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THE ROLE OF MAST CELLS IN A MOUSE MODEL OF FOCAL CEREBRAL ISCHAEMIA

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DECLARATION

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: **Date:**

Chapter 2: General methods and materials

Chapter 7: General Discussion

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Poster Communication: The role of mast cells in a murine model of focal cerebral ischaemia. *CM McKittrick, CE Lawrence and HVO Carswell.*

Abstract

Treatments for ischaemic stroke are limited and have low efficacy despite many years of promising research uncovering many potential therapeutic targets. As a result, stroke ranks as the third leading cause of death and the leading cause of disability worldwide. Mast cells have been identified as contributors to ischaemic stroke pathology, and are therefore a novel candidate for targeted stroke therapy. Indeed, genetic mast cell deficiency prior to onset of transient middle cerebral artery occlusion (tMCAo) in rats resulted in a significant reduction in blood brain barrier (BBB) permeability, oedema and neutrophil recruitment. Therefore the hypothesis of this thesis was that mast cells are active in the acute phase of experimental focal cerebral ischaemia, and interact with the brain to potentiate ischaemic pathology by release of a plethora of pro-inflammatory, vasoactive and proteolytic mediators. These mediators could degrade the BBB leading to vasogenic oedema, and may also promote an inflammatory milieu within the brain.

Using wild type C57BL6/J (WT) and, mast cell deficient, C57BL6/J Kit $^{w\text{-}sh/w\text{-}sh}$ (Kit) mice in the tMCAo model of focal cerebral ischaemia, we found a significant reduction of BBB permeability, oedema and neutrophil recruitment. Additionally, sodium cromoglycate (SCG) treatment prior to tMCAo had the added effect of reducing lesion volume in WT mice. Seventy two hours post-tMCAo, genetic mast cell deficiency did not result in reduction of any of the parameters analysed. Investigation of protein expression in brain homogenates, twenty minutes and forty-five minutes post-tMCAo, indicated that tumour necrosis factor-alpha (TNF-α) was not responsible for the pathology seen in the WT mice, as it was found at similar concentrations in both mouse strains. However, endothelin-1, endoglin and matrixmetaloproteinase-9 were highly expressed in the WT mice, but not in the Kit mice forty five minutes post tMCAo. Further studies are required to elucidate whether the absence of these mediators is responsible for the reduced pathology in the Kit mice.

In conclusion, the present study has provided an insight into mast cell responses following tMCAo, and has highlighted potential mechanisms by which mast cells mediate their response.

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid
AND	Anaphylactic degranulation
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
Ca^{2+}	Calcium ions
CBF	Cerebral blood flow
CCA	Common carotid artery
Cl^-	Chlorine ions
COX	Cyclooxygenase
CTMC	Connective tissue mast cells
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
EBA	Evans blue albumin
ECA	External carotid artery
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ET	Endothelin
ETR_A	Endothelin receptor A
ETR_B	Endothelin receptor B

FITC	Fluorescein isothiocyanate
ICA	Internal carotid artery
ICAM-1	Intracellular adhesion molecule-1
IgG	Immunoglobulin G
IL	Interleukin
IL-R	Interleukin receptor
ILT	Intraluminal thread
IP_3	Inositol triphosphate
K^+	Potassium ions
Kit	C57BL6/J $Kitw^{-sh/w-sh}$
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
$MC^{-/-}$	Mast cell deficient
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MC_T	Tryptase containing mast cells
MC_{TC}	Tryptase and chymase containing mast cells
MCP	Monocyte chemoattractant protein
MCP-1$\alpha^{-/-}$	Mice deficient in MCP-1α
MHC II	Major Histocompatibility complex type two
MMC	Mucosal mast cells
mRNA	Messenger ribonucleic acid

Na^+	Sodium ions
NE	Neutrophil elastase
NOS	Nitric oxide synthase
NMDA	N-methyl-d-aspartate
O_2	Oxygen
PBS	Phosphate buffered saline
PcomA	Posterior communicating artery
PIP_2	Phosphatidylinositol bisphosphate
PLA2	Phospholipase A2
PLC	Phospholipase C
pMCAo	Permanent middle cerebral artery occlusion
PMD	Piecemeal degranulation
ROS	Reactive oxygen species
rtPA	Recombinant tissue plasminogen activator
SCF	Stem cell factor
SCG	Sodium cromoglycate
TGF-β	tumour growth factor beta
TJ	Tight junction
tMCAo	Transient middle cerebral artery occlusion
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor

TRADD	Tumour necrosis factor receptor 1 associated death domain
TRAF2	Tumour necrosis factor receptor associated factor 2
TRECK	Toxin receptor-mediated conditional cell knockout
VEGF	Vascular endothelial growth factor
WT	C57BL6/J

Chapter 1: General Introduction

1.1 Introduction to stroke

Stroke is defined as a loss of body function due to lack of blood flow to the brain. This focal reduction directly affects the region of the brain perfused by the injured vessel, and manifests as a neurological deficit due to irreversible brain damage (Donnan *et al.,* 2008). Most affected by stroke are the elderly and individuals predisposed due to known risk factors such as history of heart disease, smoking, diabetes and hypertension (Di Carlo *et al.,* 2006; Basile *et al.,* 2008). Epidemiological studies also show that postmenopausal females are at higher risk of stroke compared to age matched males (Pozzi *et al.,* 2006).

Stroke is the cause of 10% (5.7 million) of deaths globally and this level is predicted to be maintained over the next 20 years (WHO, 2008). Prognosis is poor regardless of stroke type and half of all stroke victims are expected to die within one year (Hankey *et al.,* 2000; Andersen *et al.,* 2009). However this figure is not evenly distributed over the globe and in industrialised nations the trend is less severe than in lower income countries. This is probably due to greater availability of medical interventions of known risk factors and greater public health awareness (Donnan *et al.,* 2008).

The greatest burden of stroke is on those who survive the disease, which is around 70% (10.9 million per annum), and their caregivers. Stroke survivors may suffer physical, cognitive and psychological impairment, with many depending upon constant support from others to carry out daily tasks (Doyle *et al.,* 2004; Suh *et al.,* 2004). The mental status of the caregiver has been shown to be at risk and also to have an impact on the development of the stroke survivor (Elmstahl *et al.,* 1996; Suh *et al.,* 2004). From a global perspective, the burden on healthcare budgets to the treatment and management of stroke is approximately 4%, resources that could be effectively redistributed should stroke treatment be improved (Donnan *et al.,* 2008).

1.1.1 Ischaemic stroke

Ischaemic stroke is most commonly caused by atherothrombosis of large and small vessels or by cardioembolism (Rubattu *et al.,* 2000). Large and small vessel thrombosis occurs following blockage of an artery by an atherosclerotic or

arteriosclerotic plaque, respectively. This reduces the flow of blood through the artery and further complications can arise if the plaque becomes clotted with blood, which can cause a complete cessation of blood flow and may also lead to embolus generation (Donnan *et al.,* 2008). Cardioemboli can travel to the cerebrovasculature and become lodged wherever the vessels are too narrow to allow passage and can arise due to myocardial infarction, endocarditis or atrial fibrillation (Tonk and Haan, 2007).

1.1.2 Haemorrhagic stroke

Haemorrhagic stroke is characterised by bleeding from a ruptured artery, which can occur intracerebrally or in the subarachnoid, epidural or subdural spaces (Durukan, 2007). Intracerebral haemorrhage most commonly occurs due to effects of chronic hypertension, causing weakening of the vessels, which can also be attributed to cerebral amyloid angiopathy, a process by which protein is deposited on the vessel walls with an ensuing inflammatory response (Rensink *et al.,* 2003 and Tonk and Haan, 2007). Subarachnoid, epidural and subdural haemorrhages are caused by rupture of an aneurysm, which is a globular protrusion, positioned on a major artery (Isono *et al.*, 2002). Haemorrhagic strokes account for approximately 15% of strokes, however come with a greater risk of mortality within three months, when compared to ischaemic strokes.

1.2 Stroke treatments

The development of drugs for the treatment of stroke is targeted at recanalization of occluded vessels and protection of cells at risk of infarction within the ischaemic penumbra. This approach of recanalization has been successful with the introduction of recombinant tissue plasminogen activator (rtPA) in 1995 (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). The action of rtPA is mediated via cleavage of endogenous plasminogen to plasmin which is fibrinolytic and therefore dissolves blood clots (Billeci *et al.,* 2009). However, the use of rtPA therapy is very limited and as few as 2% of stroke patients may actually receive the treatment, which is due to the fastidious inclusion and exclusion criteria for its safe use (Albers and Olivot, 2007; Hacke *et al.,* 2008). The greatest danger of

misuse is the risk of haemorrhagic transformation, which has been shown to be the case in 2-10% of those treated. For this reason, rtPA must be administered within 3 hours of stroke onset, although recent studies have shown that this period may be extended up to 6 hours with no significant adverse outcome. However, there appears so far to be no benefit of rtPA use beyond 4.5 hours of stroke onset (Billeci *et al.*, 2009; Hacke *et al.*, 2008 and The International Stroke Trial 3 collaborative group, 2012). Nonetheless, the use of rtPA has been shown in trials to improve the outcome of stroke sufferers in terms of disability by 30% at three months post-ischaemia (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995).

A large body of research has been carried out with the aim of uncovering vital processes in the ischaemic cascade which may be a target for pharmacological intervention, offering neuroprotection. Despite showing favourable outcomes in animal models, none of the neuroprotective drugs have yet to be proven to be efficacious in the treatment of humans (Durukan, 2007). Reasons for this vary, and include timing of administration, the heterogeneity of stroke in humans and the differences in physiology and anatomy between animal and humans (Turley, 2005).

1.3 Ischaemic pathology: An overview

Following arterial occlusion, blood flow to the area directly perfused by the occluded artery is reduced by more than 80% (Hertz, 2008). This area is subject to the most severe damage following ischaemic stroke and is known as the ischaemic core, where cellular damage is mediated by events termed the ischaemic cascade (Du *et al.*, 1996). Energy failure leads to the breakdown of adenosine triphosphate (ATP) dependent functions leading to an increase of free calcium (Ca^{2+}), an increase in excitatory neurotransmitters and the production of free radicals, resulting in necrotic cell death. Further events of the ischaemic cascade, include inflammation, oedema, blood-brain barrier dysfunction and haemorrhagic transformations, which all contribute significantly to cell death and stroke outcome (**Figure 1.1**) (Brouns and

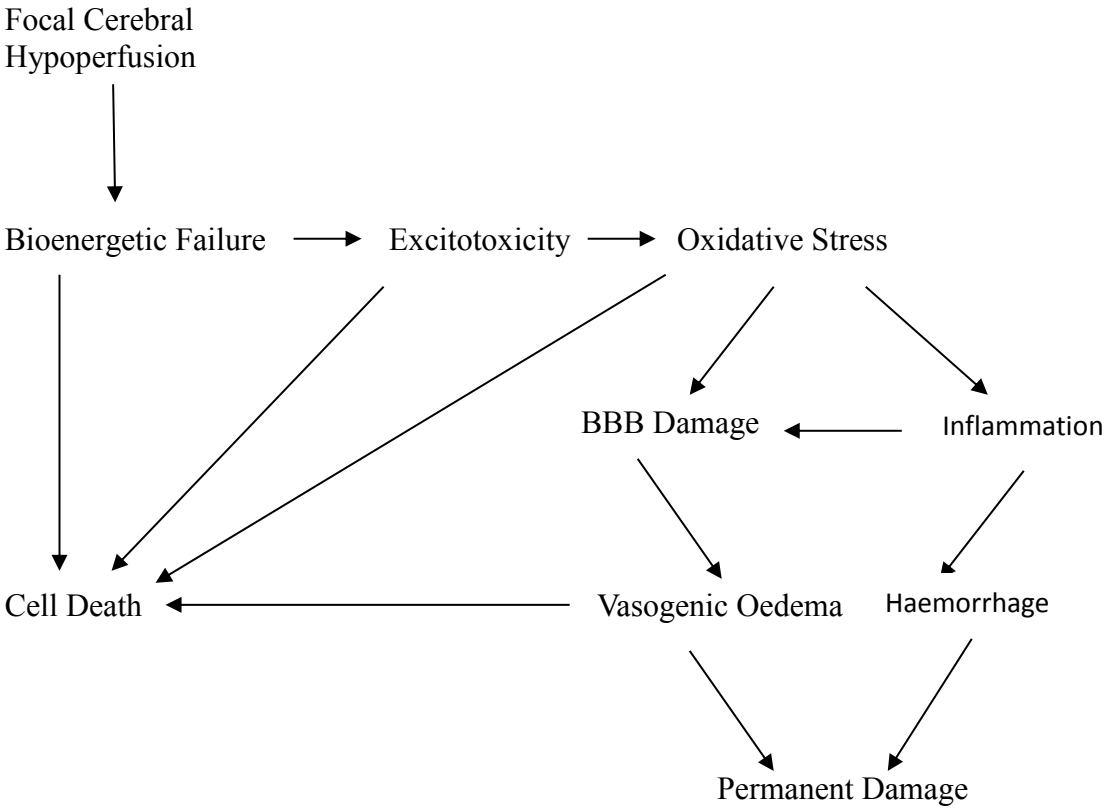


Figure 1.1: **An overview of the major pathological mechanisms following transient focal cerebral ischemia.** Figure adapted from Brouns and Deyn, 2009.

Deyn, 2009). In the surrounding areas, blood flow is less affected and is reduced by 20-40% due to collateral supply, creating an area known as the ischaemic penumbra. Within the penumbra, the cells remain viable, however are *non*-functional and are susceptible to the effects of the ischaemic cascade and tend to die from apoptosis (Phan *et al.,* 2002).

1.3.1 Bioenergetic failure

Energy metabolism in the brain is reliant on oxidative phosphorylation of glucose, and depends entirely on the systemic circulation for oxygen supply. Under ischaemic conditions, oxygen metabolism is reduced by 30-40%, causing a fall in aerobic respiration rate and forcing a 20-fold increase in lactate concentration due to anaerobic glycolysis (Frykholm *et al.,* 2000 and Hertz *et al.,* 2008). This results in a decline in ATP production from 38 to only 2 molecules per molecule of glucose. Following an ischaemic period of one hour, the concentration of cerebral ATP reaches 5-10% of base values. This quickly leads to a loss of membrane potential the failure of ATP dependent ion pumps, causing ion dyshomeostasis and loss of membrane integrity (Hata *et al.,* 2000).

1.3.2 Excitotoxicity

Anoxic depolarisation induces the release of the excitatory amino acid glutamate into the synaptic cleft, the concentration of which rapidly increases as the pre-synaptic neuron does not have energy sufficient for the active reuptake of the neurotransmitter (Turley, 2005). Subsequently, post-synaptic N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) glutamate receptors are over stimulated. This leads to a loss of ion homeostasis, due to NMDA receptor mediated Ca^{2+} influx and AMPA receptor mediated sodium (Na^{+}) and chlorine (Cl^{-}) intake, with a concurrent efflux of potassium (K^{+}) (Dirnagl *et al.,* 1999). The concentration of free cytosolic Ca^{2+} is further increased due to glutamate stimulation of metabotropic glutamate receptor, which activates phospholipase C (PLC). PLC generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) via phosphatitylinositol bisphosphate (PIP_2) cleavage. IP_3 docks on the endoplasmic reticulum and induces the release of Ca^{2+}, further increasing free cytosolic Ca^{2+}

(Turley, 2005). These pre-synaptic cells therefore undergo excessive membrane depolarisation and consequently, excessive release of glutamate, a process which spreads in waves, outward from the ischaemic core causing a spreading depression. This is a process by which the infarct core develops and recruits tissue from the penumbral region (Jarvis *et al.*, 2001).

1.3.3 Intracellular calcium increase and free radicals

Under ischaemic conditions, intracellular Ca^{2+} concentration rises by as much as 150% in neurons and facilitates the activation of many intracellular events (Kristian and Siesjo, 1998). This includes the excessive activation of enzymes such as phospholipase A2 (PLA2), calpain, cyclooxygenase (COX) and nitric oxide synthase (NOS), pathways which leads to cytotoxicity (Brouns and Deyn, 2009). The production of the free radicals, superoxide anion and nitric oxide (NO), through the activation of PLA2 and NOS, respectively, cause membrane damage and lipid peroxidation. Furthermore, superoxide combines with NO to form the toxic metabolite peroxynitrite, which directly damages deoxyribonucleic acid (DNA). Free radicals, along with Ca^{2+} accumulation at the mitochondrial membrane also leads to the formation of a mitochondrial permeability transition pore, which releases pro-apoptotic cytochrome C and further free radicals (Sims and Muyderman, 2009). Furthermore, free radicals induce the translation of pro-inflammatory cytokines which initiate the post-ischaemic inflammatory response (Clark and Valente, 2004). More direct cellular damage is mediated by Ca^{2+} activation of proteolytic enzymes which degrade cytoskeletal proteins and proteins of the extracellular matrix (ECM). For example, gelosin activation causes depolymerisation of actin and plasmin activation causes laminin degradation and consequent breakdown of the ECM (Chen and Strickland, 1997; Furukawa *et al.*, 1997).

1.3.4 Oedema

The oedematous response in the ischaemic brain can be categorised into cytotoxic and vasogenic events. Cytotoxic oedema occurs rapidly after ischaemic onset, and is mediated by the uptake of water into cells under conditions of ion dyshomeostasis

caused by energy failure and excitotoxicity, as described above (Heo *et al.*, 2005). The initial cell type to undergo cytotoxic oedema is the perivascular astrocytes, which undergo transformation after five minutes of ischaemic onset. This may be due to the presence of astrocyte specific aquaporin-4 channels and mice deficient in aquporin-4 had reduced oedematous response following MCAo (Manley *et al.*, 2000 and Kleffner *et al.*, 2008). Subsequently, neurons, oligodendrocytes, endothelial cells and nerve processes all undergo cytotoxic oedema, leading to cellular necrosis within the ischaemic core (Heo *et al.*, 2005).

A potentially more serious form of oedema, vasogenic oedema, occurs as a consequence of increased BBB permeability, where brain volume increases due to mass flow of fluid from the periphery (Ayata and Roper, 2002). This leads to an increase in intracranial pressure and causes dislocation and herniation of brain structures and impairs circulation; to such an extent that vasogenic oedema is a leading cause of death following ischemic stroke (Ayata and Roper 2002).

1.4 Inflammation

Inflammation in stroke is induced rapidly after the onset of ischaemia by a number of molecular signals. In particular, reactive oxygen species (ROS) and debris from necrotic cells, such as hyaluronic acid, heat shock proteins, messenger ribonucleic acid (mRNA) and high mobility group box one proteins activate intracellular pro-inflammatory signalling cascades. For example, hydrogen peroxide liberates the nuclear factor kappa B (NF-κB) to transcribe pro-inflammatory cytokines and chemokines such as TNF-α and monocyte chemoattractant protein-1 (MCP-1) within ischemic cells (Clark and Valente, 2004). This leads to the upregulation of adhesion molecules in endothelial cells and circulating leukocytes, causing leukocyte infiltration and activation of resident glial cells such as microglia and astrocytes. Infiltrating leukocytes potentiate the pathology of ischaemia by generation of ROS, reactive nitrogen species (RNS), various degradative enzymes, and pro-inflammatory cytokines (**Figure 1.2).**

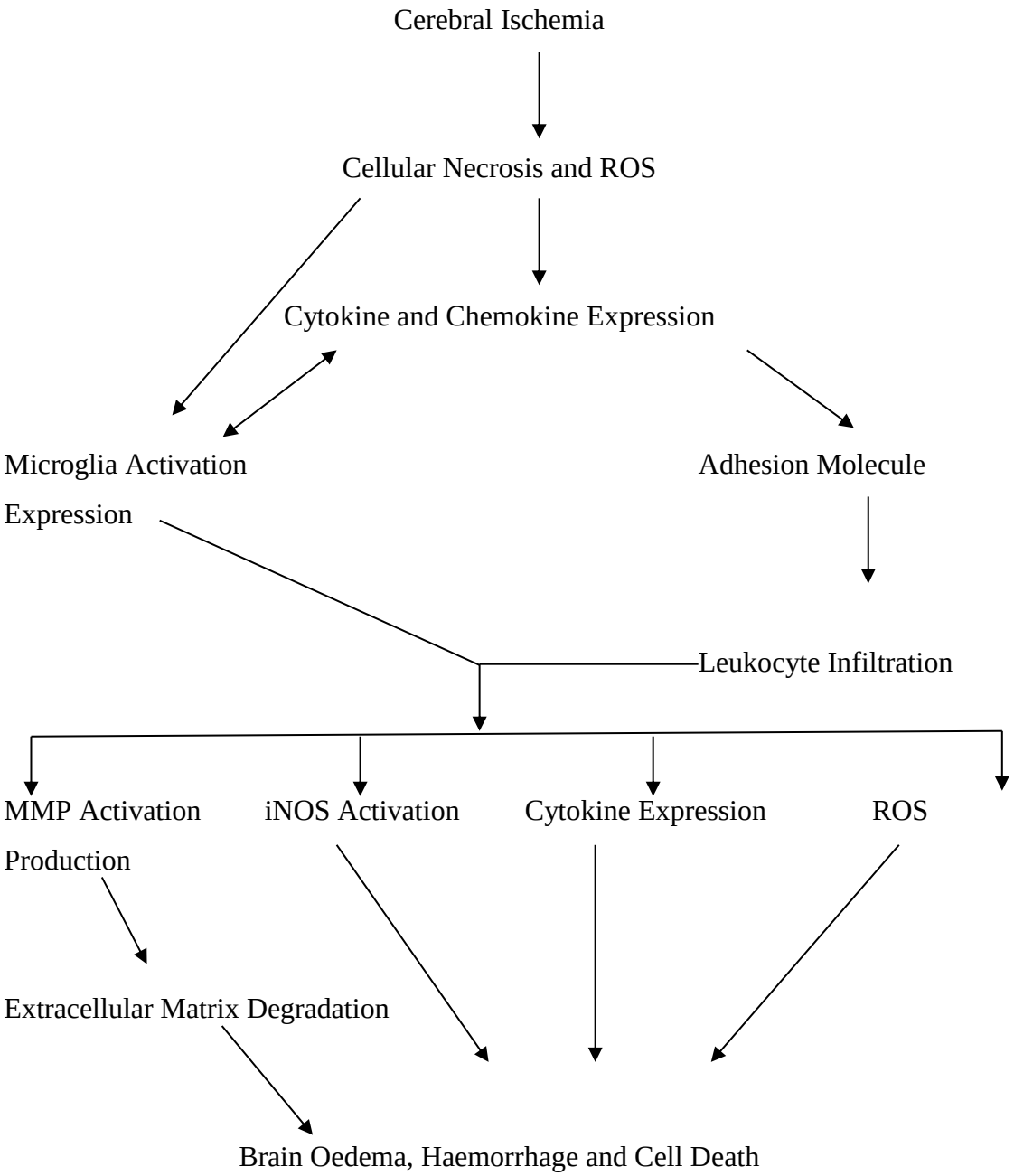


Figure 1.2: **An overview of the inflammatory response to transient focal cerebral ischemia.** Schema adapted from Wang *et al.*, 2007.

1.4.1 Cellular inflammation

Microglia, the resident phagocyte of the central nervous system (CNS) are activated 4-6 hours following onset of ischaemia and play both a protective and damaging role, depending on activation stimulus (Zhang *et al.*, 2005; Wang *et al.*, 2007). Indeed, microglial cells have the potential to secrete both pro and anti-inflammatory cytokines, as well as ROS and growth factors (Wang *et al.*, 2007). For example, recognition of dead or dying cells by microglia activates an anti-inflammatory, protective phenotype, whereas ROS and inflammatory mediators promote a cytotoxic microglia phenotype. Nonetheless, reduction of activated microglia in the ischemic penumbra following tMCAO using free radical scavenger edaravone improved neurological deficit scores and significantly reduced ischaemic infarct in rats, 24 hours post 6 hours tMCAo (Zhang *et al.*, 2005). Similarly, administration of the free radical scavenger ethyl pyruvate at various time points before and after MCAO in the Sprague-Dawley rat improved outcome and was associated with a reduction in active microglia (Yu *et al.*, 2005).

Astrocytes have a typically protective role in ischaemia, by the production of anti-inflammatory cytokines, and growth factors which contribute to BBB repair and axonal regeneration (Lively and Brown, 2007). However, evidence also suggests a damaging role of astrocytes, as they express the receptor for TNF weak inducer of apoptosis Fn14 (Saas *et al.*, 2000). A study which antagonised the Fn14 receptor found a significant reduction in infarct volume and apoptosis in the penumbra, following permanent MCAo (Yepes *et al.*, 2005). An important role for astrocytes in the post stroke brain is the formation of a glial scar, which acts to protect the brain from further ischaemic damage by creating a barrier around the damaged tissue. Although beneficial in the acute period following stroke, the scar prevents effective neurogenesis of the ischaemic tissues by neuronal stem cells, by limiting access to the damaged areas (Kahle and Bix, 2013).

The initial cell type to infiltrate the CNS from the periphery is the neutrophil, leucocytes important in the innate immune system, which transmigrate approximately 4-6 hours after ischaemic onset in the tMCAo model (Wang *et al.*, 2007). Neutrophils contribute to ischaemic pathology by producing various

degradative enzymes, ROS and to a lesser extent RNS and their role has been highlighted in a number of studies (Stowe *et al.*, 2009). In an analysis of human stroke patients, there was found to be a direct correlation between neutrophil infiltration and an increase in infarct volume (Buck *et al.*, 2008). In an animal study, the broad acting serine protease neutrophil elastase (NE), which degrades the ECM, was shown to be important in mediating ischaemic pathology (Stowe *et al.*, 2009). Mice genetically deficient in NE had a significantly reduced infarct volume and vasogenic oedema, as well as decreased BBB permeability and a reduction of leukocyte vascular adherence following tMCAO (Stowe et al., 2009). Likewise, pharmacological inhibition of NE, using compound ZN200355, led to a similar outcome in wild type mice. However, the NE null mice and those pharmacologically modulated showed no improvement in infarct volume following permanent middle cerebral artery occlusion (pMCAo), highlighting the importance of reperfusion in potentiating ischemic pathology and promoting leucocyte infiltration (Stowe *et al.*, 2009). Further leukocyte populations are observed in the ischaemic brain, with the infiltration of macrophages occurring within 24 hours after reperfusion and numbers peaking between 3 and 6 days (Petry *et al.*, 2007 and Gelderblom *et al.*, 2009). However, the role of macrophages is not currently thought to differ from microglia (Schilling *et al.*, 2003).

Current experimental evidence suggests that T lymphocytes, a subset of lymphocytes responsible for cellular adaptive immune responses, may have an important role to play in the outcome of ischaemic stroke. In a model of tMCAO utilising genetically modified mice, T cell deficiency led to a markedly improved outcome. Measures of leukocyte adherence to vessel walls, infarct volume and neurological deficit all showed an improvement in mice lacking either $CD4^+$ T cells or $CD8^+$ T cells (Yilmaz *et al.*, 2006). Lymphocytes have been recently shown to be present in the ischaemic brain as early as 24 hours post tMCAo in rats, and their numbers increase over the first week post-ischemia, in a model of pMCAo (Li *et al.*, 2005 and Gelderblom *et al.*, 2009). The specific populations of lymphocytic infiltrates were further differentiated by Gelderblom et al in 2009. There was a marked increase in both B and T lymphocytes, the latter being represented by greater numbers of CD4+ helper T cells and CD4-/CD8- regulatory T cells within the ischemic hemisphere.

However, none of the effector T cell populations detected were seen to be active and further differentiation identified small numbers of regulatory cells in the brain and an even greater proportion in the spleen (Gelderblom *et al.*, 2009). The regulatory phenotype of the T cells can be explained by the finding that dendritic cells (DC) were detected in the brain which displayed antigen presenting molecules, major histocompatibility complex class two (MHC II), without sufficient display of the requisite co-stimulatory molecule cluster of differentiation 40, required for T cell activation (Gelderblom *et al.*, 2009). The contribution of regulatory T cells in the ischemic brain has recently been shown to involve the limitation of damage induced by ischemia. In a study using regulatory T cell depleted mice, infarct volume and inflammatory cytokine production was increased following tMCAo and pMCAo. Additionally, neutrophil infiltration and microglia activation were also increased (Liesz *et al.*, 2009).

The idea that there is no adaptive immune response to CNS tissues, as indicated by a lack of T cell activation by Gelderblom *et al.*, 2009, is supported by experiments carried out by Becker and colleagues (Becker, 2009). They found that there was no indication of a polarised Th1 response when brain lymphocytes and splenocytes were exposed *ex vivo* to the CNS antigen major basic protein (MBP) following 3 hours of tMCAo. The authors measured production of interferon-gamma and tumour growth factor-β (TGF-β) using ELISPOT, and found no disparity between regulatory and effector T cells (Becker, 2009). Whether this is the case in human stroke is debatable as co-morbidity is frequent in human cases and often will provide a pro-inflammatory milieu in which polarised inflammatory responses are encouraged (Becker, 2009). Furthermore, post-ischemic infections are frequent and may contribute to the inflammatory environment. This idea was explored by inducing an inflammatory response after 3 hours of tMCAo by injecting lipopolysaccharide (LPS) intraperitoneally upon reperfusion. Mononuclear cells of the brain where shown to display a Th1 phenotype when examined after one month, whereas LPS negative animals displayed a regulatory T cell response. These findings were supported by the finding that LPS induced greater expression of both MHC II and co-stimulatory molecules (Becker *et al.*, 2005).

1.4.2 Tumour necrosis factor alpha

Animal models of ischaemia have indicated that TNF-α contributes significantly to pathology in the acute phase, however is required for resolution of ischaemic injury and is also involved in inducing ischaemic tolerance. During the ischaemic period TNF-α is produced by mostly in ischaemic neurons and both resident and infiltrating immune cells. The mechanisms by which TNF-α interact with ischaemic brain to confer either protection or pathology are many and varied, and include the induction of necrosis or apoptosis of ischaemic cells, potentiation of excitotoxicity through glutamate production, activation of pro-inflammatory cytokines and chemokines and vascular adhesion molecule up-regulation, in the acute period of stroke (Watters and O'Connor and O'Connor, 2011).

The detrimental effects of TNF-α was shown by inhibition of TNF-α by ICV injection of anti-TNF-α monoclonal antibody (mAb) upon reperfusion following 1 hour of middle cerebral artery occlusion (MCAo) was shown to have a protective effect in a mouse model (Yang *et al.*, 1998). The treated mice had a 28% smaller infarct size, whilst intracellular adhesion moloecule-1 (ICAM-1) expression was also greatly reduced in endothelial cells, when compared to controls (Yang *et al.*, 1998). In a similar fashion, intraventricular administration of anti-TNF-α mAb 30 minutes prior to MCAo in hypertensive rats and also 3 and 6 hours after reperfusion reduced infarct volume by 20% when compared to controls (Barone *et al.*, 1997).

There is body of evidence that indicates TNF-α to be neurotoxic in the ischaemic brain by enhancement of glutamate excitotoxicity or by direct engagement of the TNFR1 (TNF receptor 1), leading to apoptosis (Watters and O'Connor, 2011). The activation of TNFR on microglia, by microglia derived TNF-α produced in response to ischaemia induce excitotoxicity, induces an increase in glutamate production via increased glutimanase expression. Additionally, TNF-α mediated dysfunction of the astrocytic glutamate transporter results in an excess of the excitatory neurotransmitter within the synaptic cleft, potentiating neuronal excitotoxic cell death. Furthermore, this mechanism of cell death is enhanced by a reduction of inhibitory gamma-aminobutyric acid (GABA) receptors on the surface of neurons exposed to TNF-α (Watters and O'Connor, 2011).

It is thought that the protective effects of TNF-α are mediated through induction of pro-angiogenic factors, such as vascular endothelial growth factor and fibroblast growth factor-2 (Goukassian *et al.*, 2007 and Watters and O'Connor 2011). In a model of hind limb ischaemia, TNFR1 was found to be essential for tissue neovascularisation after a 7 to 10 day recovery period. Mice deficient in TNFR1 had low tissue perfusion and capillary density, attributed to lower tissue and endothelial cell expression of vascular endothelial growth factor and fibroblast growth factor-2, which could be rescued by adoptive transfer of bone marrow from wild type mice (Goukassian *et al.*, 2007). In TNFR knockout mice, lesion volume was significantly greater after 1 hour tMCAo followed by 24 hours of reperfusion compared to wild type controls. However, mice deficient in TNFR2 were similarly affected, indicating a protective role for TNFR1 in stroke. Additionally, TNF-α deficient mice were more vulnerable to effects of ischaemia (Gary *et al.*, 1998). Soluble and membrane bound TNF-α and its receptors, TNFR1 and TNFR2, are known to increase in expression in the first 3 hours of ischaemic stroke on multiple cell types including neurons, astrocytes, microglia, endothelial cells and vascular smooth muscle (Mahaddi *et al.*, 2011). The fate of any cell type expressing TNF-α receptors is dependent upon the isotype presented on the cell surface. Indeed, TNFR1 is associated with induction of cell death through its death domain accessory protein TNFR1 associated death domain (TRADD), whilst TNFR associated factor 2 (TRAF2) signalling on TNFR2 is anti-inflammatory and neuroprotective (**Figure 1.3**). This is considered a general rule of TNF-α signalling, though TNFR1 can also signal through TRAF2, most likely following pre-conditioning, and the outcome from stroke may be dependent on the expression level of each of the receptors (Wang *et al*., 2007 and Watters and O'Connor 2011).

1.4.3 Interleukin-1

A much studied cytokine in the pathogenesis of stroke is the pro-inflammatory mediator interleukin (IL)-1, which is produced mainly by microglia in the brain, and also from hematopoietic sources, the exact cell types of which remain unidentified, following experimental stroke (Denes *et al.*, 2013). IL-1 expression is seen to increase in the first 30-min of ischemia, and acts to reinforce the inflammatory

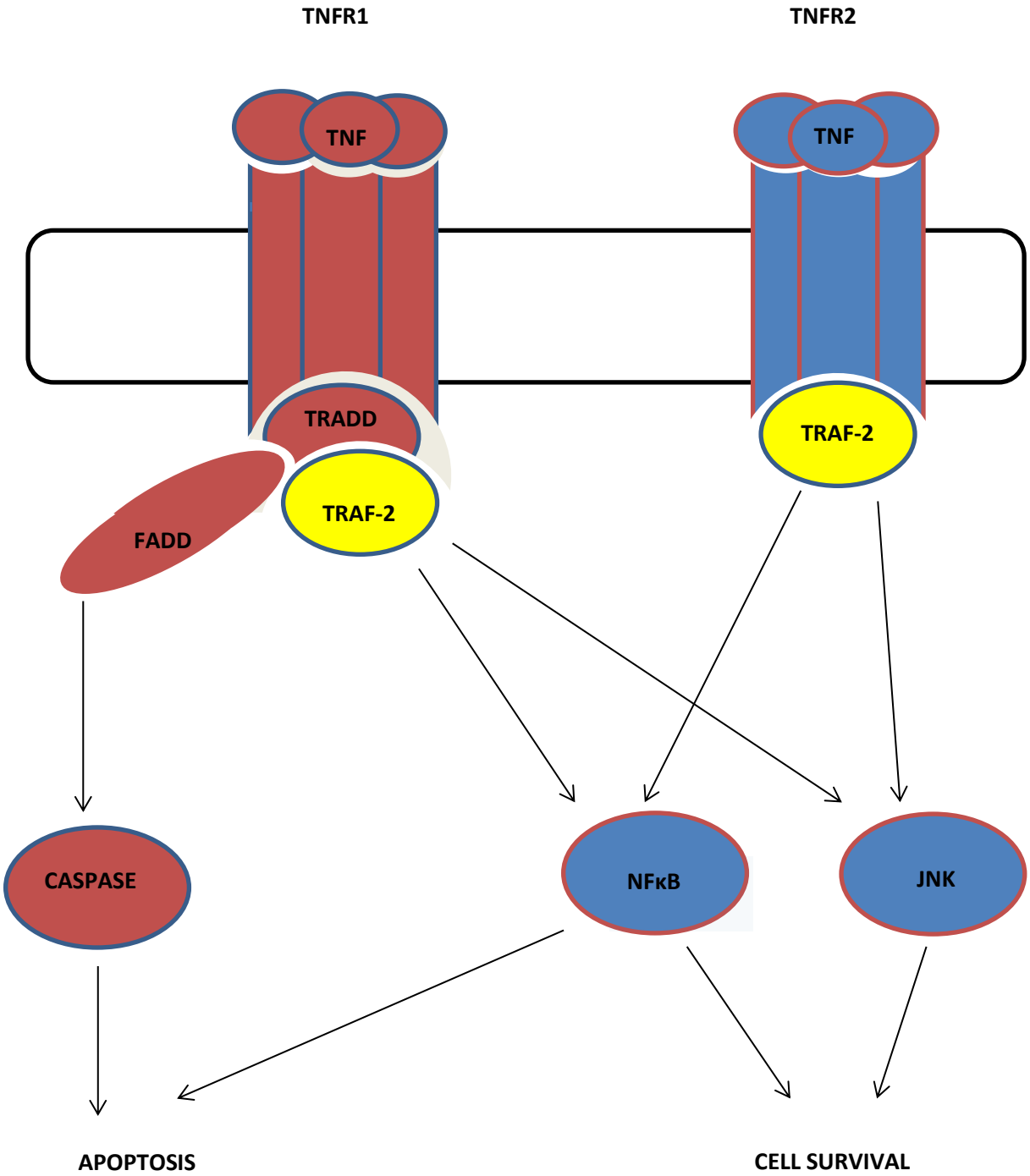


Figure1.3: Schematic of the outcomes from tumour necrosis factor-α receptor activation. Abbreveations, FADD, Fas associated death domain; JNK, Janus kinase; NFκB, nuclear factor κB; TNF-α receptor associated factor-2; TNFR, tumour necrosis factor receptor; TRADD, TNFR associated death domain; TRAF-2,. Figure adapted from Watters and O'Connor 2011.

response by activation of NF-κB via the IL-1 receptor (IL-1r). The damaging role of IL-1 was demonstrated in mice deficient in the IL-1r antagonist, a natural product of microglia, upon activation. Following 15 minutes of MCAo in the mice, a significantly greater infarct volume was measured in the IL-1R antagonist deficient animals compared to the wild type (Pinteaux *et al.,* 2006). In a mouse model of hypoxic ischaemia, IL-R1 deficiency led to less severe oedema and a 60% smaller infarct volume compared to wild type (Basu *et al.,* 2005). The inflammatory response in the IL-r1deficient mice was also significantly depressed compared to wild type, as indicated by reduced mRNA expression of IL-1β, IL-1α, IL6,TNF-α and monocyte-colony stimulating factor (Basu *et al.,* 2005).

1.4.4 Interleukin-10 and tumour growth factor beta

Anti-inflammatory mediators such as TGF-β and IL-10 are also active in the ischaemic brain and act to suppress the production and action of pro-inflammatory cytokines. Injection of IL-10, 30 min prior to and 3 hours after pMCAo, in spontaneously hypertensive rats decreased the ischaemic damage. Intravenous administration led to a 40% smaller infarct size, whilst intracerebroventricular (ICV) administration resulted in a 20% smaller infarct region (Spera *et al.,* 1998). A similar result was found in mice overexpressing TGF-β, where a 36% decrease in infarct volume was observed following 30 minutes of MCAo. Furthermore, the treated mice also expressed significantly lower concentrations of the chemokines MCP-1 and macrophage inflammatory protein-1α (Pang *et al.,* 2001).

1.4.5 Chemokines

The production of chemokines during, and after, ischemic stroke is known to promote ischaemic pathology. Indeed, overexpression of MCP-1 in mice undergoing pMCAo with partial reperfusion displayed a 50% increase in infarct volume. Furthermore, macrophage infiltration was significantly increased in the MCP-1 overexpressing mice (Chen *et al.,* 2003). A study of the neutrophil chemoattractant IL-8 in a tMCAo indicated that this chemokine exacerbates injury. Inhibition of IL-8 using repertaxin, an IL-8 receptor antagonist, significantly reduced both infarct

volume and the infiltration of neutrophils, which was optimal when repertaxin was administered intravenously 2 hours after reperfusion onset (Garau *et al.,* 2005).

1.4.6 Endothelin-1

Endothelin (ET) 1 is produced predominantly by activated endothelial cells, although a number of other cells types produce this potent vasoconstrictor, including mast cells, smooth muscle cells, neurons, astrocytes, microglia and leucocytes. The actions of ET-1, as well as the other isoforms, ET-2 and ET-3, is responsible for maintaining vascular tone and remodelling, promoting smooth muscle proliferation, maintaining the ECM and regulating water and Na^+ excretion, through ET receptor A (ETR_A). ETR_B, is active in clearing soluble ET-1 from the circulation. Vasodilatory effects of ET-1 are mediated via ETR_{B1}, whilst ETR_{B2} is also involved in mediating vascular constriction (Leung *et al.,* 2004 and Moldes *et al.,* 2012). The effects of ET-1 is dependent on the receptor with which it engages, and also the location of the receptor (Frommer and Muller-Ladner 2008) (**Figure 1.4**) In stroke, ET-1 contributes to disease in humans, with plasma concentrations around 4 times higher in the 72 hours following ischaemic stroke compared to healthy controls, which correlated with poor neurological outcome (Ziv *et al.*, 1992). In mice overexpressing ET-1 in the brain, lesion volume and neurological deficit were markedly increased following 2 hours tMCAo and 22 hours of reperfusion (Leung *et al.*, 2004). The potency of ET-1 in the brain is evident in the fact that is used to model stroke by application to the MCA or stereotaxic injection into the tissue surrounding the MCA. Furthermore, intracortical injection of ET-1 causes prolonged reduction of local blood flow for up to 48 hours and produces a local and discrete ischaemic lesion (Macrae *et al*., 1993 and Sharkey *et al.,* 1993).

1.4.7 Matrix metalloproteinases

As a broad ranging group containing 25 known members, the MMPs are zinc dependent proteases which are implicit in mediating pathological effects of multiple diseases of both the CNS and the periphery (Romi *et al.,* 2012). The most recent literature has identified the gelatinases, MMP-9 and MMP-2, as being important mediators of stroke pathology. They are produced and secreted by most cell types in

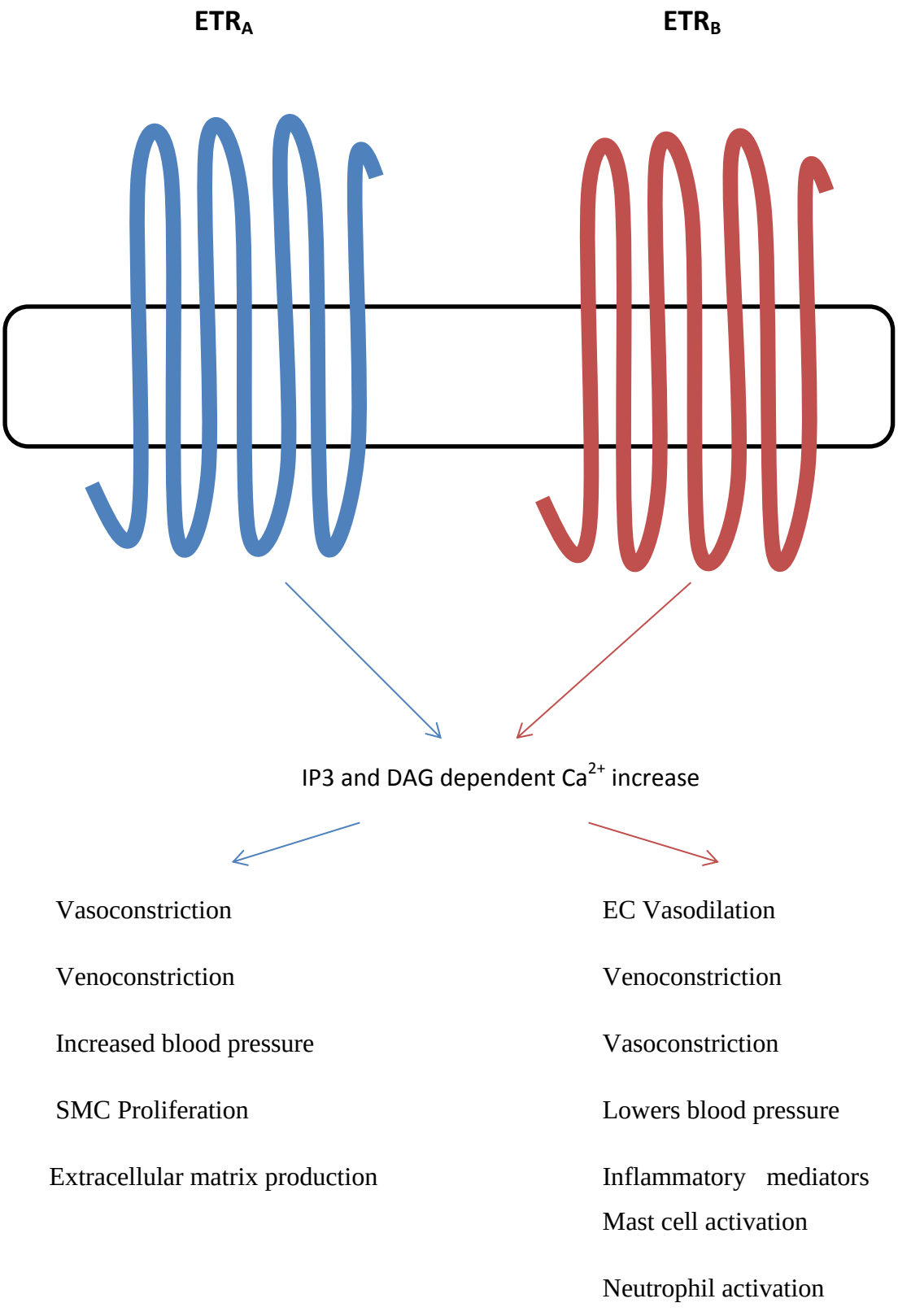


Figure 1.4: Outcomes from endothelin receptor activation. Abbreveations, DAG, diacylglycerol; EC, endothelial cell; ETR, endothelin receptor; IP3, inositol triphosphate; SMC, smooth muscle cell. Figure adapted from McKinsey, 2007.

the ischaemic brain, including mast cells, in an inactive form which is rapidly converted to the active substrate, a process which can be halted by tissue inhibitor of metalloproteinase (TIMP). In the early stages of stroke, MMP-2 and MMP-9 degrade the ECM, particularly laminin, type IV collagen and fibronectin and cause disassociation of tight junction proteins such as occludin and claudin. As a result, increased MMP levels after stroke are correlated with increased vasogenic oedema and haemorrhagic transformation (Sandoval and Witt, 2008).

Both *in vivo* and *in vitro* models of ischaemia have implicated MMP-2 and MMP-9 as an important mediator of BBB breakdown. Endothelial cells cultured in a monolayer were more permeable to fluorescein isothiocyanate (FITC) labelled dextran after 2 hours of oxygen glucose depravation (OGD) by reduction of occludin expression, which could be rescued by treatment with a specific MMP inhibitor, SB-3CT (Liu *et al.,* 2012). Furthermore, a rat model of MCAo confirmed the results from the OGD endothelial cell studies. After 2 hours MCAo, FITC-dextran leakage into the brain tissue was detected in the ischaemic core, and was absent from the brain tissue out with the injured region. In line with these findings, micro dialysis of the same region immediately after the ischaemic period confirmed the increased levels of both extracellular MMP-2 and MMP-9 (Liu *et al.,* 2012). Previous work had already identified mast cells as a source of post ischaemic MMP-2 and MMP-9 in the rat brain, following 60 minutes tMCAo with 3 hours of reperfusion. In mast cell deficient $WsRc^{Ws/Ws}$ rats, gelatinase expression was reduced by around 60% within the ischaemic hemisphere and by around 70% within the microvasculature compared to wild type controls. The expression was greatly increased within the ischaemic hemisphere by ICV injection of the mast cell degranulating compound, 48/80, prior to onset of MCAo in wild type rats. Additionally, stabilisation of mast cells prior to ischaemia by ICV injection of sodium cromoglycate (SCG), a mast cell stabiliser, reduced microvascular localisation of MMP-2 and MMP-9 compared to saline treated controls. Finally, mast cell and neuronal expression of gelatinase was strongly increased following ischaemia, which was not observed in control tissue samples. In all groups analysed, there was a positive correlation between increased activity of MMP-2 and MMP-9 and cerebral oedema, supporting the concept that

MMP mediated BBB breakdown is critical in the development of brain swelling (Mattila *et al.,* 2011).

1.4.8 Endoglin

Endoglin (Eng) is expressed abundantly on vascular endothelial cells, were it acts as a modulator of angiogenesis, as a TGF-β receptor (TGFR) accessory protein (**Figure 1.5**). It is also expressed on other cell types, including macrophages, pericytes and vascular smooth muscle cells (Docherty *et al.,* 2006). Evidence indicates that TGFR signalling through Eng is necessary for production of endothelial NOS, as Eng+/- mice are resistant to acetylcholine induced vasodilation, and promotes cell survival by suppression of apoptosis (Jerkic *et al.,* 2004). In this regard it is commonly found to be increased in expression in a variety of malignant tumours. Additionally, Eng increases vascular smooth muscle proliferation, pericyte migration and production of ECM components (Diez-Marquez *et al.,* 2002) Eng is also found in the circulation in its soluble form, sEng, which acts as a scavenger of TGF-β. Under hypoxic conditions both human and mouse vascular endothelium markedly increase Eng expression, whilst in CD1 mice the protein is found in abundance within the ischaemic hemisphere, 28 days after 90 minutes tMCAo (Li *et al.,* 2003 and Zhu *et al.,* 2003). Suppression of Eng in endothelial cells increases susceptibility to apoptosis under hypoxic conditions, which is exacerbated by addition of TGF-β. This suggests that by engaging with TGF-β, Eng protects the vascular endothelium from the pro-apoptic action of TGF-β (Li *et al.,* 2003).

Analysis of Eng from plasma samples of patients admitted to hospital suffering acute ischaemic stroke found a strong link between circulating protein levels and stroke outcome. Compared to healthy controls, stroke patients had a significantly greater number of cells expressing Eng and the pro-coagulation molecule phosphatidylserine. When the stroke patients were divided into mild (NIHSS score <5) and moderate to severe (NIHSS score >5) more specific cell types expressing Eng were identified as increased in the stroke patients and this was correlated to increased lesion volume (Simak *et al.,* 2006).

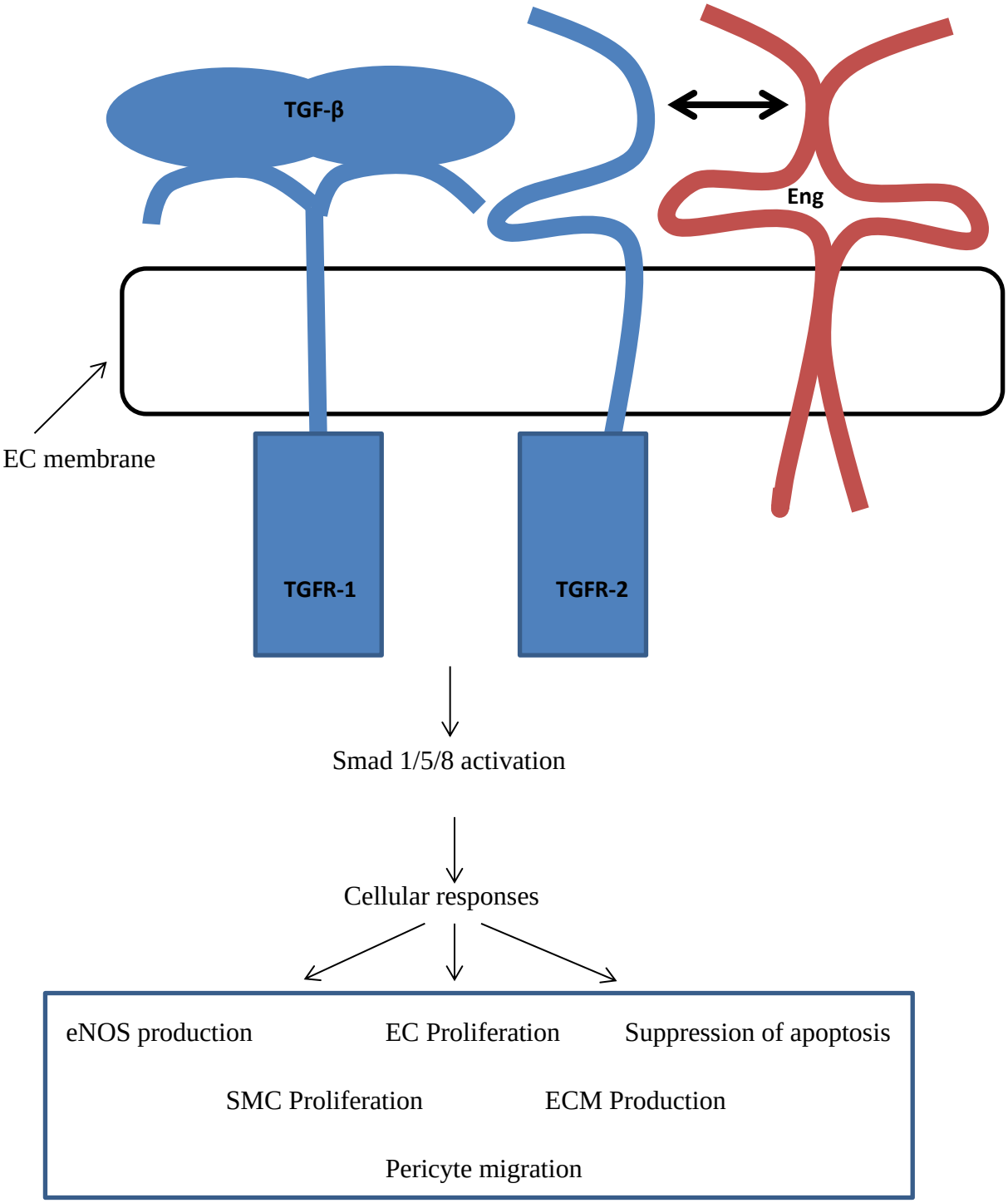


Figure 1.5: Outcomes from endoglin mediated tumour growth factor receptor activation. Abbreviations, EC, endothelial cell; ECM, extra cellular matrix; Eng, endoglin; eNOS, endothelial nitric oxide synthase; SMC, smooth muscle cell; TGFR, tumour growth factor receptor. Adapted from Laurens and ten Dijke, 2012.

1.5 BBB

The BBB serves as a diffusion barrier, which segregates the brain from the systemic circulation and is composed of five distinct subunits, i.e. endothelial cells, ECM, pericytes, astrocytes and neurons, which work in concert as a functional body known as the neurovascular unit (NVU) (see **Figure 1.6**) (Sandoval and Witt, 2008). Failure of any of the subunits leads to dysfunction of the BBB, which is defined as an increase in paracellular permeability so much so that serum products are no longer excluded from the CNS (Strbian *et al.,* 2008).

1.5.1 BBB functional anatomy

The primary mediators of BBB permeability are the endothelial cells which surround the cerebral vasculature and prevent the diffusion of solutes, and contain active transport mechanisms to allow the absorption of nutrients and the excretion of toxic metabolites. Furthermore, the expression of a number of peptidases allows the degradation of serum proteins with neuroactive properties, such as substance P and neurotensin (Witt *et al.,* 2001). Central to the permeability of the endothelial cells are tight junctions (TJ), which form a seal between neighbouring endothelial cells and are anchored to the cytoskeleton via interactions of accessory proteins, such as zonular occludin, with actin. The TJ of neighbouring cells then interact to form a barrier impermeable to molecules with a molecular weight greater than 180 daltons (Wolburg and Lippoldt, 2002) (**Figure 1.7**). The maturation, maintenance and stability of the BBB are dependent on pericytes, which are thought to modulate the BBB via cell signalling mechanisms. Indeed, pericytes produce TGF-β, which initiates the proliferation and differentiation of endothelial cells as well as inducing the production of ECM proteins (Von Tell *et al.,* 2006). The intercellular contact also indicates that pericytes have a regulatory control of endothelial cells, and they also express contractile proteins, suggesting that they help maintain myogenic tone under fluctuating blood pressure (Bandopadhyay *et al.,* 2001 and Von Tell *et al.,* 2006).

Astrocytes have similarities with pericytes in that they influence endothelial cells and TJ through secreted factors, which are facilitated by end-foot process contact. Furthermore, astrocytic end-foot processes regulate the flow of water through the

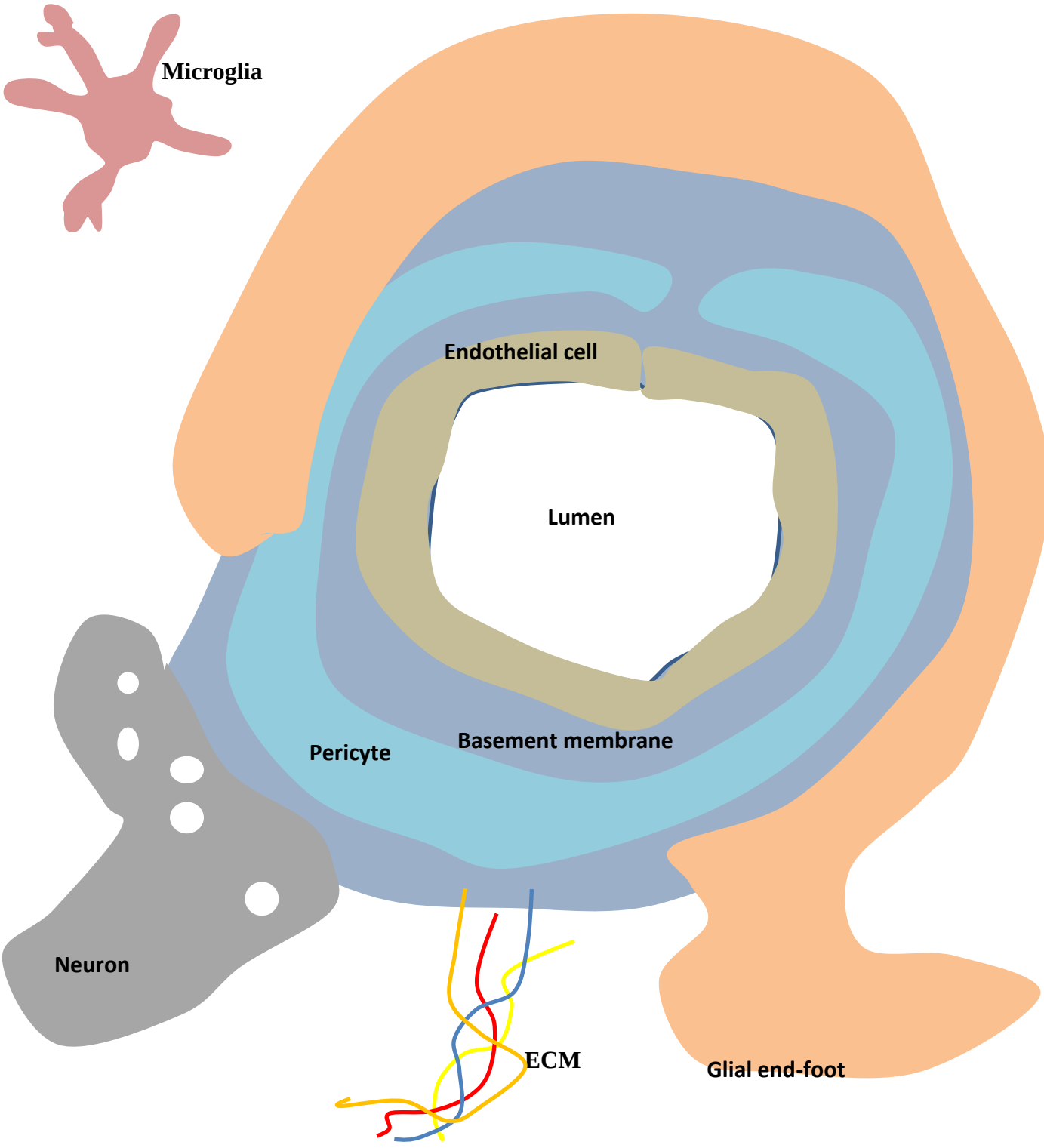


Figure 1.6: Schematic of a transverse section of the neurovascular unit of the blood brain barrier. Abbreviations, ECM, extra cellular matrix. Adapted from Skaper, 2012.

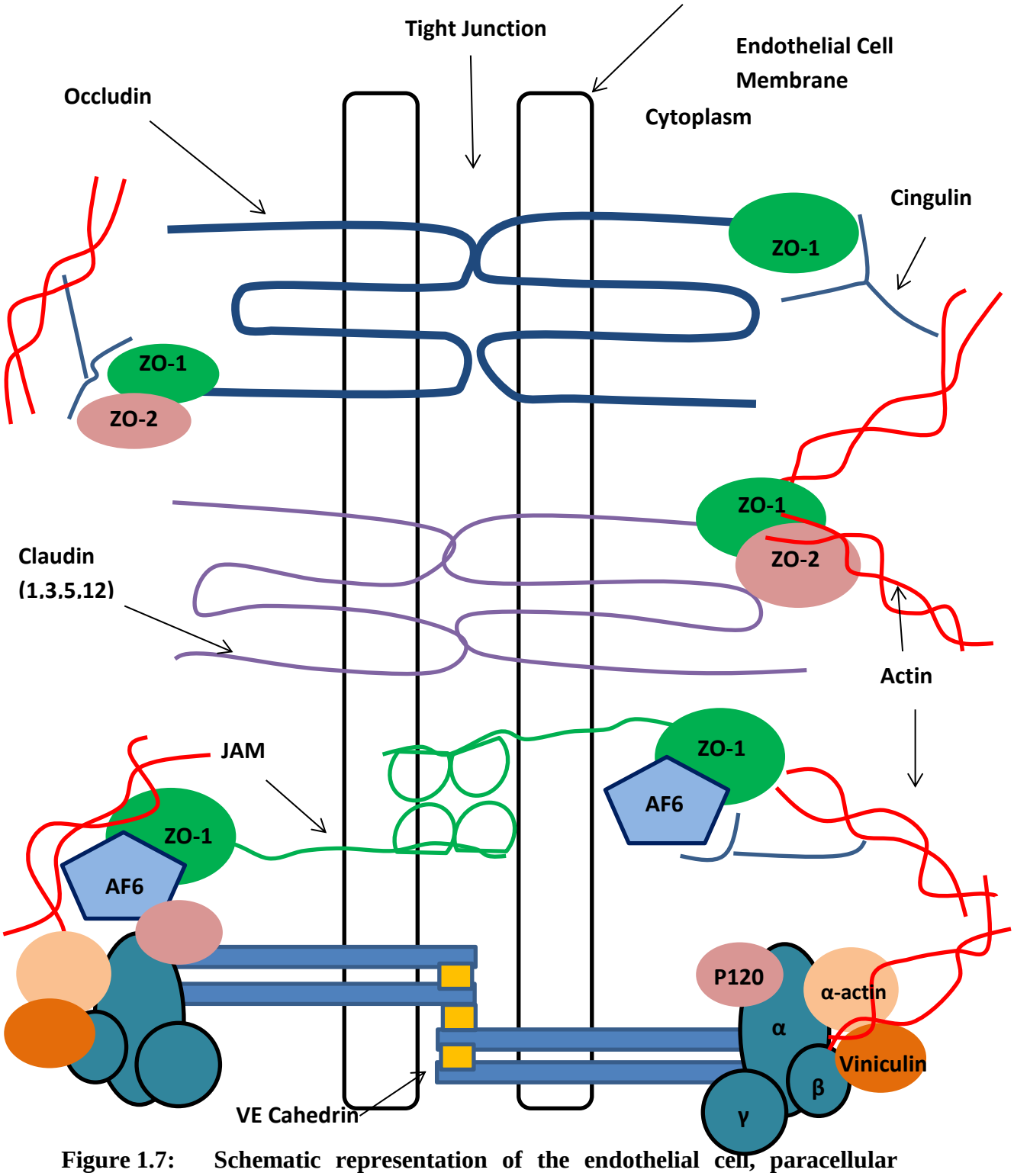


Figure 1.7: Schematic representation of the endothelial cell, paracellular tight junction of the blood brain barrier. Abbreveations, JAM, junctional adhesion molecule; VE, vascular endothelial and ZO, zonular occludin;. Schema adapted from Brouns and Deyn, 2008.

BBB, via aquaporin-4 channels (Kleffner *et al.*, 2008). Important in the control of BBB function is the neurotransmitter signalling via astrocyte end-foot processes and perhaps by direct endothelial cell contact (Rancillac *et al.*, 2006). Contact between the neuron and astrocyte is bidirectional as neuronal synaptogenesis has been shown to be astrocyte dependent *in vitro* (Ullian *et al.*, 2001). Support, separation and cellular communication within the NVU is mediated by the ECM, which attaches to the cells via integrins and dystroglycans. Integrin-ECM contact provides a conduit for anchorage and inward signal propagation which regulate proliferation, differentiation, cytoskeleton organisation and ion channel gating (Giancotti and Ruoslahti, 1999). Dystroglycan associates with ECM components laminin, perlecan and agrin, bridging the extracellular environment with the internal cytoskeleton and is thought to be important in cell development (del Zoppo *et al.*, 2006).

1.5.2 BBB dysfunction in ischaemic stroke

BBB dysfunction induced by transient cerebral ischaemia has been shown to occur after as little as 25 minutes of reperfusion. In a model of tMCAo in Sprague-Dawley rats, 90 minutes of occlusion caused an increase in BBB permeability, which remained in a continuously open state over a period of five weeks (Strbian *et al.*, 2008). In this model, an initial sharp increase in permeability to EBA occurred within the initial 12 hours, where it peaked and slowly became less permeable over the next week. From one week onwards, the permeability of the BBB again was sharply increased before slowly becoming less permeable at five weeks post tMCAo. Throughout the entire period of analysis the BBB permeability of the operated rats was significantly greater than sham operated animals who displayed no detectable Evans Blue within the brain (Strbian *et al.*, 2008). Initial opening of the BBB can be mediated by the effects of the ischemic cascade, such as Ca^{2+} and ROS increase, on the NVU. Blocking Ca^{2+} in an *in vitro* model of hypoxia/aglycaemia led to increased expression of occludin, which was found to be mostly localised to the cell membrane. In the absence of Ca^{2+} blockade, occludin was localised within the cytoplasm, negating its function as a TJ protein (Brown and Davis, 2005). Another *in vitro* model of the BBB where ROS were introduced displayed increased permeability caused by claudin-5 and occludin dysfunction, as well as rearrangement

of the cytoskeleton. ROS also increased the migration of monocytes across the BBB (Schreibelt *et al.*, 2007).

Mediators of post-ischaemic inflammation also contribute to BBB leakage, for example, MMP-9 activation caused degradation of laminin and increased BBB permeability to immunoglobulin G (IgG) following MCAo by embolism (Kelly *et al.*, 2006). The MMP-9 mediated ECM degradation is also associated with increased degradation of zonular occludin-1 and resultant increased BBB permeability due to a disruption of TJ protein, actin binding (Asahi *et al.*, 2001). TNF-α administered intracerebrally induced an increase in BBB permeability to radiolabelled sucrose, which was concurrent with an increased expression of MMP-9 activation (Candelario-Jalil *et al.*, 2007).

1.6 Mast cells

Mast cells are involved in a number of normal physiological functions such as immunity to extracellular pathogens and angiogenesis, as well as being implicated in multiple pathological processes. Indeed, mast cells are postulated to mediate a number of functions through both direct effector mechanism as well as via modulation of other cell types (**Figure 1.8**). For example, TNF-α and MCP-1α released by mast cells promote pro-inflammatory phenotypes in other cell types, such as macrophages and neutrophils, whereas in helminth infections mast cells are essential for the induction of Th2 responses which are required for helminth expulsion. Additionally, mast cell secretion of phospholipases, carboxypeptidases and other stored granules can cause direct cytotoxicity to invading pathogens (Collington *et al.*, 2011 and Moon *et al.*, 2010).

Mast cells are derived from bone marrow and released into the circulation as $CD34^+$, KIT^+, $CD13^+$ undifferentiated precursors, which migrate to tissues and subsequently mature under local factors (Metcalfe, 2008). Differentiation, proliferation and survival have been shown to be influenced by IL-3 (only present in rodents), IL-4, IL-5, IL-9, IL-10, GM-CSF, and NGF and stem cell factor (SCF) (Morales *et al.*, 2010). These mediators are also stored and released by mast cells upon activation indicating a self-regulatory mechanism, a process by which mast cells are able to

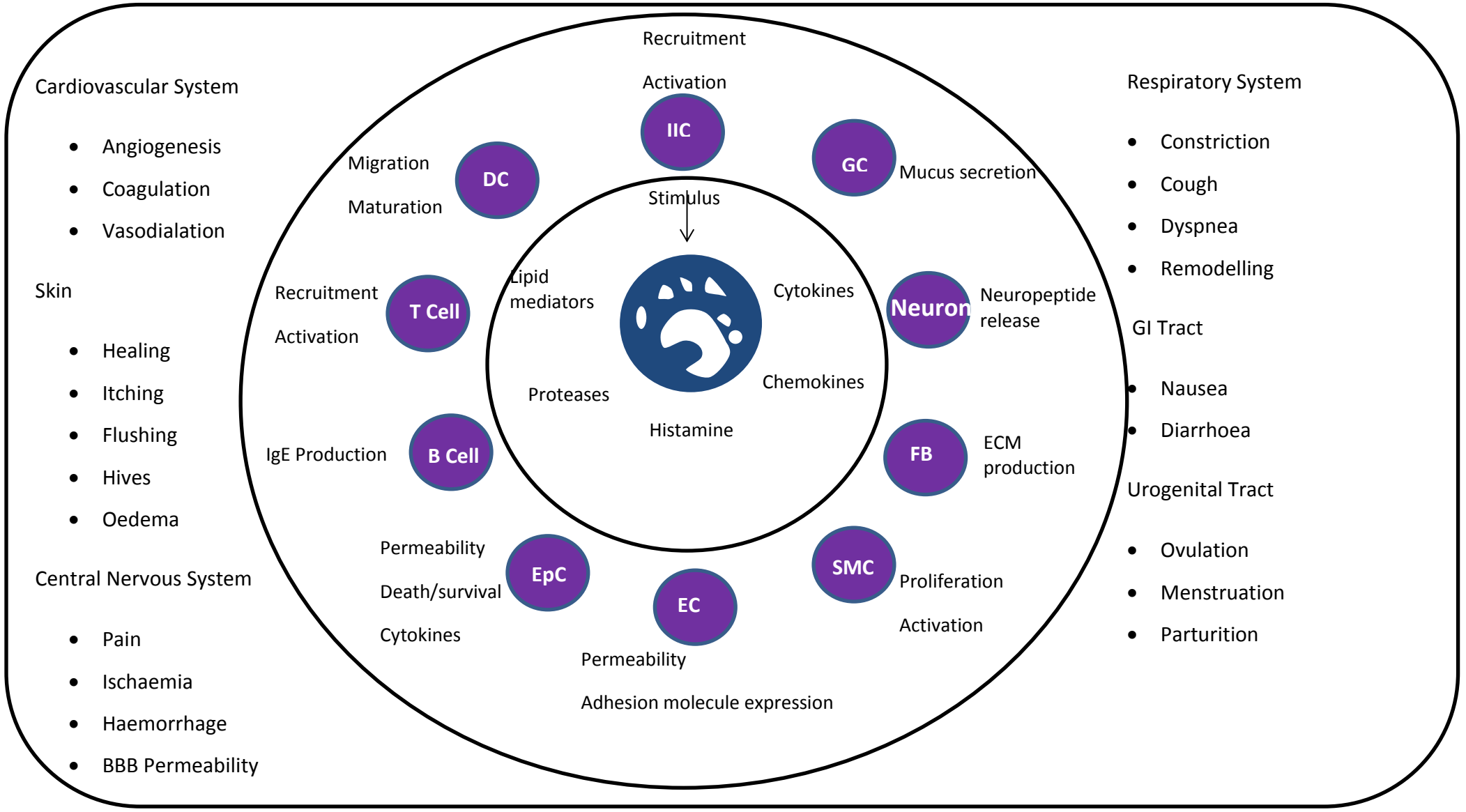


Figure 1.8: The role of mast cells in normal and aberrant physiological processes. Mast cell derived mediators influence cell function, which translates to changes at the organ and system levels. Abbreveations, DC, dendritic cell; EC, endothelial cell; EpC, epithelial cell; FB; fibroblast; GC, goblet cell; GI, gastrointestinal IIC, innate immune cells; SMC, smooth muscle cell;. Adapted from Moon *et al*, 2010.

survive for months to years within tissue through suppression of apoptosis (Morales *et al*., 2010). The most important of these mediators is SCF, the ligand for the KIT receptor, which is vital for the survival of mast cells.

There are two distinct phenotypes of mast cells found in humans, tryptase containing (MC_T) and tryptase and chymase containing (MC_{TC}). MC_T are concentrated to the airways and small bowel submucosa, whilst MC_{TC} reside in the skin and small bowel mucosa. In rodents there are also two phenotypes, mucosal type (MMC) found in the gut and connective tissue mast cells (CTMC) found in the skin and peritoneal cavity, (Metcalfe, 2008) (**Table 1.1**). It is not known whether mast cells of the brain represent a distinct phenotype; however the lack of KIT receptor on brain mast cells of C57BL/6J mice, Sprague-Dawley rats and Ring doves is evidence that they may be distinct (Shanas *et al.*, 1998).

During pathological events, including focal cerebral ischaemia and hypoxia-ischemia, mast cell hyperplasia is evident, which can be due to proliferation of local mast cells under appropriate stimulation or migration of populations from other sites (Bischoff and Sellge, 2002; Biran *et al.*, 2007). This migration of mast cells was witnessed in the Wistar rat brain, were fluorescence labelled cells were infused and subsequently observed in the brain parenchyma under confocal microscopy (Silverman *et al.*, 2000).

1.6.1 Mast cell activation and secreted mediators

Mast cell activation can be facilitated by a vast array of stimulants, as depicted in **Table 1.2**. Under conditions of ischaemic stroke the mechanism of mast cell activation is not currently known, however the current knowledge on the pathology of stroke gives an indication. A shift in pH caused by lactatacidosis may be responsible for mast cell activation, and similarly the increase in ICP following vasogenic oedema could trigger activation (Metz *et al.*, 2007 and Strbian *et al.*, 2009). The response of the mast cell depends upon the activation stimulus, where mediator release can be either slow, piecemeal degranulation (PMD), or explosive, anaphylactic degranulation (AND), (Dvorak 2005). Besides the rate of content extrusion, PMD and AND can be distinguished as AND leads to loss of membrane

Feature	Mouse Mast Cells	Human Mast Cells
Phenotype	MMC, CTMC	MC_T, MC_{TC}, MC_C
Proteoglycans	MMC: chondroitin sulphate di-B, A, E. CTMC: heparin, chondroitin sulphate E.	Heparin, chondroitin sulphate A and E.
Proteases	MMC: MMCP-1 and 2 CTMC: MMCP-3, 4, 5, 6 and 7	Tryptase-α, β and γ, chymase.
Biogenic Amine	MMC: Histamine (1 pg/cell), serotonin CTMC: Histamine (≥15pg/cell), serotonin.	Histamine, serotonin.
Growth factors	IL-3 and SCF	SCF
MHC Expression	MHC I: BMMC MHC II: BMMC, PCMC	MHC I: constitutively expressed MHC II: HMC-1.
NOS	iNOS, eNOS.	nNOS, iNOS and eNOS.

Table 1.1: **Comparison of some features of mouse and human mast cells.** Abbreviations, BMMC, bone marrow cultured mast cell; CTMC, connective tissue mast cell; eNOS, endothelial NOS; HMC-1, human mast cell line; iNOS, inducible NOS; MC_C, chymase containing mast cell; MC_T, tryptase containing mast cell; MC_{TC}, tryptase-chymase containing mast cell; MHC, major histocompatibility complex; MMC, mucosal mast cell; MMCP, mouse mast cell protease; NOS, nitric oxide synthase; nNOS, neuronal NOS and PCMC, peritoneal cavity derived mast cell;. Adapted from Moon *et al.*, 2010.

Ligand	Receptor
IgE	FcεR1, FcγR11, FcγR111 and galectin-3
C3a, C5a, C3b and C4b	C3aR, C5aR, CR3 and CR4
Lipopolysaccharide	Toll-like receptor 4
Stem Cell Factor	KIT
Substance P, NGF	Via respective G protein coupled receptors
Heat, Pressure, pH and Vibration	Via indirect mechanisms
TNF-α, IL-1 and IL-12	Via cognate receptors

Table 1.2: Mechanism of mast cells activation. Abbreveations, C, compliment; FcR, crystallisable fragment receptor; IgE, immunoglobulin E; IL, interleukin; NGF, nerve growth factor; TNF, tumour necrosis factor;. Adapted from Galli and Tsai, 2008.

integrity with subsequent fragmentation (Dvorak, 2005). Upon activation, mast cells release a number of active mediators as depicted in **Table 1.3**.

1.6.2 Mast cells in ischaemic stroke

The vascular location of mast cells within the brain and the broad array of mediators stored within and *de novo* synthesised, gives an initial indication that mast cells may be involved in the pathogenesis of stroke (**Figure 1.9**). Indeed, in the mammalian brain, mast cells are found predominantly at perivascular locations in the dura mater, leptomeninges, choroid plexus, thalamus, hypothalamus, mesencephalon and the olfactory bulb (Silver *et al.,* 1996). This perivascular location means mast cells are ideal candidates to regulate the BBB, and many of the mast cell mediators have been shown to affect BBB integrity. For example, histamine has been shown *in vitro* to increase the permeability of endothelial cells by rearrangement of the cytoskeleton and the accessory protein vascular endothelial-cahedrin (Andriopoulou *et al.,* 1999). The mast cell secreted chemokine MCP-1α, has also been shown *in vitro* to alter the distribution of occludin, zonular occludins and claudin-5 following five hours of OGD followed by reoxygenation to mimic ischaemia/reperfusion. Addition of MCP-1α mAb to the medium reduced the permeability of the endothelial cells to FITC labelled albumin (Dimitrijevic *et al.,* 2006).

Assessment of mast cell mediators has also been carried out *in vivo*. Mice deficient in MCP-1α (MCP-1$\alpha^{-/-}$) displayed an improved outcome following thirty minutes of tMCAo, when compared to their wild type littermates. Post-mortem analysis of brain water and electrolyte content indicated a lesser degree of brain swelling and BBB permeability in the MCP-1$\alpha^{-/-}$ mice. The volume of infarct was also significantly reduced in the genetically modified mice, and there was also a reduction in infiltrating leukocytes. Finally, the wild type mice displayed a greater expression of the pro-inflammatory cytokines TNF-α and IL-1β (Dimitrijevic *et al.,* 2007). More direct analysis of mast cell contribution to cerebral pathology was assessed in an animal model of hypoxic-ischemia. The mast cell stabilising agent SCG was administered to Wistar rats thirty minutes prior to exposure to a hypoxic gas mixture for a period of ninety minutes (Jin *et al.,* 2007). The authors found that mast cell

Mediator Class	Mediators
Pre-formed	Histamine, serotonin, heparin, neutral proteases (tryptase and chymase, carboxypeptidase, cathepsin G), major basic protein, acid hydrolases, peroxidase, phospholipases, TNF-α.
Lipid Mediators	LTB4, LTC4, PGE2, PGD2, PAF
Cytokines	TNF-α, TGF-β, IFN-α, IFN-β, IL-1α, IL-1β, IL-5, IL-6, IL-13, IL-16, IL-18.
Chemokines	CXCL8, I-309 CCL1, MCP-1 CCL2, MIP-1αS, MIP1β, MCP-3, RANTES, eotaxin.
Growth Factors	SCF, M-CSF, GM-CSF, FGF, VEGF, NGF.

Table 1.3: Mast cell derived mediators from human cell lines. Abbreveations, C, chemokine; FGF, fibroblast growth factor; GM-CSF, granulocyte monocyte colony stimulating factor; IFN, interferon; IL, interleukin; LT, leukotriene; MCP, mast cell protease; M-CSF, monocyte colony stimulating factor; MIP, macrophage inflammatory protein; NGF, nerve growth factor; PG, prostaglandin; PAF, platelet activating factor; RANTES, regulated and normal t cell expressed and secreted; SCF, stem cell factor; TGF, tumour growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor;. Adapted from Metcalfe, 2008.

stabilisation significantly reduced neuronal cell death and reduced the activation of astrocytes and microglia (Jin *et al.,* 2007). Direct evidence that mast cells are important in transient cerebral ischaemia came from work carried out in Wistar rats subjected to one hour of tMCAo occlusion followed by three hours of reperfusion (Strbian *et al.,* 2006). The study compared the effects of ischaemia on genetically manipulated rats which were mast cell deficient ($MC^{-/-}$), and also rats treated with SCG and the mast cell secretagogue, compound 48/80. There was no real difference in the infarct volumes between any of the groups, however the $MC^{-/-}$ rats had a 58% reduction in brain swelling and the 48/80 treated animals had 89% more swelling compared to the control. These findings directly correlate to the data collected regarding BBB permeability. The $MC^{-/-}$ rats exhibited a significantly lower permeability to Evans blue albumin (EBA), as did the SCG treated rats, whereas the 48/80 treated rats displayed significantly more EBA leakage. Neutrophil infiltration was also measured and was found to be significantly lower in the $MC^{-/-}$ rats and SCG rats compared to their respective controls. Perhaps unsurprisingly, the 48/80 compound rats displayed an increase in neutrophil infiltration, however this increase was not found to be statistically significant (Strbian *et al.,* 2006).

1.7 Modelling stroke in animals

The study of stroke pathology and the testing of potential therapeutics are aided greatly by the availability of a broad range of animal models of focal cerebral ischaemia (**Table 1.4**). Models have been established in multiple species, including rats, mice, dogs, cats, rabbits, pigs and non-human primates, and are designed to replicate as much as possible the human condition, however for practical and ethical reasons some concessions must be made (Liu and McCullough 2010). The vast majority of basic and pre-clinical research is carried out in rats, which are readily available in large numbers at a reasonable cost and are a large enough size to permit the technically demanding surgical induction of stroke and also to permit monitoring of physiological variables such as temperature, blood pressure and blood gas concentrations. Additionally, the rat has a similar cerebrovasculature network to humans, with a complete circle of Willis, although there are some confounding factors to consider. For example, there are distinct differences in neuroanatomy and

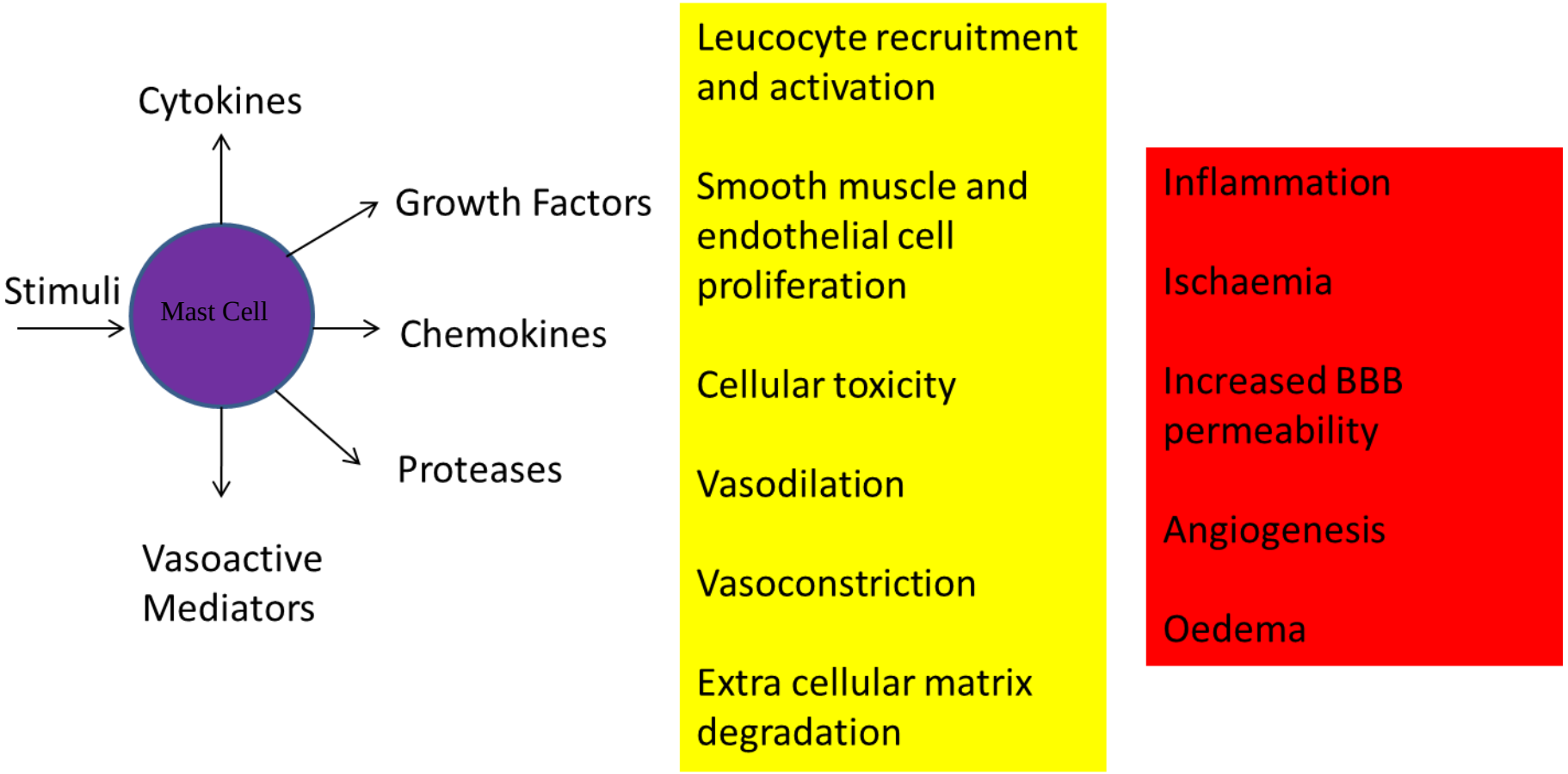


Figure 1.9: Potential role of mast cells in cerebral ischaemia. Upon activation, mast cells release an array of mediators which act at the cellular and tissue level to induce pathobiological mechanisms of cerebral ischaemia. Adapted from Moon *et al.*, 2010.

Model	Advantage	Disadvantages
ILT occlusion of MCA	Reproducible infarct, permanent or transient ischaemia achievable, less technically demanding than other models.	No visual confirmation of occlusion (Laser Doppler flowmetry available). Filament can introduce variability.
MCA electrocoagulation	Highly reproducible, low mortality can alter infarct topography by occluding multiple vessels or by changing occlusion position. Visual confirmation of occlusion.	Reperfusion not possible, technically challenging.
ET-1 topical application or microinjection	Reproducible injury, occlusion visually confirmed. Guide cannula permits induction of ischaemia in conscious animals.	Technically challenging, ET-1 potency can be unreliable among batches. Unable to investigate thrombolysis.
Photochemical occlusion of MCA	Reproducible, visual confirmation, thrombolysis studies possible and low mortality.	Some pathology very different from human condition, small injury not compatible with behavioural studies.
Thrombin injection	Reproducible, visually confirmed, thrombolysis studies possible and low mortality.	Technically challenging, secondary ischaemia caused by incomplete dissolution of clot and incompatible with behavioural studies due to small injury.

Table 1.4: The advantages and disadvantages of selected major rodent models of focal cerebral ischaemia. Abbreviations, ET-1, endothelin-1; ILT, intraluminal thread; MCA, middle cerebral artery;. Table adapted from Macrae, 2011.

connectivity between rats and humans as well as large differences in cerebral perfusion rates (Macrae, 2011 and Kraft *et al.,* 2012). There are four well established methods of inducing ischaemic stroke through occlusion of the MCA, which is the most commonly occluded artery in human stroke, in animals and involves reduction of flow to the striatum and most of the cerebral cortex. The MCA can be mechanically obstructed by insertion of an intraluminal filament or by direct electrocoagulation as described below. Alternatively, thromboembolism, photothrombosis and ET-1 can be used to induce ischaemia of the MCA territory (Macrae, 2011).

1.7.1 Intraluminal thread model

The intraluminal thread (ILT) model is the most commonly used model of stroke and can be performed by insertion of a silicone coated or heat blunted filament through the internal carotid artery. The filament traverses the artery until it reaches the origin of the MCA, where it can be left in position for varying lengths of time and subsequently removed or alternatively it can be left in place permanently. Removal of the filament to allow reperfusion is popular a method of modelling stroke, as it is an attempt to replicate vascular recanalization in human stroke. This artificial reperfusion is more rapid and less complicated than drug induced clot dissolution, and it permits the study of ischaemia/reperfusion injury. The extent of injury produced by the intraluminal thread model can vary considerably, depending on the length of the occlusion period, if the filament is removed, the period of reperfusion, the filament type used and also the species and strain of animal (Liu and McCullough, 2010).

In the WT mouse, increasing the occlusion period from 15 to 30 minutes increases lesion volume 6-fold, after a period of 24 hours of reperfusion (McColl *et al.,* 2004). This is due in part to poor collateral perfusion caused by an incomplete circle of Willis, which puts at risk deeper brain structures out with the MCA territory including the hippocampus, thalamus and hypothalamus. Indeed, the majority of WT mice have none or only one posterior communicating artery (PcomA), which link the anterior and posterior circulation, via the posterior cerebral artery and the superior cerebellar artery to complete the circle of Willis (McColl *et al.,* 2004). The volume

of the ischaemic lesion varies further amongst different strains of mice, which again have unique cerebrovascular architecture, causing up to a fivefold difference in lesion volume under identical conditions between different strains (Barone *et al.*, 1994).

The ischaemic lesion develops to its maximal volume by 72 hours after reperfusion commences, and is thought to evolve at a higher rate in the mouse model than in rats. In rats undergoing 60 minutes of tMCAo, the lesion is fully developed by 24 hours and the damage remains consistent after one week. Where the filament is not removed, the infarct develops over a longer period and the injury is less severe (Liu and McCullough 2010). Indeed, in Long Evans rats, reperfusion of the occluded vessel for up to 24 hours following 2-5 hours of occlusion resulted in lesions of more than double the volume of animals where the filament was left in place for 24 hours. This indicates that the majority of the ischaemic damage in tMCAo models is due to reperfusion, whilst damage in pMCAo is purely a result of ischaemia (Aronowski *et al.*, 1997).

The extent of damage in the ILT model can vary greatly, depending on the type of filament used to occlude the vessel, which vary in length and coating. Silicone coated filaments, when inserted block the origin of the posterior communicating artery, anterior choroidal artery hypothalamic artery and the anterior communicating artery, as well as the MCA, whereas blunted filaments block mainly the MCA origin (Durukan, 2007). The filament used also yields varying results between strains and species. In rats, coated sutures increase lesion volume but also decrease variability, whereas in the mouse consistency of lesion volume is unaffected by coating the filament and lesion volume has been reported to be similar (Belayev *et al.*, 1996 and Huang *et al.*, 1998). What has been proven to be crucial to reproducibility of the model is the diameter of the filament in relation to the weight of the animal. In w mice weighing 22-24g, a suture diameter of between 170mm and 180mm were found to give the most consistently reproducible infarcts, probably through reduction of residual flow around the filament (Tureyen *et al.*, 2005).

1.7.2 Animal selection

As mentioned previously, the most popular animal model of stroke is ILT in the rat. However, the versatility of the mouse, which is amenable to genetic manipulation to allow the study of individual molecules in stroke pathology, has seen these animals used more frequently in recent history. The most popular strain of mouse in use is the WT, which is vulnerable to tMCAo by ILT as described in the previous section, and, of relevance to this work, is the background strain of the most often used mast cell deficient mouse model, the C57BL6/J Kitw$^{-sh/w-sh}$ (Kit) mouse. The Kit mutation first arose around 30 years ago by spontaneous mutation of the transcription regulatory elements of the start site of c-kit transcription, a cell surface receptor for stem cell factor vital for mast cell maturation and survival (Grimbaldeston *et al.,* 2005 and Moon *et al.,* 2010). This mutation was then backcrossed to the WT background through ten generations, to produce a strain with phenotypic advantages over other available strains. Other strains, such as the Kit$^{w/w-v}$ are anaemic and sterile, so are difficult and expensive to breed, have leukocyte deficiencies and are susceptible to spontaneous diseases such dermatitis and squamous papilloma, problems not faced by the Kit strain. However, compared to the WT background strain the Kit mice suffer from splenomegaly, cardiomegaly, and thrombocytosis and are neutrophilic (Nigrovic *et al.,* 2008) (**Table 1.5**). The greatest advantage of the Kit mice over other mast cell deficient strains of mice is that the deficiency can be repaired by engraftment of mast cells cultured from bone marrow of the WT mouse. This is made possible as the mutation is on the mast cell, whilst in other strains the mutation involves the kit signalling molecule, SCF (Grimbaldeston *et al.,* 2005).

1.8 Hypothesis

The hypothesis to be tested here is that mast cells are active in the acute phase of experimental focal cerebral ischaemia, and interact with the brain to potentiate ischaemic pathology. The plethora of pro-inflammatory, vasoactive and proteolytic mediators stored by and synthesised by mast cells, could degrade the BBB leading to vasogenic oedema, and may also promote an inflammatory milieu within the brain and periphery.

Genotype	Mutation	Phenotype
W-sh/W-sh	Inversion of regulatory element upstream of Kit coding region, reducing its expression	White coat, fertile, lack anaemia, backcrossed to WT background, more severe mast cell deficiency than W/W^v mice. Mast cells can be restored from BMMC transfer.
W/W^v	Heterozygous. Point mutation produces truncation of Kit (W). Point mutation in tyrosine domain of Kit (W^v, W^{v-}viable).	White coat, sterile, anaemic and neutropenic. Restoration of mast cells by BMMC less than 1% of wild type. Lack interstitial γδT cells.
SI/SI^d	Heterozygote. SCF coding region deleted (SI). Deletion of cell surface SCF (SI^d).	White coat, sterile and anaemic. Restoration of mast cells by BMMC less than 1% of wild type.
mi/mi	Mutation of regulatory element of Kit transcription factor, MITF.	Oseteoperosis and decreased expression of other MITF dependent genes such as MCCP, tryptase, granzyme B and tryptophan hydrolase. Can restore mast cells by adoptive transfer of BMMC.

Table 1.5: Comparison of the major mouse models of mast cell deficiency. Abbreviations, BMMC, bone marrow cultured mast cells; MITF, microphthalmia-associated transcription factor SCF, stem cell factor;. Table adapted from Moon *et al.*, 2010.

1.9 Objectives

The following objectives were met to investigate the involvement of mast cells in a mouse of focal cerebral ischaemia:

- Investigate the effect of genetic mast deficiency on BBB damage, oedema, and neutrophil infiltration following 4 hours of reperfusion after 45 minutes tMCAo.
- Investigate the effect of pharmacological mast cell stabilisation on BBB damage, oedema, lesion size and neutrophil infiltration following 4 hours of reperfusion after 45 minutes tMCAo.
- Determine the effect of genetic mast cell deficiency on serum and brain tissue concentration of TNF-α, and the expression of an array of proteins at various time points during the acute period following tMCAo.
- Investigate the effect of genetic mast cell deficiency on BBB damage, oedema and lesion size; 72 hours post 45 minutes tMCAo

Chapter 2: General Methods and Material

2.1 *In vivo* studies

2.1.1 Animal source

All experiments were carried out on adult male C57BL/6 (WT) or C57BL6/J Kit $^{w\text{-}sh/w\text{-}sh}$ (Kit) mice. WT mice were either bred in-house or purchased from Charles River Laboratories as specified in each study. Kit mice were originally sourced from Jackson Laboratories, USA and the colony maintained in-house. Prior to surgery animals were housed in their home cage in a temperature controlled environment, with a 12 hour light dark cycle and were given access to food and water *ad libitum*. Following surgery animals were individually housed, soft diet and water were placed in the cage, and they were monitored three times daily. Animals found to be moribund through excessive weight loss (>20% of start weight) or those unable to move and/or correct gait were terminated and excluded, as specified in each study. The regulations, as specified by the Animals (Scientific Procedures) Act (1986) where strictly adhered to throughout and were carried out under local ethical approval and the appropriate Home Office licence (Project Licence No. PPL 60/3775; Personal licence 60/11900). All procedures were carried out in accordance with ARRIVE and STAIR guidelines where possible.

2.1.2 Animal preparation for surgery

Male WT or Kit mice aged 10-12 weeks and weighing 25-30g were placed in a Perspex box and anaesthesia was induced by inhalation of 3% isoflurane/1000ml/min oxygen (O_2) for approximately 2-3 minutes. Mice were transferred to a face mask and anaesthesia was reduced to 2% isoflurane/1000ml/min O_2, which was maintained for 20 minutes and subsequently reduced to 1.5% isoflurane/1000ml/min O_2 for the remainder of the procedure. Excess isoflurane and O_2 were actively scavenged using an aldosorber unit (Shirley Alder & Co, Cardiff, UK). Body temperature was monitored using a rectal probe and maintained at 37 ± 0.5 C$^{\circ}$ with an automatic heat mat (Harvard Apparatus, Kent, Edenbridge, UK).

2.1.3 Laser doppler flowmetry

Collateral perfusion of the MCA territory was measured in one study throughout the surgical procedure using laser Doppler flowmetry (Moor Instruments, Axminster, UK). The animal was laid on its right hand side and securely taped to the operating surface. An incision midway between the ear and eye exposed the left skull for probe placement. Using Bregma as a reference point, the probe was glued in place approximately anterior/posterior -1mm, medial/lateral +2.5mm and readings were recorded at five minute intervals throughout the procedure.

2.1.4 ILT model of tMCAo

Lignocaine (5% w/v), a topical local anaesthetic, was applied to the neck area of the mouse, prior to making a midline neck incision. The neck incision was opened further with rounded blunt scissors to fully expose the submandibular gland. The submandibular gland was then dissected and retracted onto tissue which was kept moist throughout surgery with sterile saline. The left common carotid artery (LCCA) and its bifurcation to the left external carotid artery (LECA) and left internal carotid artery (LICA) were exposed by blunt dissection of the overlaying connective tissue using tweezers and Tri-swabs (Royem Scientific, Luton, UK) (**Figure 2.1**). Next, the left superior thyroid artery, a branch of the ECA, was electro-coagulated using a diathermy probe (Eschmann Equipment, Lancing, UK) and dissected. The left occipital artery, lying across the LICA was also electro-coagulated with great care taken not to disrupt the nearby vagus nerve (**Figure 2.1 B**).

A ligature (6-0 silk thread) (Henry Schein, Nitside, UK) was placed under the LECA by dissecting the underlying connective tissue with 90˚ tweezers and passing the thread through with tweezers. The ligature was double knotted distally from the bifurcation of LCCA. A second thread was placed under the LICA by first passing a thread under LCCA and then passing the trailing end under the LECA. This thread was left untied and taped to the operating surface. A third thread was placed under the LECA and loosely tied at the LCCA-LECA bifurcation point, and a final thread was placed under the LCCA, distal from the bifurcation point and was left untied.

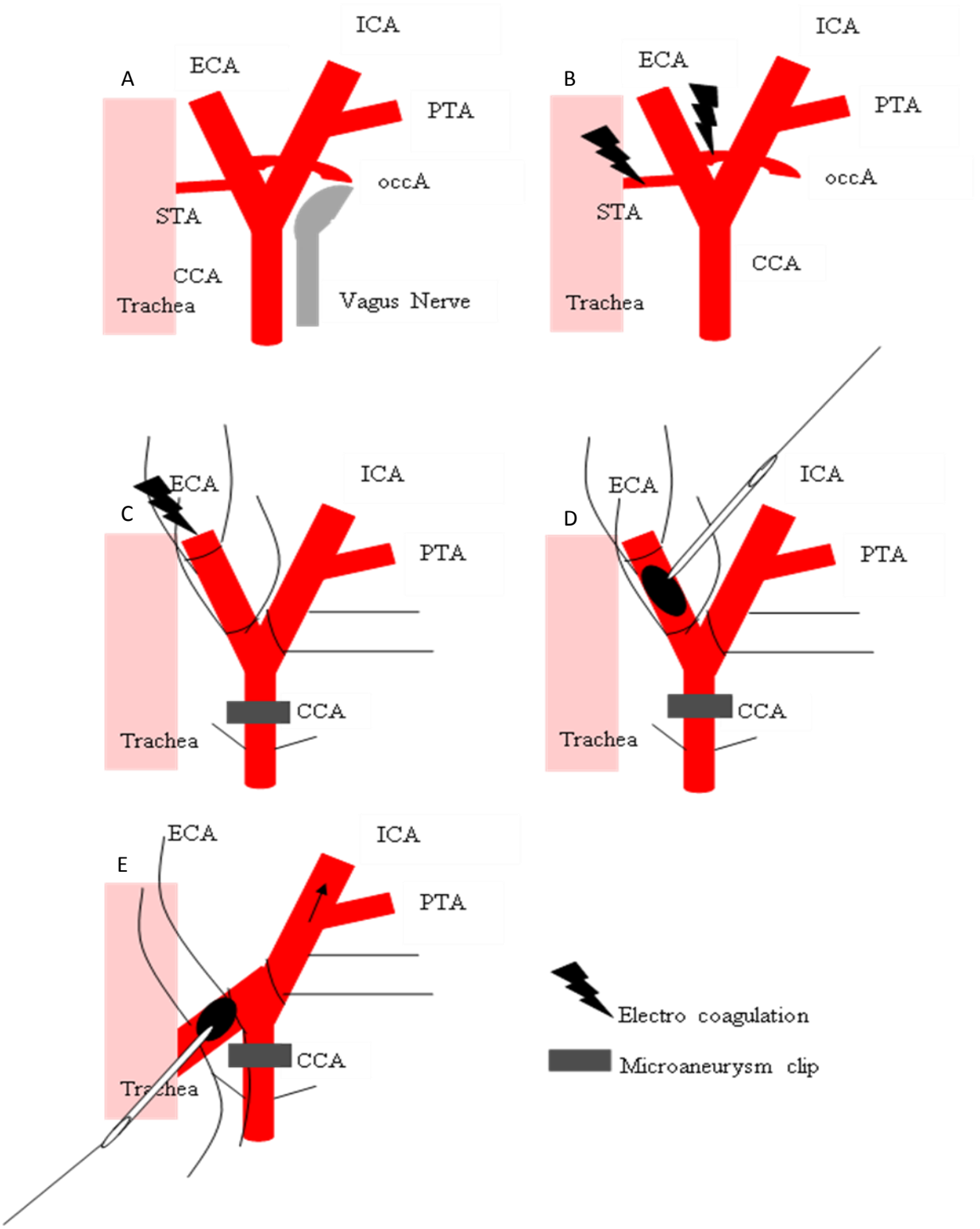


Figure 2.1: Representation of the Intraluminal filament occlusion of the middle cerebral artery model. Described in section **2.1.4.** Abbreviations, ICA, internal carotid artery; ECA, External carotid artery; PTA, pterygopalatine; occA, occipital artery; STA, superior thyroid artery;

The segment of vessel distal to the first ligature on the LECA was electrocoagulated and was followed by the placement of a 13 mm microaneurysm clip on the LCCA, distal to its bifurcation point (**Figure 2.1 C**). Tension was applied to the thread around the LICA to cease blood flow along the LICA, and the tense thread was taped down to the operating surface. Next, a small incision was made on the abluminal surface of the LECA, and a silicon-rubber coated monofilament (20 mm length of which 9 mm is coated with silicone giving an overall diameter of 0.23 +/- 0.01 mm) (Doccol Corporation, Sharon, USA) inserted approximately 9 mm until its tip sat at the bifurcation with the LCCA and LICA. The filament was held in place by tightening the loosely tied thread on the LECA (**Figure 2.1 D**). The coagulated area distal to the ligature on the LECA was cut and pulled to give an angle into the LICA. Tension on the thread around the LICA was released and the filament was advanced until resistance was met, indicating the filament had reached the origin of the middle cerebral artery (**Figure 2.1 E**). At this point the filament was tied in place and remained for 45 minutes, unless otherwise stated. The filament was then carefully removed and the insertion incision electro-coagulated and the microaneurysm clip removed. The submandibular gland was replaced to its original position and the surgical wound sealed with 5-0 suture. Finally the isoflurane was turned off and the mouse turned on its side to receive 100% O_2 for 2 minutes and given 500 µl sterile saline (Henry Schein, Nitside, UK) intraperitoneally. For animals undergoing sham operation, all procedures were followed as described. However, the filament was removed immediately after insertion and not left in place.

2.1.5 Stereotaxic surgery

In one study SCG (Sigma, Poole, UK) (75µg in 2µl saline) or sterile saline (Henry Schein,Nitside, UK) alone were injected intracerebroventricularly by stereotaxic injection immediately prior to onset of MCAo. Mice were mounted onto a stereotaxic frame (Harvard Apparatus, Edenbridge, UK) and secured in the prone position using ear bars. Lidocaine 5% was applied to the scalp and an incision made along the midline. The injection site was established using the co-ordinates, anterior/posterior -0.6mm medial/lateral, +1mm, using Bregma as a zero reference point, on the surface of the ipsilateral hemisphere. A burr hole was made over the

injection site and a 32 gauge needle attached to a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) was inserted 2.4mm, using the surface of the dura for zero reference. Over a two minute period 2µl of SCG or vehicle was injected, and the needle was left in place for a further two minutes. The needle was then slowly retracted and the wound sutured.

2.1.6 Clarks neurological deficit

In one study, animals were evaluated at 24 hours, 48 hours and 72 hours post-tMCAo using a system developed to detect both general and focal deficits in the mouse following MCAo (Clark *et al.,* 1997). General deficits were assessed in a $4m^2$ open field without interference and observations were recorded regarding appearance of fur, ears, eyes, posture, spontaneous activity and epileptic activity as described in **Table 2.1**. For focal deficit assessment, mice were subject to a number of motor function tasks as follows as described in **Table 2.2**. Body symmetry, walking and circling behaviour were all assessed in an open field. To assess sloping desk climbing, mice were placed on a coarse 45^o angled surface, whilst forelimb symmetry was analysed with the mouse hanging from its tail and the forelimbs were allowed to contact the desk to measure mandatory circling behaviour. Finally, the whisker response was observed following stimulation in an open field. Each animal was given a score for each of the individual observations and the score was accumulated to give and overall severity assessment. A maximum score of 28 was achievable for each category, however a score of greater than 21 in either the general or focal deficit was considered so severe that the animal be excluded from the study and euthanized.

2.17 Termination and tissue collection

At the end of each experiment animals were killed by injection of 200µl of sodium pentobarbital (Henry Schein, Nitside, UK), followed by cardiac puncture. Typically, blood was collected and stored overnight at 4˚C, before being centrifuged at 2000g to separate serum. The serum was taken and stored at -20˚C until used. The brain was then immediately removed and either flash frozen or homogenised as described in section **2.2.1.1** and **2.2.1.2**.

Score	Fur	Ears	Eyes	Posture	Spontaneous Activity	Epileptic Seizures
0	Fluffy, clean, tidy	The ears are normally stretched latero-posteriorly and respond to the noises.	Open, clean, following quickly any movements around the animal	Upright on 4 legs, with its back parallel to desk and balanced on 4 legs	Alert and explores continuously	No seizures
1	Localized piloerection and/or dirty fur limited to one or two areas of the body	One or both ears are positioned lateral without being stretched behind.	Open and with a mucosal and watery dirt, following slowly any movements	Stooped when walking and lowers to gain balance	Looks alert but is calm and listless	Hyperactivity with tendency to climb cage walls (scores 3)
2	Piloerection or dirty fur in more than two areas of the body	As score 1, but unable to respond to the noises.	Open with mucosal dark dirt	Head or a part of the trunk lies on the desk	Starts and stops to explore slowly and iteratively	Aggressive, nervouos and staring (scores 6)
3	N/A	N/A	Oval opening of the eyes, with mucosal dirt	Lies on side but can still straighten with some effort	Lethargic, numb and moves on site	Extremely excitable, frenzy, seizures after stimulation (scores 9)
4	N/A	N/A	Shut.	Lies unable to straighten up	Still, moving occasionally on handling	Epileptic seizures, breathing variations and loss of consciousness Score 12

Table 2.1: Criteria used to assess and score animals on general neurological deficits. Deficit was assesed at 24 hours, 48 hours and 72 hours after transient middle cerebral artery occlusion. Animals were assessed as described in **section 2.1.6.**

0	Both sides are raised from the desk, the 4 legs are symmetrical under the body and the tail is straight	Normal walking, flexible, symmetric and quick	Climbs quickly	Turns quickly to left and right	Forelimbs are extended towards the desk, vigorously moving	Extends both forelimbs equally	Symmetrical response. Looks toward stimulation and moves away from source of stimulation
1	Mild asymmetry. Lies on ipsilateral side and tail is bent	Still, mechanical walking. Stooped and reduced speed	Climbs slowly with a lot of effort	Turns preferentially to one side	Mild asymmetry with ipsilateral forelimb not completely extended	Extends both forelimbs but starts to circle to one side preferentially	Delayed response on ipsilateral side with normal response on contralateral
2	Moderate asymmetry. Lies on ipsilateral side with limbs extended laterally and tail is bent	Slight limp, asymmetric movements	Can only keep position on sloping desk	Turns to one side although inconstantly	Moderate asymmetry ipsilateral forelimb is kept on trunk with mild contortion of the body towards ischaemic side	Turns slowly to one side	No response on ipsilateral side with normal response on contralateral side
3	Clear asymmetry. Body is bent and ischaemic side lies on desk	Severe limp, the animal often falls, clear walking deficit	Slowly falls towards the bottom	Turns constantly to one side	Extreme asymmetry with ipsi-lateral forelimb clearly adhered to trunk	Turns slowly to one side without completing a full circle	No response on Ipsilateral side with delayed response on contralateral side
4	Extreme asymmetry. Body and tail are bent and ischaemic side lies on the desk	Not moving. Movement after stimulation is limited to three steps	Falls immediately unable to avoid it	Swings slowly on site or remains still	Slow or no movement of the forelimbs	Animal is still and the anterior part of the trunk lies on the desk with few slow movements	No response on either side

Table2.2: Criteria used to assess focal neurological deficits. Deficits were assessed at 24 hours, 48 hours and 72 hours following 45 minute transient middle cerebral artery occlusion. Animals were assessed as described in **section 2.1.6.**

2.2 Histology

2.2.1 Tissue sample preparation

2.2.2 Fresh freezing

Brains were removed and fresh frozen in isopentane (Fisher Scientific, Loughborough, UK) cooled to -42˚C on dry ice and subsequently stored at -20˚C until used. The tissue was embedded in M-1 embedding matrix (Thermo Electron Corporation, Milton Keynes, UK), and 20 μm sections were cut on a cryostat with low profile microtome blades, and then mounted on to glass slides. Coronal sections were collected at eight regions of interest (as described by Osborne *et al.*, 1987 and adapted for the mouse) which are positioned at 6.02 mm, 4.48 mm, 3.94 mm, 3.34mm, 2.86 mm, 1.98 mm, 1.00 mm and -0.8 mm Interaural (IA), using the Allen Reference Atlas (Dong, 2008) for reference (**Figure 2.2**).

2.2.3 Brain homogenate preparation

Upon removal, brains were placed in 1% protease inhibitor (in 20mM TRIS, pH 7.4) (Merck Chemicals, Nottingham, UK), at a 5 times w/v ratio, and kept on ice. The hemispheres were separated and homogenised with a hand held homogeniser until the sample was cloudy and contained no visible pieces of tissue. Triton X-100 (Sigma, Poole, UK) was added to samples to a concentration of 1% and aliquots were stored at -70˚C. Prior to use, samples were centrifuged at 2000g for ten minutes and the supernatant removed for analysis.

2.2.4 Haematoxylin and eosin staining

Haematoxylin and eosin staining was carried out to identify areas of ischaemic damage. Eosin stains connective tissue and cytoplasm pink, whilst haematoxylin stains nuclei dark blue (see **Figure 2.3**). Coronal tissue sections on glass microscope slides were initially post fixed in 4% PFA (Sigma, Poole, UK) for 20 minutes, then rinsed in tap water. Slides were then stained in Haematoxylin (1% w/v in absolute ethanol) (Sigma, Poole, UK) for 2 minutes, before 2 minutes of differentiation in acid alcohol (1% v/v HCL in absolute ethanol) (Sigma, Poole, UK) followed by a water wash and Scott's tap water for 2 minutes. The slides were then immersed for 2

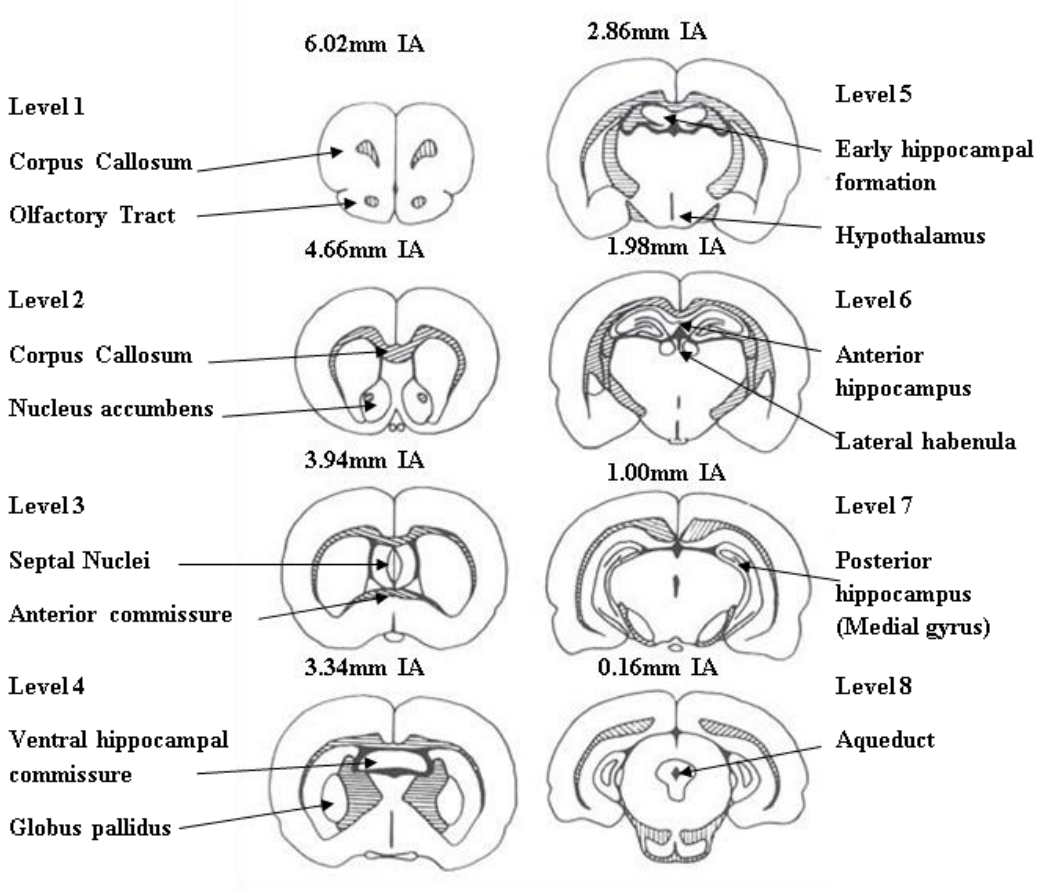


Figure 2.2: The eight distinct regions collected for quantification of lesion and oedema volume. The anatomical landmarks for each section are labelled as well as the distance from the interaural line (Adapted from Osborne *et al.*, 1987).

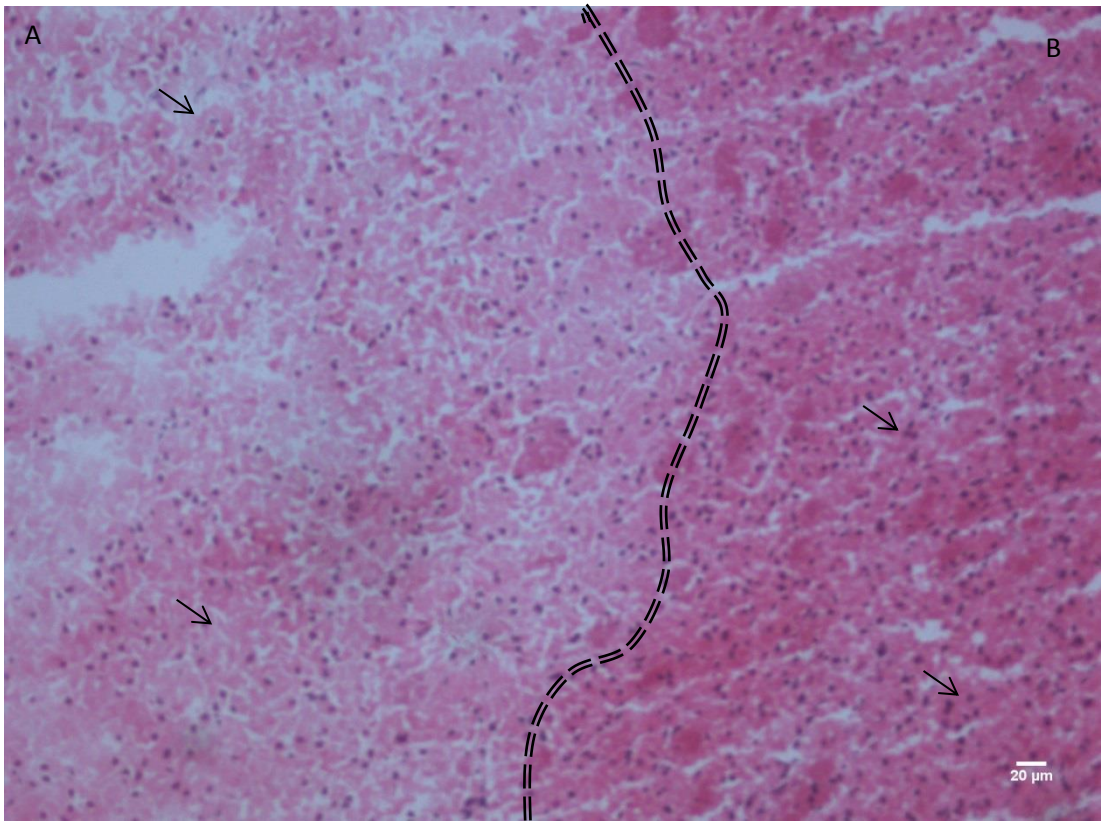


Figure 2.3: Ischaemic lesion on Haematoxylin and Eosin stained coronal tissue sections Areas of ischaemic lesion were identified on Haematoxylin and Eosin stained coronal tissue sections at eight distinct brain regions, shown in **Figure 2.2,** in tissue 4 hours after 45 minutes tMCAo. Damaged tissue, shown in image A has large neuronal loss and pale disrupted parenchyma (arrows). Healthy tissue in image B contains an abundance of neurons and intensely stained intact cytoplasmic structure (arrows).

minutes 30 seconds in Eosin Y (1% w/v aqueous) (Sigma, Poole, UK) and then rinsed in tap water until the water ran clear. The tissue was then dehydrated through 70%, 90%, 100% and an additional 100% ethanol (Sigma, Poole, UK) for 3 minutes each before clearing in two changes of histoclear for 3 minutes each. Slides were then mounted with a coverslip using DPX mounting medium (Sigma, Poole, UK).

2.2.5 Measurement of Ischaemic Damage and Oedema

Haematoxylin and Eosin stained sections, representative of the forebrain (see **Figure 2.2**), were analysed for measurement of lesion and oedema using a densitometer (MCID, InterFocus Imaging Ltd, Cambridge, UK). For each section, the ipsilateral and contralateral hemispheric areas were measured, as well as areas of ischemic pathology represented by regions of pallor. For confirmation, ischemic damage was assessed under a light microscope through identification of regions containing pyknotic and necrotic neurons (**Figure 2.3**). The volumes of each hemisphere and of the lesion were calculated by graphing the area of each of the eight sections against their IA distance. The area under the curve was then calculated; where Y intersected X at 7.9 mm IA and 0.1 mm IA, to produce the hemispheric and lesion volumes. Oedema volume was expressed as a percentage increase in the ipsilateral hemisphere over the contralateral volume as follows: (ipsilateral hemisphere volume - contralateral hemisphere volume)/contralateral hemisphere volume x 100.

2.2.6 Toluidine Blue Staining

Toluidine blue metachromasia was used to identify mast cells in tissue (**Figure 2.4**) (Overman *et al*., 2012). For each animal 24 sections were stained, two from each of the regions described in section **Figure 2.2**, with additional sections from regions 2.86 mm, 1.98 mm, 1.00 mm and 0.16 mm interaural, due to the observation that mast cells numbers were higher in the posterior regions of the brain. The tissue was defrosted overnight at 4˚C and then fixed in 4% paraformaldehyde for 20 minutes. Slides were washed briefly in dH_2O and then stained for 15 minutes in 0.1% acidic (HCl, pH 1) Toluidine blue for 15 minutes. Another wash in dH_2O, followed by 3 minutes in Eosin Y (1% w/v aqueous) (Sigma, Poole, UK), which was washed clear in dH_2O. The tissue was then dehydrated in 70% Ethanol for 5 minutes, followed by

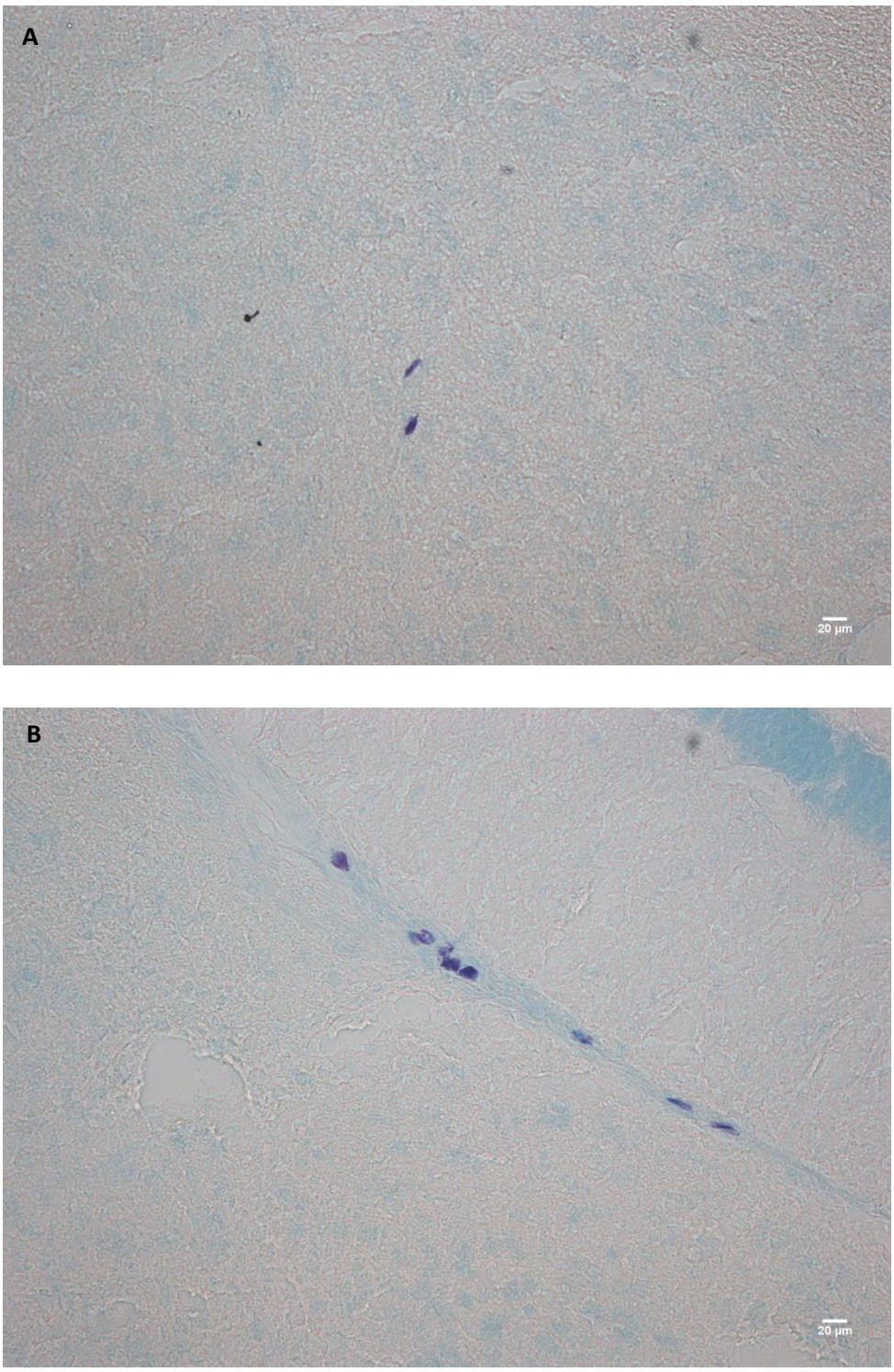


Figure 2.4: Mast cells stained in naive brain tissue with toluidine blue. Mast cells within tissue (image A) and those located perivascularly (image B) were stained on tissue sections located 1.98 mm IA, with acidic Toluidine blue using a light microscope at 200X magnification.

90% and 2 exchanges of 100% ethanol for 5 minutes each. They were then cleared in two 5 minute incubations in histoclear and then mounted with DPX. Cells were manually counted under a light microscope (x200 magnification), and expressed as mast cells per mm^2. This method could not reliably determine whether or not perivascular mast cells were inside or outside of the vessel.

2.3 Immunofluorescent staining

2.3.1 Immunoglobulin G measurement

Integrity of the BBB was measured by labelling endogenous immunoglobulin G within brain tissue, which would normally be absent under healthy conditions. Tissue was stained using an adapted method of Chen *et al.*, 2009, by direct immunofluorescence at the regions 3.94mm IA and 2.86mm IA. Frozen tissue sections were defrosted overnight in the fridge and sections were fixed in cold acetone (4˚C) for 20 minutes. Sections were encircled with a wax pen then washed in 1% Triton X (in PBS,pH 7.4) for 15 minutes before being washed with PBS, 2 x 5 minutes. Blocking buffer (20% normal goat serum) was applied for 20 minutes at room temperature, and then tapped off. Sections were incubated at room temperature for 1 hour with FITC-conjugated goat anti-mouse IgG (prepared in 5% normal goat serum) (1:250) (Abcam Ltd, UK), or horse anti-mouse IgG (1:250) (Abcam Ltd, Cambridge, UK) before washing off with PBS (pH 7.4) for 2 x 5 minutes. Vectashield® (Vector Laboratories, Peterborough, UK), containing the nuclear stain4',6-diamidino-2-phenylindole (DAPI) was applied to each section of tissue and a cover slip applied. Negative controls were incubated in the absence of antibody.

Stained sections were analysed using an upright epifluorescent microscope (Nikon Eclipse E600) at 200x magnification, at excitation wavelengths of 405 nm and 488 nm for DAPI and FITC respectively along with Metamorph imaging software. A fluorescent threshold was set by reduction of the exposure time of the negative controls until no signal was detected. Thereafter, wherever a fluorescent signal was detected within the ipsilateral hemisphere an image was acquired (see **Figure 2.5**). Acquired images were then analysed using Image J software, and areas of FITC fluorescence highlighted using the threshold tool (**Figure 2.6**). The threshold was set

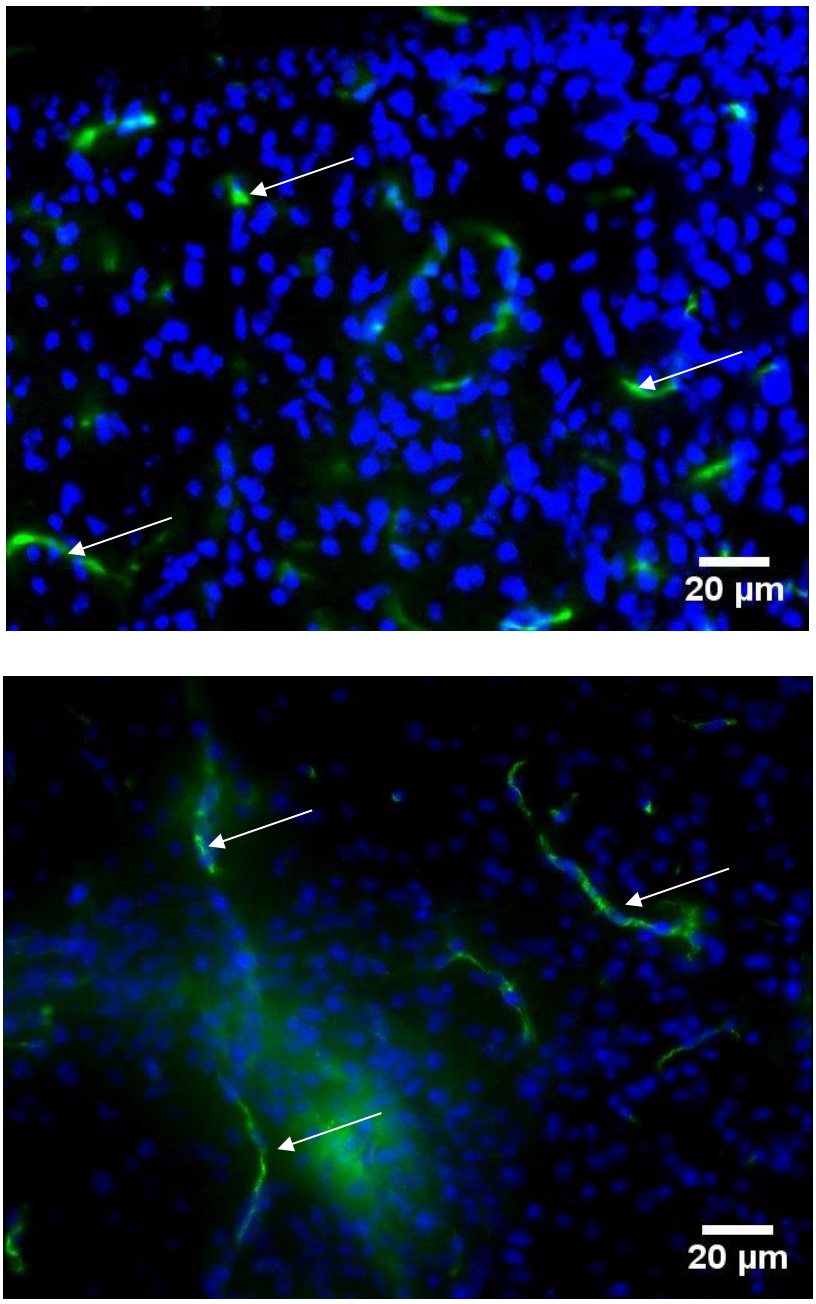


Figure 2.5: Endogenous immunoglobulin G stained in brain regions 3.94 mm and 2.86mm IA. Staining is seen within intact vessels in the contralateral hemisphere at 3.94mm IA (A, white arrows). Image B shows regions where immunoglobulin G has leaked into the tissue, within the ipsilateral hemisphere (white arrows) of a WT mouse which had undergone 45 minute tMCAo, followed by four hours of reperfusion. Images were acquired on a nikon Eclipse upright epifluorescent camera at 200 X magnifications and at wavelength of 488nm.

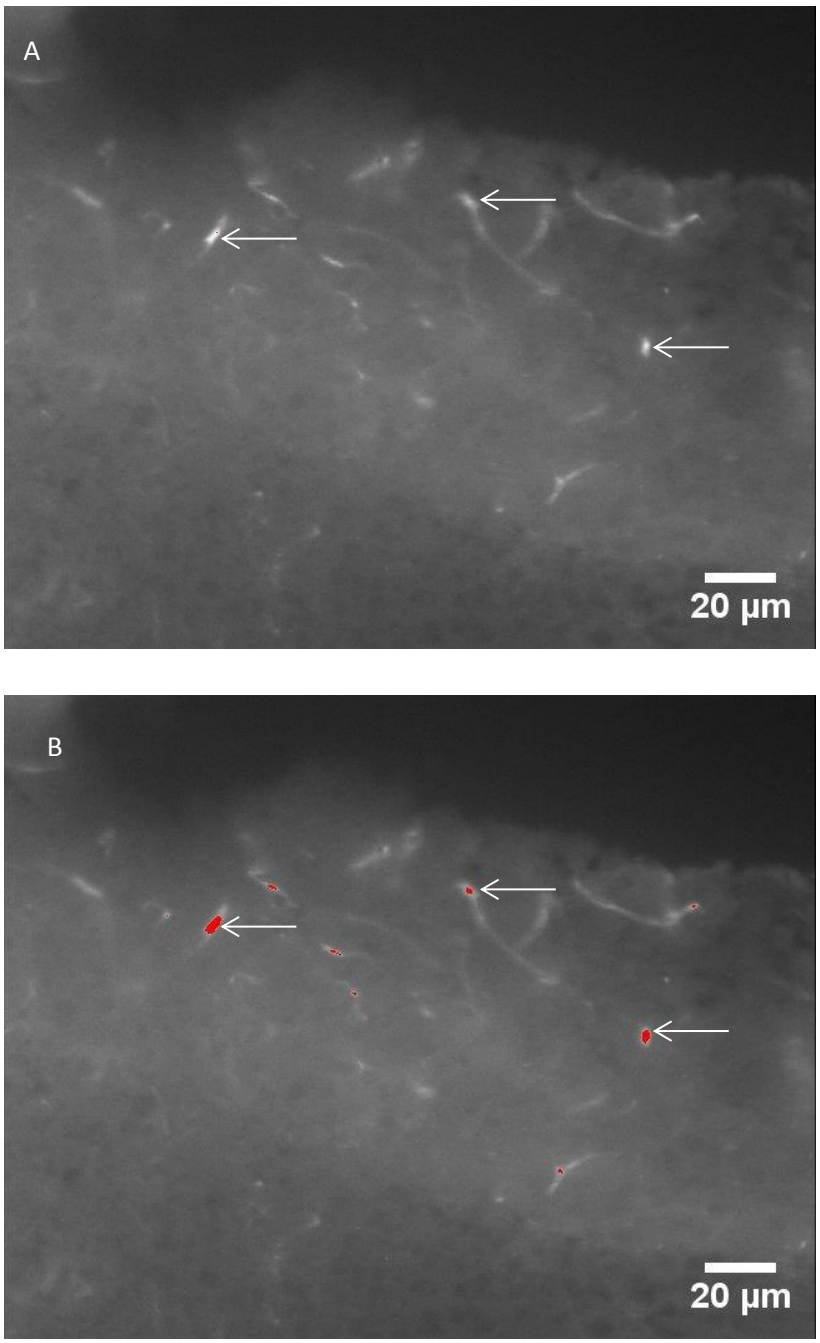


Figure 2.6: Native images of immunostained immunoglobulin G. Images were acquired using an epifluorescent microscope at 488 nm (200X magnification) on tissue sections stained by direct immunofluorescence for endogenous IgG, were analysed using Imagej software. Areas of fluorescence were identified (image A arrows) and then highlighted using the threshold tool (image B arrows) and the area and density measured, using the analyse particles tab. The threshold for identifying fluorescence was kept consistent for all tissue sections analysed.

and subsequently used for analysis of all images acquired. Highlighted areas were measured, with pixel size set at 0.0335 μm, using the 'analyse particles' tool which outputs the area and pixel density for each image. For each tissue section the total area in which IgG was detected was measured, and the integrated density, which is calculated by dividing the area in which IgG was detected by the density of the fluorescence, was recorded.

2.3.2 Neutrophil quantification

Immunofluorescent staining was performed to identify and quantify neutrophil infiltration post-tMCAo in the regions 3.94mm IA and 1.98mm IA. Neutrophils were labelled using scs-j, a custom rabbit polyclonal antibody (1:1000 dilution) (Gifted by Allan laboratory, Manchester, UK), whilst nuclei within cells were stained using Vectashield® (Vector Laboratories, Peterborough, UK), containing 4', 6-diamidino-2-phenylindole (DAPI), which binds to DNA (Favilla *et al.*, 1997). The frozen tissue sections were defrosted overnight in the fridge, before being post fixed in cold acetone (4 ˚C) for 20 minutes. Tissue sections were encircled with a wax pen and then washed for 15 minutes in 1% Triton X (in PBS, pH 7.4), followed by 2 washes in PBS for 5 minutes each. Blocking buffer (20% normal goat serum) (Vector Laboratories, Peterborough, UK) was applied for 20 minutes at room temperature, and was tapped off and replaced with primary antibody preparation (1:1000, in 5% normal goat serum). Primary antibody was omitted from negative controls. After 1 hours incubation period the primary antibody was cleared with 2 x 5 minute washes in PBS. Secondary antibody, FITC-conjugated goat anti-rabbit (1:250) (Abcam Ltd, Cambridge, UK) was applied for 1 hour in the dark before being washed off with PBS for 5 minutes. A drop of Vectashield® (Vector Laboratories, Peterborough, UK) was added to each tissue section and a cover slip applied. The slides were stored in the dark prior to viewing using an upright epifluorescent microscope (Nikon Eclipse E600), at excitation wavelengths of 405 nm and 488 nm for DAPI and FITC respectively. Cells fluorescently marked (**Figure 2.7**) in both hemispheres were quantified in duplicate sections and the mean number of cells expressed as cells/mm^2.

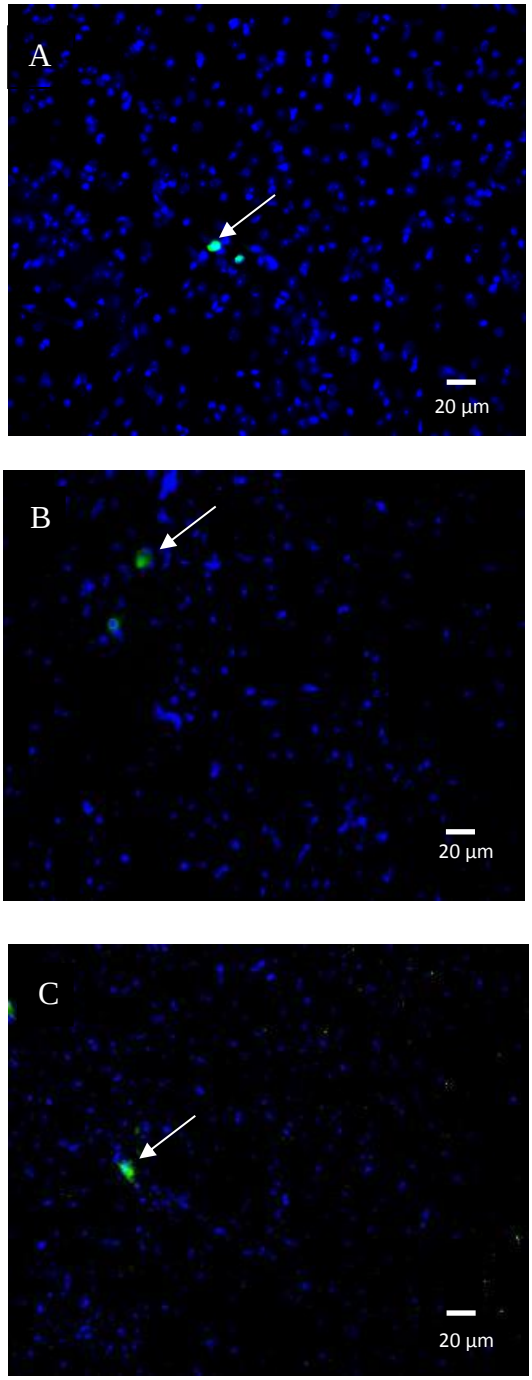


Figure 2.7: Neutrophils stained by indirect immunofluorescence. Neutrophils were identified in both the ipsilateral and contralateral hemispheres at two regions (white arrows). Images A and B are taken from the region 3.94mm IA, whilst C is from the region 1.98mm IA. All images were acquired from the penumbral region of the ipsilateral hemisphere of animals which underwent 45 minutes tMCAo with 45 minutes of reperfusion, and were acquired on a Nikon eclipse upright epifluorescent camera at 200X magnification and at 488nm.

2.4 Protein and cytokine analysis

2.4.1 Protein concentration assay

Protein concentration in brain homogenates was determined using the Bio-Rad Protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK) following manufacturer's guidelines. Dye reagent concentrate was diluted in four parts distilled water and passed through a 0.2µm sterilising filter. BSA standard was serially diluted to give a concentration range from 0.03mg/ml to 0.5mg/ml. 10µl of each sample and standard were transferred in triplicate to a 96 well dish. Samples were diluted 1:100 with dye reagent and standards diluted 1/20 in dye reagent. The plate was incubated at room temperature for 5 minutes and the absorbance of each sample then read at 595nm using a spectrophotometer. The protein concentration of each sample was determined from the absorbance values of the protein standards, using linear regression, R^2=0.97.

2.4.2 Enzyme linked immunosorbent assay

The concentration of TNF-α was measured in serum samples and brain homogenates using a commercially available set, Mouse TNF-α set 1 (BD Biosciences, Oxford, UK). The assay was carried out according to the manufacturers' guidelines. Wells of a 96 well plate were coated with 100µl of capture antibody (1:250), diluted in coating buffer (0.2M sodium phosphate, pH 6.5). The plate was then sealed and incubated overnight at 4˚C. Wells were aspirated and washed 3 times with 300µl/well wash buffer (PBS with 0.05% Tween 20). After last wash, the plate was inverted and blotted on absorbent paper to remove any residue. The plate was blocked with 200µl/ well assay diluents (10% foetal calf serum) and incubated at room temperature for 1 hour. Wells were then aspirated and washed 3 times with 300µl/well wash buffer. After last wash, the plate was inverted and blotted on absorbent paper to remove any residue. Into appropriate wells, 100µl of each standard dilution, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 64.5 pg/ml, 31.3 pg/ml and 15.6 pg/ml, and samples (1/5 dilution) all prepared in assay diluent, were transferred. The plate was sealed and incubated at room temperature for 2 hours before washing as previously described. Working detector (detection antibody,

1:1000 with streptavidin-HRP, 1:250) was added to each well, 100µl each and incubated for 30 minutes at room temperature. The plate was again washed 7 times as previously described before 100 µl substrate solution (Tetramethylbenzidine) (Sigma, Poole, UK) was added to each well and incubated at room temperature for 30 minutes in the dark. Finally, 50 µl of stop solution (2 N H_2SO_4) was added to each well and the absorbance read at 450 nm. The TNF-α concentration of each sample was determined from the absorbance values of the TNF-α standards, using linear regression, R^2=0.99.

2.4.3 Mouse angiogenesis proteome profiler

Relative expression of an array of proteins associated with angiogenesis was measured in tissue homogenates using a commercially available proteome profiler kit ARY015 (R&D Systems, Abingdon, UK). The protocol was carried out following the manufacturer's guidelines as follows. The array membrane was blocked for one hour on a rocking platform using blocking buffer provided and then aspirated. Samples (300µg protein/ml), containing detection antibody cocktail (1:250) were applied to the membrane and incubated overnight at 4˚C on a rocking platform. The membrane was then washed 3 x 10 minutes in 20 ml of wash buffer provided and then incubated with streptavidin-HRP conjugate at room temperature for 30 minutes. Again, the membrane was washed 3 x 10 minutes in 20 ml wash buffer and the blotted dry and placed on a plastic sheet protector. Next, the membrane was overlaid with 1 ml Chemical Reagent mix provided in a dark room. The membrane was removed from the plastic sheet protector and wrapped in cling film and any air bubbles smoothed out. Finally the membrane was placed in an autoradiography cassette and exposed to x-ray film for multiple periods between 1-10 minutes.

2.5 Statistics

Comparisons between two groups were carried out using Student unpaired t-test with significance set at <0.05. Blood flow measurements were analysed using a mixed model analysis of variance (ANOVA). Comparisons between multiple groups were analysed by one-way ANOVA with Bonferronis correction. Pearson's correlation coefficient was used where appropriate.

Chapter 3:

The effect of genetic mast cell deficiency on blood brain barrier permeability, oedema, lesion volume and neutrophil recruitment in the acute period, post transient middle cerebral artery occlusion.

3.1 Introduction

Identifying novel therapeutic targets for the treatment of cerebral ischaemia is of paramount importance given the current shortfall in effective interventions, and recent evidence suggests that mast cells may prove to be a viable target (Strbian *et al.,* 2006; Hacke *et al.,* 2008). Mast cells have already been shown to be important in a rat model of cerebral ischaemia. Indeed the evidence indicates that mast cells worsen oedema, increase BBB permeability and enhance the recruitment of neutrophils to the ischaemic brain (Strbian *et al.,* 2006). It is also clear that mediators stored within, and synthesised by mast cells worsen outcome in animal models of cerebral ischaemia. Global knock-out of cytokines and chemokines involved in promoting and maintaining inflammatory responses, such as TNF- α, IL-1β, IL-8 and MCP-1, as well as vasoactive mediators such as histamine, have been shown to have reduced injury in rodent models of cerebral ischaemia (Wang *et al.,* 2007). As described in **Table 1.2**, all of the aforementioned mediators are either stored in mast cells, ready for release upon activation or *de novo* synthesised in the period following activation (Metcalfe, 2008).

The study of mast cell behaviour in a mouse model of ischaemia as opposed to the rat will greatly enhance our ability to dissect the molecular mechanisms involved in the development of ischaemic pathology, and potentially aid in the identification of novel therapeutic targets. The mast cell deficient mice used in this study, Kit mice, are very well characterised and have few deficiencies other than splenomegaly, cardiomegaly, and thrombocytosis and are neutrophilic, along with a complete deficiency of mast cells. Unlike Wistar$^{ws/ws}$ rats used in the Strbian studies (Strbian *et al.,* 2006), which are anaemic and sterile, the Kit mice breed readily. They are therefore suitable for the investigation of molecular aspects of mast cell function by cross breeding with mice deficient in molecules of interest, for example TNF-α. Alternatively, the mast cell deficiency can be repaired by adoptive transfer of cultured mast cells from WT mice or from mice with a gene of interest mutated, creating 'mast cell knock-in mice' (Niwa *et al.,* 1991 and Grimbaldeston *et al.,* 2005). This gives the Kit mouse an advantage over other mast cell deficient mouse strains, such as the WBB6F(1)-W/W(v) which are also sterile, or the WBB6F(1)-

Sl/Sl(d) strain which cannot accept a mast cell engraftment (Yoshino *et al.,* 2012) (see **Table 1.5**). Therefore, Kit mice are an ideal candidate for studying the *in vivo* function of mast cells in a model of focal cerebral ischaemia, a possibility that has yet to be examined.

3.2 Hypothesis and aims

The general hypothesis of this thesis is that mast cell numbers increase and contribute to pathology in a mouse model of focal cerebral ischaemia, tMCAo, by increasing BBB permeability, oedema volume, lesion volume and neutrophil recruitment. Therefore, the aim of this study was to determine whether genetic mast cell deficiency resulted in reduced BBB permeability to IgG, reduction in oedema and lesion volume and reduced neutrophil recruitment to the brain, four hours post tMCAo.

3.3 Protocol

All experiments were carried out on 10-12 week old male WT mice (bred in-house) or Kit mice (Jackson Laboratories, USA), weighing 25-30g. Mice underwent 45 minutes tMCAo followed by 4 hours of reperfusion (see **section 2.1**). At termination brains were immediately removed and flash frozen in isopentane at -42°C. The brains were sectioned and analysed as described in chapter 2 for lesion volume (**section 2.2.6**), BBB damage (**section 2.3.2**) and neutrophil recruitment (**section 2.3.1**). The tMCAo experiments were carried out in a randomised fashion, and the experimenter was blinded to tissues being analysed by coding each individual animal.

3.4 Results

3.4.1 Mast cell numbers increased in the ischaemic hemisphere 4 hours post tMCAo.

Mast cell populations were manually counted in coronal tissue samples of naive WT controls, WT sham operated animals, and WT tMCAo subjects (n=8). Initial comparison of the mast cell population of each group showed no evident hyperplasia in this experimental model (**Figure 3.1 A**). Observations from the topography of the

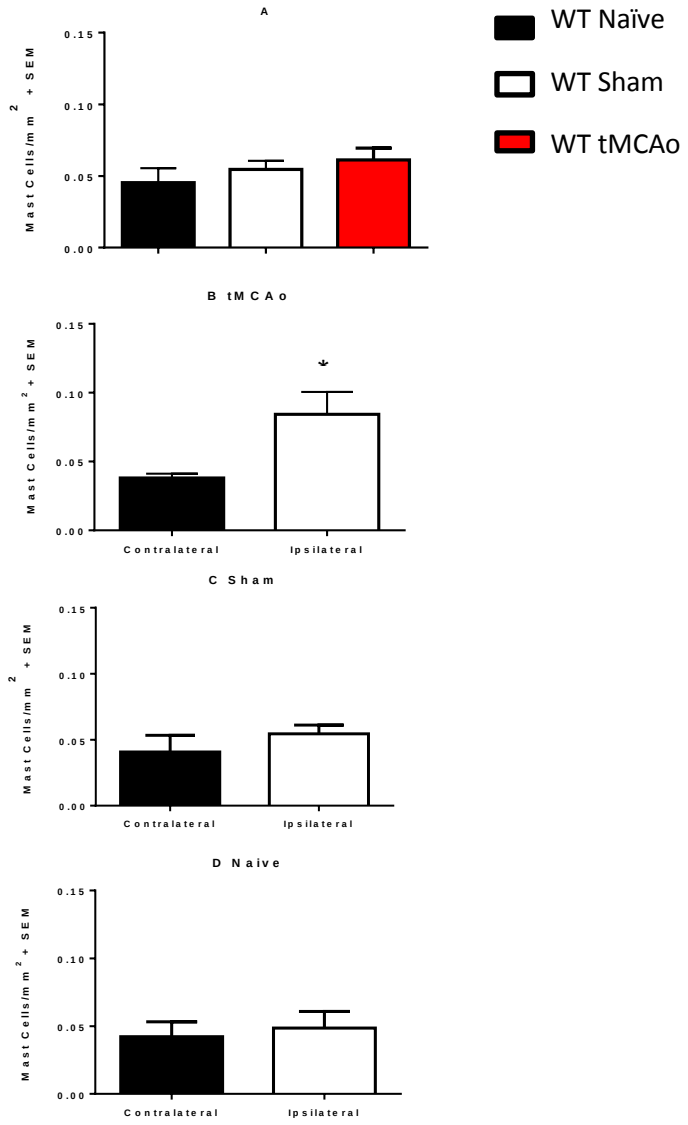


Figure 3.1: Quantification of mast cells in tissue sections following transient middle cerebral artery occlusion. Mast cells were stained in 24 coronal tissue sections, as described in **section 2.2.4** using acidic Toluidine blue. Mast cells were counted in tissue sections from WT naïve controls (n=3), WT sham operated animals (n=5), and WT tMCAo subjects (n=8) (A). Mast cells were also compared between the ipsilateral and contralateral hemisphere of WT tMCAo mice (B), WT sham (C) and WT naïve controls (D). Mast cell numbers were calculated per mm^2 of tissue. Data are expressed as mean + SEM, and analysed by unpaired Students t-test, *$p<0.05$

the mast cells gave an indication that there was some active involvement. Indeed, there appeared to be a greater abundance of mast cells in the ipsilateral hemisphere of the WT tMCAo mice, compared to the contralateral side. Indeed, enumeration of mast cells showed this increase to be statistically significant (see **Figure 3.1 B**). This difference in mast cell numbers between the two hemispheres was not observed in either the naive control group or sham treated animals (**Figure 3.2 C and D**). In all groups the mast cells seemed to be evenly distributed throughout the tissue, however in the WT tMCAo mice, perivascular mast cells appeared to be more widely distributed among the different regions than in control and sham treated animals, particularly in the regions 2.86 mm, 1.98 mm, 1.00 mm and 0.16 mm IA. This perceived difference in perivascular mast cell population was not however found to be statistically significant, and there was also no difference in perivascular distribution between the ipsilateral and contralateral hemispheres (**Figure 3.2**).

3.4.2 BBB permeability was significantly decreased in Kit mice.

The integrity of the BBB was assessed by identification of endogenous IgG within the injured brain. Direct labelling of IgG with a fluorescent antibody in tissue sections from the region of the septal nuclei and the region of the hypothalamus, was quantified in terms of integrated density and area of staining.

At 4 hours post tMCAo, staining of endogenous IgG showed a significant reduction of intensity in Kit mice. Compared to the WT, the Kit mice had a significant reduction of IgG accumulation at the region 2.86 mm IA. However, there was no distinction between the two groups at 3.94 mm IA (**Figure 3.3 A**). Likewise, the total area of IgG measured did not prove to be statistically significantly different between the groups and was similar at each region (**Figure 3.3 B**).

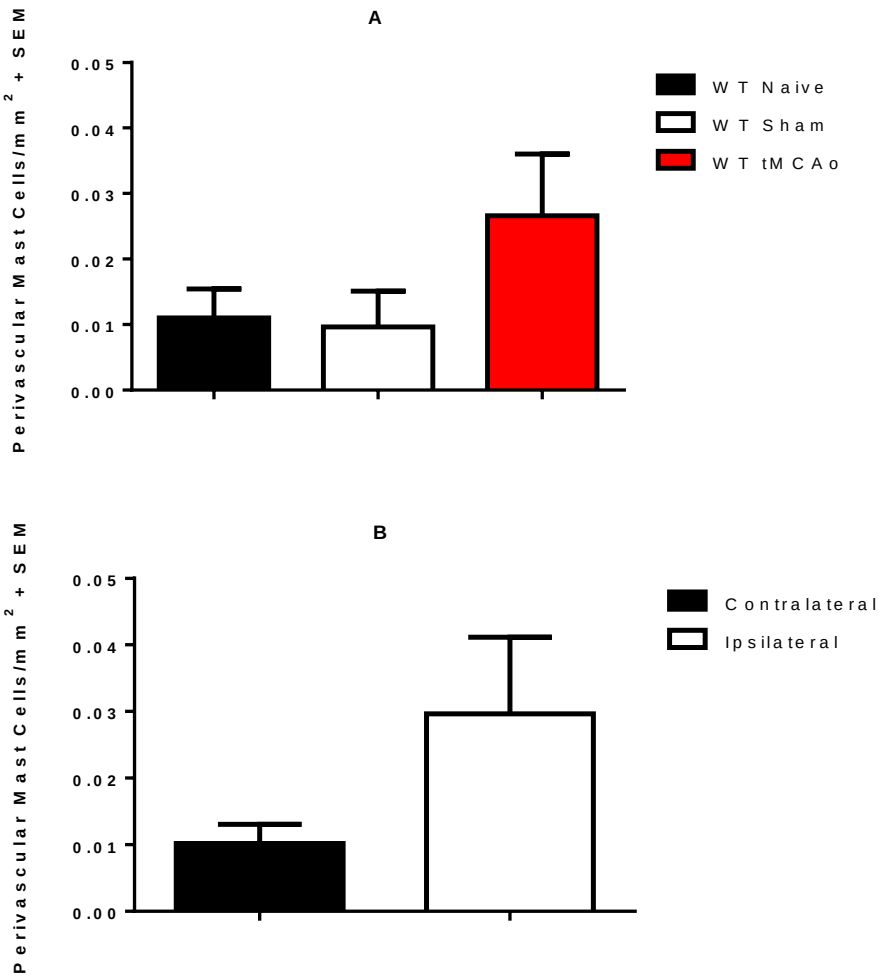
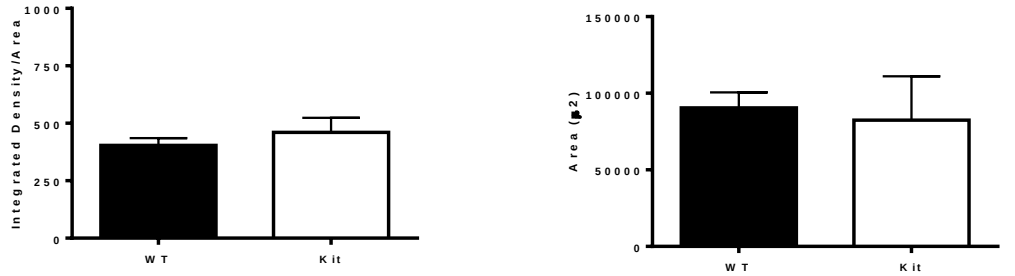


Figure 3.2: Quantification of perivascular mast cells following transient middle cerebral artery occlusion. Perivascular mast cells stained using acidic toluidine blue were compared between the ipsilateral and contralateral hemisphere of WT tMCAo mice (n=8) (A), WT sham (n=5) (B) and WT naïve controls (n=3) (A). Mast cells located perivascularly were also compared between the ipsilateral and contralateral hemispheres of WT tMCAo subjects (B). For each animal 24 tissue sections were analysed and mast cell numbers were calculated per mm^2 of tissue. Data are expressed as mean + SEM, and analysed by unpaired Students t-test.

A



B

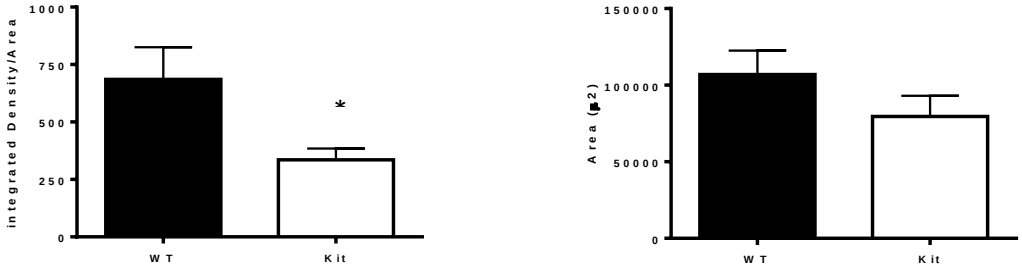


Figure 3.3: Density and area of endogenous immunoglobulin G staining. Endogenous immunoglobulin G was detected in brain tissue of WT (n=8) and Kit (n=7) mice, following 45 minute tMCAo with 4 hours of reperfusion, using indirect immunofluorescence at two regions, (A) (3.94mm IA, level of the septal nuclei) and (B) (2.86mm IA, level of the hypothalamus). The density of fluorescence and area of staining were quantified and are represented as mean + SEM. Unpaired Students t-test, *$p<0.05$.

3.4.3 Oedema was significantly reduced in Kit mice, and correlated to reduced BBB permeability.

Oedema was measured in haematoxylin and eosin stained coronal tissue sections from eight brain regions spanning the territory of the MCA. The area of both the ipsilateral and contralateral hemispheres were measured using a densitometer and were then graphed against their distance from the IA line. The area under the curve produced a volume for each hemisphere, and oedema was expressed as a percentage increase of the ipsilateral hemisphere over the contralateral volume.

The results revealed a significant reduction in the swelling of the ipsilateral hemisphere in the Kit mice compared to the WT group ($p<0.05$) (**Figure 3.4 A**). Swelling was reduced by 52.2% in the Kit mice. Analysis of region specific decreases indicated that posterior sections of the brain were less severely swollen in the Kit mice. In particular, at the level of the anterior hippocampus, 1.98 mm IA, oedema was significantly reduced in the Kit mouse strain compared to the WT mice ($p<0.05$) (**Figure 3.4 B**). Analysis of the relationship between BBB permeability and oedema indicates that there was a strong positive correlation (**Figure 3.4 C**).

3.4.4 Lesion volume was not reduced in Kit mice.

The ischaemic lesion was measured in haematoxylin and eosin stained coronal tissue sections from eight distinct brain regions, representative of the MCA territory in each mouse strain. Regions of pallor, indicating tissue loss, were identified in the ipsilateral hemisphere and measured using a densitometer. The values were then graphed against their distance from the IA line, with the distances 7.9 mm and 0.1 mm equal to zero. The volume of the lesion was ascertained from the area under the curve and expressed as a percentage of the ipsilateral hemispheric volume.

There was no difference in the volume of lesion between the WT and Kit mouse strains (**Figure 3.5 A**). In both mouse strains the lesion was similarly distributed throughout the eight regions analysed (**Figure 3.5 B**).

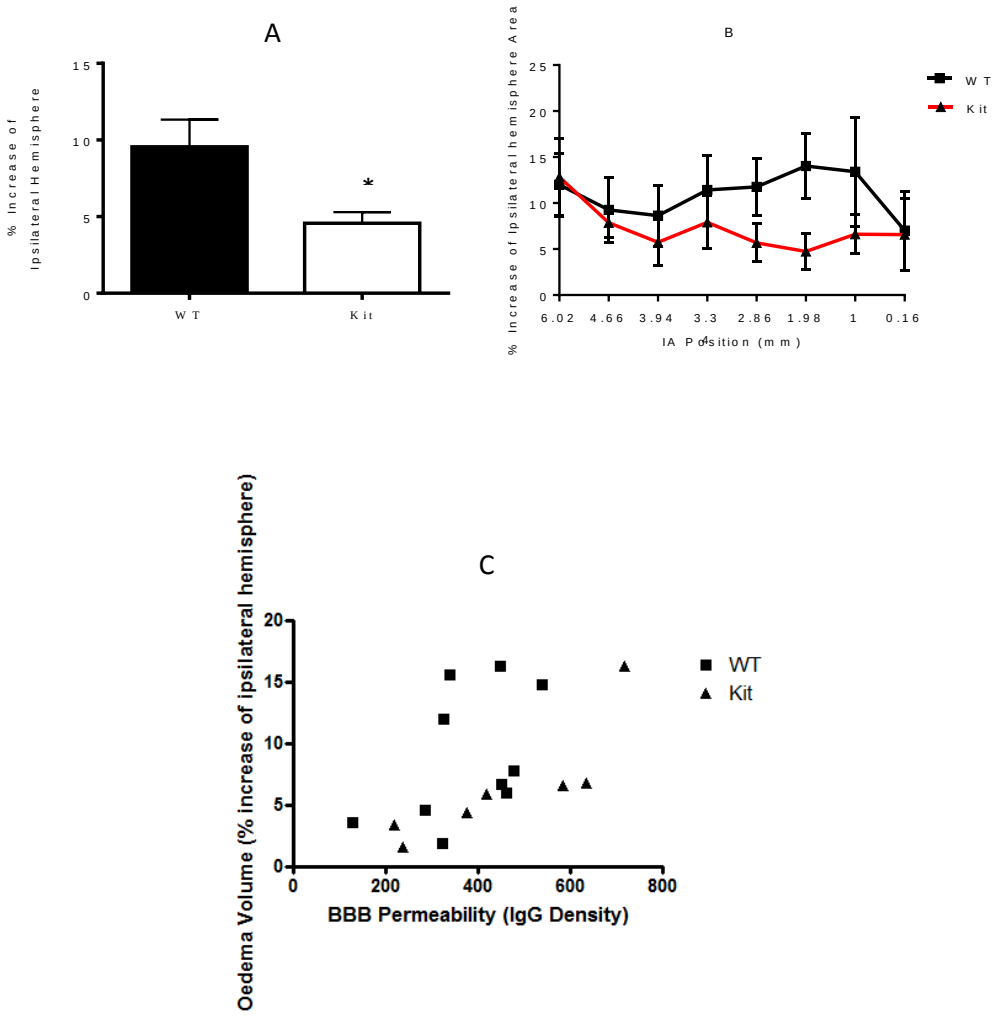


Figure 3.4: Oedema measurements and correlation with blood brain barrier permeability in the brain of WT and WT Kit mice subject to 45 minutes tMCAo followed by 4 hours of reperfusion. The volume of oedema was taken as a percentage increase in volume of the ipsilateral hemisphere over the contralateral side (A). Regional distribution of oedema is shown in graph (B), whilst correlation of BBB permeability and oedema is represented in panel (**C**). Data are represented as mean+SEM. Unpaired Student's t-test A , *$p<0.05$. B was analysed by two-way ANOVA, $p<0.05$, and Pearson's correlation was used for graph C, $P<0.05$, $R^2=0.4948$.

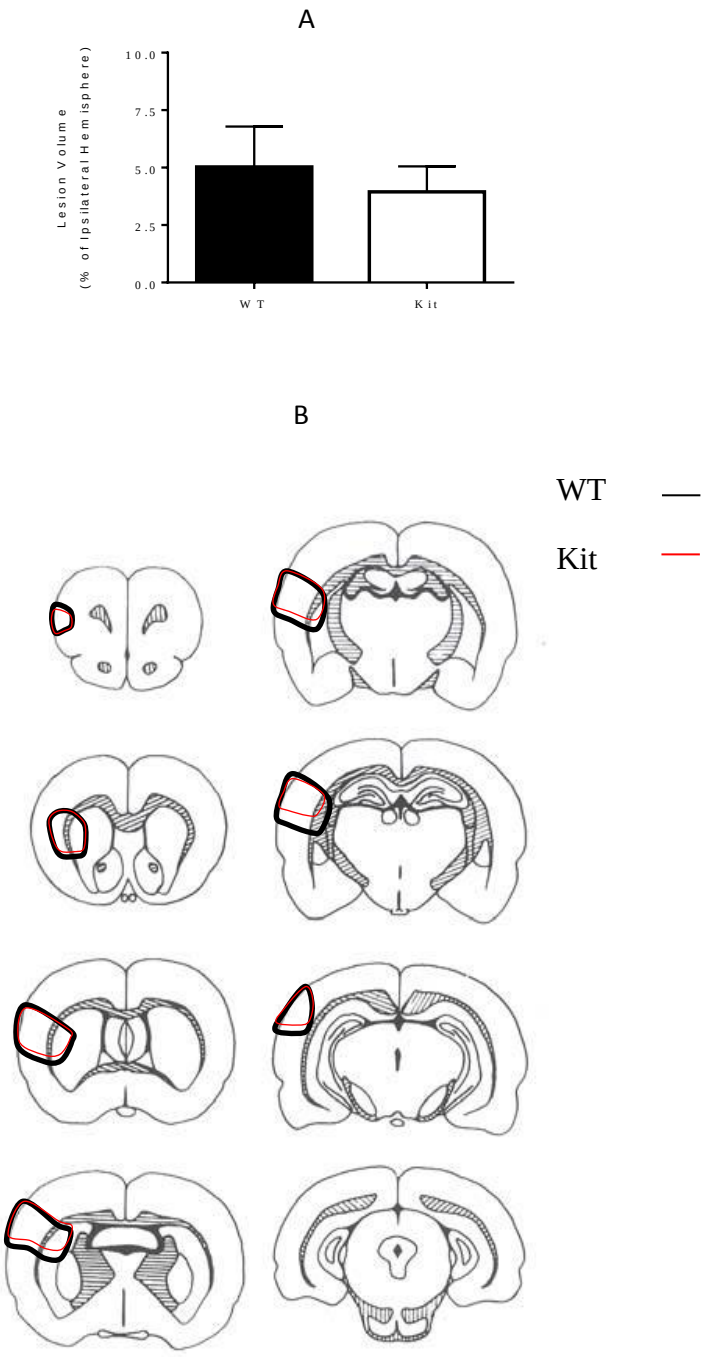


Figure 3.5: Ischaemic lesion volume and distribution. Ischaemic lesion volume in the brain of WT (n=8) and Kit (n=7) (A) mice subject to 45 minutes tMCAo followed by 4 hours of reperfusion. The area of the lesion and ipsilateral hemisphere were quantified from eight distinct regions throughout the brain and the lesion volume was measured as a percentage of the ipsilateral hemispheric volume. Topography and distribution of the lesion is depicted in image B. Data are represented as mean + SEM. Unpaired Student's t-test, *$p<0.05$.

3.4.5 Neutrophil recruitment was significantly decreased in Kit mice

Neutrophil recruitment was assessed by indirect immunofluorescent staining of cells using a custom anti-neutrophil antibody, scs-j. At 2 regions, 3.94 mm and 1.98 mm IA, cells were counted on duplicate tissue sections and the mean number was expressed as a ratio of the area of the tissue section. To account for oedema in the ipsilateral hemisphere, the area of the contralateral hemisphere was used to represent both sides of the brain.

At the region 3.94 mm IA, neutrophil recruitment was significantly decreased in the Kit, tMCAo operated mice compared to the WT tMCAo group (**Figure 3.6 A**). The Kit tMCAo operated mice had 57.7% fewer neutrophils than WT tMCAo operated mice and had similar numbers to the WT and Kit sham groups. Furthermore, the WT tMCAo mice had significantly greater numbers of neutrophils than the sham operated animals of both the WT and Kit mouse groups. However, analysis at the region 1.98mm IA indicated only a small reduction in neutrophil recruitment in Kit mice. The reduction was not statistically significant, and again sham operated mice from both strains had a similar level of neutrophil recruitment (**Figure 3.6 B**).

3.5 Discussion.

The data presented demonstrates that mast cells had a negative impact in this experimental model of stroke, and backs up previous findings in a similar model in the rat (Strbian *et al.,* 2006). Genetic deficiency of mast cells resulted in the significant reduction of BBB permeability, oedema and neutrophil accumulation, compared to WT subjects. Furthermore, an increase in the mast cell population in the ipsilateral hemisphere of occluded animals reinforced the idea that mast cells have a role in disease outcome.

The presence of mast cells in the healthy and injured brain has been reported in many studies, including the current study, for over a century, with evidence of involvement under numerous conditions (Moon *et al.,* 2010). The evidence presented here indicates that the response of mast cells, at this early stage of disease progression, is restricted to those present within the brain as there was no overall increase in the mast cell population compared to naive control or sham treated animals**.** However,

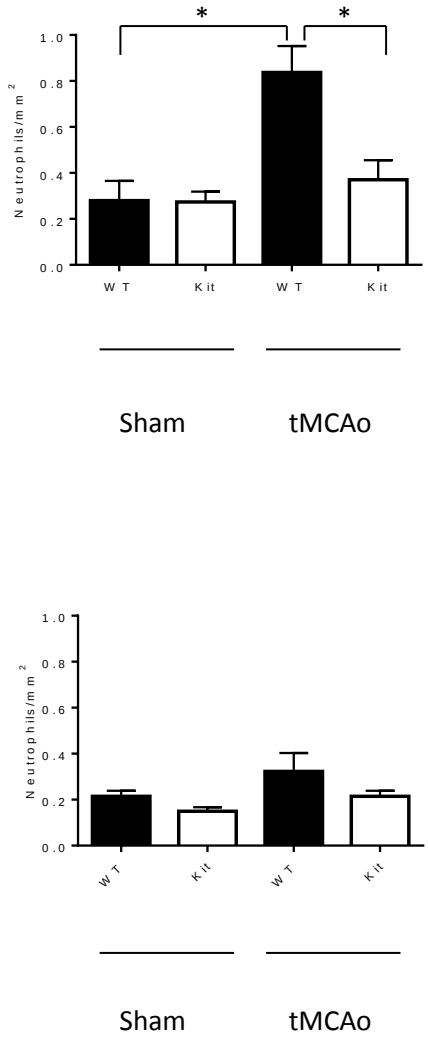


Figure 3.6: Quantification of Neutrophil recruitment to the region of the septal nuclei and anterior hippocampus. WT (n=6) and Kit (n=6) mice were subject to 45 minutes tMCAo followed by 4 hours of reperfusion or sham operation (n=4). Neutrophils were quantified on coronal sections stained using indirect immunofluorescence, at the regions 3.94mm IA (A), and 1.98mm IA (B). Data represented as mean + SEM and analysed by ANOVA, *$p<0.05$.

the population within the ipsilateral hemisphere of the WT mice increased significantly compared to the contralateral hemisphere. The source of this increase was likely to be the contralateral hemisphere or perhaps the abundant population within the leptomeninges. Rapid movement of mast cells through vessels has been shown previously in a study where fluorescent peritoneal mast cells where injected into the jugular vein of healthy rats, and where found to be present in the brain after one hour (Silverman *et al.,* 2000). Given the pro-inflammatory environment of the brain during and after the ischaemic event, there is potential for the mast cell migration to be augmented by chemoattractant mechanisms, possibly through many of the mediators released by activated mast cells (Chen *et al.,* 2003, Wang *et al.,* 207 and Metcalfe *et al.,* 2008). Indeed, eotaxin and CCXR2, both released by mast cells, have been shown to increase mast cell infiltration to sites of injury in models of heart and lung fibrosis, possibly through upregulation of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) (Hallgren *et al.,* 2007 and Zweifel *et al.,* 2010).

The role of mast cells in inducing cellular infiltration was evident with the marked reduction in neutrophil recruitment to the Kit mouse brains following tMCAo, at the region of the septal nuclei. Neutrophils are widely known to be the first cell type to infiltrate sites of injury and inflammation in many disease states, due to their abundance within the circulation, and migrate in response to IL-8, which is secreted by activated mast cells (Garau *et al.,* 2005). Once in the brain, neutrophils contribute to stroke pathology by releasing ROS, and degrading the ECM through production of NE (Allen *et al.,* 2012). A similar outcome was found after genetic deficiency of mast cells in the rat led to a significant reduction in neutrophil migration into the brain after experimental stroke. However, in this work, genetic mast cell deficiency reduced significantly the number of neutrophils within the brain, but not the intravascular population. This data could be interpreted as an indication that mast cells are important in promoting neutrophil diapadesis, but not necessarily chemotaxis. Therefore, targeting mast cells in stroke treatment may alleviate neutrophil induced injury by preventing their migration into the brain (Strbian *et al.,* 2006).

The effect of mast cell deficiency was seen in the vascular response of the Kit mice, where there was a reduction in BBB permeability. The lower density of IgG leakage into the brain indicated a reduction in BBB permeability in the Kit mice. Although there was no change in area of staining, the migration of the IgG through the neuropil is restricted and it therefore accumulates at the vessel, which was reflected in the increased density of the molecule. This decrease in BBB damage was only seen at the region of the hypothalamus and not at the level of the septal nuclei. The only evidence from this work that may explain this is the trend of increased perivascular mast cells in the posterior regions of the brain, which supports the idea that mast cells are indeed responsible for the increase in BBB permeability in the region of the hypothalamus.

What is evident is that there was a hugely significant reduction in the degree of swelling of the ipsilateral hemisphere in the Kit mice compared their WT counterparts. The precise mechanisms by which these events have occurred are not yet clear, however as discussed in previous sections, both mast cells and neutrophils can potentially increase the BBB permeability by release of mediators which can directly degrade the ECM, or in the case of mast cells, which are in close contact with the vasculature, directly affect the cells ensheathing the vessels (Buck *et al.*, 2008, Stowe *et al.*, 2009 and Lindsberg *et al.*, 2010). Furthermore, the regional specific attenuation of BBB permeability, seen at the level of the hypothalamus, was mirrored in the degree of oedema within the posterior regions of the brain, where there was a significant reduction in the Kit mice at the level of the anterior hippocampus. Indeed analysis of the relationship between BBB permeability and oedema shows that as BBB permeability increased, oedema volume increased.

The decrease in BBB permeability and oedema, and attenuated neutrophil recruitment in the Kit mice had no real impact on the lesion volume at this early time point. Decreased BBB permeability will reduce the exposure of the cerebral tissue and vessels to many potentially harmful mediators within the circulation, such as vasoactive neurotransmitters, complement, autoantibodies and autoreactive T-cells, whereas, a reduction of neutrophils in the ischaemic brain should offer some neuroprotection (Sandoval and Witt, 2008 and Becker, 2009). Furthermore, we

might expect that mast cells themselves contribute to the lesion development through release of their own mediator content (Strbian *et al.*, 2006). Additionally, increased oedema leads to secondary ischaemic insults by compression of vessels globally (Ayata and Ropper, 2002). A possible explanation for this discrepancy is the time point measured post-ischaemia, as the lesion will continue to increase and recruit tissue at risk into the ischaemic core. Extension of the reperfusion period to 72 hours or beyond will determine whether mast cell deficiency has any real impact on lesion development (Liu *et al.*, 2012).

In conclusion, the main findings of this chapter are that mast cells play a harmful role in the development of ischaemic pathology in the acute reperfusion period, following tMCAo in the mouse. In this model, genetic deficiency of mast cells led to a reduction in BBB permeability, oedema volume and neutrophil recruitment. Additionally, the number of mast cells present in the ipsilateral hemisphere increased following tMCAo, indicative of mast cell involvement in disease progression. This work highlights mast cells as a potential target for stroke therapy; however more work is required to identify mechanisms of action of mast cells in this model.

Chapter 4:

The effect of mast cell stabilisation prior to transient middle cerebral artery occlusion on blood brain barrier permeability, oedema, neutrophil recruitment and lesion volume.

4.1 Introduction

Data from the previous study in chapter 3, confirmed that mast cells increase pathology in the tMCAo ILT model of experimental focal cerebral ischaemia in mice. The data has also given some insight into the potential function of mast cells *in vivo,* where oedema was significantly reduced and region specific reductions of BBB permeability and neutrophil recruitment were seen in mast cell deficient Kit mice compared to WT controls. To support this data and to identify a potential therapeutic avenue, one strategy is to investigate whether mast cells can be pharmacologically manipulated to yield a similar outcome.

The most widely used drug for the investigation of mast cell function *in vivo* and *in vitro* is SCG, which prevents mast cell degranulation, and thus the release of mast cell mediators. The mechanism of action of the drug is poorly understood, however evidence from *in vitro* treatment of human, rat and mouse mast cells indicates that it prevents Ca^{2+} influx across the cell membrane, which would normally initiate the release of cellular content, possibly through stabilisation of G protein coupled receptor 35 (Spataro and Bosmann, 1976 and Yang *et al.,* 2010).

The effects of SCG administration have been studied previously, in a rat model of tMCAo. SCG injection prior to ischaemia caused a significant reduction in BBB disruption, oedema and neutrophil recruitment (Strbian *et al.,* 2006). Although there was no detailed analysis on mast cell populations in this study, SCG treatment has been shown in other studies to affect the mast cell population. In a rat model of small intestine ischaemia, SCG administration during the ischaemic period reduced the number and activation of mast cells compared to untreated controls. The drug treated group also exhibited attenuated tissue oedema and inflammatory cytokine production (Huang *et al.,* 2012). Studies in a mouse model of vein graft disease also support the action of SCG *in vivo*. Disease progression in this model is dependent on the activation of mast cells via the complement component C5a, which was completely blocked by the administration of SCG (de Vries *et al.,* 2012).

4.2 Hypothesis and aims

Mast cell stabilisation, using SCG, will reduce BBB permeability, oedema and neutrophil recruitment in the acute phase of experimental tMCAo in WT mice, but not in vehicle treated controls. Furthermore, SCG treatment will have no effect on mast cell deficient Kit mice. Therefore, the aim of this study was to determine whether mast cell stabilisation prior to onset of forty five minute tMCAo, resulted in reduced BBB permeability to IgG, reduction in oedema and lesion volume and reduced neutrophil recruitment to the brain, four hours post tMCAo.

4.3 Protocol

All experiments were carried out on WT (Charles River Laboratories, UK) or Kit mice (Jackson Laboratories, USA). Animals were injected ICV with SCG (75µg in 2µl) or 0.9% saline (2µl), prior to commencement of tMCAo procedure. The mice underwent 45 minutes of tMCAo followed by 4 hours of reperfusion, after which brains were removed and flash frozen. The brain tissue was then analysed to determine lesion volume, oedema, BBB disruption and neutrophil recruitment as described in **sections 2.1, 2.2 and 2.3**. The tMCAo experiments were carried out in a randomised fashion, and the experimenter was blinded to tissues being analysed by coding each individual animal.

4.4 Results

4.4.1 SCG treatment of WT mice resulted in a significant reduction of BBB permeability.

The integrity of the BBB was assessed by identification of endogenous IgG within the injured brain. Direct labelling of IgG with a fluorescent antibody in tissue sections from the region of the septal nuclei and the region of the hypothalamus, was quantified in terms of integrated density and area of staining.

Measurement of endogenous IgG leakage into the brain indicated that there was a significant reduction in BBB permeability in SCG treated WT mice at the level of the hypothalamus, were the density of IgG staining was markedly reduced compared to vehicle treated controls (**Figure 4.1 B**). This response was not repeated at the level of the septal nuclei, where the density of IgG staining was similar between the two groups of animals (**Figure 4.1 A**). Likewise, the total area stained at each region was similar between the drug treated group and vehicle treated controls (**Figure 4.1 A and B**). To confirm the specificity of SCG as a mast cell stabilising agent, experiments were repeated in Kit mice. At both of the regions analysed, the region of the septal nuclei and the region of the hypothalamus, there was similar density and area of IgG leakage regardless of treatment in Kit mice (**Figure 4.2**).

4.4.2 Oedema volume was significantly reduced in SCG treated WT mice.

The development of post ischaemic vasogenic oedema of the ipsilateral hemisphere was measured from eight frozen tissue sections, representative of the MCA territory, stained with haematoxylin and eosin. The volume of oedema was expressed as an increase in ipsilateral hemisphere volume over the volume of the contralateral hemisphere.

In WT mice, there was a significant reduction in oedema ($p<0.05$) of around 60% following SCG treatment compared to the group injected with vehicle (**Figure 4.3 A**). Oedema was similar in Kit mice, regardless of whether SCG or vehicle was injected (**Figure 4.3 B**).

4.4.3 Lesion volume was significantly decreased in SCG treated WT mice.

To assess the impact of SCG on post ischaemic lesion development, tissue loss (areas of pallor) was measured in Haematoxylin and Eosin stained frozen tissue sections using a densitometer. Lesion volume was expressed as a percentage of the ipsilateral hemispheric volume.

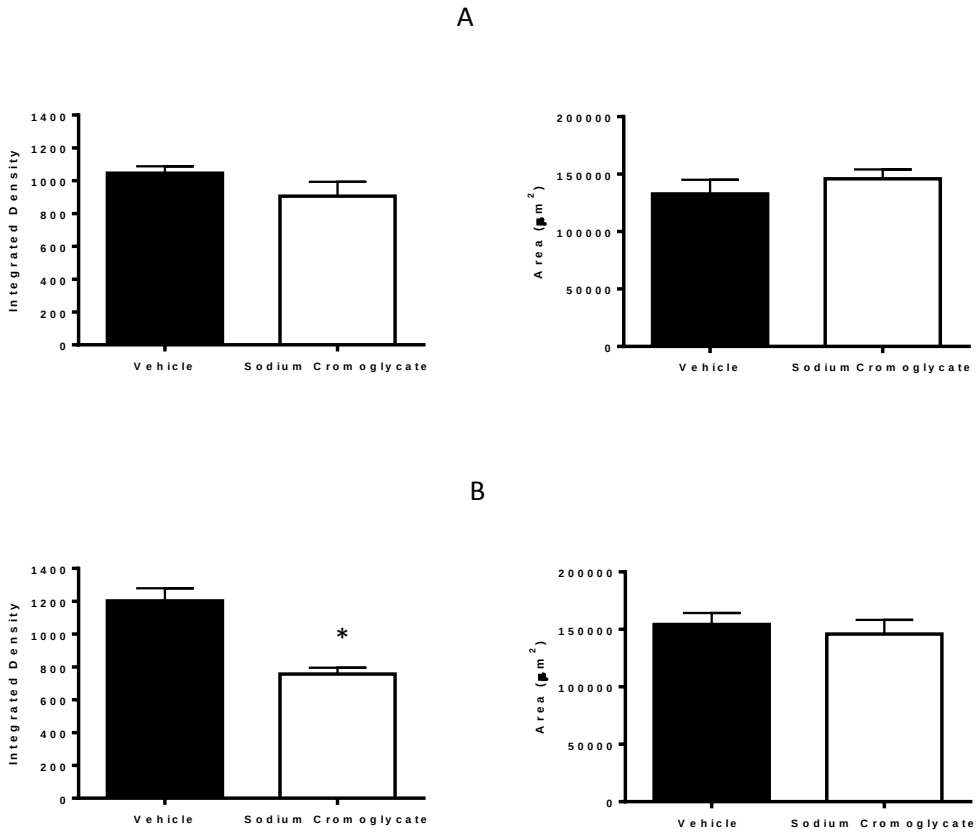
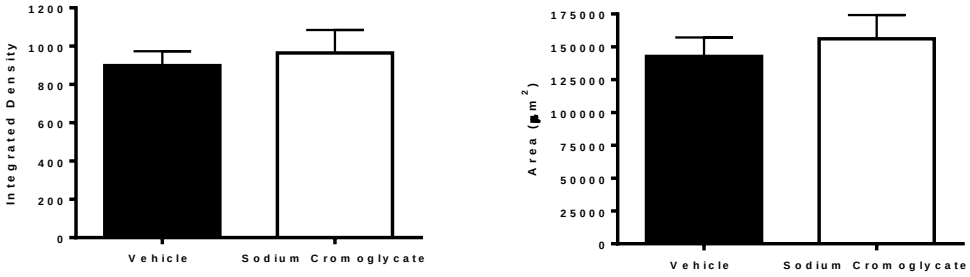


Figure 4.1: Quantification of Blood brain permeability to endogenous Immunoglobulin G in WT mice. WT mice were treated with sodium cromoglycate (n=6) or vehicle (n=6), prior to 45 minutes of transient middle cerebral artery occlusion with 4 hours of reperfusion. Immunoglobulin G was detected by direct immunofluorescence at the region of the septal nuclei (A) and the hypothalamus (B) and the density and area were measured. Data are represented as mean+SEM and analysed by unpaired Student's t-test. *$p<0.05$.

A



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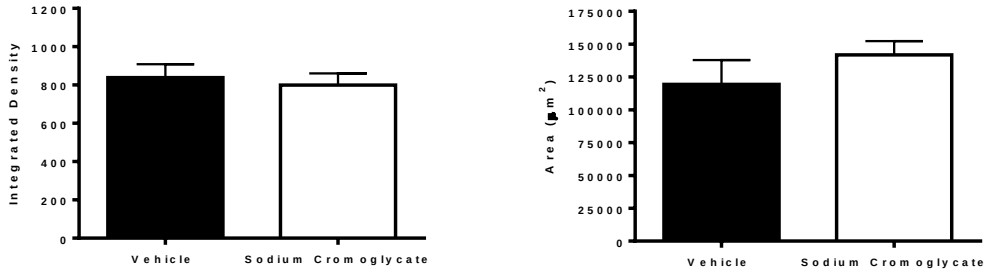

Figure 4.2: Quantification of Blood brain permeability to endogenous Immunoglobulin G in Kit mice. Kit mice (n=6) were treated with sodium cromoglycate or vehicle, prior to 45 minutes of transient middle cerebral artery occlusion with 4 hours of reperfusion. Immunoglobulin G was detected by direct immunofluorescence at the region of the septal nuclei (A) and the hypothalamus (B) and the density and area were measured. Data are represented as mean+SEM and analysed by unpaired Student's t-test.

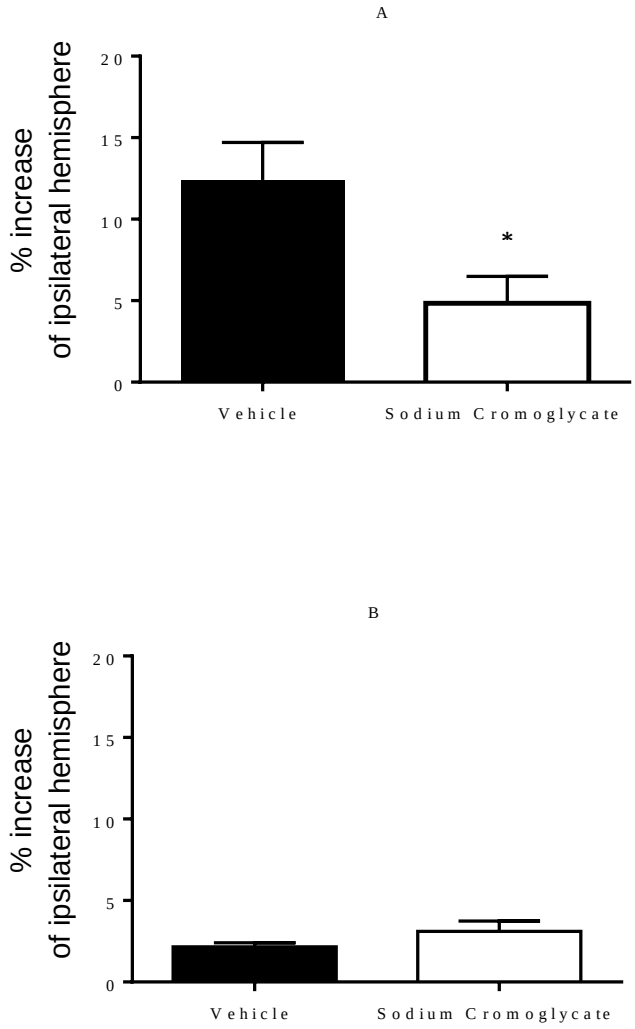

Figure 4.3: Measurement of Oedema in WT and Kit mice treated with sodium cromoglycate or vehicle, prior to onset of 45 minutes tMCAo followed by 4 hours of reperfusion. The area of the ipsilateral and contralateral hemisphere was quantified from haematoxylin and Eosin stained sections from eight distinct regions throughout the MCA territory of WT (n=7) (A) and Kit mice (n=7) (B). The degree of oedema was measured as a percentage increase in volume of the ipsilateral hemisphere over the contralateral side. Data are represented as mean+SEM. Unpaired Students t-test, *$p<0.05$.

Lesion volume in WT mice treated with SCG was significantly reduced compared to their vehicle treated littermate ($p<0.05$) s. The mean lesion volume was reduced by around 50% in the SCG treated group, compared to the vehicle treated group (**Figure4.4 A**). Drug treatment had no real effect on the lesion volume of Kit mice compared to vehicle treated controls (**Figure 4.4 B**).

4.4.4 Recruitment of neutrophils was significantly decreased by SCG treatment in WT mice.

To assess the effect of SCG treatment on inflammation and leukocyte recruitment, neutrophils were quantified at two brain regions. Neutrophils were identified and quantified in 20µm thick frozen sections, taken from the region of the septal nuclei and the anterior hippocampus using indirect immunofluorescent staining. The total number of cells counted in each section was then expressed as a ratio of area.

Quantification of neutrophils at the region of the septal nuclei revealed a significant decrease in the population following SCG treatment in WT mice compared to vehicle treated controls. The neutrophil population was reduced by around one third, from $0.65/mm^2$ to $0.45/mm^2$ ($p<0.05$) (**Figure 4.5 A**). However, within the region of the anterior hippocampus there was no change in neutrophil numbers between either the drug treated animals or vehicle treated controls (**Figure 4.5 B**). SCG treatment of Kit mice revealed no change in neutrophil recruitment to the injured brain. At the region of the septal nuclei and anterior hippocampus, the neutrophil population was similar in SCG treated animals compared to vehicle treated controls (**Figure 4.6 A and B**).

4.5 Discussion

The effect of pharmacological stabilisation of mast cells on the outcome of tMCAo was evaluated in this study. We found that treatment by ICV injection with SCG prior to onset of tMCAo, significantly reduced BBB permeability, oedema, lesion development and recruitment of neutrophils to the brain in WT mice, when compared

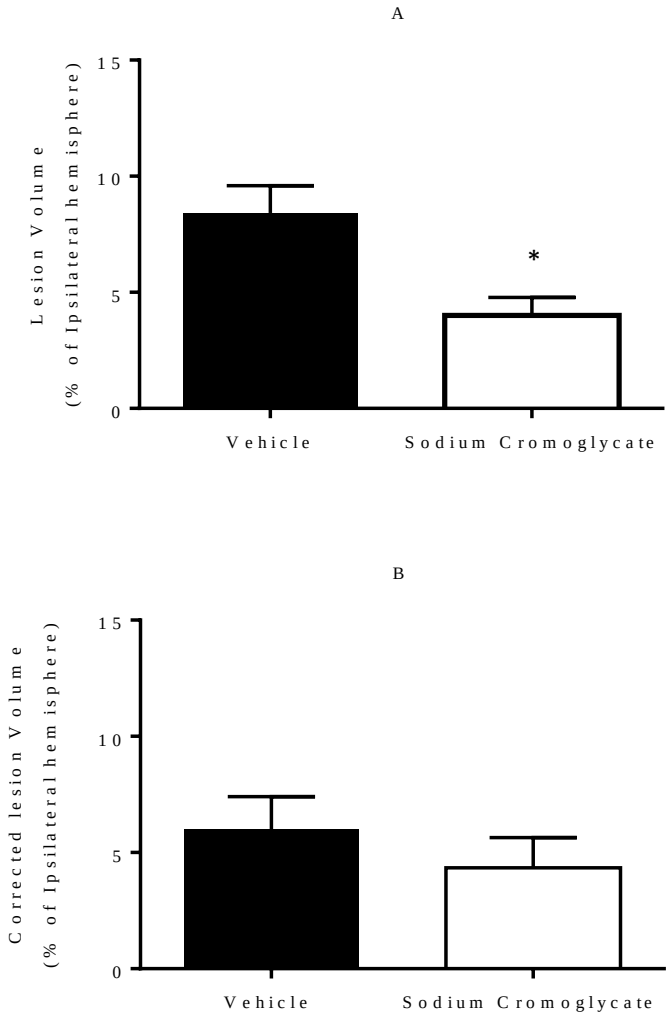

Figure 4.4: Ischaemic lesion volume in WT and Kit mice treated with sodium cromoglycate or vehicle, prior to onset of 45 minutes tMCAo followed by 4 hours of reperfusion. The area of the lesion and ipsilateral hemisphere was quantified from eight distinct regions throughout the MCA territory of WT (n=7) (A) and Kit (n=7) (B). The lesion volume was measured as a percentage of the ipsilateral hemispheric volume. Data are represented as mean + SEM. Unpaired Student's t-test, *$p<0.05$.

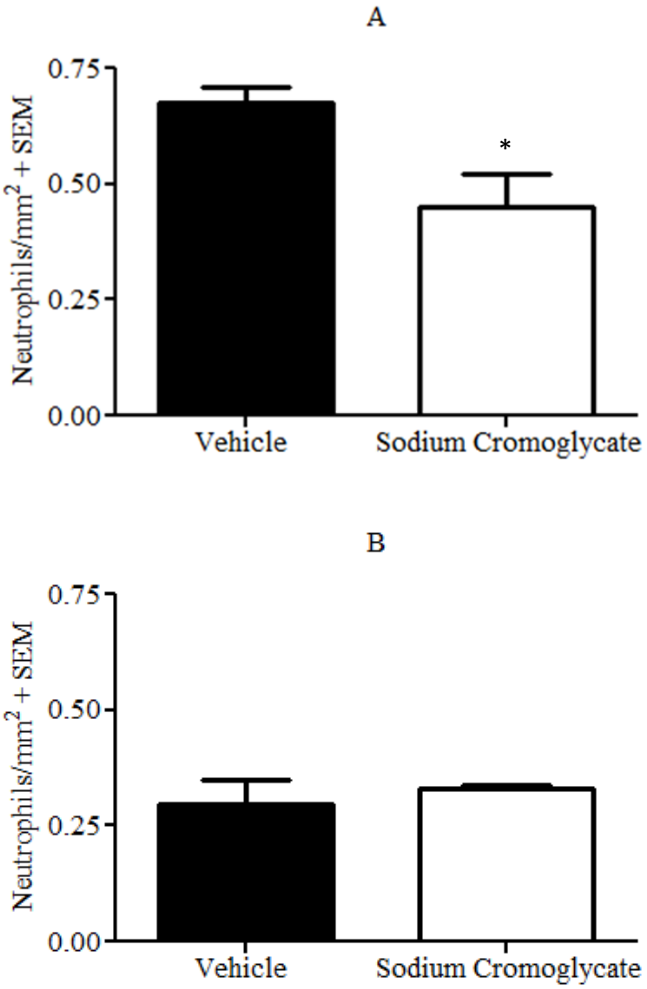

Figure 4.5: Quantification of neutrophil recruitment in WT mice treated with sodium cromoglycate or vehicle, prior to onset of 45 minutes tMCAo followed by 4 hours of reperfusion. Neutrophils were quantified on 20µm thick frozen coronal sections from WT mice (n=6) stained by indirect immunofluorescence at the region of the septal nuclei (A) and the anterior hippocampus (B). Data represented as mean + SEM and analysed by unpaired Student's t-test. *$p<0.05$.

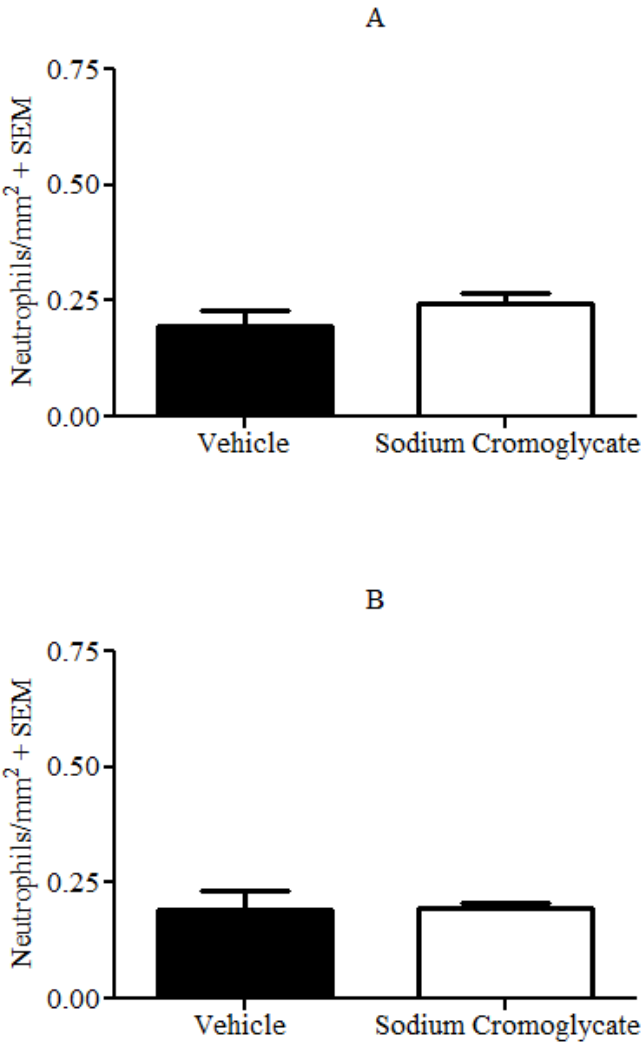

Figure 4.6: Quantification of neutrophil recruitment in WT Kit mice treated with sodium cromoglycate or vehicle, prior to onset of 45 minutes tMCAo followed by 4 hours of reperfusion. Neutrophils were quantified on 20µm thick frozen coronal sections stained by indirect immunofluorescence at the region of the septal nuclei (A) and the anterior hippocampus (B) from WT Kit mice (n=6). Data represented as mean + SEM and analysed by unpaired Student's t-test.

to saline treated controls. Furthermore, ICV SCG injection had no effect on outcome from tMCAo in Kit mice compared to saline treated littermates.

Mast cells are resident in the brain, in close proximity to the vasculature were their mediator content is capable of causing vascular damage, particularly by degradation of the ECM of the BBB, and promoting inflammation through release of various cytokines and chemokines (Huang *et al.,* 2012). This makes mast cells an ideal target for intervention in experimental stroke. We found in our previous study in Chapter 3 that Kit mice displayed a region specific reduction in BBB permeability and neutrophil recruitment and oedema was also significantly reduced compared to WT controls. A similar outcome has been found in this study, where BBB permeability was significantly reduced at the region of the hypothalamus, and global oedema was markedly attenuated, in the drug treated WT mice, compared to saline treated controls. Similarly, Strbian *et al* 2006 showed that ICV injection of SCG prior to onset of tMCAo significantly reduced BBB permeability and oedema, compared to vehicle treated control rats. Also, rats injected with the mast cell secretatouge, 48/80 had a marked increase in BBB permeability and oedema compared to controls (Strbian *et al.*, 2006). In a model of haemorrhagic stroke, pre injection of SCG ICV reduced the development of oedema to the level of saline treated controls at 30 minutes and 24 hours after injury. Importantly, SCG treatment improved neurological deficit and reduced mortality to 0%, compared to 45% in the control group at 24 hours (Strbian *et al.*, 2007). Prevention of oedema development by administration of SCG has also been demonstrated in a mouse model of gout. Injection of SCG prior to the induction of disease led to a significant reduction in swelling of the affected paw by almost 70%. Additionally, selective inhibition of mast cell derived mediators' histamine, serotonin and tryptase greatly reduced the degree of paw swelling (Hoffmeister *et al.*, 2011). Further work in rats has given more support to a mast cell specific role in mediating BBB breakdown and oedema development post tMCAo. The mast cell produced endopeptidase, MMP-9, was significantly reduced in activity in animals injected ICV with SCG prior to tMCAo. This outcome was associated with reduced oedema and BBB breakdown compared to wild type mice and those injected with 48/80 (Matilla *et al.*, 2011).

The inflammatory response to tMCAo in this model was alleviated at the level of the septal nuclei by pre injection of SCG, compared to saline treated WT controls. Indeed, recruitment of neutrophils to the brain at the level of the septal nuclei was significantly reduced, which was seen in the previous study in chapter 3. The mechanism by which mast cell stabilisation might reduce neutrophil recruitment is not addressed here, however it is well known that mast cells release a range chemotactic mediators and pro-inflammatory cytokines which promote the recruitment and transmigration of neutrophils across the vascular wall, including IL-8, IL-1 and TNF-α (Strbian *et al.*, 2006). Transmigration of neutrophils into the injured brain across activated endothelium causes a shift in phenotype, with the release of neurotoxic proteases, NE and extracellular traps which may contribute to neuronal loss (Buck *et al.*, 2008 and Allen *et al.*, 2012). This may offer some explanation as to why SCG treatment greatly improved lesion volume in the WT mouse compared to the saline treated group, which is not observed in comparison between Kit and WT mice in chapter 3. Some evidence suggests an enhanced anti-inflammatory response after SCG treatment, as both intravascular and extravascular populations of neutrophils were reduced in brains of rats given the drug prior to MCAo compared to saline treated controls. In the same study, genetically mast cell deficient rats had a significant reduction in extravascular neutrophils, whilst intravascular numbers were similar to wild type controls. This indicates that SCG may inhibit neutrophil chemotaxis independently of mast cells. Additionally, SCG injection prior to hypoxic-ischaemia in rats lead to reduced astrocyte and microglia activation and also reduced neuronal loss (Jin *et al.*, 2009). However in the current study, SCG had no influence on neutrophil recruitment in Kit mice, which supports the specificity of the drug for mast cells. Nonetheless, attenuation of neutrophil recruitment, due to mast cell stabilisation, may therefore be, in part, responsible for the improvement in lesion development in the SCG treated mice.

In conclusion, the specificity and the effectiveness of SCG in acting as a mast cell stabiliser can be seen in the results of this study, as an improvement was recorded in all outcomes measured in WT mice treated with the drug prior to tMCAo, compared

to saline treated controls. Furthermore, the drug had no effect, as expected, on the outcome from tMCAo in Kit mice, where the extent of pathology was similar in drug treated and saline treated controls. Although this work is very preliminary, it does identify a potential option for therapy in stroke which would be a repurposing of a currently available drug, which is far more appealing than developing a novel drug. SCG, in various formulations, has been used in human medicine for over 30 years and is used to treat a range of diseases including asthma, allergic rhinitis, conjunctivitis and mastocytosis, all disease where mast cell function leads to pathology (Hendrix *et al.*, 2013). Also, a number of clinical trials have been performed in other disease states such as nephritis, chronic lung disease and dermatitis which have produced results showing an improved outcome following SCG treatment (Sur and Scandale, 2010 and Feily *et al.*, 2012). This abundance of evidence indicates that the drug is a viable option for stroke treatment in terms of safety and suitability for use as a mast cell stabiliser, and further research is warranted to unlock the drugs full potential.

Chapter 5:

Investigation of the concentration of tumour necrosis factor-α, and an array of mediators associated with angiogenesis in brain tissue post transient middle cerebral artery occlusion.

5.1 Introduction

From the results of the previous two studies we concluded that mast cells have a detrimental role in the development of BBB breakdown, oedema and lesion development and neutrophil recruitment following transient focal cerebral ischaemia in the WT mouse. Indeed, genetic mast cell deficiency and pharmacological stabilisation can prevent the damaging effect of the mast cells. What remain unclear are the mechanisms by which mast cells interact with the ischemic brain to promote the ischaemic pathology in the previous studies. Also, it is unknown whether differences exist in cerebral perfusion between the WT and Kit mouse strains which may contribute to disease progression.

Reduction of CBF in experimental stroke is highly variable depending on mouse strain used and method of stroke induction. Infarct volumes can differ up to five-fold during global ischaemia between mouse strains, with differences in vascular anatomy thought to be responsible (Barone *et al.,* 1993). The vascular architecture of WT mice is responsible for the susceptibility of this strain to the intraluminal thread model. The circle of Willis was found to be complete in only 10% of WT mice due to the presence of both PcomAs. Only one PcomA was present in 60% of mice, whilst 30% had no PcomAs (McColl *et al.,* 2004). Given that mast cells have a close relationship to the cerebrovasculature, and have been found in close proximity to growing vessels within the neonatal rat brain, it is possible that differences exist in the Kit strains vascular anatomy, compared to the WT mice (Silverman *et al.,* 2000). Moreover, we might also expect that upon activation that vasoactive mediators produced by mast cells will contribute to cerebral hypoperfusion. Measurement of CBF within the MCA territory during the occlusion period, using laser Doppler flowmetry, should identify any improvements in the collateral perfusion of Kit mice.

Experimental evidence implicates TNF-α in multiple pathological pathways in the ischaemic brain, with involvement in necrosis, apoptosis, BBB damage and leukocyte recruitment (Watters and O'Connor, 2011). In the acute ischaemic period the concentration of TNF-α is elevated in the WT mouse brain, starting with the first hour and peaking at around 12 hours, and is maintained at a high level for around 24 hours (Sieber *et al.,* 2011). The deleterious effects of TNF-α can be inhibited by

injection of neutralising antibody, either before onset of ischaemia or upon reperfusion resulting in reduced lesion size, oedema and neurological deficit in mouse and rat models of tMCAo (Barone *et al.*, 1997 and Yang *et al.*, 1998). Moreover, injection of TNF-α ICV before induction of experimental stroke enhances lesion size and oedema and also worsens neurological deficit (Barone *et al.*, 2007). Given that TNF-α is stored within mast cells ready to be released upon activation (Metcalfe, 2008), it is a clear candidate for mediating the effects of mast cells in this model. Therefore, tissue concentration of TNF-α will be measured in brain homogenates after 20 minutes and 45 minutes of tMCAo.

Increased expression of a whole raft of mediators occurs in the early phase of ischaemia, many of which have been implicated in mediating pathology, including cytokines, chemokines, growth factors, various enzymes and vasoactive hormones (Wang *et al.*, 2007). Many of the mediators expressed in this period can be produced by mast cells, positioned near to or on blood vessels, which release a broad range of mediators capable of influencing the development of oedema, lesion, BBB degradation and inflammation (Strbian *et al.*, 2006). For example, mice deficient in the chemokine RANTES, which is produced by mast cells though not exclusively, had a much improved outcome after tMCAo compared to wild type controls. BBB permeability, infarct volume, leukocyte recruitment and circulating pro-inflammatory cytokine levels were all significantly attenuated in mice deficient in RANTES compared to wild type controls (Terao *et al.*, 2008). Considering mast cells can extrude their granule content in a very short period of time, it is reasonable to investigate whether mast cell derived mediators contribute significantly to the early onset of ischaemic pathology. An array of proteins related to angiogenesis, many of which are implicated in worsening BBB breakdown, oedema, lesion development and inflammation, post stroke, will be profiled in brain homogenates after 20 minutes and 45 minutes of tMCAo.

5.2 Hypothesis and aims

In the acute period of tMCAo, mast cells produce and release a broad range of mediators, including TNF-α, which may play a harmful role in the development of ischaemic pathology. Expression of TNF-α and other mediators involved in

enhancing ischaemic pathology, will be decreased in the brain tissue of Kit mice compared to wild type controls in the acute phase of tMCAo. Furthermore, improved regional CBF in Kit mice may account for the reduced pathology in this strain following tMCAo.

5.3 Protocol

All experiments were carried out on 10-12 week old male WT (bred In-house) or Kit mice (Jackson Laboratories, USA) weighing 25-30g. The regulations, as specified by the Animals (Scientific Procedures) Act (1986) where strictly adhered to throughout and were carried out under the appropriate Home Office licence. Mice underwent either 20 minutes tMCAo followed by 5 minutes of reperfusion or 45 minutes tMCAo followed by 45 minutes of reperfusion, and blood flow was monitored throughout using a laser Doppler flowmetry probe positioned anterior/posterior -1 mm, medial/lateral +2.5 mm, using Bregma as reference as described in **section 2.1**. At termination brains were removed and homogenisedas described in **section 2.2.3**. Protein concentrations of the ipsilateral hemispheres were determined, before being analysed by ELISA to determine TNF-α concentration. Serum from blood was also analysed for TNF-α concentration by ELISA. Finally, a proteome profiler array identified the relative expression of 53 proteins related to angiogenesis, within brain homogenates as described in **section 2.4**. The tMCAo experiments were carried out in a randomised fashion, and the experimenter was blinded to tissues being analysed by coding each individual animal.

5.4 Results

5.4.1 Cerebral blood flow was similar in WT and Kit mice during tMCAo.

To assess collateral perfusion of the MCA territory in both mouse strains, laser Doppler flowmetry was employed throughout the tMCAo period, and readings were recorded at five minute intervals throughout the procedure.

CBF was similarly affected in both WT and Kit mice undergoing tMCAo, whether for 20 minutes (**Figure 5.1 A**) or 45 minutes (**Figure 5.1 B**). Following placement of the microvascular clip on the LICA, blood flow dropped to around 60% of baseline levels. Perfusion was further decreased upon insertion of the filament, to

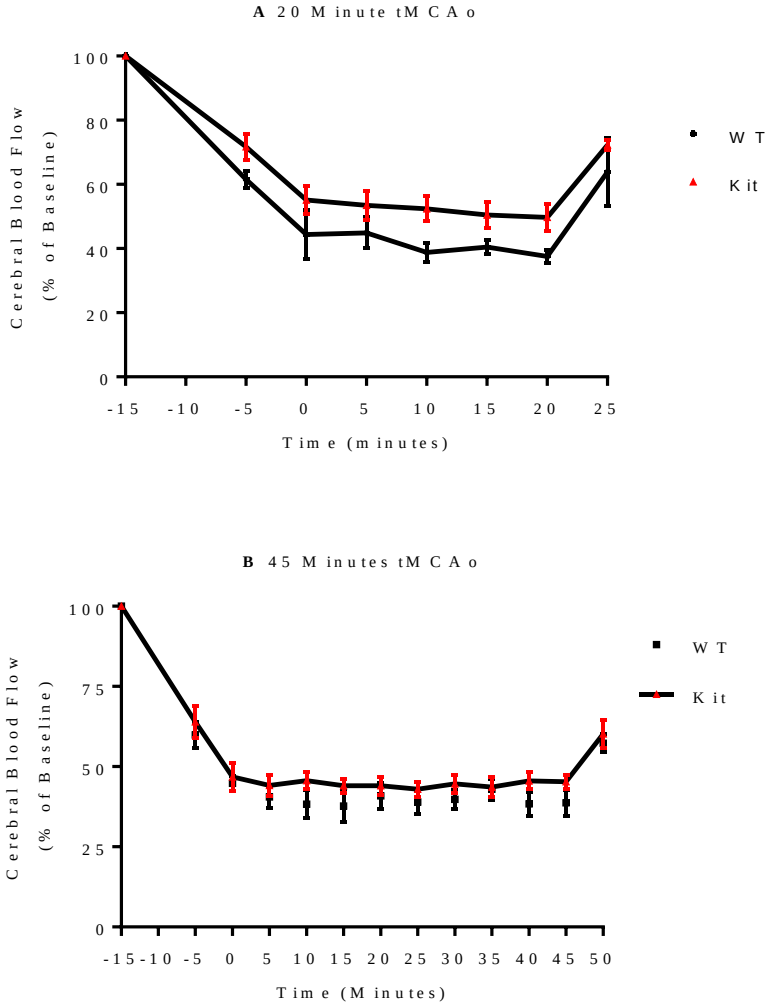

Figure 5.1: Cerebral blood flow in the middle cerebral artery territory during MCAo. The relative cerebral perfusion of the middle cerebral artery territory in WT and Kit mice undergoing 20 minutes (A, n=6) or 45 minutes (B, n=6) tMCAo was recorded using laser Doppler flowmetry. Readings were recorded prior to onset of ischemia and at 5 minute intervals throughout. Data are expressed as % of baseline blood flow. Analysed using a mixed model repeated measures ANOVA.

45% of normal flow. After five minutes of occlusion, the CBF dropped again to around 40% of baseline level where it remained stable throughout the occlusion period. Removal of the intraluminal thread restored blood flow to a rate similar to that prior to insertion.

5.4.2 Concentration of TNF-α in brain homogenates was not reduced in Kit mice following tMCAo.

The concentration of TNF-α was measured in homogenates of brain tissue from WT and Kit mice, by ELISA. Analysis was normalised to account for brain protein content and expressed as picograms of TNF-α/mg of protein.

After 20 minutes of tMCAo and 5 minutes of reperfusion, TNF-α concentration was similar in WT and Kit mice. Furthermore, TNF-α concentration was similar to that exhibited by sham operated animals of both strains (**Figure 5.2 A**). In mice subject to 45 minutes tMCAo and 45 minutes reperfusion, concentration of TNF-α was not significantly different between any group analysed (**Figure 5.2 B**). No TNF-α was detectable in serum samples at either time point analysed.

5.4.3 Relative expression of endothelin-1, matrix mettaloprotease-9 and endoglin were decreased in Kit mice.

To identify potential mediators of ischaemic pathology, the proteomic profile was determined from brain homogenates against an array of 53 proteins related to angiogenesis, many of which are linked to worsened ischaemic pathology, on a nitrocellulose membrane. The pixel density of each protein was determined and expressed as a percentage of the pixel density of a positive control.

The relative expression of 53 proteins was similar in WT and Kit mice after 20 minutes of ischaemia, as there was no significant difference in expression level and no proteins were expressed exclusively by either mouse strain (**Figure 5.3 A**). However, after 45 minutes of tMCAo the proteomic profile between the two mouse strains is strikingly different (**Figure 5.3 B**). In WT mice, ET-1, MMP-9 and Eng were expressed in all subjects tested and were not detected in tissue from Kit mice (**Figure 5.4 A**). Eng was most prominently expressed, with levels around 40% of the

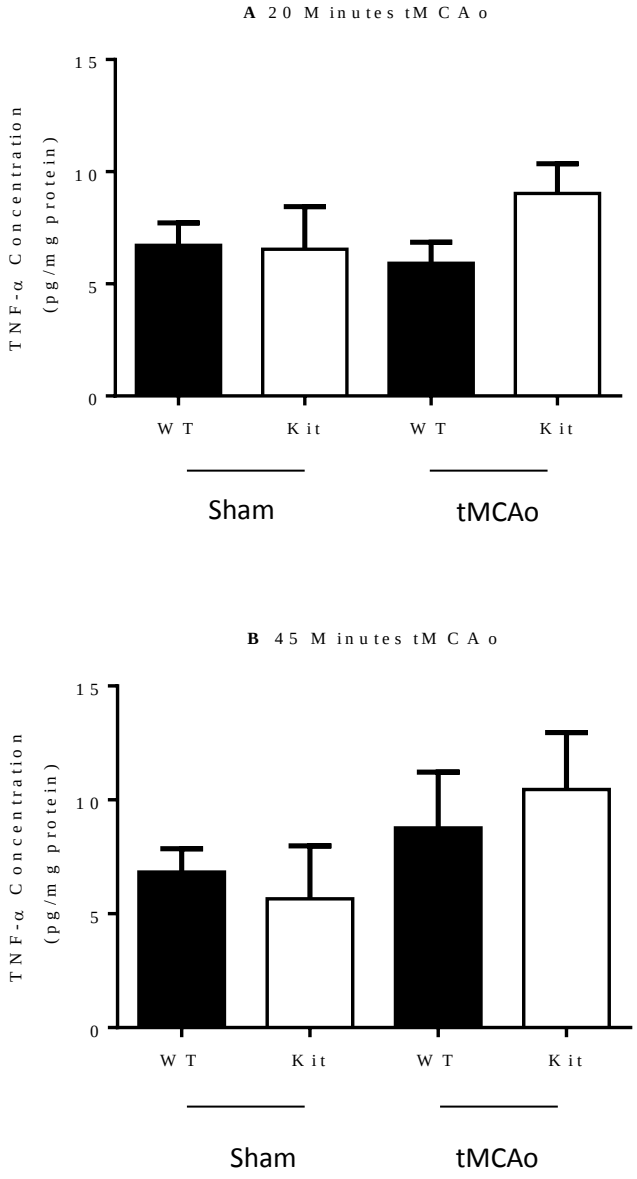

Figure 5.2: Concentration of TNF-α in brain homogenates following tMCAo. The concentration of TNF-α was measured by ELISA in brain homogenates of WT and Kit mice after 20 minutes tMCAo with 5 minutes of reperfusion (n=6) or sham operation (A) and after 45 minutes tMCAo with 45 minutes of reperfusion (n=6) or sham operation (n=4) (B). The concentration of TNF-α is expressed as pg/mg protein and represented as mean+SEM. Analysed by one-way ANOVA.

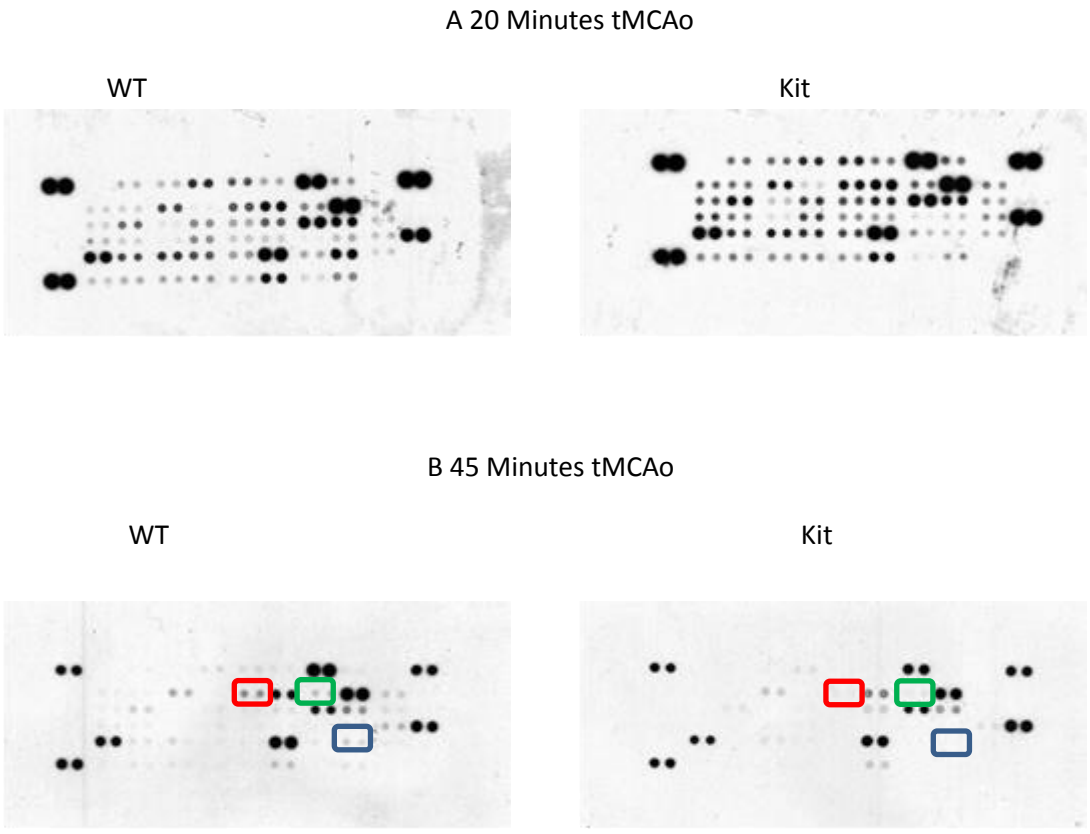

Figure 5.3: Proteome profile array of mediators involved in angiogenesis. Expression of an array of mediators related to angiogenesis was assessed in WT and Kit mice following 20 minutes tMCAo with 5 minutes reperfusion (A, n=2) and 45 minutes tMCAo with 45 minutes reperfusion (B, n=4). The spots highlighted indicate meditators expressed in WT mice, which are not expressed in the Kit strain.

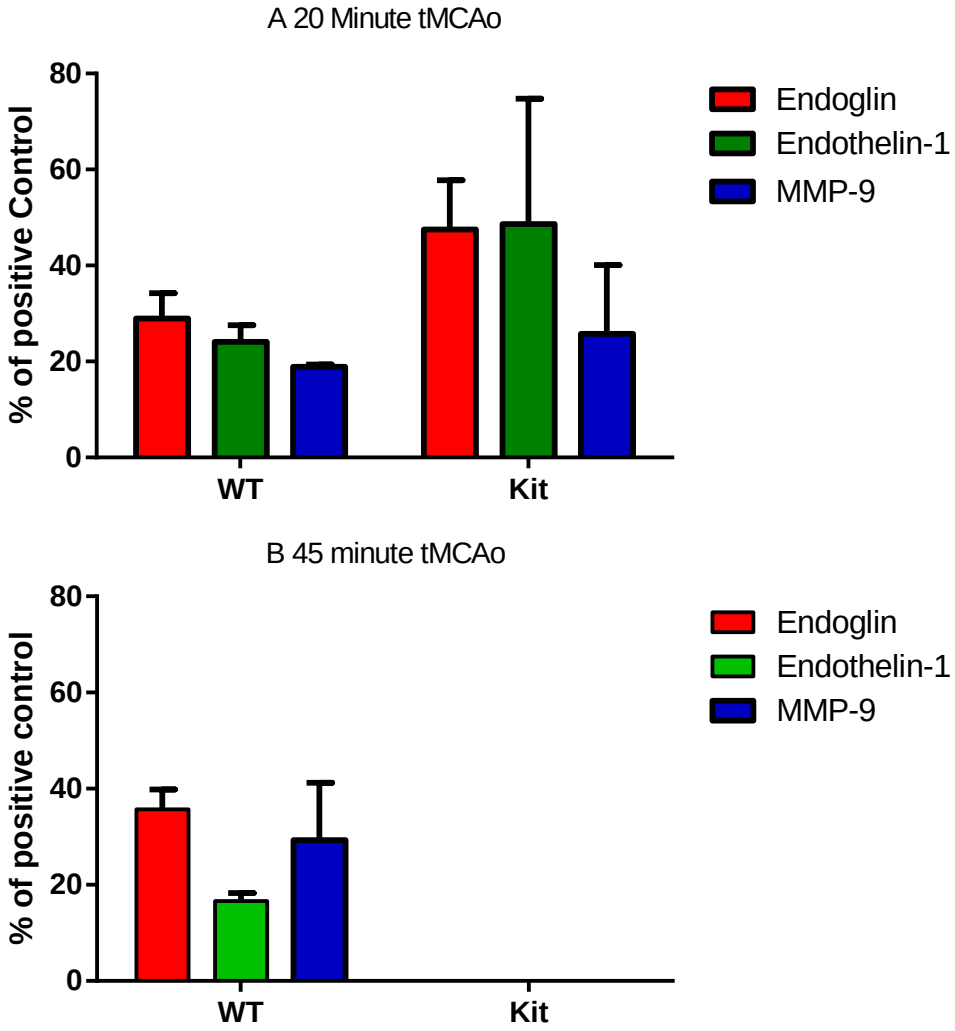

Figure 5.3: Expression level of endoglin, endothelin-1 and Matrixmetalloproteinase-9 following tMCAo. The relative expression of each mediator was measured from brain homogenates in WT and Kit mice after twenty minute tMCAo with five minutes of reperfusion (A) (n=2) and forty five minute tMCAo with forty five minutes of reperfusion (B) (n=4), using a proteome profiler array kit. The pixel density of each protein was measured and expressed as a percentage of the pixel density of the positive control.

positive control, whilst MMP-9 expression was 25% of the positive control. Finally, ET-1 expression was approximately 18% of the control spot (**Figure 5.4 B**).

5.5 Discussion

The current study examined collateral perfusion, expression profile of TNF-α and an array of proteins in the acute period of ischaemic pathology. This work has demonstrated that in mice subject to 20 or 45 minutes tMCAo, there is no improvement in collateral perfusion of MCA territory and there is no reduction in the tissue concentration of TNF-α in the brain of Kit mice compared to WT controls. However, ET-1, MMP-9 and Eng expression is abolished in Kit mice following 45 minutes tMCAo, whereas they are expressed in WT mice.

Perfusion of the MCA territory is similar in both mouse strains throughout the period of occlusion, indicating that improved CBF is not a determining factor in the outcome from tMCAo in Kit mice during the occlusion period. Extension of CBF analysis beyond the period of occlusion may give a different outcome, particularly considering the decreased expression of ET-1 in the 45 minutes of reperfusion. In this regard, the WT mice may be susceptible to secondary ischaemic events caused by ET-1 induced vasoconstriction during the reperfusion period.

The data obtained from the TNF-α ELISA experiments suggest that mast cell derived TNF-α is not responsible for the ischaemic pathology in the acute phase. In experimental stroke, the temporal profile of TNF-α expression varies depending on the model and animal used (Wang *et al.*, 2007). In WT mice, 30 minutes of tMCAo induced a steady increase with no real dramatic rise in expression in the time frame analysed in this study (Sieber *et al.,* 2011). In the current experiment the concentration was similar in both mouse strains and was not significantly different between mice subject to tMCAo or sham controls. However, the TNF-α concentration found in the tissue samples of sham operated mice was over two times greater than the level expected in a WT mouse aged three months (Sieber *et al.,* 2011). An explanation for this discrepancy is the use of isoflurane to anesthetise the mice. Inhalation of 1.4% isoflurane along with 100% oxygen for two hours causes TNF-α in brain tissue to increase two-fold, which is maintained for at least twenty

four hours (Wu *et al.,* 2012). In the current study, isoflurane was used at 2%, with 100% oxygen, for the initial twenty minutes of the procedure before being reduced to 1.5%, where it was maintained for the duration of the experiment, approximately 1.5 hours. Also, sham operated mice were subject to the same invasive procedure, except filament insertion, and had the same isoflurane exposure as occluded mice. However, in the animals subject to 90 minutes of ischaemia there was an increase in TNF-α concentration from that measured after 25 minutes, in particular the WT group, which was not witnessed in the sham animals whose levels remained stable. The profile of TNF-α expression beyond the time points analysed will give a better indication of whether mast cells do contribute a significant amount of TNF-α.

Protein expression analysis has identified ET-1 as a potential mediator of pathology in this model as it was expressed only by WT and not Kit mice after 90 minutes of ischaemia. ET-1 is one of the most potent vasoconstrictors identified, and is produced by, among others, vascular endothelium, smooth muscle cells and neurons, as well mast cells, following stimulus from various mediators including cytokines and numerous hormones (Piechota *et al.,* 2011). In both human and experimental stroke, ET-1 is elevated and considered a biomarker of poor outcome. Indeed, plasma concentration of ET-1 is increased 4 fourfold in stroke patients compared to healthy controls, which correlates to increased neurological deficit (Zhu *et al.,* 2003). ET-1 is thought to actively contribute to lesion development, BBB disruption and oedema. In a rat model of 90 minute tMCAo, blockade of the ETR_A by injection an inhibitor, Clasozenstan, 30 minutes after occlusion led to a much improved outcome compared to controls. Measurement of oedema and infarct volume showed a significant reduction after 24 hours, 72 hours and also after one week. Additionally, somatosensory recovery was much improved at the three time points analysed (Moldes *et al.,* 2012). In mice overexpressing ET-1, BBB damage, infarct, oedema and neurological deficit were all significantly greater than in their wild type littermates following two hours tMCAo and four hours of reperfusion. Additionally, there was a significant decrease in expression of the tight junction accessory protein occludin, concomitant with BBB breakdown. Increased ET-1 expression was also associated with up-regulation of aquaporin-4 channels, which regulate water uptake through the BBB, and implicates ET-1 in mediating oedema development (Lo *et al.,*

2005). Therefore, ET-1 remains a strong candidate for mediating mast cell dependent ischaemic pathology.

The expression of the gelatinase, MMP-9, was also completely absent in Kit mice after 90 minutes of ischaemia but increased in WT controls. MMP-9 is produced by activated mast cells, as well as numerous other cell types, and has already been implicated in mediating pathology in experimental and human stroke through proteolytic breakdown of the ECM (Ulrich *et al.,* 2013). In mast cell deficient rats subject to 1 hour tMCAo and three hours of reperfusion, MMP-9 activity was significantly reduced on the microvasculature. This correlated to a marked reduction in BBB permeability to EBA and a significant decrease in cerebral oedema (Mattila *et al.,* 2011). Studies using MMP-9 deficient mice also showed a significant improvement in BBB disruption and oedema, with the tight junction protein zonular occludin expression significantly greater than wild type controls (Asahi *et al.,* 2001). These findings merit further investigation in to the contribution of mast cell derived MMP-9 as a potential mediator of ischaemic pathology.

Eng is a receptor accessory protein for the anti-inflammatory cytokine TGF-β, and is found abundantly on proliferating endothelial cells (Zhu *et al.,* 2003). It is expressed in WT, but not Kit mice after 90 minutes of ischaemia. Very little research into the role of Eng in stroke has been carried out, however the consensus is that it is a marker of poor outcome and is associated with the vascular response to ischaemia and the number of Eng positive endothelial cells increases with stroke severity (Dziewulska and Rafałowska, 2006 and Simak *et al.,* 2006). In brain vascular endothelial cells cultured in hypoxic conditions, Eng expression is significantly increased after incubation for 4, 8, 26 and 24 hours. In the same study, Eng was also found to significantly increase within the infarct core after 28 days of recovery from tMCAo (Zhu *et al.,* 2003). In the present model, the increased expression of Eng may therefore be an indicator of responsive vascular endothelium, undergoing angiogenesis, whereas its absence in Kit mice may point to a less stressed microvasculature.

The results from this study have given some insight into potential mediators through which mast cells might cause injury in this model of tMCAo. We have shown that

mast cells appear not to release a significant level of TNF-α in the very early stages of ischaemia. Additionally, after 90 minutes of ischaemia, mast cells either produced ET-1, MMP-9 and Eng, or stimulate their production by other cells. What this data indicates is that mast cells may not be necessary for the immediate expression of the mediators identified, but that they are required for continued expression. Further work is warranted to investigate in more detail the contribution of these three mediators in this model, focusing on the temporal profile of mediator release and mechanisms by which mast cells may influence their production.

Chapter 6:

The effect of genetic mast cell Deficiency on blood brain barrier permeability, oedema, infarct volume and neurological deficit in the subacute period, post transient middle cerebral artery occlusion.

6.1 Introduction

Despite the success of the previous studies in identifying mast cells as modifiable modulators of ischaemic pathology and identifying mediators through which they might exert their affects, it remains untested whether any mast cell intervention would have any longer term benefit. To this point, only the very acute period of ischaemia has been studied, where mast cell deficiency or pharmacological stabilisation resulted in a much improved outcome from tMCAo by reducing BBB permeability, oedema and neutrophil recruitment. However, the pathobiological events associated with cerebral ischaemia are highly dynamic and occur over days to weeks, with the environment within the brain constantly changing (Dirnagl *et al.,* 1999). How mast cells might behave in the subacute and chronically injured ischaemic brain remains entirely unexplored.

In rodent models of ischaemic stroke, the ischaemic lesion develops and increases in volume, recruiting tissue at risk in the penumbra in to the ischaemic core, for up to around 72 hours (Liu *et al.,* 2012). During this period there are also fluctuations in the degree and extent of inflammation, oedema and BBB opening, events in which mast cells may play a role in orchestrating. The opening of the BBB and the ensuing oedema may be events where mast cells are particularly important. Mast cells are known to be involved in all stages of vascular remodelling, from breakdown of the tight junctions and cell death caused by inflammatory responses, to cellular proliferation and neovascularisation through release of an array of cytokines, growth factors and vasoactive mediators, such as TNF-α, vascular endothelial growth factor (VEGF), histamine, chymase, tryptase and perlecan, among others (Sandoval and Witt, 2008, Moon *et al.,* 2010). Whether mast cells remain an important modulator of the pathobiological processes occurring in the days after tMCAo will help in evaluating the validity of mast cells as a target for post stroke intervention.

The most important measure of the outcome from experimental stroke is the functional recovery and mortality rate of the animals. Improvements in lesion volume or alleviation of any of the multiple pathological pathways in the post tMCAo brain are irrelevant if there is no real measurable benefit to the animal (Hossmann, 2008). The acute nature of the studies performed until now, with a

maximum recovery period of 4 hours of reperfusion, has not permitted full evaluation of neurological deficit, whilst mortality has been negligible. Therefore, it is important to evaluate whether the attenuation of stroke pathology in the Kit mice seen in the previous studies has any real benefit to the animal.

6.2 Hypothesis

In the subacute period, 72 hours post tMCAo, mast cells will contribute to the on-going pathobiological events, including BBB opening, oedema and lesion development. Attenuation of these stroke pathologies in Kit mice will improve neurological function and decrease mortality compared to mast cell competent WT controls. Randomisation of the tMCAo experiments was carried out and the experimenter was blinded to the identity of the animals during analysis by assigning each animal a code.

6.3 Protocol

All experiments were carried out on 10-12 week old male WT mice (Bred in-house) or Kit (Jackson Laboratories, USA) weighing 25-30g. Mice underwent 45 minutes tMCAo followed by 72 hours of reperfusion as described in **section 2.1.4**. At termination, blood was collected and brains immediately removed and flash frozen in isopentane at -42C. The brains were sectioned and analysed for development of oedema, lesion volume and BBB damage as described in **section 2.**2. Additionally, neurological deficit was assessed at 24 hours, 48 hours and 72 hours post tMCAo using Clark's deficit, a system developed to detect both general and focal deficits in the mouse following MCAo (Clark *et al.,* 1997) as described in **section 2.1.6**.

6.4 Results

6.4.1 Neurological deficit was not improved in Kit mice, however mortality was reduced.

Neurological deficit was assessed in animals at 24 hours, 48 hours and 72 hours post-tMCAo using a system developed to detect both general and focal deficits in the mouse following MCAo (Clark *et al.,* 1997). General deficits were assessed in a $4m^2$ open field without interference and observations were recorded regarding appearance

of fur, ears, eyes, posture, spontaneous activity and epileptic activity. For focal deficit assessment, mice were subject to a number of motor function tasks. Animals were removed from the study if a score of greater than 21 was achieved in either of the tests.

At the three time points assessed, there was no difference in focal or general deficits between the two groups of mice (**Figure 6.1**). There were no animals omitted from either group due to severe deficit, however mortality in the WT group was 25%, a total of two animals, compared to 0% in the Kit group. All deaths in the WT group occurred during the first 24 hours and were attributed to severe oedema. This conclusion was met after ruling out haemorrhagic transformation and observation of an enlarged ipsilateral hemisphere. Sham operated animals scored 0 on neurological testing and mortality was also absent.

6.4.2 BBB permeability was not reduced in Kit mice.

At 72 hours post tMCAo, there was no difference in BBB permeability between WT and Kit mice. The density and area of fluorescently stained endogenous IgG leakage was similar at both the region of the septal nuclei and the region of the hypothalamus in both groups (**Figure 6.2**).

6.4.3 Oedema development was not attenuated in Kit mice.

Oedema was measured in haematoxylin and eosin stained coronal tissue sections from eight brain regions spanning the territory of the MCA. The area of both the ipsilateral and contralateral hemispheres were measured using a densitometer and were then graphed against their distance from the IA line. The area under the curve produced a volume for each hemisphere, and oedema was expressed as a percentage increase of the ipsilateral hemisphere over the contralateral volume.

The volume of brain oedema at 72 hours post tMCAo was not reduced in Kit mice, compared to WT mast cell competent mice. There was a non-significant increase in the ipsilateral hemisphere volume to around 16% in the Kit mice, compared to 12% in the wild type group, which is contrary to the hypothesis (**Figure 6.3 A**).

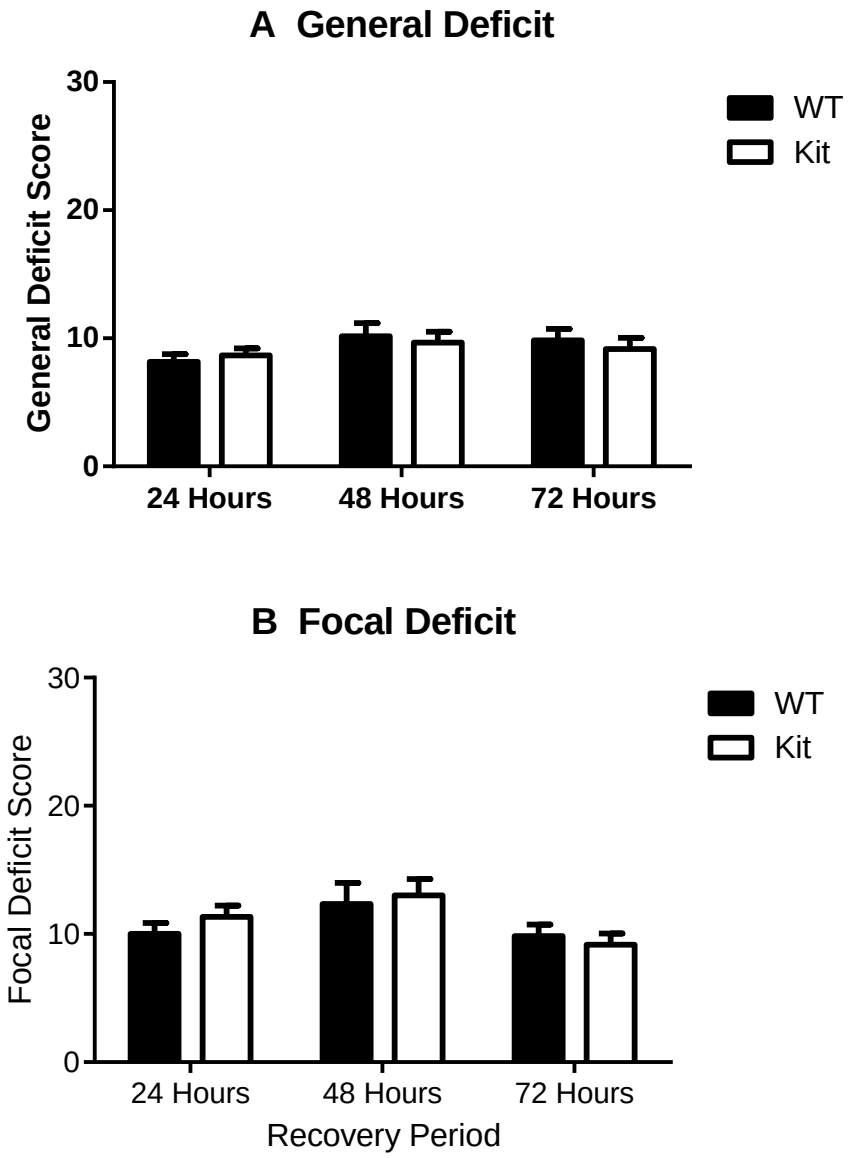

Figure 6.1: General and focal deficits assessed at 24, 48 and 72 hours post 45 minute tMCAo. In WT (n=6) and WT Kit (n=6) mice general (A) and focal (B) deficits were assessed. The deficits were assessed on a scale from 0 (healthy) to 28 (severe and moribund). Data are represented as mean + SEM, analysed using 2-way ANOVA

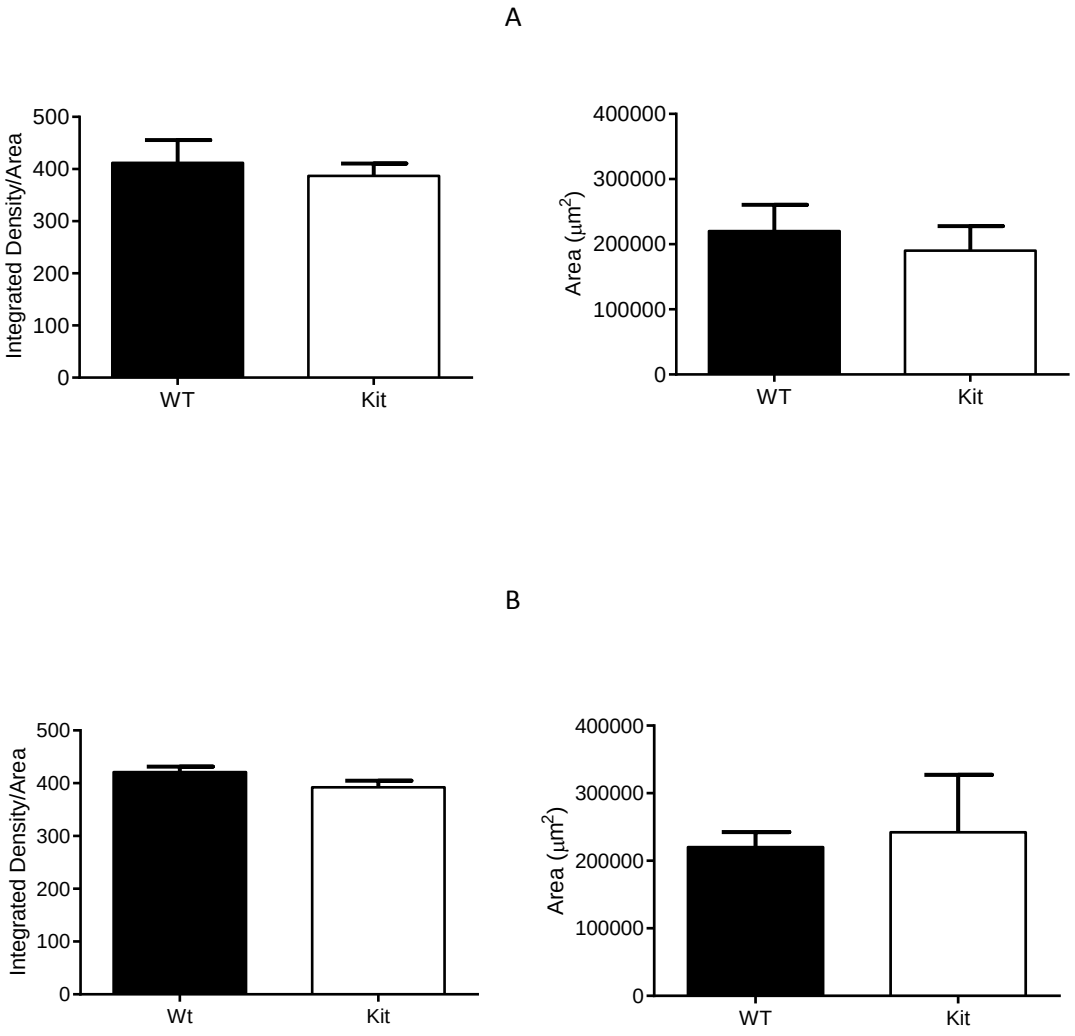

Figure 6.2: Measurement of density and area of Endogenous immunoglobulin G detected in brain tissue of WT and Kit mice, following 45 minute tMCAo with 72 hours of reperfusion. Indirect immunofluorescence at two regions, A (3.94mm IA, level of the septal nuclei) and B (2.86mm IA, level of the hypothalamus) in WT (n=6) and Kit (n=6). The density of fluorescence and area of staining were quantified and are represented as mean + SEM. Unpaired Students t-test.

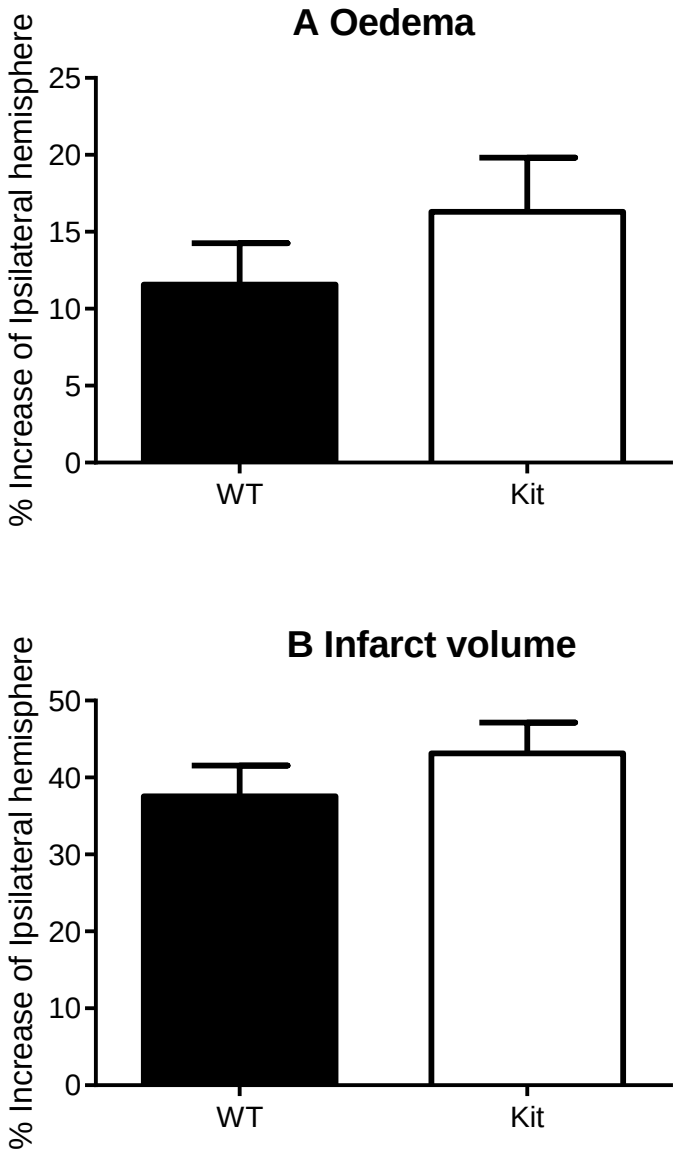

Figure 6.3: Oedema and infarct volume in WT and Kit mice subject to 45 minutes tMCAo followed by 72 hours of reperfusion. The area of the ipsilateral and contralateral hemisphere was quantified in tissue sections from WT (n=6) and Kit (n=6) mice at eight distinct regions and the degree of oedema was taken as a

percentage increase in volume of the ipsilateral hemisphere over the contralateral side (A). The lesion volume was measured as a percentage of the ipsilateral hemispheric volume (B). Data are represented as mean + SEM. Unpaired Student's t-test

6.4.4 Ischaemic infarct was not reduced in Kit mice.

Ischaemic lesion volume was assessed in haematoxylin and eosin stained coronal tissue sections from eight distinct brain regions, representative of the MCA territory in each mouse strain. Regions of pallor, indicating tissue loss, were identified in the ipsilateral hemisphere and measured using a densitometer. The values were then graphed against their distance from the IA line, with the distances 7.9 mm and 0.1 mm equal to zero. The volume of the lesion was ascertained from the area under the curve and expressed as a percentage of the ipsilateral hemispheric volume.

The infarct volume was similar in both mouse strains at 72 hours post tMCAo, with an overall ipsilateral hemispheric infarct volume of around 39% in wild type WT mice, compared to 43% in the Kit group (**Figure 6.3 B**).

6.5 Discussion

This study reports, for the first time, on the role of mast cells in the subacute period following tMCAo in mice. We have found that there was no difference in BBB permeability, oedema or lesion volume at 72 hours post ischaemia between WT and Kit mice. Additionally, we have found that neurological deficit was similar between both groups at 24 hours, 48 hours and 72 hours following onset of tMCAo. However, during the recovery period, mortality rate was 25% in the WT mice whilst Kit had 100% survival. This data indicates that mast cell targeted intervention may improve survival in the post stroke period.

The response of the BBB to ischaemia reperfusion is multiphasic and has been shown to fluctuate between a highly permeable and a relatively stable state for up to five weeks after tMCAo in rats. The initial increase in BBB permeability occurs in as little as 25 minutes and peaks at around 6 hours, before gradually stabilising by 36 hours. A second increase in opening then occurs at 72 hours and it remains open at

2, 3 and 4 weeks before gradually improving by week 5 (Strbian *et al.,* 2008). Importantly, during this initial period of BBB opening, vasogenic oedema develops and is maintained at a hazardous state for up to 5 days post ischaemia in animal models (Sandoval and Witt, 2008). Of most relevance to this work is the very early increase in permeability, during the first 24 hours, when animal mortality occurred in the WT mouse group. Mast cell activation is rapid and may contribute to the initial phase of BBB opening, which would be absent in the Kit mice who had no mortality during this period. It remains unclear what role mast cell might play in the phase of BBB remodelling beyond the recovery period of this study, and how mast cell intervention beyond 72 hours might affect outcome.

The development of the ischaemic lesion in rodent models of tMCAo reaches its maximum volume by 72 hours of reperfusion, and in this study there was no difference between the two animal groups at this point. This would imply that any mast cell mediated damage has no real consequence on the outcome for the animals and that between the very acute period post tMCAo, studied in the previous chapters, and the 72 hour end point that mast cells are not participating in pathology. This would tie in with evidence from a model of traumatic brain injury, where after 24 hours the mast cell population was significantly reduced compared to sham operated controls. The likely explanation for this is mass degranulation of the mast cell population in response to injury, with a subsequent re-population within the following week (Hendrix *et al.,* 2013). How the mast cells might participate in the chronic stages of stroke remains to be seen, however there is evidence to argue for both protective and negative effects. It is likely that mast cells would participate in the continuous opening of the BBB in the weeks following stroke, however they may also play an anti-inflammatory role as was shown by reduction of T-cell infiltration and microglia activation in chronic traumatic brain injury (Strbian *et al.,* 2008 and Hendrix *et al.,* 2013).

The data presented here indicates that mast cells are not important mediators of injury in the subacute period post tMCAo. Any mast cell mediated effects following tMCAo are likely to be limited to the acute period, as was observed in the previous studies and given the mortality rate in the mast cell competent mice during the acute

phase in this study. How mast cells might behave in the chronic period post tMCAo needs further investigation.

Chapter 7: General Discussion

7.1 Discussion of main findings and future work

7.1.1 The mast cell population increased in the ischaemic hemisphere after tMCAo.

The population of mast cells in the ischaemic hemisphere of WT mice increased markedly after four hours of reperfusion when compared to sham operated or naïve control mice following 45 minute tMCAo. Additionally, there was a non-significant increase in mast cells located perivascularly at this time point.

Mast cells are resident immune competent cells of the CNS and are found in close proximity to the cerebrovasculature, where they are ideally positioned to respond to any insult. Furthermore, an increase in mast cells to the site of injury is found in experimental models of CNS disease, including hypoxia-ischaemia and MCAo. At the site of injury, mast cell degranulation is evident, with release of mediator content which includes cytokines, growth factors, chemokines and various enzymes, among others (Jin *et al.,* 2009). Indeed, in a rat model of hypoxic ischaemia, mast cells containing and releasing TNF-α where found to increase in population in the first 48 hours after injury onset. Meanwhile, in a rat model of tMCAo, mast cells positively stained for MMP-9 are found abundantly at the site of injury four hours after onset (Jin *et al.,* 2009 and Mattila *et al.,* 2011).

The degranulation of mast cell and the release of their contents can lead to the pathological events in the ischaemic brain, including increased BBB permeability, enhanced local inflammation and vasogenic oedema, and these outcomes were reduced in severity in the Kit mice in the current study four hours after tMCAo. Beyond the acute phase of this experimental disease, the involvement of mast cells is less clear, as there is no difference in outcome between WT and Kit mice. This may be due to overt degranulation of the mast cell population, which is subsequently repopulated over the following week or so. To test this hypothesis, a full histological examination of mast cells in tissue from the 72 hour reperfusion period, as described in **section 2.2.4**, would be necessary.

Whilst the initial increase in mast cells in the WT mice was clearly indicative of mast cell involvement in this model, what was less clear from the results are the

mechanisms by which the mast cells are enhancing post tMCAo pathology. The proteomic analysis from chapter 5 would indicate that TNF-α was not the cause, although it has been shown previously to contribute to onset of ischaemia induced injury in a rat model (Jin *et al.,* 2007 and Jin *et al.,* 2009). The reduced expression of ET-1, Eng and MMP-9 in the Kit mice after forty five minutes of reperfusion from forty five minute tMCAo gave an indication of when and how mast cells might be involved in disease progression. Indeed, the expression profile after twenty minutes of ischaemia was similar in both strains, indicating that mast cells may not be the driving force behind expression of these mediators. Mast cells appear to be involved in maintaining the on-going expression of these mediators which, as discussed later in this chapter, can promote the ischaemic pathology in this model.

The literature indicates that mast cells within the brain of the WT mouse brain are potentially distinct from those of the rat and probably other species as the mast cell population is generally heterogeneous in terms of mediator content, surface receptors and survival mechanisms (Shanas *et al.,* 1998 and Gali and Tsai, 2008). A full characterisation of the brain mast cells of the WT mouse is therefore necessary before we can fully understand the function of mast cells in this model. This could be achieved using traditional immunohistochemical techniques by targeting candidate receptors and mediators contained on or within the mast cells. However, the use of genetic tools may be favourable, such as microarray or RT-PCR which could identify whole cell gene expression (Haenisch *et al.,* 2013). This would be a useful tool not just for characterising the mast cell phenotype, but also for identifying altered gene expression under experimental conditions, post tMCAo in WT mice. Such a study would provide yet further information about when and by what mechanisms mast cells are active in the ischaemic brain.

7.1.2 Mast cells contributed to BBB opening post tMCAo.

We found that genetic mast cell deficiency or SCG injection into the brain prior to 45 minutes tMCAo led to a region specific reduction in BBB permeability after four hours of reperfusion. However, on the third day of recovery there was no difference between the WT and Kit mice in terms of BBB opening in this model.

Mast cells contain and release upon activation an array of mediators which can potentially decrease the integrity of the BBB through degradation of ECM and tight junction proteins by both direct and indirect mechanisms (Matilla *et al.*, 2011). Of particular significance to these studies is the mast cell derived endopeptidase MMP-9, which was found to be expressed in the brain of WT but not Kit mice after 45 minutes of reperfusion. MMP-9 is capable of digesting most of the ECM components including laminin, fibronectin, vitronectin and collagen, and also the tight junction proteins occludin and claudin. Additionally, mast cell derived chymase, tryptase and cathepsin G act to activate MMP-9 and also MMP-2, which is also active post ischaemia and produced by endothelium and smooth muscle, from their inactive forms, as well as directly degrading the BBB independent of MMP-9 (Heo *et al.*, 2005 and Lindsberg *et al.*, 2010). Aside from the proteolytic mechanisms of mast cell induced BBB breakdown, vasoactive mediators such as histamine and growth factors such as VEGF are released after activation and are known to increase BBB permeability (Heo *et al.*, 2005).

In the subacute recovery period following tMCAo, mast cells do not appear to have had any impact on the integrity of the BBB. Indeed both WT and Kit mice had similar levels of BBB permeability to endogenous IgG after 72 hours reperfusion following 45 minutes of tMCAo, and this level of IgG staining was similar to that after four hours of reperfusion. Interestingly, the level of IgG staining in the study in chapter 4 was considerably higher than the other two studies despite the same reperfusion period as chapter 3 of four hours. This difference may have been due to the trauma caused to the brain tissue and vasculature following the injection of SCG, which may have in turn permitted an increase of endogenous IgG leakage into the tissues. Perhaps using a high molecular weight tracer such as EBA to evaluate BBB permeability would negate this confounding factor. Additionally, the extent of BBB permeability after 72 hours of reperfusion may have been masked by the mortality within the WT group. Had these mice survived we may have seen a difference when compared to the Kit mice. Also, as the cause of death was attributed to excessive oedema, the oedema volumes at this time point may also be underestimated.

It may be assumed that after the initial BBB opening in the acute recovery period that the mast cell population is exhausted from overt degranulation and is no longer capable of influencing the BBB. There is some evidence to support this idea from studies in ischaemic stroke and traumatic brain injury in rats. After one day of recovery from traumatic brain injury there was a dramatic decrease in the numbers of mast cells in the brain compared to uninjured controls, and the population remained low at the fourth day of recovery. Also, evaluation of BBB opening for 5 weeks following 90 minutes tMCAo in rats indicated a gradual repair of the BBB after an initial surge in opening during the first six hours of reperfusion. Interestingly, it took around one week for the mast cell population to be restored to normal levels following traumatic brain injury, whilst the BBB in rats who underwent tMCAo was again highly permeable after one week of reperfusion (Strbian *et al.,* 2008 and Hendrix *et al.,* 2013). Of course, the alternative to this is that mast cells are essential for maintaining the integrity of the BBB in the period beyond the 72 hour reperfusion time point analysed. It is known that mast cells are important modulators of angiogenesis, and in their absence normal BBB repair mechanisms may be impaired.

A better understanding of the temporal profile of mast cell contribution to BBB opening will increase our understanding of when any mast cell directed intervention may be appropriate. Longitudinal study of BBB permeability post tMCAo could be carried out using tracers such as Gd-DTPA or EBA, which can be detected by MRI or Xenogen IVIS respectively, and can be evaluated for acute, subacute and chronic stages of the disease similar to that undertaken by Strbian *et al.,* 2008. Comparing the profile of BBB opening between the WT and Kit mice will highlight the stages at which mast cells are important or indeed dispensable.

7.1.3 Mast cells potentiated oedema development post tMCAo

The development of oedema, post tMCAo, was potentiated by the presence of mast cells within the brain. Indeed genetic deficiency or pharmacological stabilisation of mast cells greatly reduced oedema after four hours of reperfusion. However, after a reperfusion period of 72 hours, oedema volume was similar in both WT mice and Kit mice.

Vasogenic oedema occurs due to mass movement of fluids into the brain tissue through a compromised BBB, and results in herniation of brain structures, disruption to axonal connections and compression of vessel, leading to ischaemia (Ayata and Ropper, 2002). The extent of oedema, around a 10% increase in the ipsilateral hemisphere volume, was similar in WT mice after both 4 hours and 72 hours of reperfusion, and at the latter time point Kit mice were affected to a similar degree. This could indicate that oedema develops at a slower rate in the absence of mast cells, which are likely contributing through degradation of the BBB. This idea is supported by the decreased permeability of the BBB in Kit mice in the acute recovery period, and the positive correlation found between increased BBB opening and larger oedema volumes. Additionally, in WT mice recovering to 72 hours there was 25% mortality within the first 24 hours, which was attributed to oedema, whilst there was no mortality in the Kit mice. Therefore, mast cells appear to be causal to this increased mortality by mediating the development of brain oedema in the period of reperfusion between 4 and 24 hours.

To affirm this idea that mast cells are contributing to oedema during the reperfusion period beyond four hours, we require some empirical evidence. Similar to the longitudinal study proposed for BBB opening analysis, oedema can be tracked over time using real time *in vivo* MRI, and could be measured concurrently to the BBB permeability. Extraction of hemispheric volumes from both ipsilateral and contralateral sides would allow quantification of oedema (Lu *et al.*, 2012).

7.1.4 Mast cells increased neutrophil recruitment post tMCAo.

The recruitment of neutrophils to the brain post tMCAo was significantly attenuated at the region of the septal nuclei following either genetic mast cell deficiency or pharmacological stabilisation, using SCG, compared to WT mice or saline treated controls, respectively.

The consequences of this attenuation of neutrophil recruitment is difficult to definitively state given the data available, however, as discussed in previous sections, neutrophils can contribute extensively to stroke pathology. Indeed, mice deficient in NE exhibit reductions in both BBB permeability and lesion volume following

tMCAo (Stowe *et al.,* 2009). However, there was no improvement in final infarct volume at 72 hours of reperfusion in this model despite a significant reduction in neutrophil recruitment in the acute period. This may indicate that the neutrophil attenuation was modest and may not have been sufficient to have any biological significance. Nonetheless, the extent of neutrophil attenuation may have been beneficial to the stability of the BBB during the acute reperfusion period.

The time point measured in this study, 4 hours, is really at the early stages of neutrophil recruitment and involvement in cerebral ischaemia and was not sufficient to fully assess the contribution of neutrophils to BBB permeability and subsequent oedema during the period up to 24 hours, where the mortality occurred in the WT mice. However, the reduction in neutrophil recruitment to the brain post tMCAo in the Kit mice highlights a potential role of mast cells in orchestrating recruitment of other leukocytes not analysed here. Indeed, a full repertoire of immune competent cells has been shown to infiltrate the ischaemic hemisphere in the hours and days post tMCAo, with the potential to be either damaging or protective (Gelderblom *et al.,* 2009). Since mast cells are resident in the brain and capable of responding rapidly to produce an array of chemokines and cytokines which promote recruitment, vascular adhesion, diapadesis and activation of leukocytes, they may therefore act as a beacon to promote inflammation during cerebral ischaemia. This concept is supported by the finding that mast cells were activated prior to microglia in a rat model of hypoxia-ischaemia, and that mast cell stabilisation using SCG reduced both microglia and astrocyte activation in the subacute and chronically injured brain (Jin *et al.,* 2009).

Analysis of neutrophil infiltration in the period after four hours would really highlight whether or not mast cells significantly contribute to their recruitment and activation, or if other inflammatory stimuli within the ischaemic brain is sufficient and compensatory to mast cells. When neutrophils enter the brain during ischaemia, they adopt a neurotoxic phenotype and degranulate to release proteases such as cathepsin-G, NE and also MMP-9. This change can be detected using immunostaining methods to label mediators released (Allen *et al.,* 2012). An alternative to this is to image neutrophils *in vivo*, which would allow for repeated

measures in the same animals over time, reducing the number of animals required, and provide more information than the snap shot provided by more traditional staining methods. Bioluminescence of neutrophils can be identified *in vivo* by injection of luminol. Luminol exploits myeloperoxidase activity of the cells, which oxidises luminol and emits a detectable blue light which can be visualised using the Xenogen IVIS (Gross *et al.*, 2009).

7.1.5 Mast cells were not important for post tMCAo lesion development

The absence of mast cells in Kit mice did not lead to a reduction in lesion volume at 4 hours post tMCAo, or in infarct volume after 72 hours of reperfusion. However, stabilisation of mast cells prior to onset of tMCAo did lead to a significant reduction in lesion volume after 4 hours of reperfusion.

The significant improvements in BBB permeability and oedema, together with the reduction of neutrophil infiltration after four hours of reperfusion would be expected to translate to a reduction in the ischaemic lesion volume. A more stable BBB should reduce exposure to potentially toxic circulating proteins, and offer some neuroprotection. Additionally, alleviation of oedema could reduce lesion development by preventing vascular compression, resulting in secondary ischaemia, and herniation (Ayata and Roper, 2002 and Sandoval and Witt, 2008). Furthermore, attenuation of neutrophils should negate some of the neurotoxic effects of neutrophil recruitment. In the acute period there was no improvement in lesion volume, which may be due to the evolution of ischaemic damage, which increases over time. However, the infarct at 72 hours post reperfusion is similar in both the mouse strains indicating that mast cells are not vital in the development of ischaemic lesion.

Nonetheless, there was a significant reduction in lesion volume in WT mice injected with SCG compared to vehicle treated controls in the acute recovery period. As discussed in chapter 4, this may be due to non-specific anti-inflammatory effects of SCG which has been shown to inhibit microglia and astrocyte activation as well as prevent neutrophil transmigration (Strbian *et al.*, 2006 and Jin *et al.*, 2009). Improved immunohistochemical analysis of the neutrophil population within the brain post tMCAo could help identify the numbers of neutrophils crossing the BBB

following SCG treatment. The technique used in these studies could not differentiate between neutrophils located intravascular or those located extravascular. Comparison of these populations between SCG and vehicle treated WT mice would give an indication of whether or not neutrophils were behaving differently in the presence of SCG and thus potentially affecting lesion development. Although there was no difference between the lesion volume in Kit mice regardless of whether they received SCG or vehicle, the lesion volume was very small, so perhaps there was no room for improvement.

7.1.6 Mast cell dependent pathology may be mediated by endoglin post tMCAo.

In WT mice, Eng was expressed in the ipsilateral hemisphere, after 45 minutes of reperfusion following 45 minute tMCAo. However, in Kit mice Eng expression was attenuated, and was not detectable using the proteomic profiler used. The reduced expression of Eng may have been responsible for the improved outcome during the acute period in the Kit mouse strain.

Eng is expressed predominantly on endothelial cells, and is increased in expression during angiogenesis, in which it contributes to smooth muscle and endothelial proliferation, pericyte migration and production of ECM proteins (Diez-marquez *et al.*, 2002). Under ischaemic conditions Eng expression is increased on mouse endothelium, and is also found abundantly in the ischaemic hemisphere 28 days after pMCAo in mice (Li *et al.*, 2003). In this model the absence of Eng in the Kit mouse brain may be indicative of a less responsive vascular endothelium, due to a reduction of injury to the BBB, as seen with the decreased permeability during the acute reperfusion period. This reduction in Eng expression may also give further backing to the concept that mast cells are important modulators of inflammation post tMCAo, and may be directing turnover of the injured microvasculature. Furthermore, the reduction of Eng in the period between twenty minutes of ischaemia and forty five minutes tMCAo in the Kit mice indicates that mast cells help maintain the up-regulation of Eng expression. Identification of the specific Eng isoforms expressed would help to clarify its role in this setting. The membrane bound Eng can undergo

proteolytic cleavage to form a soluble protein, which acts a scavenger of TGF-β. This leads to impairment of angiogenesis and may be a mechanism by which the BBB of mast cell competent WT mice is compromised (Zhu *et al.*, 2003). The isoforms of Eng are readily identifiable in tissue and serum by ELISA or RT-PCR (del Rey *et al.*, 2013).

7.1.7 Mast cell dependent pathology may be mediated by endothelin-1 post tMCAo.

The potent vasoconstrictor ET-1 was expressed in the brain of WT mice forty five minutes post tMCAo. However it was not expressed in the Kit mouse strain, which may have caused the reduction in stroke pathology in these mice in the acute recovery period.

ET-1 is produced by many cell types including endothelial cells, neurons and mast cells and is important for maintaining vascular tone and remodelling. It acts through its two receptors ETR_A and ETR_B, which exert a range of biological functions such as SMC proliferation, vasoconstriction, vasodilation, and ECM production, depending on the receptor engaged and the cell type expressing the receptor (Frommer and Muller-Ladner 2008). In both experimental and human stroke, ET-1 is widely regarded to increase injury severity by contributing to lesion development, BBB disruption and oedema. In mice overexpressing ET-1, BBB permeability was increased, as was oedema and lesion volume compared to wild type controls after four hours reperfusion following two hours tMCAo (Lo *et al.*, 2005). Therefore it may be assumed that in the current study, that ET-1 contributed to the development of BBB disruption and oedema. This may have been due to secondary ischaemic events caused by the vasoconstrictor properties of ET-1, and perhaps continued monitoring of blood flow throughout the reperfusion period, using multiple laser Doppler flowmeter probes, would identify any haemodynamic effect. Furthermore, identification of the expression profile of the ETR subtypes and the cells expressing the receptors, using immunostaining, would give an indication of what effects this increased ET-1 expression might be having.

The development of oedema post ischaemia has been shown in animal models to be dependent on ET-1 expression which is thought to increase aquaporin4 channel expression on astrocytic end-feet, facilitating water uptake across the BBB (Lo *et al.*, 2005 and Moldes *et al.*, 2012). This may be the case in this model as we saw an increase in oedema in the WT mice expressing ET-1 in the acute recovery period. Analysis of aquaporin4 gene expression by Western blot or RT-PCR in brain homogenates may give an indication if this is the case, particularly if the expression is lower in the Kit mice. Whether or not any mast cell contribution to ET-1 production has any real impact on the outcome from tMCAo is probably a more important question and can be answered by using ETR antagonists. Introduction of both ETR_A and ETR_B antagonists, Clasozenstan and BQ-788 respectively, post tMCAo may well improve the outcome in WT mice whilst the Kit strain may be unaffected by the antagonists, with a similar outcome to vehicle treated animals.

7.1.8 Mast cell dependent pathology may be mediated by matrixmettaloproteinase-9 post tMCAo.

MMP-9 is expressed in brain homogenates from both WT and Kit mice after twenty minutes tMCAo, with five minutes of reperfusion. However, after forty five minutes of reperfusion following forty five minutes tMCAo MMP-9 is no longer detectable in the Kit mice.

The degradation of the ECM post tMCAo is an important step in the development of increased BBB permeability and vasogenic oedema, and the endopeptidase MMP-9 has been implicated as an important mediator in this process. Indeed, MMP-9 is known to degrade claudins and occludin, leading to disassociation of endothelial cell tight junctions, thus increasing permeability of the BBB (Lindsberg *et al.*, 2010). We saw a reduction in this model of both BBB permeability and oedema in the Kit mice, which may be a result of reduced MMP-9 expression. Immunostaining of the tight junction proteins, for example claudin or occludin, will help confirm the role of MMP-9 in this model, as we would expect to see reduced staining in the WT mice if MMP-9 were important.

To achieve a more integrated view of how MMP-9 is influencing the outcome in our model, a more direct approach is required. We know that in the absence of mast cells, MMP-9 expression was reduced upon reperfusion after forty five minutes of tMCAo in Kit mice, although it is expressed prior to this after twenty minutes tMCAo. We also know that mast cells produce MMP-9 following tMCAo in rats (Matilla *et al.*, 2011). Therefore the important question is, does mast cell derived MMP-9 significantly influence pathology in this model? The most conclusive result would come from the use of 'mast cell knock in-mice', where mast cells cultured from MMP-9 deficient mice are adoptively transferred to Kit mice. We would then a have a mouse deficient in mast cell derived MMP-9, and could measure the outcome from tMCAo and compare to wild type controls.

7.1.9 Mast cell derived tumour necrosis factor alpha was not an important mediator mast cell dependent pathology post tMCAo.

There was no decrease in production of TNF-α in brain homogenates following tMCAo in Kit mice compared to WT or indeed sham operated mice of either strain. This was evident at both five minutes after a twenty minute tMCAo, and also forty five minutes post forty five minute tMCAo. This indicates that at these time points, TNF-α was not an important mediator, and that mast cells were not a significant source of this cytokine.

The role of TNF-α in experimental stroke is not clear cut, as it has been shown to be protective and damaging in animal models. TNFR subtype appears to dictate whether TNF-α elicits protective or detrimental responses, with TNFR1 activation leading to cell death and TNFR2 activation promoting survival. Indeed, animals deficient in TNFR are more vulnerable to ischaemia, and whilst seemingly paradoxically, the TNF-α knockout mouse recover better from experimental stroke (Gary *et al.*, 1998). Nonetheless, TNF-α does promote post-ischaemic inflammation and contributes to excitotoxic cell death in both animal models and *in vitro* assays (Watters and O'Connor, 2011). However, in this model it does not appear to be responsible for ischaemic pathology related to mast cells or otherwise.

Given the abundance of evidence for a role of TNF-α in cerebral ischaemia it is unlikely that the results obtained here are a definitive measure of TNF-α in this model and perhaps refining or adapting our method of detection will yield more informative results. It may be the case that TNF-α was concentrated within the ischaemic territory and by homogenising the entire hemisphere the cytokine has been diluted. By dissecting out the territory of the MCA and measuring TNF-α within, we may detect a different result. As discussed previously, timing of analysis may be an issue, with TNF-α not yet fully involved in the on-going pathology. It must be considered that TNF-α has to be synthesised, and is perhaps not stored within brain mast cells of this mouse. Of course it may also be true that there is no overt degranulation of mast cells by forty five minutes post tMCAo, and beyond this point TNF-α may well be abundantly detectable. This highlights the need for characterisation of these cells and further supports the idea that the brain mast cells are phenotypically distinct from other known populations.

As described in the previous section for MMP-9, 'mast cell knock-in' mice may be the optimal method for identifying a role for mast cell derived TNF-α in this model. Should mast cell derived TNF-α be important in mediating ischaemic pathology we would expect to see a reduction in BBB permeability, oedema and lesion volume as well as a general reduction in inflammation.

7.1.10 SCG treatment prior to onset of tMCAo improved outcome

The use of the mast cell stabilising agent SCG ameliorated ischaemic pathology in WT mice, as described in chapter four. Administration by ICV injection prior to onset of ischaemia resulted in attenuation of BBB permeability, oedema, lesion development and neutrophil recruitment. This indicates a real avenue towards mast cell targeted stroke therapy, as SCG is currently used for treatment of various mast cell related disorders and is therefore both safe and efficacious (Sur and Scandale, 2010).

Before making any leap towards using SCG in humans there is still much to understand about if and when any intervention may be appropriate. Further studies in this model will certainly help to identify the correct use of the drug. There is no

information available on the consequences of mast cell stabilisation or indeed genetic deficiency, on the long term recovery from tMCAo. It is well accepted that mast cells are important modulators of angiogenesis and have anti-inflammatory properties, as well as being involved in tissue remodelling (Moon *et al.,* 2010). Extension of the recovery period to at least twenty eight days post tMCAo will help identify any deficiencies in this respect. Furthermore, continuous monitoring of BBB opening and oedema, as described in previous sections, will aid in identifying when mast cells are involved and intervention with SCG may be worthwhile. Intervention with SCG at these times identified may improve outcome in the chronic stage for the animals, or may identify a requirement for mast cells in the advanced stages of stroke recovery.

The drug was administered prior to ischaemia in these studies, which is clearly not relevant to its use in the treatment of human stroke. Injecting the drug after the onset of reperfusion will give a more clear indication of its true potential. As identified in the last section, it is not clear when mast cells become active after tMCAo so injection would have to be tested at multiple time points in multiple groups of animals. Furthermore, the length of time the drug is active is unknown in this setting, so multiple doses should be administered. A study carried out in a rat model of hypoxia-ischaemia, where the drug was administered at one, four and twenty four hours after insult, showed SCG to be effective in stabilising the mast cell population. There was minimal increase in mast cell population or TNF-α production between four and twenty four hours, indicating that drug may well be active for at least twenty hours (Jin *et al.,* 2009).

It is worth mentioning that there are other mast cell stabilising agents available for use in animal models, and others which are already in use in humans. The c-kit receptor antagonists mastinib and imatinib can inhibit degranulation, however as there is evidence to suggest brain mast cells do not express c-kit receptor these drugs are inappropriate. Targeting of specific mast cell mediators, such as chymase, is also possible using nafamostat mesilate (Kennedy *et al.,* 2013). However this approach is historically ineffective in stroke therapy, where individual mediators are unlikely to contribute a great deal amongst a vast array of pathological processes.

7.1.11 Discussion of the suitability of Kit mice for modelling stroke.

Although the data from these studies indicate a harmful role for mast cells in this model of focal cerebral ischaemia, the results must be interpreted with some caution with regards the suitability of the model of mast cell deficiency used, the Kit mouse strain. As mentioned in **section 1.8.2,** the Kit mouse strain has a number of abnormalities which must be taken into consideration. Indeed, these mice suffer from splenomegaly, cardiomegaly, and thrombocytosis and are neutrophilic (Nigrovic *et al.*, 2008).

Of most relevance to this work is the neutrophilic phenotype displayed by the Kit mouse strain, which is likely a mechanism of compensation due to the deficiency of mast cells (Nigrovic *et al.*, 2008). In this work, we have shown that Kit mice had a region specific reduction of neutrophils, at the level of the septal nuclei, post tMCAo compared to the WT strain. Clearly this data does not support this observed phenotype in the Kit strain, as we would have expected an increase in the neutrophil population compared to the WT mice. However, it must be considered that the neutrophils, regardless of their reported abundance within the circulation, require a stimulus to initiate recruitment to the injured brain. We could therefore interpret this data as supportive of the idea that mast cells are important in the process of neutrophil recruitment. Despite reportedly being abundant within the circulation, recruitment of neutrophils to the brain is still significantly impaired when mast cells are genetically depleted.

The Kit mice used in this study suffer from cardiomegaly, which is associated with mild hypertension, and may have an influence on the recovery from tMCAo (Chan *et al.*, 2005 and Nigrovic *et al.*, 2008). In the context of stroke, blood pressure is an important physiological parameter which can greatly influence CBF in the acute stroke period. Hypotension can result in a reduction in CBF which potentiates ischaemia and increases growth of the lesion, which ultimately increases neurological deficit. Hypertension during the acute recovery period increases the risk of oedema and haemorrhagic transformation (Goerlick and Aiyagari, 2013). Despite the potential mild hypertension in the Kit mice, it did not lead to either increased oedema or worsened neurological deficit, or indeed increased infarct

volume in this study. Additionally, CBF was similar in Kit and WT mice throughout the occlusion period.

To overcome the limitations of the Kit mouse model the use of 'mast cell knock-in' techniques are useful. By reconstituting the mast cell population with either BMMC or by full bone marrow transplant, we can repair the mast cell deficiency (Grimbaldeston *et al.,* 2005). This would confirm whether or not any differences between Kit and WT mice were due to mast cell deficiency. We would expect no differences in the post stroke pathology analysed between the mast cell knock-in and WT mice. Additionally, the use of novel mouse models of mast cell deficiency would be desirable. The selective ablation of mast cells can be achieved using toxin receptor-mediated conditional cell knockout (TRECK) technology. In this system, mast cells display the diphtheria toxin receptor which when activated by injection of diphtheria toxin, depletes mast cells *in vivo* (Sawaguchi *et al.,* 2012). Mas-TRECK mice, which adopt this system, are therefore another candidate model for investigating the role of mast cells in our model of focal cerebral ischaemia. Unlike the Kit mice, Mas-TRECK mice have few reported abnormalities other than elevated serum IgE and IL-4 expression (Sawaguchi *et al.,* 2012).

At present the Kit mice are the most robust and most widely used model of mast cell deficiency available. However with development of new models there is potential in future to better understand the role of mast cells in stroke without having to overcome confounding abnormalities present in the existing models.

7.2 Clinical relevance.

The ultimate aim of researching stroke in animal models is to identify potential therapies which can be translated to the treatment of human disease. From animal models of ischaemia, over one thousand potential therapeutics have been identified, of which none have proven effective in the human condition. The only real effective treatment available in acute stroke which can reduce injury severity is rtPA, which was initially developed for myocardial ischaemia, and has fastidious inclusion criteria for its use. For this reason, along with increasing prevalence of stroke and

the aging population, it is essential that new stroke therapies are developed (Albers and Olivot, 2007)

The targeting of immune cells in stroke treatment is not a new concept, and drug targeting of leukocyte attachment to the vascular membrane, anti-ICAM-1 therapy, failed clinical trials. This was due to idiopathic antibody production causing complement and neutrophil activation resulting in a 22% mortality rate of patients treated, compared to 16% of placebo treated controls (Sherman *et al.,* 2001). Neutrophil infiltration post ischaemia has also been targeted and made it to clinical trials where there was no improvement in the treatment group. The fact that evidence points to mast cells being active before the initial leukocytes arrive or before microglia become involved in disease progression, makes them a very attractive therapeutic target (Wang *et al.,* 2007 and Lindsberg *et al.,* 2010).

The major drawback of rtPA therapy is risk of haemorrhagic transformation, which may be partly mediated by mast cells. The activation of mast cells *in vitro* has been demonstrated to occur in the presence of rtPA, and mast cells have fibrinolytic activity, through release of heparin (Lindsberg *et al.,* 2010). In a rat model of tMCAo, ICV injection of SCG after onset of reperfusion and immediately prior to infusion of rtPA, reduced the occurrence of haemorrhage caused by rtPA therapy alone by up to 96%. The significance of this study is that it was performed within the current time window permitted for use of rtPA of four and half hours post stroke onset (Strbian *et al.,* 2007).

In order to receive rtPA treatment, stroke patients must undergo MRI to confirm an ischaemic stroke has indeed occurred, which would allow for assessment of BBB permeability. If there is increased BBB permeability then the use of SCG would be very straightforward and could simply be injected, as it would gain access to the brain readily. This evidence shows that mast cells are a well-defined target for stroke therapy and that there is reliable and safe treatment option waiting to be fully explored and utilised.

7.3 Conclusions.

The data presented in this thesis indicates that mast cell numbers increase in the acute period of reperfusion following tMCAo and are potentially modulators of post tMCAo pathology. Genetic mast cell deficiency lead to significant reductions in BBB permeability, oedema and neutrophil recruitment compared to WT mice. Additionally, mast cell stabilisation using SCG, prior to onset of tMCAo conferred protection from these outcomes as well as reducing lesion volume. The harmful effects of mast cells may have been mediated by Eng, ET-1 and MMP-9, as they were expressed in the WT mouse brain, but not in the brain of Kit mice post tMCAo. In the subacute period following tMCAo, mast cells do not appear to be instrumental in the development of pathology as the outcomes measured were similar between the WT and Kit mouse strains.

This data adds to a growing body of evidence regarding the detrimental role of mast cells in animal models of cerebral ischaemia, and has highlighted mast cells as a potential therapeutic target for stroke therapy. Based on the work done in this thesis we can make sound *a priori* formulated hypotheses, and design studies with enough power to really define the role of mast cells in experimental stroke.

Chapter 8: References

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