhibit angiogenesis, and has been reported to sequester VEGF₁₆₅ and interfere with its receptor binding and downstream signalling pathway (Vandercappellen et al., 2011). Therefore it may act as a counter-balance to the pro-angiogenic environment in the post-stroke brain. Its role in the post-stroke brain has not been investigated and it may be a potential candidate for future studies.

ADAMTS1 and thrombospondin-2

ADAMTS1 and thrombospondin-2 are both anti-angiogenic and in addition to the decreased levels of VEGF in Gal-3^{-/-} mutants, these molecules were up-regulated in both naïve and post-stroke brains in Gal-3^{-/-} compared to Gal-3^{+/+} mice. ADAMTS1 is an ECM metalloproteinase which binds VEGF₁₆₅ and prevents its binding to VEGFR2 (Luque et al., 2003). ADAMTS1 also possess a thrombospondin-1 motif which along with thrombospondin-2 are the 2 members of the thrombospondin family which have anti-angiogenic activity (Porter et al., 2005). The anti-angiogenic activity is thought to be mediated via CD36-induced endothelial apoptosis. Thrombospondins are ECM proteins which, like galectins, mediate cell-cell and cell-ECM interactions (Lu and Kipnis, 2010). Astrocytes-secreted thrombospondin-1 and -2 promote synaptogenesis during development (Christopherson et al., 2005), and thrombospondin-1 maintains SVZ and SGZ proliferation and neuronal differentiation (Lu and Kipnis, 2010). Thrombospondin-1 also mediates OPC adhesion and migration *in vitro* (Scott-Drew and French-Constant, 1997). Deletion of Gal-3 does not change the rate of SVZ proliferation both constitutively

and post-stroke, therefore the up-regulation of thrombospondins in Gal-3^{-/-} mutants may potentially be a compensatory mechanism for the loss of Gal-3.

IGFBP

The 5 IGFBPs detected by the proteome array were up-regulated by stroke. Gal-3^{-/-} mutants had higher baseline levels of IGFBP compared to Gal-3^{+/+} wildtypes, and the levels increased further following stroke, especially IGFBP-2 and IGFBP-3. IGFBPs bind IGF with high affinity and regulate the bioavailability of free IGF (Jogie-Brahim et al., 2009). IGF-1 is a growth factor which protects against ischaemic injury and improves long term functional outcome (Zhu et al., 2008). IGF-1 is up-regulated in reactive astrocytes in the ischaemic striatum and enhances SVZ NSPC proliferation (Yan et al., 2006). IGF-1 also has angiogenic properties, and over-expression by adenoviral vectors in the ischaemic striatum increases post-stroke vascular density and endothelial proliferation (Zhu et al., 2009). In contrast, IGFBP-3 inhibits IGF-1 stimulated NSPC proliferation *in vitro* (Kalluri and Dempsey, 2011). Competitive inhibition of IGFBPs with a non-bioactive IGF-1 analogue significantly decreases stroke lesion size, possibly due to increased free IGF-1 (Loddick et al., 1998). There is also evidence that IGFBPs may have IGF –independent effects. For example, IGFBP-2 / $\alpha 5$ integrin interaction mediates cell migration in IGFBP-2-overexpressing gliomas (Mendes et al., 2010).

Prolactin

Prolactin is a pituitary hormone that plays an important role in lactation but its role in males is less well understood. Prolactin was present in the normal male brain and was down-regulated after stroke. Surprisingly, prolactin was virtually undetectable in Gal-3^{-/-} mutants, constitutively or after stroke. It is difficult to speculate the significance of this,

however there is literature which suggests that prolactin promotes SVZ proliferation during both maternal and paternal-bonding (Larsen and Grattan, 2010; Mak and Weiss, 2010). Prolactin increases OPC proliferation and enhanced repair of demyelinating lesions (Gregg et al., 2007), and in a recent study, Gal-3 deletion results in defective myelination and oligodendrocyte differentiation (Pasquini et al., 2011). *In vitro*, exogenous prolactin also promoted human neural stem cell proliferation and migration (Pathipati et al., 2011).

6.4. Conclusion

Gal-3 is a pleiotropic molecule which mediates different aspects of the cellular and molecular response to ischaemic stroke. Loss of Gal-3 did not affect neurological outcomes and infarct volumes after stroke. Post-stroke SVZ cytoarchitectural reorganisation and cell proliferation were also preserved. However, Gal-3 was required for the angiogenic response in the ischaemic striatum. VEGF levels were decreased in the ischaemic brain in Gal-3^{-/-} mutants, and this observation was associated with high levels of anti-angiogenic proteins. Interestingly, although the loss of Gal-3 led to an increased number of ectopic neuroblasts in the ischaemic striatum, the pro-proliferative interaction between ectopic neuroblasts and the vasculature was lost in Gal-3^{-/-} mutants, suggesting defective vascular basement membrane signalling. Thus, in addition to its role in constitutive neuroblast migration, Gal-3 may have an important role in the vascular remodelling in the ischaemic striatum post-MCAO, as it transforms into an environment which supports neurogenesis.

Chapter 7

Discussion

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The central focus of this thesis has been to understand the changes in the adult neural stem cell niche after ischaemic stroke. Using the intraluminal filament model of MCAO in mice, a detailed examination of the cytoarchitectural reorganisation in the post-stroke SVZ was carried out. Reactive astrogliosis with disruption of glial tube migratory scaffold, increased vascular density and ependymal cells with defective motile cilia function were demonstrated as potential mechanisms that interrupted the integrity of the stem cell niche. At a molecular level, the role of Gal-3 in these changes in the post-stroke SVZ, as well as tissue remodelling in the ischaemic striatum were investigated. Gal-3 was found to contribute to the regulation of neuroblast emigration into the ischaemic striatum, in addition to its role to maintain constitutive neuroblast migration. After stroke, Gal-3 was required for the angiogenic response in the ischaemic striatum, and facilitated the proliferative interaction between ectopic neuroblasts and striatal blood vessels. This was associated with a decrease in the level of VEGF, and an increase in anti-angiogenic proteome profile in Gal-3^{-/-} brains after stroke. However, in loss-of-function studies using Gal-3^{-/-} mutants, the features of cytoarchitectural reorganisation after stroke were not regulated by Gal-3. Together, these results indicate that the SVZ as a neural stem cell niche, and the ischaemic striatum as the target of post-stroke neurogenesis, undergo significant changes in response to stroke, and Gal-3 regulates distinct processes in these two brain regions.

7.1. An integrated view of the current work

The SVZ is an exciting therapeutic target in attempts to develop neurorestorative strategies following ischaemic stroke. Although the mechanism by which the SVZ response reduces infarct volume and improves neurological outcomes has not been fully elucidated, it is worthwhile to compartmentalise the elements of SVZ-mediated repair and

post-stroke neurogenesis in order to better understand the regulation of these processes. SVZ-mediated repair and post-stroke neurogenesis can be broadly divided into (1) proliferation, (2) migration, (3) survival, (4) differentiation and (5) function (Ohab and Carmichael, 2008). Based largely on experimental stroke models in multiple animal species, now including the studies presented in this thesis, there is good understanding of the sequence of events. Stroke is associated with robust increase in SVZ cell proliferation which remains elevated for weeks and months (Thored et al., 2006). Many of these neural precursors are able to leave their normal migratory pathway and localise near the ischaemic lesion. The majority of these cells die, while a small proportion survive locally and differentiate into mature neurons with evidence of synapse formation and electrophysiological activity. Fundamental changes to the regulation of the SVZ potentially affect each of these processes.

The tight regulation of the neural stem cell niche by cellular and molecular components of the SVZ is well-documented, and as the data in this thesis demonstrate, they are unlikely to be passive bystanders in SVZ-mediated post-stroke neurogenesis. The interaction and response of these regulatory components in the SVZ with the ischaemic brain environment potentially translate into the observed increase in cell proliferation and neuroblast emigration. In this thesis, significant cytoarchitectural reorganisation in the post-stroke SVZ, affecting SVZ astrocytes, the vasculature and ependymal cells, was demonstrated. The affected cellular components and their co-ordinated functions are critical for the maintenance of the neural stem cell niche. For instance, ependymal cells which regulate multiple aspects of cell proliferation and migration in the SVZ assumed a reactive astroglial phenotype with evidence of motile cilia dysfunction. Normal CSF flow is required to maintain neuroblast migration in the SVZ-RMS-OB axis (Sawamoto et al., 2006). Molecules such Shh is found in the CSF, and disruption of CSF flow may perturb

its molecular equilibrium at the brain / CSF interface. Shh regulates the regional heterogeneity of astrocyte-like stem cells and their progenies (Ihrle et al., 2011). Astrocyte-like stem cells in the SVZ have primary cilia which contact the ventricular surface (Mirzadeh et al., 2008), and Shh-receptors Patched1 and Smoothened that regulate downstream Shh signalling are located on primary cilia (Han et al., 2008). There is also evidence that primary cilia may perform mechanosensory and chemosensory functions (Han and Alvarez-Buylla, 2010). For instance, primary cilia in kidney epithelial cells respond to fluid flow by passively bending, resulting in influx of calcium ions (Louvi and Grove, 2011). Therefore, CSF disruption may have multiple effects on the neurogenic niche. Interestingly, the loss of Gal-3 results in dysmorphic primary cilia in kidney epithelial cells, while acute knockdown of Gal-3 by siRNA lead to cyst formation similar to those observed in polycystic kidney disease, a primary ciliopathy (Koch et al., 2010). Therefore, although SVZ proliferation was not affected in Gal-3^{-/-} mice, Gal-3 may regulate cell fate specification via primary cilia-dependent mechanisms.

Given the considerable heterogeneity of SVZ neural precursors (Merkle et al., 2007; Young et al., 2007), it is not known whether different NSPCs and neuroblasts with different fate choices respond similarly to stroke. If differences exist, are they cell-autonomous or dependent on niche interactions i.e. does the niche play a role to regulate the cell type(s) that migrates ectopically? This may serve as a mechanism by which the niche can determine cell survival and fate in the ischaemic striatum. In addition, the observation that the SVZ contributes astrocytes and oligodendrocyte-lineage cells to the ischaemic striatum (Li et al., 2010c) further raises the question whether these cells can interact directly or indirectly with emigrating neuroblasts to influence their migration, survival and differentiation.

A novel way to consider post-stroke neurogenesis may be to view it as a recapitulation of embryonic development. Neuroblasts migrate, survive, differentiate and functionally integrate in the ischaemic environment. In Chapter 6, neuroblasts were also found to undergo a considerable degree of cell division. Neuroblasts thus perform the full spectrum of their normal biological activities in an alien environment which does not normally support adult neurogenesis. Therefore, has the ischaemic striatum in effect become an alternate neurogenic zone which supports the steps of adult neurogenesis? In addition, striatal and cortical reactive astrocytes have been reported to become de-differentiated following injury (Buffo et al., 2008) and potentially resemble their neurogenic SVZ counterparts. Taking this into account, the ischaemic striatum is not only the destination of SVZ-derived neurons, but may also harbour cells with stem-cell properties of self-renewal and multipotency. A provocative suggestion may be that the ischaemic striatum has transformed into a *de facto* adult neurogenic niche; albeit considerably less robust than the SVZ or SGZ, but insofar as it is an environment which may retain self-renewing and multipotent cells and support the steps of adult neurogenesis.

Gal-3 was a suitable candidate molecule because whereas its expression was largely confined to the SVZ in the normal brain, it became highly expressed in ischaemic brain tissue. It was therefore well positioned to influence striatal remodelling as it became an environment that supported “adult neurogenesis”. Paradoxically, in contrast to its role to maintain normal neuroblast migration, deletion of Gal-3 increased the number of neuroblasts in the ischaemic striatum. The dynamic migratory defects observed by 2-photon time-lapse microscopy in Gal-3^{-/-} neuroblasts may have conferred a greater capacity to respond to the “exit” signal after stroke. Another possibility is that Gal-3, through its ECM interactions contributes to tissue integrity in the post-stroke striatum and

that the loss Gal-3 resulted in tissue that was more amenable to proteolytic digestion and neuroblast emigration.

Thirty percent of ectopic neuroblasts in the striatum after stroke were associated with blood vessels and loss of Gal-3 changed the interaction between neuroblasts and the vasculature. NSPCs in the SVZ proliferate in close proximity to blood vessel (Shen et al., 2008; Tavazoie et al., 2008). Similarly, in wildtype mice, ectopic neuroblasts in the striatum underwent cell division preferentially in contact with blood vessels, while in Gal-3^{-/-} mutant they did not. Although the deletion of Gal-3 did not affect overall neuroblast proliferation in the striatum, this shift suggests that Gal-3 may play a role in basement membrane signalling between ectopic neuroblasts and the vasculature in the ischaemic striatum. As discussed previously, Gal-3 has been shown to mediate integrin receptor binding to ECM ligands (Kariya et al., 2010; Saravanan et al., 2009), and it will be interesting to test if Gal-3 exerted similar effects at the vascular basement membrane between neuroblasts and ECM components both in the SVZ and the ischaemic striatum.

Gal-3 was required for post-stroke angiogenesis in the ischaemic striatum, and the loss of Gal-3 was associated with decreased levels of VEGF and increased anti-angiogenic proteome profile. Gal-3 has been shown to regulate angiogenesis via an extracellular mechanism by stabilisation of VEGFR-2 receptor expression on endothelial cell (Markowska et al., 2011). In contrast, the data in this thesis indicate that Gal-3 may regulate VEGF transcriptional and/or translational products, potentially via an intracellular mechanism associated with the HIF-1 α signalling pathway (Zeng et al., 2007). Counter-intuitively, Gal-3 deletion did not affect post-stroke angiogenesis in the SVZ, and the post-stroke SVZ in Gal-3^{-/-} mice exhibited equivalent increase in vascular density compared to wildtypes.

The loss of Gal-3 does not influence SVZ cell proliferation and the cytoarchitectural reorganisation in the post-stroke SVZ. However, the regulation of the interaction between ectopic neuroblast and blood vessels by Gal-3, and the role of Gal-3 in post-stroke angiogenesis in the ischaemic striatum suggest that it contributes to the novel post-stroke neurogenic environment in the striatum. It is difficult to speculate the cause of the disparate functions of Gal-3 in the SVZ and the striatum in health and disease, although the differences emphasise 2 important points: (1) Gal-3, as a pleiotropic molecule, has different functions depending on its localisation and surrounding environment with which it interacts, and (2) there are distinct differences between the post-stroke SVZ and striatum such that arguably similar biological processes (e.g. post-stroke angiogenesis) in the 2 locations may be mediated via independent mechanisms. The present findings further suggest that within neural tissue, Gal-3 function may be altered radically following tissue ischaemia. Indeed, MMP-9, which is up-regulated after stroke (Lee et al., 2006; Doverhag et al., 2010), has been shown to cleave Gal-3 and significantly alter its carbohydrate-binding affinity and ability to polymerise into oligomers (Ochieng et al., 1998). Gal-3 may therefore be present and function as distinct bio-active molecules in the ischaemic striatum compared to the post-stroke or normal SVZ.

7.2. Limitations

Disturbance of normal CSF flow, disruption of the glial tube migratory scaffold and changes in the vasculature have independently been shown to alter the neural stem cell niche and regulate adult neurogenesis (Ihrie and Alvarez-Buylla, 2011). These features of cytoarchitectural reorganisation are demonstrated this thesis in the context of stroke,

together with increase in neural cell proliferation and ectopic neuroblast migration into the ischaemic striatum. A previous study by Ohab and colleagues showed defective neuroblast migration to the OB after focal cortical ischaemic stroke (Ohab et al., 2006). The cytoarchitectural reorganisation may contribute at least in part to the altered SVZ neurogenesis after stroke. However, direct causal evidence to link these features to changes in cell proliferation and migration has not been provided. Future experimental work will need to directly examine the effect of cytoarchitectral reorganisation on adult neurogenesis, and the molecular mechanisms involved. A potential first step would be to perform 2-photon time-lapse microscopy on whole mounts to assess dynamic features of neuroblast migration, and test the effect of naïve versus post-stroke CSF. Moreover, the pathogenesis of ischaemic stroke is complex and the mechanism which causes the cytoarchitectural changes observed is unknown. Hypoxia, inflammatory cytokines and direct mechanical effects are but a few possibilities. Based on the current results, it is also difficult at this juncture to distinguish between pathological changes as part of the stroke pathology versus adaptive processes of SVZ-mediated endogenous repair.

As a study of the cytoarchitectural reorganisation following stroke, electron microscopy (EM) and immuno-EM provides ultrastructural validation. Despite the compelling evidence of ependymal expression of GFAP, immuno-EM will provide definitive confirmation. EM will also allow detailed examination of the ependymal basal bodies and provide further information regarding the potential mechanism of PCP disruption, and whether the rotational (beating) orientation of the ciliary basal bodies (Mirzadeh et al., 2010b) are also misaligned. Moreover, EM studies will permit close examination of the vascular basement membrane, the glial-vascular interaction and integrity of the blood brain barrier in the post-stroke SVZ.

In vitro neurosphere assays and *in vivo* Ara-C infusion studies suggest that astrocyte-like stem cells are activated and become more proliferative after stroke (Zhang et al., 2004). Doetsch and colleagues reported that the infusion EGF drove transit amplifying cells towards a more stem-like state and increased the number of cells which contacted the ventricle (Doetsch et al., 2002). Using whole mounts, an initial aim in this thesis was to examine whether the number of astrocyte-like stem cells which contacted the ventricles increased after stroke by quantifying the number of GFAP apical contacts. However, the unexpected finding that post-stroke ependymal cells expressed high levels of GFAP rendered this impossible. The ratio of proliferating cells in the SVZ did not change after stroke and ~85% were in the neuronal lineage (Mash-1 and/or Dcx+). This suggested that there was a proportional increase in the proliferation of astrocyte-like stem cells. Scanning EM may have a role to examine if apical contacts increased after stroke. In addition, emerging techniques of identifying NSC populations in varying state of quiescence versus activation by fluorescence-activated cell-sorting (Pastrana et al., 2009; Beckervordersandforth et al., 2010), may pave way for determining whether niche astrocytes can become NSCs after stroke.

My study of Gal-3 has relied on the use of a Gal-3^{-/-} mice which have targeted deletion of the Gal-3 by homologous recombination. As a loss-of-function experimental paradigm, Gal-3 is absent from the point of conception in Gal-3^{-/-} mice. There have been reports that knockout animals frequently exploit redundant pathways to compensate for lost gene functions (Doetschman, 1999) and families of molecules that exhibited such redundancy include FGF (Zhou et al., 1998), caspases (Los et al., 1999), intermediate filaments (Wilhelmsson et al., 2004), and synucleins (Chandra et al., 2004). Gal-3 and Gal-1 have redundant function as pre-mRNA splicers (Vyakarnam et al., 1997), while Gal-1 and Gal-

8 may have overlapping function in plasma cell formation (Tsai et al., 2011). It is possible that the development of redundant pathways in Gal-3^{-/-} mice have masked processes normally regulated by Gal-3 in the current studies. For example, SVZ cell proliferation is unchanged in Gal-3^{-/-} brains constitutively and after stroke *in vivo*. However, preliminary results from the Szele Laboratory using the neurosphere assay shows that addition of recombinant Gal-3 may increase NSPC proliferation *in vitro* (R. James and F. Szele unpublished results). In the traumatic brain injury gene array performed by the Szele Laboratory, Gal-1 and Gal-9 gene expression were also highly up-regulated. Gal-1 and Gal-9 have been implicated in many similar biological processes as Gal-3, such as cell proliferation, cell adhesion and inflammation, with shared molecular partners (e.g. β 1 integrin; Liu and Rabinovich, 2005; Wiersma et al. 2011). Gal-1 also promotes SVZ cell proliferation and mediates stroke outcome (Sakaguchi et al., 2006; Ishibashi et al., 2007). Therefore studies using acute gain- and loss- of function strategies such as infusion of Gal-3 recombinant protein, over-expressing vectors, siRNA, or functional blocking antibodies will further clarify Gal-3 function. In addition, conditional gene deletions by a promoter-specific tamoxifen inducible Cre system (e.g. loxP-flanked deletion of Gal-3 specifically in GFAP+ astrocytes with temporal control; Hirrlinger et al., 2006) may help to refine our understanding. It will also be important to investigate the functional interaction of different members of the galectin family in the SVZ and after stroke.

Post-stroke tissue inflammation was assessed based on the morphology and proliferation of reactive astrocytes and microglia and significant differences in the post-stroke brain between wildtype and Gal-3^{-/-} brains was not found. In support of this assessment, the levels of apoptosis in the ischaemic striata were similar. Nevertheless, direct measurements of inflammatory mediators such as IL-1 β and TNF α would be useful to confirm the similar degrees of inflammatory response. However, it may be more

worthwhile to perform these measurements in future acute gain- or loss- of Gal-3 function MCAO experiments if significant differences in infarct volume or neurological outcome are observed, rather than in samples from the current cohort of wildtype and Gal-3^{-/-} mice.

In the present studies, mice were sacrificed after a maximum of 14 days post-MCAO. A small number of neuroblasts were found in the ischaemic penumbra which acquired mature neuronal phenotype, although the numbers were too small to perform meaningful statistical comparison. Therefore, even though the number of ectopic neuroblasts in the ischaemic striatum increased in Gal-3^{-/-} brains compared to wildtypes, their eventual fate was uncertain. Loss of Gal-3 may impact significantly on their survival, differentiation or integration. Future work will need to examine the medium and long term survival and differentiation of ectopic neuroblasts in order to fully appreciate the role of Gal-3 in post-stroke neurogenesis. Similarly, the loss of pro-proliferative interaction between the vasculature and neuroblasts in the Gal3^{-/-} ischaemic striatum, and its molecular basis, need to be investigated. As already discussed, this may be mediated via an integrin-dependent mechanism.

7.3. Future directions

Over the past decade of intense research, much attention has focused on mitogens and chemotactic molecules originating from the site of ischaemic injury which are believed to diffuse to the SVZ and result in increased cell proliferation and neuroblast recruitment towards the injured brain regions. There have been attempts to identify transcriptional changes in the SVZ although these have again focused on mitogens and cytokines (Liu et al., 2006, 2007a). I envisage this thesis will emphasise the importance of both the SVZ niche and the ischaemic brain tissue as inextricable components of post-stroke

neurogenesis, and that understanding of these changes and their targeted manipulation may lead to the development of novel neurorestorative therapies.

The role of the ependyma as a metabolic barrier warrants further research. Although ependymal cells do not have tight junctions, there is evidence that they regulate the passage of fluid and molecules across the brain / CSF interface (Del Bigio, 1995). It will be interesting to determine how ependymal transport function is altered by stroke, and the extent to which it affects the SVZ niche. The biochemical composition of CSF changes after stroke, and choroid plexus cells secrete anti-inflammatory and neuroprotective molecules (Matsumoto et al., 2010), further strengthening the case for a renewed focus on the CSF. Clinical stroke increases the risk of neuropathologies such as Alzheimer's disease (Honig et al., 2003) and idiopathic communicating hydrocephalus (Krauss et al., 1996; Boon et al., 1999). Chronic ependymal dysfunction may contribute towards the pathogenesis of these conditions by dysregulation of fluid transport and metabolic exchange between the brain parenchyma and CSF. Similarly, vascular changes in the post-stroke SVZ and altered astroglial endfeet suggest possible changes in blood brain barrier integrity, and ultrastructural or functional studies will be useful to determine whether molecules from the systemic circulation may have increased access to the SVZ and CNS in general after stroke (Jiang et al., 2008).

Gal-3 remains an enigmatic molecule and in addition to its effect on constitutive neuroblast migration and angiogenesis and neural precursor / vascular interaction in the ischaemic striatum, it is likely to facilitate additional aspects of neural stem cell niche regulation. As mentioned above, acute gain- and loss- of Gal-3 function experiments with *in vivo* and *in vitro* approaches are strategies to elucidate its potential function in vascular basement membrane signalling. In addition, the Szele Laboratory is also examining the role of Gal-3 in T-cell recruitment in a Theiler's murine encephalomyelitis model

(TMEV) of multiple sclerosis, and comparison of Gal-3's role between ischaemic stroke and TMEV in the brain will provide further clues regarding its role in neuroinflammation. HIF-1 α has been implicated in the neuroprotective effects of ischaemic preconditioning (Harten et al., 2010) and is an upstream regulator of both VEGF and Gal-3. Loss of Gal-3 is associated with decreased levels of VEGF post-stroke. The potential interaction between these 3 molecules in relation to stroke neuroprotection and the neural stem cell niche will be a worthy topic for further research.

This thesis proposes that the ischaemic brain regions can be regarded as a neural stem cell niche and that the logical steps which have been taken to understand adult neurogenesis in the SVZ and SGZ can be similarly applied. Although the SVZ origin of striatum-bound neuroblasts has been clearly demonstrated (Yamashita et al., 2006; Hou et al., 2008) very little is known about their fate choice, heterogeneity or proliferative potential. It is not known whether astrocyte-like stem cells and transit amplifying cells also migrate ectopically into the ischaemic striatum. If they did, would they retain the capacity to self-renewal and proliferate respectively? In the presumably hostile environment of the ischaemic striatum (Ekdahl et al., 2009), ectopic neuroblasts do not survive well. From a therapeutic perspective, what strategies could enhance their survival and differentiation? Lastly, what are their intended functions? Given their innate programmed fate to become GABAergic interneurons, is it sensible to believe that in order to mediate clinical improvement and brain repair, neuroblasts in the ischaemic striatum need to differentiate and synaptically integrate in to the neural network? Or are these cells possibly better suited to mediate neuroprotection via immunomodulation and trophic support with a high rate of turnover? Alternatively, could SVZ-derived cells potentially serve to mediate neural plasticity in a similar manner to their normal function in the OB? These are some of the important questions that await the scientific community.

REFERENCES

- ACAS (1995). Endarterectomy for asymptomatic carotid artery stenosis. Executive Committee for the Asymptomatic Carotid Atherosclerosis Study. *JAMA* 273, 1421–1428.
- Aguirre, A. A., Chittajallu, R., Belachew, S., and Gallo, V. (2004). NG2-expressing cells in the subventricular zone are type C-like cells and contribute to interneuron generation in the postnatal hippocampus. *J Cell Biol* 165, 575–589.
- Ahmad, N., Gabius, H.-J., Andre, S., Kaltner, H., Sabesan, S., Roy, R., Liu, B., Macaluso, F., and Brewer, C. F. (2004). Galectin-3 Precipitates as a Pentamer with Synthetic Multivalent Carbohydrates and Forms Heterogeneous Cross-linked Complexes. *J. Biol. Chem.* 279, 10841–10847.
- Akahani, S., Nangia-Makker, P., Inohara, H., Kim, H. R., and Raz, A. (1997). Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res.* 57, 5272–5276.
- Altman, J., and Das, G. D. (1965). Post-Natal Origin of Microneurons in the Rat Brain. *Nature* 207, 953–956.
- Andrikopoulos, S., Blair, A. R., Deluca, N., Fam, B. C., and Proietto, J. (2008). Evaluating the glucose tolerance test in mice. *American Journal of Physiology - Endocrinol Metab* 295, 1323–1332.
- Anton, E. S., Ghashghaei, H. T., Weber, J. L., McCann, C., Fischer, T. M., Cheung, I. D., Gassmann, M., Messing, A., Klein, R., Schwab, M. H., et al. (2004). Receptor tyrosine kinase ErbB4 modulates neuroblast migration and placement in the adult forebrain. *Nat. Neurosci* 7, 1319–1328.
- Argüeso, P., and Panjwani, N. (2011). Focus on molecules: galectin-3. *Exp. Eye Res.* 92, 2–3.
- Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 8, 963–970.
- Asahi, M., Huang, Z., Thomas, S., Yoshimura, S., Sumii, T., Mori, T., Qiu, J., Amin-Hanjani, S., Huang, P. L., Liao, J. K., et al. (2005). Protective effects of statins involving both eNOS and tPA in focal cerebral ischemia. *J Cereb Blood Flow Metab* 25, 722–729.
- Attwell, D., Buchan, A. M., Charpak, S., Lauritzen, M., MacVicar, B. A., and Newman, E. A. (2010). Glial and neuronal control of brain blood flow. *Nature* 468, 232–243.
- Bacigaluppi, M., Pluchino, S., Jametti, L. P., Kilic, E., Kilic, U., Salani, G., Brambilla, E., West, M. J., Comi, G., Martino, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132, 2239–2251.
- Baird, T. A., Parsons, M. W., Phan, T., Butcher, K. S., Desmond, P. M., Tress, B. M., Colman, P. G., Chambers, B. R., and Davis, S. M. (2003). Persistent poststroke hyperglycemia is independently associated with infarct expansion and worse clinical outcome. *Stroke* 34, 2208–2214.

- Banizs, B., Pike, M. M., Millican, C. L., Ferguson, W. B., Komlosi, P., Sheetz, J., Bell, P. D., Schwiebert, E. M., and Yoder, B. K. (2005). Dysfunctional cilia lead to altered ependyma and choroid plexus function, and result in the formation of hydrocephalus. *Development* 132, 5329–5339.
- Barber, P. A., Hoyte, L., Colbourne, F., and Buchan, A. M. (2004). Temperature-regulated model of focal ischemia in the mouse: a study with histopathological and behavioral outcomes. *Stroke* 35, 1720–1725.
- Barkho, B. Z., Munoz, A. E., Li, X., Li, L., Cunningham, L. A., and Zhao, X. (2008). Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. *Stem Cells* 26, 3139–3149.
- Batista-Brito, R., Close, J., Machold, R., and Fishell, G. (2008). The distinct temporal origins of olfactory bulb interneuron subtypes. *J. Neurosci* 28, 3966–3975.
- Beck, H., Acker, T., Wiessner, C., Allegrini, P. R., and Plate, K. H. (2000). Expression of Angiopoietin-1, Angiopoietin-2, and Tie Receptors after Middle Cerebral Artery Occlusion in the Rat. *Am J Pathol* 157, 1473–1483.
- Beck, H., and Plate, K. (2009). Angiogenesis after cerebral ischemia. *Acta Neuropathol* 117, 481–496.
- Beckervordersandforth, R., Tripathi, P., Ninkovic, J., Bayam, E., Lepier, A., Stempfhuber, B., Kirchhoff, F., Hirrlinger, J., Haslinger, A., Lie, D. C., et al. (2010). In vivo fate mapping and expression analysis reveals molecular hallmarks of prospectively isolated adult neural stem cells. *Cell Stem Cell* 7, 744–758.
- Bederson, J. B., Pitts, L. H., Germano, S. M., Nishimura, M. C., Davis, R. L., and Bartkowski, H. M. (1986a). Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* 17, 1304–1308.
- Bederson, J. B., Pitts, L. H., Tsuji, M., Nishimura, M. C., Davis, R. L., and Bartkowski, H. (1986b). Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* 17, 472–476.
- Belkin, A. Granulocyte-colony Stimulating Factor for Stem Cells Therapy for Acute Ischemic Stroke (STEMTHER). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2009. Available from: <http://clinicaltrials.gov/ct2/show/NCT00901381> NLM Identifier: NCT00901381.
- Belvindrah, R., Hankel, S., Walker, J., Patton, B. L., and Müller, U. (2007). Beta1 integrins control the formation of cell chains in the adult rostral migratory stream. *J. Neurosci* 27, 2704–2717.
- Benraiss, A., Chmielnicki, E., Lerner, K., Roh, D., and Goldman, S. A. (2001). Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J. Neurosci* 21, 6718–6731.
- Bernier, P. J., Vinet, J., Cossette, M., and Parent, A. (2000). Characterization of the subventricular zone of the adult human brain: evidence for the involvement of Bcl-2. *Neurosci Res* 37, 67–78.
- Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Felzi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K.-ichi, et al. (1994). Galectins: A family of animal beta-galactoside-binding lectins. *Cell* 76, 597–598.

- Bolteus, A. J., and Bordey, A. (2004). GABA release and uptake regulate neuronal precursor migration in the postnatal subventricular zone. *J. Neurosci.* *24*, 7623–7631.
- Boon, A. J. W., Tans, J. T. J., Delwel, E. J., Egeler-Peerdeman, S. M., Hanlo, P. W., Wurzer, H. A. L., and Hermans, J. (1999). Dutch Normal-Pressure Hydrocephalus Study: the role of cerebrovascular disease. *J Neurosurg* *90*, 221–226.
- Bosnell, R. A., Kincses, T., Stagg, C. J., Tomassini, V., Kischka, U., Jbabdi, S., Woolrich, M. W., Andersson, J., Matthews, P. M., and Johansen-Berg, H. (2011). Motor practice promotes increased activity in brain regions structurally disconnected after subcortical stroke. *Neurorehabil Neural Repair* *25*, 607–616.
- Boutin, C., Hardt, O., de Chevigny, A., Coré, N., Goebbels, S., Seidenfaden, R., Bosio, A., and Cremer, H. (2010). NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. *Proc. Natl. Acad. Sci. U.S.A.* *107*, 1201–1206.
- Bovetti, S., Hsieh, Y.-C., Bovolin, P., Perroteau, I., Kazunori, T., and Puche, A. C. (2007). Blood vessels form a scaffold for neuroblast migration in the adult olfactory bulb. *J. Neurosci.* *27*, 5976–5980.
- Brill, M. S., Ninkovic, J., Winpenny, E., Hodge, R. D., Ozen, I., Yang, R., Lepier, A., Gascon, S., Erdelyi, F., Szabo, G., et al. (2009). Adult generation of glutamatergic olfactory bulb interneurons. *Nat Neurosci* *12*, 1524–1533.
- Brinjikji, W., Rabinstein, A. A., Kallmes, D. F., and Cloft, H. J. (2011). Patient Outcomes With Endovascular Embolectomy Therapy for Acute Ischemic Stroke. *Stroke* *42*, 1648–1652.
- Buchan, A. M., Xue, D., and Slivka, A. (1992). A new model of temporary focal neocortical ischemia in the rat. *Stroke* *23*, 273–279.
- Buffo, A., Rite, I., Tripathi, P., Lepier, A., Colak, D., Horn, A.-P., Mori, T., and Götz, M. (2008). Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. *Proc. Natl. Acad. Sci. U.S.A* *105*, 3581–3586.
- Buffo, A., Rolando, C., and Ceruti, S. (2010). Astrocytes in the damaged brain: Molecular and cellular insights into their reactive response and healing potential. *Biochem. Pharmacol* *79*, 77–89.
- Bulkley, G. B. (1987). Free radical-mediated reperfusion injury: a selective review. *Br. J. Cancer Suppl.* *8*, 66–73.
- Burns, T. C., Catherine M. Verfaillie, and Walter C. Low (2009). Stem cells for ischemic brain injury: A critical review. *J Comp Neurol* *515*, 125–144.
- Cajal, S. R. (1991). *Cajal's Degeneration and Regeneration of the Nervous System*. New York. Oxford University Press.
- Calzà, L., Giardino, L., Pozza, M., Bettelli, C., Micera, A., and Aloe, L. (1998). Proliferation and phenotype regulation in the subventricular zone during experimental allergic encephalomyelitis: in vivo evidence of a role for nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A* *95*, 3209–3214.

- Camby, I., Belot, N., Rorive, S., Lefranc, F., Maurage, C. A., Lahm, H., Kaltner, H., Hadari, Y., Ruchoux, M. M., Brotschi, J., et al. (2001). Galectins are differentially expressed in supratentorial pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas and glioblastomas, and significantly modulate tumor astrocyte migration. *Brain Pathol* 11, 12–26.
- Canning, P., Glenn, J. V., Hsu, D. K., Liu, F. T., Gardiner, T. A., and Stitt, A. W. (2007). Inhibition of advanced glycation and absence of Galectin-3 prevent blood-retinal barrier dysfunction during short-term diabetes. *Exp Diabetes Res* 2007, 51837.
- Capela, A., and Temple, S. (2002). LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* 35, 865–875.
- Carlen, M., Meletis, K., Goritz, C., Darsalia, V., Evergren, E., Tanigaki, K., Amendola, M., Barnabe-Heider, F., Yeung, M. S. Y., Naldini, L., et al. (2009). Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. *Nat Neurosci* 12, 259–267.
- Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* 407, 249–257.
- Carmichael, S. T. (2005). Rodent Models of Focal Stroke: Size, Mechanism, and Purpose. *NeuroRx* 2, 396–409.
- Cayre, M., Canoll, P., and Goldman, J. E. (2009). Cell migration in the normal and pathological postnatal mammalian brain. *Prog Neurobiol* 88, 41–63.
- Chandra, S., Fornai, F., Kwon, H.-B., Yazdani, U., Atasoy, D., Liu, X., Hammer, R. E., Battaglia, G., German, D. C., Castillo, P. E., et al. (2004). Double-knockout mice for α - and β -synucleins: Effect on synaptic functions. *Proc. Natl. Acad. Sci. U.S.A* 101, 14966–14971.
- Chauveau, F., Moucharraffie, S., Wiart, M., Brisset, J.-C., Berthezène, Y., Nighoghossian, N., and Cho, T.-H. (2010). In vivo MRI assessment of permanent middle cerebral artery occlusion by electrocoagulation: pitfalls of procedure. *Exp Transl Stroke Med* 2, 4.
- Chen, H.-Y., Fermin, A., Vardhana, S., Weng, I.-C., Lo, K. F. R., Chang, E.-Y., Maverakis, E., Yang, R.-Y., Hsu, D. K., Dustin, M. L., et al. (2009). Galectin-3 negatively regulates TCR-mediated CD4⁺ T-cell activation at the immunological synapse. *Proc. Natl. Acad. Sci. U.S.A* 106, 14496–14501.
- Chen, J., Braet, F., Brodsky, S., Weinstein, T., Romanov, V., Noiri, E., and Goligorsky, M. S. (2002). VEGF-induced mobilization of caveolae and increase in permeability of endothelial cells. *Am J Physiol Cell Physiol* 282, C1053–C1063.
- Chen, J., Li, Y., Wang, L., Lu, M., Zhang, X., and Chopp, M. (2001). Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci* 189, 49–57.
- Chen, J., Zhang, C., Jiang, H., Li, Y., Zhang, L., Robin, A., Katakowski, M., Lu, M., and Chopp, M. (2005). Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab* 25, 281–290.
- Chen, R.-L., Balami, J. S., Esiri, M. M., Chen, L.-K., and Buchan, A. M. (2010). Ischemic stroke in the elderly: an overview of evidence. *Nat Rev Neurol* 6, 256–265.

- Chen, R. L., Balami, J. S., Young, C. C., Chiou, S. H., Chien, Y., Chen, L. K., Szele, F. G., and Buchan, A. M. (2011). Stem cell therapy for ischaemic stroke in the elderly. In *Advances in Medicine and Biology*, Volume 38. Nova Publishers.
- Chen, Y., Ito, A., Takai, K., and Saito, N. (2008). Blocking pterygopalatine arterial blood flow decreases infarct volume variability in a mouse model of intraluminal suture middle cerebral artery occlusion. *J Neurosci Methods* 174, 18–24.
- Chiaramello, S., Dalmasso, G., Bezin, L., Marcel, D., Jourdan, F., Peretto, P., Fasolo, A., and Marchis, S. D. (2007). BDNF/ TrkB interaction regulates migration of SVZ precursor cells via PI3-K and MAP-K signalling pathways. *Eur J Neurosci* 26, 1780–1790.
- Chiasson, B. J., Tropepe, V., Morshead, C. M., and van der Kooy, D. (1999). Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. *J Neurosci* 19, 4462–4471.
- Chojnacki, A. K., Mak, G. K., and Weiss, S. (2009). Identity crisis for adult periventricular neural stem cells: subventricular zone astrocytes, ependymal cells or both? *Nat Rev Neurosci* 10, 153–163.
- Christopherson, K. S., Ullian, E. M., Stokes, C. C. A., Mallowney, C. E., Hell, J. W., Agah, A., Lawler, J., Mosher, D. F., Bornstein, P., and Barres, B. A. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120, 421–433.
- Colnot, C., Fowles, D., Ripoche, M.-A., Bouchaert, I., and Poirier, F. (1998a). Embryonic implantation in galectin 1/galectin 3 double mutant mice. *Dev Dyn* 211, 306–313.
- Colnot, C., Ripoche, M. A., Milon, G., Montagutelli, X., Crocker, P. R., and Poirier, F. (1998b). Maintenance of granulocyte numbers during acute peritonitis is defective in galectin-3-null mutant mice. *Immunology* 94, 290–296.
- Colognato, H., Baron, W., Avellana-Adalid, V., Relvas, J. B., Baron-Van Evercooren, A., Georges-Labouesse, E., and French-Constant, C. (2002). CNS integrins switch growth factor signalling to promote target-dependent survival. *Nat. Cell Biol.* 4, 833–841.
- Colognato, H., French-Constant, C., and Feltri, M. L. (2005). Human diseases reveal novel roles for neural laminins. *Trends Neurosci.* 28, 480–486.
- Comte, I., Kim, Y., Young, C. C., van der Harg, J. M., Hockberger, P., Bolam, P. J., Poirier, F., and Szele, F. G. (2011). Galectin-3 maintains cell motility from the subventricular zone to the olfactory bulb. *J. Cell. Sci.* 124, 2438–2447.
- Comte, I., Tran, P. B., and Szele, F. G. (2007). Techniques and strategies to analyze neural progenitor cell migration. *Curr Pharm Biotechnol* 8, 177–185.
- Connolly, E. S., Winfree, C. J., Stern, D. M., Solomon, R. A., and Pinsky, D. J. (1996). Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia. *Neurosurgery* 38, 523–531; discussion 532.
- Conover, J. C., and Notti, R. Q. (2007). The neural stem cell niche. *Cell Tissue Res* 331, 211–224.
- Cooper, A. (1836). Some experiments and observations on tying the carotid and vertebral arteries, and the pneumo-gastric, phrenic and sympathetic nerves. *Guys Hosp Rep* 1, 457–475.
- Cooper, D. N. W. (2002). Galectinomics: finding themes in complexity. *Biochim Biophys Acta* 1572, 209–231.

- Cooper, O., Isacson, O. (2004) Intrastriatal transforming growth factor alpha delivery to a model of Parkinson's disease induces proliferation and migration of endogenous adult neural progenitor cells without differentiation into dopaminergic neurons. *J Neurosci* 24, 8924–8931.
- Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., et al. (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* 367, 455–459.
- Curtis, M. A., Kam, M., Nannmark, U., Anderson, M. F., Axell, M. Z., Wikkelsø, C., Holtas, S., van Roon-Mom, W. M. C., Bjork-Eriksson, T., Nordborg, C., et al. (2007). Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science* 315, 1243–1249.
- Daadi, M. M., Davis, A. S., Arac, A., Li, Z., Maag, A.-L., Bhatnagar, R., Jiang, K., Sun, G., Wu, J. C., and Steinberg, G. K. (2010). Human neural stem cell grafts modify microglial response and enhance axonal sprouting in neonatal hypoxic-ischemic brain injury. *Stroke* 41, 516–523.
- Dagher, S. F., Wang, J. L., and Patterson, R. J. (1995). Identification of galectin-3 as a factor in pre-mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1213–1217.
- De Marchis, S., Fasolo, A., Shipley, M., and Puche, A. (2001). Unique neuronal tracers show migration and differentiation of SVZ progenitors in organotypic slices. *J. Neurobiol.* 49, 326–338.
- Deacon, R. M. J. (2006). Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. *Nat Protoc* 1, 118–121.
- Debray, C., Vereecken, P., Belot, N., Teillard, P., Brion, J.-P., Pandolfo, M., and Pochet, R. (2004). Multifaceted role of galectin-3 on human glioblastoma cell motility. *Biochem Biophys Res Commun* 325, 1393–1398.
- Deierborg, T., Roybon, L., Inacio, A. R., Pesic, J., and Brundin, P. (2010). Brain injury activates microglia that induce neural stem cell proliferation ex vivo and promote differentiation of neurosphere-derived cells into neurons and oligodendrocytes. *Neuroscience* 171, 1386–1396.
- Del Bigio, M. (2010). Ependymal cells: biology and pathology. *Acta Neuropathologica* 119, 55–73.
- Del Bigio, M. R. (1995). The ependyma: a protective barrier between brain and cerebrospinal fluid. *Glia* 14, 1–13.
- Detante, O. Intravenous Stem Cells After Ischemic Stroke (ISIS). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2009- [cited 2011/09/10]. <http://clinicaltrials.gov/ct2/show/NCT00875654> NLM Identifier: NCT00875654.
- Dimitrijevic, O. B., Stamatovic, S. M., Keep, R. F., and Andjelkovic, A. V. (2007). Absence of the chemokine receptor CCR2 protects against cerebral ischemia/reperfusion injury in mice. *Stroke* 38, 1345–1353.
- Dirnagl, U. (2010). Rodent Models of Stroke 1st ed. New York. Humana Press.

- Dirnagl, U., Group, Members of the MCAO-SOP. (2010). Standard operating procedures (SOP) in experimental stroke research: SOP for middle cerebral artery occlusion in the mouse. *Nature Precedings*. Available at: <http://precedings.nature.com/documents/3492/version/2>.
- Dizon, M., Szele, F., and Kessler, J. A. (2010). Hypoxia-ischemia induces an endogenous reparative response by local neural progenitors in the postnatal mouse telencephalon. *Dev. Neurosci.* 32, 173–183.
- Doetsch, F. (2003). The glial identity of neural stem cells. *Nat Neurosci* 6, 1127–1134.
- Doetsch, F., and Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. *Proc. Natl. Acad. Sci. U.S.A* 93, 14895–14900.
- Doetsch, F., Caillé, I., Lim, D. A., García-Verdugo, J. M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703–716.
- Doetsch, F., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.* 17, 5046–5061.
- Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J.-M., and Alvarez-Buylla, A. (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron* 36, 1021–1034.
- Doetschman, T. (1999). Interpretation of phenotype in genetically engineered mice. *Lab. Anim. Sci.* 49, 137–143.
- Doverhag, C., Hedtjärn, M., Poirier, F., Mallard, C., Hagberg, H., Karlsson, A., and Sävman, K. (2010). Galectin-3 contributes to neonatal hypoxic-ischemic brain injury. *Neurobiol Dis* 38, 36–46.
- Doyle, K. P., Yang, T., Lessov, N. S., Ciesielski, T. M., Stevens, S. L., Simon, R. P., King, J. S., and Stenzel-Poore, M. P. (2008). Nasal administration of osteopontin peptide mimetics confers neuroprotection in stroke. *J Cereb Blood Flow Metab* 28, 1235–1248.
- Du, C., Hu, R., Csernansky, C. A., Hsu, C. Y., and Choi, D. W. (1996). Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? *J Cereb Blood Flow Metab* 16, 195–201.
- Ekdahl, C. T., Kokaia, Z., and Lindvall, O. (2009). Brain inflammation and adult neurogenesis: The dual role of microglia. *Neuroscience* 158, 1021–1029.
- Elola, M. T., Wolfenstein-Todel, C., Troncoso, M. F., Vasta, G. R., and Rabinovich, G. A. (2007). Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell Mol Life Sci* 64, 1679–1700.
- Emsley, J. G., and Hagg, T. (2003). $\alpha 6 \beta 1$ integrin directs migration of neuronal precursors in adult mouse forebrain. *Exp. Neurol* 183, 273–285.

- Endres, M., Namura, S., Shimizu-Sasamata, M., Waeber, C., Zhang, L., Gomez-Isla, T., Hyman, B. T., and Moskowitz, M. A. (1998). Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J Cereb Blood Flow Metab* 18, 238–247.
- Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.-M., Nordborg, C., Peterson, D. A., and Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nat Med* 4, 1313–1317.
- Feigin, V. L., Lawes, C. M., Bennett, D. A., and Anderson, C. S. (2003). Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol* 2, 43–53.
- Feng, Y., and Walsh, C. A. (2001). Protein-Protein interactions, cytoskeletal regulation and neuronal migration. *Nat Rev Neurosci* 2, 408–416.
- Fiorella, D. J., Turk, A. S., Levy, E. I., Pride, G. L., Woo, H. H., Albuquerque, F. C., Welch, B. G., Niemann, D. B., Aagaard-Kienitz, B., Rasmussen, P. A., et al. (2011). US Wingspan Registry. *Stroke* 42, 1976–1981.
- Fujii, M., Hara, H., Meng, W., Vonsattel, J. P., Huang, Z., and Moskowitz, M. A. (1997). Strain-related differences in susceptibility to transient forebrain ischemia in sf-129 and C57Black/6 mice. *Stroke* 28, 1805–1811.
- Fukumori, T., Oka, N., Takenaka, Y., Nangia-Makker, P., Elsamman, E., Kasai, T., Shono, M., Kanayama, H., Ellerhorst, J., Lotan, R., et al. (2006). Galectin-3 regulates mitochondrial stability and antiapoptotic function in response to anticancer drug in prostate cancer. *Cancer Res.* 66, 3114–3119.
- Fukushi, J.-ichi, Makagiansar, I. T., and Stallcup, W. B. (2004). NG2 proteoglycan promotes endothelial cell motility and angiogenesis via engagement of galectin-3 and alpha3beta1 integrin. *Mol. Biol. Cell* 15, 3580–3590.
- Ganat, Y., Soni, S., Chacon, M., Schwartz, M. L., and Vaccarino, F. M. (2002). Chronic hypoxia up-regulates fibroblast growth factor ligands in the perinatal brain and induces fibroblast growth factor-responsive radial glial cells in the sub-ependymal zone. *Neuroscience* 112, 977–991.
- Ganong, W. (2005). Review of Medical Physiology 22nd ed. New York. McGraw-Hill Medical.
- Garcia, A. D. R., Doan, N. B., Imura, T., Bush, T. G., and Sofroniew, M. V. (2004). GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat Neurosci* 7, 1233–1241.
- Garcia, J. H., Yoshida, Y., Chen, H., Li, Y., Zhang, Z. G., Lian, J., Chen, S., and Chopp, M. (1993). Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol* 142, 623–635.
- George, M. G., Tong, X., Kuklina, E. V., and Labarthe, D. R. (2011). Trends in stroke hospitalizations and associated risk factors among children and young adults, 1995–2008. *Ann Neurol.* 70, 713–721.

- Ghashghaei, H. T., Weber, J., Pevny, L., Schmid, R., Schwab, M. H., Lloyd, K. C. K., Eisenstat, D. D., Lai, C., and Anton, E. S. (2006). The role of neuregulin–ErbB4 interactions on the proliferation and organization of cells in the subventricular zone. *Proc. Natl. Acad. Sci. U.S.A* *103*, 1930–1935.
- Gleason, D., Fallon, J. H., Guerra, M., Liu, J.-C., and Bryant, P. J. (2008). Ependymal stem cells divide asymmetrically and transfer progeny into the subventricular zone when activated by injury. *Neuroscience* *156*, 81–88.
- Gleeson, J. G., Lin, P. T., Flanagan, L. A., and Walsh, C. A. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* *23*, 257–271.
- Goings, G. E., Kozlowski, D. A., and Szele, F. G. (2006). Differential activation of microglia in neurogenic versus non-neurogenic regions of the forebrain. *Glia* *54*, 329–342.
- Goings, G. E., Sahni, V., and Szele, F. G. (2004). Migration patterns of subventricular zone cells in adult mice change after cerebral cortex injury. *Brain Res* *996*, 213–226.
- Gonzalez-Perez, O., Romero-Rodriguez, R., Soriano-Navarro, M., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (2009). Epidermal growth factor induces the progeny of subventricular zone type B cells to migrate and differentiate into oligodendrocytes. *Stem Cells* *27*, 2032–2043.
- Gotts, J. E., and Chesselet, M.-F. (2005). Vascular changes in the subventricular zone after distal cortical lesions. *Exp. Neurol* *194*, 139–150.
- Gould, E., Reeves, A. J., Fallah, M., Tanapat, P., Gross, C. G., and Fuchs, E. (1999). Hippocampal neurogenesis in adult Old World primates. *Proc. Natl. Acad. Sci. U.S.A.* *96*, 5263–5267.
- Gregg, C., and Weiss, S. (2003). Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells. *J. Neurosci* *23*, 11587–11601.
- Gregg, C., Shikar, V., Larsen, P., Mak, G., Chojnacki, A., Yong, V. W., and Weiss, S. (2007). White matter plasticity and enhanced remyelination in the maternal CNS. *J. Neurosci.* *27*, 1812–1823.
- Gross, C. G. (2000). Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci* *1*, 67–73.
- Guirao, B., Meunier, A., Mortaud, S., Aguilar, A., Corsi, J.-M., Strehl, L., Hirota, Y., Desoeuvre, A., Boutin, C., Han, Y.-G., et al. (2010). Coupling between hydrodynamic forces and planar cell polarity orients mammalian motile cilia. *Nat Cell Biol* *12*, 341–350.
- Gunton, J. E., Kulkarni, R. N., Yim, S., Okada, T., Hawthorne, W. J., Tseng, Y.-H., Roberson, R. S., Ricordi, C., O’Connell, P. J., Gonzalez, F. J., et al. (2005). Loss of ARNT/HIF1 beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* *122*, 337–349.
- Gura, T. (2001). Pot-bellied mice point to obesity enzyme. *Science* *294*, 2071–2072.
- Han, Y.-G., and Alvarez-Buylla, A. (2010). Role of primary cilia in brain development and cancer. *Curr Opin in Neurobiol* *20*, 58–67.

- Han, Y.-G., Spassky, N., Romaguera-Ros, M., Garcia-Verdugo, J.-M., Aguilar, A., Schneider-Maunoury, S., and Alvarez-Buylla, A. (2008). Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. *Nat Neurosci* *11*, 277–284.
- Hanna, J. H., Saha, K., and Jaenisch, R. (2010). Pluripotency and Cellular Reprogramming: Facts, Hypotheses, Unresolved Issues. *Cell* *143*, 508–525.
- Harten, S. K., Ashcroft, M., and Maxwell, P. H. (2010). Prolyl hydroxylase domain inhibitors: a route to HIF activation and neuroprotection. *Antioxid. Redox Signal.* *12*, 459–480.
- Harvey, J., and Rasmussen, T. (1951). Occlusion of the middle cerebral artery; an experimental study. *AMA Arch Neurol Psychiatry* *66*, 20–29.
- Hayashi, T., Noshita, N., Sugawara, T., and Chan, P. H. (2003). Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. *J. Cereb. Blood Flow Metab* *23*, 166–180.
- Heinrich, C., Blum, R., Gascón, S., Masserdotti, G., Tripathi, P., Sánchez, R., Tiedt, S., Schroeder, T., Götz, M., and Berninger, B. (2010). Directing astroglia from the cerebral cortex into subtype specific functional neurons. *PLoS Biol* *8*, e1000373.
- Hess, D. C., and Borlongan, C. V. (2008). Cell-based therapy in ischemic stroke. *Expert Rev Neurother* *8*, 1193–1201.
- Hirrlinger, P. G., Scheller, A., Braun, C., Hirrlinger, J., and Kirchhoff, F. (2006). Temporal control of gene recombination in astrocytes by transgenic expression of the tamoxifen-inducible DNA recombinase variant CreERT2. *Glia* *54*, 11–20.
- Ho, M. K., and Springer, T. A. (1982). Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. *J. Immunol.* *128*, 1221–1228.
- Holmes, K., Roberts, O. L., Thomas, A. M., and Cross, M. J. (2007). Vascular endothelial growth factor receptor-2: Structure, function, intracellular signalling and therapeutic inhibition. *Cellular Signalling* *19*, 2003–2012.
- Honig, L. S., Tang, M.-X., Albert, S., Costa, R., Luchsinger, J., Manly, J., Stern, Y., and Mayeux, R. (2003). Stroke and the risk of Alzheimer disease. *Arch Neurol* *60*, 1707–1712.
- Hosomi, N., Lucero, J., Heo, J. H., Koziol, J. A., Copeland, B. R., and del Zoppo, G. J. (2001). Rapid differential endogenous plasminogen activator expression after acute middle cerebral artery occlusion. *Stroke* *32*, 1341–1348.
- Hou, S.-W., Wang, Y.-Q., Xu, M., Shen, D.-H., Wang, J.-J., Huang, F., Yu, Z., and Sun, F.-Y. (2008). Functional integration of newly generated neurons into striatum after cerebral ischemia in the adult rat brain. *Stroke* *39*, 2837–2844.
- Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* *5*, 1806–1814.
- Howells, D. W., Porritt, M. J., Rewell, S. S., O'Collins, V., Sena, E. S., van der Worp, H. B., Traystman, R. J., and Macleod, M. R. (2010). Different strokes for different folks: the rich diversity of animal models of focal cerebral ischemia. *J Cereb Blood Flow Metab* *30*, 1412–1431.

- Hoyte, L., Kaur, J., and Buchan, A. M. (2004). Lost in translation: taking neuroprotection from animal models to clinical trials. *Exp Neurol* 188, 200–204.
- Hu, H., Tomasiewicz, H., Magnuson, T., and Rutishauser, U. (1996). The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. *Neuron* 16, 735–743.
- Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265, 1883–1885.
- Hughes, R. C. (2001). Galectins as modulators of cell adhesion. *Biochimie* 83, 667–676.
- Hunter, A. J., Hatcher, J., Virley, D., Nelson, P., Irving, E., Hadingham, S. J., and Parsons, A. A. (2000). Functional assessments in mice and rats after focal stroke. *Neuropharmacology* 39, 806–816.
- Ihrie, R. A., and Alvarez-Buylla, A. (2011). Lake-front property: A unique germinal niche by the lateral ventricles of the adult brain. *Neuron* 70, 674–686.
- Ihrie, R. A., Shah, J. K., Harwell, C. C., Levine, J. H., Guinto, C. D., Lezameta, M., Kriegstein, A. R., and Alvarez-Buylla, A. (2011). Persistent sonic hedgehog signaling in adult brain determines neural stem cell positional identity. *Neuron* 71, 250–262.
- Imitola, J., Raddassi, K., Park, K. I., Mueller, F.-J., Nieto, M., Teng, Y. D., Frenkel, D., Li, J., Sidman, R. L., Walsh, C. A., et al. (2004). Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1 α /CXC chemokine receptor 4 pathway. *Proc. Natl. Acad. Sci. U.S.A.* 101, 18117–18122.
- Inacio, A. R., Ruscher, K., Leng, L., Bucala, R., and Deierborg, T. (2010). Macrophage migration inhibitory factor promotes cell death and aggravates neurologic deficits after experimental stroke. *J Cereb Blood Flow Metab.* 4, 1093–1106.
- Ishibashi, S., Kuroiwa, T., Sakaguchi, M., Sun, L., Kadoya, T., Okano, H., and Mizusawa, H. (2007). Galectin-1 regulates neurogenesis in the subventricular zone and promotes functional recovery after stroke. *Exp Neurol* 207, 302–313.
- Jablonska, B., Aguirre, A., Raymond, M., Szabo, G., Kitabatake, Y., Sailor, K. A., Ming, G.-L., Song, H., and Gallo, V. (2010). Chordin-induced lineage plasticity of adult SVZ neuroblasts after demyelination. *Nat Neurosci* 13, 541–550.
- Jacques, T., Relvas, J., Nishimura, S., Pytela, R., Edwards, G., Streuli, C., and French-Constant, C. (1998). Neural precursor cell chain migration and division are regulated through different β 1 integrins. *Development* 125, 3167–3177.
- Jacquet, B. V., Salinas-Mondragon, R., Liang, H., Therit, B., Buie, J. D., Dykstra, M., Campbell, K., Ostrowski, L. E., Brody, S. L., and Ghashghaei, H. T. (2009). FoxJ1-dependent gene expression is required for differentiation of radial glia into ependymal cells and a subset of astrocytes in the postnatal brain. *Development* 136, 4021–4031.
- Jaenisch, R., and Young, R. (2008). Stem Cells, the Molecular Circuitry of Pluripotency and Nuclear Reprogramming. *Cell* 132, 567–582.
- James, R., Kim, Y., Hockberger, P. E., and Szele, F. G. (2011). Subventricular zone cell migration: lessons from quantitative two-photon microscopy. *Front Neurosci* 5, 30.

- Jankovski, A., Garcia, C., Soriano, E., and Sotelo, C. (1998). Proliferation, migration and differentiation of neuronal progenitor cells in the adult mouse subventricular zone surgically separated from its olfactory bulb. *Eur J Neurosci* 10, 3853–3868.
- Ji, J. F., He, B. P., Dheen, S. T., and Tay, S. S. W. (2004). Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. *Neurosci Lett.* 355, 236–240.
- Jiang, H.-R., Al Rasebi, Z., Mensah-Brown, E., Shahin, A., Xu, D., Goodyear, C. S., Fukada, S. Y., Liu, F.-T., Liew, F. Y., and Lukic, M. L. (2009). Galectin-3 deficiency reduces the severity of experimental autoimmune encephalomyelitis. *J Immunol.* 182, 1167–1173.
- Jiang, Y., Deacon, R., Anthony, D. C., and Campbell, S. J. (2008). Inhibition of peripheral TNF can block the malaise associated with CNS inflammatory diseases. *Neurobiol. Dis.* 32, 125–132.
- Jin, K., Minami, M., Lan, J. Q., Mao, X. O., Batteur, S., Simon, R. P., and Greenberg, D. A. (2001). Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc Natl Acad Sci U S A.* 98, 4710–4715.
- Jin, K., Sun, Y., Xie, L., Peel, A., Mao, X. O., Batteur, S., and Greenberg, D. A. (2003). Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. *Mol Cell Neurosci* 24, 171–189.
- Jin, K., Wang, X., Xie, L., Mao, X. O., and Greenberg, D. A. (2010). Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice. *Proc. Natl. Acad. Sci. U.S.A* 107, 7993–7998.
- Jin, K., Wang, X., Xie, L., Mao, X. O., Zhu, W., Wang, Y., Shen, J., Mao, Y., Banwait, S., and Greenberg, D. A. (2006). Evidence for stroke-induced neurogenesis in the human brain. *Proc. Natl. Acad. Sci. U.S.A* 103, 13198–13202.
- Jin, K., Zhu, Y., Sun, Y., Mao, X. O., Xie, L., and Greenberg, D. A. (2002). Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A* 99, 11946–11950.
- Jogie-Brahim, S., Feldman, D., and Oh, Y. (2009). Unraveling Insulin-Like Growth Factor Binding Protein-3 Actions in Human Disease. *Endocr Rev* 30, 417–437.
- Johanson, C., Stopa, E., McMillan, P., Roth, D., Funk, J., and Krinke, G. (2011). The distributional nexus of choroid plexus to cerebrospinal fluid, ependyma and brain: toxicologic/pathologic phenomena, periventricular destabilization, and lesion spread. *Toxicol Pathol* 39, 186–212.
- Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisén, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96, 25–34.
- Kalaria, R. N., Cohen, D. L., Premkumar, D. R. D., Nag, S., LaManna, J. C., and Lust, W. D. (1998). Vascular endothelial growth factor in Alzheimer's disease and experimental cerebral ischemia. *Mol. Brain Res* 62, 101–105.
- Kalluri, H. S. G., and Dempsey, R. J. (2011). IGFBP-3 inhibits the proliferation of neural progenitor cells. *Neurochem. Res* 36, 406–411.

- Kandel, E., Schwartz, J., and Jessell, T. (2000). *Principles of Neural Science* 4th ed. New York. McGraw-Hill Medical.
- Kaneko, N., Marín, O., Koike, M., Hirota, Y., Uchiyama, Y., Wu, J. Y., Lu, Q., Tessier-Lavigne, M., Alvarez-Buylla, A., Okano, H., et al. (2010). New neurons clear the path of astrocytic processes for their rapid migration in the adult brain. *Neuron* 67, 213–223.
- Kaplan, M. S., and Hinds, J. W. (1977). Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science* 197, 1092–1094.
- Kariya, Y., Kawamura, C., Tabei, T., and Gu, J. (2010). Bisecting GlcNAc residues on laminin-332 down-regulate galectin-3-dependent keratinocyte motility. *J. Biol. Chem* 285, 3330–3340.
- Kazanis, I., and ffrench-Constant, C. (2011a). Extracellular matrix and the neural stem cell niche. *Dev Neurobiol* 71, 1006–1017.
- Kazanis, I., Lathia, J. D., Vadakkan, T. J., Raborn, E., Wan, R., Mughal, M. R., Eckley, D. M., Sasaki, T., Patton, B., Mattson, M. P., et al. (2010). Quiescence and activation of stem and precursor cell populations in the subependymal zone of the mammalian brain are associated with distinct cellular and extracellular matrix signals. *J. Neurosci.* 30, 9771–9781.
- Kazanis, I., and ffrench-Constant, C. (2011b). The number of stem cells in the subependymal zone of the adult rodent brain is correlated with the number of ependymal cells and not with the volume of the niche. *Stem Cells Dev* 21, 1090–1096.
- Kempermann, G. (2002). Why new neurons? Possible functions for adult hippocampal neurogenesis. *J. Neurosci* 22, 635–638.
- Kerever, A., Schnack, J., Vellinga, D., Ichikawa, N., Moon, C., Arikawa-Hirasawa, E., Efird, J. T., and Mercier, F. (2007). Novel extracellular matrix structures in the neural stem cell niche capture the neurogenic factor fibroblast growth factor 2 from the extracellular milieu. *Stem Cells* 25, 2146–2157.
- Kernie, S. G., and Parent, J. M. (2010). Forebrain neurogenesis after focal Ischemic and traumatic brain injury. *Neurobiol Dis* 37, 267–274.
- Kim, G. H., Mocco, J., Hahn, D. K., Kellner, C. P., Komotar, R. J., Ducruet, A. F., Mack, W. J., and Connolly, E. S. (2008). Protective effect of C5a receptor inhibition after murine reperfused stroke. *Neurosurgery* 63, 122–125; discussion 125–126.
- Kim, Y., and Szele, F. G. (2008). Activation of subventricular zone stem cells after neuronal injury. *Cell Tissue Res* 331, 337–345.
- Kim, Y., Comte, I., Szabo, G., Hockberger, P., and Szele, F. G. (2009). Adult mouse subventricular zone stem and progenitor cells are sessile and epidermal growth factor receptor negatively regulates neuroblast migration. *PLoS ONE* 4, e8122.
- Kirschenbaum, B., Doetsch, F., Lois, C., and Alvarez-Buylla, A. (1999). Adult subventricular zone neuronal precursors continue to proliferate and migrate in the absence of the olfactory bulb. *J. Neurosci.* 19, 2171–2180.

- Koch, A., Poirier, F., Jacob, R., and Delacour, D. (2010). Galectin-3, a novel centrosome-associated protein, required for epithelial morphogenesis. *Mol. Biol. Cell* 21, 219–231.
- Koizumi, H., Higginbotham, H., Poon, T., Tanaka, T., Brinkman, B. C., and Gleeson, J. G. (2006). Doublecortin maintains bipolar shape and nuclear translocation during migration in the adult forebrain. *Nat Neurosci* 9, 779–786.
- Koizumi, J.-ichi, Yoshida, Y., Nakazawa, T., and Ooneda, G. (1986). Experimental studies of ischemic brain edema. *Jpn. J. Stroke* 8, 1–8.
- Kojima, T., Hirota, Y., Ema, M., Takahashi, S., Miyoshi, I., Okano, H., and Sawamoto, K. (2010). Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. *Stem Cells* 28, 545–554.
- Kokaia, Z., Thored, P., Arvidsson, A., and Lindvall, O. (2006). Regulation of Stroke-Induced Neurogenesis in Adult Brain--Recent Scientific Progress. *Cereb. Cortex* 16, i162–i167.
- Kokovay, E., Goderie, S., Wang, Y., Lotz, S., Lin, G., Sun, Y., Roysam, B., Shen, Q., and Temple, S. (2010). Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. *Cell Stem Cell* 7, 163–173.
- Kondziolka, D., Steinberg, G. K., Wechsler, L., Meltzer, C. C., Elder, E., Gebel, J., Decesare, S., Jovin, T., Zafonte, R., Lebowitz, J., et al. (2005). Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J. Neurosurg.* 103, 38–45.
- Kondziolka, D., Wechsler, L., Goldstein, S., Meltzer, C., Thulborn, K. R., Gebel, J., Jannetta, P., DeCesare, S., Elder, E. M., McGrogan, M., et al. (2000). Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* 55, 565–569.
- Kovacs, Z., Ikezaki, K., Samoto, K., Inamura, T., Fukui, M., Kawamata, T., and Finklestein, S. P. (1996). VEGF and flt: Expression time kinetics in rat brain infarct. *Stroke* 27, 1865–1873.
- Kowalska, M. A., Rauova, L., and Poncz, M. (2010). Role of the platelet chemokine platelet factor 4 (PF4) in hemostasis and thrombosis. *Thromb. Res.* 125, 292–296.
- Krauss, J. K., Regel, J. P., Vach, W., Droste, D. W., Borremans, J. J., and Mergner, T. (1996). Vascular risk factors and arteriosclerotic disease in idiopathic normal-pressure hydrocephalus of the elderly. *Stroke* 27, 24–29.
- Kronenberg, G., Gertz, K., Cheung, G., Buffo, A., Kettenmann, H., Götz, M., and Endres, M. (2010). Modulation of fate determinants Olig2 and Pax6 in resident glia evokes spiking neuroblasts in a model of mild brain ischemia. *Stroke* 41, 2944–2949.
- Kuhn, H. G., Dickinson-Anson, H., and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 16, 2027–2033.
- Kuo, C. T., Mirzadeh, Z., Soriano-Navarro, M., RaÅjin, M., Wang, D., Shen, J., Å estan, N., Garcia-Verdugo, J., Alvarez-Buylla, A., Jan, L. Y., et al. (2006). Postnatal deletion of Numb/Numbl like reveals repair and remodeling capacity in the subventricular neurogenic niche. *Cell* 127, 1253–1264.

- Kwiatkowski, T. G., Libman, R. B., Frankel, M., Tilley, B. C., Morgenstern, L. B., Lu, M., Broderick, J. P., Lewandowski, C. A., Marler, J. R., Levine, S. R., et al. (1999). Effects of tissue plasminogen activator for acute ischemic stroke at one year. National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study Group. *N. Engl. J. Med* 340, 1781–1787.
- Lakhan, S., Kirchgessner, A., and Hofer, M. (2009). Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *J Transl Med* 7, 97.
- Lalancette-Hébert, M., Gowing, G., Simard, A., Weng, Y. C., and Kriz, J. (2007). Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *J. Neurosci* 27, 2596–2605.
- Larsen, C. M., and Grattan, D. R. (2010). Prolactin-induced mitogenesis in the subventricular zone of the maternal brain during early pregnancy is essential for normal postpartum behavioral responses in the mother. *Endocrinology* 151, 3805–3814.
- Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C., and Steindler, D. A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. U.S.A* 97, 13883–13888.
- Lazarov, O., Mattson, M. P., Peterson, D. A., Pimplikar, S. W., and van Praag, H. (2010). When neurogenesis encounters aging and disease. *Trends Neurosci.* 33, 569–579.
- Lee, S.-R., Kim, H.-Y., Rogowska, J., Zhao, B.-Q., Bhide, P., Parent, J. M., and Lo, E. H. (2006). Involvement of matrix metalloproteinase in neuroblast cell migration from the subventricular zone after stroke. *J. Neurosci.* 26, 3491–3495.
- Leff, J. A., and Repine, J. E. (1990). Blood cells and ischemia-reperfusion injury. *Blood Cells* 16, 183–191; discussion 191–192.
- Lehtinen, M. K., Zappaterra, M. W., Chen, X., Yang, Y. J., Hill, A. D., Lun, M., Maynard, T., Gonzalez, D., Kim, S., Ye, P., et al. (2011). The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron* 69, 893–905.
- Lein, E. S., Hawrylycz, M. J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A. F., Boguski, M. S., Brockway, K. S., Byrnes, E. J., et al. (2007). Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445, 168–176.
- Leone, D. P., Relvas, J. B., Campos, L. S., Hemmi, S., Brakebusch, C., Fassler, R., French-Constant, C., and Suter, U. (2005). Regulation of neural progenitor proliferation and survival by beta1 integrins. *J Cell Sci* 118, 2589–2599.
- Leventhal, C., Rafii, S., Rafii, D., Shahar, A., and Goldman, S. A. (1999). Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. *Mol Cell Neurosci* 13, 450–464.
- Levy, N. S., Chung, S., Furneaux, H., and Levy, A. P. (1998). Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J Biol Chem* 273, 6417–6423.
- Li, B., Piao, C.-S., Liu, X.-Y., Guo, W.-P., Xue, Y.-Q., Duan, W.-M., Gonzalez-Toledo, M. E., and Zhao, L.-R. (2010a). Brain self-protection: The role of endogenous neural progenitor cells in adult brain after cerebral cortical ischemia. *Brain Res* 1327, 91–102.

- Li, J., Siegel, M., Yuan, M., Zeng, Z., Finnucan, L., Persky, R., Hurn, P. D., and McCullough, L. D. (2010b). Estrogen enhances neurogenesis and behavioral recovery after stroke. *J Cereb Blood Flow Metab.* *31*, 413–425.
- Li, L., Harms, K. M., Ventura, P. B., Lagace, D. C., Eisch, A. J., and Cunningham, L. A. (2010c). Focal cerebral ischemia induces a multilineage cytogenic response from adult subventricular zone that is predominantly gliogenic. *Glia* *58*, 1610–1619.
- Li, L., Lundkvist, A., Andersson, D., Wilhelmsson, U., Nagai, N., Pardo, A. C., Nodin, C., Stahlberg, A., Aprico, K., Larsson, K., et al. (2007). Protective role of reactive astrocytes in brain ischemia. *J Cereb Blood Flow Metab* *28*, 468–481.
- Li, Y., Chopp, M., Chen, J., Wang, L., Gautam, S. C., Xu, Y.-X., and Zhang, Z. (2000). Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. *J Cereb Blood Flow Metab* *20*, 1311–1319.
- Lichtenwalner, R. J., and Parent, J. M. (2006). Adult neurogenesis and the ischemic forebrain. *J. Cereb. Blood Flow Metab* *26*, 1–20.
- Lim, D. A., Tramontin, A. D., Trevejo, J. M., Herrera, D. G., García-Verdugo, J. M., and Alvarez-Buylla, A. (2000). Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* *28*, 713–726.
- Lin, T., He, Y., Wu, G., Khan, M., and Hsu, C. (1993). Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. *Stroke* *24*, 117–121.
- Liu, F., and McCullough, L. D. (2011). Middle cerebral artery occlusion model in rodents: methods and potential pitfalls. *J Biomed Biotechnol* *2011*.
- Liu, F., Schafer, D. P., and McCullough, L. D. (2009a). TTC, fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by middle cerebral artery occlusion. *J. Neurosci. Methods* *179*, 1–8.
- Liu, F., You, Y., Li, X., Ma, T., Nie, Y., Wei, B., Li, T., Lin, H., and Yang, Z. (2009b). Brain injury does not alter the intrinsic differentiation potential of adult neuroblasts. *J. Neurosci.* *29*, 5075–5087.
- Liu, F.-T., and Rabinovich, G. A. (2005). Galectins as modulators of tumour progression. *Nat. Rev. Cancer* *5*, 29–41.
- Liu, G., and Rao, Y. (2003). Neuronal migration from the forebrain to the olfactory bulb requires a new attractant persistent in the olfactory bulb. *J. Neurosci.* *23*, 6651–6659.
- Liu, H. M. (1988). Neovasculature and blood-brain barrier in ischemic brain infarct. *Acta Neuropathol* *75*, 422–426.
- Liu, N.-K., and Xu, X.-M. (2006). beta-tubulin is a more suitable internal control than beta-actin in western blot analysis of spinal cord tissues after traumatic injury. *J. Neurotrauma* *23*, 1794–1801.
- Liu, X. S., Chopp, M., Santra, M., Hozeska-Solgot, A., Zhang, R. L., Wang, L., Teng, H., Lu, M., and Zhang, Z. G. (2008). Functional response to SDF1alpha through over-expression of CXCR4 on adult subventricular zone progenitor cells. *Brain Research* *1226*, 18–26.

- Liu, X. S., Chopp, M., Zhang, X. G., Zhang, R. L., Buller, B., Hozeska-Solgot, A., Gregg, S. R., and Zhang, Z. G. (2009c). Gene profiles and electrophysiology of doublecortin-expressing cells in the subventricular zone after ischemic stroke. *J Cereb Blood Flow Metab* 29, 297–307.
- Liu, X. S., Zhang, Z. G., Zhang, R. L., Gregg, S. R., Meng, H., and Chopp, M. (2007a). Comparison of in vivo and in vitro gene expression profiles in subventricular zone neural progenitor cells from the adult mouse after middle cerebral artery occlusion. *Neuroscience* 146, 1053–1061.
- Liu, X. S., Zhang, Z. G., Zhang, R. L., Gregg, S. R., Wang, L., Yier, T., and Chopp, M. (2007b). Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke. *J Neurosci Res* 85, 2120–2125.
- Liu, X. S., Zhang, Z. G., Zhang, R. L., Gregg, S., Morris, D. C., Wang, Y., and Chopp, M. (2006). Stroke induces gene profile changes associated with neurogenesis and angiogenesis in adult subventricular zone progenitor cells. *J Cereb Blood Flow Metab* 27, 564–574.
- Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ. Res.* 77, 638–643.
- Lledo, P.-M., Alonso, M., and Grubb, M. S. (2006). Adult neurogenesis and functional plasticity in neuronal circuits. *Nat Rev Neurosci* 7, 179–193.
- Loddick, S. A., Liu, X.-J., Lu, Z.-X., Liu, C., Behan, D. P., Chalmers, D. C., Foster, A. C., Vale, W. W., Ling, N., and De Souza, E. B. (1998). Displacement of insulin-like growth factors from their binding proteins as a potential treatment for stroke. *Proc Natl Acad Sci U S A* 95, 1894–1898.
- Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. *Science* 264, 1145–1148.
- Lois, C., and Alvarez-Buylla, A. (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* 90, 2074–2077.
- Longmore, M., Wilkinson, I., and Török, E. (2002). *Oxford Handbook of Clinical Medicine* 5th ed. London. Oxford University Press.
- Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1999). The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. *Immunity* 10, 629–639.
- Louvi, A., and Grove, E. A. (2011). Cilia in the CNS: the quiet organelle claims center stage. *Neuron* 69, 1046–1060.
- Lu, Y. B., Iandiev, I., Hollborn, M., Korber, N., Ulbricht, E., Hirrlinger, P. G., Pannicke, T., Wei, E. Q., Bringmann, A., Wolburg, H. et al. (2011). Reactive glial cells: increased stiffness correlates with increased intermediate filament expression. *FASEB J* 25, 624–631.
- Lu, Z., and Kipnis, J. (2010). Thrombospondin 1—a key astrocyte-derived neurogenic factor. *The FASEB J* 24, 1925–1934.
- Luo, J., Shook, B. A., Daniels, S. B., and Conover, J. C. (2008). Subventricular zone-mediated ependyma repair in the adult mammalian brain. *J. Neurosci* 28, 3804–3813.

- Luo, Y., Cai, J., Xue, H., Miura, T., and Rao, M. S. (2005). Functional SDF1 α /CXCR4 signaling in the developing spinal cord. *J Neurochem* 93, 452–462.
- Luque, A., Carpizo, D. R., and Iruela-Arispe, M. L. (2003). ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. *J Biol Chem* 278, 23656–23665.
- Luskin, M. B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11, 173–189.
- Lyden, P.D., Hemmen, T.M. and Grotta, J.C. The Intravascular Cooling in the Treatment of Stroke 2/3 Trial (ICTuS2/3). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2010- [cited 2011/09/10]. Available from: <http://clinicaltrials.gov/ct2/show/NCT01123161> NLM Identifier: NCT01123161.
- Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T., and Springer, T. A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. U.S.A* 95, 9448–9453.
- Macas, J., Nern, C., Plate, K. H., and Momma, S. (2006). Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *J. Neurosci* 26, 13114–13119.
- MacDougall, N. J. J., and Muir, K. W. (2011). Hyperglycaemia and infarct size in animal models of middle cerebral artery occlusion: systematic review and meta-analysis. *J Cereb Blood Flow Metab* 31, 807–818.
- Mackay, J., and Mensah, G. A. (2004). The Atlas of Heart Disease and Stroke 1st ed. Geneva. World Health Organization.
- Maeda, K., Hata, R., and Hossmann, K. A. (1998). Differences in the cerebrovascular anatomy of C57black/6 and SV129 mice. *Neuroreport* 9, 1317–1319.
- Mak, G. K., and Weiss, S. (2010). Paternal recognition of adult offspring mediated by newly generated CNS neurons. *Nat Neurosci* 13, 753–758.
- Markowska, A. I., Jefferies, K. C., and Panjwani, N. (2011). Galectin-3 modulates cell surface expression and activation of VEGF receptor 2 in human endothelial cells. *J Biol Chem* 286, 29913–29921.
- Marti, H. J. H., Bernaudin, M., Bellail, A., Schoch, H., Euler, M., Petit, E., and Risau, W. (2000). Hypoxia-induced Vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am J Pathol* 156, 965–976.
- Marti-Fabregas, J., Romaguera-Ros, M., Gomez-Pinedo, U., Martinez-Ramirez, S., Jimenez-Xarrie, E., Marin, R., Marti-Vilalta, J.-L., and Garcia-Verdugo, J.-M. (2010). Proliferation in the human ipsilateral subventricular zone after ischemic stroke. *Neurology* 74, 357–365.
- Masuda, T., Isobe, Y., Aihara, N., Furuyama, F., Misumi, S., Kim, T.-S., Nishino, H., and Hida, H. (2007). Increase in neurogenesis and neuroblast migration after a small intracerebral hemorrhage in rats. *Neurosci Lett* 425, 114–119.

- Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R., and Flier, J. S. (2001). A transgenic model of visceral obesity and the metabolic syndrome. *Science* 294, 2166–2170.
- Matsumoto, N., Taguchi, A., Kitayama, H., Watanabe, Y., Ohta, M., Yoshihara, T., Itokazu, Y., Dezawa, M., Suzuki, Y., Sugimoto, H., et al. (2010). Transplantation of cultured choroid plexus epithelial cells via cerebrospinal fluid shows prominent neuroprotective effects against acute ischemic brain injury in the rat. *Neurosci Lett* 469, 283–288.
- Meller, R., Stevens, S. L., Minami, M., Cameron, J. A., King, S., Rosenzweig, H., Doyle, K., Lessov, N. S., Simon, R. P., and Stenzel-Poore, M. P. (2005). Neuroprotection by osteopontin in stroke. *J. Cereb. Blood Flow Metab* 25, 217–225.
- Mendes, K. N., Wang, G. K., Fuller, G. N., and Zhang, W. (2010). JNK mediates insulin-like growth factor binding protein 2/integrin alpha5-dependent glioma cell migration. *Int. J. Oncol.* 37, 143–153.
- Menn, B., Garcia-Verdugo, J. M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D., and Alvarez-Buylla, A. (2006). Origin of oligodendrocytes in the subventricular zone of the adult brain. *J Neurosci.* 26, 7907–7918.
- Mercier, F., Kitasako, J. T., and Hatton, G. I. (2002). Anatomy of the brain neurogenic zones revisited: Fractones and the fibroblast/macrophage network. *J Comp Neurol* 451, 170–188.
- Merkle, F. T., Mirzadeh, Z., and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. *Science* 317, 381–384.
- Merkle, F. T., Tramontin, A. D., Garcia-Verdugo, J. M., Alvarez-Buylla, A., and Nottebohm, F. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl. Acad. Sci. U.S.A* 101, 17528–17532.
- Messier, B., Leblond, C. P., and Smart, I. (1958). Presence of DNA synthesis and mitosis in the brain of young adult mice. *Exp Cell Res* 14, 224–226.
- Millikan, C. (1992). Animal stroke models. *Stroke* 23, 795–797.
- Mirzadeh, Z., Doetsch, F., Sawamoto, K., Wichterle, H., and Alvarez-Buylla, A. (2010a). The subventricular zone en-face: wholemount staining and ependymal flow. *J Vis Exp* 39, e1938
- Mirzadeh, Z., Han, Y.-G., Soriano-Navarro, M., García-Verdugo, J. M., and Alvarez-Buylla, A. (2010b). Cilia organize ependymal planar polarity. *J. Neurosci* 30, 2600–2610.
- Mirzadeh, Z., Merkle, F. T., Soriano-Navarro, M., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (2008). Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. *Cell Stem Cell* 3, 265–278.
- Mobley, A. K., Tchaicha, J. H., Shin, J., Hossain, M. G., and McCarty, J. H. (2009). beta8 integrin regulates neurogenesis and neurovascular homeostasis in the adult brain. *J Cell Sci* 122, 1842–1851.
- Monkkonen, K., Hakumaki, J., Hirst, R., Miettinen, R., O’Callaghan, C., Mannisto, P., and Laitinen, J. (2007). Intracerebroventricular antisense knockdown of G alpha i2 results in ciliary stasis and ventricular dilatation in the rat. *BMC Neurosci* 8, 26.

- Murase, S.-ichi, and Horwitz, A. F. (2002). Deleted in Colorectal Carcinoma and differentially expressed integrins mediate the directional migration of neural precursors in the rostral migratory stream. *J. Neurosci.* 22, 3568–3579.
- Nachtigal, M., Ghaffar, A., and Mayer, E. P. (2008). Galectin-3 gene inactivation reduces atherosclerotic lesions and adventitial inflammation in ApoE-deficient mice. *Am J Pathol* 172, 247–255.
- Nakagomi, T., Taguchi, A., Fujimori, Y., Saino, O., Nakano-Doi, A., Kubo, S., Gotoh, A., Soma, T., Yoshikawa, H., Nishizaki, T., et al. (2009). Isolation and characterization of neural stem/progenitor cells from post-stroke cerebral cortex in mice. *Eur J Neurosci* 29, 1842–1852.
- Nam, S. C., Kim, Y., Dryanovski, D., Walker, A., Goings, G., Woolfrey, K., Kang, S. S., Chu, C., Chenn, A., Erdelyi, F., et al. (2007). Dynamic features of postnatal subventricular zone cell motility: A two-photon time-lapse study. *J Comp Neurol* 505, 190–208.
- Nangia-Makker, P., Balan, V., and Raz, A. (2008). Regulation of Tumor Progression by Extracellular Galectin-3. *Cancer Microenviron.* 1, 43–51.
- Nangia-Makker, P., Honjo, Y., Sarvis, R., Akahani, S., Hogan, V., Pienta, K. J., and Raz, A. (2000a). Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am J Pathol* 156, 899–909.
- Nangia-Makker, P., Honjo, Y., Sarvis, R., Akahani, S., Hogan, V., Pienta, K. J., and Raz, A. (2000b). Galectin-3 Induces Endothelial Cell Morphogenesis and Angiogenesis. *Am J Pathol.* 156, 899–909.
- National Audit Office (2005). Reducing brain damage: Faster access to better stroke care. London. Department of Health, UK
- Nelson, P. T., Kondziolka, D., Wechsler, L., Goldstein, S., Gebel, J., DeCesare, S., Elder, E. M., Zhang, P. J., Jacobs, A., McGrogan, M., et al. (2002). Clonal human (hNT) neuron grafts for stroke therapy: neuropathology in a patient 27 months after implantation. *Am. J. Pathol.* 160, 1201–1206.
- Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 13, 9–22.
- Ng, K. L., Li, J.-D., Cheng, M. Y., Leslie, F. M., Lee, A. G., and Zhou, Q.-Y. (2005). Dependence of olfactory bulb neurogenesis on Prokineticin 2 signaling. *Science* 308, 1923–1927.
- Nguyen Ba-Charvet, K. T. N., Brose, K., Marillat, V., Kidd, T., Goodman, C. S., Tessier-Lavigne, M., Sotelo, C., and Chédotal, A. (1999). Slit2-mediated chemorepulsion and collapse of developing forebrain axons. *Neuron* 22, 463–473.
- Nieminen, J., St-Pierre, C., Bhaumik, P., Poirier, F., and Sato, S. (2008). Role of galectin-3 in leukocyte recruitment in a murine model of lung infection by *Streptococcus pneumoniae*. *J Immunol* 180, 2466–2473.
- Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409, 714–720.
- Nomura, T., Göritz, C., Catchpole, T., Henkemeyer, M., and Frisén, J. (2010). EphB signaling controls lineage plasticity of adult neural stem cell niche cells. *Cell Stem Cell* 7, 730–743.

- O'Connor, J. P. B. (2003). Thomas Willis and the background to *Cerebri Anatome*. *J R Soc Med* 96, 139–143.
- Ocbina, P. J., Dizon, M. L. V., Shin, L., and Szele, F. G. (2006). Doublecortin is necessary for the migration of adult subventricular zone cells from neurospheres. *Mol. Cell. Neurosci* 33, 126–135.
- Ochieng, J., Green, B., Evans, S., James, O., and Warfield, P. (1998). Modulation of the biological functions of galectin-3 by matrix metalloproteinases. *Biochim. Biophys. Acta* 1379, 97–106.
- Ohab, J. J., and Carmichael, S. T. (2008). Poststroke neurogenesis: Emerging principles of migration and localization of immature neurons. *Neuroscientist* 14, 369–380.
- Ohab, J. J., Fleming, S., Blesch, A., and Carmichael, S. T. (2006). A neurovascular niche for neurogenesis after stroke. *J. Neurosci.* 26, 13007–13016.
- Ohira, K., Furuta, T., Hioki, H., Nakamura, K. C., Kuramoto, E., Tanaka, Y., Funatsu, N., Shimizu, K., Oishi, T., Hayashi, M., et al. (2010). Ischemia-induced neurogenesis of neocortical layer 1 progenitor cells. *Nat Neurosci* 13, 173–179.
- Ohtaki, H., Ylostalo, J. H., Foraker, J. E., Robinson, A. P., Reger, R. L., Shioda, S., and Prockop, D. J. (2008). Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses. *Proc. Natl. Acad. Sci. U.S.A* 105, 14638–14643.
- Oliveira, F. L., Chammas, R., Ricon, L., Fermino, M. L., Bernardes, E. S., Hsu, D. K., Liu, F.-T., Borojevic, R., and El-Cheikh, M. C. (2009). Galectin-3 regulates peritoneal B1-cell differentiation into plasma cells. *Glycobiology* 19, 1248–1258.
- Ortinski, P. I., Dong, J., Mungenast, A., Yue, C., Takano, H., Watson, D. J., Haydon, P. G., and Coulter, D. A. (2010). Selective induction of astrocytic gliosis generates deficits in neuronal inhibition. *Nat Neurosci* 13, 584–591.
- Paez-Gonzalez, P., Abdi, K., Luciano, D., Liu, Y., Soriano-Navarro, M., Rawlins, E., Bennett, V., Garcia-Verdugo, J. M., and Kuo, C. T. (2011). Ank3-dependent SVZ niche assembly is required for the continued production of new neurons. *Neuron* 71, 61–75.
- Papaspyridonos, M., McNeill, E., de Bono, J. P., Smith, A., Burnand, K. G., Channon, K. M., and Greaves, D. R. (2008). Galectin-3 is an amplifier of inflammation in atherosclerotic plaque progression through macrophage activation and monocyte chemoattraction. *Arterioscler. Thromb. Vasc. Biol.* 28, 433–440.
- Paratcha, G., Ibáñez, C. F., and Ledda, F. (2006). GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream. *Mol Cell Neurosci* 31, 505–514.
- Parent, J. M., Vexler, Z. S., Gong, C., Derugin, N., and Ferriero, D. M. (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol* 52, 802–813.
- Park, C. K., Mendelow, A. D., Graham, D. I., McCulloch, J., and Teasdale, G. M. (1988). Correlation of triphenyltetrazolium chloride perfusion staining with conventional neurohistology in the detection of early brain ischaemia. *Neuropathol Appl Neurobiol* 14, 289–298.

- Paron, I., Scaloni, A., Pines, A., Bachi, A., Liu, F.-T., Puppini, C., Pandolfi, M., Ledda, L., Di Loreto, C., Damante, G., et al. (2003). Nuclear localization of galectin-3 in transformed thyroid cells: a role in transcriptional regulation. *Biochem Biophys Res Commun* 302, 545–553.
- Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L., and Dennis, J. W. (2004). Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. *Science* 306, 120–124.
- Pasquini, L. A., Millet, V., Hoyos, H. C., Giannoni, J. P., Croci, D. O., Marder, M., Liu, F. T., Rabinovich, G. A., and Pasquini, J. M. (2011). Galectin-3 drives oligodendrocyte differentiation to control myelin integrity and function. *Cell Death Differ.* 18, 1746–1756.
- Pastrana, E., Cheng, L.-C., and Doetsch, F. (2009). Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. *Proc. Natl. Acad. Sci. U.S.A* 106, 6387–6392.
- Pathipati, P., Gorba, T., Scheepens, A., Goffin, V., Sun, Y., and Fraser, M. (2011). Growth hormone and prolactin regulate human neural stem cell regenerative activity. *Neuroscience* 190, 409–427.
- Paxinos, G., and Franklin, K. B. J. (2001). *The Mouse Brain in Stereotaxic Coordinates*. San Diego. Academic Press.
- Pekny, M., Johansson, C. B., Eliasson, C., Stakeberg, J., Wallen, A., Perlmann, T., Lendahl, U., Betsholtz, C., Berthold, C. H. and Frisen, J. (1999). Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. *J Cell Biol* 145, 503–514.
- Pham, M., Helluy, X., Braeuninger, S., Jakob, P., Stoll, G., Kleinschmitz, C., and Bendszus, M. (2010). Outcome of experimental stroke in C57Bl/6 and Sv/129 mice assessed by multimodal ultra-high field MRI. *Exp Transl Stroke Med* 2, 6.
- Piao, C.-S., Gonzalez-Toledo, M. E., Xue, Y.-Q., Duan, W.-M., Terao, S., Granger, D. N., Kelley, R. E., and Zhao, L.-R. (2009). The role of stem cell factor and granulocyte-colony stimulating factor in brain repair during chronic stroke. *J Cereb Blood Flow Metab* 29, 759–770.
- Plane, J. M., Andjelkovic, A. V., Keep, R. F., and Parent, J. M. (2010). Intact and injured endothelial cells differentially modulate postnatal murine forebrain neural stem cells. *Neurobiol Dis* 37, 218–227.
- Plate, K. H., Beck, H., Danner, S., Allegrini, P. R., and Wiessner, C. (1999). Cell type specific upregulation of vascular endothelial growth factor in an MCA-occlusion model of cerebral infarct. *J. Neuropathol. Exp. Neurol* 58, 654–666.
- Porter, S., Clark, I. M., Kevorkian, L., and Edwards, D. R. (2005). The ADAMTS metalloproteinases. *Biochem J* 386, 15–27.
- Pugh, C. W., and Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 9, 677–684.
- Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol* 145, 61–83.

- Ramaswamy, S., Goings, G. E., Soderstrom, K. E., Szele, F. G., and Kozlowski, D. A. (2005). Cellular proliferation and migration following a controlled cortical impact in the mouse. *Brain Res* 1053, 38–53.
- Reichert, F., and Rotshenker, S. (1999). Galectin-3/MAC-2 in experimental allergic encephalomyelitis. *Exp Neurol* 160, 508–514.
- Reynolds, B. A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255, 1707–1710.
- Robel, S., Berninger, B., and Götz, M. (2011). The stem cell potential of glia: lessons from reactive gliosis. *Nat. Rev. Neurosci* 12, 88–104.
- Robel, S., Mori, T., Zoubaa, S., Schlegel, J., Sirko, S., Faissner, A., Goebbels, S., Dimou, L., and Götz, M. (2009). Conditional deletion of $\beta 1$ -integrin in astroglia causes partial reactive gliosis. *Glia* 57, 1630–1647.
- Robin, A. M., Zhang, Z. G., Wang, L., Zhang, R. L., Katakowski, M., Zhang, L., Wang, Y., Zhang, C., and Chopp, M. (2005). Stromal cell-derived factor 1 alpha mediates neural progenitor cell motility after focal cerebral ischemia. *J Cereb Blood Flow Metab* 26, 125–134.
- Robinson, C. J., and Stringer, S. E. (2001). The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J. Cell. Sci* 114, 853–865.
- Rogers, D. C., Fisher, E. M., Brown, S. D., Peters, J., Hunter, A. J., and Martin, J. E. (1997). Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* 8, 711–713.
- Roitbak, T., Li, L., and Cunningham, L. A. (2008). Neural stem/progenitor cells promote endothelial cell morphogenesis and protect endothelial cells against ischemia via HIF-1 alpha-regulated VEGF signaling. *J. Cereb. Blood Flow Metab* 28, 1530–1542.
- Rosa, A. I., Goncalves, J., Cortes, L., Bernardino, L., Malva, J. O., and Agasse, F. (2010). The angiogenic factor Angiopoietin-1 is a proneurogenic peptide on subventricular zone stem/progenitor cells. *J. Neurosci.* 30, 4573–4584.
- Rosenberg, G. A., Cunningham, L. A., Wallace, J., Alexander, S., Estrada, E. Y., Grossetete, M., Razhagi, A., Miller, K., and Gearing, A. (2001). Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. *Brain Res* 893, 104–112.
- Rothwell, P. M., Coull, A. J., Giles, M. F., Howard, S. C., Silver, L. E., Bull, L. M., Gutnikov, S. A., Edwards, P., Mant, D., Sackley, C. M., et al. (2004). Change in stroke incidence, mortality, case-fatality, severity, and risk factors in Oxfordshire, UK from 1981 to 2004 (Oxford Vascular Study). *Lancet* 363, 1925–1933.
- Rudd, A. G., Hoffman, A., Grant, R., Campbell, J. T., and Lowe, D. (2011). Stroke thrombolysis in England, Wales and Northern Ireland: how much do we do and how much do we need? *J Neurol, Neurosurg Psychiatry* 82, 14–19.
- Sakaguchi, M., Shingo, T., Shimazaki, T., Okano, H. J., Shiwa, M., Ishibashi, S., Oguro, H., Ninomiya, M., Kadoya, T., Horie, H., et al. (2006). A carbohydrate-binding protein, Galectin-1, promotes proliferation of adult neural stem cells. *Proc. Natl. Acad. Sci. U.S.A* 103, 7112–7117.

- Sanai, N., Tramontin, A. D., Quinones-Hinojosa, A., Barbaro, N. M., Gupta, N., Kunwar, S., Lawton, M. T., McDermott, M. W., Parsa, A. T., Manuel-Garcia Verdugo, J., et al. (2004). Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature* 427, 740–744.
- Saravanan, C., Liu, F.-T., Gipson, I. K., and Panjwani, N. (2009). Galectin-3 promotes lamellipodia formation in epithelial cells by interacting with complex N-glycans on alpha3 beta1 integrin. *J Cell Sci* 122, 3684–3693.
- Sarnat, H. B. (1995). Ependymal reactions to injury. A review. *J. Neuropathol. Exp. Neurol* 54, 1–15.
- Sasaki, E., Suemizu, H., Shimada, A., Hanazawa, K., Oiwa, R., Kamioka, M., Tomioka, I., Sotomaru, Y., Hirakawa, R., Eto, T., et al. (2009). Generation of transgenic non-human primates with germline transmission. *Nature* 459, 523–527.
- Sawamoto, K., Wichterle, H., Gonzalez-Perez, O., Cholfin, J. A., Yamada, M., Spassky, N., Murcia, N. S., Garcia-Verdugo, J. M., Marin, O., Rubenstein, J. L. R., et al. (2006). New neurons follow the flow of cerebrospinal fluid in the adult brain. *Science* 311, 629–632.
- Scott-Drew, S., and ffrench-Constant, C. (1997). Expression and function of thrombospondin-1 in myelinating glial cells of the central nervous system. *J. Neurosci. Res.* 50, 202–214.
- Shen, Q., Goderie, S. K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., and Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 304, 1338–1340.
- Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S.-M., Goderie, S. K., Roysam, B., and Temple, S. (2008). Adult svz stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell Stem Cell* 3, 289–300.
- Shinjyo, N., Ståhlberg, A., Dragunow, M., Pekny, M., and Pekna, M. (2009). Complement-derived Anaphylatoxin C3a regulates in vitro differentiation and migration of neural progenitor cells. *Stem Cells* 27, 2824–2832.
- Shyu, W.-C., Lin, S.-Z., Lee, C.-C., Liu, D. D., and Li, H. (2006). Granulocyte colony-stimulating factor for acute ischemic stroke: a randomized controlled trial. *CMAJ* 174, 927–933.
- Snappyan, M., Lemasson, M., Brill, M. S., Blais, M., Massouh, M., Ninkovic, J., Gravel, C., Berthod, F., Gotz, M., Barker, P. A., et al. (2009). Vasculature guides migrating neuronal precursors in the adult mammalian forebrain via Brain-derived neurotrophic factor signaling. *J. Neurosci.* 29, 4172–4188.
- Spassky, N., Merkle, F. T., Flames, N., Tramontin, A. D., García-Verdugo, J. M., and Alvarez-Buylla, A. (2005). Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J Neurosci* 25, 10–18.
- Spradling, A., Drummond-Barbosa, D., and Kai, T. (2001). Stem cells find their niche. *Nature* 414, 98–104.
- Stein, I., Itin, A., Einat, P., Skaliter, R., Grossman, Z., and Keshet, E. (1998). Translation of vascular endothelial growth factor mRNA by internal ribosome entry: Implications for translation under hypoxia. *Mol. Cell. Biol.* 18, 3112–3119.
- Steiner, B., Wolf, S., and Kempermann, G. (2006). Adult neurogenesis and neurodegenerative disease. *Regen Med* 1, 15–28.

- Stratmann, G., Sall, J. W., May, L. D. V., Loepke, A. W., and Lee, M. T. (2010). Beyond anesthetic properties: The effects of isoflurane on brain cell death, neurogenesis, and long-term neurocognitive function. *Anesth Analg*, *110*, 431–437.
- Stroemer, P., Patel, S., Hope, A., Oliveira, C., Pollock, K., and Sinden, J. (2009). The Neural Stem Cell Line CTX0E03 Promotes Behavioral Recovery and Endogenous Neurogenesis After Experimental Stroke in a Dose-Dependent Fashion. *Neurorehabil Neural Repair* *23*, 895–909.
- Sugiura, S., Kitagawa, K., Tanaka, S., Todo, K., Omura-Matsuoka, E., Sasaki, T., Mabuchi, T., Matsushita, K., Yagita, Y., and Hori, M. (2005). Adenovirus-mediated gene transfer of heparin-binding epidermal growth factor-like growth factor enhances neurogenesis and angiogenesis after focal cerebral ischemia in rats. *Stroke* *36*, 859–864.
- Suh, S. W., Shin, B. S., Ma, H., Van Hoecke, M., Brennan, A. M., Yenari, M. A., and Swanson, R. A. (2008). Glucose and NADPH oxidase drive neuronal superoxide formation in stroke. *Ann Neurol* *64*, 654–663.
- Sun, Y., Jin, K., Xie, L., Childs, J., Mao, X. O., Logvinova, A., and Greenberg, D. A. (2003). VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest* *111*, 1843–1851.
- Sutherland, B. A., Papadakis, M., Chen, R.-L., and Buchan, A. M. (2011). Cerebral blood flow alteration in neuroprotection following cerebral ischaemia. *J. Physiol.* *589*, 4105–4114.
- Swanson, R. A., Morton, M. T., Tsao-Wu, G., Savalos, R. A., Davidson, C., and Sharp, F. R. (1990). A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* *10*, 290–293.
- Sykora, M., Diedler, J., Poli, S., Rizos, T., Kellert, L., Turcani, P., and Steiner, T. (2011). Association of non-diabetic hyperglycemia with autonomic shift in acute ischaemic stroke. *Eur J Neurol* *19*, 84–90.
- Szele, F. G., and Chesselet, M. F. (1996). Cortical lesions induce an increase in cell number and PSA-NCAM expression in the subventricular zone of adult rats. *J. Comp. Neurol* *368*, 439–454.
- Takaba, H., Fukuda, K., and Yao, H. (2004). Substrain differences, gender, and age of spontaneously hypertensive rats critically determine infarct size produced by distal middle cerebral artery occlusion. *Cell. Mol. Neurobiol.* *24*, 589–598.
- Takenaka, Y., Fukumori, T., and Raz, A. (2004). Galectin-3 and metastasis. *Glycoconjugate Journal* *19*, 543–549.
- Tavazoie, M., Van der Veken, L., Silva-Vargas, V., Louissaint, M., Colonna, L., Zaidi, B., Garcia-Verdugo, J. M., and Doetsch, F. (2008). A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* *3*, 279–288.
- Teramoto, T., Qiu, J., Plumier, J.-C., and Moskowitz, M. A. (2003). EGF amplifies the replacement of parvalbumin-expressing striatal interneurons after ischemia. *J Clin Invest.* *111*, 1125–1132.
- Thomas, L. B., Gates, M. A., and Steindler, D. A. (1996). Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. *Glia* *17*, 1–14.

- Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Darsalia, V., Ekdahl, C. T., Kokaia, Z., and Lindvall, O. (2006). Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* 24, 739–747.
- Thored, P., Heldmann, U., Gomes-Leal, W., Gisler, R., Darsalia, V., Taneera, J., Nygren, J. M., Jacobsen, S.-E. W., Ekdahl, C. T., Kokaia, Z., et al. (2009). Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. *Glia* 57, 835–849.
- Thored, P., Wood, J., Arvidsson, A., Cammenga, J., Kokaia, Z., and Lindvall, O. (2007). Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke* 38, 3032–3039.
- Tissir, F., Qu, Y., Montcouquiol, M., Zhou, L., Komatsu, K., Shi, D., Fujimori, T., Labeau, J., Tyteca, D., Courtoy, P., et al. (2010). Lack of cadherins Celsr2 and Celsr3 impairs ependymal ciliogenesis, leading to fatal hydrocephalus. *Nat Neurosci* 13, 700–707.
- Tomasiewicz, H., Ono, K., Yee, D., Thompson, C., Goridis, C., Rutishauser, U., and Magnuson, T. (1993). Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. *Neuron* 11, 1163–1174.
- Tonchev, A. B., Yamashima, T., Sawamoto, K., and Okano, H. (2005). Enhanced proliferation of progenitor cells in the subventricular zone and limited neuronal production in the striatum and neocortex of adult macaque monkeys after global cerebral ischemia. *J. Neurosci. Res.* 81, 776–788.
- Tramontin, A. D., García-Verdugo, J. M., Lim, D. A., and Alvarez-Buylla, A. (2003). Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb. Cortex* 13, 580–587.
- Tran, P. B., Banisadr, G., Ren, D., Chenn, A., and Miller, R. J. (2007). Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. *J. Comp. Neurol.* 500, 1007–1034.
- Tsai, C. M., Guan, C. H., Hsieh, H. W., Hsu, T. L., Tu, Z., Wu, K. J., Lin, C. H., and Lin, K. I. (2011). Galectin-1 and galectin-8 have redundant roles in promoting plasma cell formation. *J. Immunol.* 187, 1643–1652.
- Tsuchiya, D., Hong, S., Kayama, T., Panter, S. S., and Weinstein, P. R. (2003). Effect of suture size and carotid clip application upon blood flow and infarct volume after permanent and temporary middle cerebral artery occlusion in mice. *Brain Res* 970, 131–139.
- Türeyen, K., Vemuganti, R., Sailor, K. A., and Dempsey, R. J. (2004). Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. *J Neurosci Methods* 139, 203–207.
- Vandercappellen, J., Van Damme, J., and Struyf, S. (2011). The role of the CXC chemokines platelet factor-4 (CXCL4/PF-4) and its variant (CXCL4L1/PF-4var) in inflammation, angiogenesis and cancer. *Cytokine Growth Factor Rev* 22, 1–18.
- Villa-Verde, D. M. S., Silva-Monteiro, E., Jasiulionis, M. G., Farias-de-Oliveira, D. A., Brentani, R. R., Savino, W., and Chammas, R. (2002). Galectin-3 modulates carbohydrate-dependent thymocyte interactions with the thymic microenvironment. *Eur J Immunol* 32, 1434–1444.

- Vyakarnam, A., Dagher, S. F., Wang, J. L., and Patterson, R. J. (1997). Evidence for a role for galectin-1 in pre-mRNA splicing. *Mol. Cell. Biol.* 17, 4730–4737.
- Walther, M., Kuklinski, S., Pesheva, P., Guntinas-Lichius, O., Angelov, D. N., Neiss, W. F., Asou, H., and Probstmeier, R. (2000). Galectin-3 is upregulated in microglial cells in response to ischemic brain lesions, but not to facial nerve axotomy. *J Neurosci Res* 61, 430–435.
- Wang, Y., Jin, K., Mao, X. O., Xie, L., Banwait, S., Marti, H. H., and Greenberg, D. A. (2007). VEGF-overexpressing transgenic mice show enhanced post-ischemic neurogenesis and neuromigration. *J. Neurosci. Res* 85, 740–747.
- Wang, Y., Nangia-Makker, P., Balan, V., Hogan, V., and Raz, A. (2010). Calpain activation through galectin-3 inhibition sensitizes prostate cancer cells to cisplatin treatment. *Cell Death and Dis* 1, e101.
- Warfield, P. R., Makker, P. N., Raz, A., and Ochieng, J. (1997). Adhesion of human breast carcinoma to extracellular matrix proteins is modulated by galectin-3. *Invasion Metastasis* 17, 101–112.
- Wei, B., Nie, Y., Li, X., Wang, C., Ma, T., Huang, Z., Tian, M., Sun, C., Cai, Y., You, Y., et al. (2011). Emx1-expressing neural stem cells in the subventricular zone give rise to new interneurons in the ischemic injured striatum. *Eur J Neurosci.* 33, 819–830.
- Weibel, M., Pettmann, B., Artault, J. C., Sensenbrenner, M., and Labourdette, G. (1986). Primary culture of rat ependymal cells in serum-free defined medium. *Brain Res* 390, 199–209.
- Weller, R. O., Mitchell, J., Griffin, R. L., and Gardner, M. J. (1978). The effects of hydrocephalus upon the developing brain: Histological and quantitative studies of the ependyma and subependyma in hydrocephalic rats. *J Neurol Sci* 36, 383–402.
- White, B.D., Nathe, R.J., Maris, D.O., Nguyen, N.K., Goodson, J.M., Moon, R.T., Horner, P.J. (2010) Beta-catenin signaling increases in proliferating NG2+ progenitors and astrocytes during posttraumatic gliogenesis in the adult brain. *Stem Cells* 28, 297–307.
- Whitman, M. C., and Greer, C. A. (2009). Adult neurogenesis and the olfactory system. *Prog Neurobiol* 89, 162–175.
- Whitman, M. C., Fan, W., Rela, L., Rodriguez-Gil, D. J., and Greer, C. A. (2009). Blood vessels form a migratory scaffold in the rostral migratory stream. *J. Comp. Neurol* 516, 94–104.
- Wichterle, H., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (1997). Direct evidence for homotypic, glia-independent neuronal migration. *Neuron* 18, 779–791.
- Wiebers, D., Adams, H., and Whisnant, J. (1990). Animal models of stroke: are they relevant to human disease? *Stroke* 21, 1–3.
- Wiersma, V. R., de Bruyn, M., Helfrich, W., and Bremer, E. (2011) Therapeutic potential of Galectin-9 in human disease. *Med Res Rev.* Available at: <http://onlinelibrary.wiley.com/doi/10.1002/med.20249/abstract>.
- Wilhelmsson, U., Li, L., Pekna, M., Berthold, C.-H., Blom, S., Eliasson, C., Renner, O., Bushong, E., Ellisman, M., Morgan, T. E., et al. (2004). Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. *J. Neurosci.* 24, 5016–5021.

- Wolfensohn, S., and Lloyd, M. (2003). *Handbook of Laboratory Animal Management and Welfare* 3rd ed. London. Wiley-Blackwell.
- World Health Organisation (2002). *The World Health Report 2002: Reducing Risks to Health, Promoting Healthy Life* Revised edition. Geneva. World Health Organization.
- Wu, W., Wong, K., Chen, J.-hui, Jiang, Z.-hong, Dupuis, S., Wu, J. Y., and Rao, Y. (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* 400, 331–336.
- Yamashita, K., Kataoka, Y., Sakurai-Yamashita, Y., Shigematsu, K., Himeno, A., Niwa, M., and Taniyama, K. (2000). Involvement of glial endothelin/nitric oxide in delayed neuronal death of rat hippocampus after transient forebrain ischemia. *Cell. Mol. Neurobiol* 20, 541–551.
- Yamashita, T., Ninomiya, M., Hernández Acosta, P., García-Verdugo, J. M., Sunabori, T., Sakaguchi, M., Adachi, K., Kojima, T., Hirota, Y., Kawase, T., et al. (2006). Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *J Neurosci* 26, 6627–6636.
- Yan, Y.-P., Kurt A. Sailor, Raghu Vemuganti, and Robert J. Dempsey (2006a). Insulin-like growth factor-1 is an endogenous mediator of focal ischemia-induced neural progenitor proliferation. *Eur J Neurosci* 24, 45–54.
- Yan, Y.-P., Lang, B. T., Vemuganti, R., and Dempsey, R. J. (2009a). Galectin-3 mediates post-ischemic tissue remodeling. *Brain Res.* 55, 826–832.
- Yan, Y.-P., Lang, B. T., Vemuganti, R., and Dempsey, R. J. (2009b). Osteopontin is a mediator of the lateral migration of neuroblasts from the subventricular zone after focal cerebral ischemia. *Neurochem. Int* 55, 826–832.
- Yan, Y.-P., Sailor, K. A., Lang, B. T., Park, S.-W., Vemuganti, R., and Dempsey, R. J. (2006b). Monocyte chemoattractant protein-1 plays a critical role in neuroblast migration after focal cerebral ischemia. *J Cereb Blood Flow Metab* 27, 1213–1224.
- Yang, G., Chan, P. H., Chen, J., Carlson, E., Chen, S. F., Weinstein, P., Epstein, C. J., and Kamii, H. (1994). Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. *Stroke* 25, 165–170.
- Yang, H. K. C., Sundholm-Peters, N. L., Goings, G. E., Walker, A. S., Hyland, K., and Szele, F. G. (2004). Distribution of doublecortin expressing cells near the lateral ventricles in the adult mouse brain. *J Neurosci Res* 76, 282–295.
- Yang, Z., You, Y., and Levison, S. W. (2008). Neonatal hypoxic/ischemic brain injury induces production of calretinin-expressing interneurons in the striatum. *J Comp Neurol.* 511, 19–33.
- Yoshikawa, G., Momiyama, T., Oya, S., Takai, K., Tanaka, J., Higashiyama, S., Saito, N., Kirino, T., and Kawahara, N. (2010). Induction of striatal neurogenesis and generation of region-specific functional mature neurons after ischemia by growth factors. *J. Neurosurg* 113, 835–850.
- Young, C. C., Brooks, K. J., Buchan, A. M., and Szele, F. G. (2011). Cellular and molecular determinants of stroke-induced changes in subventricular zone cell migration. *Antioxid. Redox Signal.* 14, 1877–1888.

- Young, K. M., Fogarty, M., Kessaris, N., and Richardson, W. D. (2007). Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J. Neurosci* 27, 8286–8296.
- Zeng, Y., Danielson, K. G., Albert, T. J., Shapiro, I. M., and Risbud, M. V. (2007). HIF-1 alpha is a regulator of galectin-3 expression in the intervertebral disc. *J. Bone Miner. Res* 22, 1851–1861.
- Zgavc, T., Ceulemans, A.-G., Sarre, S., Michotte, Y., and Hachimi-Idrissi, S. (2011). Experimental and clinical use of therapeutic hypothermia for ischemic stroke: opportunities and limitations. *Stroke Res Treat* 2011.
- Zhang, H., Vutskits, L., Pepper, M. S., and Kiss, J. Z. (2003). VEGF is a chemoattractant for FGF-2-stimulated neural progenitors. *J. Cell Biol* 163, 1375–1384.
- Zhang, R. L., Chopp, M., Gregg, S. R., Toh, Y., Roberts, C., Letourneau, Y., Buller, B., Jia, L., P Nejad Davarani, S., and Zhang, Z. G. (2009). Patterns and dynamics of subventricular zone neuroblast migration in the ischemic striatum of the adult mouse. *J. Cereb. Blood Flow Metab* 29, 1240–1250.
- Zhang, R. L., Chopp, M., Roberts, C., Jia, L., Wei, M., Lu, M., Wang, X., Pourabdollah, S., and Zhang, Z. G. (2010). Ascl1 lineage cells contribute to ischemia-induced neurogenesis and oligodendrogenesis. *J Cereb Blood Flow Metab.* 31, 614–625.
- Zhang, R. L., Chopp, M., Zhang, Z. G., Jiang, Q., and Ewing, J. R. (1997a). A rat model of focal embolic cerebral ischemia. *Brain Res* 766, 83–92.
- Zhang, R. L., LeTourneau, Y., Gregg, S. R., Wang, Y., Toh, Y., Robin, A. M., Gang Zhang, Z., and Chopp, M. (2007a). Neuroblast division during migration toward the ischemic striatum: A study of dynamic migratory and proliferative characteristics of neuroblasts from the subventricular zone. *J. Neurosci.* 27, 3157–3162.
- Zhang, R. L., Zhang, Z. G., Wang, Y., LeTourneau, Y., Liu, X. S., Zhang, X., Gregg, S. R., Wang, L., and Chopp, M. (2007b). Stroke induces ependymal cell transformation into radial glia in the subventricular zone of the adult rodent brain. *J Cereb Blood Flow Metab* 27, 1201–1212.
- Zhang, R. L., Zhang, Z. G., Zhang, L., and Chopp, M. (2001). Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience* 105, 33–41.
- Zhang, R., Zhang, Z., Wang, L., Wang, Y., Goussev, A., Zhang, L., Ho, K.-L., Morshead, C., and Chopp, M. (2004). Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. *J Cereb Blood Flow Metab* 24, 441–448.
- Zhang, Z. G., Zhang, L., Jiang, Q., Zhang, R., Davies, K., Powers, C., Bruggen, N. van, and Chopp, M. (2000). VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J. Clin. Invest.* 106, 829–838.
- Zhang, Z., Chopp, M., Zhang, R. L., and Goussev, A. (1997b). A mouse model of embolic focal cerebral ischemia. *J Cereb Blood Flow Metab* 17, 1081–1088.
- Zhao, C., Deng, W., and Gage, F. H. (2008). Mechanisms and Functional Implications of Adult Neurogenesis. *Cell* 132, 645–660.

- Zhou, M., Sutliff, R. L., Paul, R. J., Lorenz, J. N., Hoying, J. B., Haudenschild, C. C., Yin, M., Coffin, J. D., Kong, L., Kranias, E. G., et al. (1998). Fibroblast growth factor 2 control of vascular tone. *Nat Med* 4, 201–207.
- Zhu, W., Fan, Y., Frenzel, T., Gasmi, M., Bartus, R. T., Young, W. L., Yang, G.-Y., and Chen, Y. (2008). Insulin growth factor-1 gene transfer enhances neurovascular remodeling and improves long-term stroke outcome in mice. *Stroke* 39, 1254–1261.
- Zhu, W., Fan, Y., Hao, Q., Shen, F., Hashimoto, T., Yang, G.-Y., Gasmi, M., Bartus, R. T., Young, W. L., and Chen, Y. (2009). Postischemic IGF-1 gene transfer promotes neurovascular regeneration after experimental stroke. *J Cereb Blood Flow Metab* 29, 1528–1537.
- Zipfel, W. R., Williams, R. M., and Webb, W. W. (2003). Nonlinear magic: multiphoton microscopy in the biosciences. *Nat. Biotechnol.* 21, 1369–1377.
- Zivin, J. A., Fisher, M., DeGirolami, U., Hemenway, C. C., and Stashak, J. A. (1985). Tissue plasminogen activator reduces neurological damage after cerebral embolism. *Science* 230, 1289–1292.

APPENDICES

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Appendix 1. Recipes for solutions

1. Cryoprotectant
2. DNase buffer
3. Homogenisation buffer
4. PFA 4%
5. PB 0.1 M
6. PBS 0.1 M
7. Running buffer
8. TAE buffer
9. TBS 20 mM
10. Transfer buffer

Cryoprotectant

Sucrose	300 g
Ethylene glycol	300 mL
PB 0.1 M	
Final volume	1000 mL

DNase buffer

Tris-hydrochloride	Tris-HCl	10 mM (0.32 g)
Sodium chloride	NaCl	50 mM (0.58 g)
Magnesium chloride	MgCl ₂	10 mM (2 g)
Dithiothreitol	DTT	1 mM
Sodium Phosphate Dibasic Anhydrous	Na ₂ HPO ₄	14.4 g
Glycerol		100 mL
Distilled Water		
Final volume		200 mL

Homogenisation buffer

Tris Base (Adjust to pH 7.4 with HCl)	50 mM	1.21 g
NaCl	150 mM	1.75 g
EDTA	1 mM	0.06 g
SDS	0.1 %	0.2 g
Triton X 100	1 %	2 ml
dH ₂ O		
Final volume		200 ml

PFA 4% w/v

Paraformaldehyde		20 g
PBS 0.1 M		400 mL
<i>Heat to 70° C in fume cupboard on magnetic stirrer.</i>		
<i>Add 2 drops of 10 mM NaOH to clear. pH to 7.4</i>		
Final volume		500 mL

PB 0.1 M pH 7.4

Monobasic Solution A (0.2 M)		
Sodium phosphate monobasic	NaH ₂ PO ₄	27.8 g
Distilled water	dH ₂ O	Fill to 1000 mL
Dibasic Solution B (0.2 M)		
Sodium phosphate dibasic anhydrous	Na ₂ HPO ₄	28.4 g
Distilled water		
Final volume		1000 mL
PB (0.1 M)		
Monobasic Solution A (0.2 M)		140 mL
Dibasic Solution B (0.2 M)		360 mL
Distilled water		500 mL
Final volume		1000 mL

PBS 10X 1 M pH 7.4 – use at 1X

Sodium chloride	NaCl	80 g
Potassium chloride	KCl	2 g
Potassium dihydrogen phosphate	KH ₂ PO ₄	2.4 g
Sodium phosphate dibasic anhydrous	Na ₂ HPO ₄	14.4 g
Distilled water	dH ₂ O	
Final volume		1000 mL

Running buffer 10X – use at 1X

Tris base	250 mM	30.3 g
Glycine	1.92 M	144.1 g
SDS – add last	1 %	10 g
dH ₂ O		
Final Volume		1000 ml

TAE buffer 50X – use at 1X

Tris-base		242 g
Glacial acetic acid		57.1 mL
EDTA 0.5 M		100 mL
<i>for 250 mL 0.5 M EDTA pH 8</i>		
<i>46.5 g EDTA and 5.5 g NaOH pellet in fill to 250 mL</i>		
Distilled water		
Final volume		1000 mL

TBS 10X 20 mM pH 7.4 – use at 1X

Sodium chloride	NaCl	88 g
Tris-base		24 g
Distilled water	dH ₂ O	
Final volume		1000 mL

Transfer buffer 10X

Tris HCl	250 mM	30.3 g
Glycine	1.92 M	144.1 g
dH ₂ O		
Final Volume		1000 ml

Transfer buffer 1X

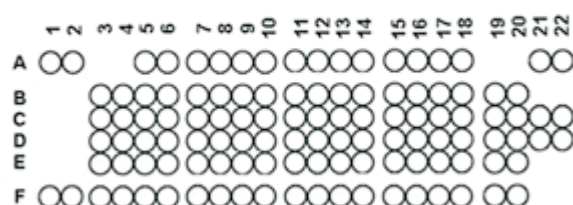
10X Transfer buffer stock	100 ml
Methanol	100 ml
dH2O	800 ml
Final Volume	1000 ml

Appendix 2. Proteome array data

Protein Name	Spot	Gal-3 +/+		Gal-3 -/-	
		CTRL	MCAO	CTRL	MCAO
		(pixel density as % of positive control)			
ADAMTS1	A5,6	0.41	0.82	1.25	2.12
Amphiregulin	A7,8	0.73	0.99	1.45	1.92
Angiogenin	A9,10	2.74	3.42	4.40	5.97
Angiopoietin-1	A11,12	1.54	1.44	2.82	2.71
Angiopoietin-3	A13,14	1.00	0.89	1.92	1.53
CXCL16	A17,18	1.62	1.49	2.86	3.96
IGFBP-10	B3,4	0.90	1.40	1.50	3.57
DLL4	B5,6	1.56	1.25	1.26	2.26
CD26	B7,8	1.79	2.71	2.66	2.18
EGF	B9,10	0.00	0.00	0.35	0.60
CD105	B11,12	1.28	2.68	3.71	2.74
Endostatin/Collagen XVIII	B13,14	5.13	10.01	9.37	12.56
Endothelin-1	B15,16	4.07	6.75	8.51	6.58
FGF-1	B17,18	63.74	77.93	81.12	79.66
FGF-2	B19,20	1.72	1.84	3.57	2.20
FGF-7	C3,4	1.40	1.43	1.66	2.23
Fractalkine CX3CL1	C5,6	3.10	2.64	3.76	3.25
GM-CSF	C7,8	0.35	0.43	0.87	0.77
HB-EGF	C9,10	2.18	2.45	3.14	1.96
HGF	C11,12	3.10	0.89	1.58	0.67
IGFBP-1	C13,14	0.50	0.91	1.60	1.39
IGFBP-2	C15,16	6.88	13.80	19.69	33.83
IGFBP-3	C17,18	2.81	7.62	8.21	23.00
IL-1α IL-1F1	C19,20	0.61	0.94	2.74	1.35
IL-1β IL-1F2	C21,22	0.35	0.89	1.85	0.77
IL-10	D3,4	1.01	2.25	1.51	2.13
IP-10 CXCL10	D5,6	0.72	0.66	1.41	1.76
KC CXCL1, CINC-1, GRO	D7,8	0.00	0.00	0.60	0.71
Leptin OB	D9,10	1.82	1.71	2.22	1.26

MCP-1 / CCL2	D11,12	0.39	0.71	1.33	0.66
MIP-1 / CCL3	D13,14	0.74	1.01	1.51	0.78
MMP-3 (pro and mature form)	D15,16	0.00	0.23	0.89	0.92
MMP-8 (pro form)	D17,18	1.30	1.63	2.30	2.80
MMP-9 (pro and active form)	D19,20	0.56	0.84	2.22	1.28
IGFBP-9	D21,22	49.44	63.59	73.38	62.93
Osteopontin	E3,4	14.81	90.27	6.38	94.61
PD-ECGF	E5,6	1.09	1.21	2.04	1.47
PDGF-AA	E7,8	3.19	4.71	4.61	4.63
PDGF-AB/PDGF-BB	E9,10	1.75	2.15	4.47	2.52
Pentraxin-3	E11,12	0.90	1.61	1.45	1.54
Platelet Factor 4 / CXCL4	E13,14	9.82	24.26	9.54	21.68
PIGF-2	E15,16	0.50	1.13	1.99	1.06
Prolactin	E17,18	24.51	8.54	2.14	1.58
Proliferin	E19,20	1.31	2.52	2.02	1.03
SDF-1 / CXCL12	F3,4	2.03	1.58	3.52	1.82
Serpin E1	F5,6	2.02	2.19	3.53	2.24
Serpin F1	F7,8	3.68	2.92	3.97	3.99
Thrombospondin-2	F9,10	0.60	0.93	2.51	4.38
Tissue Factor	A15,16	51.33	68.58	78.16	65.35
TIMP-1	F11,12	2.60	3.75	4.53	3.17
TIMP-4	F13,14	1.53	1.52	4.77	2.67
VEGF	F15,16	0.54	0.00	1.46	0.98
VEGF-B	F17,18	0.82	0.90	2.31	2.57

Mouse Angiogenesis Array



Capture antibody coordinates on the proteome array membrane.