Evaluation of the role of the melanocortin receptor system in ischemia-reperfusion induced leukocyte endothelium interaction in the brain microcirculation

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Abstract

Leukocyte recruitment following cerebral ischemia reperfusion (I/R) has been shown to amplify inflammatory processes and enhance brain injury. Potent anti-inflammatory and neuro-protective properties have been attributed to the melanocortin receptor system, thus targeting this system may provide a novel therapeutic strategy for the treatment of stroke. Melanocortin receptor (MC) subtypes MC_1, MC_3 and MC_4 have previously been shown to mediate MC anti-inflammatory actions, however their relative importance is poorly understood and may differ with the pathophysiological environment.

In this study the bilateral common carotid artery occlusion (BCCAo) mouse model of global stroke was used with intravital microscopy to investigate the potential of melanocortin targeted compounds in modulating cerebral I/R induced leukocyte recruitment. While under basal conditions the cerebral microcirculation provides a poor substrate for leukocyte recruitment, BCCAo induced significant leukocyte recruitment by 40 min of reperfusion, with leukocyte rolling increasing from 21.5 cells/mm2/min to 191.0 cells/mm2/min and adhesion from 42.1 cells/mm2/min to 282.3 cells/mm2/min. Extending reperfusion to 2 h resulted in further increases in adhesion to 1500% of sham levels. Treatment with the non-selective melanocortin agonist, α-MSH, significantly reduced I/R induced leukocyte recruitment (Rolling by 80% at 40 min and 68% at 2 h and adhesion by 68% at 40 min and 82% at 2 h). The relative contributions of the melanocortin receptor subtypes were investigated in this model using MC selective compounds and receptor mutant mice. The MC_1 selective BMS-470,539 provided most potent inhibition of leukocyte recruitment at 40 min with the MC_1 mutant e/e mice also showing enhanced leukocyte rolling and circulating TNF-α, while $MC_3^{-/-}$ mice showed no inflammatory phenotype. However by 2 h the inhibitory effects of BMS-470,539 subsided and e/e mice no longer showed abnormalities in leukocyte recruitment. On the other hand MC_3 targeted compounds were highly effective at this later time point. These investigations reveal an important role for MC_1 immediately following BCCAo with a later shift toward MC_3 mediated processes by 2 h. Due to no observed changes in receptor mRNA and that by 2 h α-MSH treatment was found to reduce NF-κβ related gene expression but not at 40min. It is likely the apparent shift in receptor importance is due to the activation of distinct signalling pathways rather than change in receptor expression.

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Abbreviations

AA	Arachidonic acid
Ab	Antibody
ABC	Avidin-biotin-peroxidase
aCSF	Artificial cerebro-spinal fluid
ACTH	Adrenocorticotropic hormone
AgRp	Agouti-related protein
ANOVA	Analysis of variance
BCCAo	Bilateral common carotid artery occlusion
BM	Basement membrane
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclooxygenase
CRs	Consensus repeats
DPBS	Dulbecco phosphate buffered saline
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESL-1	E-selectin ligand-1

FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum
fMLP	Formylated methionine-leucine-proline
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPCR	G-protein coupled receptors
h	Hours
HFRW	Histidine-Phenylalanine-Arginine-Tryptophan
HMGB1	High-mobility group box 1
HO	Heme-oxygenase
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular cell adhesion molecule
i.c.v	Intracerebroventricular
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
I/R	Ischemia-reperfusion
i. v.	Intravenous
IVM	Intravital microscopy
JAM	Junctional adhesion molecule
kDa	Kilodalton
KO	Knock-out
KPV	Lysine-Proline-Valine
LAD-I	Leukocyte adhesion deficiency type I
LER	Low expression regions of matrix protein
LFA-1	Leukocyte function-associated antigen-1

LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
M	Molar
mAb	Monoclonal antibody
Mac-1	Macrophage antigen-1
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MAPK	Mitogen-activated protein kinase
MCAo	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein 1
min	Minutes
MMP	Matrix metalloprotease
MC	Melanocortin Receptor
MRAP	Melanocortin 2 receptor accessory protein
MRS	Melanocortin Receptor System
M∅	Macrophage
Mg^{2+}	Magnesium ions
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
MW	Molecular weight
NFκ-B	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
n	Number of independent experiments performed
O_2	Oxygen
P	Probability value
PAF	Platelet-activating factor
PECAM	Platelet endothelial cell adhesion molecule

PBS	Phosphate buffered saline
PC	Pro-hormone convertase
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
PMN	Polymorphonuclear leukocyte
POMC	Proopiomelanocortin
PSGL-1	P-selectin glycoprotein ligand-1
ROS	Reactive oxygen species
rpm	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of mean
SOD	Superoxide dismutase
TBP	TATAA-box binding protein gene
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
VCAM-1	Vascular endothelial cell adhesion molecule
VE-cadherin	Vascular endothelial cadherin
VLA-4	Very late antigen-4
WT	Wild-type
WSR	Wall shear rate

Declaration of Originality

I declare that the work presented herein is the sole work of Paul Holloway. Any information derived from the published or unpublished work of others has been appropriately acknowledged in the text and referenced.

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1.0. Introduction

1.1. The global burden of Stroke

1.1.1. Aetiology

Stroke is a neurological disorder caused by the cessation or reduction of the blood supply to the brain, resulting in significant neuronal damage and rapid loss of function. Stroke may result from an ischemic (>80 % of incidence) or haemorrhagic (~13 % of incidence) event, affecting the brain in either a focal or global manner [1]. Focal ischemic stroke (~68 % of cases) results from the obstruction of either a pre-cerebral or intrinsic artery or arteriole; this may be caused by an embolus, a thrombus or an atherosclerotic plaque [1]. Whilst global stroke accounts for ~12 % of ischemic strokes and arises when blood flow to the entire brain is interrupted most often due to cardiac arrest or trauma [2].

1.1.2. Epidemiology and socio-economic impact

Stroke is the third most common cause of death, behind cardiac disorders and all cancers combined [3], killing around 53,000 people in the UK each year [4] and 5.5 million worldwide [5]. Depending on age, co-morbidity, type, and severity of stroke between 20 % and 50 % die within the first month following stroke [6]. Where stroke is not fatal, only 10 % of survivors are reported to recover completely, making stroke the most common cause of long-term physical disability in the world [4]. Deficits experienced by survivors include paralysis, impairments in memory; cognition; co-ordination and language. The great need for palliative and rehabilitative care imposes a huge socio-economic burden, with the total economic cost of stroke in the UK being estimated at £7 billion per year [7].

With stroke treatment consuming 2-4 % of global medical costs [1] the development of more effective and cost efficient therapies is essential.

1.1.3. Risk factors

Important risk factors for cardiovascular disease and stroke are; smoking, hypertension, diabetes and obesity. Levels of obesity have more than doubled in England over the last 20 years [8] and almost 40 % of men and over 30 % of women in the UK are now reported to have high blood pressure [4]. While stroke mortality has been falling in recent years, largely accredited to the rising prescription of prophylactic antihypertensive (9 fold since 1990) and lipid lowering drugs such as statins (63 fold increase since 1990) [4], many of the key risk factors for stroke continue to rise. Increasingly inactive lifestyles and an aging population mean that stroke may continue to place a major burden on society. Indeed by 2020 it is predicted that stroke and heart disease will be the leading cause of death and disability worldwide [5].

1.2. The pathophysiology of stroke

To define the pathophysiology of stroke as a reduced blood supply, leading to cellular death and damage due to energy failure, would only be to scratch the surface. Diverse and complex mechanisms underlie the pathology of stroke, with destructive events extending far beyond the initial ischemic damage. At the onset of stroke disturbance of blood flow leads to energy failure and necrotic cell death in the ischemic core [9, 10]. In hypoperfused tissues surrounding the core, excitotoxic events and oxidative stress lead to delayed necrotic and apoptotic cell death [10]. Upon reperfusion, damage may be compounded by enhanced oxidative stress and a cascade of damaging inflammatory processes [9, 11, 12].

1.2.1. Ischemic damage

The brain is particularly sensitive to ischemic injury due to its constant high energy demands; utilizing 20 % of total body oxygen consumption, and 25 % of total body glucose [13]. Neurons are more susceptible to ischemic damage than glial or vascular cells and rapidly become dysfunctional or die [11]. Within 20 seconds of global disruption of blood flow, electroencephalography (EEG) readings disappear in the mammalian brain [14, 15] and during each minute of a large vessel ischemic stroke, it is estimated that 1.9 million neurons, 14 billion synapses, and 12 km of myelinated fibres are destroyed [16].

In the ischemic core (blood flow reduced to less than 12 ml/100 g/min [17]) loss of high energy phosphate compounds leads to rapid energy failure. With cells unable to maintain active transport, tightly controlled ionic gradients are disrupted [18, 19]. As a result there is a huge intra-cellular influx of Na^+ and Ca^{2+}, leading to cell swelling, loss of membrane integrity and necrotic cell death [11].

1.2.2. The concept of the penumbra and stroke progression

The ischemic insult may often be contained and/or short lived however it leads to derangements in cellular functioning that cannot be corrected via normal homeostatic mechanisms [19]

Surrounding the infarct core exists a region of hypoperfused tissue (blood flow approximately 12 - 22 ml/100 g/min [17]) termed the 'penumbra' which although functionally compromised is not yet irreversibly damaged, potentially remaining viable for 16 to 48 h [18]. In this territory the level of ischemia is insufficient to cause immediate cell death but collateral flow maintains neurons at a viable yet critically stressed state [19]. As neurons and glial cells depolarize in the ischemic core, voltage gated Ca^{2+} channels open and glutamate is released into the extracellular space [11, 19, 20]. As a consequence, N-Methyl-D-aspartate (NMDA) receptors and metabotropic glutamate receptors are over activated in the penumbra, leading to increased intra-cellular Ca^{2+} overload and mitochondrial dysfunction [11, 20]. Indeed, it is worth noting that those areas of the brain that are acutely sensitive to ischemic damage such as the hippocampus and neocortex are particularly enriched with excitatory NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [19, 21]. These excitotoxic events also lead to the activation of Ca^{2+} dependent enzymes including; phospholipases, endonucleases and proteases [19, 20]. Activated phospholipases degrade the cell membrane producing free fatty acids including arachidonic acid (AA) [19, 20] thus triggering the production of AA metabolites such as leukotrienes, thromboxane and prostaglandins, which promote platelet aggregation and elicit a cascade of inflammatory events.

The pathological events that occur in the penumbra therefore not only cause necrotic cell death and apoptosis but also generate stress signals, chemokines and damage associated molecular pattern (DAMPs) such as high-mobility group protein B1 (HMGB1),

heat shock protein 60 (HSP60) and β-amyloid (Aβ), thus provoking post-ischemic inflammatory responses [12].

1.2.3. Reperfusion injury

In the absence of reperfusion, neuronal damage in the penumbra may become permanent and indistinguishable from the ischemic core. However where stroke is not immediately fatal, pharmacological treatments, surgical intervention or endogenous mechanisms may restore blood flow to the infracted region. Despite the clear benefit of reperfusion of blood to the ischemic tissue, this can paradoxically serve to exacerbate injury, initiating a cascade of adverse reactions [9, 22]. Indeed in a number of animal models reperfusion may produce infarct sizes larger than those observed when occlusion is permanent [22]. Diverse mechanisms underlie the neuronal damage experienced in the penumbra at reperfusion, including oxidative stress, reactive oxygen species (ROS) production, and with the restoration of blood flow circulating immune cells are recruited to the damaged tissue, initiating a destructive inflammatory cascade. Due to its potentially salvageable nature, the penumbra provides a promising target to reduce neuronal damage and improve functional outcome whilst extending the window of opportunity for therapeutic intervention [23].

1.3 The role of inflammation in stroke

Playing a central role in I/R injury and the pathology of stroke is an aberrant inflammatory response. The neuro-inflammatory response in stroke comprises of a complex cascade of events; characterised by cytokine release, increased expression of cell adhesion molecules, leukocyte recruitment and disruption of the blood-brain barrier (BBB) [24, 25].

A disproportionate inflammatory response can be particularly detrimental in the brain as it lacks robust antioxidant defences [9]. Free radicals and reactive oxygen species (ROS), released by infiltrating inflammatory cells, may be particularly damaging to at risk neurons in the penumbra. Oxidative stress increases BBB permeability and reduces bioavailability of nitric oxide (NO), a potent inhibitor of leukocyte adhesion and platelet aggregation [26]. The accumulation of immune cells in the cerebral microcirculation may also lead to occlusion of the vessel and further increase the potential for ischemic tissue

damage in the “no-reflow” phenomenon [27]. Additionally oedema may oppose arterial influx and potentially bring about further ischemia [28]. Inflammation is thus increasingly being seen as a major contributing factor in the pathogenesis of a number of conditions affecting the brain including; multiple sclerosis, meningitis, acute brain trauma, Alzheimer’s disease, Parkinson’s and stroke [29]. A detailed understanding of the events underscoring inflammation, from activation of resident cells to recruitment of circulating immune cells, amplification and eventual resolution (Figure 1.1), is therefore necessary for the development of therapeutics counteracting inflammatory reactions following stroke.

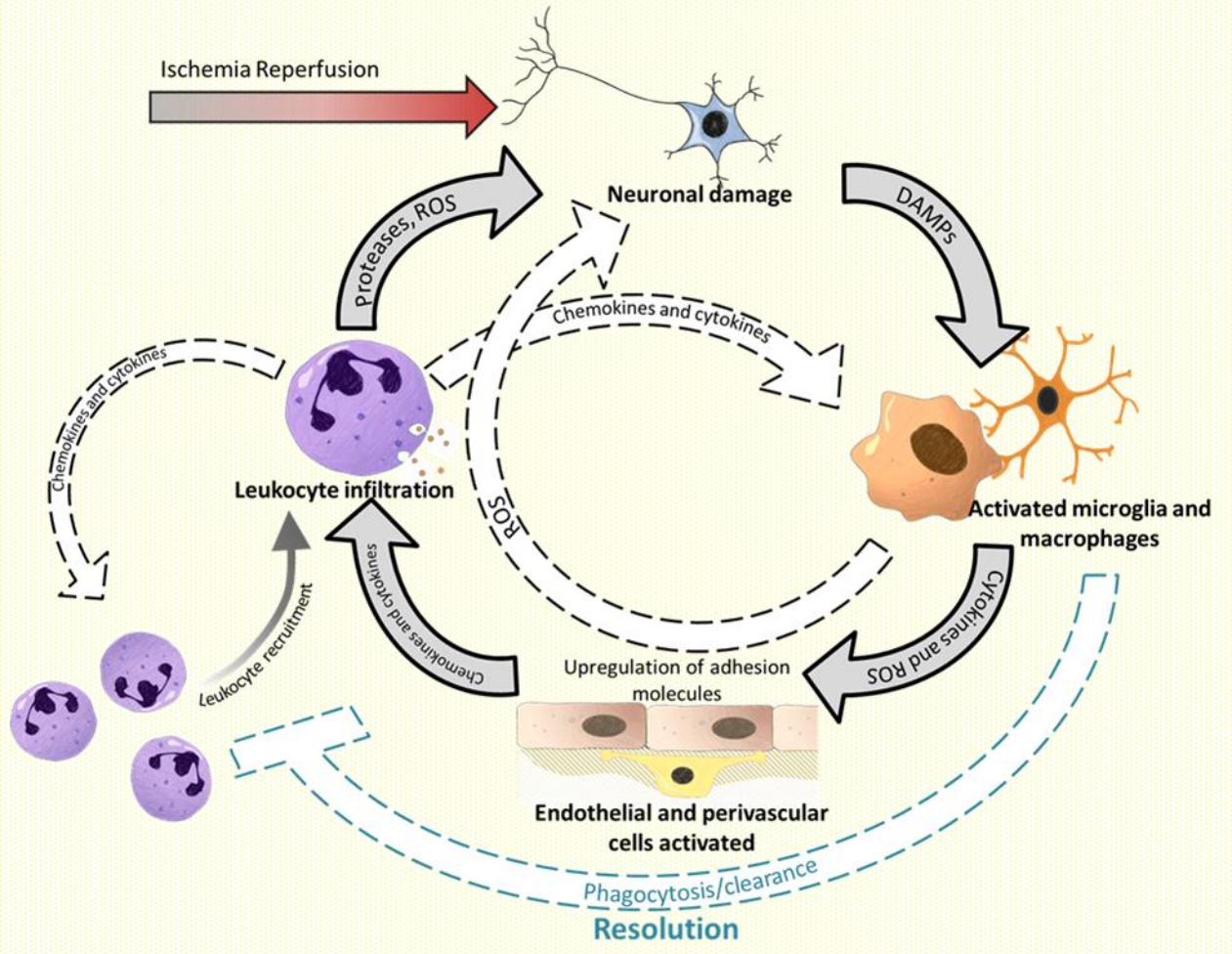

Figure 1.1 ***Inflammation following stroke*** Ischemia reperfusion injury drives neuronal damage, but also sets in motion a damaging cascade of inflammatory events. Damage associated molecular patterns (DAMPS) and stress signals activate brain resident immune cells, which in turn release cytokines and reactive oxygen species (ROS) activating perivascular cells and the endothelium. Leukocyte endothelial interactions may then allow for recruitment of blood born leukocytes which invade the brain parenchyma and release a variety of cytotoxic substances further amplifying neuronal damage. (P. Holloway, *unpublished*)

1.3.1. Resident immune cells

1.3.1.1 Microglia

Microglia are the resident immunocompetent and phagocytic cells of the brain and act as the primary immune defence in the CNS. In their ramified “resting” forms microglia extend branching processes surveying the microenvironment. Sensitive to pathological environmental changes in the brain microglia may be activated within minutes of an ischemic insult [30]. Activated microglia may functionally transform into phagocytes and release a variety of cytotoxic substances such as ROS, proteases and prostanoids whilst also secreting pro-inflammatory cytokines such as Tumour Necrosis Factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 [9, 30, 31]. However microglia may also provide neuroprotection through the production of anti-inflammatory, cytoprotective and neurotrophic molecules such as IL-10, Transforming growth factor beta (TGF-β), insulin-like growth factor I (IGF-1) and brain-derived neurotrophic factor [9, 30]. Microglia may also play a protective role by way of removing extracellular excitotoxins and via phagocytosis of infiltrating neutrophils [32].

1.3.1.2 Astrocytes

These specialised glial cells provide a crucial interplay of neurogenic and vascular elements. So intimate are these functional connections provided by astrocytes that the brain parenchyma and the blood vessels feeding it are no longer considered wholly separate entities. In 2000, the neurovascular unit (NVU) was proposed as a physiological unit [33], an important concept when discussing the pathology of stroke. Astrocyte processes interact with neurons to maintain the synaptic extracellular chemical environment. Astrocyte endfeet also contact endothelial cells (ECs) inducing BBB properties and maintaining BBB integrity whilst also regulating blood flow in response to neuronal energy demand through their release of vaso-active compounds such as prostaglandins (PGE), NO and AA metabolites [34]. Additionally these cells may store and utilise glycogen which can briefly maintain neuronal activity during hypoglycemia and may thus buffer ischemic neuronal damage.

Astrocytes may play both pro and anti-inflammatory roles following stroke [11, 34]. In response to stressors such as ROS, hypoxia and glucose deprivation or inflammatory mediators such as TNF-α and IL1-β, astrocytes may release a variety of pro-

inflammatory compounds, including IL-1β, IL-6, interferon(IFN)-α, and –β, TNF-α; and C-C motif Chemokine ligand 5 (CCL-5) [34, 35]. Astrocytes are also a major source of the potent monocyte chemoattractant (MCP-1) and neutrophil chemoattractant IL-8.

In addition to their pro-inflammatory actions astrocytes may also provide anti-inflammatory protection *via* IL-10, whilst also releasing neuro protective factors and promoting neuronal survival and thus should be considered important modulators of neuroinflammation [35].

A hallmark of CNS injury is the formation of a scar tissue by reactive astrogliosis, 7–10 days following ischemia, astrocytes form scar tissue at the borders of the infarct which persist for life of the patient [34] this scar acts as a neuroprotective barrier to inflammatory cells and infectious agents however this barrier may also prevent neuronal regrowth and recovery [34].

1.3.1.3 Perivascular macrophages

Perivascular macrophages, located between the basement membrane and glia limitans, are continuously replenished by circulating monocytes [11]. While macrophages can exist in a spectrum of different activation states they are typically divided into two phenotypically distinct populations, M1 and M2. M1 macrophages have a pro-inflammatory phenotype releasing cytokines (IL-1β, IL-12, IL-23 and TNF-α), chemokines and ROS [11, 36] driving the infiltration of blood born inflammatory cells. The other class of macrophages, M2, however possess anti-inflammatory actions and release IL-10, TGF-β and IL-1 receptor (IL-1R) antagonists promoting the resolution of inflammation [36].

1.3.1.4 Mast cells

Mast cells are perivascular cells which may rapidly respond to stimuli by releasing cytokines such as TNF-α, histamine, anticoagulants and proteases such as tryptase, Matrix metalloprotease (MMP)2 and MMP9 [11, 37]. Following experimental stroke in rats these cells have been shown to regulate BBB permeability, oedema formation and the intensity of localised neutrophil infiltration [37].

1.3.2. Blood borne immune cells

Blood born immune cells are recruited to the site of brain injury occurs in response to DAMPs and chemokines released from activated mircoglia and perivascular cells and damaged parenchymal cells. Infiltrating cells release additional chemotactic signals and inflammatory mediators and secrete toxic substances thus releasing more DAMPs and serving to amplify the inflammatory cascade.

1.3.2.1 Monocytes

Monocytes are a heterogeneous population with at least two main subtypes being described and classified by their cell surface markers. 'Classical' monocytes produce the anti-inflammatory cytokine IL-10 in response to an inflammatory challenge, whereas pro-inflammatory ‘non-classical’ monocytes release TNF-α and following stroke are recruited to the site of injury [11]. Upon infiltration into the brain parenchyma these cells functionally transform giving rise to macrophages with a ramified morphology indistinguishable from resident reactive microglia. While on the other hand activated microglia may assume an amoeboid phagocytic phenotype similar to infiltrating monocytes [32]. Due to lack of discriminating cellular markers and their functional similarities, distinguishing blood born monocytes from microglia has provided a considerable technical challenge and has confounded a complete understanding of their contribution in post-stroke inflammation. However experiments utilising chimeric mice with GFP bone marrow have shown that while the vast majority of macrophage-like cells in the brain following stroke are resident (GFP-negative) microglial cells, circulating cells are first detected on the cerebral venules at 4 to 6 h and reach peak numbers in the brain at Day 7 [38, 39].

1.3.2.2 Neutrophils

Neutrophils are the most abundant phagocytic leukocytes in the body [40]. These cells harbour cytoplasmic granules and secretory vesicles containing a variety of cytotoxic substances allowing for a rapid and indiscriminate response to pathogens. Rapidly reacting to molecular cues, neutrophils are the earliest leukocyte subtype recruited to the site of tissue damage [12, 41]. Activated neutrophils utilize NADPH oxidase to generate oxygen free radicals and hydrogen peroxide, which are released during respiratory burst, a highly destructive event aimed at the removal of pathogens. Myeloperoxidase (MPO)

is also released from azurophilic granules which catalyses the production of cytotoxic hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2). Additionally MPO may oxidize tyrosine to tyrosyl radical which in turn catalyses the oxidative cross-linking of proteins [42]. In addition to the damaging respiratory burst neutrophils also release proteases MMP8, MMP9, elastase and cathepsins causing significant cellular damage, loss of membrane integrity and disruption of the BBB [11]. Proteins released from neutrophil granules may further contribute to the inflammatory milieu by prompting the adhesion and emigration of inflammatory monocytes [43]. A number of studies have shown inhibition of this neutrophil recruitment to reduce infarct size following stroke [44].

1.4. The leukocyte recruitment cascade

The recruitment of leukocytes to the injured or infected tissue is a central element to the inflammatory response and in numerous studies has been demonstrated to act as a rate limiting step [45]. Therefore targeting this process may provide a key point of intervention in the inflammatory cascade and understanding the mechanisms governing leukocyte recruitment is vital in developing effective therapies. This recruitment process is termed 'the leukocyte adhesion cascade' and comprises a succession of adhesion and activation events, which culminate in the extravasation of leukocytes from the circulation to the site of tissue damage (Figure 1.2).

In the absence of disease the unique characteristics of the cerebral microcirculation and the presence of the BBB confer a vascular phenotype largely impermeable to blood born macromolecules. This coupled with the extremely low basal expression of adhesion molecules in comparison to other vascular beds [46], make the cerebral microvasculature a poor substrate for leukocyte adhesion and extravasation. However a profound increase in the expression of a number of adhesion molecules in the early stages of cerebral inflammation result in the capture of circulating immune cells and their eventual breach of the BBB into the brain parenchyma. These adhesion molecules and their roles in the leukocyte adhesion cascade are summarised in table 1.1.

Table 1.1. Adhesion molecules and their role in leukocyte recruitment

Family	Molecule	Expression pattern	Counter receptor	Role
Selectins	L-Selectin (CD62L)	Leukocytes (constitutively expressed)	SLe^x, P- and E-selectin	Capture and rolling, Leukocyte secondary tethering
	P-Selectin (CD62P)	platelets and ECs (Up-regulated on activation)	PSGL-1, L-selectin	Capture/rolling
	E-selectin (CD62E)	ECs (transcriptionally up-regulated on activation)	PSGL-1, L-selectin, β2 integrins	Rolling/ slow rolling
Mucin-like glycans	PSGL-1 (CD162)	Leukocytes, and activated EC	All selectins	Capture and Rolling
	ESL-1	neutrophils and myeloid cells	E-selectin	Rolling
Integrins	VLA-4 (α4; CD49d/β1; CD29)	Lymphocytes, monocytes	VCAM-1	Rolling, firm adhesion and transmigration
	Mac-1 (αM CD11b/β2; CD18)	Neutrophils, Granulocytes, NK cells, monocytes, MΦ and certain T cells (Constitutive, increased on activation)	ICAM-1	Firm adhesion and transmigration
	LFA-1 (αL; CD11a/β2; CD18)	All leukocytes (constitutive, activatable)	ICAM-1 and ICAM-2	Rolling, arrest and firm adhesion
IgSF	ICAM-1 (CD54)	EC, leukocytes, fibroblasts, epithelial cells (low constitutive, up-regulated by cytokines)	LFA-1, Mac-1	Firm adhesion and transmigration
	ICAM-2 (CD102)	EC, platelets (constitutive)	LFA-1	Firm adhesion
	VCAM-1 (CD106)	EC (low constitutive, up-regulated by cytokines)	VLA-4	Firm adhesion and transmigration
	PECAM-1 (CD31)	EC, platelets, Leukocytes	PECAM-1	Adhesion and transmigration
	JAM-A (CD321)	EC (constitutive)	LFA-1	Transmigration
	JAM-B (CD322)	EC (constitutive)	VLA-4, JAM-C	Transmigration
	JAM-C (CD323)	EC (constitutive)	JAM-B	Transmigration

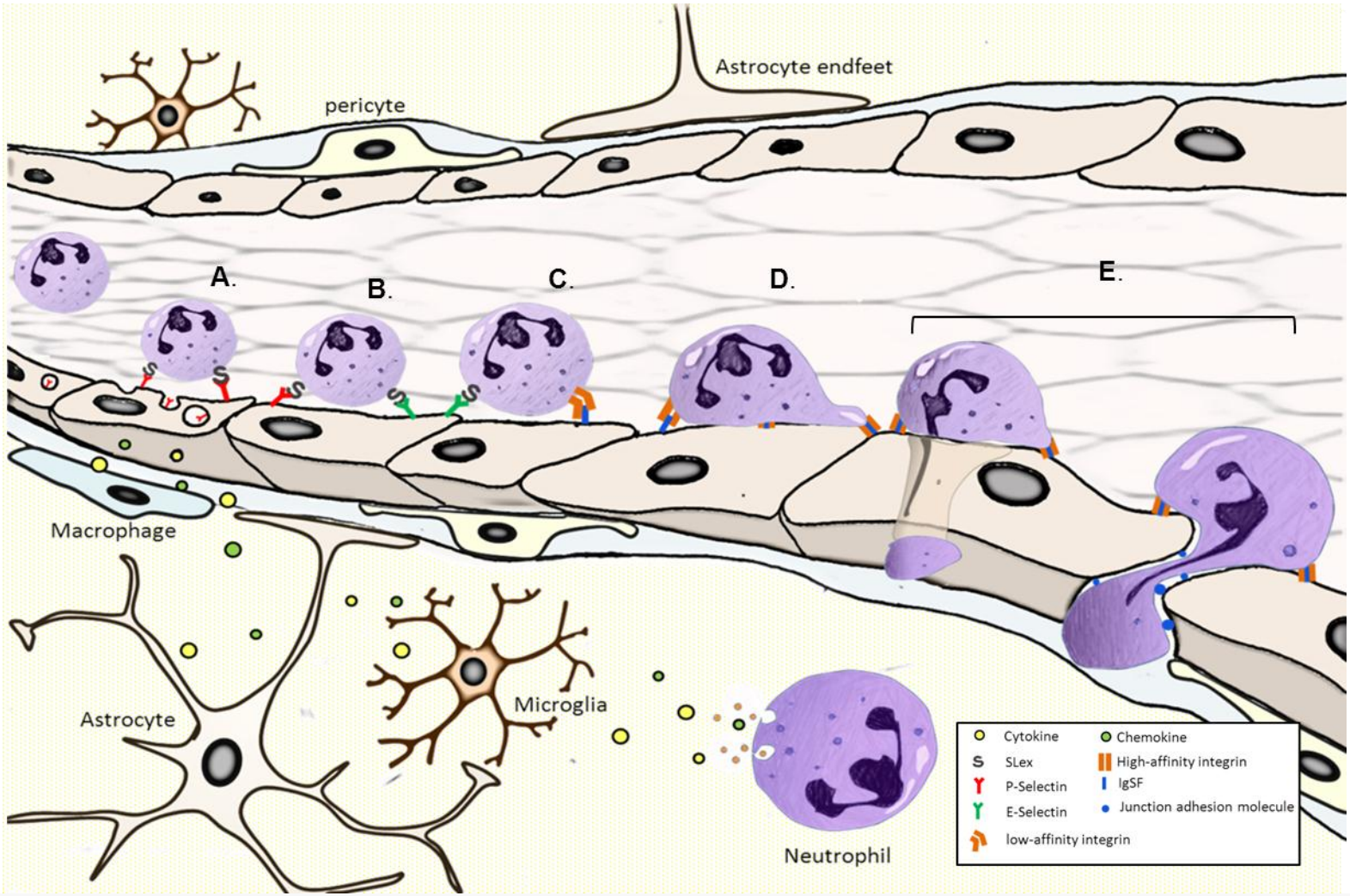

Figure 1.2. ***The sequential steps of the leukocyte adhesion cascade***. Factors released from activated or damaged resident cells cause endothelial cell activation and selectin expression. A) Capture and rolling of the leukocytes from the blood stream is mediated by endothelial selectins binding to their leukocyte mucin-like ligands. B) Subsequent Slow rolling involves the synergistic actions of E selectin to mucin binding and Integrin binding to their IgSF ligands. C) Arrest occurs upon chemokine mediated transformation of integrins to their high affinity conformation. Integrin bond strengthening and signalling mediate the recruitment of additional adhesion proteins. Mac1 – ICAM1 interactions then drive D) Crawling across the luminal surface toward preferred sites of transmigration often at sites of matrix protein low expression regions. (E) The Extravasation or diapedesis of the leukocyte may then occur either through a trans-cellular or para-cellular route. (F) Once in the brain parenchymal leukocytes release a variety of cytotoxic and pro-inflammatory substances contributing to neuronal damage and amplifying the local inflammatory response. (P. Holloway, *unpublished*)

1.4.1 Selectin-glycan interactions: Capture and rolling

The initial capture and rolling of leukocytes is mediated by the interactions of the selectins with their ligands. The selectins belong to the calcium-dependent, type I trans-membrane glycoprotein family of adhesion molecules which comprises of P-selectin, L-selectin, and E-selectin, named with relation to the cell type on which they were originally identified (Platelet, Leukocyte and Endothelium). P and E-selectin are expressed by the inflamed endothelial cells with P-selectin also being expressed by activated platelets, whereas L-selectin is constitutively expressed by most leukocytes [47]. All three selectins have a similar basic structure containing: a short cytoplasmic tail, single trans-membrane domain, a variable number of consensus repeats (CRs), an epidermal growth factor–like domain and an extracellular N-terminal lectin-like ligand binding domain [47, 48]. The CRs provide a length to the selectins necessary to extend beyond the negatively charged glycocalyx of the endothelium and permit leukocyte-endothelial cell interactions [48]. Variations between the lectin-like domains of the selectins confer selectivity for their mucin-like glycoprotein ligands such as P-selectin glycoprotein ligand-1 (PSGL-1), CD34, CD44 and E-selectin ligand-1 (ESL-1) [47]. Although a number of these mucin-like ligands are constitutively expressed on the leukocyte cell surface, selectin binding is glycosylation dependant and all known selectin ligands require enzymatic modification by a sialyltransferase and fucosyltransferase VII [47]. For selectins to be able to bind a ligand the minimal motif of a sialyl-Lewis X tetrasaccharide (SLe^x) is required. Further modifications such as sulphation are required to optimize binding to specific selectin isotopes [48, 49]. Selectin-glycan interactions are able to mediate capture and rolling of leukocytes under the conditions of blood flow due to the transient nature of these bonds, displaying high on- and off-rates. The shear stress exerted by blood flow is in fact essential to selectin binding and under static conditions the importance of selectins in leukocyte recruitment is greatly reduced, as capture and rolling may be bypassed [50].

P-selectin is the earliest adhesion molecule to be up-regulated on the luminal-endothelial surface in response to pro-inflammatory signals or noxious stimuli. Released from Weibel–Palade bodies (WPB) in endothelial cells and α-granules in platelets, the up-regulation of P-selectin is rapid and occurs independently of transcription [46, 49]. In rats, up regulation of P-selectin has been observed as early as 15 min following

ischemic insult [46]. This increase in P-selectin expression mediates the initial capture or “tethering” and rolling of leukocytes.

Subsequent to capture the transient formation of selectin–glycan bonds at the leading edge of the leukocyte and the breaking of these bonds at the trailing end allows for the rolling of the leukocyte along the endothelium. E-selectin is synthesized *de novo* in response to mediators such as IL-1β, TNF-α and LPS and expression has been observed on the endothelial cell luminal surface within 2 h of ischemic stroke in rats [51]. Despite this delayed expression of E-selectin its presence on the endothelium temporally overlaps that of P-selectin acting synergistically to decelerate rolling leukocytes. In addition L-selectin, *via* interactions with PSGL-1, may mediate leukocyte–leukocyte interactions facilitating secondary leukocyte capture. This allows leukocytes that do not express PSGL-1 variants appropriate for E-selectin or P-selectin binding, to reach sites of inflammation [47].

1.4.2 Integrins and IgSF: Slow rolling and arrest

In conjunction with selectin-mucin like glycan interactions, integrins binding to their immunoglobulin superfamily (IgSF) ligands enable the slow rolling and adhesion of leukocytes in the cerebral microvasculature within the first few hours of CNS injury [52]. The integrins are a large family of membrane spanning glycoprotein adhesion molecules expressed predominantly by leukocytes. Integrins are hetero-dimmers consisting of one α-subunit non-covalently bound to a β-subunit The integrin subfamilies found to be most relevant to leukocyte recruitment are the β1-integrin and β2-integrins [47, 53]. The immunological importance of the β2-integrins is illustrated by the phenotype of patients with leukocyte adhesion deficiency type 1 (LAD-1). These patients lack a functional β2-integrin gene and consequently display impaired wound healing and severe infectious complications [48].

Members of the IgSF expressed on the endothelial surface serve as ligands for the leukocyte integrins. In the cerebral microcirculation these include; intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1).

The process of leukocyte arrest from rolling is also mediated by integrin-IgSF interactions and is rapidly initiated in response to selectin mediated signalling and chemokine activation [47, 48, 53]. Chemokines produced by endothelial cells, platelets and perivascular cells may be secreted to the luminal surface and bound to endothelial extracellular matrix components such as heparin sulphate and glycosaminoglycans, protecting them from proteolytic cleavage and also serving to localise the chemotactic signal [47]. Detection of these chemokines by leukocytes triggers intracellular signalling and a conformational change in the integrin cytoplasmic domain [54]. This change propagates to the extracellular domain inducing a transformation from a bent low-affinity form to the extended intermediate and high-affinity conformations [55]. These integrin activation events enable leukocyte arrest on the endothelial surface [54]. Integrins on the leukocyte surface may exist in a range of these conformational states at any one time, thus allowing for a highly dynamic control of integrin affinity and leukocyte arrest [47, 54].

Experiments using genetic knockout mice have provided strong evidence for the critical role of the integrins in the inflammatory response of the cerebral microcirculation following acute cerebral ischemia. In studies by Connolly *et al.* [44] knockout mice for the integrin ligand ICAM-1 showed a significant decrease in leukocyte extravasation following MCAo which translated into a 75 % decrease in infarct volume and a 3.7-fold decrease in mortality. Additionally mice null for the integrin β subunit CD18 subjected to transient focal cerebral ischemia, displayed a 53 % decrease in infarct volume compared to $CD18^{+/+}$ mice [56]. Furthermore this effect was only observed in the presence of reperfusion.

1.4.3 Crawling and transmigration

Before crossing the walls of post capillary venules, neutrophils and monocytes have been observed to “crawl” along the endothelium seeking out preferred sites for diapedesis following chemotactic gradients [47].

Following successful recruitment to the EC luminal surface, leukocytes must then breach and transmigrate across the endothelium. Leukocytes have been reported to migrate either in a para-cellular fashion, through reorganisation and utilisation of inter-endothelial junction molecules or by a trans-cellular route involving the formation of vesiculo-vacuolar organelles consisting of ICAM-1-containing caveolae [57] In the

microvasculature of the brain, the ability of leukocytes to utilise the para-cellular route is limited due to the highly-organized inter-endothelial tight junctions of the BBB. In inflammatory pathology of the CNS, and also in the similarly immune privileged retina, the up-regulation of leukocyte trafficking has been widely documented as occurring preferentially *via* trans-cellular diapedesis pathways [58-60].

Subsequent to infiltration of the endothelial cell barrier, migrating immune cells must then penetrate the endothelial basement membrane (BM) followed by the glia limitans with its distinct basement membrane [61]. Whereas extravasation through the vascular endothelial-cell barrier may be rapid (~2–5 min), penetrating the BM takes place over a considerably longer period (5-15 min) [47]. While endothelium and endothelial BM penetration is understood to be mediated through the actions of the β_1-integrins, the mechanisms that drive the leukocyte migration across the parenchymal basement membrane are not as well characterised. However MMP–2 and MMP-9 cleavage of dystoglycan, a transmembrane protein with the role of anchoring the astrocyte endfeet to the parenchymal BM, has been shown to play an important role in this process [62]. Furthermore tissue plasminogen activator (tPA) has also recently been suggested to possibly play a role in this leukocyte migration through the parenchymal BM [60].

Upon migration into the cerebral parenchyma leukocytes may release an array of cytotoxic agents including MMPs and a further release of cytokines such as IL-1β [25, 63-65]. Indeed studies have demonstrated a correlation between increasing levels of the cytokine IL-1β and an amplified infarct severity following ischemic stroke [66].

The detrimental effects of leukocyte recruitment following stroke have been supported by several reports demonstrating reduced neurological injury in rat models of stroke, through therapeutic targeting of various adhesion molecules [67-69]. Further to this, animals with deficiency in adhesion molecules display reduced infarct volume after transient focal cerebral ischemia [12] and reduced neuronal damage in experimental autoimmune encephalomyelitis [70].

1.4.4. Time course of inflammation and stroke progression

The temporal progression of the post-ischemic inflammatory response is complex, with immune cells differentially and synergistically contributing to the developing brain injury [32]. Although the term inflammatory cascade suggests a linear sequence of events, the cellular and humoral inflammatory response following stroke in fact consists of a number of feedback loops and self-reinforcing processes with temporal overlaps leading to an amplification of the response (Figure 1.1), pushing forward a general progression of events. Figure 1.3 demonstrates the temporal pattern of chemokine and chemotactic signal release along with the relative activation and recruitment of immune cells in the brain following stroke [32, 71-77].

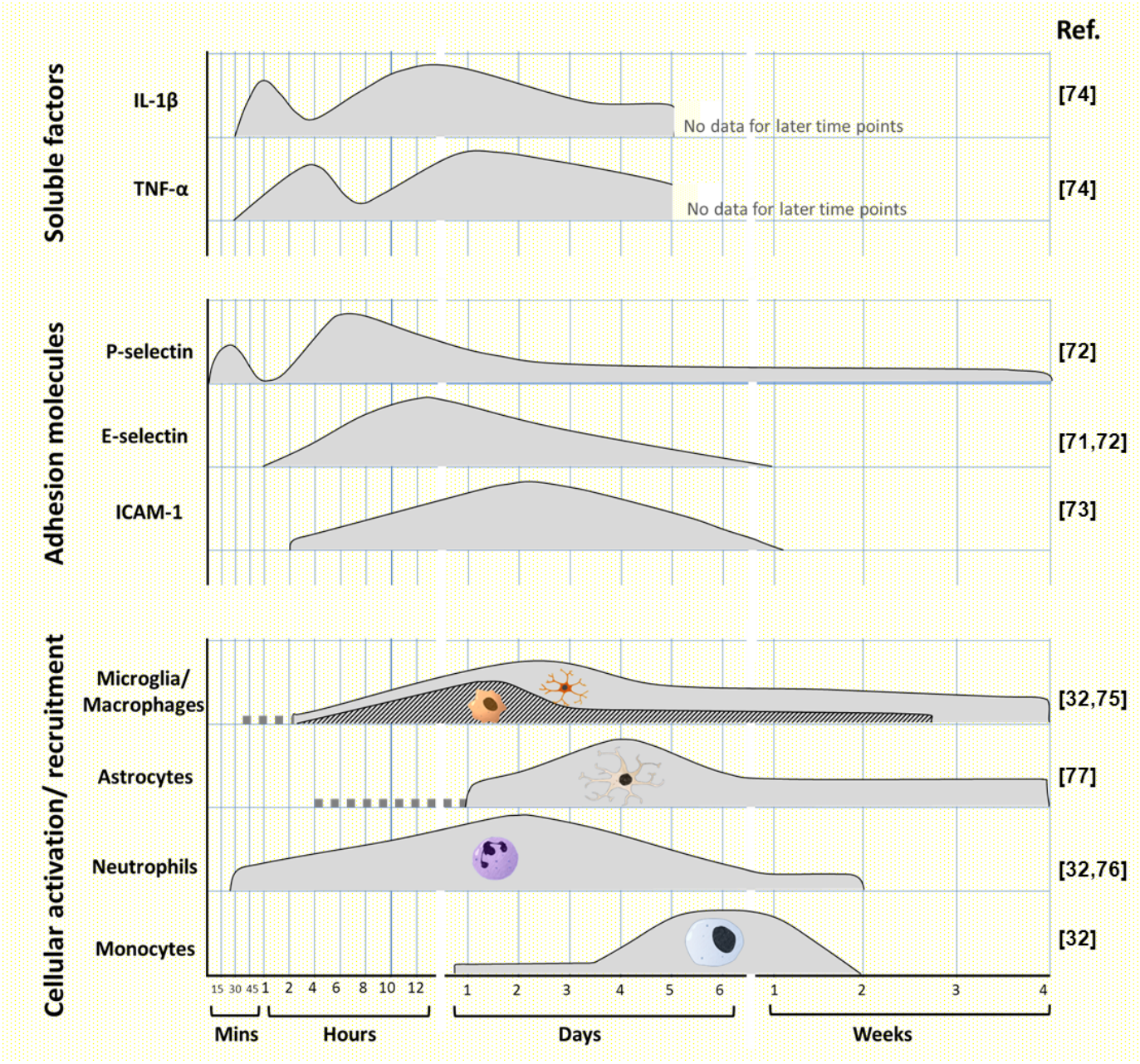

Figure 1.3 ***Time course of the inflammatory response following stroke.*** within minutes following stroke pre-formed TNF-α and IL-1β are released from resident activated cells with a second peak in these cytokines being from their release by invading immune cells and resident cell *de novo* synthesis. In response to pro-inflammatory signals endothelial adhesion molecules are up regulated, with P-selectin exocytosed from WPBs being the most rapid, with a second peak resulting from the de novo synthesis of this adhesion molecule. Microglia, can be activated (dashed lines) within minutes following stroke and may functionally transform into macrophages releasing a variety of cytokines and chemokines. While astrocyte activation and up regulation is more delayed. Neutrophils are the first blood born immune cells to arrive in the brain where they release a number of cytotoxic compounds. The arrival of monocytes is more delayed, with those giving rise to an M1 phenotype enhancing inflammatory responses but with the arrival of M2 cells making the beginnings of inflammatory resolution. Time points are taken from experimental findings in rats due to the dearth of data in human or murine stroke (references given to the right of each row). Existing data from both human and mouse studies have however been found to correlate well. P Holloway, *unpublished*.

1.4.5. Inflammatory resolution

Whist pro-inflammatory mediators orchestrate and augment the acute-phase of the inflammatory response this also initiates the release of a number of "pro-resolution" factors that promote catabasis (the return to homeostasis). Upon the arrival of macrophages and monocytes, signals are released that inhibit further leukocyte trafficking, promote the normalization of chemokine gradients and the return to a non-inflammatory environment [78]. Key players in this process include lipoxins, D series prostaglandins and resolvins [78]. Additionally alpha-melanocyte stimulating hormone (α-MSH) has been suggested as acting as an endogenous factor promoting resolution. Hijacking such endogenous systems for the resolution inflammation may prove to be extremely fruitful in halting a pathological inflammatory reaction whist retaining processes for the removal of dead cells and initiation of tissue repair.

1.5. Animal models of stroke

Due to the complex pathology of stroke and the elaborate interplay between numerous cell types which underlies the inflammatory process the disease cannot yet be adequately replicated *in vitro*. Thus for the investigation of prospective therapeutics, animal models must be utilised in combination with *in vitro* studies. Whilst non-human primates may have a brain structure and cerebral vasculature most similar to humans, ethical consideration and cost mean that experiments on primates represent only a small proportion of animals used in stroke research [79]. Due to the availability of genetically manipulated animals, rodents are currently the preferred model organisms [80]. However, generating a reproducible rodent model which accurately mimics the human condition has proven to be challenging [81].

1.5.1. Current models

There are currently less than 10 experimental models of focal stroke [81]. In such models ischemia may be produced by either introduction of a filament into the middle cerebral artery, Macrosphere embolization or photochemical induction of a common carotid artery thrombosis. Out of these middle cerebral artery occlusion (MCAo) is the

most reproducible [81]. However focal stroke models produce infarct sizes far larger than those observed in non-fatal human stroke with rodent models displaying a peak infarct volume occupying 20-40 % of the brain [82] whereas CT scans reveal this to be on average only 4.4 % in humans [83].

For the production of global ischemic stroke models there are four major arteries which may be occluded in combination, with the minimum being occlusion of both common carotid arteries (BCCAo) [80]. Such methods however require deep anaesthesia and invasive surgery, which in itself may affect physiology. Additionally animals vary in their susceptibility to ischemic damage, merely amongst rodents there exists significant variation in the wiring of major vessels supplying the brain [84], gerbils for instance do not possess a Circle of Willis [85]. Other global stroke models include the application of a neck tourniquet or the induction of cardiac arrest by administration of cold potassium chloride (KCl) [86, 87].

1.5.2. Comparison of human and rodent brain

The use of studies in mice have enabled a great insight into the pathophysiology of human stroke, however between 65 and 75 million years of evolution separate these species [88] and as such there can be huge discrepancies in physiology that must be understood before meaningful data can be extrapolated. Despite the obvious disparity in size there are a number of differences between the human and rodent brain that limit the translation of results obtained. Humans have gyrencephalic brains and relatively far greater amounts of white matter than found in the lissencephalic rodent brain. Despite white matter ischemia often being more severe than grey in humans, due to lower collateral flow, studies of white matter damage are to some extent limited in rodent models [89]. As such, in comparison to grey matter pathophysiology, the current understanding of mechanisms underlying white matter damage and the role of oligodendrocytes in I/R remains relatively incomplete [89].

In addition to macroscopic differences between human and rodent brains, cellular elements and their relative abundance also contrast. Throughout evolution the number and structural complexity of astrocytes have increased to support more sophisticated neuronal circuitry [34]. As such humans not only have larger, more functionally diverse astrocytes but the astrocytes to neuron ratio found in the human cortex is higher, 1.5:1

compared with that found in rodent cortex, 1:3 [34, 90, 91]. Due to their intimate involvement in blood flow regulation, anti-oxidative capabilities and pro-and-anti-inflammatory potential, the quantitative and qualitative differences in human and rodent astrocytes may impact stroke pathophysiology [34]. As such this is one of the many aspects that should be taken into consideration when translating stroke therapies to humans.

1.5.3. Comparison of human and rodent innate immune response

Due to the different pathological pressures placed on humans and mice differences in both the components and functioning of each species immune systems have developed. Whilst the overall organisation of the immune system is largely similar between mice and humans, it should not be taken for granted that anti-inflammatory therapies effective in mice will have the same effect in humans.

Whereas the human immune system is strongly supported by a predominance of circulating neutrophils which account for 50-70 % of all leukocytes, neutrophils only make up 10-25 % in mice who rely on a larger population of lymphocytes (75–90 %) which in humans is between 30 and 50 % [88].

Not only do the relative populations of cellular elements differ between human and murine immune anatomy but also the molecular and chemical features vary. A number of immune-related molecules including IL-8 receptor alpha (CXCR1), IL-8, neutrophil-activating peptide-2 (CXCL7), CCL18, monocyte chemoattractant protein (MCP)-4, and eotaxin-2/3 (CCL24/CCL26) have been found to be expressed in humans but not mice [88, 92, 93]. On the other hand CCL6, macrophage inflammatory protein-1 gamma (CCL9), and MCP-5 are present in mice but not in humans [88, 93]. Also in mice, P-selectin may be induced by TNF-α and lipopolysaccharide (LPS) *via* an NF-κβ regulated promoter element, however in humans P-selectin is not under the control of NF-κβ and expression levels are unaffected by TNF-α and LPS [94]. E-selectin in humans is strongly up-regulated in response to TNF-α perhaps suggesting a more prominent role in leukocyte recruitment than in mice [88].

The impact of these differences must therefore be considered when translating findings between species and in the identification of drug targets. Indeed subtle differences in the

melanocortin receptor system exists between species, the main macrophage MC receptor in mice is MC_3 where as in humans it is MC_1 [95]. Such discrepancies must therefore be taken into account when analysing data from murine models.

1.5.4 Efficiently utilising rodent models

Considering the above differences in physiology and the various limitations of the disease models efficiently using currently available models and appropriate interpretation of data is essential for an effective assessment of a candidate therapy. One pitfall in current animal research into potential treatments for stroke is the failure to address the issue of co-morbidities. Experiments are largely carried out on animals which are otherwise healthy where as in the clinic large numbers of patients present with a variety of co-morbidities such as diabetes and obesity [4]. Additionally time of administration of test compounds should be carefully assessed, as patients are often unable to receive treatments until several hours following stroke. Indeed due to the hugely variable nature of stroke it is essential to test compounds in the context of varying lengths and severity of ischemia and at differing times of reperfusion.

Whist no singular perfect model exists, a combination of *in vitro* techniques utilising human cells and effective use of different animal models is required for the exploration of potential stroke treatments.

1.6. Therapeutic strategies for the treatment of stroke

1.6.1. The challenge of developing an effective therapeutic for stroke

Despite the huge impact of stroke, current therapeutic options remain extremely limited. Surgical interventions include endarterectomy and angioplasty of the carotid artery, to mechanically clear the occluded vessel. However i.v administration of tissue plasminogen activator (t-PA) within 3 h of stroke onset remains the only approved pharmacological treatment for acute ischemic stroke in the western world. Recent studies have suggested the therapeutic time window of t-PA may be extended to 4.5 or even 9 h following stroke onset [96], although this is still under debate. Nevertheless t-PA therapy is far from adequate, with clinical audits having shown only 2 % of all patients to be eligible [97] and 6.4 % of patients developing substantial brain

haemorrhage as a complication [98, 99]. t-PA has also been demonstrated to be a significant factor in causing excitotoxic injury, promoting NMDA-mediated cell death [100] and in activating MMP-9; potentially enhancing BBB disruption, increasing cerebral inflammation and further aggravating any hemorrhagic tendencies [101].

The paucity of effective treatments has driven research into a plethora of protective compounds. Prior to 2006, a total of 1,082 prospective neuroprotective interventions have been published on the treatment of stroke [102]. The translation of these hopefuls to the clinic has however been extremely disappointing.

Neuro-restorative therapies and treatments promoting repair and recovery will not be reviewed here however for informative reviews on the use of neurogenic factors and stem cell therapies in stroke please see Chen & Chopp, 2006 [103] and Smith & Gavins, 2012 [104].

1.6.2. Promoting reperfusion

A number of novel thrombolytic compounds have been investigated (summarised in table 1.2) yet the risk of haemorrhage remains a significant obstacle when entering clinical trials. The most promising of these thrombolytics, Desmoteplase, a component of vampire bat saliva, has been shown to reduce this risk [105, 106]. Whilst these thrombolytics are targeted toward the opening of the occluded vessel and essential restoration of blood flow to the ischemic tissue, such singular treatments fail to adequately address the multiple pathogenic events that occur subsequent to reperfusion. Thus there is a great need for additional therapeutics targeted toward the treatment of ischemia I/R injury and a resolution of inflammation in the brain.

Table 1.2 ***A non-exhaustive list of therapeutic strategies and compounds that have entered clinical trials for stroke therapy*****.**

Therapeutic goal	Compound	Mechanism of action	Outcome of clinical trials	Ref.
Prevention	E-selectin	Induction of mucosal tolerance to E-selectin	Phase II study terminated	
	Ximelagatran	Thrombin inhibitor intended as a preventative of stroke to replace aspirin or warfarin	SPORTIF III and V phase III clinical trials showed high levels of Hepatotoxicity	[107]
	Perindopril	Angiotensin-converting enzyme (ACE) inhibitor	Perindopril group had a 28% relative risk reduction. Trials on-going.	
Thrombolysis and restoration of blood flow	rtPA (alteplase)	Thrombolytic serine protease which cleaves plasminogen to plasmin.	Trials successful, FDA approved. Currently in use.	[108]
	Streptokinase	A serine protease which cleaves plasminogen to plasmin	Phase III clinical trial ended due to increased risk of intra-cerebral haemorrhage	[109]
	Desmoteplase	Plasminogen activator derived from the vampire bat saliva	In 2009, phase III clinical trials began. Outcome awaited.	[105]
	Ancrod (Viprinex)	A fibrinogenolytic agent purified from pit viper venom	Phase III ESTAT trial showed Increased risk of haemorrhage	
Protection from Excitotoxicity	Clomethiazole	Positive allosteric modulator at the GABAa receptor	Clomethiazole acute stroke study (CLASS) showed no benefit	[110]
	Aptiganel Cerestat®	Glutamate antagonist, non-competitive NMDA channel blocker	Phase III trial halted due to lack of therapeutic impact.	
Protection from free-radical and ROS damage	Cerovive (NXY-059)	Nitrone-based free radical trapping agent	Promising initial results, but phase II trial showed no improvement.	[111]
	Edaravone	Free radical scavenger	Approved in Japan since 2001 with modest to large improvements in functional outcome. However remains under clinical investigation in other countries. Phase II trials showed improved functional outcome	[96, 112]
Anti-inflammatory protection	Enlimomab	Anti-ICAM-1 monoclonal antibody	Phase III trials ended due to enhanced neurological disability.	[113]
	UK-279, 276	Selective antagonist of CD11b	Phase II trials found no overall benefit.	[52]
	Minocycline	Inhibits apoptosis by attenuation of TNF-alpha. Also suggested to inhibit NF-κB translocation in microglia.	2007 pilot study showed improved outcome. However confirmation by a larger controlled double-blinded trial still required.	[114]
	Acetaminophen (Paracetamol)	Anti-inflammatory and anti-pyretic *via* inhibition of cyclo-oxygenase.	Phase I clinical trials showed no benefit. Treatment also carries risk of liver damage due to the high doses used.	[115]
	rIL-1RA	Interleukin-1 receptor antagonist	Phase II trials promising	[116]
	LeukArrest	IgG1 antibody to human CD18	Phase III trials showed no benefit.	[113]

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1.6.3. Neuroprotection

With a more detailed understanding of the pathophysiology of stroke has also come an appreciation that simply re-introducing blood flow may not be sufficient to halt stroke progression, however the damaging events triggered by ischemia might also provide opportunities for intervention, which could extend the therapeutic window. Neuroprotective strategies aim to rescue ischemic neurons from irreversible damage by targeting key components of the biochemical ischemic cascade.

Some of the most intensively studied neuroprotective candidates are the glutamate receptor antagonists [117]. Glutamate, the most abundant excitatory neurotransmitter in the CNS, plays a major role in post stroke excitotoxicity [117]. As such blocking NMDA, AMPA, KA, and metabotropic glutamate receptors has provided the therapeutic rational for a number of compounds however lack of efficacy along with potential psychiatric and cardiovascular side effects have limited their translation to the clinic [118, 119]. Furthermore such therapies also fail to act upon non-neuronal cell types such as astrocytes, microglia or vascular cells of the ischemic brain.

Failure of glutamate receptor targeted therapies emphasises the notion that NMDA-AMPA pathways make up only a subset of reactions leading to ionic imbalance. As such a number ion channel modulators have also been investigated for the treatment of stroke, including calcium (Nimodipine), sodium (Fosphenytoin) and potassium (BMS-204352) channel blockers. However to date clinical trials of these therapeutics have all terminated due to failure to show benefit [117, 119] most likely due to the diversity of mechanisms that underlie cellular damage in cerebral I/R injury.

Another strategy to achieve neuroprotection has been to quench the damaging effects of ROS using free radical scavengers. Such treatments have the benefit of protecting both cells of the brain parenchyma and the vasculature. While a number of trials with free radical scavengers have resulted in limited success, Edaravone, a low-molecular-weight antioxidant, has been approved in Japan for use as a neurovascular protective agent in ischemic stroke since 2001 (although in other countries it still remains under clinical investigation) [96]. Aside from protection against free radical induced damage Edaravone has also been shown to reduce brain oedema and high-mobility group box 1

(HMGB1) induced neuronal cell death [96], thus highlighting the importance of targeting multiple aspects of stroke pathology to achieve effective neuro-protection.

1.6.4 Anti-inflammatory treatment

Inflammatory processes following stroke cause a significant amplification of cellular damage, contributing at multiple levels to a highly cytotoxic environment and providing a significant source of ROS. As such, inhibiting this damaging inflammatory response has provided a promising therapeutic rational to achieve neuro-protection following stroke.

Anti-inflammatory therapeutic strategies for stroke have received considerable attention due to a number of potential benefits; Anti-inflammatory treatments have an extended therapeutic window, and could be administered to patients outside the time window for thrombolysis. Indeed due to the major role of inflammation in I/R injury, anti-inflammatory treatments would provide a fitting complement to current therapies, which promote reperfusion. Furthermore, anti-inflammatory strategies have also proven beneficial in haemorrhagic stroke, potentially enabling immediate treatment before imaging and exclusion of haemorrhagic stroke.

A major strategy in developing anti-inflammatory treatments has been to block pro-inflammatory mediators or adhesion molecules. Pharmacological blockade of different integrins using monoclonal antibodies has shown neuroprotection in a number of models of stroke [52]. Despite these initial promising studies, such treatments have however, failed to show efficacy in clinical trials. Failure of the anti-ICAM-1, Enlimomab, in 2001, was attributed to patients developing adverse immune responses to the mouse antibody. Furthermore integrins are also known to be involved in a number of other cellular processes such as proliferation and apoptosis and the full extent of integrin signalling function is still to be fully characterized [52, 55]. The most probable limiting factor to such therapies however, is that the inflammatory response is a robust system propagated by diverse and redundant pathways, and as such cannot be effectively subdued by neutralising just a single component. As such, harnessing endogenous mechanisms for the resolution of inflammation, which impact upon multiple elements of the inflammatory response may prove to be a fruitful strategy.

The melanocortin system has been demonstrated to be an endogenous system to counteract inflammation allowing a modulation rather than an indiscriminate termination of the inflammatory response [120].

1.7. The melanocortin receptor system

The melanocortin peptides have been shown to exert a multitude of effects on numerous physiological functions, including regulatory roles in skin pigmentation [121], energy metabolism [122], memory [123], thermoregulation [124, 125], nociception [126] and inflammation [127, 128]. Thus the melanocortin system is increasingly being recognised as a promising target for pharmacological intervention in a wide variety of disorders [129].

The well documented potent anti-inflammatory and neuro-regenerative circuits centred on the melanocortins receptors make them a particularly attractive target for the development of novel therapeutics for stroke.

1.7.1. Melanocortin peptide production

Adrenocorticotrophic hormone (ACTH) and α, β and γ melanocyte-stimulating hormones (MSH) are collectively known as the melanocortin peptides [130]. These peptides are derived from the post-translational processing of a large pre-cursor molecule known as the pro-opiomelanocortin protein (POMC) which is 241 amino acids in humans [131] and 209 in mouse and rat [132, 133]. POMC comprises of three main domains; the N-terminal “pro-γ-MSH” containing the sequence corresponding to γ-MSH; the central “ACTH domain”, covering both the ACTH and α-MSH regions and the “C-terminal β-lipotropin”, which provides the β-MSH peptide (Figure 1.4).

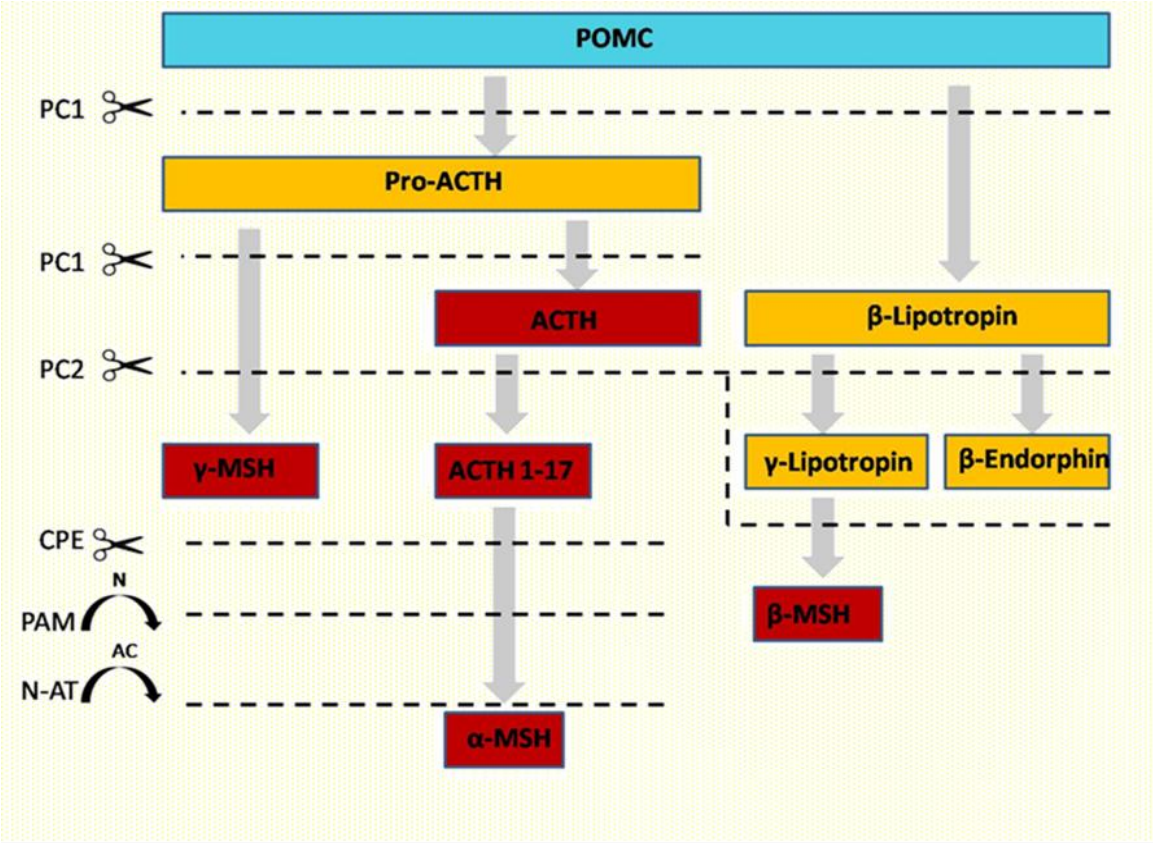

Figure 1.4 ***The post-translational processing of POMC*** Pro-opiomelanocortin protein is sequentially cleaved pro-hormone convertases and carboxypeptidase E to form the melanocortin peptides ACTH, β-MSH and γ-MSH. α-MSH is produced by amidation and acetylation of ACTH 1-17.

A number of enzymes including pro-hormone convertases 1 and 2 (PC1 and PC2) and carboxypeptidase E mediate the processing of POMC [134, 135]. PC1 may cleave POMC to produce pro-$ACTH_{1–39}$ and β-lipotropin. Further PC1 mediated processing of pro-$ACTH_{1–39}$ may then lead to the formation of $ACTH_{1–39}$ [136]. PC2 generates γ-MSH, $ACTH_{1–17}$ and β-endorphin [136]. $ACTH_{1–17}$ may then be cleaved by carboxypeptidase E, amidated by Peptidyl-glycine α-amidating monooxygenase (PAM) and acetylated by n-acetyltransferase (N-AT) to form α-MSH [130]. This processing of the POMC protein is tissue-specific and extremely versatile; enabling the regulation of melanocortin activity through altering their ratios and composition in response to hormonal, neuronal and environmental stimuli [134, 137]. For example in the corticotrophs of the anterior pituitary, PC1 is the predominate enzyme thus POMC processing biases ACTH

production. On the other hand in the pituitary pars intermedia, PC2 expression accounts for α-MSH and β-endorphin generation [120, 138]. Further enzymatic modifications of the POMC derived peptides such as phosphorylation, glycosylation and methylation have also been found to be of particular importance in their biological activity, regulation and binding specificity [139].

The melanocortin peptides all share an invariant sequence of four amino acids, His-Phe-Arg-Trp (HFRW), corresponding to the minimum sequence required for receptor binding and being a major determinant of their biological activity [120, 130]. This discovery has led to the production of a number of potent and selective melanocortin agonists and antagonists (summarised in table 1.3). Such selective melanocortin active peptides have proved to be extremely useful tools in elucidating the roles of specific melanocortin receptor subtypes.

More recently the C-terminal tripeptide sequence of α-MSH, Lysine-Proline-Valine (KPV), has also been shown to exert anti-inflammatory actions, although these effects were found to be independent of receptor binding [140]. The sequences of key melanocortin compounds and the two distinct pharmacophores are illustrated in table 1.4.

Table 1.3: ***A non-exhaustive list of melanocortin based compounds; characterisation of receptor binding properties and relevant pre-clinical data***

Class	Hormone/ Compound	Agonist (+) Antagonist (-)	Relative affinity for MC subtype	Pre-clinical data	refs
Endogenous peptides	α-MSH	+	MC1>3>>4>>5	↓ TNF-α and IL1-β expression following cerebral I/R in mice. improved recovery in CNS-lesioned rats	[134, 141-143]
	β-MSH	+	MC1>3>4>>5	↓NF-kB activation and ↓iNOS activity in LPS induced brain inflammation	[141, 144]
	γ-MSH	+	MC1>3>>4=5	↓ NO production in mouse model of acute brain inflammation	[141, 144]
	ACTH	+	MC1>3>4>5	↓TNF-α, IL-6 and NO in murine microglial cell lines challenged with LPS. Currently in phase III trials for haemorrhagic shock.	[120, 134]
	Agouti signalling protein (ASP)	-	MC4>1>>5>3	↑ inflammatory response to picryl chloride challenge in mice over expressing ASP	[128, 145]
Truncated peptides	α-MSH 6–9 (HFRW)	+	No reported selectivity	↓ PMN accumulation in crystal-induced mouse peritonitis.	[140]
	MSH 11–13 (KPV)	Does not bind		↓Activation of NF-κB following central injection of LPS. ↓ PMN accumulation in crystal-induced mouse peritonitis	[140]
Synthetic peptides based on His-Phe-Arg-Trp invariant sequence	NDP-α-MSH (Melanotan-I, Afamelanotide)	+	MC1>3>4> 5	↑ neuroprotection and functional recovery in murine and gerbil BCCAo and in rat MCAo stroke models.	[134, 146-148]
	Melanotan-II	+	MC4>1>>5>3	↑ Nerve regeneration following crush lesion in rat.	[134]
	[D-TRP^{8}]-γ-MSH	+	MC3>>5>4	↓Leukocyte recruitment, and chemokine generation following IR in mouse mesentery.	[127]
	SHU9119	- at $MC_{3/4}$ + at $MC_{1/5}$	MC4>1>5>3	exacerbates in LPS induced fever	[130, 149]
	HS014	-	MC4>>5>3>1	Blocks the anti-inflammatory effects of β-MSH in mouse brain inflammation	[144, 149]
Small molecule	THIQ	+	MC4>>1>>3>5	Weakly ↓ LPS-induced nitric oxide overproduction in brain tissue.	[150]
	BMS-470539	+	MC1>>4>5 no measurable activity at MC_2 or MC_3	↓ leukocyte trafficking in the mouse mesentery in LPS-induced inflammation and I/R injury.	[151]

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Table 1.4: ***The amino acid sequences of key melanocortin peptides: two distinct pharmacophores***

	Amino acid																						
Peptide	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val											
ACTH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val	Gly	Lys	Lys	Arg	Arg	Pro	Val	Lys	Val	Tyr	Pro
[Dtrp^{8}]-γ-MSH		Tyr	Val	Met	Gly	His	Phe	Arg	D-Trp	Asp	Arg	Phe	Gly											
NDP-αMSH (Melanotan I)	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val											
Melanotan II				Nle	Asp	His	D-Phe	Arg	Trp	Lys														
SHU9119				Nle	Asp	His	D-Nal	Arg	Trp	Lys														
HSO14				Cys	Glu	His	D-Nal	Arg	Trp	Gly	Cys	Pro	Pro	Lys	Asp									
KPV											Lys	Pro	Val											
						Conserved MC binding sequence				.	**KPV sequence**													

Amino acids forming a ring structures are circled

1.7.2. The melanocortin receptors

Five melanocortin receptors have been identified (MC_1–MC_5) and subsequently cloned. The differing tissue expression profiles and affinities for the melanocortin peptides are summarised in table 1.5. The MCs belong to class A subfamily 13 of the rhodopsin-like G protein-coupled receptors [120]. These seven trans-membrane receptors are the smallest known G protein coupled receptors, possessing a small second extracellular loop and short amino and carboxy terminal ends [120]. The MCs share a high sequence homology, with MC_4 and MC_5 showing 60 % identity [130]. With the exception of MC_2, which is activated exclusively by ACTH, each MC can be activated by any of the endogenous melanocortin peptides. All MCs are positively coupled to adenylate cyclase via G_s and activate cAMP based signalling pathways. MC activation has also been shown to initiate other pathways *via* increasing intracellular calcium and secondary activation of inositol triphosphate, mitogen-activated protein kinase (MAPK) signalling has also recently been reported [152]. As with other G-protein-linked receptors the MCs also possess recognition sites for protein kinase C and/or protein kinase A [130, 153].

1.7.2.1 MC_1

The MC_1 was the first melanocortin receptor to be cloned and originally investigated for its role in melanogenesis. MC_1 has since been demonstrated to be expressed in many non-pigmentary cells including neutrophils, monocytes, micro-vascular ECs and astrocytes [120]. Anti-inflammatory and immune-regulatory functions for the MC_1 have since been demonstrated in a number of studies. Both MS05 and MS09 are selective MC_1 agonists and have been shown to down regulate the expression and secretion of E-selectin, VCAM and ICAM in human vascular ECs stimulated with TNFα [154, 155]. Conversely antibody neutralisation of MC_1 increases both the basal and LPS-stimulated production of TNF-α [156]. Additionally the excessive production of the MC_1 MC_4 antagonist agouti protein, as with agouti allele “lethal yellow” mice, incurs an enhanced inflammatory response to picryl chloride [128].

1.7.2.2 MC_2

MC_2 is exclusively activated by ACTH and is hence often referred to as the ACTH receptor. MC_2 mediates steroidogenesis and is expressed in the zona glomelurosa (the site of mineralcorticoid production) and zona fasciculate (the site of glucocorticoid

production) of the adrenal cortex [120]. Mutations in this receptor are associated with familial glucocorticoid deficiency, resulting in ACTH insensitivity, frequent childhood infection and hyperpigmentation, due to increased circulating ACTH acting on cutaneous MC_1.

1.7.2.3 MC_3

The MC_3 expression occurs in a wide variety of cell types and has been demonstrated in the hypothalamic paraventricular nucleus, arcuate nucleus, cortex, thalamus, and hippocampus of the brain and also in peripheral tissues, macrophages and ECs [120, 134]. MC_3 stimulation has been demonstrated to activate both cAMP and inositol phospholipid-Ca^{2+} mediated signalling pathways [120]. MC_3 activation has been shown to be important in modulating feeding, autonomic functions, and inflammation [130]. The role of the MC_3 in the inflammatory response has been illustrated by use of the MC_3 null mouse. These mice present with an exacerbated inflammatory reaction in the microvasculature, suggesting the existence of a tonic anti-inflammatory signal centred on the MC_3 [127]. Additional experiments by Leoni *et al,.* demonstrated that pharmacological treatment with the selective MC_3 agonist [D-Trp^8]-γ-MSH resulted in reduced leukocyte recruitment following I/R injury in the WT mouse mesenteric microcirculation and these effects were confirmed to be absent in MC_3 null mice [127].

1.7.2.4 MC_4

The MC_4 is the main melanocortin receptor of the brain, and thought to be exclusively found in the CNS [120]. In addition to G_s/cAMP signalling a number of recent studies have provided evidence that MC_4 can couple to all three major classes of G proteins, G_s, $G_{i/o}$, and G_q, and may activate MAPK signalling [157]. Although the most studied effects of this receptor subtype are in its control of food intake, metabolism, and sexual function, MC_4 (with a potential involvement of MC_3) has also been shown to activate centrally mediated anti-inflammatory adrenergic and cholinergic pathways *via* the spinal cord and vagus nerve respectively [134, 158].

1.7.2.5 MC_5

The MC_5 shows a relatively ubiquitous expression pattern and has important roles in exocrine gland (particularly sebaceous gland) function [159]. A limited amount of data also points towards immunomodulatory roles in B and T lymphocytes [160-162].

Table 1.5 ***Characterisation of the melanocortin receptor sub types; expression and function***

Receptor subtype	Affinity for the natural ligands	Signal transduction	Function	Distribution	Ref.
MC_1	α-MSH > β-MSH ≥ ACTH, γ-MSH	**G_s-cAMP-PKA** and G_q –PLC- Ca^{2+}	Pigmentation Anti-inflammatory Anti-pyretic	Melanocytes, ECs, glial cells, fibroblasts, macrophage/monocytic cells, B lymphocytes, neutrophils, Natural killer cells, a subset of cytotoxic T-cells, keratinocytes, periaqueductal grey matter	[120, 121, 158, 163]
MC_2	ACTH	**G_s-cAMP-PKA**	Steroidogenesis	Adrenal cortex, Dermis, sebaceous glands, endocrine glands, ECs	[164, 165]
MC_3	γ-MSH β-MSH ≥ ACTH, α-MSH	**G_s-cAMP-PKA** and G_q –PLC- Ca^{2+}	Cardiovascular Anti-inflammatory, energy homeostasis	Brain(hypothalamic paraventricular nucleus, arcuate nucleus, cortex, thalamus, hippocampus) heart, Macrophages, ECs,	[127, 141, 166]
MC_4	β-MSH ≥ α-MSH ACTH > γ-MSH	**G_s-cAMP-PKA** G_q –PLC-Ca^{2+} and G_i	Feeding control, energy homeostasis, sexual arousal, anti-pyretic regulation of nociception	Brain (hypothalamic paraventricular nucleus, arcuate nucleus, cortex, thalamus, hippocampus)	[134, 167-169]
MC_5	α-MSH ≥ β-MSH ≥ ACTH > γ-MSH	**G_s-cAMP-PKA** Jak/STAT	Control of sebaceous secretion Possible role in immune regulation	Exocrine glands T and B Lymphocytes,	[159]

Proposed main signalling pathway is shown in bold

1.7.2.6 Receptor knockout mice

Knockout animals have provided a wealth of information and insight into the workings of particular genes. These have also proved useful tools in elucidating the many functions of the melanocortins both in health and disease. Table 1.6 summarises the different phenotypes of mice bearing melanocortin related gene mutations.

Table 1.6: ***Melanocortin receptor knockout and mutant mice and their phenotype***

Mouse strain	Background strain	Mutation	Phenotype	Ref.
Recessive yellow (e/e)	-	A frameshift mutation in the MC gene causes a single nucleotide deletion at position 549; resulting in MC_1 being unable to functionally couple to adenylate cyclase and activate cAMP signalling.	Yellow coat colour. Reduced nociception and increased analgesic responsiveness to morphine-6-glucuronide. Aggravated inflammatory response in a model of inflammatory bowel disease.	[127, 170, 171]
MC_3 KO	C57BL/6J	The entire MC_3 coding region was deleted by homologous recombination with a cassette.	Increased fat mass, hyperleptinaemic and reduced lean mass despite being hypophagic and retaining normal metabolic rates. Male MC_3KO mice also develop mild hyperinsulinaemia. Increased leukocyte recruitment in the mesenteric microcirculation following I/R injury indicates an enhanced inflammatory response.	[172]
MC_4 KO	C57BL/6J	homologous recombination with a neomycin-resistance gene deleted virtually all the MC_4 coding sequence	Obese, hyperphagic, significant hyperinsulinaemia, altered metabolic rate but normal lean body mass.	[167]
$MC_{3/4}$ double KO	MC_3KO and MC_4KO cross		Significantly more obese than MC_4 KO	[173]
MC_5 KO	C57BL/6J	A 650 bp ApaI/MscI fragment of the single coding exon of the MC_5 gene was deleted by homologous recombination.	Impaired sebaceous lipid production, defective cutaneous water repulsion and thermoregulation.	[159, 174]
POMC-null mutation	129SvEv × C57BL/6	Entire third exon replaced by a neomycin-resistance cassette; depleting all POMC derived peptides.	Yellow coat, obesity, adrenal insufficiency and abnormal HPT axis.	[175]

1.8. The melanocortin receptor system as a target for the treatment of stroke

1.8.1. The anti-inflammatory potential of the melanocortin receptor system

The multiple protective physiological effects of the melanocortins make them an attractive target for the treatment of stroke. A large number of studies have demonstrated efficacy of melanocortin treatments in a variety of models of both acute and chronic inflammatory conditions. Indeed ACTH is currently used as a supportive therapy for human gouty arthritis [176]. The potent anti-inflammatory actions of the melanocortins have however primarily been shown to be independent of steroidogenesis and attributed to the activation of MC_1 and MC_3 with centrally activated anti-inflammatory circuits also being attributed to MC_4 [120, 134].

1.8.1.1 Multiple mechanisms of action

The anti-inflammatory actions of the melanocortins are mediated by direct effects on cells of the immune system but also by an indirect effect, acting on the function of resident, non-immune cells [177]. Melanocortin receptor activation provides a multifaceted anti-inflammatory response, inhibiting a pro-inflammatory milieu, whilst also affecting inflammatory cell proliferation, activity and migration. Indeed such anti-inflammatory influences are not only exerted through the reduction of pro-inflammatory mediators (TNF-α, IL-1β, IL-8, IL-2, INF-γ, iNOS and COX-2) and adhesion molecules (ICAM-1, VCAM-1 and E-selectin) but also through the enhanced expression of the anti-inflammatory, pro-resolving mediators such as IL-10, superoxide dismutase 2 (SOD2) and heme oxygenase-1 (HO-1) [142].

The melanocortins mediate such diverse anti-inflammatory actions through inhibiting the translocation of transcription factor Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB) to the nucleus [134, 142]. Ubiquitous and highly evolutionary conserved, NF-κB is considered a master regulator of inflammation and is involved in the regulation of more than 150 target genes. NF-κB dysregulation has been linked to a number of inflammatory diseases, particularly in disorders of the brain [178]. Under basal conditions NF-κB is sequestered in the cytoplasm through its interaction with inhibitory IκB proteins which mask the nuclear localization signal *via* their ankyrin repeat domains [179]. A number of stimuli, such as inflammatory cytokines or ROS, initiate NF-

κB signalling. Upon activation the IκB kinase enzyme complex (IKK-α, IKK-β and NF-κB essential modulator (NEMO)) phosphorylates IκB on specific serine residues targeting it for ubiquitination and subsequent proteasomal degradation [179]. Nuclear localization signals on NF-κB are thus exposed, enabling nuclear translocation, binding to DNA κB elements, and gene transcription. Melanocortins may inhibit NF-κB by preventing IKK activation, either directly by cAMP or *via* cAMP activation of PKA. It is also possible that melanocortin signalling may also interfere with NF-κB signalling by activation of cAMP-response element binding protein (CREB). For maximal activation, NF-κB must bind co-activators such as CREB-binding protein (CBP) [179]. CREB activation leads to its nuclear localisation where it may compete with NF-κB for the co-activator CBP and thus inhibit NF-κB mediated transcription [180]. Melanocortin signalling induces CREB activation, this mechanism could therefore provide an additional level of control over NF-κB (figure 1.5).

In addition to the cAMP dependant inhibition of NF-κB the melanocortins may also initiate other, less well defined, anti-inflammatory pathways (Figure 1.5). Manna and colleagues have shown α-MSH stimulation to induce the cleavage of the IL-8 receptors CXCR1 and CXCR2 on the neutrophil cell surface, *via* release of elastase acting in an autocrine fashion [181]. While the exact mechanisms underpinning this effect have yet to be defined, this process was shown to be mediated *via* the MC_1 and independent of cAMP accumulation [181].

Some melanocortin peptides have additionally been shown to exert anti-inflammatory effects independent of their receptor binding. The α-MSH terminal tri-peptide KPV is able to reduce the production of TNF-α, IL-6, and NO induced by a number of proinflammatory stimulus [95]. Furthermore KPV has been found to reduce NF-κB activation both *in vitro* [95] and *in vivo* [182] and reduce PMN recruitment in a model of murine crystal-induced peritonitis [140]. However a strong body of evidence demonstrates that KPV does not bind to any melanocortin receptor [140, 183-185] and that KPV treatment fails to induce cAMP accumulation [140, 185]. Instead, structural similarities between KPV and the IL-1β antagonist KPT indicate that KPV may act as an IL-1R antagonist. In agreement with this, KPV reduces IL-1β-induced, but not TNF-α induced, NF-κB activation [95] and both KPV and α-MSH have been revealed to displace surface binding of radio-labelled IL-1β [95, 186].

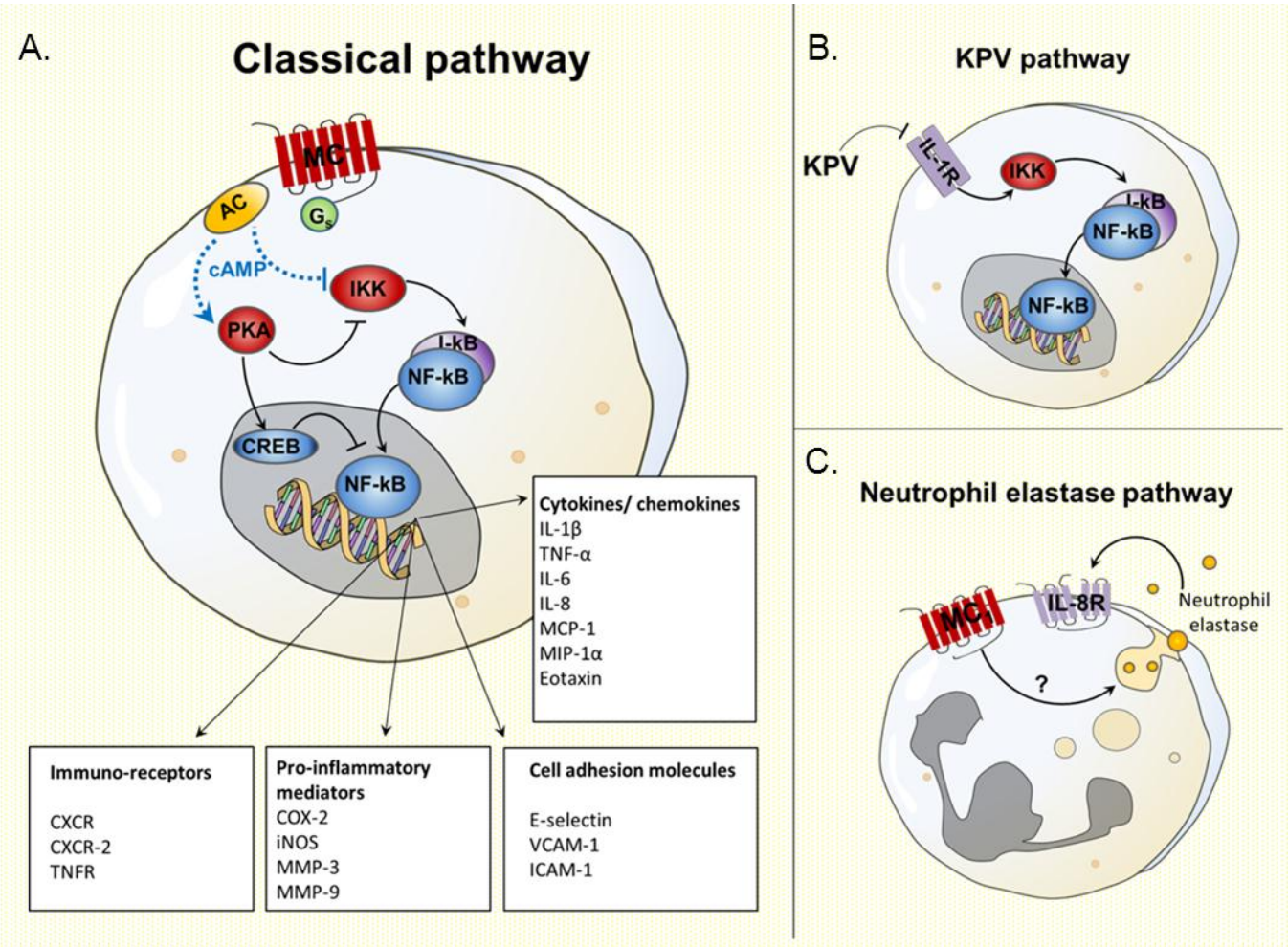

Figure 1.5. ***Proposed mechanisms for MC anti-inflammatory activity***. A) The classical anti-inflammatory pathway centred on the melanocortin receptors is the inhibition of NFκ-β translocation to the nucleus. Receptor activation increases cAMP levels via G_s and activation of adenylate cyclase (AC). cAMP may directly inhibit IKK activity or induce PKA mediated IKK inhibition preventing the degradation of I-κB and NFκ-β translocation to the nucleus. cAMP accumulation may also activate CREB which has been shown to inhibit NFκ-β activity by competing for CBP, thus preventing NF-KB mediated pro-inflammatory gene transcription (a non-exhaustive list of NFKB genes inhibited by MCs is shown). Less well characterised mechanisms for MC activity also exist. B) KPV has been proposed to work via competitively antagonising IL-1 receptor thus preventing IL-1 mediated effects such as NFκ-β activation. C) In neutrophils activation of MC_1 may cause the release of neutrophil elastases from granuels by an uncharacterised, cAMP independent pathway. Elastases may then cleave neutrophil cell surface IL-8 receptors CXCR1 and CXCR2. (P. Holloway, *unpublished)*

1.8.1.2 An endogenous anti-inflammatory circuit

Whist melanocortins may have anti-inflammatory actions on leukocytes, leukocytes themselves are also a source of melanocortins [158] suggesting that the melanocortins may play a role in a self-limiting anti-inflammatory loop. Additionally *In vitro*, NF-κB activation directly stimulates the transcription of POMC the melanocortin precursor [187].

The melanocortins may also be considered to promote the effective resolution of inflammation, sparing some of the beneficial repair initiating effects of inflammation, rather than simply ending the process. An advantage of melanocortins over many other anti-inflammatory agents, such as glucocorticoids, is that they do not hamper the anti-microbial activities of immune cells, indeed they have been shown to enhance them [96]. Melanocortin receptor activation has been shown to enhance macrophage phagocytosis, not only of zymosan particles but also efferocytosis of apoptotic neutrophils, thus promoting resolution. Mice bearing a non-functional MC_1 have also showed delayed clearance of infection in a bacterial colitis model [171]. These findings may be of particular importance in the treatment of immune-compromised patients and patients presenting with co-morbidities. Indeed following a marked acute inflammatory response, stroke patients often present with delayed immunodepression 1 to 2 days following stroke, associated with a higher susceptibility to respiratory and urinary tract infections. Modulating rather than terminating the inflammatory response may thus be highly beneficial [11].

1.8.2. Melanocortins in the brain

The anti-inflammatory actions of the melanocortin peptides have been proven effective in a number of models of brain inflammation. However in addition to this these peptides have displayed a number of remarkable behavioural [134], anti-pyretic [188], neurotrophic and neuroprotective effects in the brain [189, 190] whilst also modulating nociception [191], metabolism [192] and reducing peripheral inflammation [193] through centrally mediated pathways.

1.8.2.1 Modulation of inflammation in the brain

Treatment with MC agonists has been shown to improve disease severity in a number of disorders of the brain where inflammation plays a critical role, including animal models of

stroke [146, 169] and EAE [194]. Polymorphisms in the MC_1 gene have also recently been related to poor outcomes in multiple sclerosis [195]. Markedly reduced concentrations of circulating α-MSH have also been noted to coincide with unfavourable outcomes in patients following acute brain injury of both traumatic and vascular origins [196].

In addition to these actions on local inflammation in the brain, centrally administered melanocortin peptides have also been shown to supress inflammation responses in the periphery *via* a vagus nerve-mediated cholinergic anti-inflammatory pathway [147, 193, 197]. These effects have been shown to be mediated by the melanocortin stimulation of vagal cholinergic pathways and the protective actions of acetylcholine *via* peripheral nicotinic acetylcholine receptors. Such mechanisms may be beneficial in the treatment of stroke as it has been shown focal brain ischemia can cause significant adverse effects not only in the brain but to also contribute to multiple organ damage and dysfunction, in particular to the liver [147].

1.8.2.2 Antipyretic activity

The melanocortins have been shown to display potent antipyretic activities, which could prove to be an additional beneficial effect in the treatment of stroke. Subsequent to acute stroke patients often develop an elevated body temperature, this has been shown to exasperate outcomes and correlate with the severity of stroke [198]. Furthermore lowering the general body temperature has been seen as a promising neuro-protective therapy and is an aim of a number of treatments for stroke such as paracetamol treatment and induced therapeutic hypothermia [199-201]. While the exact mechanisms underlying the neuro-protective effects of hypothermia are poorly understood, reduced temperature, has been proposed to suppress cerebral oxygen consumption, decrease intracellular lysosomal enzyme activity, lower free radical formation and inhibition of excitatory neurotransmitter release [117, 201].

In reducing a pyrogen-induced fever, α-MSH has been shown to, on a molar basis, exhibit a potency 25,000-fold greater than paracetamol when injected into the cerebral ventricular [188] while central administration of the MC antagonist SHU9119 to febrile rats exacerbated fever. Furthermore MCs are expressed in key hypothalamic thermoregulatory centres and during fever, the concentration of α-MSH has been shown

to naturally increase by 2–3-fold, suggesting an endogenous role in control of fever [188, 202].

1.8.2.3 Nociception

The melanocortins have also been shown to modulate the perception of pain in a functionally antagonistic manner to the endogenous opioidergic system in the brain [191]. Such observations have led to the understanding that melanocortins and endorphins initiate opposing actions, thus the POMC system is particularly important in regulation of homeostasis by the differential production of melanocortin and endorphin peptides [134]. Whereas Opioids induce analgesia, inhibit sexual behaviour and have amnesic properties, the melanocortins promote sexual arousal, antagonize morphine-induced analgesia and enhance short term memory [134].

The administration of melanocortin receptor agonists has been shown to be pro-nociceptive in a number of studies. Additionally treatment with the MC_4 antagonist SHU9119 produces analgesic effects in rats, surprisingly in a more potent manner than the μ-opioid receptor agonist, morphine [126]. These may have implications in highlighting melanocortin agonists as potential analgesic drugs, but also emphasises the importance of selective agonists of the MCs in the treatment of cerebral inflammatory conditions, to avoid enhancing nociception.

1.8.2.4 Feeding behaviour and metabolism: Obesity as a stroke risk factor

The first evidence for a role of the melanocortin peptides in the regulation of human appetite came in 1998 with description of two children possessing mutations of the POMC gene resulting in defects of ACTH and α-MSH synthesis [203]. The children displayed severe metabolic derangement hyperphagia and obesity. Since then an increasing body of evidence demonstrates the importance of the CNS melanocortin receptor, MC_4, in obesity. Whilst beyond the scope of this thesis, the MC_4 effects of both on lipid metabolism and feeding behaviour may be of particular interest in the development of drugs targeting obesity, a major risk factor of stroke [204]. These anorectic effects may also prove to be of some small benefit in controlling weight gain, which can occur subsequent to stroke. However, although the feeding and anorectic effects of melanocortins have been postulated to occur solely through the actions of

MC_4, a recent study in rat models of stroke has disappointingly shown no beneficial effects of selective stimulation of the MC_4 by the agonist RY767 [205].

1.8.2.5 Neurotrophic actions

The neurotrophic properties of the melanocortin peptides are well documented. They have been shown to stimulate turnover of acetylcholine in the hippocampus and enhance neurotransmission. α-MSH is considered a neurotrophic factor both in foetal development and in adulthood [190]. Several studies have also shown improved functional recovery following CNS and peripheral nerve lesions in rats when treated with melanocortins [189]. More recently the immediate early gene Zif268 has been associated with these actions. Zif268 is a gene known to be involved in injury repair and memory formation and is dependent on synaptic activity [146]. Melanocortins have been suggested to initiate neuronal repair mechanisms and promote synaptic plasticity *via* MC_4 mediated induction of Zif268 [146]. The melanocortin mediated recruitment of astrocytes to the site of injury may also help regulate ionic balances and remove excess excitatory amino acids, whilst enhancing the release of astrocytes derived trophic factors and promoting neuronal growth and survival [206].

Protective roles of the melanocortins in neuro-degeneration have been exemplified by improved functional outcome in animal models of stroke and diabetic neuropathy upon treatment with melanocortin peptides [134]. In dogs melanocortin treatment has been shown to improve the recovery of auditory evoked potentials following brain ischemia/reperfusion [207]. Treatment with NDP-α-MSH has also shown to improve the recovery of behavioural functions, spatial learning and memory following BCCAo global stroke in gerbils [169].

1.8.3. Melanocortins in ischemia reperfusion

The protective actions of the melanocortins have been documented in animal models of I/R injury in a variety of organs including; heart [208], renal ischemic injury in mice and rats [209], intestinal I/R [210] and cerebral I/R in mice [211] gerbils [169] and dogs [207].

Furthermore, recent investigations have found NDP-α-MSH treatment to induce protective phenotypic changes in the heart similar to those achieved in ischemic preconditioning [212]. Ischemic preconditioning is an intrinsic protective phenotype

induced by repeated brief episodes of ischemia and involves CREB phosphorylation and STAT3 activation. In the rat heart NDP-α-MSH was found to promote a powerfully cardio-protective environment *via* the activation of the IL-6/STAT3 signalling pathway whilst also inducing suppressor of cytokine signalling 3 (SOCS3), a powerful cytokine antagonist [212]. Ischemic preconditioning has also recently been shown to be beneficial in the brain [213]. Attempts to reveal the molecular and cellular mechanisms underlying the neuroprotection provided by ischemic preconditioning have identified MAPK signalling to play a key role and the up regulation of the anti-apoptotic Bcl-2 protein to also be a major determinant [213]. Bcl-2 has been shown to be up regulated in astrocytes treated with α-MSH [214] additionally MC_4 activation has been shown induce MAPK signalling pathways [157], these effects may therefore prove beneficial in stroke.

The pleiotropic neurovascular protective and anti-inflammatory actions of the melanocortins, described above, thus make these receptors a promising therapeutic candidate to address the aberrant inflammatory response proceeding stroke.

Hypothesis

Given the wealth of data demonstrating a pathological role of inflammation following cerebral I/R and enhanced leukocyte recruitment to exacerbate neuronal damage, it is proposed that inhibiting leukocyte recruitment in the cerebral microcirculation is a valid therapeutic strategy to suppress post stroke inflammation and progression of neuronal injury.

Considering that melanocortin treatments have previously been shown to reduce the expression of adhesion molecules E-selectin, VCAM and ICAM on endothelial cells *in vitro* and in models of lung inflammation, peritonitis and both mesenteric and cremasteric I/R melanocortin compounds reduce *in vivo* leukocyte accumulation, it is hypothesised that melanocortin treatments will be effective in abrogating I/R induced leukocyte recruitment in the cerebral microcirculation. Given that melanocortin receptor activation has been shown to inhibit nuclear translocation of NF-κβ it is expected that reductions in leukocyte recruitment will also coincide with reduced expression of pro-inflammatory signalling molecules such as TNF-α whilst also enhancing expression of pro-resolving molecules such as IL-10.

Previous studies have shown MC_1 and MC_3 to be the melanocortin receptor subtypes which initiate anti-inflammatory signalling, with MC_4 also activating centrally mediated anti-inflammatory signals to the periphery. As such it is likely that either MC_1 or MC_3 may be crucial in mediating any anti-inflammatory actions observed with a possible involvement of MC_4 should the peripherally administered melanocortin compounds reach the brain parenchyma. Due to the low BBB permeability of peptide melanocortin compounds it is however proposed that either MC_1 or MC_3 will be the key mediators of any observed inhibitory actions. Given that a wealth of *in vivo* and *in vitro* data support roles for both MC_1 and MC_3 it is however possible that both these receptors may play a role. Conflicting data on the principle anti-inflammatory receptor in different disease models suggest that the role of melanocortin receptors may vary within the different physiological micro-environment and/or pathological state. Thus determining which of these receptors is most important to anti-inflammatory actions following I/R in the unique micro-environment of the cerebral microcirculation would provide a novel contribution to the field

Aims

This study utilises the murine BCCAo model of global stroke and intravital microscopy, with an aim to assess leukocyte recruitment following cerebral I/R and to ascertain the therapeutic value of a number of melanocortin active compounds in modulating this neuro-inflammatory response. These investigations will focus on establishing the principle anti-inflammatory melanocortin receptor subtype in the context of cerebral I/R induced leukocyte recruitment.

The first aim of this project is thus to establish and characterise the inflammatory response following BCCAo. As such leukocyte-endothelial interactions will be quantified in wild type (C57BL/6) mice using IVM, in terms of leukocyte rolling, rolling velocity and leukocyte adhesion. Circulating levels of pro-inflammatory cytokines and the activation of resident microglia will also be established and investigations made at multiple time points of reperfusion to investigate the temporal progression of leukocyte recruitment.

In order to assess the melanocortin receptor system as a potential therapeutic target in inhibiting cerebral I/R induced leukocyte recruitment, an initial aim is to establish the relative expression of each melanocortin receptor in the brain and blood. Treatments using the endogenous non-selective melanocortin receptor agonist α-MSH will then be given at different time points in order to determine whether activation of the MRS can modulate cerebral leukocyte recruitment and levels of circulating cytokines following I/R.

The aim of determining the receptor subtype/subtypes responsible for any anti-inflammatory effects observed will then be achieved through the use of selectively active pharmacological compounds (MC_1 agonist BMS-470539, MC_3 agonist and $MC_{3/4}$ antagonist SHU9119). Receptor mutant animals (MC_1 mutant e/e mice and $MC_3^{-/-}$ mice) will also be used with the aim of ascertaining the endogenous importance of these receptors to I/R induced leukocyte recruitment and give further insight to the therapeutic potential of targeting these receptors.

A further aim of this project is to establish the mechanism by which the melanocortins inhibit leukocyte recruitment, as such NF-κβ related genes will be assessed to find if treatments inhibit activation, and the cell type responsible for mediating melanocortin effects on leukocyte recruitment determined using a number of *in vitro* assays. These assays will also help to achieve the final aim of this project; to establish whether the melanocortin mediated inhibition of leukocyte recruitment observed in mice is also effective in human cells.

2.0. Materials and methods

2.1. Materials

The general materials and reagents used are listed in tables 2.1 and 2.2

Table 2.1: General materials

Material	Supplier	Location
96-well plates	VWR	Leicestershire, England
Falcon tubes (15ml and 50ml)	VWR	Leicestershire, England
Microcentrifuge tubes (1.5ml)	VWR	Leicestershire, England
Needles (26 G)	Becton Dickinson	Oxford, England
PE10 tubing	Intramedic	Oxford, England
Pipette tips (20 µl, 1-200 µl and 200-1000 µl)	VWR	Leicestershire, England
Serological Pipettes (5ml and 10ml)	VWR	Leicestershire, England
Syringes (1 ml and 20ml)	Becton Dickinson	Oxford, England

Table 2.2: General reagents

Reagent	Supplier	Location
All chemicals (unless otherwise stated)	Sigma Aldrich	Pool, England
α-MSH	Abcam-Ascent scientific	Cambridge, England
β-mercaptomethanol	Sigma Aldrich	Pool, England
Bovine Serum Albumin	Sigma Aldrich	Pool, England
BMS 470539	Tocris	Bristol, England
[Dtrp^{8}]-y-MSH	Sigma Aldrich	Pool, England
Heparin (from porcine intestinal mucosa)	Sigma-Aldrich	Pool, England
KPV	Bachem	Bubendorf, Switzerland
Physiological Saline	Baxter Healthcare	Thetford, England
SHU9119	Tocris	Bristol, England
Sodium Pentobarbital (Euthatal)	Merial Animal Health	Harlow, England

2.2. *In vivo* procedures

2.2.1. Animals

All Housing conditions and experimental procedures were in strict accordance with Home Office Regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986) under the project licence 70/6973. Male C57BL/6 mice (20-28g body weight) were purchased from Charles River, male Recessive yellow (e/e) and $MC_3^{-/-}$ mice (20-32g body weight) were kindly provided by Professor Mauro Perretti from the William Harvey Research Institute, London, England. All animals were maintained on a 12 h light/dark cycle with an ambient temperature between 21-23°C with access to standard chow pellet diet and water ad libitum. Drug treatments were all administered by intraperitoneal (i.p) injection at 10 µg in 100 µl of saline, with exception to BMS 470539 which was given at 15 µM. Drug dose was chosen with regards to current literature to minimise animal usage in does response experiments (see table 4.1 for references). Treatments were either given at 30 min before BCCAo or at the start of reperfusion.

2.2.2. Global stroke model: Bilateral Common Carotid Artery occlusion (BCCAo)

2.2.2.1. BCCAo

Mice were anaesthetised using an i.p injection of 100 ml/kg sodium pentobarbital. The animal was then positioned in a dorsal recumbence under a dissecting microscope with its head toward the surgeon and secured in this position using tape. A rectal thermometer was inserted and animals were maintained throughout the procedure close to 37.5 ^{o}C using a heat mat. A skin incision of approximately 1-2 cm^2 was then made over the ventrolateral neck using surgical scissors. The salivary glands were exteriorised and blunt dissection either side of the trachea allowed visualisation of the common carotid arteries. The anatomical location of the common carotid arteries are shown in Figure 2.1. Sham animals then proceeded to the cranial window step omitting artery occlusion. In BCCAo groups both arteries were then occluded using aneurysm clips (Fine Science Tools) for five min. Prior to removing the clips a small amount of saline was applied to avoid damaging the vessels whist removing the clips.

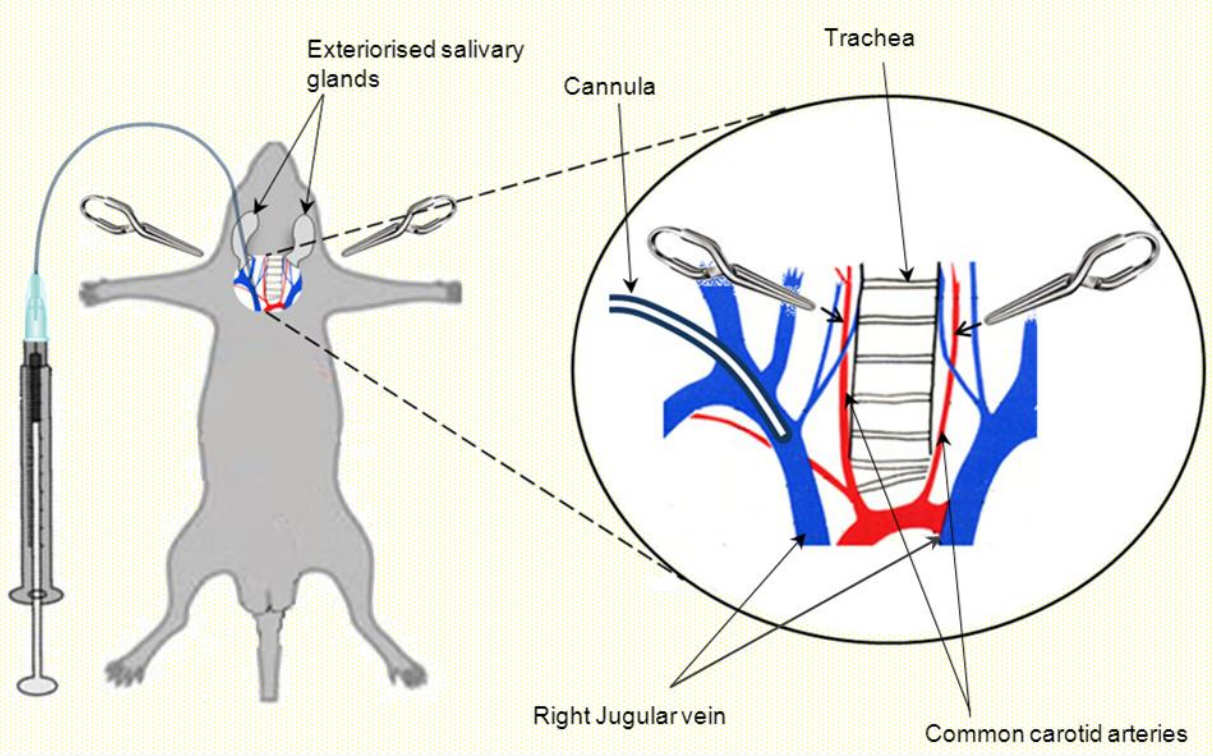

Figure 2.1. *BCCAo*. Diagram showing the location of the trachea, common carotid arteries and jugular veins. (P. Holloway, *unpublished*)

2.2.2.2. Cannulation

Blunt dissection was used to expose the right jugular vein. A small section of the vessel was cleaned of connective tissue using curved forceps and a length of 4-0 braided silk suture was passed underneath the vessel. The suture was then cut into two sections and one piece moved toward the cranial end of the vessel and tied tightly to occlude blood flow. The caudal suture was then tied in a loose knot. Whist the vessel was stabilised by lifting the cranial suture a small incision was then made in the vessel between the two sutures using a 26 gauge needle, before inserting a cannula, consisting of a 27 gauge needle and a length of polyethylene tubing (PE-10) attached to a syringe containing saline. Withdrawal of blood into the tubing confirmed successful insertion. The cannula was then secured in place by tightening the caudal ligature around the cannula.

2.2.2.3. Cranial window

Animals were placed in a stereotactic frame in a sphinx position and their heads securely positioned using the ear bars. An area of skin on the scalp was removed using surgical scissors to expose the parietal bone and then the underlying periosteum was removed using forceps. A high-speed micro-drill (1/4 bit, Fine Science Tools) was used to thin an area of the skull to one side of the midline approximately 5x5 mm in circumference, drilling intermittently and periodically applying cold saline to minimize heat induced activation and tissue damage. Once the skull was sufficiently thinned the island of bone was carefully removed using forceps. Artificial cerebrospinal fluid (aCSF; NaCl 131.9 mM, $CaCl_2$ 1.26 mM, $CaCl_2.2H_2O$ 1.26 mM, KCl 2.95 mM, $MgCl_2.6H_2O$ 0.64 mM, $MgCl_2$ 0.5 mM, $(NH_2)2CO$ 6.69 mM, $C_6H_{12}O_6$ 3.69 mM) was used to suffuse the exposed brain before a 12 mm circular cover slip was carefully positioned over the window.

2.2.2.4. Intravital microscopy

Five min prior to IVM, 100 µl of rhodamine-6G (0.02 % in saline) was injected *via* the cannulated jugular in order to fluorescently label leukocytes. The cerebral microcirculation was visualized using an Olympus BW61WI microscope with water-immersion objective lens; magnification x 40 (LUMPlan FI/IR, Olympus) and a 12V 100 W halogen light source through a CY3 filter. Sections of un-branched pial venules 100 µm in length and 30-70 µm in diameter were selected and a Photometrics® CoolSnap™ HQ2 colour camera (Intelligent Imaging Innovations) was used to record 2 min time-lapse videos using Slidebook 4.2 software (Intelligent Imaging Innovations Inc).

2.2.2.5. Video analysis

Videos were analysed manually offline, in terms of leukocyte rolling cell flux, leukocyte rolling velocity and adhesion of the leukocytes. Leukocytes travelling along the venular wall at a velocity much less than the central line blood flow were defined as rolling cells. White blood cell velocity (V_{WBC}) was calculated by advancing the video frame by frame and measuring the distance the leukocyte travels along the venular wall within a given period of time and expressed as µm/sec. Leukocytes that remained stationary on the venular wall for 30 seconds or longer were considered to be adherent. Assuming the

vessel as being cylindrical the number of rolling and adherent leukocytes were calculated per mm^2 of vessel per min using the following equations:

$$\text{No. Rolling leukocytes/mm}^2 = \frac{\text{Number of rolling cells}}{\text{Vessel diameter}/1000}$$

$$\text{No. Adherent leukocytes/mm}^2 = \frac{\text{Number of adherent cells}}{\text{Vessel diameter} \times \pi \times 100 / 10^6}$$

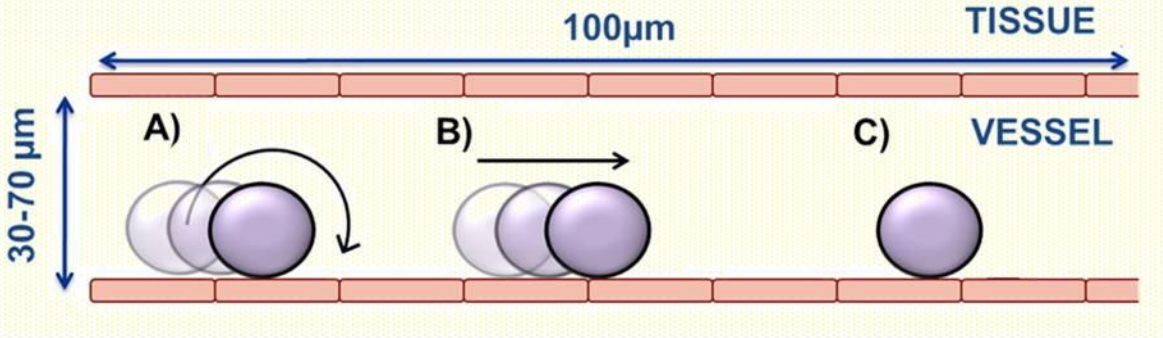

Figure 2.2; ***The parameters for Intravital video analysis***. A) Rolling cell flux; the number of cells rolling on the vessel wall past a chosen point. B) Rolling velocity; the speed of cell rolling (µm/sec) C) Adherent Cells; number of cells that remain stationary on the vessel wall for 30 seconds or longer.

2.2.3 Plasma protein leakage

To assess vessel integrity and BBB compromise plasma protein leakage was measured using IVM. Following IVM of leukocyte endothelial interactions the mouse remained under the microscope and was injected i.v with 250 mg/Kg of fluoroscein isothiocyanate (FITC)- conjugated albumin (5mg/µl saline Sigma-Aldrich). 5 minutes following FITC-albumin injection a vessel of 30-70µm in diameter was viewed in an area clear of other

vessels and a snapshot taken using the FITC channel (excitation 450-490 nm; emission 535-620 nm). Albumin leakage was quantified during offline analysis of snapshots using ImageJ software (National Institute of Health, USA). Mean fluorescence intensity was measured across three selected areas inside the vessel (internal, black squares), immediately outside the vessel (external, white squares), and areas with no obvious fluorescence (background, dashed white square) (Figure 2.3). Averages were taken of the three measurements for each area and albumin leakage determined using the following calculation:

$$\frac{(\text{external fluorescence} - \text{background})}{(\text{internal fluorescence} - \text{background})} \times 100.$$

:

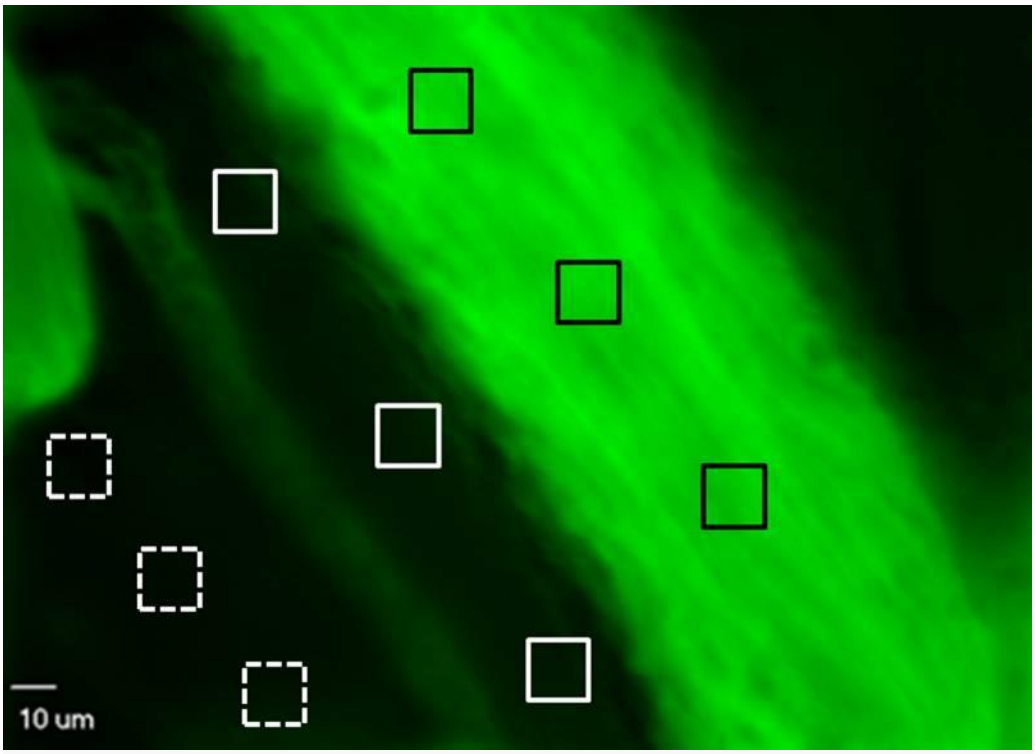

Figure 2.3. ***Measurement of plasma protein extravasation***. Protein extravasation was measured by the leakage of FITC conjugated albumin. Fluorescent intensity was measured immediately outside the vessel (white boxes) and the background (Dashed white boxes) subtracted. This value was then divided by the internal fluorescence (black boxes) - background.

2.2.4. Neutrophil depletion

Neutrophil depletion in C57BL/6 mice was achieved by i.p injection (10 ml/kg) of anti-mouse PMN serum (diluted in sterile saline 1:10, Gentaur, Product Number: AIA31140) daily for two days prior to BCCAo surgery. Control animals were injected with IgG isotype matched serum (Gentaur, Product Number: AIS403). PMN depletion was confirmed 48 h following injection by total white blood cells count using blood withdrawn from the tail vein diluted 1:20 in Turk's solution using. WBC differential counts were obtained from blood smears stained with Wright-Giemsa (Sigma Aldrich). Both counts were compared with samples taken immediately before the first injection of anti-mouse PMN serum. In all cases PMNs were found to be depleted by >85 %

2.2.5. Tissue and serum sample collection

A number of different *in vitro* tests were performed on samples collected from experimental animals. As such tissue and blood samples were collected in a number different ways, according to the experimental requirements:

2.2.5.1. ELISA, Bead Array and Western blot

Blood samples were taken by cardiac puncture under terminal anaesthesia (sodium pentobarbital, 100 mg/kg) using a heparinised syringe and then centrifuged at 12,000 RPM at 4 °C for 6 min. The supernatant was collected and snap frozen in liquid nitrogen and stored at -80°C. Brains and other tissues were removed, snap frozen and stored at -80°C.

2.2.5.2. Immunohistochemistry

At the conclusion of experiments animals were injected with an i.p. overdose of 200 mg/ml sodium pentobarbital. Following confirmation of respiratory arrest the thoracic cavity was opened and the right atrium cut open before perfusing animals through the left ventricle with 20 ml PBS over 5 min. The brain was then collected and immediately transferred to a bath of isopentane on dry ice, and left to freeze before transferring to -80 °C for storage

2.2.5.3. qRT-PCR

Following sham or BCCAo surgery blood and brain samples were taken and immediately transferred to RNAprotect Animal Blood Tubes or RNAse Later (Qaigen) respectively and stored at 4 °C until use for RNA isolation.

2.2.6. Quantification of brain oedema

Brain oedema was determined using the wet/dry method. Briefly, brains were removed rapidly and weighed. Samples were then incubated for 48 h at 70 °C until fully dehydrated and then re-weighed. The % brain water determined by the following calculation was taken as a representative of brain oedema.

$$\% \text{ Brain water} = [(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%$$

2.3. *In vitro* procedures

2.3.1. Western blotting

Frozen brain samples were defrosted and homogenized in NP-40 buffer (10 % glycerol, 1 % nonidet P-40, 2 mM Ethylenediaminetetraacetic acid (EDTA), 137 mM NaCl, 20 mM Tris HCl, pH 8), 6 µl/mg of tissue, containing EDTA-free protease inhibitor (1 tablet per 10 ml; Roche). Samples were then incubated at 4 °C under constant agitation for 1 h. 1 ml of the homogenized sample was transferred to a 1.5 ml eppendorf and centrifuged at 4 ^{o}C, 13,000 RPM, for 10 min. The supernatant was then removed to a clean aliquot and a Nanodrop ND-1000 spectrophotometer (Labtech international) set to read absorbance at 280 nm was used to determine sample protein content. Samples were diluted with the sample buffer to a concentration of 2 mg/ml. Loading buffer (Invitrogen) containing 4 % β-mercaptomethanol (Sigma Aldrich) was then added to the sample 1 part per 3 parts of sample. Samples were then incubated either for 5 min at 70 ^{o}C when probing for receptor proteins or for 3 min at 95 °C for all other proteins. 26 µl of each sample corresponding to 20 µg of protein was then loaded into a NuPAGE® Novex Bis-Tris Gel (invitrogen). 10 µl of SeeBlue® (Invitrogen) was used as a pre-stained protein standard.

Electrophoresis and transfer to a nitrocellulose membrane, 0.45 µm pore size (invitrogen), was performed according to the manufacturer's instructions using XCell *SureLock*® Mini-Cell, NuPAGE running buffer, XCell II™ Blot Module Kit and NuPAGE transfer buffer (Invitrogen).

The nitrocellulose membrane was removed from the blot module and successful protein transfer checked by washing with Ponceau S (Sigma-Aldrich) for 30 seconds to visualise protein bands. The membrane was then washed with water to remove excess Ponceau S and then incubated at room temperature with gentle rocking for 30 min in blocking buffer (5 % BSA in TBS-T; 50 mM TRIS buffer, 150 mM NaCl, 0.05 % Tween 20 adjusted to pH 7.4 using HCl). Blocking buffer was then discarded and the membrane incubated with the primary antibody diluted in blocking buffer (antibody concentrations given in table 2.3). All primary antibody incubations were done overnight at 4 °C with gentle rocking. The primary antibody was then poured away and following 3 x 5 min

washes in TBS-T was incubated with the corresponding secondary antibody for 1 h at room temperature. Following secondary antibody incubation membranes were washed 3 x 5 min in TBS-T. Excess TBS-T was blotted off the membrane and the membranes were then covered with 2 ml of Pierce ECL Western Blotting Substrate (Thermoscientific) and left for 30 seconds before excess substrate was removed. The membranes were then positioned in a Hypercassette™ Autoradiography Cassette (GE Healthcare), exposed to Fuji X-ray film RX (Fisher Scientific) and the films developed in Kodak developer and fixer (Sigma Aldrich). Developed films were then scanned and the mean integrated intensity of each band measured using image J software.

Membranes were stripped by 10 min incubation at room temperature in glycine stripping buffer (0.2 M glycine, 3.47 mM sodium dodecyl sulphate, 1 % Tween 20, pH 2.2). The stripping buffer was then discarded and membranes incubated for a further 10 min in fresh buffer. Membranes underwent 2 x 20 min incubations in PBS followed by 2 x 10 min incubations in TBS-T, all at room temperature. Blots were then re-probed. Blots were normalised for loading against Alpha-tubulin expression.

Table 2.3: Antibodies used for western blot analysis

Antibody to;	Reacts with	Raised in	Company	Working Conc.
MC_1 receptor	Mouse, Human	Rabbit	Sigma Aldrich (M9193-50UL)	1:1000
MC_3 receptor (a)	Mouse, Rat	Rabbit	Sigma Aldrich (M4937)	1:2000
MC_3 receptor (b)	Mouse, Rat, Human	Rabbit	Acris-antibodies (AP10124PU-N)	1:500
Cyclooxygenase 2	Mouse, Rat, Human	Rabbit	Abcam (ab15191)	1:1000
α-Tubulin	Mouse, Rat, Human	Chicken	Sigma Aldrich (SAB3500023)	1:1000
Rabbit IgG (HRP conjugated)	Rabbit IgG	Goat	Sigma Aldrich (A9169)	1:12000
Chicken IgG (HRP conjugated)	Chicken IgG	Rabbit	Sigma Aldrich (A9046)	1:12000

2.3.2. ELISA

A Quantikine® Colorimetric Sandwich ELISA kit (R&D Systems) were used for the analysis TNF-α cytokine expression in mouse serum samples and was performed according to the manufactures guidelines. Optical density was read at 450 nm with wavelength correction at 540 nm. TNF-α levels were determined using a standard curve (Figure 2.4). Readings were also normalised to sample protein content, determined using a Bradford assay as previously described [215].

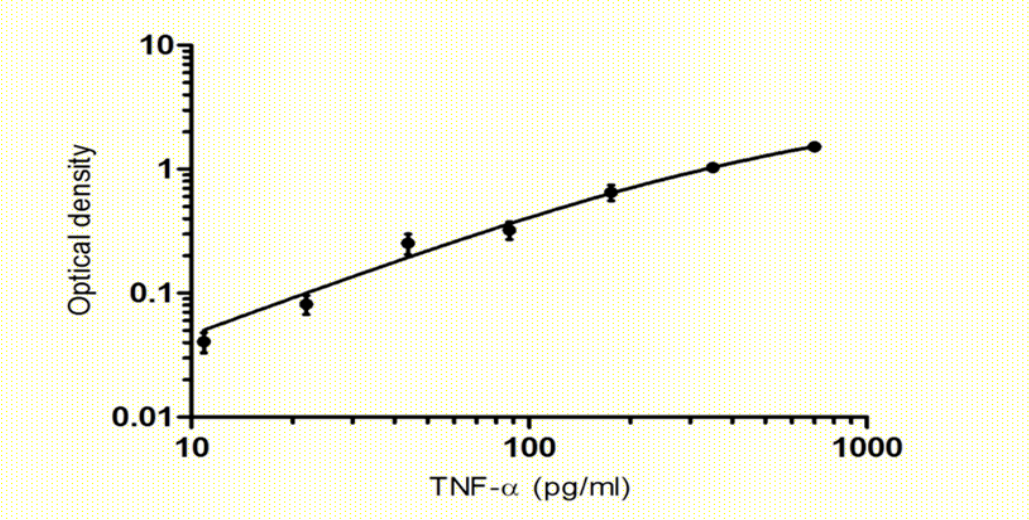

Figure 2.4. ***TNF-α ELISA standard curve***. Serial dilutions of recombinant mouse TNF-α were assayed using the Quantikine® Colorimetric Sandwich ELISA kit to create a 7 point standard curve with a range of 700-10.9pg/ml and a 0 pg/ml standard. TNF-α content of unknowns were extrapolated from their optical density using the standard curve. Minimum detection limit was determined as the lowest significant increase on the standard curve and as such is 10.9 pg/ml.

2.3.4. Cytometric Bead Array

Quantitative detection of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 was performed on mouse serum samples using a BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences). In this assay soluble analytes are captured by antibodies conjugated to beads of known size and fluorescence thus allowing the analytes to be detected and quantified using flow cytometry. In brief, flow cytometer settings were optimised for the bead assay using cytometer set up beads (BD biosciences). Capture beads for each of the 6 cytokines were mixed and then added to 50 µl of each serum sample tested. 50 µl of detection reagent was then added and the mixture incubated for

2 h, allowing the formation of capture bead+ analyte + detection reagent complexes. The detection reagent contains phycoerythrin conjugated antibodies, which produce a fluorescent signal proportionate to the amount of bound analyte thus allowing for specific quantification by flow cytometary. A standard curve for each cytokine was setup covering a defined set of concentrations from 20 to 5,000 pg/mL. Samples were run at a high flow rate on a dual laser BD FACSCalibur using BD CellQuest pro software for acquisition. The flow cytometer was set to count 2100 events per sample (300 for each cytokine) and gated for FSC vs SSC using the negative control bead sample. Results were analysed and quantified with reference to the standard curve using FCAP Array software (version 3, Soft Flow, Inc.).

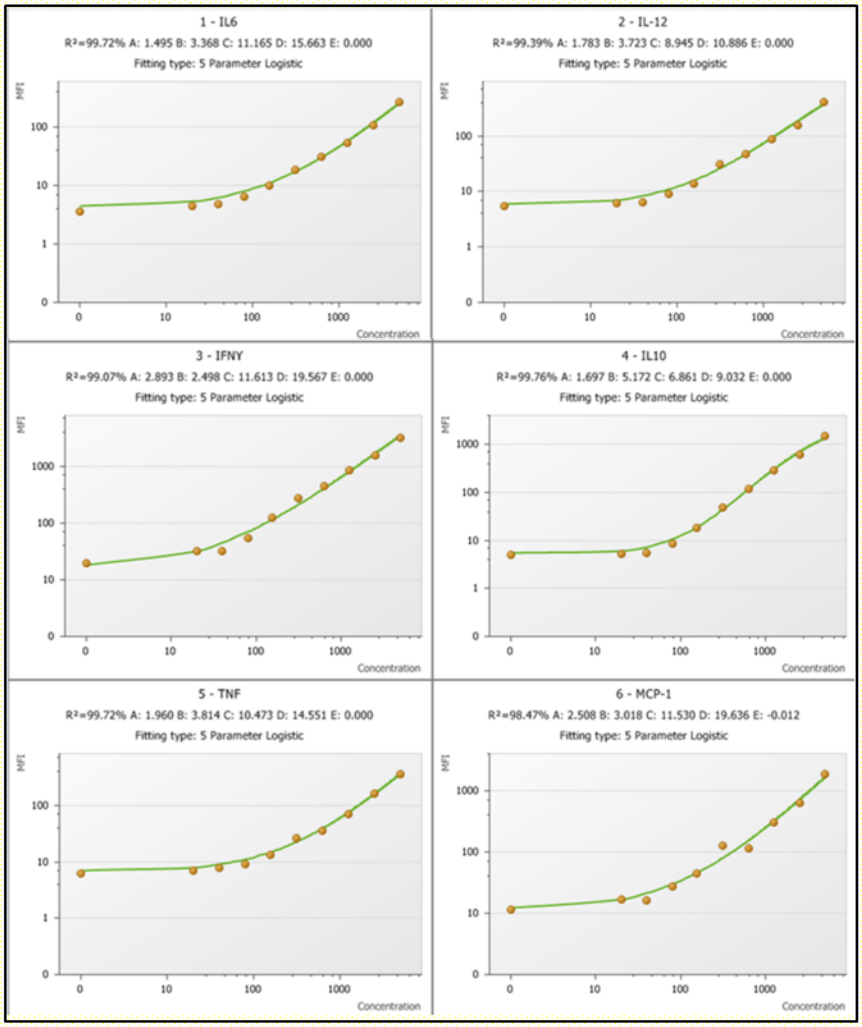

Figure 2.5. ***Cytometric bead array standard curves***. Curves were generated using FCAP array software for IL-6, IL-12, INF-γ, IL-10, TNF-α and MCP-1 with a range from 20 to 5,000 pg/mL.

2.3.5. Immunohistochemistry

2.3.5.1. Sample preparation

Before sectioning frozen brain samples were embedded in an aluminium foil mould filled with OCT (Sakura) and the OCT allowed to freeze on dry ice. Samples were then transferred to a cryostat and mounted using OCT before obtaining 12 µm sections along the coronal plane. Sections were transferred to positively charged microscope slides and stored at -20 °C.

2.3.5.2. Staining

Brain sections used to stain neutrophils were fixed for 30 min in acetone, while sections used for IBA-1 staining were first fixed in 4 % paraformaldehyde in PBS for 30 min before rinsing with PBS and undergoing endogenous peroxidase removal through a 15 min incubation in ice cold methanol containing 1 % H_2O_2. Slides were then rinsed in PBS before blocking with PBS + 2 % BSA + 4 % goat serum for 30 min at room temperature. Sections were then coated with primary antibody in antibody diluent (PBS + 1 % BSA + 2 % goat serum) and incubated overnight at 4 °C in a humidified chamber. Rat anti mouse Gr-1 (abcam ab2557) was used at 1:50 to stain neutrophils. Rabbit anti IBA-1 (Wako) was used to stain for activated microglia and was used at 1:200 in antibody diluent as determined by previously performed optimisation experiments. Negative control slides were incubated with antibody diluent only. Following incubation the primary antibody was removed and slides rinsed three times with PBS. Sections used for neutrophil staining were incubated in the dark for 45 min with Alexa Fluor® *488* Goat Anti-Rat (invitrogen) at 1:1000 before washing in PBS and mounting with vectorsheild + DAPI (Vectorlabs). Slides were then sealed with clear nail varnish and viewed under DAPI and FITC fluorescent channels. Sections used for microglial staining were incubated for 45 min with biotinylated goat anti-rabbit secondary antibody (Vectorlabs) used at a concentration of 1:200 in antibody diluent. Avidin Biotin Complex (ABC) solution (Vectorstain elite ABC kit, Vectorlabs) was then prepared by mixing 5 µl of reagent A and 5 µl of reagent B in 1 ml of ABC buffers as per the manufacturer's instructions. The ABC solution was then allowed to stand for 30 min for A-B complexes to form. The secondary antibody was then removed from the sections before washing the slides with PBS. 200 µl of ABC solution was applied to each section and incubated for 1 h at room temperature in a humidified chamber. The slides were then rinsed with

PBS before visualising antibody staining using 3, 3'-diaminobenzidine (Vector ImmPACT DAB peroxidase substrate, Vectorlabs) applied for 1.5 min. Reaction of the chromogen was stopped by removing developer from slides and placing them into distilled water. Following a brief rinse in distilled water, nuclear staining was achieved using Mayer's Haemalum solution (Vectorlabs), 1.5 min incubation followed by 5 min in running tap water. A 30 second incubation in 0.1 % $NaHCO_3$ in dH_20 was used as a blueing agent to help differentiate nuclear and antibody staining. Sections were dehydrated in increasing concentrations of ethanol (1 min in 70 %, 1 min in 90 %, 1 min in 100 %, 1 min in 100 %) followed by a 4 min incubation in xylenes and coverslip applied with DPX xylene based mount (VWR).

For analysis of staining, DAB positive cells for microglia or FITC fluorescent cells for neutrophils were counted in four randomly chosen fields of view at x40 magnification in four different brain regions across at least four brain sections from each animal, at least 50 µm apart, taken between 1 mm and 3 mm posterior to the bregma (Figure 2.6).

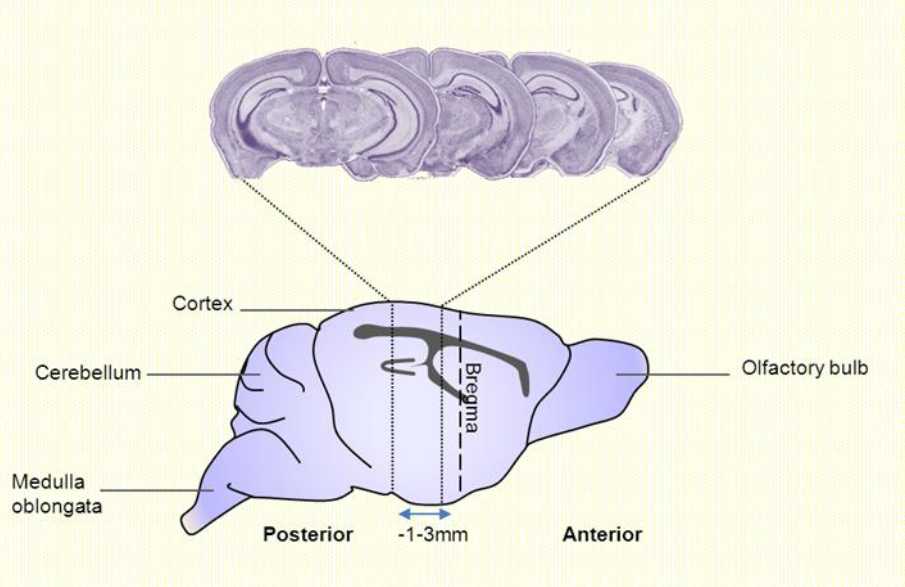

Figure 2.6. ***Location of mouse brain slices used for analysis of microglial activation*** Brain slices were taken between 1mm and 3mm posterior to the bregma

2.3.6. Quantitative RT-PCR

2.3.6.1 Brain and blood RNA isolation

Total RNA was isolated from homogenates of whole brain using an RNeasy Mini RNA purification kit (Qiagen). Briefly; samples were lysed and homogenised in a denaturing guanidine-thiocyanate–containing buffer and, following the addition of ethanol, the sample was applied to an RNeasy Mini spin column. Tubes were briefly centrifuged allowing total RNA to bind to the silica-based membrane, contaminants were removed during a series of wash steps and the RNA eluted in RNase-free water.

For whole blood RNA isolation, RNeasy® Protect Animal Blood Kit (Qiagen) was used following the manufacturer's instructions. In brief, blood cells were lysed and intracellular RNA stabilised through incubation in RNAprotect solution (Qiagen). Samples were then homogenized by centrifugation through QIAshredder spin columns before centrifuging in RNeasy MinElute columns, where total RNA was bound to the silica membrane. RNA was then eluted in RNase-free water. Concentration and purity of RNA was determined by measuring the absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (Labtech international). The ratio between the absorbance at 260 nm and 280 nm was used as an estimate of RNA purity and was found to be between 1.9 and 2.1 indicating an absence of contaminants.

2.3.6.2 qRT-PCR

Quantitative Real-Time PCR (qRT-PCR) was employed to measure the expression of $MC_{1\text{-}5}$, TNF- α and IκB-α mRNA in both brain and blood samples from experimental C57BL/6 mice. One step qRT-PCR was performed following the manufactures instructions using a QuantiFast SYBR green one step RT-PCR kit (Qiagen) with QuantiTect primer assays, containing forward and reverse primers for MC_1, MC_2, MC_3, MC_4 and MC_5, TNF-α and IκB-α (Qiagen) as detailed in table 2.4. A panel of reference genes, chosen using criteria set out by Radonic *et al.* [216] and cross referenced with Refgenes Genvestigator online database (Nebion) were tested and the coefficient of variance calculated for each reference gene. TATAA-box binding protein (TBP) was found to be the most suitable, showing no significant variation in expression across all samples tested, and was used in all subsequent calculations to adjust for uncontrolled variability between samples when determining relative gene expression.

Table 2.4: Primers used for qPCR.

Protein name	Gene name	Assay name	Cat No.
MC_1 receptor	*Mc1r*	Mm_Mc1r_SG	QT00305011
MC_2 receptor	*Mc2r*	Mm_Mc2r_2_SG	QT01066338
MC_3 receptor	*Mc3r*	Mm_Mc3r_1_SG	QT00264404
MC_4 receptor	*Mc4r*	Mm_Mc4r_1_SG	QT00280861
MC_5 receptor	*Mc5r*	Mm_Mc5r_1_SG	QT01166494
IκB-α	*Nfkbia*	Mm_Nfkbia_1_SG	QT00134421
TNF-α	*Tnf*	Mm_Tnf_1_SG	QT00104006
TBP	*Tbp*	Mm_Tbp_1_SG	QT00198443

All primers were obtained from Qiagen.

Reactions containing 50 ng of template RNA were run in triplicate using an MX3000P real time cycler (Stratagene) under the following cycler conditions: Reverse transcription at 50 °C for 10 min, Activation at 95 °C for 5 min then 40 cycles of: 95 °C for 10 seconds and 60 °C for 30 seconds for denaturation and combined annealing/extension. Fluorescence data collection was performed during the annealing/extension phase. SYBR Green I was detected at 521 nm with excitation at 494 nm. ROX dye, contained in the master mix, was used as a passive reference dye to compensate for non-PCR related changes in fluorescence and to provide a baseline to which the signal was normalised. Dissociation curves were performed at the end of each reaction confirming the presence of a single PCR product per reaction (Figure 2.7).

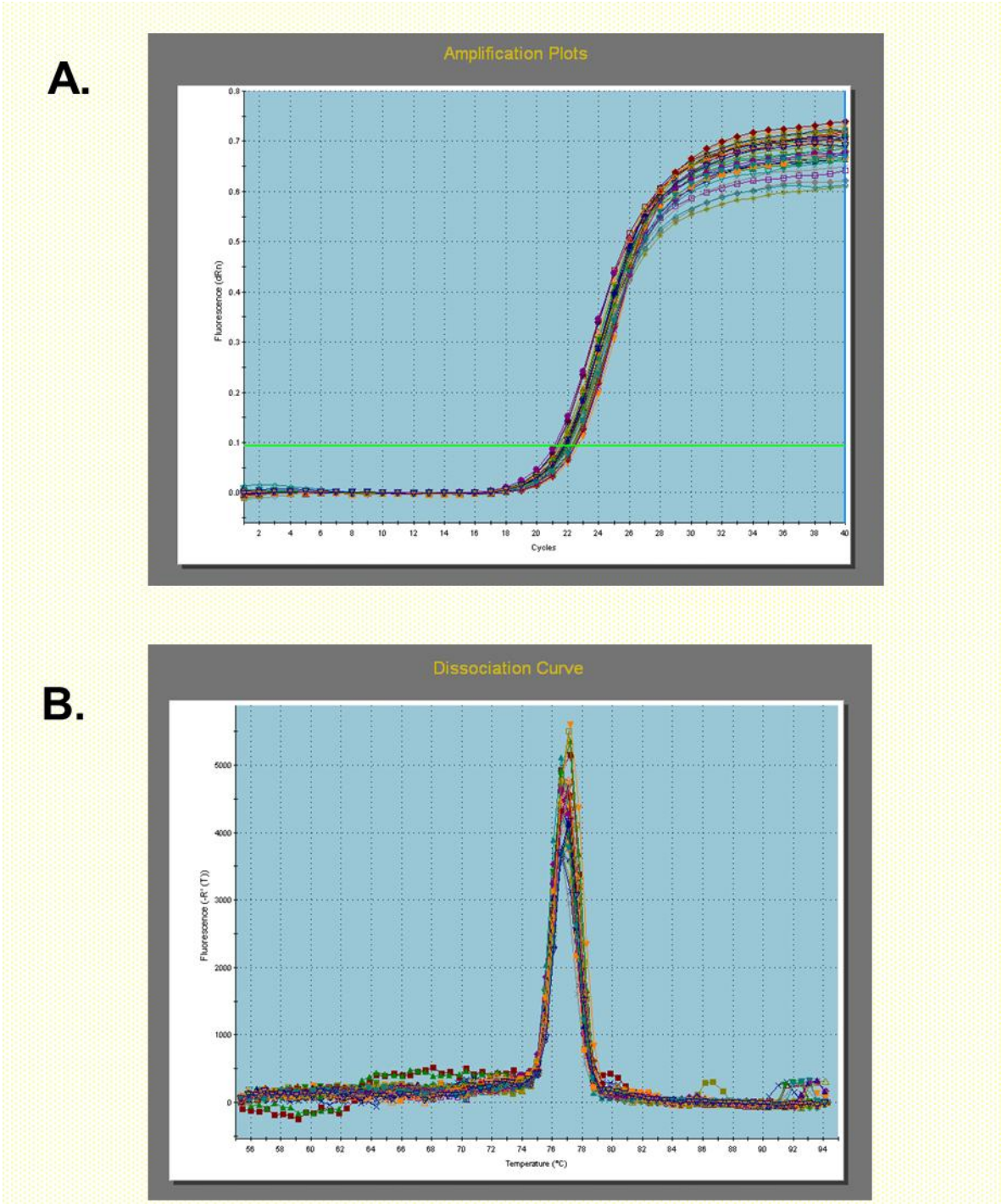

Figure 2.7. ***qPCR amplification plots and dissociation curve***. Successful amplification of a transcript showed a sigmoidal shaped amplification curve (A) with a geometric, linear and plateau phase. Amplification curves were plotted with ROX correction and the threshold was set to 0.1. CT values collected from upper part of the log-linear phase. B) Dissociation curves showing a single spike confirmed a single PCR product. Any amplification not displaying these characteristics was excluded from data analysis.

2.3.6.3 PCR efficiency

For relative quantification of gene expression using the ΔΔCT method the amplification efficiencies of the target and reference genes must be comparable. Thus the efficiency of the PCR reaction for each gene of interest was calculated by performing a standard curve using 100 ng, 10 ng, 1 ng and 0.1 ng of template RNA for each primer and run using the standard PCR conditions as detailed above. CT values were collected and the difference between CT of target and reference genes plotted against the logarithm of RNA loaded. A line of best fit was plotted using Microsoft Excel 2010 software and a slope of less than 0.1 for each target gene confirmed that the amplification efficiency was comparable and thus suitable for analysis using the ΔΔCT method (Figure 2.8).

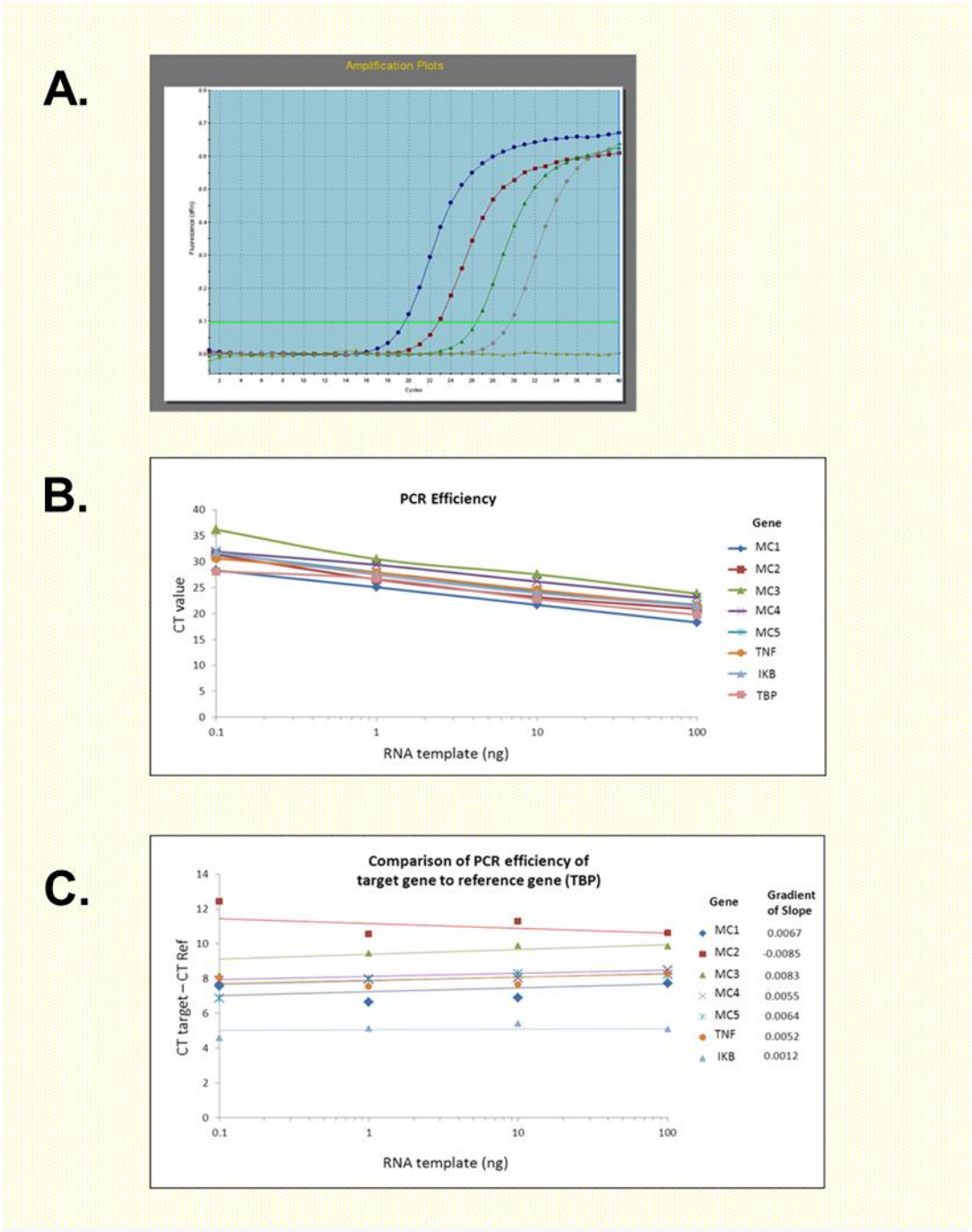

Figure 2.8. ***Comparing PCR efficiencies of target and reference gene.*** Different concentrations of RNA template were used to create standard curves for each primer set used. A) shows representative amplification curves for 100, 10, 1, 0.1 and 0ng of RNA template. B) shows the standard curves of each primer set used. C) To determine whether the chosen reference gene (*Tbp*) had a comparable PCR efficiency to the target genes, the difference between target and reference gene CT value was plotted against RNA concentration. All genes tested displayed a slope of less than 0.1 suggesting *Tbp* to be an appropriate reference gene for use in ΔΔCT gene expression analysis.

2.3.6.4 Data analysis of gene expression

Triplicate CT values for each sample were averaged and then the mean CT value taken for each treatment group. Data analysis was performed using the Comparative CT Method (ΔΔCT Method) as previously described [147] by using the following equation:

$$2^{-\Delta\Delta CT} = [(\text{CT gene of interest} - \text{CT reference gene})\text{Sample A}] - [(\text{CT gene of interest} - \text{CT reference gene})\text{Sample B}]$$

Where A is the treated group, B is the sham operated control group and $2^{-\Delta\Delta CT}$ = fold change. Data represents the mean ± SEM of the fold change in RNA expression compared to the sham operated control group.

2.3.7. Neutrophil isolation from human blood

Blood collection and leukocyte isolation was performed with ethical approval from the NHS Research Ethical Committee, reference number: 2009, 04/Q0401/40. Control samples were taken from healthy individuals with no history of recent acute or chronic illness from Hammersmith hospital, London for chemotaxis assays or at the William Harvey Research Institute, London for flow chamber assays. Blood was collected from the anterior cubital fossa with a 21G needle into a syringe containing 1 part of 3.2 % sodium citrate to every 9 parts of blood. All samples were collected and processed within 4 h.

2.3.7.1. Histopaque method

For chemotaxis assays neutrophils were isolated using a sodium diatrizoate polysucrose density gradient using Histopaque 1119 and histopaque 1077 (Sigma Aldrich). Blood samples mixed with equal volumes of RPMI medium (Sigma Aldrich) were then layered on top of the histopaque gradient and samples centrifuged at 1800 rpm for 30 min at room temperature. The resulting neutrophil rich layer was removed and mixed with equal volumes of RPMI. Samples were centrifuged at 1500 rpm for 15 min and the resulting supernatant discarded. Contaminating erythrocytes were lysed in 7.5 mL of ice cold water before 2.5 mL of 3.6 % saline was added to restore isotonicity. Samples were then centrifuged at 1800 rpm for 10 min, the supernatant aspirated and remaining pellet re-

suspended. Neutrophils contained in the resulting pellet Cells were counted in a haemocytometer using a 1 in 10 dilution in turks solution (Sigma Aldrich) and samples made to the desired concentration in RPMI for immediate use in the chemotaxis assay.

2.3.7.2. Dextran method

For flow chamber assays neutrophils were isolated using dextran sedimentation and gradient-centrifugation. Following collection blood samples were centrifuged at 800 rpm for 20 min at room temperature. The upper layer containing the platelet rich plasma was then removed and discarded before slowly adding 10 ml of PBS over the remaining layer. 8 ml of 6 % dextran solution (Sigma Aldrich) was then added before mixing by inversion. Cells were allowed to sediment at room temperature for 15 min. The resulting top layer was collected added to fresh 50 ml falcon tubes containing 10 ml of Histopaque 1077 (Sigma Aldrich). Samples were then centrifuged at 1500 rpm for 30 min. The resulting supernatant and buffy coat were aspirated off. The remaining pellet was then re-suspended and 9 ml of ice cold saline added before adding 1 ml of 10X hanks solution (Sigma Aldrich) to restore isotonicity. Samples were pooled and the volume made up to 40 ml with PBS before centrifuging at 1000 rpm for 10 min at room temperature. The supernatant was then aspirated off and the pellet re-suspended in 4 ml of PBS. Cells were then made up to a concentration of $1x10^7$ cells/ml using PBS and kept on ice until use

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2.3.8. Flow chamber assay

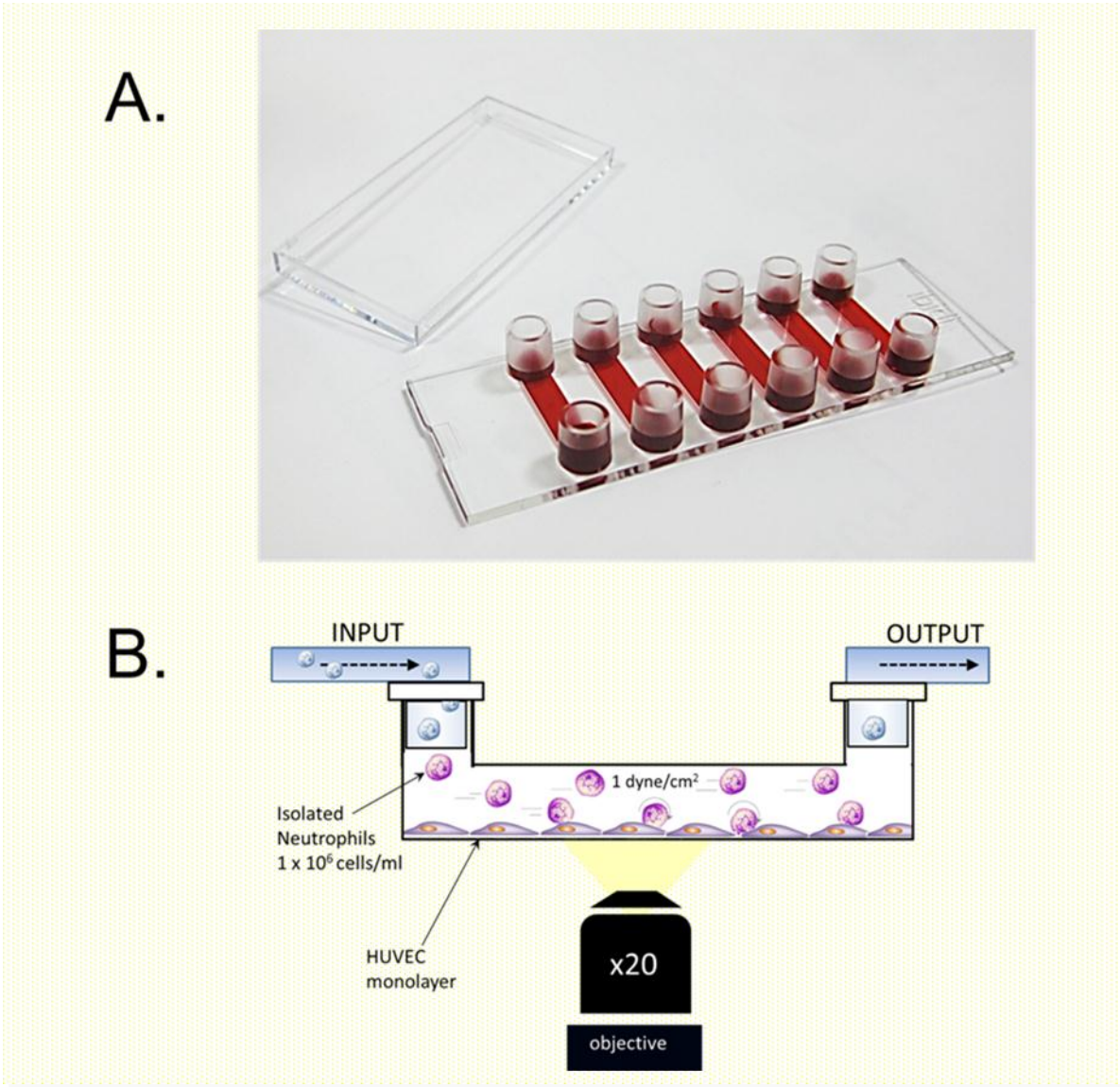

Figure 2.9. ***The IBIDI flow chamber system.*** A) Photograph of the disposable plastic IBIDI µ-Slide VI$^{0.4}$. B) Cross section diagram of one of the six flow chambers in the µ-Slide VI$^{0.4}$, showing how isolated blood cells may be perfused over an endothelial monolayer grown in the chamber. (P. Holloway, *unpublished*)

Primary human umbilical vein endothelial cells (HUVECs) were isolated at the William Harvey Research Institute, London by collagenase digestion. Cells were cultured at 37.5 °C, 5 % CO_2 and 90 % humidity in Endothelial Cell Growth Medium (ECGM) and used at passage 2 for all experiments. The day preceding the experiment, HUVECs were

detached using 0.05 % Trypsin/0.02 % EDTA solution (Sigma Aldrich) and seeded in µ-Slide VI$^{0.4}$ flow chamber slides (IBIDI) at a concentration of 2.5x10^6 cells/ml, 100 µl per channel. Cells were incubated overnight at 37.5 °C, 5 % CO_2 and 90 % humidity. On the day of the experiment 100 % confluence of the monolayer was confirmed microscopically, non-confluent cultures were not used. ECs were stimulated by spiking the media with TNF-α 10 ng/mL (R&D systems) and incubated for 4 h before commencing flow. For α-MSH treated ECs, the corresponding wells were treated with 1, 10 or 100 µg/ml of α-MSH, 2 h prior to flow, all other wells were treated with saline. Isolated neutrophils (1x10^7 cells/ml) were either pre-treated with saline or α-MSH at concentrations of 1 or 10 µg/ml for 30 min before flow. The flow chamber slide was positioned under a Nikon Eclipse TE3000 inverted microscope fitted with a x20 phase contrast objective (Nikon). The IBIDI µ VI 0.4 flow chamber slides were kept at a constant temperature during the course of the experiment using an ibidi Heating System (IBIDI) set to 37.5 °C. Ten min before flow, neutrophils, contained in 50 ml falcon tubes, were transferred to a water bath set at 37.5 °C. Immediately before flow commenced isolated human neutrophils were diluted 1 in 10 from the stock concentration with Dulbecco's PBS supplemented with Ca^{2+} and Mg^{2+} containing 0.1 % BSA PBS + Ca^{2+} + Mg^{2+} (1 M) to give a final concentration of 1x10^6 cells/ml. Neutrophils were flowed over the endothelial monolayer using a programmable 6 syringe pump (Stoelting) to provide a shear stress of 1 dyne/cm^2. The flow required to expose cells to this shear stress was calculated using the below calculation, assuming a laminar flow profile in a µ-Slide VI$^{0.4}$ flow slide.

$$\text{Flow rate [ml/min]} = \frac{\text{Shear Stress [dyne/cm}^2\text{]}}{\text{Viscosity of medium [dyn.s/cm}^2\text{]} \times 176.1}$$

Initial flow was maintained for 8 min, then at least 3 random fields per treatment were recorded for 10 sec each. The number of rolling, firmly adherent (cells stationary for 10 sec or longer) and transmigrated cells (those changing from bright phase to dark phase) were quantified during offline analysis using Image-Pro Plus software (Media Cybernetics). These parameters were then given as an average number of cells per 10 second frame at x 20 magnification.

2.3.9. Chemotaxis

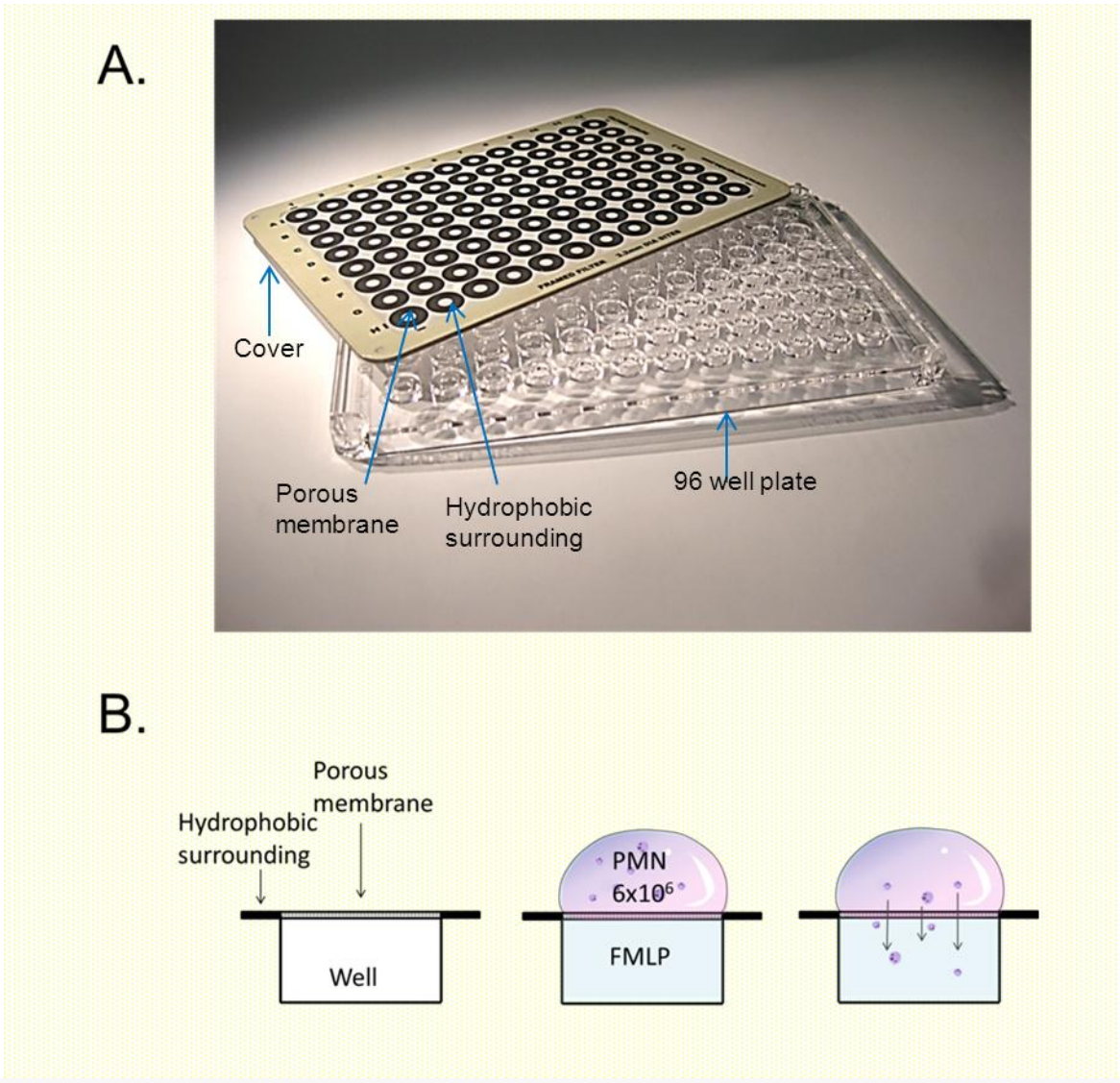

Figure 2.10. ***The chemotaxis assay.*** A) Photograph of the neuroprobe™ chemotaxis plate, showing the 96 well plate and detachable membrane cover. B) Cross section diagram of individual wells of the neuroprobe chemotaxis plate. A chemoattractant is placed in the bottom well before attaching the membrane cover. Isolated leukocytes are then pipetted onto the upper surface of the membrane, hydrophobic surrounds prevent liquid from spilling into neighbouring wells. The plate is then incubated to allow chemotactic migration across the membrane. The membrane is then removed and cells that have migrated into the well are stained and counted. (P. Holloway, *unpublished*)

Chemotaxis assays were performed using neuroprobe 96 well disposable plates (Neuro Probe Inc.) with 3 μm pore size. Neutrophils isolated from human blood using the histopaque method as described above, were used at a concentration of 4 x 10^6 cells/ml, previously determined as the optimum concentration for this assay during initial calibration experiments performed in our laboratory. Cells were incubated for 10 min at 37.5 °C, 5 % CO_2 either with RPMI alone or α-MSH (0.1, 1.0 or 10 μg/ml) in RMPI. FMLP 10^{-6} M in RPMI + 0.1 % BSA was used as a chemo-attractant and placed in the bottom wells (27 μl per well), whilst RPMI + 0.1 % BSA was used as a control. The top chamber was filled with 25 μL of neutrophils. Cells were incubated for 1.5 h at 37.5 °C in 5 % CO_2. Following incubation the remaining fluid was removed from the top wells using cotton buds. 25 μL of ice cold EDTA .was then applied to the membrane, left for 5 min before being removed. Plates were centrifuged at 1500 rpm for 5 min. The number of migrated cells in each well was then counted in a haemocytometer using Turks solution.

2.4. Statistics

Results are expressed as mean ± one standard error. Data was analysed either by ANOVA with Bonferroni test for post hoc analyses or student T-test for comparison of only two groups, using Graph pad prism5 computer software. $p<0.05$ was considered to be statistically significant.

Group sizes for *in vivo* experiments were determined based on power calculations performed using G* power 3 software (Heinrich Heine University of Dusseldorf) using an alpha value of 0.05 and effect size of 1.04. Effect size (δ) was obtained from preliminary experiments to this project and calculated using the maximum likelihood estimator of Cohen's δ. Calculations to determine δ are given in appendix page 196. To obtain statistical power of 0.8, thus giving an 80% chance of correctly rejecting the null hypothesis, avoiding a type II error, to determine a difference between two groups, a minimum of 4 animals was calculated to be required in *in vivo* BCCAo experiments and as such a minimum n of 4 has been used for *in vivo* experiments, unless specifically stated otherwise (section 5.2.3). Power calculations have not been used for *in vitro* experiments for which a minimum n of 3 has been used.

3.0. Characterising the inflammatory response in the BCCAo murine model of Global stroke

3.1. Introduction

During the course of this project the bilateral common carotid artery occlusion (BCCAo) murine model of global stroke has been utilised to investigate the effects of the melanocortins on leukocyte endothelial interactions following stroke.

Inflammation is a major feature in the pathology of a wide variety of diseases and as such numerous methods have been used to obtain inflammatory read-outs when assessing pathophysiology and potential therapeutics. Leukocyte recruitment has frequently been used as a quantitative parameter to assess the cellular inflammatory response. Leukocyte recruitment has been investigated through their accumulation and the production of local oedema in; the synovial space in arthritic models, peritoneal cavity in models of peritonitis and also in inflamed air pouch models [217, 218]. Utilising intravital microscopy however enables direct, real-time measurement of leukocyte and endothelial cell activation and behaviour localised to a specific micro-environment. Leukocyte recruitment is governed by an exquisitely complex web of inflammatory signalling events and as such IVM may provide an insight into the inflammatory milieu through the measurement of just a few parameters (rolling, velocity, adhesion and in some preparations emigration) [219].

During this chapter IVM has been used along with traditional IHC and cytokine analysis to gain an insight into cellular inflammatory events and characterise the acute neuro-inflammatory response following global cerebral I/R (by BCCAo) to enable the subsequent investigation of the melanocortin receptor system (MRS) as a potential intervention.

3.2. Results

3.2.1. BCCAo causes an increase in leukocyte endothelial cell interactions in the cerebral microcirculation

The effects of cerebral I/R injury on leukocyte recruitment in the cerebral microvasculature was investigated by inducing 5 minutes of global blood flow occlusion by BCCAo before allowing for varying periods of reperfusion and visualising leukocyte recruitment in the pial post capillary venules using IVM (Figure 3.1).

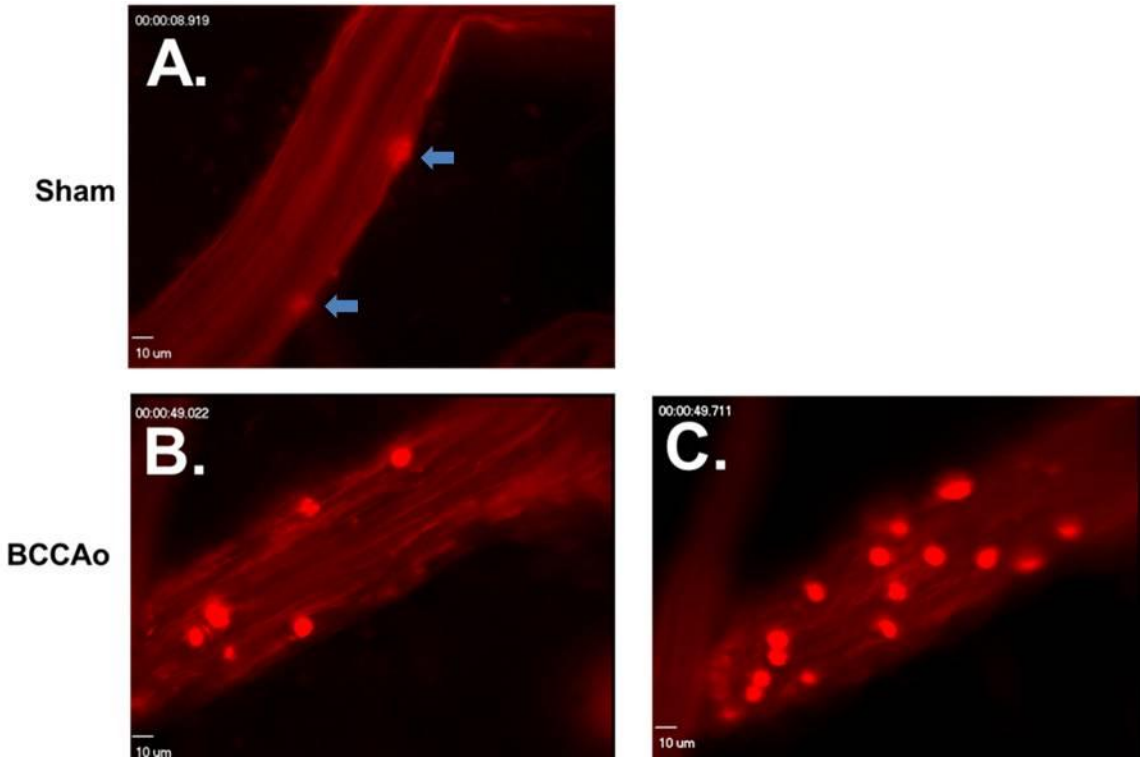

Figure 3.1 ***BCCAo increases leukocyte recruitment in the cerebral microcirculation.*** (A) In sham operated animals relatively few leukocytes (marked by blue arrows) could be seen interacting with the endothelium of post-capillary venules. (B) 5 min of ischemia produced by BCCAo followed by 40 min of reperfusion was sufficient to incur a large increase in leukocyte-endothelial interactions. (C) Allowing for 2 h of reperfusion showed a further increase in cell adhesion

Leukocyte recruitment was quantified in terms of cell rolling, rolling velocity and adhesion (Figure 3.2). While sham surgery, omitting vessel occlusion, produced little to no leukocyte recruitment (rolling = 21.5 ± 6.9 cells/mm^2/min, adherence = 42.1 ± 14.4 cells/mm^2/min) 5 min of BCCAo was found to induce significant increases in both leukocyte rolling (191.0 ± 31.49 cells/mm^2/min) and adherence (282.3 ± 49.4 cells/mm^2/min) by 40 minutes of reperfusion (earliest time point tested). The average velocity of the rolling cells observed also showed a trend toward a reduction following BCCAo. Reperfusion time was then extended to 2 h in this model to investigate the progression of inflammatory processes. This resulted in a further increase in leukocyte-endothelium interactions with adhesion significantly increasing to 659.7 ± 115.6 cells/mm^2/min, approximately 1500 % the level of adhesion observed in sham animals. While no further statistically significant increase in rolling was observed when reperfusion was extended to 2 h, there was an apparent trend toward an increase. Velocity of these cells remained low in the BCCAo 2 h reperfusion group but was not significantly reduced in comparison to sham operated groups. In sham operated animals left for 2 h before IVM imaging, all four IVM parameters of rolling, WBC velocity and adhesion remained unchanged when compared to the 40 min sham group ruling out the possibility that the enhanced leukocyte recruitment in the BCCAo 2 h group was due to extended time following the beginning of surgery.

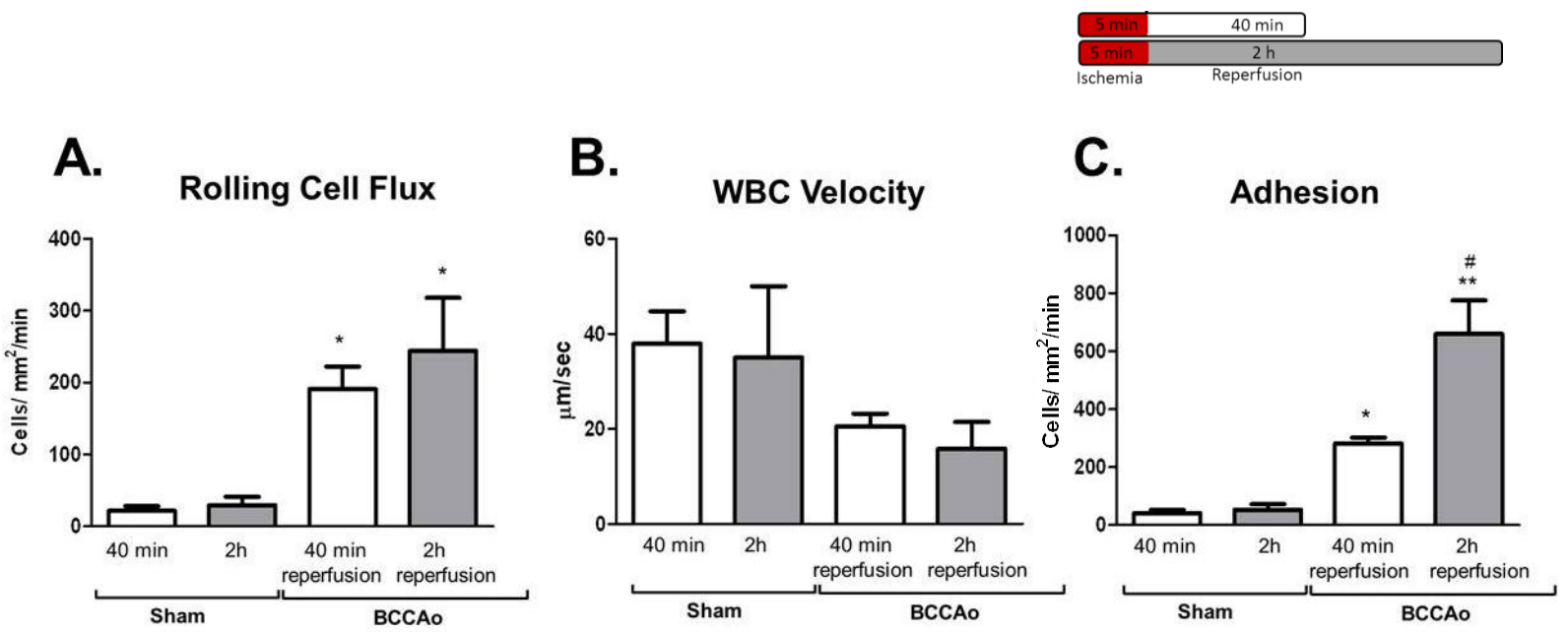

Figure 3.2. ***Leukocyte recruitment increases with time of reperfusion.*** Histograms showing leukocyte endothelial interactions as assed by IVM following 5 min BCCAo and either 40 min (white bars) or 2h (grey bars) of reperfusion. Leukocyte recruitment was quantified in terms of (A) the number of cells rolling along the vessel wall (rolling cell flux) (B) the speed at which those cells roll (WBC velocity) and (C) those cells stationary for 30 second or longer (Adhesion). Values represent mean +/- SEM. BCCAo 40 min reperfusion groups n = 6. 2h reperfusion groups n = 4. * denotes significance to sham P<0.01 ** = P<0.001. # denotes significance to BCCAo 40 min reperfusion group. Results from Sham and BCCAo animals shown here have subsequently been used as saline treated (at the start of reperfusion) controls for pharmacological investigations in section 4.0.

3.2.2. Experimental depletion of neutrophils in the BCCAo model

The rhodamine 6G die used to fluorescently label leukocytes during IVM is non-specifically taken up by all leukocyte populations, thus in order to determine whether the cells found interacting with the endothelium in this model were neutrophils (as supported by previous investigations), a sub set of mice were experimentally depleted of neutrophils before undergoing BCCAo surgery. In these animals, neutrophils were found to be depleted by approximately 84 % when compared to levels prior to treatment with anti-PMN serum (see appendix figure A1 and Table A1 for white blood cell counts). While the serum was supplied as an anti-PMN serum, levels of other PMN (eosinophils and basophils) remained at a relatively low level (0.5 -1 % of the total WBC population) and was unchanged following anti-PMN treatment. The absolute levels of other blood born immune cells also showed no significant changes following the PMN depletion treatment regime, thus this protocol can be regarded as specifically depleting neutrophils. Control animals were treated with IgG matched control serum and total WBC counts and differential WBC counts showed no significant changes compared to pre-treatment levels.

When subjected to BCCAo it was found that the number of cells found recruited to the pial venules of neutrophil depleted mice was significantly attenuated compared to IgG control treated mice (Figure 3.3). Leukocyte rolling was found to be reduced by 81 % and adhesion by 76 %. This is consistent with the majority or all of those cells observed interacting with the vessel wall being neutrophils.

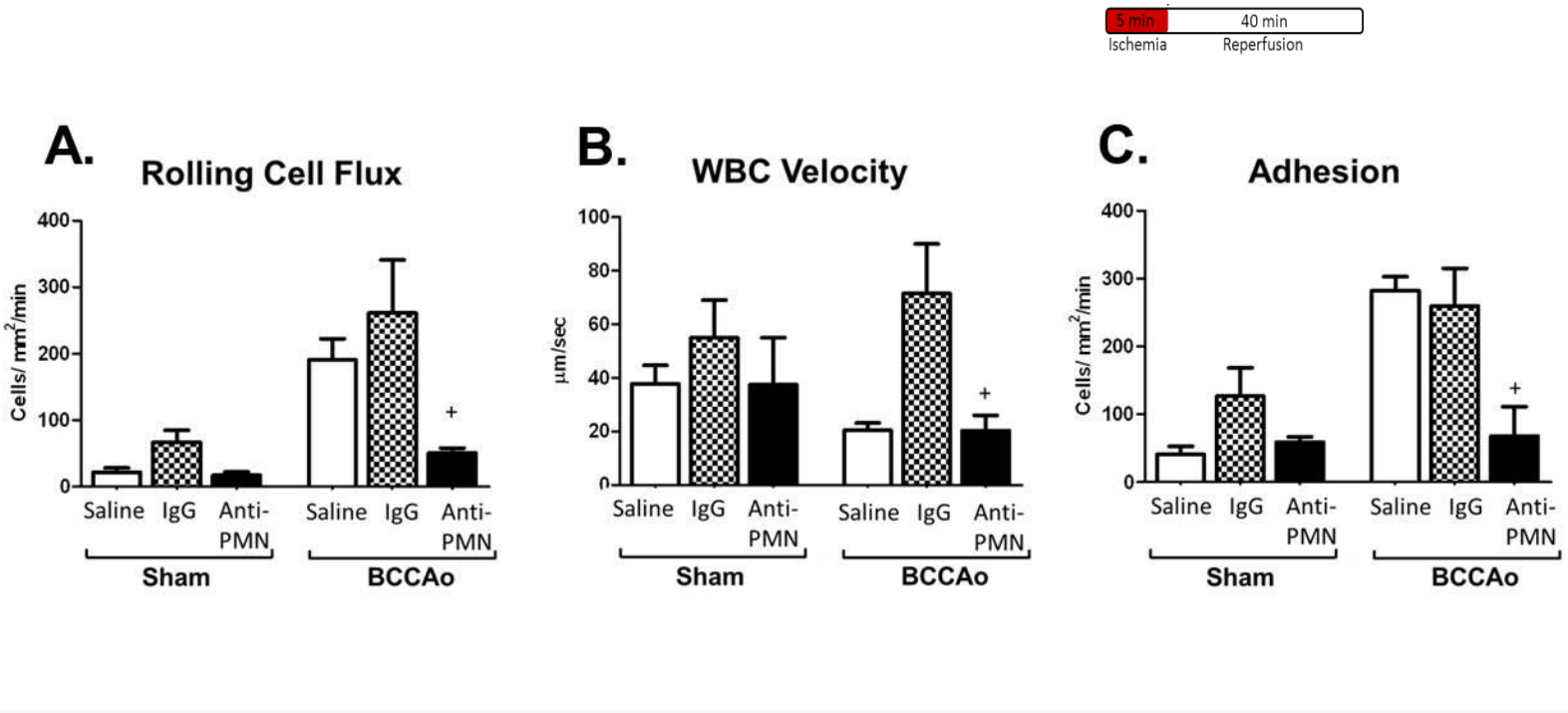

Figure 3.3. ***PMN depletion prevents leukocyte recruitment following BCCAo.*** C57BL/6 mice were treated with anti-PMN serum to deplete circulating neutrophils (Black bars) for two days before BCCAo or sham surgery. IgG matched serum treated mice were used as a control (checked bars). Following surgery IVM was used to assess the rolling (A) velocity (B) and adhesion (C) of leukocytes. Depleting mice of neutrophils was found to significantly inhibit the number of observed leukocytes interacting with venules of the cerebral microcirculation. Values represent mean +/- SEM. All IgG and anti-PMN treated groups n = 4. + denotes statistical significance to IgG group $P<0.01$.

3.2.3. BBB integrity and brain oedema.

The BBB confers a restrictive phenotype that is, under normal conditions, impermeable to macro molecules such as albumin. However under certain pathological inflammatory conditions the BBB can become compromised and allow un-regulated diffusion of molecules and plasma protein leakage into the surrounding parenchyma causing brain oedema. In order to evaluate the effect of the BCCAo model on BBB integrity and permeability FITC conjugated albumin was injected i.v and pial venules analysed for leakage of the fluorescent signal outside of the vessel using IVM. Brain oedema was also investigated by measuring the brain water content using the wet/dry method. Following 5 min of BCCAo at both 40 min and 2 h of reperfusion no significant BBB permeability was observed with the entire fluorescent signal being retained within the vessels observed (Figure 3.4 A). Furthermore no elevation in brain water content (indicative of oedema) was detected, suggesting that at the time points tested there was no breakdown of the BBB (Figure 3.4 B).

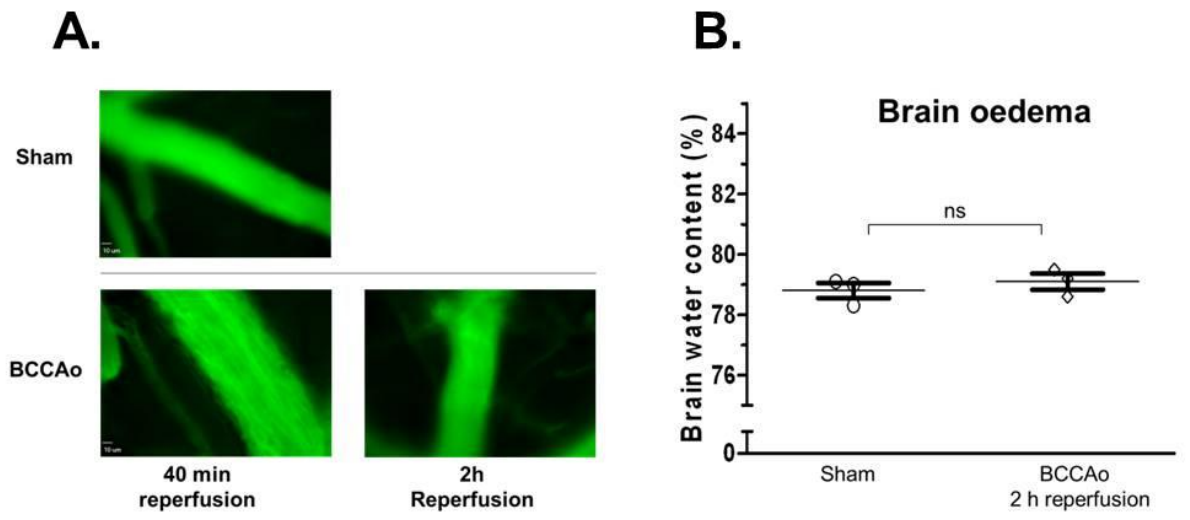

Figure 3.4. ***BBB integrity and brain oedema.*** A) representative fluorescent IVM images of cerebral venules following i.v injection of FITC labelled albumin. Vessels show no significant leakage of the dye in sham or BCCAo treated animals at both 40 min and 2h. B) Brain oedema was investigated using the wet/dry method following 5 min of BCCAo and 2h of reperfusion. No significant increases in brain water content were observed compared to sham operated animals.

3.2.4. Investigating brain neutrophil infiltrates

Previous investigations in our lab (H. K. Smith pending publication) have used the myeloperoxidase (MPO) assay as an indirect method to quantify brain PMN content. These investigations have demonstrated no significant increases in MPO enzyme activity in the brain in this BCCAo model indicative of a lack of neutrophil extravasation. To confirm this IHC has been employed to directly stain for neutrophils present in the brain parenchyma (Figure 3.5). The specificity of the antibody used (Rat anti-mouse Gr-1, AB2557, Abcam) was confirmed in blood smears where cells with multilobulated nuclei were specifically stained, with approximately 10 % of all nucleated cells being stained as is consistent with previously reported C57BL/6 blood neutrophil content (5-20 %). However across 10 sections taken between 1 mm and 3 mm posterior to the bregma in all mice tested, no positively stained neutrophils were observed thus supporting the previous findings.

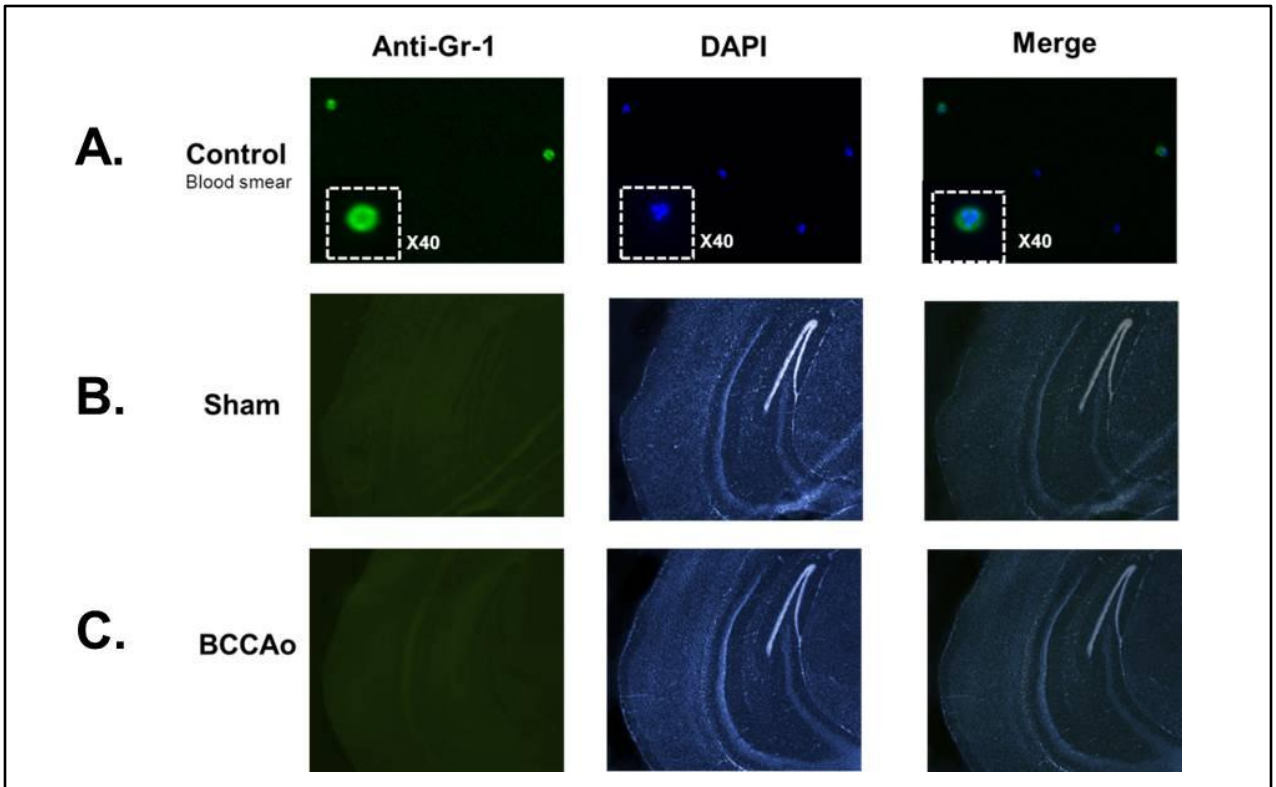

Figure 3.5. ***Neutrophil IHC in brain sections.*** 12µm frozen brain sections were stained with anti-Gr-1 antibody to stain for neutrophil. No Gr-1 positive cells were observed across 10 sections for each group tested n=3. A) Representative images of blood smears taken with a x10 objective show specific staining of neutrophils using the anti-Gr-1 antibody. Only cells with lobed nuclei were stained (x40 magnified inserts). (B,C) Representative x10 images of brain slices from (B) sham operated and (C) BCCAo 2h reperfusion c57BL/6 mice.

3.2.5. Microglial activation

To investigate the activation of the main immune competent brain resident cells, the microglia, preliminary experiments have used IBA-1 immuno-fluorescent staining in frozen brain sections with an IBA-1 positive cell count being performed in 4 different brain regions (Figure 3.6). At 40 min following BCCAo no significant changes in number or morphology of IBA-1 positive microglia were detected when compared to sham operated animals. However by 2 h of reperfusion a small but statistically significant increase in IBA-1 staining was observed in the hippocampus. These cells were typically found to display a more bushy morphology with shorter, thicker processes than the ramified cells found in sham operated animals (micrograph insert figure 3.6), these changes are indicative of the early stages of activation. However in the other areas of the brain assessed for IBA-1 staining no increases were detected following BCCAo

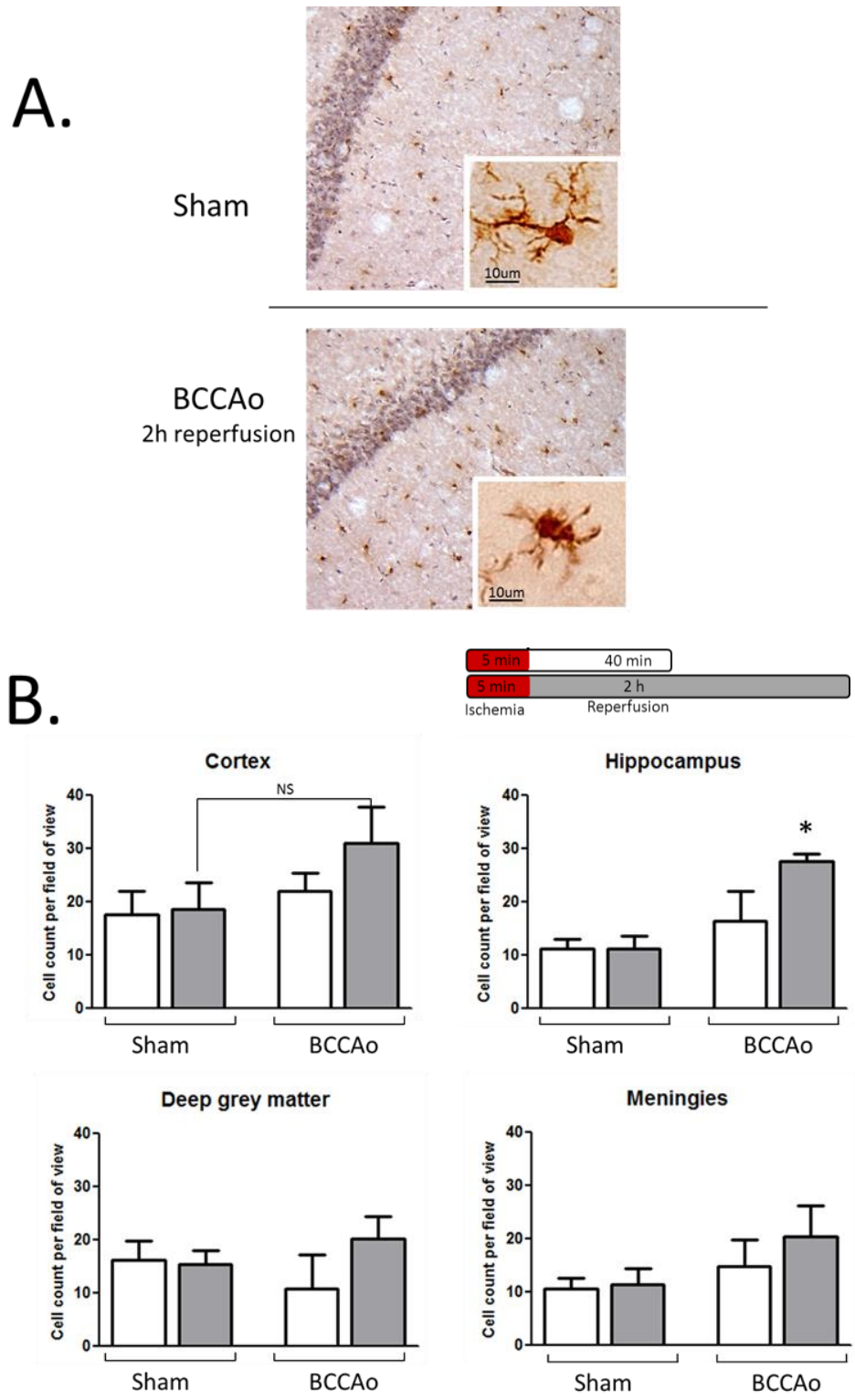

Figure 3.6. ***Microglial activation following BCCAo.*** BCCAo was found to induce a significant increase in IBA-1 positive cells in the hippocampus following 2 h of reperfusion. A) representative x20 micrographs on IBA-1 stained brain sections from sham and BCCAo 2 h reperfusion mice. B) Histograms representing mean ± SEM of IBA-1 positive cell counts across four different brain regions assessed. Only in the hippocampal region of the brain was a statistically significant increase in IBA-1 expression detected. N=3 for all groups. * denotes significance to sham $P < 0.05$.

3.2.6. Brain COX-2 expression

In order to further investigate the inflammatory environment in the brain parenchyma following BCCAo levels of the key inflammatory enzyme COX-2 were assessed in the brain by western blot analysis of whole brain homogenates (Figure 3.7). While there was a slight tend toward increased brain COX-2 by 2 h of reperfusion, this was not statistically significant and no conclusions can be drawn from the current results. As such further studies will need to be conducted with greater n numbers to reveal whether there is indeed any change in COX-2 expression by 2 h reperfusion.

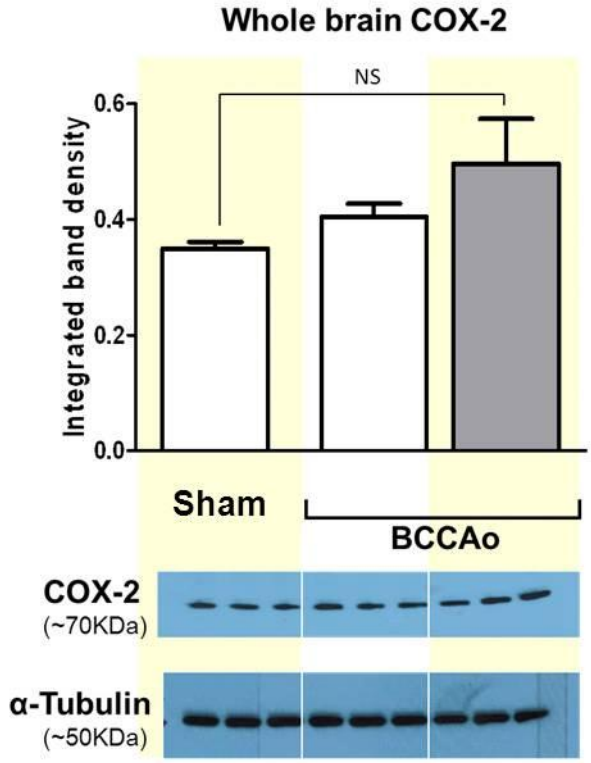

Figure 3.7. ***Western blot analysis of whole brain COX-2 expression.*** Whole brain homogenates from C57BL/6 mice subjected to sham surgery or BCCAo with 40 min (white bars) or 2h (grey bars) of reperfusion were assessed for COX-2 expression by western blot analysis. Histogram and representative blots of three separate western blot experiments. For each group n=3

3.2.7. Cytokine response to BCCAo

In order to investigate the effects of BCCAo on circulating levels of soluble inflammatory signalling molecules, an ELISA for the key inflammatory mediator TNF-α was performed along with multi-cytokine analysis using the cytometric bead array (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70). minutes following 5 min of BCCAo with levels remaining below the reliable detectable range of the kit (defined as the first statistically significant change on the standard curve). However by 2h of reperfusion TNF-α levels were significantly elevated, above the detection limit, with levels rising to 11.3 pg/ml compared to the control value of 4.2pg/ml (Figure 3.8).

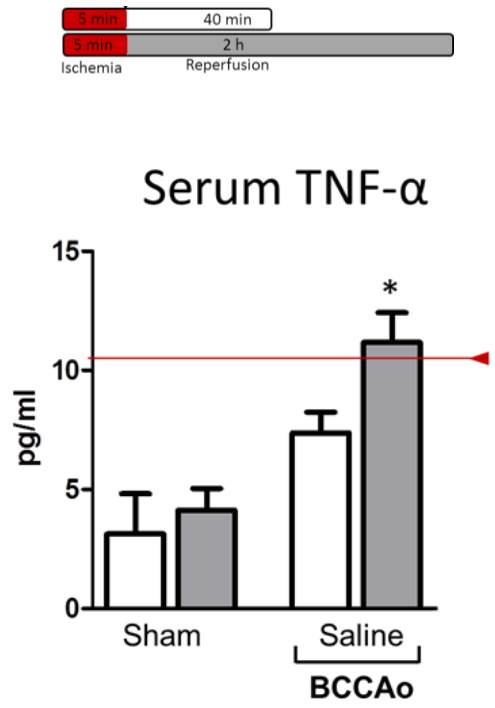

Figure 3.8. Serum *TNF-α.concentrations detected by ELISA* TNF-α levels in serum samples from C57BL/6 mice subjected to sham surgery or BCCAo with 40 min (white bars) or 2h (grey bars) of reperfusion. N= 3 for all groups. * denotes significance to sham P<0.01. Red line indicates reliable detection limit of the kit.

Cytometric bead arrays (CBA) showed no detectable level of IL-12p70 or IFN-γ protein expression across all samples tested. While low levels of IL-6 were detected in serum samples (at the lower detection range of the kit used), these remained unchanged following BCCAo. MCP-1 was undetectable in both sham groups but was increased to a statistically significant level following BCCAo and 2 h of reperfusion to 49.94 pg/ml, this was however still below the limit of detection of the CBA as stated in the manufacturers guidelines (52.7 pg/ml). TNF-α levels detected by the CBA supported the results from the quantikine ELISA, showing a significant increase following BCCAo 2 h reperfusion to 17.69 pg/ml. The anti-inflammatory cytokine IL-10 was at levels below the detectable range in sham operated and BCCAo 40 min groups nevertheless by 2 h of reperfusion there was an induction of IL-10 expression to 34.7 pg/ml which however was not statistically significant (P>0.05).

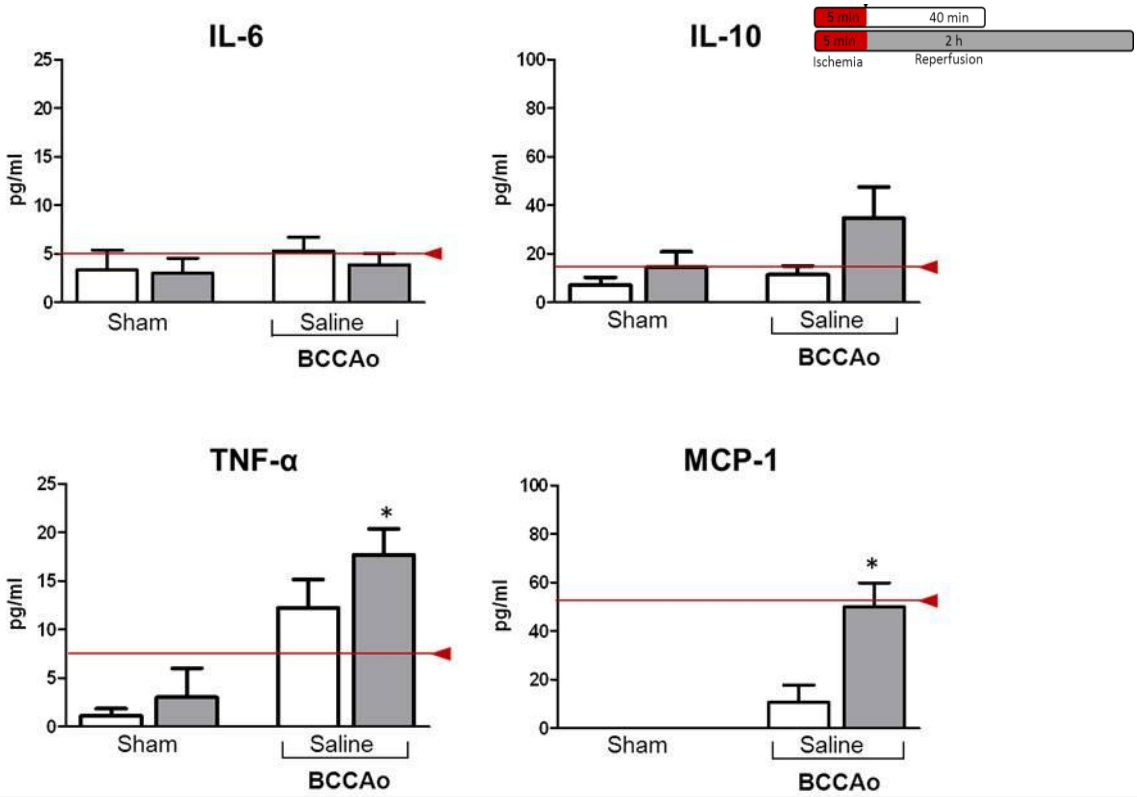

Figure 3.9. ***Serum cytokine levels detected by CBA***. Levels of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 protein were analysed using the CBA in serum samples from C57BL/6 mice subjected to Sham surgery or BCCAo with reperfusion times of 40 min or 2h. No detectable levels of IL-12p70 or IFN-γ were detected across all samples tested. Histograms represent mean ±SEM of (A) IL-6 (B) IL-10 (C) MCP (D) TNF-α. Red lines represent the reliable limit of detection for each cytokine tested. N=3 for all groups. P<0.01

3.2.8. Melanocortin receptor expression

To allow for an investigation into the MRS as a potential therapeutic target in this murine model, expression levels of each of the melanocortin receptors were assessed in both the whole brain and blood of C57BL/6 mice. Expression was tested at both 40 min and 2 h of reperfusion along with BCCAo mice treated with the endogenous melanocortin peptide α-MSH at the start of reperfusion, to test for any ligand mediated change in receptor expression. Western blot analysis initially confirmed MC_1 and MC_3 expression in brain showing consistent expression levels across all treatments (appendix figure A2). However, upon using tissues from $MC_3^{-/-}$ mice as negative controls, no difference in staining using the MC_3 antibody (Sigma Aldrich M4937) was observed between WT and $MC_3^{-/-}$ tissues. PCR confirmed absence of the MC_3 transcript in the $MC_3^{-/-}$ mice (appendix figure A3). Similar results were obtained when using an alternative MC_3 antibody from a different supplier (Acris antibodies AP10124PU-N). Thus suggesting both MC_3 antibodies tested display non-specific binding to protein at the same molecular weight as predicted for the MC_3. In the absence of a specific antibody for the MC_3 qRT-PCR was used to quantify the expression of all 5 melanocortin receptors at the mRNA level. qRT-PCR analysis of RNA isolated from blood confirmed the presence of all MC_1 MC_3 MC_4 and MC_5 but failed to detect any MC_2 transcript (figure 3.10). No significant changes in receptor expression were detected across all treatments for MC_1, MC_3 and MC_5. However the MC_4 transcript was found to be significantly decreased following α-MSH treatment at 40min of reperfusion, showing an approximate 5 fold reduction compared to the 40min sham operated group. However MC_4 levels did not remain suppressed and α-MSH treated groups showed no significant difference at 2 h reperfusion to the saline treated group.

qRT-PCR revealed mRNA expression of all 5 melanocortin receptors in C57BL/6 brains (Figure 3.11), with CT values suggesting MC_4 to be by far the most abundant transcript (data not shown). Relative quantification of MC expression showed no significant changes in receptor expression in response to all treatments tested, with invariant MC_1 and MC_3 expression being consistent with the results from western blot analysis.

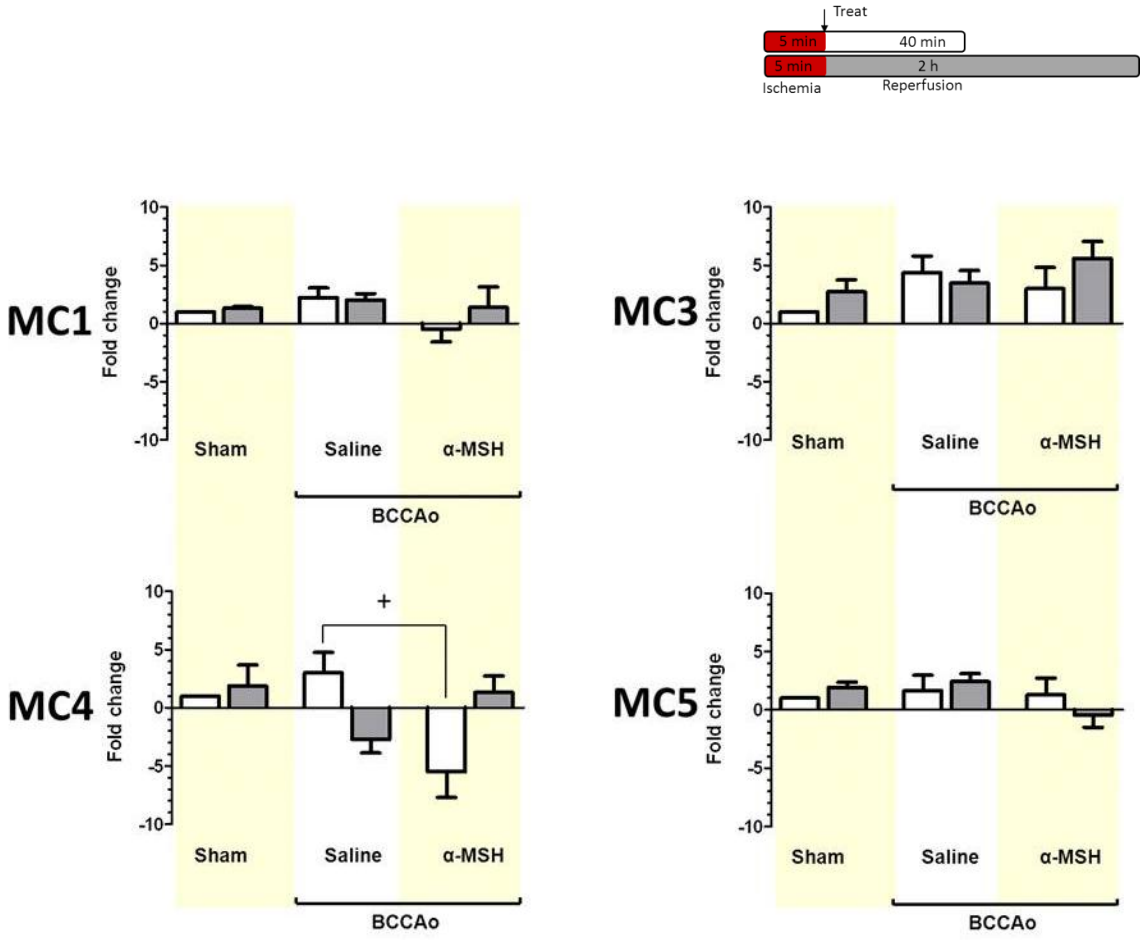

Figure 3.10. ***Total blood MC mRNA expression.*** Total blood RNA was isolated from whole blood of C57BL/6 mice subjected to Sham surgery, BCCAo saline treated or BCCAo α-MSH treated (10µg i.p at the start of reperfusion) with reperfusion times of 40 min or 2h. qRT-PCR used to quantify the mRNA levels of $MC_{1\text{-}5}$. Values represent mean ± SEM of fold change in expression compared to 40 min sham operated control. No MC_2 mRNA was detected in blood. N = 3 for all groups. + denotes statistical significance P<0.01

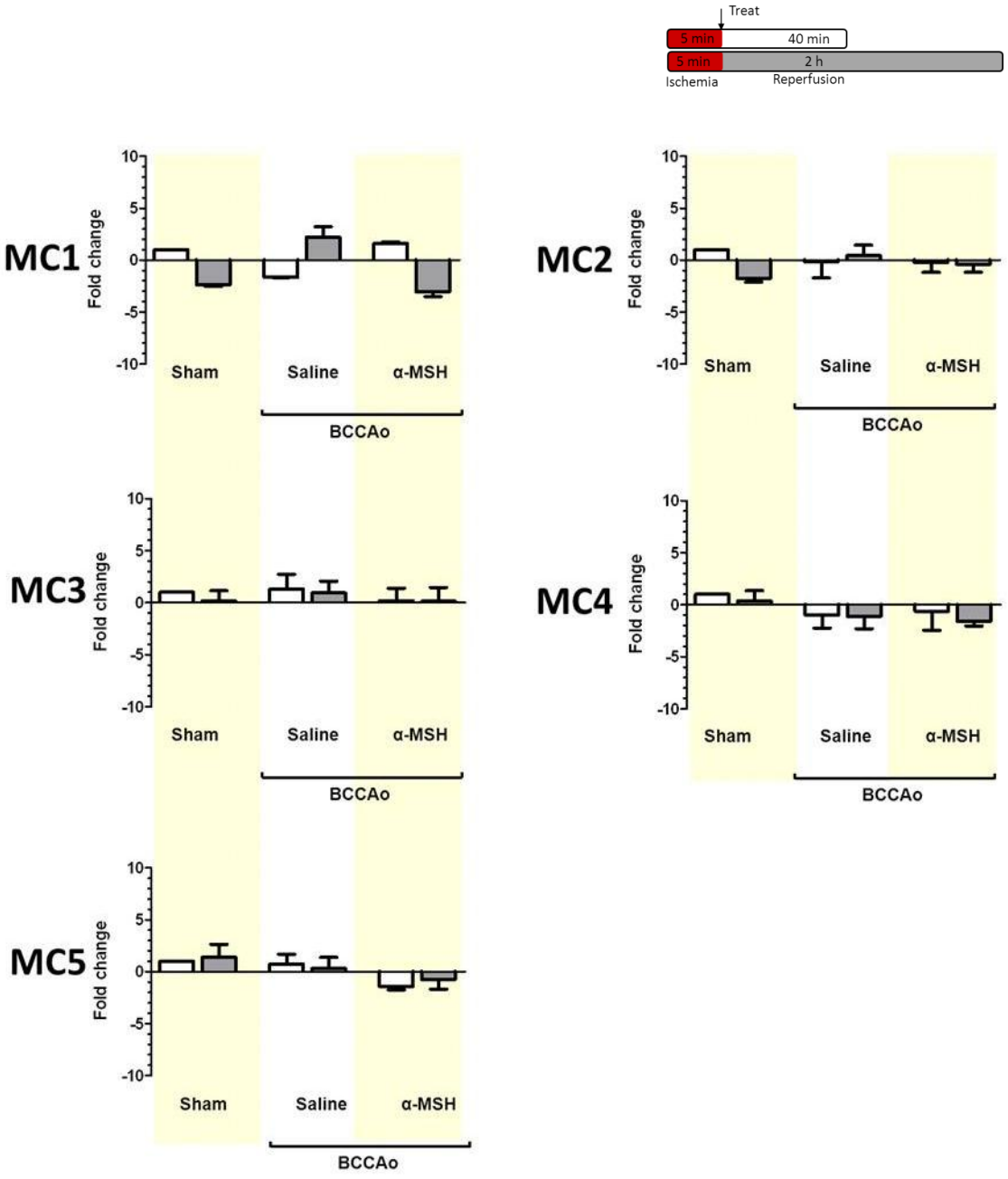

Figure 3.11. ***Total brain MC mRNA expression*** Total brain RNA isolated from experimental animals was assessed for $MC_{1\text{-}5}$ mRNA using qRT-PCR. Values represent mean ± SEM of fold change in expression compared to 40 min sham operated control. N = 3 for all groups.

3.3. Discussion

The aim of these investigations were to validate and characterise the leukocyte recruitment response in a murine model of global cerebral I/R injury. Experiments using intravital microscopy to visualise leukocyte-endothelial interactions in the pial venules of the brain demonstrated that as early as 40 minutes following 5 minutes of global ischemia there is a significant increase in leukocyte recruitment (Figure 3.1 and 3.2). While rhodamine 6G fluorescent labelling of cells is unable to distinguish between leukocyte sub populations, neutrophil depleting experiments demonstrated that, in line with previous investigations, at this acute phase of the inflammatory response the recruited cells consisted largely or even entirely of neutrophils (Figure 3.3). Extending the time of reperfusion time to 2 h resulted in a further enhancement of leukocyte recruitment with adhesion of leukocytes reaching approximately 1500 % that of sham operated animals however by this time point no extravasated neutrophils were recorded. Accompanying the observed progression of leukocyte recruitment was a significant increase in total circulating blood levels of the pro-inflammatory cytokine TNF-α along with an activation of brain resident microglia.

3.3.1. The BCCAo model as a tool to investigate I/R induced leukocyte recruitment.

In this study C57BL/6 mice have been used in a BCCA model of global stroke. The C57BL/6 strain was selected not only as it is the background strain for many genetically modified mice, but also due to its sensitivity to cerebral ischemic injury. C57BL/6 mice generally have hypoplastic posterior communicating arteries resulting in an incompletely formed circle of Willis and reduced collateral blood flow [220]. Studies by Yang *et al.* revealed that of seven different commonly used mouse strains C57BL/6 mice were the most susceptible to cerebral ischemia following BBCAo [221] producing reliable neurological and histopathological evidence of ischemic neuronal death [221]. The poorly developed posterior communicating artery found in C57BL/6 mice results in an approximate 90 % reduction in cerebral blood flow following BCCAo which is highly reproducible. BALB/c mice on the other hand have been found to have variable degrees of anastomosis resulting highly variable reductions in blood flow (between 70-90 %) following BCCAo.

The C57BL/6 strain thus allowed the BCCAo model to be used as a simple and reproducible model of cerebral ischemia reperfusion injury in which leukocyte recruitment could be investigated.

3.3.2. Length of reperfusion and the time course of the inflammatory response

Contrasting to the microcirculation of peripheral tissues constitutive leukocyte endothelial interactions in the pial vessels of the brain is almost entirely absent, however in response to phlogistic stimuli cerebral vessels may support a rapid increase in leukocyte recruitment [222, 223]. In this model while few leukocyte-endothelial interactions were observed in sham animals, significant increases in both rolling and adhesion of leukocytes was observed in the pial venules as early as 40 min into reperfusion. Neutrophil depletion studies demonstrated this recruitment of leukocytes to be largely, if not entirely, consisting of neutrophils. Previous investigations have revealed neutrophils to be the earliest leukocytes to respond to ischemic damage, with their levels of recruitment correlating with infarct volume [224]. Increases in the levels of circulating monocytes have also been reported following cerebral I/R however this is typically observed as a delayed response occurring approximately 3 days post-stroke [225]. In keeping with the results of the present study, Ishikawa *et al*. have also demonstrated rolling and firm adhesion of leukocytes in cerebral venules at 40 min of reperfusion in C57BL/6 mice but in this case following 1 h of BCCAo [27]. The levels of leukocyte recruitment reported by Ishikawa *et al.* are comparable to those found in this study despite the large difference in magnitude of ischemic insult (1 h BCCAo compared to 5 min used in these investigations). This model of 5 min BCCAo was previously developed in our lab (H. K. Smith *et al.,* pending publication) to provide a sufficiently robust inflammatory response while minimising mortality following ischemia. The relatively short time of ischemia required thus suggests post ischemic inflammatory responses to be highly sensitive and rapidly triggered. Indeed Ritter *et al.* have revealed leukocyte adhesion to cerebral venules within just 15 min of reperfusion following MCAO (2h of ischemia) [76]. However, to my knowledge, the findings of the present investigations still represent the earliest time point following initiation of the ischemic insult (45 min) at which leukocytes have been observed to adhere to cerebral microvessels. While these observations of leukocyte adhesion are seemingly earlier than expected from

investigations of integrin expression, recent studies have revealed integrin up regulation at an earlier stage that previously predicted. Morrison *et al.,* have recently demonstrated that following stroke significant increases in neutrophil CD11b (the protein subunit that pairs with CD-18 to form the heterodimeric integrin macrophage-1 antigen (Mac-1)) expression was induced within just 15 min of reperfusion [226]. Neutrophil expression of CD11b has been shown to be an indicator of neutrophil activation allowing cells to firmly adhere to the endothelium through interactions with ICAM-1 [226]. Indeed Mac-1 deficient mice have been shown to be protected against cerebral ischemia and CD11b antibodies have been investigated as potential treatments for stroke.

At the acute phase of the inflammatory response that has been investigated in this study no parenchymal extravasated neutrophils were observed in IHC analysis of brain sections. Previously, investigations in our laboratory (H. K. Smith, pending publication) have indirectly quantified PMN accumulation in the brain by measuring the activity of the azurophilic granule enzyme myeloperoxidase (MPO). These studies failed to demonstrate any significant increase in brain MPO activity indicating a lack of neutrophil extravasation. As such in the present investigations direct IHC was used as an alternative method to verify that neutrophils are not present in the brain parenchyma by 2 h of reperfusion. A rat antibody which specifically binds the murine Ly-6G and Ly-6C components of the myeloid differentiation antigen Gr-1 to label neutrophils was used to stain brain slices. While this antibody was able to specifically label PMN in blood smears no staining was observed in all brain slices analysed. However confirmation of antibody staining in a positive control of neutrophil infiltration in the brain tissue would be needed along with a more thorough search across all brain regions to confirm the absence of infiltrates in this model. Previous investigations show the earliest increase in parenchymal neutrophil infiltrates to be at 6 h (earliest point tested) [227, 228] however most studies focus on infiltration at 24 h post ischemia. Indeed, while neutrophil infiltration into other tissues has been observed as early as 30 min [229] the BBB poses a considerable obstacle, and migration across the basement membrane alone has been documented to take 15 min [47]. Thus a lack of infiltration at this stage is in keeping with the findings of others. While these results are by no means conclusive of a lack of leukocyte recruitment at this acute phase, the results do support prior MPO investigations.

3.3.3. BBB integrity

The BBB allows for the stringent control of blood born molecules entering the brain, by regulating the active transport of nutrients and the efflux of toxic molecules, inhibiting paracellular diffusion, and regulating the trans-endothelial passage of pathogens and circulating leukocytes, thus serving to maintain cerebral homeostasis [230]. The breakdown of the BBB following stroke is a complex multi-phasic phenomenon and is characterised by an early disruption of tight junctions in response to endothelial calcium signalling, oxidative stress and MMP-9 release from resident cells [230, 231]. The recruitment of activated leukocytes marks an increase in ROS and MMP along with inflammatory cell diapedesis, aggravating vasogenic oedema and increasing the risk of haemorrhagic transformation [230].

In this model FITC conjugated albumin assessment of cerebral vascular permeability and brain water content assessment of oedema, revealed no disruption of blood brain barrier by 2 h following 5 min BCCAo. As such this model could not be used, at the time points tested, for an assessment of BBB disruption following stroke in subsequent investigations of the MRS. Previous studies have shown MCAo and BCCAo in both mice and rats to induce significant leakage of cerebral vessels [232-234]. However the leakage responses of vessels has been shown to differ with the fluorescent dye used to label albumin [235]. Indeed a number of tracers used in previous investigations have now been demonstrated to contribute to BBB compromise themselves. The widely used evans blue, for example, has been found to inhibit glutamate uptake and may thus exacerbate excitotoxicity following cerebral I/R and perturb the physiological environment of the NVU [236, 237]. As such this may account for a certain level of inherent methodological bias toward greater reported BBB compromise [236]. Extended imaging with FITC fluorescent dyes has also been shown to induce phototoxicity. microvascular dysfunction and leakage in a number of tissues (although not yet reported in the BBB) [235]. In the present study the effects of epiillumination were minimised by only subjecting animals to one brief period of fluorescent imaging. More detailed investigations, beyond the scope of this thesis could also establish the effect of BCCAo on specific BBB junctional molecules such as occludins and ZOs however a recent study by Krueger *et al.* provides convincing evidence that the ultrastructual morphology of tight junctions is not entirely representative of BBB failure [236]. Their findings show that 25 h

after embolic stroke in rats tight junctions remain regularly maintained despite a massive leakage of FITC-albumin in these regions predominantly resulting from dysfunction of the endothelium rather than tight junctions [236]. Thus while the lack of any BBB disruption found in the current investigations using FITC-albumin, are not likely to be due to a lack of assay sensitivity, investigations into endothelial function following ischemia may provide useful insights.

It is likely that increased lengths of ischemia or extending the time of reperfusion would result in significant levels of BBB disruption detectable by FITC-albumin in this model. The earliest reported observation of BBB disruption in rats is within 60 min of reperfusion following BCCAo (30 min of BCCAo, visualised by FITC-dextran) [233] In mice the earliest reported BBB disruption following BCCAo is at 48 h in mice (25 min BCCAo, visualised with evans blue) [232]. In the current study BBB dysfunction has been assessed at a relatively early time point following BCCAo (40 min earliest time point tested). At this early phase of the neuro-inflammatory cascade 5 min of BCCAo is not sufficient to induce BBB permeability. This lack of any significant BBB disruption found in the current investigations is thus in keeping with the relatively short ischemic insult and lack of any neutrophil infiltration into the neurophil as observed by preliminary IHC investigations (Figure 3.5). It is thus likely that BBB dysfunction in experimental stroke is affected not only by the method of measurement but also the duration of ischemia and reperfusion and the experimental models used.

3.3.4. Activation of resident brain microglia

Microglia represent the resident immune-competent cells of the brain and are highly sensitive to pathological changes in the brain [30]. In preliminary studies on microglial activation, microglia were stained using IBA-1 which has previously been shown to be up-regulated in activated microglia in response to cerebral pathology [238]. By 40 min of reperfusion while there was a trend toward an increase in activation (as detected by IBA-1 staining) this was however not significant, suggesting that even a mild activation in resident cells can initiate a cascade of signalling resulting in a significant leukocyte recruitment. By 2 h of reperfusion the activation of microglia was however shown to be significantly increased specifically in the hippocampus; a region shown to be particularly

sensitive to ischemic damage. This correlates with previous findings in a rat model of BCCAo in which a hippocampus specific up regulation of IBA-1 was detected [239].

Activated microglia may release a variety of cytotoxic substances such as ROS and proteases and treatment with the microglial inhibitor tetracycline has been shown to protect hippocampal neurones following global ischemia in gerbils [240]. Furthermore a number of studies have revealed correlations between microglial activation and CNS cytokine levels with infarct severity [30]. The mild and localised activation of microglia observed in the present study coincided with an apparent trend toward elevated whole brain COX-2 expression, indicative of an enhanced pro-inflammatory environment. COX-2 is a powerful inducer of inflammation; mediating the synthesis of bioactive prostanoids and inducing up-regulation of numerous cytokines including IL-1β and TNF-α [241] [242]. This inflammatory mediator is not only produced by inflammatory cells but also by CNS neurons and COX-2 inhibitors have previously been shown to provide protection in the ischemic hippocampus [242]. While COX-2 up-regulation was not found to be significant the results obtained in this study may reflect that analysis was performed on whole brain homogenates. Utilising IHC to investigate expression in discrete areas may provide a greater insight into the regulation of COX-2 in the brain following BCCAo particularly in the vulnerable hippocampal region where the highest microglial activation was observed.

3.3.5. Cytokine response to BCCAo

Investigations into circulating cytokine levels following BCCAo using ELISA and CBA demonstrate a significant increase in serum TNF-α by 2 h of reperfusion but not at 40 min. This increase in coincided with the significant increase in leukocyte adherence and trend toward an increase in cell rolling in the pial microvasculature at 2 h.

TNF-α exerts potent pro-inflammatory and pro-coagulant effects, stimulating up regulation of leukocyte adhesion molecules, release of IL-1, platelet-activating factor and endothelin [243]. TNF-α can contribute to stroke progression at multiple levels through; increased ROS *via* the induction of cyclooxygenase (COX) and NADPH oxidase, aggravating vascular permeability and risk of hemorrhagic transformation by activation of MMPs, enhancing excitotoxicity and promoting leukocyte infiltration (as reviewed by Hallenbeck, 2002 [243] and McCoy & Tansey 2008 [244]) .

Synthesised as a monomeric transmembrane protein and inserted into the membrane as a homo-trimer. This membrane bound form (tmTNF) may be released by proteolytic cleavage to form soluble (sol)TNF thus allowing for a rapid up regulation of circulating levels. Both tm and sol forms display biological activity and signal *via* two receptors TNFR1 (CD120a) and TNFR2 (CD120b), with the more ubiquitously expressed TNFR1 binding solTNF and TNFR2 showing preferential binding to tmTNF [244].

The early rolling and adherence of cells observed in this study may be in response to the cleavage of pre formed TNF-α from the membrane which occurs over 20 min-2 h, as opposed to its *de novo* synthesis. In concordance with the current observations, rodent models of stroke have previously shown increased mRNA levels of TNF-α within 1 h of ischemia with protein expression being detected within 2−6 h of ischemic onset [245, 246]. Similarly TNF-α levels have been shown to be elevated in the blood and CSF of stroke patients and support a pathological role for TNF-α in stroke [243], [247]. Microglia have previously been highlighted as the major acute source of TNF-α following cerebral I/R in mice [248] and as such may contribute to the increased levels observed at 2 h in this study. However immunoreactivity has also been shown in neurons, astrocytes, endothelial cells, macrophages and leukocytes [243] [244] and as such the source of increased circulating TNF-α is difficult to determine.

While TNF-α plays an important role in inducing other inflammatory mediators, with TNF-α deficient mice showing decreased expression of chemokines and adhesion molecules following experimental I/R, TNF-α may also act in an autocrine positive feedback, increasing its own secretion [243]. Thus the early levels of TNF-α increase observed in the present study may represents the beginnings of a potentiating of TNF signalling and induction of other pro-inflammatory molecules.

In addition to signalling through TNFRs, TNF-α may also exhibit reverse signal transduction. Following the cleavage of tmTNF by the sheddase TNF-α converting enzyme (TACE; ADAM17) and release of solTNF, the free TNF-α intracellular domain may be released and initiate intracellular signalling to stimulate the production of pro-inflammatory cytokines [244]. Thus the physiological significance of increased serum soluble TNF-α as investigated in the present study, may be two fold. However the exact

in vivo significance of such TNF-α reverse signalling events are still to be fully ascertained.

While BCCAo was found to induce significant increases in TNF-α, no statistically significant increases within the reliable detection range of the kits used were detected in all other serum cytokines tested (IL-6, IL-10, IL-12, IFN-Ɣ and MCP-1).

Although MCP-1 levels increased significantly at 2 h following BCCAo, and MCP-1 has previously been shown to be increased following experimental stroke aggravating ischemic damage [249], the levels reported here remained below reliable detection range of the CBA and below a biologically relevant concentration. MCP-1 induces the recruitment of monocytes along with other leukocytes sub sets and mRNA and protein levels of MCP-1 have previously been shown to peak at around 24 h inducing monocyte recruitment by 48 h, thus fitting with the lack of any early detection reported here. Indeed many monocyte chemotactic signals require *de novo* synthesis and are thus released at a later time point than neutrophil active factors which are released by rapid exteriorisation or proteolytic cleavage [43]. Furthermore neutrophils recruited to the site of tissue injury have been shown to induce endothelial secretion of MCP-1 and monocyte recruitment through their release of proteinase-3 [43]. It is thus likely that at later time points MCP-1 levels will increase in the current model.

At 2 h reperfusion there was a trend toward an increase in IL-10 levels which was within the detection range of the kit but not statistically significant. This cytokine has been reported to contribute to anti-inflammatory processes and provide a favourable environment for neurogenesis, with low serum levels of IL-10 correlating to poor neurological outcome in stroke patients. Able to suppress cytokine receptor activation and expression whilst also inhibiting IL-1β and TNF-α, IL-10 is induced at the late stage of inflammation and promotes its resolution. As such it is likely that investigations at later time points of reperfusion following BCCAo would reveal increased IL-10 expression.

IL-6 is a pleotropic cytokine with both pro and anti-inflammatory actions having been reported. While serum levels of IL-6 have been shown to correlate with infarct volume [9], Clark *et al.,* have revealed no difference in neurological outcome or infarct size in animals lacking IL-6 following I/R suggesting no direct involvement in pathological

events following stroke [250]. Expression tends to follow that of TNF-α in stroke with IL-6 increases first detected in serum, and then in brain [250], therefore correlating with the lack of any increase seen in the present study at 2 h.

No levels of the pro-inflammatory and immunomodulatory chemoattractant IL-12 or the immunostimulatory interferon IFN-γ were detected at the time points tested in this model. In line with the present findings, as molecules interfacing the innate and adaptive immune system increases in IFN-γ and IL-12 following stroke are later than TNF-α and are largely produced by macrophages and B and T-cells.

While many of the cytokines tested were at low or below detectable levels in serum this does not entirely exclude a role in influencing the local inflammatory environment. The blood contains a number of proteases which help to contain the spread of local inflammatory mediators, Duffy antigen/chemokine receptor (DARC) on eurythrocytes may also act to bind and internalize a variety of cytokines and soluble receptors for certain cytokines help to sequester their signalling, preventing a fully systemic immune response. Thus serum cytokine levels may not always correlate with the magnitude of inflammatory events in localised microenvironment at the site of tissue injury. Indeed this differential cytokine expression in serum and the local environment has been demonstrated in human inflammatory conditions [251]. The benefit of utilising IVM to assess leukocyte recruitment in conjunction with analysis of inflammatory mediators, as performed in this project, is that localised inflammatory responses can be assessed independently of an entirely systemic response.

3.3.6. Limitations of the model

3.3.6.1.The pial microcirculation as a model of deeper cortical vessels

In this model, due to limitations in the depth of imaging possible using standard fluorescent IVM, leukocyte-endothelial interactions have been investigated in the pial vessels of the brain and been taken as representative of events in the deeper cortical vessels and the cerebral microvasculature as a whole. A number of differences between pial and cortical venules exist, pial venules lack the astrocyte ensheathment characteristic of cortical vessels [252] which could conceivably have an effect on inflammatory responses. However pial and parenchymal microvessels are understood to

share many features, such as ultra-structural features and tight junctions along with similar electrical resistance and permeability to tracers [252]. As such these vessels are often used as models of deeper inaccessible cortical venules and by enlarge considered adequate. Indeed while the exact contribution of leukocytes migrating through pial vs. parenchymal vessels to cerebral damage is unknown, pial vessels, lacking a Virchow-Robin space and glial limitans, may provide an easier route for transmigrating leukocytes to navigate, and could conceivably represent the major port of entry in early or mild inflammatory events. Nevertheless the possibility cannot be excluded that the observations in this study could be unique to pial micro-vessels.

One further drawback due to the limited depth of imaging possible is the inability to directly visualise leukocyte migration into the brain parenchyma. However increased leukocyte-endothelial interactions in the pial vessels have been shown to correlate with subsequent elevated leukocyte infiltration in the brain parenchyma as a whole. Treatments with prospective therapeutics which inhibit leukocyte rolling and adhesion have also similarly been shown to reduce leukocyte infiltration into the parenchyma in *in vivo* models [71], whist also reducing the damaging effects of neutrophil emigration [71]. Thus, while in the current study at the acute time points tested no parenchymal neutrophils have been observed, leukocyte adhesion is a prerequisite for transmigration and is indicative of subsequent infiltration. With the advancement of technology and the introduction of confocal and multi-photon intravital microscopy it has become possible for a greater depth of vision allowing the researcher to explore inflammatory events in the deeper cortical vessels. Such investigations have allowed real time visualisation of leukocyte migration in the brain. While the use of this minimally invasive technique is still in its infancy for the study of leukocyte recruitment, future investigations using this technique will undoubtedly provide an insight into the relevance of the pial microcirculation in modelling the deeper vessels along with a greater understanding of transmigration and leukocyte motility inside the brain parenchyma.

3.3.6.2. Distinguishing leukocyte subsets

In this study rhodamine 6G has been used to fluorescently label leukocytes, this dye has been previously shown to also label platelets when used at high concentrations however the clear difference in size makes these two cell types easily distinguishable. Rhodamine however cannot distinguish between leukocyte sub sets (lymphocytes, neutrophils,

basophils, or monocytes). While in this study neutrophil depletion has been used to help identify these cells, there is also the possibility that fluorescently labelled antibodies could be introduced to specifically label and track the recruitment of individual leukocyte populations. This approach however carries a number of draw backs. Varying affinity of different antibodies means that only a proportion of cells may be visualised, and *ex vivo* separation/staining procedures may activate cells. Furthermore antibodies for specific labelling should be selected with care, a number of antibodies used to selectively label leukocytes target cell surface molecules that are themselves involved in the adhesion process and would therefore affect the very parameters that are being measured. The monoclonal antibody, RB6-8C5, that binds Gr-1 (specifically Ly6C and Ly6G), has been an important investigational tool to assess neutrophil function [253]. At high doses this antibody has been used to experimentally deplete neutrophils, however more recently, low doses have frequently been used to endogenously label neutrophils for IVM [225]. During studies exploring the impact of submaximal neutrophil depletion Wang *et al.* recently observed that while low doses of RB6-8C5 did not reduce levels of circulating neutrophils, the treatment caused an almost complete inhibition of neutrophil migration [254]. Subsequent investigations revealed that the antibody inhibited the ability of neutrophils to react to chemotactic stimuli, interfering in previously undiscovered interactions between Ly6G and the β2 integrins CD11a and CD11b [253, 254]. Thus while lacking the ability to differentiate between leukocyte sub populations; the use of rhodamine in the present study avoids a host of potential confounding factors.

3.3.7. Receptor expression

In order to investigate the MRS as a therapeutic target in this cerebral I/R model, expression levels of the melanocortin receptors were first investigated in both the brain and blood of C57BL/6 mice, to confirm the presence of the receptors and also to monitor the effect of cerebral ischemia and peptide treatment on receptor expression. In these investigations MC_1 and MC_3 expression was initially detected in whole brain homogenates which remained consistent across all treatments. Detection of MC_3 protein in samples from $MC_3^{-/-}$ mice (confirmed as such by PCR) however put the specificity of this antibody into question. Due to the relatively high homology between melanocortin receptors (particularly in the TM1, TM3 and TM7 regions) [255] and similarity in molecular weight it may be that these antibodies are cross reacting with other

melanocortin receptors present. The reliability of commercially available MC_3 antibodies has also since been put into question by Kathpalia *et al.* [256]. During their investigations into the natriuretic effect of γ-MSH at Stanford University Kathpalia *et al.* tested an array of commercially available MC_3 antibodies (including the Sigma Aldrich antibody used in this project). In both western blot analysis and IHC all antibodies tested were unable to specifically detect MC_3 in mouse brain or kidney showing apparent expression in $MC_3^{-/-}$ animals [256]. These antibodies and the ones used the present project have previously been used in studies characterizing MC_3 expression in a number of different tissues and disease models. These recent findings could cast doubt on the reliability of the findings with regards to MC_3 expression in these studies, although the studies were done in conjunction with compounds which are reported to show selectivity for the MC_3 and also looked at the effects of disease progression/exacerbation in $MC_3^{-/-}$ mice compared to their wild type counterparts.

During this project the lack of a reliable and specific antibody for the MC_3 has thus limited the analysis of MC protein expression by western blot analysis or immunohistochemistry. However to investigate any transcriptional changes in melanocortin receptor expression qRTPCR has been utilised. These studies show no significant changes in the blood or brain levels of MC transcript in response to BCCAo at either 40 min or 2 h of reperfusion. However upon treatment with the pan receptor agonist α-MSH blood levels of MC_4 mRNA were found to be significantly reduced by 40 min of reperfusion.

While these investigations demonstrate that BCCAo does not induce a transcriptional modulation of MCs in either brain or blood, there is still the possibility that MCs are regulated post transcriptionally. GPCR expression is highly responsive, and receptor phosphorylation may mediate rapid desensitization and internalisation. Receptor agonists may induce GPCR internalisation and regulation of membrane localisation represents a key point at which ligand responses may be modulated. For instance treatment with various melanocortin agonists has been observed to result in desensitisation and internalisation of MC_4 [257]. Internalisation studies have not however yet been performed with other MCs. A number of GPCRs are now also understood to require accessory proteins for their function and membrane trafficking. MC_2 has previously been shown to require the accessory protein, melanocortin 2 receptor

accessory protein (MRAP) to allow for membrane localisation and also its signalling [258]. Since then a second MRAP (MRAP2) has been discovered, which also facilitates membrane localisation but imparts the receptor with an extremely low affinity for its agonist [259, 260]. Taken together, these accessory proteins may provide a fine tuning of MC_2 functioning. No such receptor specific associated proteins have been yet discovered for the anti-inflammatory receptors on which the investigations of this thesis focus, the MC_1 and MC_3. However recently co-immunoprecipitations by Chan *et al.,* indicate both MRAP and MRAP2 are in fact, able to interact with all five melanocortin receptors [261]. While MRAP enhances MC_2 function, it has also been found to inhibit MC_1, MC_3, MC_4 and MC_5 in cAMP response assays and reduce the surface expression of MC_4 in CHO cells co-expressing MRAP and MCs [261]. MRAP2 on the other hand reduces MC_3 MC_4 and MC_5 signalling but has no effect on MC_1. Whether or not these novel interactions influence receptor functioning and expression *in vivo* and indeed if cerebral ischemia affects MRAP regulation has yet to be determined. However in the absence of specific antibodies it is difficult to definitively assess the effect of BCCAo on MC regulation and function.

3.4. Summary

Using this 5 min BCCAo model with reperfusion, a significant and temporally dependant level of leukocyte recruitment has been observed. This recruitment of blood born cells coincided with significantly increased levels of circulating TNF-α. While no evidence of BBB breakdown or neutrophil infiltration into the brain was observed by 2 h of reperfusion, BCCAo was found to induce mild but statistically significant activation of resident immune competent glial cells (IBA-1 positive microglia)which was found to be localised exclusively to the hippocampus. Investigations into melanocortin receptor expression also indicated the presence of multiple melanocortin receptors in both brain and blood. As such this model provides multiple parameters (leukocyte rolling and adhesion, blood TNF-α levels and microglial activation) at which to assess the MRS as a therapeutic intervention in the acute phase neuro-inflammatory response following cerebral I/R.

3.5. Future Directions

While this thesis focuses on the acute inflammatory response following BCCAo and the early recruitment of leukocytes, to further expand on the data within it would be interesting to assess functional outcome following BCCAo. Extending reperfusion to 24 h could allow for the investigation of any functional impairment that may arise using behavioural tests, histological examination could also be used to evaluate neuronal cell death. Indeed evaluations of neuroprotection (beyond the scope of this thesis) could be made by assessing differential cell death in the vulnerable CA regions of the hippocampus.

4.0. Investigation into the effects of melanocortin agonists and antagonists on leukocyte endothelial interactions following BCCAo.

4.1. Introduction

Leukocyte recruitment in the CNS is a key process in the pathogenesis of neuro-inflammatory diseases including Stroke, MS and infection. Furthermore recent studies have implemented leukocyte recruitment in other neuropathologies including Parkinson's disease and epilepsy, thus intervening in this neuro-inflammatory process may provide a valuable therapeutic opportunity.

Activation of the melanocortin receptor system has previously been shown to provide neuroprotection in a number of models of cerebral ischemia reperfusion injury [169, 207, 211] and has demonstrated impressive inhibitory actions on leukocyte recruitment in a variety *of in vivo* inflammatory situations [127, 140, 151]. However the relative contribution of MC subtypes in mediating these protective effects remains under contention and their effects on leukocyte recruitment has not yet been assessed in the cerebral microcirculation.

In this chapter a number of MC agonists and antagonists have been utilised for a pharmacological investigation into the effects of melanocortin treatments on leukocyte recruitment following cerebral I/R. Furthermore these studies have been used to help elucidate the relative importance of MC subtypes in mediating these effects.

4.2. Results

4.2.1. Treatment with α-MSH reduces BCCAo induced leukocyte endothelial interactions

To investigate the effects of activating the MRS on leukocyte recruitment a single 10 µg i.p dose of α-MSH, an endogenous pan receptor agonist (with exception to MC_2, which is exclusively activated by ACTH), was given 30 min before initiation of BCCAo. Prophylactic treatment with α-MSH was found to provide potent inhibition of BCCAo induced leukocyte recruitment (Figure 4.1), reducing both rolling and adhesion of cells (Figure 4.2).

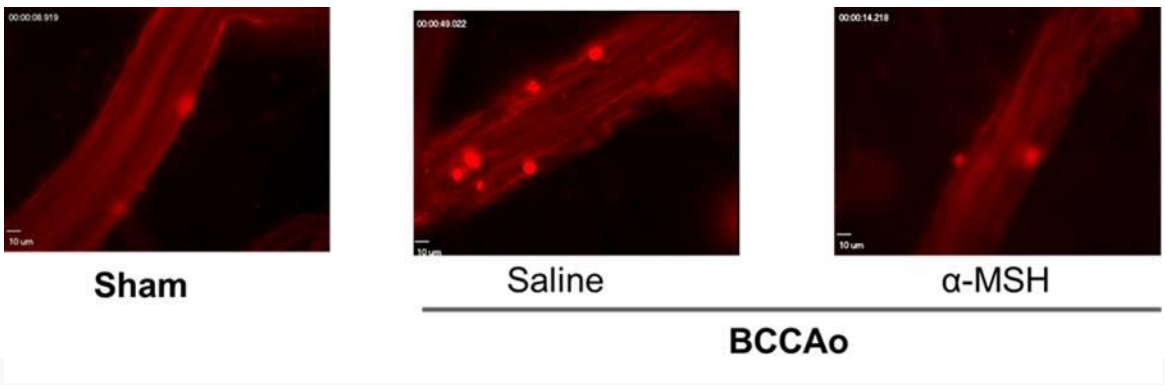

Figure 4.1 ***α-MSH treatment reduces BCCAo induced leukocyte recruitment*** Representative IVM stills of pial microvessels following sham surgery or BCCAo with 40 min of reperfusion treated 30 min prior to ischemia with saline or 10µg of α-MSH.

To investigate whether α-MSH treatment would be effective when given following stroke, treatments were brought forward to the start of reperfusion and effects on leukocyte recruitment at 40 min and 2 h following BCCAo were compared to treatments 30 min prior to BCCAo (Figure 4.2). Treatment with α-MSH at the start of reperfusion provided similar levels of protection as pre-treatment, reducing rolling by 80 % (from 191.0 ± 31.49 cells/mm^2/min to 38.25 ± 8.93) and adhesion by 68 % (from 282.3 ± 20.15

cells/mm^2/min to 90.35 ± 34.96) (Figure 4.2 B). Both levels of adhesion and rolling achieved in BCCAo animals treated either prophylactically or at the start of reperfusion were found not to be significantly different from those found in sham operated animals. By 2 h of reperfusion however the effects of a single i.p dose of α-MSH 30 min prior to ischemia had dissipated, no longer providing significant reductions in rolling or adhesion (Figure 4.2 A). Treatments given at the start of reperfusion on the other hand maintained a potent inhibitory influence on leukocyte recruitment (Figure 4.2 B). Compared to saline treated BCCAo animals at 2 h reperfusion rolling was reduced by approximately 68 % (from 243.87 cells/mm^2/min to 79.34 cells/mm^2/min) and leukocyte adhesion reduced by 82 % (from 659.70 cells/mm^2/min to 121.50 cells/mm^2/min). The velocity of rolling cells was found to be increased at 2 h with α-MSH treatment compared to saline. This increase countered the trend toward a reduction in leukocyte rolling velocity observed following BCCAo and the levels were not significantly different to sham operated groups (Figure 4.2 B). Future experimental treatments were therefore limited to the more effective and clinically relevant time of treatment, at the start of reperfusion.

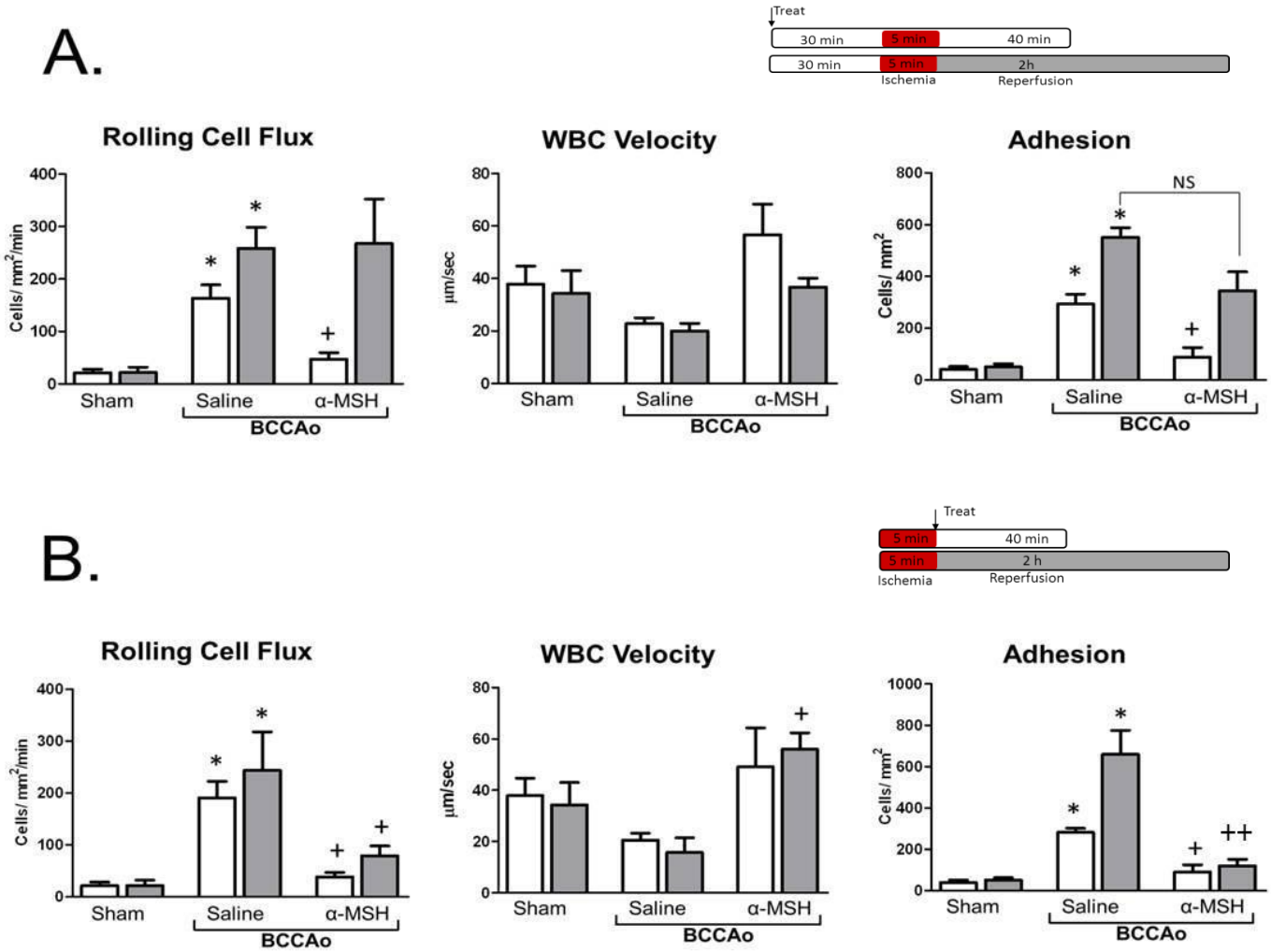

Figure 4.2. ***The effect of α-MSH treatment on BCCAo induced leukocyte recruitment at different time points .*** Leukocyte endothelial interactions were assed using IVM at either 40 min or 2h following 5 min BCCAo. Treatments of 10 µg α-MSH were given i.p either (A) prophylactically 30 min before BCCAo or (B) at the start of reperfusion Values represent mean +/- SEM. N = 6 for all 40 min groups, n = 5 for 2 h α-MSH group and n = 4 for all other 2 h treatment groups. Statistical analysis was performed across different treatments within the same time of reperfusion. * denotes significance to sham P<0.01, ** = P<0.001. + denotes significance to saline treated BCCAo group P<0.01, ++ = P<0.001.

4.2.2. The effect of MC selective treatments on leukocyte recruitment

With the aim of determining the relative contributions of melanocortin receptor sub types to the observed reductions in leukocyte recruitment, pharmacologically selective compounds were tested in the BCCAo model (Figure 4.3). Selective activation of MC_1 by a single i.p injection of 15 μM/Kg BMS-470,539 at the start of reperfusion was shown to provide a potent inhibition of BCCAo induced leukocyte recruitment at 40 min reperfusion. These effects were comparable in magnitude to those achieved with α-MSH treatment, with both rolling being reduced by 69 % (from 191.0 cells/mm^2/min to 58.1 cells/mm^2/min) and adhesion being suppressed by 80 % (from 282.3 cells/mm2/min to 58.0 cells/mm2/min) these levels achieved were comparable to those found in sham operated animals. However, BMS-470539 treatment becomes less effective by 2 h, with neither the reduction in rolling or adhesion being statistically significant when compared to the vehicle treated group. Treatment with the MC_3 agonist [$DTRP^8$]-γ-MSH was found to significantly inhibit adhesion (57 % reduction) but not the number of rolling cells at 40 min reperfusion. Contrasting the effects mediated by BMS-470539 which appear to dissipate by 2 h reperfusion, the effect of [D-TRP^8]-γ-MSH on adhesion by 2 h becomes more highly significant compared to vehicle treated BCCAo animals ($P<0.001$), reducing BCCAo induced adhesion by approximately 90 %. Additionally a trend toward a reduction in the number of rolling cells was observed in [D-TRP^8]-γ -MSH treated animals at 2 h following BCCAo compared to vehicle treated animals.

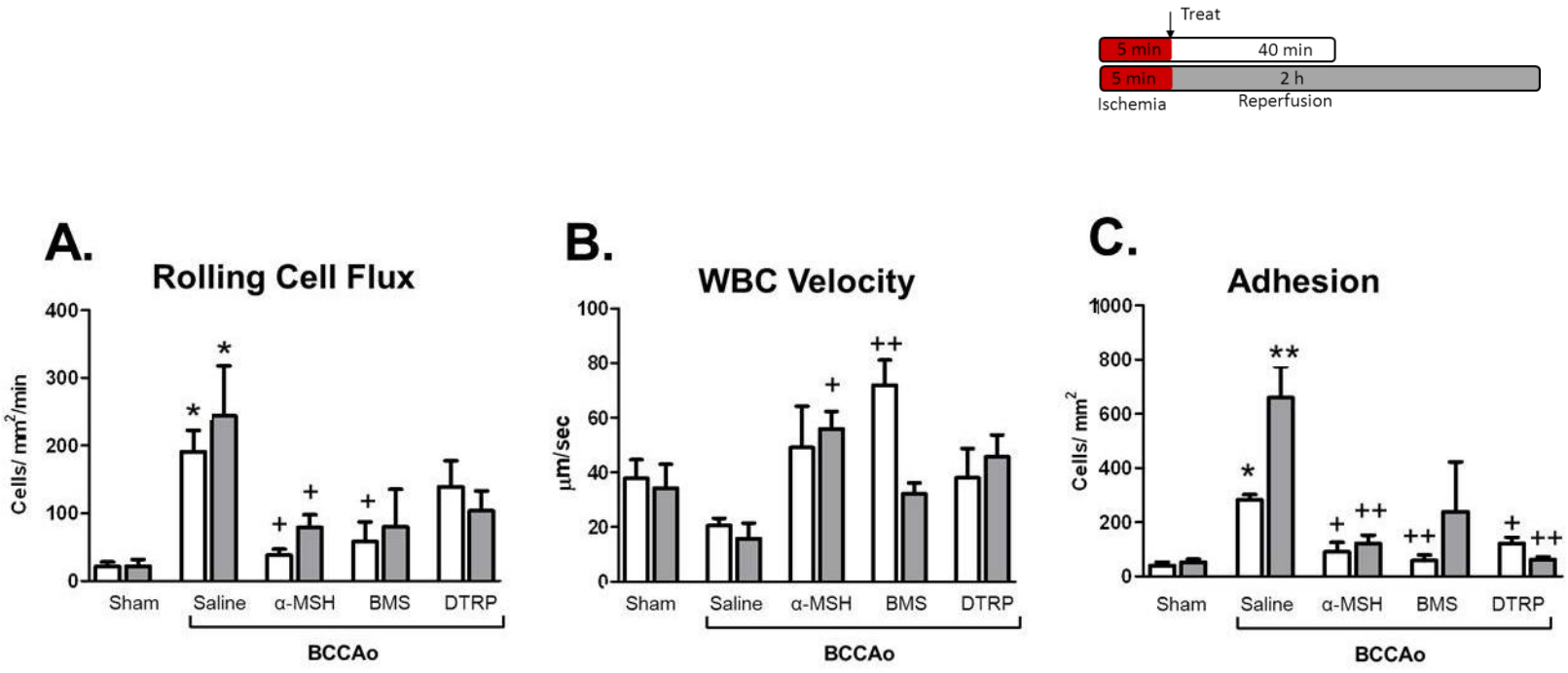

Figure 4.3. ***The effect of pharmacologically selective compounds on leukocyte recruitment following BCCAo*** Leukocyte endothelial interactions were assed using IVM in terms of (A) rolling cell flux, (B) WBC velocity and (C) adhesion, at either 40 min or 2 h following 5 min BCCAo. Treatments were given i.p at the start of reperfusion using either the endogenous pan receptor agonist α-MSH, the MC_1 selective BMS-470539 (BMS) or the MC_3 agonist [D-TRP^8]-γ-MSH (DTRP). Values represent mean +/- SEM. Statistical analysis was performed across different treatments within the same time of reperfusion. BCCAo 40 min reperfusion groups n = 6. α-MSH treated 2 h reperfusion group n = 5 all other 2 h reperfusion groups n = 4. * denotes significance to sham P<0.01, ** = P<0.001, + denotes significance to saline treated BCCAo group P<0.01, ++ = P<0.001.

4.2.3 The effect of α-MSH treatment on NF-κB related genes

With the aim of investigating whether the observed reductions in leukocyte recruitment following α-MSH treatment was due to the 'classical' melanocortin NF-κB inhibition pathway, the mRNA expression of two NF-κB related genes has been measured using qRT-PCR. RNA levels of TNF-α and IκB were investigated in both blood and brain of animals undergoing BCCAo surgery with 40 min or 2 h reperfusion and treated with saline or α-MSH at the start of reperfusion (Figures 4.4 and 4.5).

In blood there was a slight trend toward an increase in TNF-α mRNA expression at 2h following BCCAo with levels rising 2.4 fold from control levels. This nevertheless was not found to be statistically significant, as such further investigations with greater n numbers are required to allow for any firm conclusion to be drawn. IκB levels showed a trend toward an increase at the earliest time point tested following stroke and by 2 h following BCCAo this level showed statistical significance with a 25 fold increase in blood IκB transcript from control levels. Following α-MSH treatment this increase in IκB was significantly inhibited at 2 h reperfusion but not at 40 min of reperfusion.

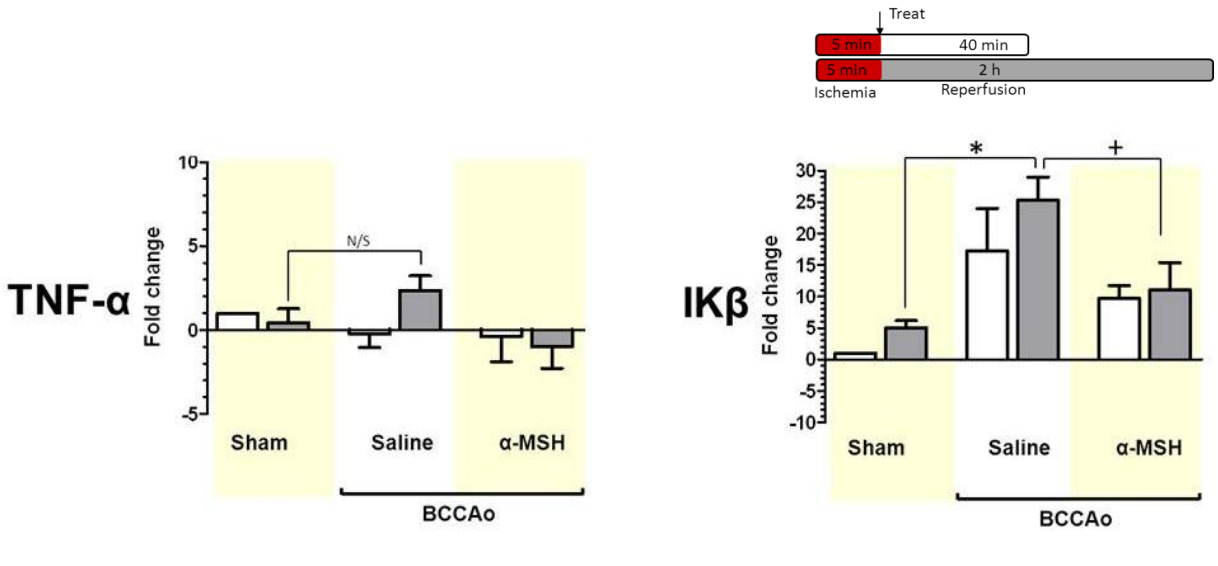

Figure 4.4. ***Blood mRNA levels of TNF-α and IκB following BCCAo and α-MSH treatment*** Change in blood levels of TNF-α and IκB mRNA transcript as detected by qRT-PCR. Data analysis was performed using the ΔΔCT Method and values expressed as fold change from control. N = 3 for all groups. * denotes significance to sham P<0.01. + shows dignificance to saline treated BCCAo group P<0.05

While not statistically significant whole brain levels of both TNF-α and I-κB showed trends toward an increase following BCCAo. A greater insight into these effects could be gained by preforming analysis on a larger study group.

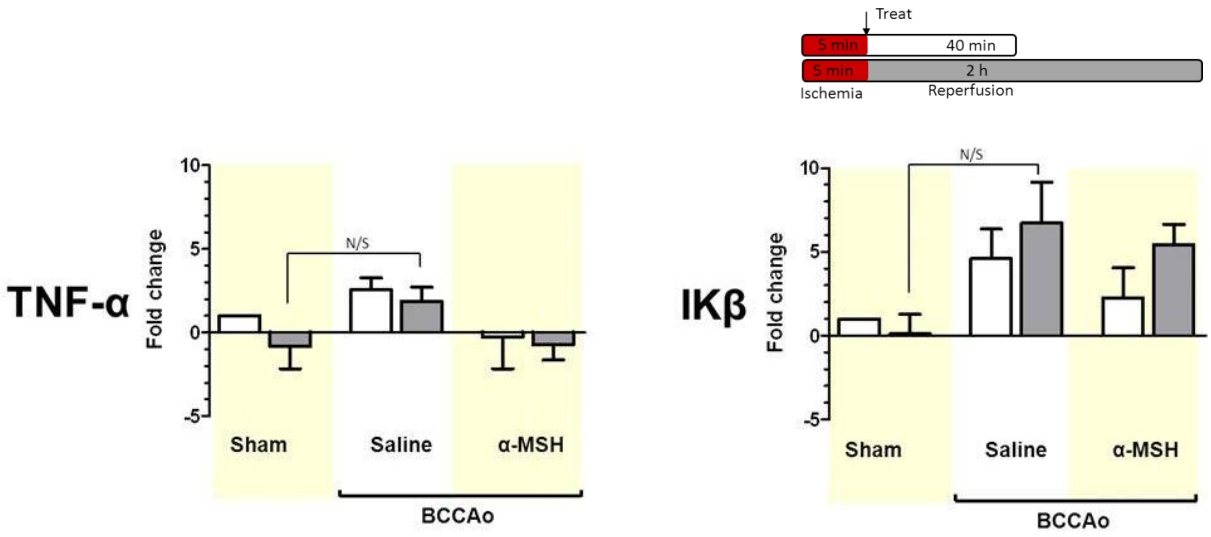

Figure 4.5. Brain *mRNA levels of TNF-α and IκB following BCCAo and α-MSH treatment* Change in whole brain levels of TNF-α and IκB mRNA transcript as detected by qRT-PCR. Data analysis was performed using the ΔΔCT Method and values expressed as fold change from control. N = 3 for all groups.

4.2.4 The impact of melanocortin agonists on microglial activation and circulating cytokines

In order to gauge the mechanism by which α-MSH treatments reduce leukocyte recruitment in this model, IHC has been used in preliminary studies to investigate whether treatment suppresses the BCCAo induced microglial activation. Treatment at the start of reperfusion with 10 µg α-MSH was however not detected to suppress increased microglial activation at 2 h post BCCAo, with no significant differences in IBA-1 expression in saline or α-MSH treated groups (Figure 4.6).

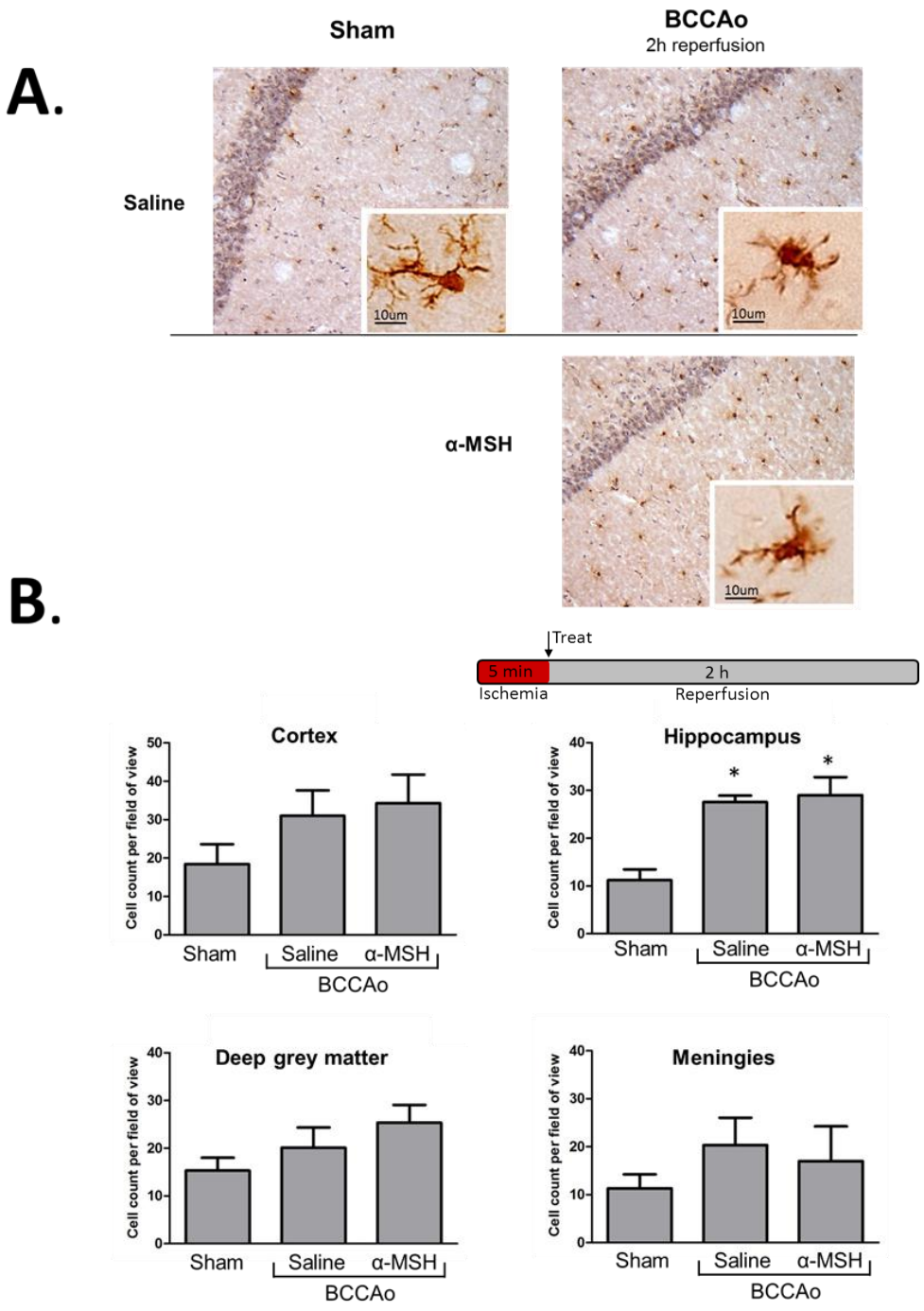

Figure 4.6. ***Hippocampal microglial activation is not significantly reduced by α-MSH treatment*** Frozen brain sections were stained with IBA-1 to measure microglial activation. A) representative X20 micrographs of IBA-1 stained brain slices. B) IBA-1 positive cells were counted per field of view in four different brain areas. No significant changes in IBA-1 staining was observed between saline and α-MSH treated groups. N=3 for all groups

Both ELISA and CBA were utilised to investigate the action of different melanocortin agonists on levels of circulating cytokines (Figures 4.7 and 4.8). All melanocortin agonists tested were able to prevent the up regulation of TNF-α which occurred 2 h following BCCAo, as detected by ELISA. The CBA confirming these findings, however with the exception of the BMS-470539 group where the TNF-α reduction detected using the CBA was not statistically significant.

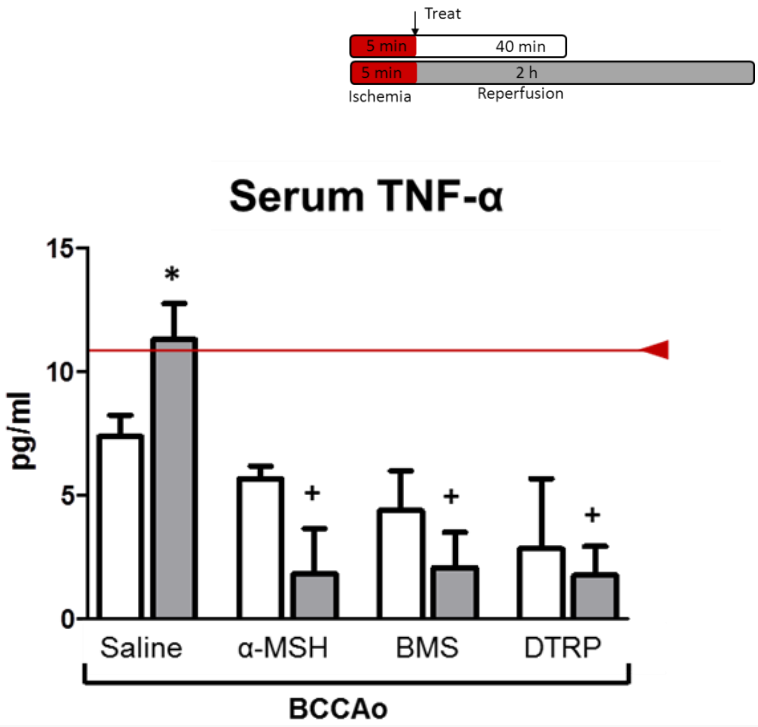

Figure 4.7. ***ELISA measurements of serum TNF-α following treatment with melanocortin agonists*** Animals undergoing BCCAo were treated with either saline, α-MSH, BMS-470539 or [D-TRP^{8}]-γ-MSH at the start of reperfusion and serum levels of TNF-α assessed using ELISA. Melanocortin treatments were found to abolish the BCCAo induced rise in serum TNF-α as seen in saline groups. N = 3 for all groups. * denotes significance to sham (not shown) P<0.01. + shows significance to 2h BCCAo saline treated group P<0.01. red line represents the reliable detection limit of the ELISA kit used.

Across the different melanocortin treatments both IFN-Ɣ and IL-12 remained undetectable in serum. Both α-MSH and [D-TRP^{8}]-γ-MSH did however induce a significant increase in the anti-inflammatory cytokine IL-10 by 2 h of reperfusion. Levels of the pro-inflammatory chemokine MCP-1 were also significantly reduced across all melanocortin treatments, although these levels were below the reliable detection range of the CBA. While IL-6 levels remained unchanged following treatment with BMS-470539 (BMS), both α-MSH and [D-TRP^{8}]-γ-MSH were found to cause significant increases in serum IL-6 levels by 2 h.

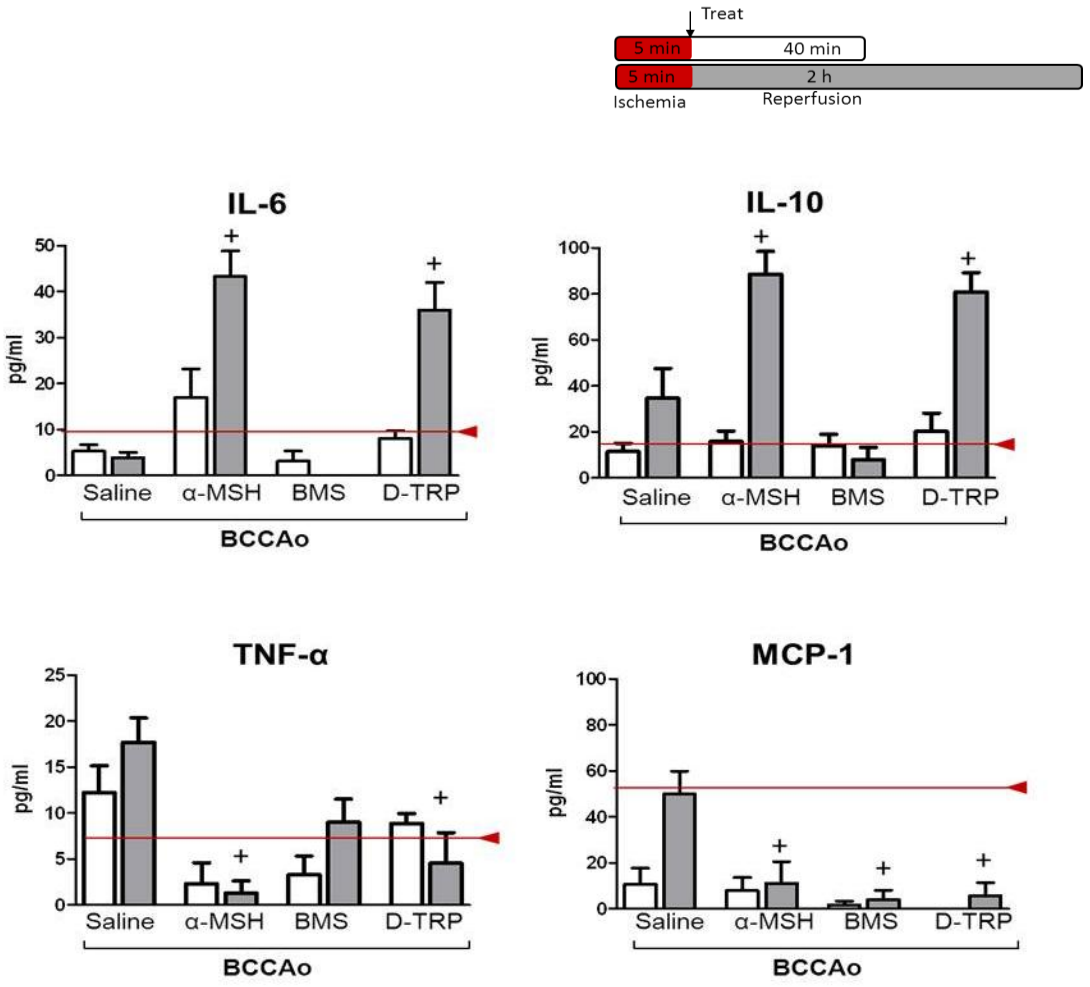

Figure 4.8. ***CBA detection of serum cytokines: the effect of melanocortin agonist treatment following BCCAo*** BCCAo groups were treated i.p at the start of reperfusion with saline, α-MSH, BMS-470539 (BMS) or [D-TRP^{8}]-γ-MSH. Serum levels of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 protein were then tested using CBA at 40 min or 2 h of reperfusion. N = 3 for all groups. + denotes significance to saline treated BCCAo group $P<0.01$. Red lines represent the reliable limit of detection for each cytokine tested

4.2.5. Utilising the $MC_{3/4}$ antagonist, SHU9119, to further investigate the anti-inflammatory role of MC subtypes

While BMS-470539 demonstrates almost entirely selective activity for MC_1, [$DTRP^8$]-γ-MSH has been shown to bind and activate other melanocortin receptors [262] (table 4.1). Furthermore MC_1 binding studies and cAMP accumulation assays have not been published for [$DTRP^8$]-γ-MSH. Thus, in order to further examine the roles of specific MC subtypes on leukocyte recruitment, investigations have been made using co-treatments with the $MC_{3/4}$ antagonist SHU9119. Mice subjected to 5 min BCCAo with 40 min or 2 h of reperfusion were treated at the start of reperfusion with α-MSH or [$DTRP^8$]-γ-MSH in combination with 10 µg SHU9119 (Figure 4.9).

Competitive antagonism at MC_3 and MC_4 using SHU9119 failed to abrogate the anti-inflammatory influence of α-MSH or [D-TRP^8]-γ-MSH on leukocyte recruitment at 40 min of reperfusion. On the contrary, co-treatment of [D-TRP^8]-γ-MSH with SHU9119 caused a more highly significant reduction in leukocyte adhesion than did [D-TRP^8]-γ-MSH alone. However, by 2 h of reperfusion co-administration of SHU9119 was found to blunt the α-MSH induced reductions in rolling and adhesion, and almost completely abolish the effects of [D-TRP^8]-γ -MSH when compared to singular treatments of either peptide.

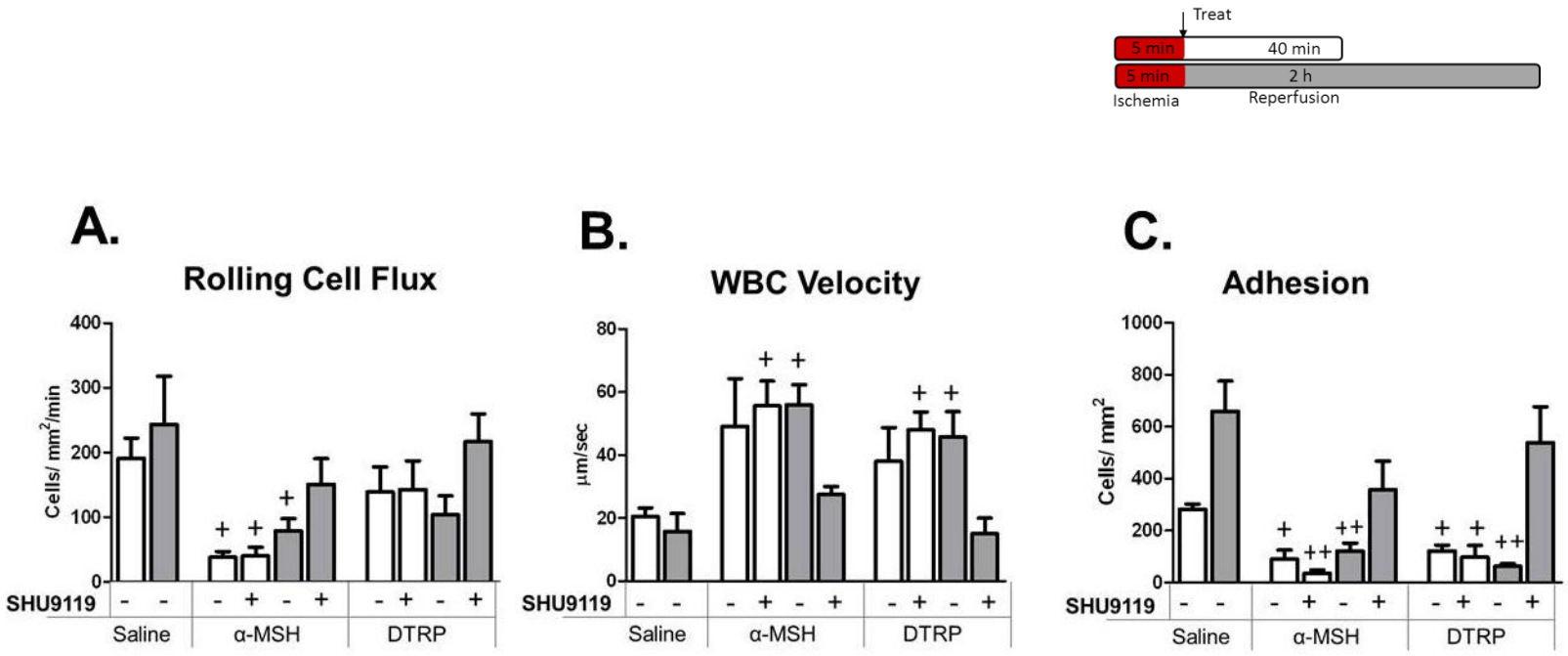

Figure 4.9. ***Effects of co-treatment with the $MC_{3/4}$ antagonist SHU9119 on the actions of melanocortin agonists.*** Leukocyte-endothelial interactions following BCCAo with 40 min or 2 h reperfusion in terms of (A) Rolling cell flux (B) WBC velocity and (C) Adhesion. C57BL/6 mice were treated at the start of reperfusion with α-MSH or [$DTrp^8$]-γ-MSH (10 µg i.p) either alone or in combination with the $MC_{3/4}$ competitive antagonist SHU9119 (10 µg). α-MSH 40 min co-treatment group n = 6 all other co-treatment groups n = 4. Singular treatment 40 min groups n = 6, 2 h groups n =4. Statistical analysis was performed comparing different treatments within the same time of reperfusion. + represents statistical significance to saline treated BCCAo group $P<0.01$. ++ = $P<0.001$.

In order to further investigate the lack of any inhibitory effects of SHU9119 when co-administered with melanocortin agonists at 40 min of reperfusion, treatments of SHU9119 were given alone and leukocyte recruitment assessed at both 40 min and 2 h following BCCAo (Figure 4.10.). At 40 min of reperfusion antagonism of $MC_{3/4}$ by a single i.p dose of SHU9119 given at the start of reperfusion, was found to significantly reduce leukocyte adhesion by 59 % (from 282.3 cells/mm^2/min to 116.0 cells/mm^2/min) and increase WBC velocity by 83 % (from 20.5 cells/mm^2/min to 37.7 cells/mm^2/min) . These effects were however lost when reperfusion was extended to 2 h.

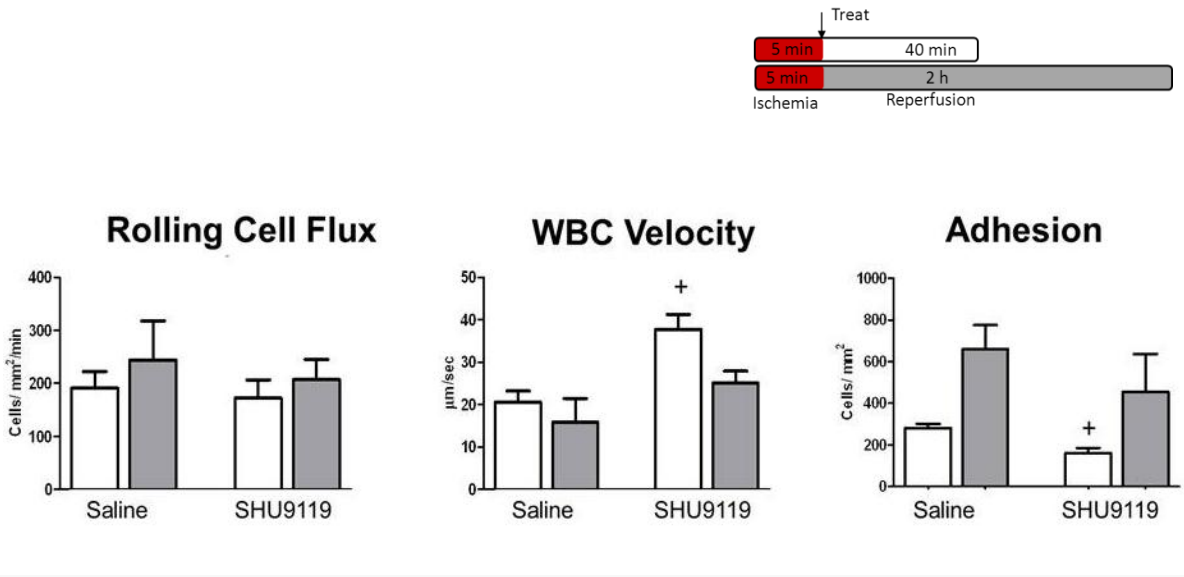

Figure 4.10. ***Effect of SHU9119 treatment on IVM parameters*** Following BCCAo animals were either treated i.p with saline or 10µg of SHU9119. Leukocyte recruitment parameters were then measured at 40 min or 2 h of reperfusion. N= 6 for 40 min reperfusion groups and n = 4 for the 2 h group. Statistical analysis was performed between groups of the same time point. + denotes significance to saline treated group P<0.01.

While the focus of these investigations has been on the MC mediated actions of melanocortin treatments and to elucidate involvement of different MCs, MC independent actions of α-MSH have previously been reported. These actions are proposed to be initiated by the N terminal tripeptide KPV acting perhaps as an IL-1R antagonist. In order to determine whether any of the observed effects of the α-MSH treatments given may be via receptor independent actions, the influence of KPV treatments on I/R induced leukocyte recruitment has been measured (Figure 4.11). By 40 min of reperfusion a single i.p dose of KPV (10μg) was able to significantly reduce BCCAo induced leukocyte adhesion by 77 % and increase WBC velocity by approximately 3 fold, a trend toward reduced leukocyte rolling (55 %) was also observed. However these effects were not maintained until 2h of reperfusion, with KPV treatment failing to influence any of the parameters measured.

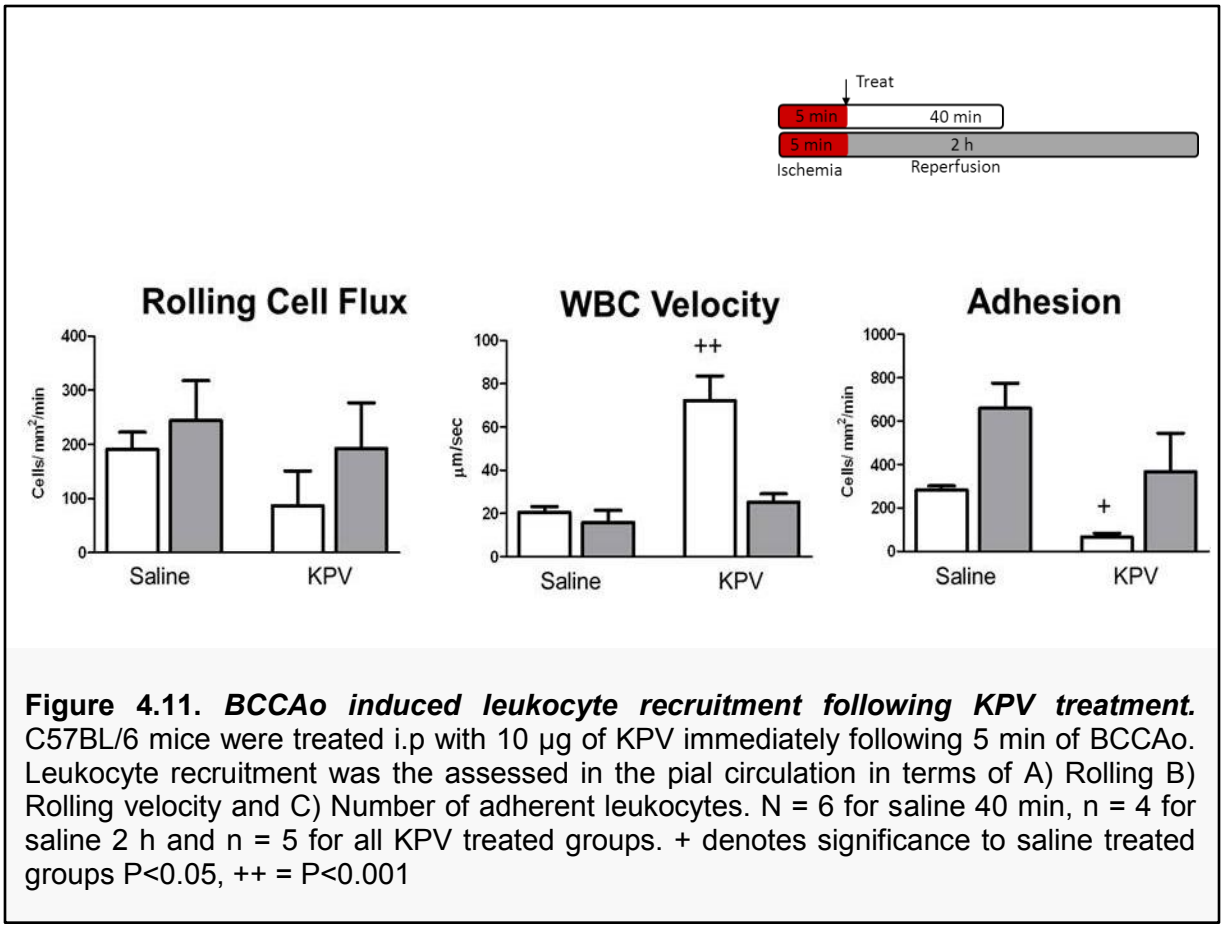

Figure 4.11. ***BCCAo induced leukocyte recruitment following KPV treatment.*** C57BL/6 mice were treated i.p with 10 μg of KPV immediately following 5 min of BCCAo. Leukocyte recruitment was the assessed in the pial circulation in terms of A) Rolling B) Rolling velocity and C) Number of adherent leukocytes. N = 6 for saline 40 min, n = 4 for saline 2 h and n = 5 for all KPV treated groups. + denotes significance to saline treated groups $P<0.05$, ++ = $P<0.001$

4.3. Discussion

4.3.1. The anti-inflammatory effects of alpha-MSH

Using BCCAo as a model of cerebral I/R induced inflammation the present investigations have demonstrated potent inhibitory actions of exogenously administered α-MSH on leukocyte recruitment. With a single i.p injection resulting in an almost complete abrogation of BCCAo induced leukocyte recruitment, causing an 80 % reduction in rolling and 69 % reduction in number of adherent cells. α-MSH has previously been shown to reduce leukocyte recruitment in monosodium urate (MSU) crystal induced inflammation [263], LPS challenge and also following I/R injury in the mesentery. Consistent with these experimental findings, in humans a negative correlation has been observed in the synovial fluid of arthritic patients between α-MSH concentrations and neutrophil number. Indeed in light of observations that plasma α-MSH concentrations increases in patients following various inflammatory insults including myocardial infarction, infectious disease and endotoxin administration [120] and that higher melanocortin levels have been shown to correlate with better outcome in stroke [264] there is growing evidence that the melanocortins are part of an endogenous system to bring about the resolution of inflammation.

In the current investigations α-MSH was able to significantly reduce leukocyte recruitment at 40 min following BCCAo when either administered prophylactically or at the start of reperfusion. However while a single dose of α-MSH given at the start of reperfusion provided significant inhibition of leukocyte recruitment at 2 h following BCCAo, given 30 min before BCCAo the peptide had lost these effect.

The half-life of iv administered full-length α-MSH has been found to be relatively short with reports of measurements from just 7 min when given i.v in rats [265] to 39 min in blood [266], with one report measuring 92 min in rabbit plasma [267] . This is most likely due to the presence of several serum proteases, with discrepancies between *in vivo* and *in vitro* half-lives perhaps indicating that extra-vascular or endothelial derived proteases may provide a major contribution in the metabolism of α-MSH. As discussed by Wilson *et al.* α-MSH half-life is thus likely to vary in different physiological and pathological environments with differing levels of proteases having a large effect on α-MSH longevity [265]. Indeed in the context of inflammation increased circulating proteases may further

limit the half-life of α-MSH *in vivo*. The duration of the physiological effects of α-MSH has been estimated at 1.5 h for its anti-pyretic activities [95]. While α-MSH is likely to initiate downstream signalling events that extend the physiological influence of the peptide beyond its longevity, in the presence of a continuing inflammatory process in the current model, these effects appeared to subside by 2.5 h following peptide administration. Thus it is likely that 2.5 h represents the extent of anti-inflammatory protection provided by a single i.p dose of 10 μg α-MSH.

4.3.2. Central effects of α-MSH and microglial activation

Microglia rapidly respond to pathogenic environmental changes in the brain and release a variety of cytotoxic and pro-inflammatory signals immediately following ischemia. However at later time points these cells may promote recovery through the release of neurotrophins and transforming growth factor-β1 [18]. Microglia express melanocortin receptors (predominantly MC_1) and previous reports show α-MSH to reduce brain TNF-α (of which microglia are the main source [248]) following i.c.v administration of LPS [268]. Furthermore *in vitro* LPS stimulated microglial cell lines (N9) treated with α-MSH have been shown to reduced production of TNF-α, IL-6 and NO [269] and when activated by LPS and IFN-ϒ microglia have been shown to produce α-MSH which may act in an autocrine fashion [269].

The lack of any effect on microglial activation in the present investigations may be indicative of α-MSH treatments not reaching the brain parenchyma. α-MSH has previously be shown to cross the BBB (albeit to a very limited degree) when administered intravenously [270]. A number of previous reports have demonstrated peripherally administered α-MSH to affect aspects CNS function [271, 272]. Huang *et al.* have shown i.p injection of α-MSH to suppress LPS mediated fever through actions on the brain in rats, the authors suggest the peptide may circumvent the BBB through circumventricular organs, which lack a tight BBB [271]. However no investigations were made into brain α-MSH concentration. Indeed, investigations into BBB permeability have given the most optimistic estimation of brain tissue entry of α-MSH following i.v administration to be 1-2 % of the circulating levels [270]. It may be still be that such low levels are able to influence brain functions however, given the results obtained herein, the single i.p injection administered in these investigations is unlikely to have such far

reaching effects. Indeed the lack of any BBB disruption observed in this model would further hamper α-MSH transit into the neurophil. Moreover while α-MSH administration induced a decrease in MC_4 expression in blood (as detected by qRT-PCR) the lack of any effects of on MC_4 expression in the brain could also be taken to support solely peripheral effect of α-MSH in this model. In support of this conclusion and fitting the observed reduction in leukocyte recruitment in the present study, investigations by Kastenbauer *et al.* have previously shown systemic administration of α-MSH but not intracisternal to reduce the CNS leukocyte infiltration in experimental pneumococcal meningitis, suggesting leukocyte recruitment to be inhibited by α-MSH from the blood side [273]. In light of the lack of any central effects detected in the current model and the poor BBB permeability of α-MSH it is unlikely that the observed actions on leukocyte recruitment are mediated via central MC_4 anti-inflammatory signalling.

4.3.3. Pharmacological investigation of MC involvement in modulating leukocyte recruitment

Of the five melanocortin receptors, anti-inflammatory actions have been attributed to MC_1, MC_3 and also to some extent MC_4 which mediates centrally activated anti-inflammatory circuits. Each of these receptors have been shown to be expressed at varying levels in the brain, with MC_4 being exclusively found in the CNS, while MC_1 and MC_3 have also been shown to be expressed on circulating immune cells and endothelial cells (Table 1.5). Numerous studies have provided compelling evidence for a role of both MC_1 and MC_3 in the endogenous control of leukocyte recruitment and as pharmacological targets for its modulation. However the exact role of the MC subtypes in mediating anti-inflammatory protection remains unclear.

Having demonstrated potent inhibitory effects of α-MSH treatment in this BCCAo model of cerebral I/R induced leukocyte recruitment, selective synthetic melanocortin agonists have been investigated in the same model to elucidate the main receptor type mediating these effects. Table 4.1 shows the pharmacological properties and doses of each melanocortin compound used.

4.3.3.1. The effect of BMS and DTRP on leukocyte recruitment

Treatment with the small molecule MC_1 selective compound, BMS-470539, provided significant inhibition of both leukocyte rolling and adhesion while activation of MC_3 using

[D-TRP^8]-γ-MSH only reduced levels of leukocyte adhesion suggest a less potent inhibitory effect from targeting MC_3 (Figure 4.3). However by 2 h following BCCAo despite having a more favourable pharmacokinetic profile BMS-470539 had lost its inhibitory actions while the action of [D-TRP^8]-γ-MSH seemed to have been enhanced. Thus suggest a role for MC_1 in initiating the anti-adhesive actions on leukocyte recruitment in the immediate early stages of reperfusion and that MC_3 has a longer pharmacodynamic effect in this model and is able to more effectively suppress leukocyte recruitment at later time points following the pathological event and/or the time of drug administration.

4.3.3.2. The dual roles of SHU9119

SHU9119 was used during these investigations as an $MC_{3/4}$ competitive antagonist for use in co-treatment studies to further investigate the role of specific MC subtypes in supressing cerebral leukocyte recruitment. Unexpectedly, by 40 min or reperfusion co-treatment with SHU9119 failed to inhibit not only the α-MSH induced inhibition of leukocyte recruitment but also the [D-TRP^8]-γ-MSH mediated effects. In fact SHU9119 was found to enhance these actions, and when administered alone SHU9119 was able to reduce leukocyte adhesion at 40 min following BCCAo. However by 2 h SHU9119 had lost these effects when administered alone, and in co-treatments was able to inhibit α-MSH and block [D-TRP^8]-γ-MSH effects. Thus supporting a role for $MC_{3/4}$ in mediating later effects on leukocyte adhesion.

While it was expected that by 40 min SHU9119 would abrogate the effects of α-MSH and [D-TRP^8]-γ-MSH by inhibiting MC_3 anti-inflammatory signalling, and when administered alone would enhance the I/R induced inflammatory response by blocking endogenous signalling, the observed early actions of SHU9119 may be explained by its seldom reported agonist actions at MC_1 and MC_5 (table 4.1).

SHU9119 was first synthesised by amino acid substitutions in the super potent MC agonist NDP-α-MSH [274] and has since regularly been used in numerous *in vitro* and *in vivo* studies as an antagonist at MC_3 and MC_4. In addition to competitively antagonising both murine and human MC_3 and MC_4 with minimal agonist activity, SHU9119 has also been found to be a potent antagonist at the frog MC_1. However SHU9119 also possesses, often overlooked, agonistic activity at human and mouse MC_1 and MC_5.

[274]. Indeed in experiments performed on B16 cell lines co-expressing MC_1, MC_3, and MC_4, SHU9119 treatment resulted in a net increase in cAMP signalling, despite acting as an antagonist to MC_3 and MC_4 [275]. Thus the reduction in leukocyte adhesion observed with SHU9119 treatment alone, can be explained by its agonistic actions at the anti-inflammatory receptor MC_1 and perhaps also partly due to agonist like activity at MC_3. MC_5 has not yet been detected on neutrophils, KO mice do not show an inflammatory phenotype, and no studies have yet revealed a role for MC_5 in supressing innate inflammation, however the receptor has been shown to be expressed on T and B lymphocytes. As such, given that SHU9119 may also act as an agonist at MC_5, a role for this receptor should not be entirely ruled out. Indeed a recent paper by Joseph *et al.,* airs caution in interpreting data from studies using the MC_3 agonist [D-TRP^8]-γ-MSH in murine cells due to agonistic activity at MC_5. [D-TRP^8]-γ-MSH was originally developed using the endogenous peptide $γ_2$-MSH which shows preferential binding to human (h) MC_3 and MC_4, modification of Trp to the D-isomer was found to cause a 100 fold increase in selectivity for MC_3 over MC_4 and a 250 fold increase in activity at hMC_3 compared to hMC_5 [262]. While studies on selectivity at the murine receptors were incomplete, [D-TRP^8]-γ-MSH has since been widely used as an MC_3 selective agonist at the murine MC_3. Joseph *et al*., have however recently demonstrated that [D-TRP^8]-γ-MSH is in fact a more potent inducer of cAMP signalling at murine MC_5 than it is at MC_3 [262]. Nevertheless the physiological significance of this revelation is still unclear due to a limited understanding of MC_5 in inflammation. Taking into account the lack of selectivity provided by [D-TRP^8]-γ-MSH and that SHU9119 also activates MC_5 any potential role for MC_5 in reducing post BCCAo leukocyte recruitment could only be present at the early stages. In co-treatment studies SHU9119 is able to inhibit the [D-TRP^8]-γ-MSH mediated effects at 2 h reperfusion, despite both SHU9119 and [D-TRP^8]-γ-MSH working as agonists at MC_5. Furthermore by 40 min of reperfusion while [D-TRP^8]-γ-MSH treatment reduces adhesion and SHU9119 fails to block the effects of [D-TRP^8]-γ-MSH, these peptides have no inhibitory actions on leukocyte rolling, indicating an early role for MC_1 in inhibiting leukocyte recruitment which perhaps also effects processes uniquely involved in leukocyte rolling, while MC_3 induces more prominent effects by 2 h.

Table 4.1; Doses and pharmacological properties of melanocortin compounds

Compound	Dose (i.p)	Ref. for dose	MC binding affinity IC50 (nM)				cAMP accumulation EC50 (nM)				Ca^{2+} accumulation EC50 (nM)				Pharmo-dynamic half life	Pharmo-kinetic halflife	Refs.
			MC_1	MC_3	MC_4	MC_5	MC_1	MC_3	MC_4	MC_5	MC_1	MC_3	MC_4	MC_5			
α-MSH	10μg	[211, 263]	4.3 (316*)	19 (18)	19 (19)	120 (96)	0.45 (0.70)	0.73 (2.10)	1.6 (2.6)	19 (2.0)	- (4.3)	- (1.3)	- (0.5)	- (1.3)	~2h	7-39 min	[262, 265, 273, 276-279]
BMS-470539	15μM /kg	[280]	120 (-)	- (-)	- (-)	- (-)	28 (11.6)	N/A	2600	4400	- (-)	- (-)	- (-)	- (-)	~8 h	1.7 h	[280, 281]
[D-TRP^8]-γ-MSH	10μg	[127]	- (-)	6.7 (-)	600 (-)	340 (-)	- (120)	0.33 (90)	100 (5580)	82 (48)	- (-)	- (-)	- (-)	- (-)	-	-	[262, 282]
SHU9119	10μg	[263]	0.7 (110)	0.23 (1.8)	0.06 (0.1)	0.09 (32)	0.04 (0.04)	2.8 (-)	N/A (-)	0.12 (0.4)	- (-)	- (-)	- (-)	- (-)	-	-	[274, 277, 278]
KPV	10μg	[283]	Does not bind any of the melanocortin receptors				Does not induce cAMP signalling				▲	- (-)	- (-)	- (-)	-	-	[284]

Binding assays competitive binding for 125I-NDP-α-MSH

Values show results from assays using human MCs. Data from mouse receptor studies are given in parenthesis.

N/A = no detectable activity

- = no data available

*Moller *et al.* performed binding studies on cells transfected with the murine MC_3, MC_4 and MC_5, however MC_1 studies were performed on a mouse melanoma cell line which over expresses MC_1, thus the level of receptor expression and conditions may be different compared to the other receptors and as such the obtained value should be taken with caution, especially since it does not fit with the human receptor binding data.

▲ increased calcium observed in MC_1 transfected cells but not quantified.

4.3.3.3 The temporal switch from MC_1 to MC_3 mediated effects

Convincing evidence has been provided for the anti-inflammatory actions of both MC_1 and MC_3 mediated signalling in a number of different *in vivo* and *in vitro* models.

Both MS05 and MS09 selective MC_1 agonists have been shown to down regulate the expression and secretion of E-selectin, V-CAM and I-CAM in human vascular endothelial cells stimulated with TNF-α [154, 155]. Conversely antibody neutralisation of MC_1 increases both basal levels and LPS-stimulated production of TNFα [156]. The anti-inflammatory action of α-MSH has also been shown to be reduced by MC_1 siRNA-transfection in RAW264.7 macrophages [285]. *In vivo,* the MC_1 agonist BMS-470539 has also been shown to reduce cytokine accumulation and leukocyte recruitment in a model of lung inflammation [280]. Additionally BMS-470539 has been shown to reduce leukocyte recruitment in both mesenteric I/R and PAF activated cremasteric vessels [151]. Moreover in both these vascular beds these effects were found to be absent in recessive yellow (e/e) MC_1 mutant mice [151]. In agouti allele "lethal yellow" mice the excessive production of the MC_1 MC_4 antagonist agouti protein, has also been demonstrated to incur an enhanced inflammatory response to picryl chloride [128].

On the other hand, treatment with the MC_1 selective MS05 failed to reduce MSU activation of macrophages and IL1-β release, while γ_2-MSH significantly reduced these effects and remained potent in macrophages isolated from e/e mice [263]. *In vivo,* the $MC_3^{-/-}$ mouse has been shown to display higher levels of leukocyte adhesion and emigration in the mesenteric microcirculation following I/R, while the e/e response was not significantly different from WT mice [127]. Additionally, in a model of peritonitis, treatment with the MC_3 agonists γ_2-MSH and Melanotan II both reduced PMN accumulation, effects which were blocked by antagonism with SHU9119 but not the MC_4 selective antagonist HS024 [263]. Furthermore these anti-inflammatory effects were again maintained in e/e mice [263].

The above investigations provide a complex picture by which the role of MC_1 and MC_3 in modulating leukocyte recruitment varies not only with pathological stimulus but also with the vascular bed. Given that the $MC_3^{-/-}$ mouse displayed an inflamed phenotype while e/e mice showed none, yet in the exact same mesenteric I/R model that MC_1 agonist BMS-470539 was effective in reducing inflammation, it may be that these receptors

show different endogenous physiological roles but that both may be harnessed pharmacologically. While others have demonstrated varying roles for MC_1 and MC_3 in different inflammatory models and differential roles in the physiological and therapeutic responses, the present study is the first to report a temporal influence on receptor role. Thus adding an additional layer of complexity to the role of the MRS in the inflammatory process.

It might be tempting to conclude that the apparent switch in significance of receptor subtype suppressing leukocyte recruitment, is due to receptor up regulation or increased receptor internalisation, however as detailed in chapter 3 no change in receptor mRNA was detected in blood or brain for both MC_1 and MC_3. While this does not exclude effects on receptor localisation and regulation at the protein level, it should be noted that that only a single dose of each drug was given at the start of reperfusion, and it should not be assumed that there are still sufficient circulating levels of the drug to activate newly up-regulated receptors at later stages of reperfusion. In fact the short half-life of α-MSH and [D-TRP^8]-γ-MSH might suggest otherwise.

The apparent value of targeting MC_1 to reduce early rolling and adhesion and MC_3 to reduce adhesion at later stages in reperfusion may be a reflection of different signalling pathways being initiated by the different receptors and/or drugs.

4.3.4. The NF-κB pathway in reducing leukocyte recruitment

Since pharmacological investigations reveal an apparent change in the importance of receptor subtype as reperfusion progresses yet qRT-PCR experiments showed no change in receptor expression across these time points, an initial investigation has been performed into whether these treatments were acting via NF-κB signalling.

The inhibition of NF-κB regulated transcription is a key element to the broad protective actions of the melanocortins, allowing for the inhibition of a plethora of cytokines, chemokines and adhesion molecules under its control. These effects are however transcriptionally dependant, with previous investigations showing NF-κB DNA binding only after 30 min following TNF-α stimulation followed by gene transcription 30 min later [286]. As such NF-κB induced transcription is unlikely to mediate the rapid effects on leukocyte recruitment observed, at least at 40 minutes, following BCCAo. To investigate

the effects of BCCAo and α-MSH treatments on NF-κB activation, qRT-PCR experiments have been performed to determine transcript levels of a key NF-κB controlled cytokine TNF-α along with the NF-κB regulator IκB. Bottero *et al.* have previously shown mRNA levels of IκB to correlate extremely well with NF-κB translocation as assessed by northern blot and gene reporter assays [286] with levels increasing within 30 min of NF-κB DNA binding. As such the real-time PCR measurement of IκB mRNA has been proposed as a rapid, sensitive, and powerful method to quantify NF-κB transcriptional activity [286].

Consistent with the early anti-inflammatory action of melanocortins not having been mediated by NF-κB inhibiting actions, both TNF-α and IκB transcript levels were found to be unchanged at 40 min following BCCAo in both brain and blood. Thus the production of molecules and signalling orchestrating these early leukocyte recruitment events are likely to be independent of gene transcription and likewise are the inhibitory actions of melanocortin treatments.

However by 2 h of reperfusion qRT-PCR revealed levels of IκB transcript to be significantly elevated in blood (by 25 fold) and TNF-α levels showed a trend toward an increase of 2.4 fold which were both supressed by α-MSH treatment (although not significantly in the case of TNF-α). While brain levels of both TNF-α and IκB were not significantly elevated, there was a strong trend toward an increase. However unlike in the blood where α-MSH reduced IκB levels by approximately 60 %, treatment failed to induce any change in IκB expression in the brain. This again suggests that in this model the peptide did not reach the brain parenchyma but instead exerted its action on blood borne or endothelial elements. The IκB inhibition observed in blood reveals that, 2 h following administration, α-MSH treatments are able to reduce BCCAo induced genetic responses, this effect is likely due to inhibition of NF-κB translocation to the nucleus as previously reported by others. Taken in conjunction with the pharmacological investigations these later effects inhibiting inflammatory gene transcription may be mediated by MC_3 signalling or at least it is unlikely that these effects are mediated by MC_1 or MC_4.

The fact that melanocortin treatments were able to significantly inhibit leukocyte recruitment at 40 min after BCCAo despite no detectable change in NF-κB activation

may be explained by other anti-inflammatory pathways centred on the melanocortin receptors. Indeed the observation of melanocortin anti-inflammatory influences in the absence of any transcriptional effect is not unique to the present study.

In zymosan stimulated primary peritoneal macrophages the α-MSH analogue AP214 reduced IL-1β and TNF-α release despite having no effect on mRNA levels of these cytokines [287]. α-MSH treatment has also been shown to reduce CD14 expression in LPS stimulated macrophages by shedding of the receptor from the cell surface and not via transcriptional mechanisms [288]. While the exact mechanism underlying these effects were not explored, antibody treatments revealed these effects to be mediated via MC_1, and α-MSH induced release of proteases was proposed as a speculative mechanism [288].

A seemingly similar mechanism that has been more thoroughly investigated is the α-MSH induced cleavage of the IL-8 receptors CXCR1 and CXCR2 on the neutrophil cell surface [181]. This was found to be mediated by MC_1 yet was independent of cAMP signalling and having no effect on mRNA levels. Instead this occurred *via* release of neutrophil elastase acting in an autocrine fashion on cell surface chemokine receptors, resulting in significant reductions in neutrophil chemotactic ability and oxidative burst [181]. As discussed in chapter 1, this demonstrated a novel MC_1 anti-inflammatory pathway independent of any action on NF-κB activity. Furthermore given that CD14 shedding has also been observed in response to MC_1 activation, this mechanism may even extend to proteolytic regulation of other chemokine receptors, thus helping to explain some of the reported rapid transcriptionally independent anti-inflammatory actions of melanocortin active compounds.

While the understanding of NF-κB independent MC effects is still in its infancy, these mechanisms may fit well with the early MC_1 mediated effects on leukocyte rolling observed here. While no temporal studies have yet been performed investigating these anti-inflammatory mechanisms it is entirely feasible that, due to the lack of transcriptional involvement, these pathways are more rapid than the classical inhibition of NF-κB nuclear translocation. As such it could be proposed that in the present model activation of MC_1 results in a rapid reduction in leukocyte recruitment via NF-κB independent

pathways while MC_3 provides more delayed protection through inhibition of NF-κB translocation.

4.3.5. Receptor independent actions of melanocortins.

It has been noted that some of the anti-inflammatory actions of α-MSH remain effective even with extremely low sub-picomolar concentrations of the agonist. In accordance to the experimentally derived binding affinity of α-MSH for the MCs very few, if any, receptors would be occupied [95], thus suggesting possible anti-inflammatory pathways independent of the melanocortin receptors. These receptor independent events have largely been attributed to the C-terminal tripeptide signal of α-MSH, KPV.

As such, in order to investigate whether the KPV sequence may have an influence over leukocyte recruitment in the BCCAo model and thus if the observed actions of α-MSH could also be via MC independent pathways, KPV treatments have been tested.

KPV was found to significantly reduce leukocyte recruitment at 40 minutes following cerebral I/R. Thus it appears that the KPV sequence of α-MSH does influence leukocyte recruitment and that MC independent mechanisms may be at least partly responsible for the anti-inflammatory actions of α-MSH. However by 2 h while still showing a trend toward a reduction the significant reduction in leukocyte adhesion observed at 40 minutes was lost.

KPV has been previously demonstrated to inhibit the elevation of a number of cytokines and reduce NF-κB activation in *in vitro* and *in vivo* inflammatory models [182] [95]. Furthermore in a murine model of peritonitis KPV also reduced PMN recruitment [140]. In the absence of melanocortin receptor binding [140, 183-185] and that KPV treatment fails to induce cAMP accumulation [140, 185], the mechanisum by which KPV exerts these effects is still incompletely understood. However, structural similarities between KPV and the IL-1β antagonist KPT indicate that KPV may act as an IL-1R antagonist. Indeed both KPV and α-MSH have been shown to displace surface binding of radio-labelled IL-1β, and inhibit IL-1 mediated effects [95, 186].

Synthesised as a precursor molecule which is proteolyticaly cleaved, IL-1β formation and release is rapid and independent of *de novo* synthesis. This pro-inflammatory

cytokine plays a key role in the inflammatory pathology of stroke, and is elevated early following brain injury [289]. The observed rapid reduction in leukocyte adhesion observed in the current model following KPV administration may be a result of KPV inhibiting signalling of the rapidly released IL-1β. Indeed IL-1R activation has been shown to be required for neutrophil recruitment [290] and an IL-1R antagonist is currently undergoing clinical trials to combat post stroke inflammatory reactions [116]. However in the present investigations the effects of KPV treatment diminish by 2 h post BCCAo. It may be that the single does given in this study is not enough to effectively antagonise a sufficient number of chemokine receptors as the inflammatory process progresses and serum levels of cytokines rise.

At the time of writing, no data on the exact pharmokinetics of KPV in blood has been published or indeed has there been any investigation into the possibility of endogenously formed KPV from α-MSH. A number of proteases are present in the circulation, which have previously been postulated to be responsible for the limited half-life of α-MSH [95]. Different proteases show different specificities for their substrate, elastase cleaves the bond after a small non-polar residue such as glycine, which separates the core α-MSH sequence from KPV. Since neutrophil elastases are elevated in inflammatory situations including stroke it could be possible that endogenous (or even exogenously administered) α-MSH is cleaved to give rise to KPV with both α-MSH (1-10) and KPV working cooperatively to resolve inflammatory events. Binding studies showing α-MSH to displace IL-1β suggest that the KPV sequence may be active even in the full $\alpha\text{-MSH}_{(1\text{-}13)}$. Whether or not KPV signalling occurs *in vivo* as part of α-MSH or as a cleavage product would require further investigation, nevertheless in the present model it would seem likely the KPV signalling is in part responsible to the early α-MSH mediated effects.

The observation that KPV is effective at reducing early leukocyte recruitment in a similar fashion to the MC_1 targeted BMS-470539 is interesting. In the present model, while α-MSH may signal *via* the KPV sequence is a possibility, [D-TRP^8]-γ-MSH however does not contain this amino acid sequence (Table 1.4) and nor does the molecular structure of BMS-470539 mimic it, and as such are unlike to act via the same pathway. The similarities between KPV and BMS-470539 in the observed actions on leukocyte recruitment and duration of effect, could be a result of both pathways inhibiting cytokine/

chemokine signalling (via blocking IL-1β or via cleavage of receptors) rather than their production via NF-κB.

4.3.6. Melanocortins effect on Cytokine expression

Investigations into cytokine levels following BCCAo revealed that all melanocortin agonists tested reduced the I/R induced rise in TNF-α circulating protein levels by 2 h while also reducing MCP-1 (however these levels remained below the accurate detectable range of the kit).

Up-regulated following stroke, TNF-α has also been recognised as a valuable potential target in stroke with recent observational studies in humans showing promise for TNF-α inhibitor Etanercept [291]. In the present model the non-selective α-MSH proved to be the most potent inhibitor of TNF-α and BMS-470539 the least (with only ELISA analysis and not CBA detecting a significant decrease). Melanocortin treatments have previously been shown to potently inhibit TNF-α via inhibiting NF-κB. As such the reduced TNF-α levels found with BMS-470539 at 2 h post stroke would suggest that BMS-470539 also activates pathways inhibiting NF-κB as well as rapid actions on leukocyte recruitment.

As cytokine investigation was performed on seum samples and not whole blood, it may be inferred that TNF-α levels being measures are of the soluble form rather than membrane bound. As with many molecules governing inflammatory process, there is a duality in TNF-α function. While generally considered detrimental in acute inflammatory responses, TNF-α may promote repair and tissue remodelling at later time points with anti-TNF treatments preventing hippocampal neurogenesis two weeks following cerebral ischemia [18, 244]. It has also been suggested that solTNF signalling through TNFR1 may mediate the damaging inflammatory actions of TNF-α while tmTNF signals through TNFR2 could provide protection [18, 244]. While the reduction in solTNF-α found in this model is likely to be entirely beneficial, considering α-MSH reduces TNF-α mRNA it would also be interesting to investigate the effects on levels of the potentially protective tmTNF-α.

The observed reduction in TNF-α mRNA transcript in the blood following α-MSH treatment (although not statistically significant) may account for the decrease in circulating TNF-α found with all melanocortin treatments, as occurring by NF-κB

inhibition. However in the CBA assay (while not significant) trends toward reduced TNF-α are observed with α-MSH and BMS-470539 treatment as early as 40 min post BCCAo. Thus, inhibition of TNF-α cleavage and release should not be entirely ruled out as an explanation for reduced circulating TNF-α, as such future investigations into melanocortin regulation of TACE could prove interesting. Especially given that inhibitors of this enzyme have proven to be protective in experimental stroke [292].

In the current investigations, surprisingly, both α-MSH and [D-TRP^8]-γ-MSH (but not BMS-470539) were found to increase IL-6 levels at both 40 min and 2 h following BCCAo. IL-6 is a pleiotrophic cytokine displaying a duality of roles. On the one hand IL-6 is an endogenous pyrogen with chemotactic activity and increased levels have been shown to correlate with poor outcome in stroke patients [18]. Whereas, IL-6 KO mice show no protection following experimental stroke [250], under certain conditions IL-6 has also been shown to reduce oxidative stress and preserve mitochondrial function [212] with up-regulation of IL-6 and its receptor being shown to accelerate nerve regeneration following trauma. Furthermore IL-6 can activate the hypothalamic-pituitary-adrenal axis and stimulate cortisol production thus stimulating anti-inflammatory signalling

A number of reports show melanocortin active peptides to reduce IL-6 expression [269, 287]. However others have reported the contrary with melanocortins inducing IL-6 expression [293]. This discrepancy seems to be explained by the findings of Lindberg *et al.* in a human microglial cell line that rather than enhancing the inflammatory release of IL-6 (in this case in response to Aβ), α-MSH elevates the basal levels [294]. Since in the present investigations melanocortin treatments were not given to sham operated animals it cannot be concluded that this is the case in the *in vivo* situation, however that BCCAo was not found to induce IL-6 alone, could support the findings of Lindberg. Furthermore Lindberg *et al*. found the increase in IL-6 to be primarily mediated via MC_3 thus perhaps explaining why IL-6 induction was not observed in BMS-470539 treated animals.

While in the present model α-MSH is able to increase IL-6, α-MSH has also been demonstrated to antagonise IL-6 induced fever. This ability to both induce IL-6 expression while blocking detrimental IL-6 induced pathways may illustrate the benefit of targeting endogenous pathways for the pro-resolving modulation of inflammatory processes over an indiscriminate termination of inflammation. Indeed IL-6 up regulation

has also been observed in the rat heart following α-MSH administration inducing a phenotype similar to ischemic preconditioning. Further investigations will need to be made into the exact role of α-MSH mediated increase in IL-6 in the present model, and furthermore whether BCCAo induces IL-6 at later stages in the inflammatory cascade.

Both α-MSH and [D-TRP^8]-γ-MSH were found to significantly induce the anti-inflammatory cytokine IL-10 by 2 h following BCCAo. However BMS-470539 treatment did not reveal any up-regulation of IL-10 at the time points tested. It may be that MC_1 prevents early rolling and represses immediate early pro-inflammatory effects while MC_3 may be more prominent in initiating pro-resolving ques. Indeed Vulliémoz *et al.* have found that SHU9119 inhibition of $MC_{3/4}$ signalling did not affect the pro-inflammatory cytokine response, but was found to decrease the pro-resolving IL-10 [295].

4.4. Summary

Investigations utilising a number of melanocortin active compounds have revealed potent inhibitory actions of the melanocortins on leukocyte recruitment following cerebral I/R with corresponding reduction in circulating inflammatory cytokines and up regulation of the anti-inflammatory IL-10. However no effect on microglial activation was observed and the findings suggest treatments act via peripheral effects and as such are independent of MC_4.

In mediating inhibition of leukocyte recruitment MC_1 seemingly provided greater inhibition at the immediate early stage following BCCAo, being able to initiate signalling which reduced leukocyte rolling as well as adhesion. However allowing for progression of reperfusion to 2 h revealed these effects to dissipate with MC_3 signalling providing more effective modulation of leukocyte recruitment. These effects were supported by findings from co-treatments with the $MC_{3/4}$ antagonist $MC_{1/5}$ agonist SHU9119.

While analysis of melanocortin receptor mRNA showed no regulatory changes between 40 min and 2 h of reperfusion, NF-κB genes were only found to be inhibited at 2 h and not 40 min. Thus, suggesting NF-κB independent mechanisms centred on MC_1 to mediate the early suppression of leukocyte recruitment. Differences in cytokine regulation between MC_1 targeted activation and MC_3 targeted/non-specific activation also support the hypothesis that MC_1 initiates distinct signalling pathways early following

reperfusion under the conditions tested. Whereas MC_3 activation was found mediated more sustained anti-inflammatory protection most likely via inhibition of NF-κB activation.

4.5. Future Directions

This work has provided a unique insight into the melanocortin receptors involved in modulating leukocyte recruitment in the cerebral microcirculation. In addition to understanding the receptor subtype mediating these effects it may also be beneficial to gain a greater understanding of the exact signalling mechanisms initiated. Further work could also explore the novel finding of a temporal switch in receptor importance, and confirm the proposed differences in signalling by assessing NF-κB activation following administration of MC_1 and MC_3 selective drugs in WT and receptor mutant mice. Indeed development of agonists not only activating the desired receptor but displaying "directed signalling" and activating the desired pathway may provide a fine tuning of melanocortin based drugs. In order to fully corroborate these findings it is also important that later time points of reperfusion be assessed, along with later treatment times to assess the potential for delayed treatment.

In order to investigate whether melanocortin treatments elicit these anti-inflammatory effects via direct actions on leukocytes or through modulating the functioning of endothelial cells, perivascular components or neurons, experiments could be performed using melanocortin treatments on autologous blood. Blood samples could be taken from mice prior to BCCAo surgery and immune cells isolated and fluorescently labelled. These cells could then be treated with melanocortin compounds before being re-introduced into the animal following BCCAo. Treated leukocytes could thus be distinguished from untreated and the level of leukocyte recruitment recorded.

As with all pharmacological investigation, studies can be limited to the selectivity of compounds tested and as such future investigations could use newly developed more selective compounds such as the MC_4 selective agonist recently synthesised by Eliasen *et al.* [296]. However at the time of writing no new selective MC_3 compounds have been produced. In the present study while the pharmacological studies have provide some interesting insights this work has also been backed up by work in receptor mutant animals to provide a robust body of evidence.

5.0. Investigations utilising receptor mutant mice

5.1. Introduction

The production of knockout mice along with the use of strains bearing specific genetic abnormalities has provided scientists with a powerful tool to investigate of the role of various molecules in the physiological maintenance of homeostasis as well as in disease progression. The production of mice lacking specific adhesion molecules has been critical in understating the discrete roles of CAMs in the leukocyte recruitment cascade. Melanocortin receptor KO mice have also demonstrated critical roles for MC_4 in metabolism and food intake, MC_1 in pigmentation and a role for MC_3 in adiposity and modulating inflammatory processes.

While the pharmacological investigations detailed in chapter 4 have revealed a possible temporally dependant switch in receptor importance to reducing leukocyte recruitment, such studies are limited not only by the pharmacodynamic profile and relative selectivity of the compounds used but also in that they fail to give an insight into the endogenous functioning of the MRS. Thus results from pharmacological studies have been substantiated by utilising a genetic approach to investigating the MRS. In this chapter mice bearing a mutation incurring a non-functional MC_1 (recessive yellow (e/e) mice) and $MC_3^{-/-}$ mice have been used to investigate the endogenous role of melanocortin receptors in modulating leukocyte recruitment following global cerebral ischemia. The phenotypic responses of these animals to pathological challenge has been compared to wild type (WT) C57BL/6 mice along with their ability to respond to therapeutically administered exogenous α-MSH has also been assessed.

5.2. Results

5.2.1. MC1 mutant mice display enhance leukocyte recruitment 40 min following BCCAo

Leukocyte endothelial interactions in the cerebral microcirculation following BCCAo were investigated in both MC_1 mutant e/e mice and $MC_3^{-/-}$ mice at 40 min of reperfusion (Figure 5.1). In the absence of a pathological challenge sham operated mice showed no significant differences between WT, e/e and $MC_3^{-/-}$ strains. However a slight trend toward an increase in rolling and adhesion of leukocytes was observed in e/e mice, conducting further experiments could provide an insight into whether this is a subtle effect or simply due to variation within the group. Following induction of 5 min global ischemia there was however a pronounced increase in leukocyte rolling which in e/e mice was over and above that found in WT and $MC_3^{-/-}$ mice. Whereas in MC_3 mice there was a trend toward enhanced leukocyte rolling following BCCAo (60 % increase compared to WT mice), in e/e mice levels of leukocyte rolling (539.3 cells/mm^2/min) were found to be 282.5 % of that found in WT. Leukocyte adherence also showed a slight but not significant trend toward an increase in the e/e mice (326.3cells/mm^2/min) compared to WT (282.3 cells/mm^2/min) while in $MC_3^{-/-}$ mice no change in adhesion was observed. This is fitting with the results obtained during pharmacological investigations showing MC_1 to be important in regulating leukocyte recruitment at 40 min following BCCAo and that this receptor has a particular influence over leukocyte rolling. A comparison of data from pharmacological studies and the results from MC mutant mice is shown in tables 5.1

Therapeutically administered α-MSH remained active in reducing leukocyte endothelial interactions in mice lacking an MC_3, suggesting melanocortin receptors other than MC_3 are able to propagate anti-inflammatory responses to α-MSH. In e/e mice α-MSH treatment failed to significantly reduce leukocyte rolling. While there was a trend toward reduced rolling following α-MSH treatment, leukocyte rolling still remained at a level that was greater than that found in BCCAo WT mice treated with saline alone (259.1 cells/mm^2/min compared to 191 cells/mm^2/min in WT saline treated). α-MSH treatment in e/e mice was nevertheless able to significantly inhibit the number of leukocytes that become adherent to the vessel wall (>80 % reduction). While $MC_3^{-/-}$ mice showed a trend toward increased rolling velocity compared to wild type animals there were no significant alterations in WBC rolling velocity found across all treatments and mouse strains tested.

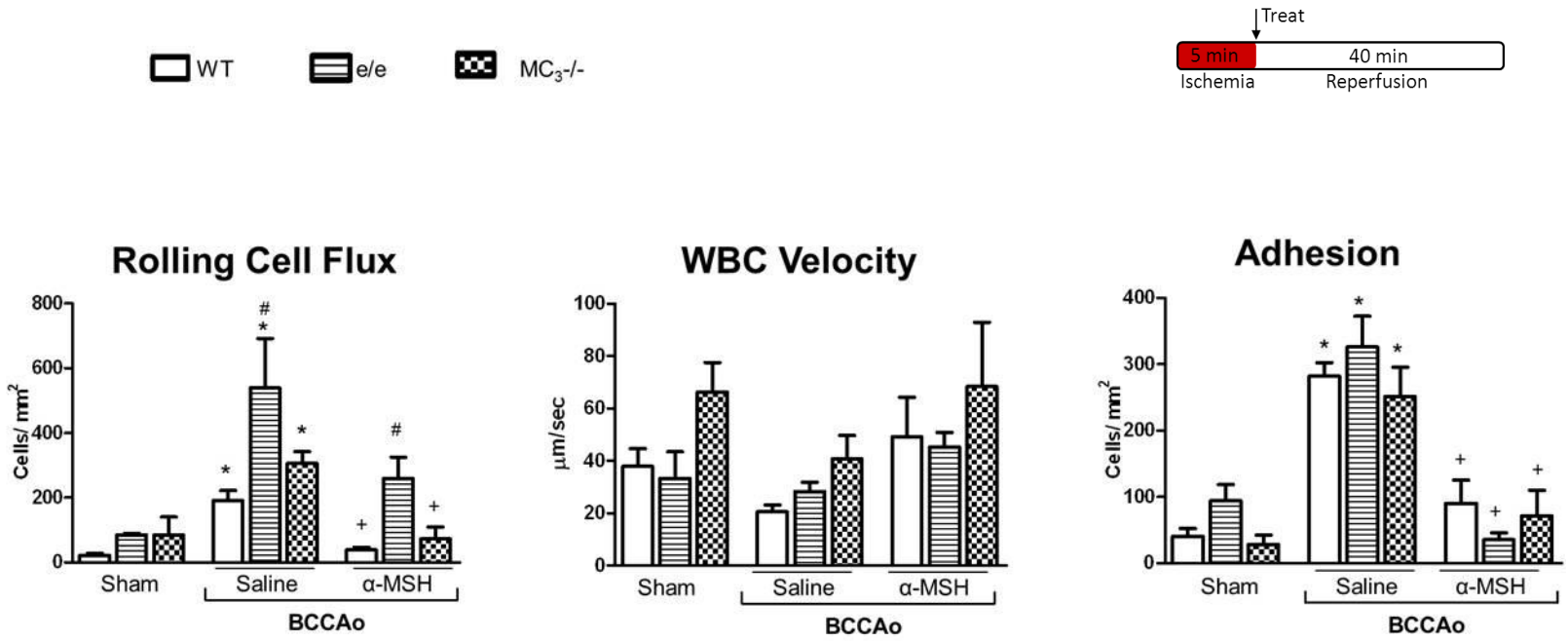

Figure 5.1. ***Comparison of leukocyte recruitment in WT, e/e and $MC_3^{-/-}$ mice at 40 min after BCCAo.*** Wild type C57BL/6 (white bars), MC_1 mutant e/e (lined bars) and $MC_3^{-/-}$ (checked bars) mice were subjected to sham or BCCAo surgery with 40 min reperfusion and treatment with either saline or 10µg α-MSH at the start of reperfusion. Leukocyte recruitment in the cerebral microcirculation was quantified in terms of (A) rolling cell flux (B) WBC rolling velocity and (C) Number of adherent cells. WT groups n = 6, e/e and $MC_3^{-/-}$ sham group n = 3 all other groups n = 4. Statistical analysis was performed between mouse strains, within sham or saline treated groups to determine the phenotypic differences between strains, # denotes significance to WT $P<0.01$. Statistical analysis was also performed between sham, saline and α-MSH treatment groups within the same strain. * shows significance to sham group $P<0.05$ and + shows significance to saline treated BCCAo group $P<0.05$.

Tables 5.1. Comparison of IVM parameters from wild type pharmacological studies and receptor mutant mice at 40 min reperfusion

Rolling (cells/mm^2/min)

Strain	Sham	BCCAo			
		Saline	α-MSH	BMS	DTRP
Wild Type	21.5 ± 6.9	191.0 ± 31.5	58.1 ±10.4	58.1 ± 29.3	139.1 ± 38.6
MC1 mutant e/e	85.48 ± 4.1	539.3 ± 153.1	259.1 ±65.8	-	-
MC3-/-	84.18 ± 56.1	305.4 ± 37.14	72.2 ± 37.4	-	-

Velocity (µm/sec)

Strain	Sham	BCCAo			
		Saline	α-MSH	BMS	DTRP
Wild Type	37.9 ± 6.8	20.5 ± 2.7	66.7 ± 7.3	72.1 ± 9.1	38.1 ± 10.7
MC1 mutant e/e	33.3 ±10.1	28.3 ± 3.6	45.3 ± 5.6	-	-
MC3-/-	66.2 ± 11.2	53.2 ± 6.8	68.33 ± 24.5	-	-

Adhesion (cells/mm^2/min)

Strain	Sham	BCCAo			
		Saline	α-MSH	BMS	DTRP
Wild Type	42.1 ± 14.4	282.3 ± 49.4	90.35 ± 34.96	58.0 ± 20.7	121.2 ± 23.7
MC1 mutant e/e	94.6 ± 24.0	326.3 ± 46.3	35.5 ± 10.4	-	-
MC3-/-	28.2 ± 14.3	251.5 ± 44.5	71.1 ± 39.1	-	-

5.2.2. Cytokine expression

The serum protein expression of TNF-α (by ELISA) was measured in both e/e and $MC_3^{-/-}$ experimental animals and compared to levels found in WT.

At 40 min following BCCAo serum levels of TNF-α were found to be significantly elevated in e/e mice compared to levels in WT and $MC_3^{-/-}$ mice. These levels in e/e mice at 40 min following BCCAo were found to be greater than those reached in WT following 2 h of reperfusion. TNF-α levels in $MC_3^{-/-}$ mice however were not found to be significantly different from WT at the time point tested, also remaining below the detectable range of the ELISA kit.

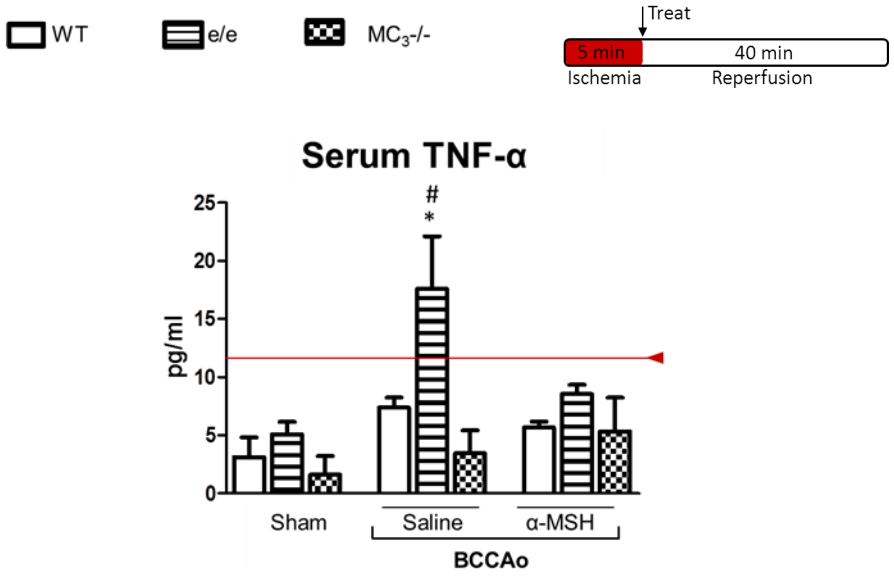

Figure 5.2. ***Comparison of serum TNF-α levels in e/e and $MC_3^{-/-}$ mice.*** Serum samples from WT (white bars), e/e (striped bars) and $MC_3^{-/-}$ (checked bars) mice subjected to BCCAo with 40 min reperfusion were analysed for protein levels of TNF-α by ELISA. N = 3 for all groups. * denotes significance to sham groups $P<0.01$, # denotes significance to WT $P<0.05$.

5.2.3. Leukocyte recruitment in recessive yellow e/e mice following 2h reperfusion.

With an aim to investigate whether the temporal shift in receptor importance observed during pharmacological investigations was also present as a physiological phenomenon, recessive yellow e/e mice were also investigated in the 2 h reperfusion model to see if the inflammatory phenotype persisted.

While at 40 min following BCCAo e/e mice displayed significantly higher levels of leukocyte rolling than WT, by 2 h of reperfusion no significant differences in the leukocyte recruitment response could be observed between e/e mice and WT. Across all parameters measured and across all treatment groups no significant differences were found between the two strains. There was however a trend toward reduced leukocyte adhesion following BCCAo in e/e mice, greater n numbers will be required to determine if this is a true effect or an artefact due to the low n numbers used. Indeed due to limited availability of the e/e mouse strain and the lack of alternative suppliers it has only been possible to obtain an n of 3 for 2 h sham and α-MSH BCCAo groups, whereas power calculations (section 2.4) recommend a minimum group size of 4. As such caution should be taken in the interpretation of the results from these two groups. However in the present investigations it does appear that the lack of any enhanced rolling and adhesion at 2 h following BCCAo in e/e mice (n = 4) again mirror the results of the pharmacological investigations at 2 h, which also demonstrate the MC_1 selective BMS-470539 loses its anti-inflammatory potency by 2 h of reperfusion. Comparisons of pharmacological data and results of investigations in e/e mice at 2 h reperfusion are given in tables 5.2.

Due to limited availability of the $MC_3^{-/-}$ strain no studies have been performed at 2 h of reperfusion using the $MC_3^{-/-}$ mice.

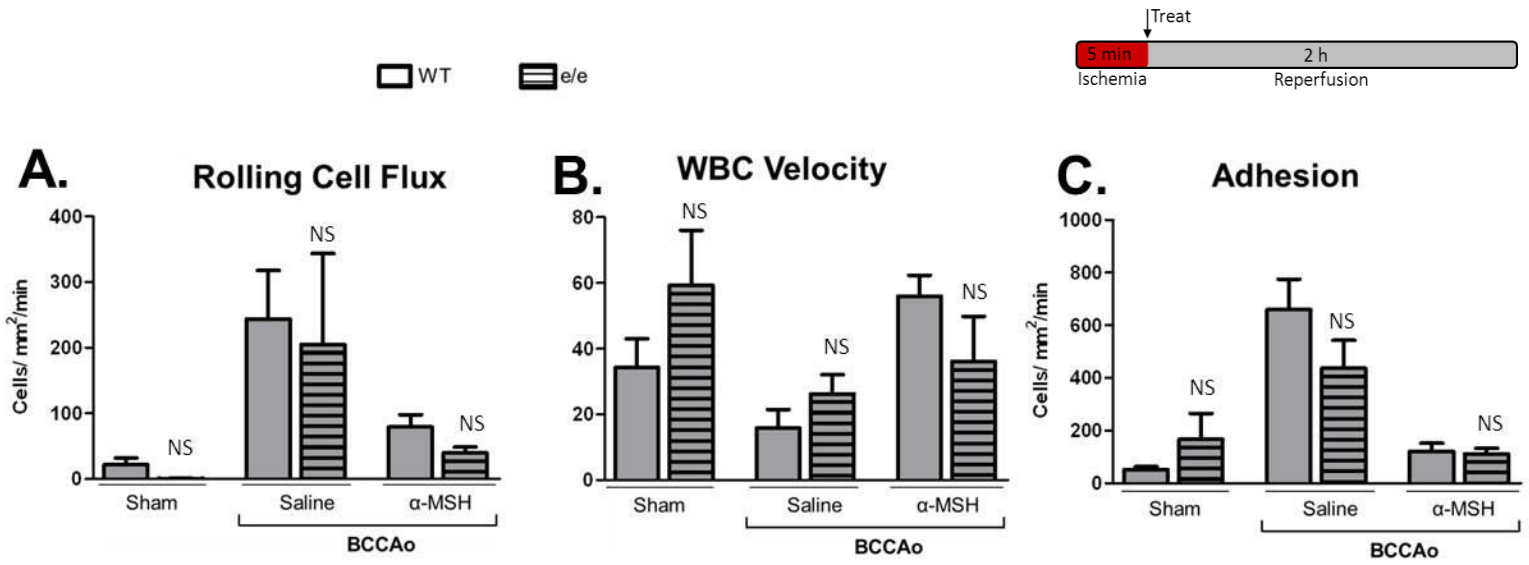

Figure 5.3. ***Leukocyte recruitment in e/e mice following 2h reperfusion*** Wild type C57BL/6 (white bars) and e/e (lined bars) mice were subjected to sham or 5 min BCCAo and treated with saline or 10μg α-MSH at the start of reperfusion .leukocyte recruitment was quantified at 2h of reperfusion in terms of (A) rolling cell flux (B) WBC rolling velocity and (C) Number of adherent cells. N = 4 for WT sham and saline groups and e/e saline group, n = 5 for WT α-MSH group and n = 3 for sham and α-MSH e/e groups Statistical analysis was performed between mouse strains, within the same treatment group to evaluate phenotypic differences between strains. NS denotes no statistical significance to WT group (P>0.05)

Tables 5.2. Comparison of IVM parameters from wild type pharmacological studies and e/e mice at 2 h reperfusion

Rolling (cells/mm^2/min)

Strain	Sham	BCCAo			
		Saline	α-MSH	BMS	DTRP
Wild Type	21.9 ± 10.1	243.9 ± 74.0	79.3 ± 18.5	80.1 ± 55.3	103.7 ± 29.4
MC1 mutant e/e	0.63 ± 0.5	204.7 ± 138.6	39.86 ± 8.8	-	-

Velocity (μm/sec)

Strain	Sham	BCCAo			
		Saline	α-MSH	BMS	DTRP
Wild Type	34.3 ± 8.7	15.8 ± 5.7	55.9 ± 6.4	32.2 ± 4.0	45.8 ± 8.0
MC1 mutant e/e	59.3 ± 16.7	26.3 ± 5.8	36.7 ± 13.8	-	-

Adhesion (cells/mm^2/min)

Strain	Sham	BCCAo			
		Saline	α-MSH	BMS	DTRP
Wild Type	51.9 ± 11.3	659.7 ± 115.6	121.5 ± 30.5	238.3 ± 184.9	61.75 ± 10.10
MC1 mutant e/e	166.7 ± 98.3	437.2 ± 106.1	112.3 ± 20.4	-	-

5.2.4. TNF-α levels in e/e mice at 2 h of reperfusion

TNF-α levels measured in serum of e/e mice following BCCAo and 2 h of reperfusion revealed a drop in the enhanced response seen at 40 minutes. This appeared to correlate with the observed reduction in leukocyte recruitment parameters, as ELISA detection of TNF-α serum levels showed no significant difference between e/e and WT animals. However, despite a trend toward an increase, due to the large variability in e/e sham animals (as reflected by the large error bars) the elevation in TNF-α levels following BCCAo in e/e mice was not statistically significant. α-MSH treatment however produced significant reduction in both WT and e/e mice.

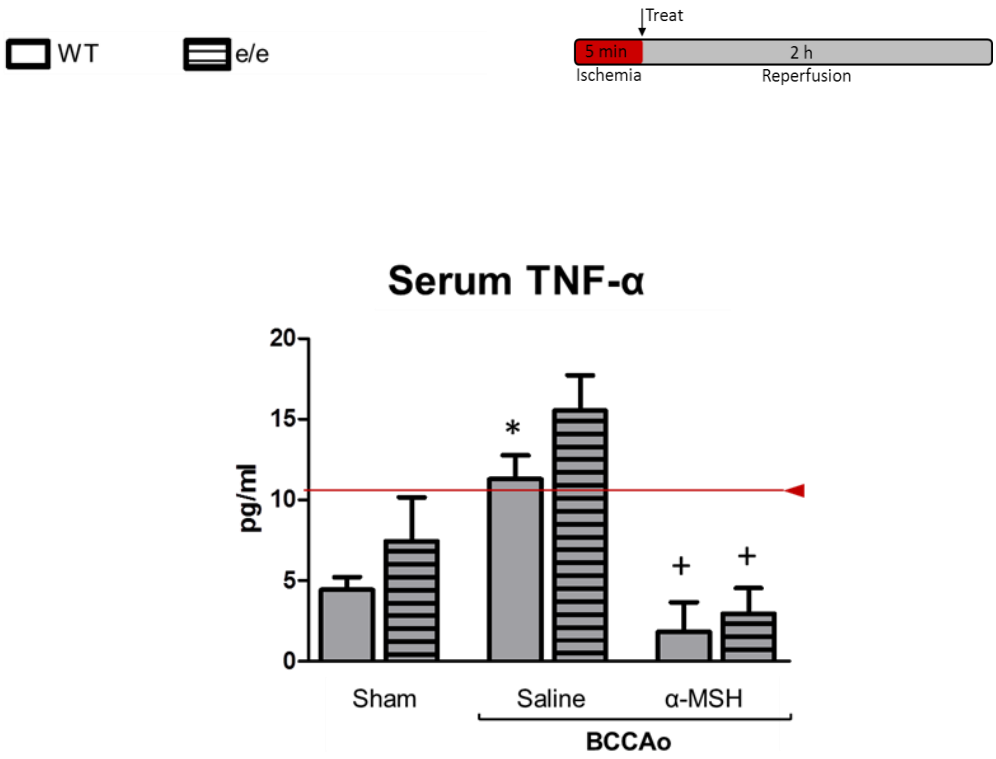

Figure 5.4. ***TNF-α levels in e/e mice at 2 h reperfusion.*** Serum samples from WT (white bars) and e/e (striped bars) mice subjected to BCCAo with 2 h reperfusion were analysed for protein levels of TNF-α by ELISA. N = 3 for all groups * denotes significance to sham groups $P<0.01$, + denotes significance to BCCAo $P<0.05$.

5.3. Discussion

Investigations in e/e and $MC_3^{-/-}$ mice have revealed an enhanced inflammatory response in the MC_1 mutant e/e mouse at 40 min of reperfusion with elevated leukocyte recruitment and serum TNF-α levels. MC_3 mice on the other hand showed no abnormalities in leukocyte recruitment or TNF-α regulation at this early time point. The inflammatory phenotype of the e/e mouse was however not maintained as the post I/R inflammatory response progressed with leukocyte recruitment and TNF-α levels being unchanged compared to WT at 2 h reperfusion. This work supports an early role for MC_1 in reducing post I/R inflammatory responses.

5.3.1. Phenotype of melanocortin receptor mutant mice

In the present model e/e mice displayed enhanced leukocyte rolling and inability to regulate leukocyte recruitment at the very onset of the inflammatory response along with elevated levels of the pro-inflammatory cytokine TNF-α.

Leukocyte differential counts have shown no significant differences in circulating PMN or monocyte levels in e/e or $MC_3^{-/-}$ mice under basal conditions [127, 297]. It is therefore unlikely that the observed enhanced leukocyte recruitment in e/e mice is simply due to a higher basal number of circulating neutrophils, especially as leukocyte rolling and adherence in sham e/e mice were not significantly different from that in WT. Inflammatory events have been shown to induce elevated blood levels of immune cells, indeed TNF-α alone has been shown to induce neutrophilia [298], it is therefore possible that cerebral I/R may induce a greater increase in circulating neutrophils in e/e mice than wild type, thus explaining the elevated levels of leukocyte-endothelial interactions. However mesenteric I/R has been previously observed to increase the number of circulating PMN levels to a similar degree in e/e mice as in WT [297]. Therefore the observed exacerbated leukocyte recruitment in e/e mice is likely to be a result of the lack of acute MC_1 mediated anti-inflammatory signalling. Furthermore the lack of any significant phenotype in sham operated e/e mice suggest anti-inflammatory signalling via MC_1 not to be tonic but that they are reactive to pathological stimuli.

These findings support the conclusions drawn from pharmacological investigations that MC_1 is important in enabling rapid anti-inflammatory responses. Furthermore these

results demonstrate that endogenous melanocortins may provide this immediate anti-inflammatory response via MC_1, and that MC_1 is particularly important in regulating the rolling stage of leukocyte recruitment.

During the pharmacological studies the observation that BMS-470539 and α-MSH reduced rolling as well as adhesion was taken as being a more potent anti-inflammatory action. However given that in e/e mice only rolling, but not adhesion, was significantly enhanced and that α-MSH was still able to reduce adhesion (but not rolling), it may be that MC_1 plays a critical role in inhibiting the expression of adhesion molecules important for leukocyte rolling. Such a mechanism would fit with the early time of the observed effect, one that might be assumed to be transcriptionally independent due to the rapidity of onset. Selectins, which mediate leukocyte capture and rolling, are rapidly up-regulated to the endothelial surface in inflammatory reaction. Indeed exocytosis of weible palade bodies (WPB) is induced within minutes of hypoxia, resulting in increased endothelial P-selectin expression and the release of the neutrophil chemoattractant IL-8 along with other inflammatory, pro-thrombotic and vasoactive factors [299]. It is possible that MC_1 signalling inhibits this rapid exocytosis of WPBs. In fact α-MSH has been shown to reduce the release of histamine a potent initiator of WPB exocytosis, and that this effect is mediated via MC_1 [300]. Furthermore TNF-α has been shown to stimulate WPB release in a dose-dependent manner. Thus the observed enhanced TNF-α in e/e mice may have implications for MC_1 mediated effects on WPB exocytosis, P-selectin expression and thus leukocyte rolling. While a unique aspect of MC_1 signalling appears to be on the cleavage of chemokine receptors, no reports have yet shown melanocortins to induce the shedding of receptors involved in WPB exocytosis.

While elevated TNF-α levels observed may play a role in mediating leukocyte recruitment, the actions of this cytokine may also enhance the pathological potential of invading neutrophils. TNF-α is known to prime neutrophils allowing maximal neutrophil degranulation and NADPH oxidase activity following neutrophil activation [298]. Neutrophil priming by cytokines may also cause a reduced deformability of cells causing a higher retention in capillary beds, and perhaps more likely to cause vessel plugging and secondary ischemia [298]. Thus the melanocortin mediated control over TNF-α levels may be particularly beneficial in regulating leukocyte recruitment in the brain following I/R.

5.3.2. Conflicting views on the roles of MC_1 and MC_3: a difference in timing, micro-environment and pathology.

A number of apparently conflicting reports have been made regarding the role of melanocortin receptors in MC_1 mutant and MC_3 null mice. The $MC_3^{-/-}$ mouse has been shown to display an enhanced inflammatory phenotype, with increased leukocyte recruitment following mesenteric I/R [127]. Since the widely studied endogenous α-MSH shows preferential binding to MC_1, the discovery of a physiological role for MC_3 in regulating inflammation, may potentially implicate a role for endogenous MC_3 ligand γ-MSH in suppressing inflammation. However relatively few studies have as yet been performed assessing the physiological influence of γ-MSH in controlling inflammation. On the other hand in the same mesenteric I/R model the MC_1 selective BMS-470539 provided potent anti-inflammatory amelioration [151] yet the e/e mice showed no inflammatory phenotype [127]. This complex scenario, discussed in chapter 4, led some to postulate that while both MC_1 and MC_3 are both expressed in mesentery, MC_3 provides physiological anti-inflammatory protection by endogenous mechanisms (and as such also provides a pharmacological target) while MC_1 serves no significant endogenous anti-inflammatory role in this model but that this receptor could also be pharmacologically utilised to reduce inflammation.

However a number of studies since, while showing important pharmacological roles for MC_3, have failed to detect an endogenous role in the $MC_3^{-/-}$ mouse, with no evident exacerbation of inflammatory response observed. Protective effects of AP214 in zymosan induced peritonitis were found to be lost in $MC_3^{-/-}$ mice but not in e/e mice [287] however in this model no physiological role for each receptor was observed, with zymosan induced neutrophil recruitment being at comparable levels to WT [287]. Also, in a model of lung inflammation MC_3 activation by [D-TRP^8]-γ-MSH reduced eosinophil and lymophocyte accumulation 24 h following a 2 week immunization protocol, with these effects being lost in $MC_3^{-/-}$ mice. However no inflammatory phenotype was observed in either $MC_3^{-/-}$ or e/e animals in the absence of pharmacological intervention. These studies suggest that the observed inflammatory phenotype of the $MC_3^{-/-}$ mouse in mesenteric I/R may be a tissue/ pathology specific phenomenon.

In the present study however no inflammatory phenotype was observed in the $MC_3^{-/-}$ mouse at the time point tested nevertheless pharmacological results, as discussed in

chapter 4, suggest that any $MC_3^{-/-}$ phenotype may become obvious with extended periods of reperfusion. Indeed that the enhanced inflammatory response detected at 40 min following I/R in e/e mice subsided by 2 h would suggest that endogenous compensatory mechanisms are initiated, of which MC_3 could be central. Suggesting the importance of endogenous MC_3 anti-inflammatory signalling may be unique to I/R as a phlogistic stimulus.

Unlike in mesenteric I/R (35 min I, 90 min R) [127] in the present study an endogenous anti-inflammatory role for MC_1 was detected, with e/e mice displaying enhanced leukocyte rolling and elevated serum TNF-α. This may point toward differential roles for melanocortin receptors in a tissue specific and/or temporally dependant manner. Indeed while the present study demonstrates an early role for MC_1 potentially switching to MC_3 as reperfusion progresses, the I/R protocol used by Leoni *et al.* is, temporally speaking, from the onset of ischemia more in line with the 2 h reperfusion studies performed here. As such the observed inflammatory phenotype of the $MC_3^{-/-}$ mouse following mesenteric I/R and apparently normal e/e mouse fits with the results and predictions made in the present study.

The observation of an endogenous role for MC_1 is not however unique to the current investigations. e/e mice have been shown to display an exacerbated inflammatory phenotype in two distinct models of colitis (dextran sodium sulphate induced and bacterial) [171]. In these studies by Maaser *et al.* the colon of untreated e/e mice showed no phenotype under basal conditions, suggesting MC_1 is not essential for maintenance of homeostasis in the absence of challenge. This is reflected in the present findings where e/e mice showed similar levels of leukocyte recruitment in sham operated as shown by WT mice, also fitting with the apparent lack of phenotype observed in humans bearing MC_1 mutations (with exception to hair and skin pigmentation). In DSS colitis models however it was found that the resultant inflammatory reactions caused the eventual death of all e/e mice while all WT animals survived. In the present study however it appears the inflammatory phenotype of e/e mice quickly dissipates as MC_3 mechanisms compensate (looking at even later time points would however be interesting in the current model). The apparent differences in magnitude of the MC_1 role between these two models could be a reflection of the cell types involved. Transplantation of MC_1+ bone marrow into e/e mice has been shown to provide no improvement in DSS

induced colitis and as such reveals a critical role for non-haematopoietic cells, in this model most likely epithelial cells. In the present model the most likely effector cells of the melanocortins effects are endothelial cells and/or leukocytes (and perhaps also a role for perivascular cells).

In light of the above investigation and the results obtained in this thesis it seems that a hypothesis put forward by Montero-Melendez *et al.* is more in keeping with the present I/R model that:

"*MC_1 could be indicated as the physiologic receptor, with an almost ubiquitous distribution in a variety of cell types, whereas MC_3 could be a "stress receptor" up-regulated in conditions of tissue injury or inflammation*." [287].

Adding to this hypothesis with respect to the results obtained herein: MC_1, as the physiologic receptor, is able to rapidly respond to stimuli via NF-κB independent mechanisms whereas MC_3 mediates a delayed but more prolonged effect.

With the physiological and pharmacological importance of melanocortins varying with both vascular bed and pathology, a universal statement on the importance of MC subtypes in providing anti-inflammatory protection across different pathologies cannot be made, it seems the specific pharmacological target must be determined within the specific pathological environment. And while the above statement (with amendments) seems accurate in the context of I/R, in light of the current findings that MC importance may switch, further investigations into the temporal regulation of melanocortin signalling in inflammation will undoubtedly need to be performed.

5.3.3. Cautionary notes on drawing conclusions solely from studies using e/e mice

As with any study using full KO animals one potential drawback in the use of KO mice is that removal of physiologically important genes may lead to compensatory mechanisms. In fact Montero-Melendez *et al., h*ave previously shown that while in wild type and e/e isolated macrophages zymosan challenge induced no change in MC_1 MC_3 or MC_5 expression, in $MC_3^{-/-}$ inflammatory challenge led to marked gene activation for MC_1 and MC_5 [287] perhaps indicating compensatory regulation of other MC in the absence of MC_3.

However in the context of the present findings, with regards to the receptor mutant e/e mouse there is an extra consideration that should be noted when drawing conclusions. Since the MC_1 mutation in the e/e mice merely renders the receptor incapable of coupling to adenylate cyclase (the principle MC signalling pathway) as previously discussed alterative signalling pathways have been demonstrated to couple to MCs and to my knowledge no investigations into Ca^{2+} or other alternative signalling pathways have been performed on MC_1 receptors bearing the MC_1 e/e mutation. However in human cells, while cAMP signalling was reduced, ERK1/ERK2 MAPK remained unaffected in melanocytes bearing MC_1 defects associated with red hair and fair skin [301]. Should the MC_1 e/e mutation render the receptor entirely in capable of intra cellular signalling it should not be ignored that simply the presence of the receptor may still be able to alter physiological responses. One possible explanation for the enhanced inflammatory response observed in the e/e mice during these investigations is that MC_1 may be binding endogenous α-MSH (or exogenous in the case of treated mice) and acting as a melanocortin sink and suppressing the endogenous anti-inflammatory actions. In a similar fashion to how soluble TNF-α receptors may inhibit the inflammatory response or how DARC may “mop up” inflammatory cytokines, so non-functional MCs could enhance inflammation by binding anti-inflammatory melanocortins. This possibility could be ruled out by pharmacologically blocking the MC_1 binding site using a competitive MC_1 selective antagonist co-administered with α-MSH in the e/e mice. However while the endogenous agouti signalling peptide shows preferential binding to MC_1 where it acts as an inverse agonist, to my knowledge at the time of writing no truly selective MC_1 antagonist is yet commercially available.

To further complicate the task of elucidating the role of individual melanocortin receptors in eliciting an anti-inflammatory response Mandrika.*et al*. have recently reported that MC may stabilize their signalling upon binding α-MSH by forming homo and hetero-dimers [285, 302]. In the context of the e/e mouse, MC_1 may still retain the ability to form heterodimers with MC_3 to enhance and transmit an intracellular signal. Indeed the existence of such functionally enhancing receptor dimers adds an additional dimension to MC signalling and could help to explain the current confusion surrounding which MCs mediate specific physiological responses. However, while both MC_1 and MC_3 have been shown to be co-expressed in monocytes and neutrophils, formation of heterodimers has yet to be demonstrated in an *in vivo* setting or in non-transfected cells.

While the pharmacological evidence amassed during the course of this project supports the role of MC_1 observed in the current investigations in e/e mice, the above limitations should not be completely over looked. Indeed investigations using e/e mice could in future help to determine cAMP independent mechanisms of signalling through MC_1 and KO mice may also allow for an insight into the roles of hetero-dimerization in melanocortin mediated physiological effects.

5.3.4. Chance behavioural observations and the influence of peripheral wounds to cerebral leukocyte recruitment.

An interesting chance observation made during the course of these investigations was that e/e mice appeared to display signs of increased anxiety and aggression when compared to WT and $MC_3^{-/-}$ mice, despite identical housing conditions. This observation has not been quantified and is beyond the scope of the investigations in this thesis however, e/e mice often showed signs of fighting and over-grooming. As such some e/e mice had to be housed separately. Those animals bearing fight wounds or any injuries were excluded from the above results, with those animals that were included in the study having been physically examined prior to surgery and found to show no signs of fighting or injury. However some animals carrying wounds to the tail and back did undergo BCCAo surgery with interesting results (appendix Figure A4). These animals displayed highly increased leukocyte rolling and adhesion far beyond any levels previously observed during the course of this thesis. It is likely that these effects are not limited to the MC_1 receptor mutant mice as these effects are maintained (and enhanced) at 2 h reperfusion. Instead these observations may relate to the role of co-morbidities in enhancing inflammatory responses. Systemic inflammation, such as infection, not only increases the risk of stroke but is also associated with poor outcome. Furthermore systemic inflammation has been previously shown to aggravate neutrophil infiltration in the brain and enhance stroke pathology [303]. McColl *et al.* have shown that administration of IL-1β before initiating experimental stroke caused a threefold increase in ischemic damage and that this increase was completely ameliorated by depletion of neutrophils [304]. Thus it is likely that in the injured animals observed in this report, their previous inflammatory status has served to prime and augment the post BCCAo inflammatory responses. Importantly in these injured animals α-MSH treatment still appeared to have some effect reducing leukocyte recruitment however investigations

into the role of prior inflammation enhancing post stroke inflammation is beyond the scope of this thesis.

5.4. Conclusions

During this chapter a novel endogenous role for MC_1 in mediating early anti-inflammatory responses has been revealed, with e/e mice displaying enhanced leukocyte recruitment and elevated serum TNF-α. On the other hand $MC_3^{-/-}$ animals showed a comparatively normal phenotype at 40 min post BCCAo. In line with the pharmacological studies the importance of MC_1 however seemed to diminish as post stroke inflammatory processes progressed. While no data has yet been obtained in the $MC_3^{-/-}$ at 2 h, pharmacological studies would suggest that MC_3 mediated signalling predominates in this later stage of reperfusion and that MC_3 compensatory mechanisms may account for the lack of e/e inflammatory phenotype at 2 h.

5.5. Future Directions

Regretfully, due to a lack of availability of $MC_3^{-/-}$ mice and an absence of alternative suppliers of mice bearing the same genetic modification, no studies at the later time of 2 h reperfusion have yet been conducted in these animals. Data from the e/e mice coupled with pharmacological investigations have in these investigations provide a robust body of data to support the hypotheis of immediate early anti-inflammatory effects being modulated by MC_1 with a temporal change in receptor importance occurring as reperfusion progresses. However further investigations using the $MC_3^{-/-}$ mice would help to corroborate these findings and confirm that MC_3 mediates transcriptionally dependant mechanisms important in supressing the progression of inflammatory events. As such it is intended that these experiments be conducted in our lab when the animals become available.

While investigations into stress are not within the scope of this thesis the initial chance observations of the e/e genotype on their behavioural phenotype with regards to stress/aggression may provide an interesting avenue of research. The other initial

finding that wounds from fighting enhances leukocyte recruitment in the brain following BCCAo also warrants further investigation. Given the known role of systemic inflammation on enhancing brain inflammation, investigating the therapeutic potential of the melanocortins in the context of co-morbidities would provide an interesting progression of the present study.

6.0. Translational potential; investigating the effects of melanocortin treatments on human leukocyte function

6.1. Introduction

Due to the complex pathology and multiple pathways and systems underpinning neuronal damage in stroke, animal models are undoubtedly required to investigate novel therapeutics. However, when assessing prospective therapies it is important to understand and explore the differences between the animal and human drug target/system. The melanocortin receptor system displays a number of disparities in organisation and function between humans and rodents. The most highly expressed melanocortin receptor on human macrophages is MC_1 where as in mice it is MC_3 [95]. Furthermore a number of melanocortin agonists and antagonists show different potencies and sub-receptor selectivity in MCs from different species (table 4.1). While [D-TRP^8]-γ-MSH displays a relative selectivity toward MC_3 in humans, this compound displays differential binding affinities toward murine MCs [262]. Additionally, while SHU9119 can act as an agonist at human and mouse MC_1 in the frog MC_1 the same compound acts as a powerful antagonist [274].

In order to assess whether melanocortin based treatments would prove effective at reducing leukocyte-endothelial interactions in the context of human cells, experiments were performed using two *in vitro* assays of neutrophil and endothelial cell function using human cells. The flow chamber has thus been used as a model of leukocyte recruitment to endothelial cells under physiological flow and in addition the chemotaxis assay has been used to further investigate the effect of melanocortins on a key process in neutrophil trafficking.

6.2. Results

6.2.1. Flow chamber

In order to model the leukocyte recruitment cascade *in vitro* using human cells the flow chamber assay has been employed. In the flow chamber assay, isolated human neutrophils from healthy donors were perfused over HUVEC monolayers activated with TNF-α 4 h prior to commencing flow. Neutrophils interacting with the endothelial monolayer were quantified in terms of; total number of captured cells, number of cells rolling, number of adherent cells (those stationary for 10 sec or longer) and also number of cells transmigrating across the endothelial monolayer (those cells turning from bright phase to dark phase). While allowing for an assessment of the melanocortins effects on the recruitment of human neutrophils, this model also enabled further dissection of the melanocortins effects by allowing neutrophils and HUVECs to be treated with α-MSH separately.

Treating HUVEC monolayers for 2 h prior to flow with concentrations of α-MSH up to 100 µg/ml failed to induce any significant reduction in neutrophil capture, rolling, adherence or transmigration following TNF-α stimulation (Figure 6.1). However, there was a slight trend toward reductions in adhesion and transmigration. On the other hand treating neutrophils with just 10 µg/ml (a comparable dose to that used during the *in vivo* studies) resulted in significant reductions in both neutrophil adherence (68 % reduction) and their transmigration (67 % reduction) when compared to cells treated with saline alone. Moreover α-MSH treatments also resulted in a trend toward a reduction in neutrophil capture (54 % reduction at 10 µg/ml).

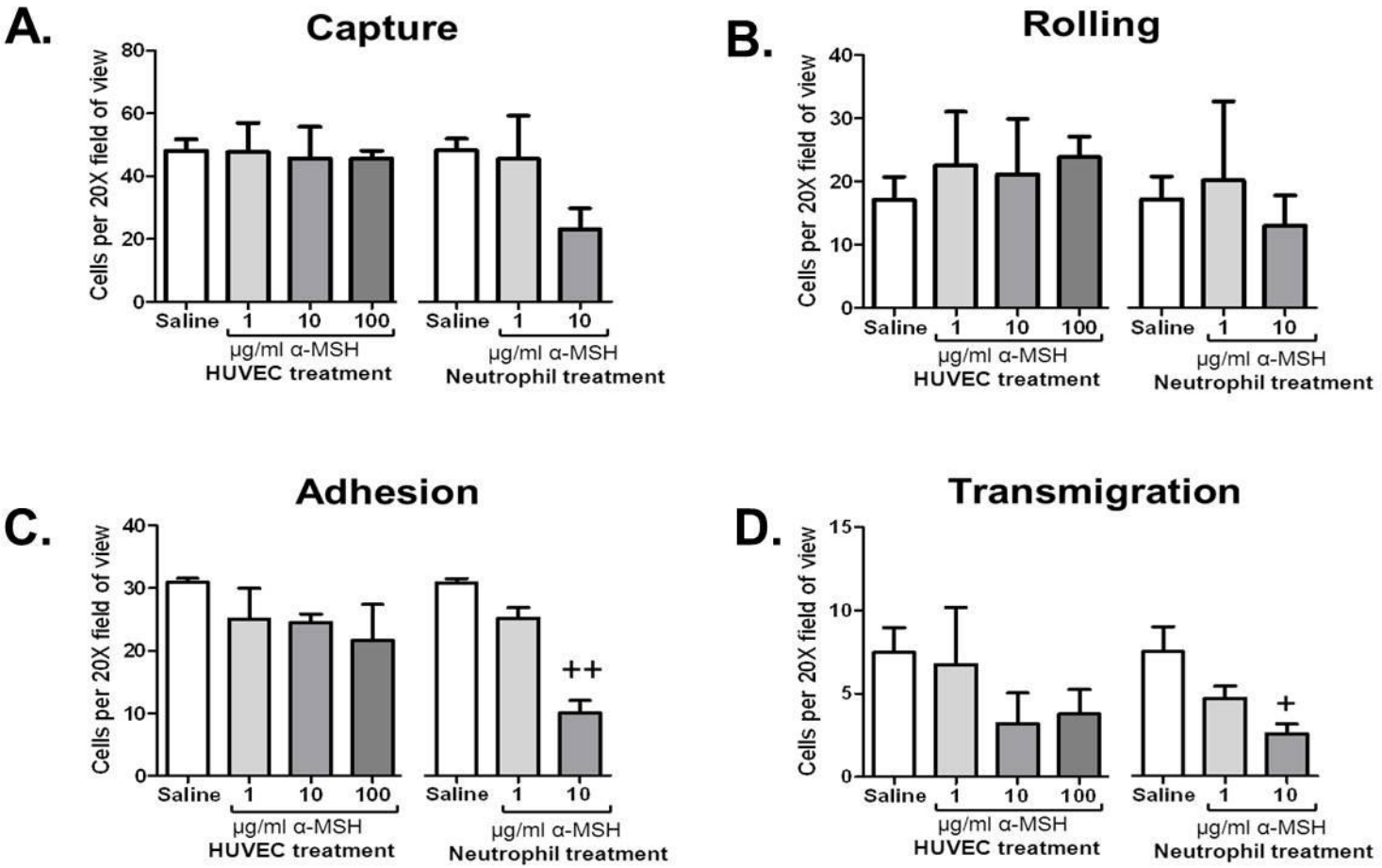

Figure 6.1. ***Neutrophil recruitment to TNF-α stimulated HUVEC monolayers.*** Isolated human neutrophils ($1x10^6$ cells/ml) were perfused over TNF-α stimulated (10 ng/ml, 4 h) confluent HUVEC monolayers. Either neutrophils or HUVEC monolayers were pre-treated with saline containing 0, 1,10 or 100 µg/ml of α-MSH. Leukocyte recruitment was quantified in terms of (A) Capture (all cells interacting with the monolayer) (B) the number of rolling cells, (C) number of adherent cells (cells stationary for 10 sec or longer) and (D) the number of cells transmigrating across the HUVEC monolayer. N = 4 for all groups. + denotes significance to saline treated group $P < 0.05$. ++ = $P < 0.001$

6.2.2. Chemotaxis assay

To investigate the influence of different melanocortin treatments on the chemotactic functionality of neutrophils the neuroprobe^{TM} chemotaxis assay has been used. Neutrophil chemotactic activity was assessed by their ability to cross a pours membrane into a lower chamber containing the potent chemo-attractant FMLP.

While pre-treatments with 1, 10 and 100 µg/ml of α-MSH did not result in a significant reduction in cell migration, a trend toward a reduction was observed (Figure 6.2 A). Treatment with the MC_3 agonist [D-TRP^8]-γ-MSH also failed to significantly reduce neutrophil migration ($P>0.05$), however there was a strong trend toward a reduction (from 1.6 million cells/ml to 1.1 million cells/ml) which with higher n numbers may prove significant (Figure 6.2 B). On the other hand, selective activation of MC_1 by treatment with 100 µg/ml of the small molecule agonist BMS-470539, caused a highly significant reduction in directed neutrophil chemotaxis (from 1.6 million cells/ml to 0.4 million) (Figure 6.2 C). This was a reduction of approximately 75 % from the number of cells migrating toward FMLP when treated with saline alone and was not significantly different from the number of cells found to randomly migrate (bottom well containing medium alone).

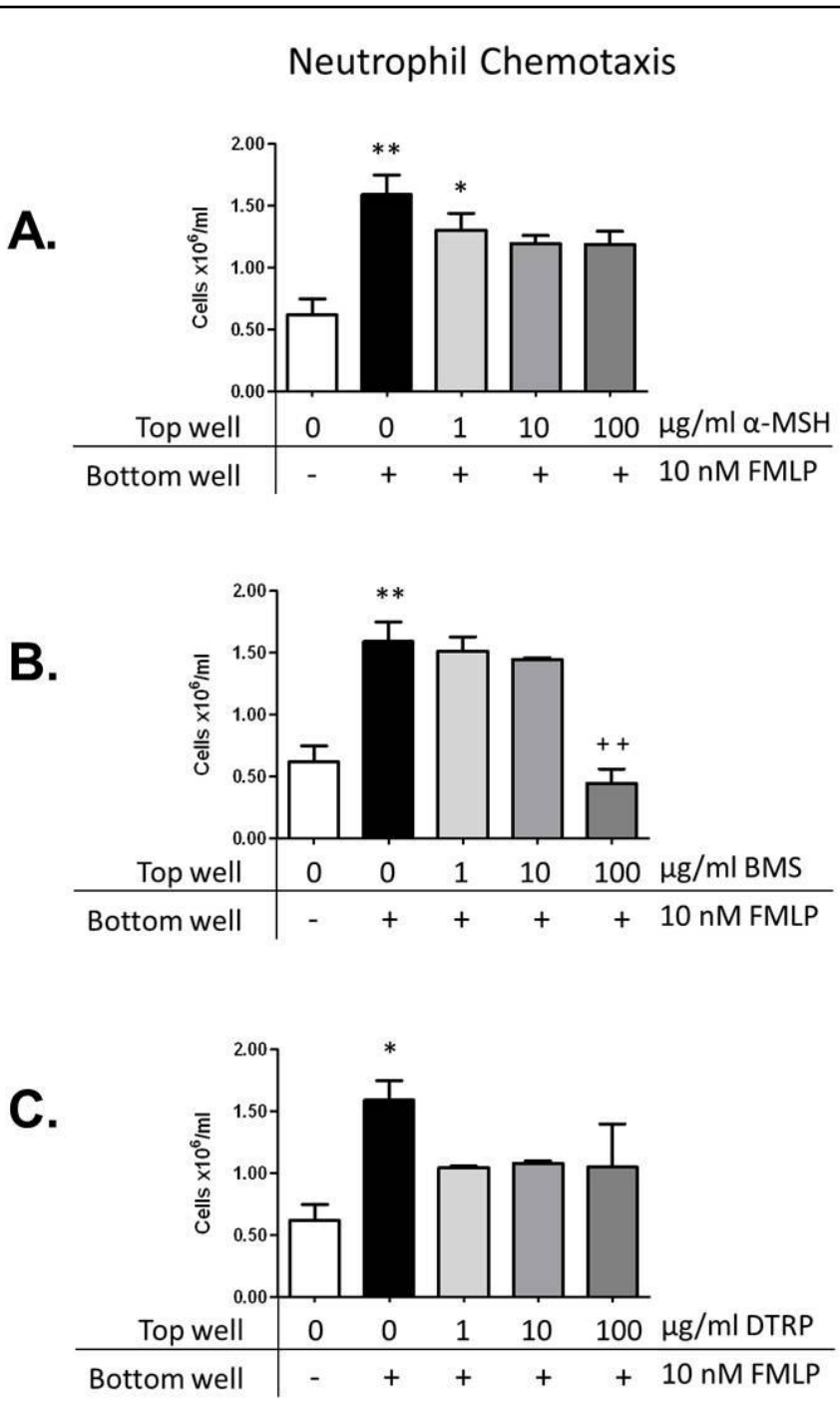

Figure 6.2. ***Neutrophil chemotaxis to FMLP: the effect of melanocortin agonists.*** Levels of neutrophil chemotaxis found to migrate across a porous membrane in the neuroprobe^{TM} chemotaxis plate toward the chemoattractant FMLP (10nM). Isolated neutrophils (4 x 10^6 cells/ml) were pre-treated 10 min prior to application to the top well of the chemotaxis plate with varying concentrations of either (A) α-MSH (B) BMS-470539 or (C) [D-TRP^8]-γ-MSH. Plates were then incubated for 1.5h before assessing neutrophil migration. Control and FMLP groups n= 5, α-MSH groups n = 5, BMS-470539 and [D-TRP8]-γ-MSH groups n = 3. * denotes significance to un-stimulated group P<0.05. **= P<0.001 ++ denotes significance to vehicle treated group P< 0.001.

6.3. Discussion

The present *in vitro* investigations into human cell inflammatory functioning have shown α-MSH treatments to suppress neutrophil recruitment to HUVEC monolayers via actions on neutrophils and MC_1 targeted treatments to significantly reduce neutrophil chemotactic activity.

6.3.1. The flow chamber model

The flow chamber assay has been used here as an *in vitro* model of human leukocyte recruitment and diapedesis under inflammatory conditions. While this model lacks the influence of pathological signalling originating from perivascular, glial and neuronal cells, as found *in vivo* in cerebral I/R, an inflammatory vascular environment has been modelled using TNF-α stimulation. Using TNF-α reflects the elevated TNF-α seen in the BCCAo mouse model used throughout this thesis. Additionally, since TNF-α acts as a key pro-inflammatory signal which is rapidly up regulated in human stroke, TNF-α stimulation provides a simplified but relevant inflammatory model to perform initial experiments to assess the translational potential of the *in vivo* findings.

6.3.1.1. The relative roles of the leukocyte and endothelium: site of action

Flow chamber experiments revealed that pre-treatment of isolated human neutrophils but not the HUVEC monolayer provided inhibitory actions on the adhesion and transmigration of neutrophils under physiological flow conditions. Both MC_1 and MC_3 are expressed on neutrophils and endothelial cells, however from the current investigations it would appear that inhibitory actions of α-MSH are mediated via modulation of neutrophil function, since only treatments of neutrophils and not of HUVECs resulted in significant reductions in leukocyte recruitment. This fits with the *in vivo* results which suggested melanocortin actions to take place on vascular or circulating cells rather than on parenchymal elements.

While most investigations have previously been performed on the melanocortins effects on endothelial adhesion molecules, the present investigations demonstrate endothelial

treatments with α-MSH to have limited effects on leukocyte recruitment at the time point and does tested.

Endothelial cells have previously been shown to express MC_1 MC_2 and MC_3 (Table 1.5) and α-MSH has been shown to reduce VCAM and E-selectin in an LPS stimulated human microvascular cell line. Furthermore MC_1 selective compounds have been shown to reduce the expression of E-selectin V-CAM and I-CAM in TNF-α stimulated human endothelial cells [154, 155]. However in the present model, aside from an 8 min exposure to α-MSH when the treated neutrophils were perfused over the endothelial cells, α-MSH demonstrated inhibitory actions on leukocyte recruitment in the absence of any endothelial cell treatment. Thus, as extremely rapid actions on HUVECs (within 8 min) are unlikely, the inhibition of leukocyte recruitment appears to be due to α-MSH modulation of neutrophil adhesion molecule expression.

Interestingly α-MSH treatment was able to inhibit leukocyte recruitment when given prior to any stimulation of neutrophils, suggesting that α-MSH treatment either prevents subsequent activation and/or modifies the constitutive ability of neutrophils to interact with the activated endothelium.

Constitutively expressed on the leukocyte cell surface the adhesion molecule, L-selectin, mediates leukocyte recruitment to the endothelial surface with L-Selectin and ICAM-1 also being able to act synergistically to mediate firm adhesion [305]. One unique property of L-selectin is that this adhesion molecule can be rapidly cleaved (within minutes) from the cell surface. Endoproteolyticly released L-selectin may then also act as a molecular buffer, by binding endothelial ligands and moderating leukocyte-endothelial interactions [306]. Several non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be able to induce both *in vivo* and *in vitro* down regulation of neutrophil cell surface L-selectin expression through L-selectin shedding [307]. Furthermore, *in vivo* neutrophil shedding of L-selectin has been shown to inhibit their migration into the inflamed peritoneum and conversely inhibition of L-selectin shedding increased it [306]. In the TNF-α activated cremaster, inhibiting L-selectin shedding was also found to have surprising effects on leukocyte recruitment events subsequent to rolling, by increasing leukocyte adhesion and transmigration [305].

While no effects on L-selectin have, as yet, been described following melanocortin treatments, in light of melanocortins effects on the shedding of CXCR1, CXCR2 and CD14 it is possible that L-selectin shedding may provide an additional mechanism by which melanocortin treatments provide anti-inflammatory protection.

Treatments with α-MSH have however been demonstrated to reduce MSU crystal induced CD11b expression on neutrophils. The Mac-1 integrin subunit, CD11b, is up regulated early in response to inflammatory stimuli [226] and plays an important role in mediating leukocyte adhesion, chemotaxis and migration. In TNF-α activated cremaster venules fMLP induced leukocyte recruitment has been shown to be critically dependant on Mac-1 expression [308]. It is thus possible that reduced cell surface expression of CD11b allows for the inhibition of leukocyte adhesion and migration observed in the present model. However further investigation into effects on the topography of neutrophil cell surface protein expression (especially with respect to Mac-1, L-selectin, LFA-1 and the mucin-like-glycans) will have to be made to understand the exact mechanisms underlying the presently observed neutrophil dependant effects.

6.3.1.2. Limitations of the model

Neutrophils have been shown to be a major contributing factor to the post stroke inflammatory response and neutrophil depletion experiments performed in this project point toward neutrophils being the primary cell type recruited in the BCCAo model. As such, a simplified *in vitro* assay has been used to assess the action of melanocortins specifically on neutrophil recruitment. However, while investigations into just a single isolated population of leukocytes provides a clear picture and high reproducibility, allowing for a focused dissection of cellular and molecular mechanisms, this is at the expense of relevance. This present assay lacks the *in vivo* influence of other circulating cell types, not only other sub types of leukocytes but also platelets and erythrocytes. Leukocyte-leukocyte interactions have been shown to mediate secondary recruitment of cells which may lack the necessary adhesion molecules needed to interact directly with the endothelium. Therefore where an experiment may show no recruitment of an isolated population of leukocytes on the endothelial surface, it may be that *in vivo* a number of these leukocytes are in fact recruited to the endothelial through secondary interactions with other leukocytes present in the blood.

In addition to other leukocytes, platelets may have significant influence over leukocyte recruitment. Indeed platelet P-selectin and not endothelial P-selectin has been demonstrated to be critical to neutrophil infiltration in a model of post-ischemic renal failure [309].

Erythrocytes have also possibly been neglected due to their lack of nucleus and inability to divide (and thus be cultured), however considering erythrocytes outnumber PMN in normal blood by a thousand fold [310], purely due to their sheer numbers any seemingly small role in the inflammatory process could prove highly influential. It is has now been established that erythrocytes enhance leukocyte-endothelial interactions by forcing the larger leukocytes away from the central flow to the periphery and vessel wall. Recent studies have shown erythrocytes to be capable of inducing a number of acute stage pro-inflammatory effects particularly in response to hypoxia. Under hypoxic conditions erythrocytes may generate and release H_2O_2 which has been shown to diffuse into endothelial cells, increase endothelial Ca^{2+}, activation and up regulation of surface P-selectin [311]. As such erythrocytes have been shown to play a significant role in hypoxic lung inflammation. Furthermore erythrocytes have been shown to express the decoy CC and CXC chemokine receptor, DARC. Important for chemokine clearance erythrocyte DARC reduced extra cellular levels of CXCL1 and CXCL2/3 and inhibited PMN migration into the alveolar space in a murine model of lung inflammation [312]. Moreover in the presence of erythrocytes, immunoreactive IL-8 rapidly disappears from plasma. Therefore erythrocytes may provide both a pro- and anti-inflammatory influence. As such undertaking investigations using whole blood and different mixed populations of circulating cells could provide an interesting progression of the present investigations.

6.3.2. Melanocortin effects on neutrophil chemotaxis

Neutrophils express numerous chemokine receptors on their cell surface and in response to chemotactic stimuli neutrophils undergo activation of the cytoskeletal machinery and cell polarisation to produce a leading actin rich lamella and the uropod to the rear [313] thus allowing for directed actin dependant motility. Accompanying these events are membrane ruffling, lysosomal enzyme release and ROS production [313].

The most widely studied chemotactic response is the fMLP activation of neutrophil motility. The fMLP receptor is highly expressed on the neutrophil surface (with estimates

of 50,000 receptors per cell) [313] and demonstrates a high affinity for its ligand; the bacterial fragment and self-stress signal fMLP (originating from degraded bacterial or mitochondrial proteins respectively).

In the present study, just 10nM of fMLP was able to induce significant directed neutrophil chemotaxis. While pre-treating with α-MSH and [D-TRP^8]-γ-MSH resulted in strong trends toward reducing neutrophil chemotaxis, selective activation of MC_1 using 100 µg of BMS-470539 was able to significantly reduce neutrophil chemotaxis by greater than 75 %.

α-MSH and $(CKPV)_2$ treatments have previously been shown to inhibit neutrophil chemotaxis to both IL-8 and fMLP [314, 315]. Furthermore both α-MSH and KPV have also been shown to inhibit chemotaxis to a complex mix of inflammatory mediators in chemotaxis assays toward supernatants from MSU-stimulated monocytes [316]. These responses have been shown to be dependent on elevation of intra-cellular cAMP [314, 315]. Elevated cAMP is associated with inhibition of a number of neutrophil responses such as candidacidal activity, phagocytosis, super oxide production and AA release [317]. Treatment with cAMP elevating factors such as forskolin, isoprotereno and PGE_1 have also previously been shown to inhibit neutrophil chemotaxis to fMLP. Thus it is likely that the inhibition of chemotaxis observed upon melanocortin treatment is in response to MC activation and adenylate cyclase mediated cAMP up regulation. Since recent reports have shown MC_1 to be the most highly expressed MC on human neutrophils [181] it is thus fitting that the MC_1 selective BMS-470539 showed significant reductions in neutrophil chemotaxis following treatment. Given the current understanding of α-MSH effects on neutrophil chemotaxis, it is thus likely that these effects are mediated through MC induced elevation in cAMP.

Paradoxically some inducers of chemotaxis such as fMLP and LTB also elevate cAMP levels although only transiently [317] The extent and duration of cAMP accumulation may thus apparently mediate opposing effects on neutrophil function. Indeed an emerging body of evidence suggests that the degree of cAMP accumulation may result in distinct outcomes [287, 318]. While the melanocortin agonist AP214 elevates cAMP and enhances macrophage efferocytosis of apoptotic neutrophils, cAMP elevating compounds such as forskolin inhibit efferocytosis [287]. The forskolin cAMP response

has however been shown to be 10 times greater than the relatively modest increase provided by AP214 [287] and indeed Low levels of cAMP elevation have been shown to inhibit efferocytocis [318]. Additionally cAMP levels induced by α-MSH treatment have been shown to be similar to that of AP214, thus it may be that not only is the specific signalling pathway important to the effect of the melanocortins but also the magnitude and duration of that signal.

While a trend toward a reduction in neutrophil chemotaxis was observed following α-MSH and [D-TRP^{8}]-γ-MSH treatment with the current n number and under the present conditions, drug dose and time point, this remained statistically non-significant. However previous reports have demonstrated α-MSH to inhibit neutrophil chemotactic ability. Nevertheless in the literature the efficacy of α-MSH to inhibit neutrophil migration to FMLP gradients appears to be under contention. While both Catania *et al.* [314] and Capsoni *et al*. [315] have previously reported α-MSH to reduce neutrophil chemotaxis to both IL-8 and FMLP, Manna *et al.* have however shown that α-MSH reduces IL-8 induced migration but not fMLP induced migration [181]. The disparity in these findings may be due to different treatment regimens, with Catania *et al.* and Capsoni *et al.* investigating melanocortin effects on neutrophil chemotaxis at an earlier stage and toward higher concentrations of FMLP (10^{-8} M for 90 min) compared to Manna *et al.* (10^{-10} M for 2 h). The fact both Catania and Capsoni used isolated blood neutrophils and Manna *et al.* utilised HL-60 (human monocyte) cell lines induced to differentiate into neutrophils in the presence of dimethyl sulfoxide, may also have an effect on neutrophil phenotype and cell responses.

Although no significant effects of α-MSH were observed in the present study, a strong trend toward an inhibitory effect was observed. With one of the melanocortin compounds tested providing significant inhibition of fMLP chemotaxis, it would seem that both the methodology used and results obtained in the present study fit more closely with those of Catania and Capsoni. The fact that Manna *et al*. did not observe any suppression of fMLP induced chemotaxis at the later time points than Catania and Capsoni may even further reflect a temporal shift in MC receptor function, as has been reported during this thesis.

To my knowledge, at the time of writing, no studies of the effect of BMS-470539 or [D-TRP^8]-γ-MSH on neutrophil chemotactic ability has yet been published. While at this stage these findings are only the result of preliminary experiments and greater n numbers may reveal inhibitory roles for both [D-TRP^8]-γ-MSH and α-MSH, the observed effects of BMS-470539 represent a novel finding, further implicating MC_1 as an effector of neutrophil functioning.

6.3.2.1. Relevance of the model

The fMLP chemotaxis assay is a well-established and reproducible technique to assess leukocyte chemotactic function. While fMLP is largely referenced as a bacterial product, being ancient endosymbiotic bacteria, mammalian mitochondria also initiate protein synthesis with an N-formylmethionine residue, and upon tissue damage degrading mitochondria release N-formylmethionine containing breakdown products [319].

In the context of sterile inflammation, while the study of cytokines that amplify inflammatory signals have been extensively studied the molecules responsible for initiating inflammatory reactions originating from the damaged tissue (Alarmins and DAMPs) are less well characterised [320]. Outside of the cell, mitochondrial elements are recognised as “non-self” and may act as a link between tissue injury and inflammation. As such mitochondrial proteins likely represent proximal mediators which are released from necrotic cells at the site of tissue injury and act as a trigger for local and systemic sterile inflammation [320] .

FMLP mediated neutrophil activation has been shown to be dependent on initiation of TNF-α release and TNFR functioning [321], thus demonstrating a link between damage recognition and inflammatory signalling suggesting TNF-α may either mediate or amplify the fMLP initiated signal.

While endogenous release of fMLP and other N-formylated peptides has not yet been quantified following stroke, mitochondrial dysfunction plays a central role in the ischemic cascade. It is thus plausible that these peptides may be highly relevant in post I/R inflammation as chemoattractant DAMPs following stroke. Indeed as well as on circulating leukocytes fMLP receptors have also been identified on microglia and

astrocytes [322] and as such fMLP could be an important component in initiating post stroke inflammatory responses

Hence the present model used represents neutrophil responses to inflammatory initiating signals rather than a propagating/amplifying cytokine signal. As such the particular role of MC_1 found in this study may further fit with the very early role found in the *in vivo* investigations. These initial results on neutrophil chemotaxis demonstrate the MC_1 activation may counteract the very early chemotactic responses of neutrophils to tissue damage. Future investigations could also assess the role of particular MCs in modulating leukocyte functioning in response to molecules in the amplification phase of inflammation, especially considering the apparent temporal switch in MC importance.

6.4. Conclusions

In vitro investigations have revealed anti-inflammatory efficacy of melanocortin treatments in human cells. The inhibitory actions of α-MSH on leukocyte endothelial interactions observed in the flow chamber model are supportive of the results obtained in the *in vivo* stroke model and builds confidence as to the potential to translate melanocortin treatments into a human inflammatory disease context. This work has also pinpointed these effects to specifically be mediated by melanocortin treatments acting on neutrophils rather than the endothelium. Whether the same holds true in the *in vivo* setting and at different time points following activation by different stimulus has however, yet to be determined. The chemotaxis assay has been used to further characterise the effect of melanocortin treatments on neutrophil functioning. In these pharmacological studies an important role for MC_1 was revealed in inhibiting neutrophil chemotaxis to fMLP. This work thus shows that melanocortin treatments are not only active in modulating human cell leukocyte recruitment but can modulate multiple aspects of neutrophil functioning in response to different stimulus.

6.5. Future Directions

This work could be furthered to give a greater insight into role of individual MCs, by the use of blocking antibodies to the melanocortin receptors. HUVECs and neutrophils could again be treated separately with blocking antibodies for different MCs before giving non selective melanocortin treatments. Alternatively blocking antibodies could be used to study the endogenous production and influence of the melanocortins. However such work would rely on the specificity of such antibodies which is currently under some conjecture.

Another, more physiological relevant approach that could be pursued, to further investigate the role of MC subtypes in supressing neutrophil recruitment, would be to take advantage of natural MC mutations in humans. Loss of function MC_1 mutations are relatively common in humans, arising in ~80 % of individuals with red hair and fair skin [323, 324]. Since MC_1 mutations confer these easily observable physical characteristics, during blood donor recruitment people with this phenotype could be actively encouraged to participate. PCR genotyping for four common MC_1 variants; D84E, R151C, R160W and D294H, could then be used to further inform results obtained from *in vitro* assays allowing an insight into the role of MC_1 in neutrophil function.

Investigating the role of MC_3 and MC_4 using this strategy would however be a more complex task as these mutations are much rarer and are associated with an obese phenotype. In humans, the MC_3 missense variants Thr6Lys and Val81Ile have been related with reduced *in vitro* expression of the receptor and diminished functionality [325] and is associated with childhood obesity, and elevated body fat percentage in comparison to non-carriers [326]. While genetic mutations of MC_4 represent the biggest monogenic cause of childhood obesity, these are again rare mutation. In a study by Farooqi *et al.* from a group of 500 patients with severe childhood obesity, just 5.8 % displayed mutations in MC_4 with only 6 individuals being homozygous [327]. Such investigations would be further hampered by the difficulties of extrapolating which findings are due to direct effects of MC dysfunction on immune functioning from those that are secondary to the metabolic dysfunction and the multitude of conditions associated with obesity.

While the flow chamber model used in the present investigation uses TNF-α (a key pro-inflammatory cytokine up regulated following stroke) to activate endothelial cells, this model could further tailored to more faithfully mimic stroke. Varying levels of glucose and oxygen deprivation could be used to activate endothelial cells, as previously performed in dermal microvascular endothelial cells by Lee *et al.*[328]. Furthermore brain microvascular cells could be seeded rather than the more readily available HUVECs. While the current model used demonstrates a proof of principle, confirming melanocortin action on leukocyte recruitment in human cells, this model also provides a useful tool for further research into the actions of the melanocortins.

The current investigations using the chemotaxis assay could also be built upon to analyse the chemotactic functioning of neutrophils in response to a number of different chemoattractants such as IL-8. Indeed following stroke a number of chemotactic factors are released by both resident and infiltrating immune cells, such as; Neutrophil Activating Protein 3 (NAP-3, CXCL1) [329], Macrophage Inflammatory Protein-1α (MIP-1α, CCL3) [330], IL-8 (CXCL8) [331] and fractalkine (CXC3CL1)[332] each of which could be investigated in this assay. In the more complex *in vivo* situation these molecules create a pro-inflammatory milieu with multiple signals influencing leukocyte functioning. Examining more complex chemotactic solutions could provide an interesting insight into how different chemical signals could act to enhance or inhibit the chemotactic outcome. In order to model this complex chemical microenvironment experiments could be performed substituting the chemo-attractant in the bottom chamber with culture medium from cultured neuronal or glial cells that have been deprived of glucose and oxygen.

Another avenue of research could be to introduce a mixed population of leukocytes to the top level of the plate. Indeed many chemoatractants display differential chemotactic potencies and selectivity for different leukocyte populations, for instance MCP-1 (CCL2) attracts monocytes but not neutrophils whereas CXCL1 recruits neutrophils but not monocytes. The effect of melanocortin treatments on the composition of leukocyte populations recruited could be indicative of therapeutic value. While neutrophil recruitment is largely considered a pathological event in stroke and the arrival of monocytes leading to an M1 phenotype may enhance inflammatory responses, the recruitment of monocytes assuming an M2 phenotype marks the beginnings of

inflammatory resolution and is associated with the clearance of apoptotic cells and the release of IL-10 and pro-resolution factors. Differential expression of receptors on sub sets of monocytes mean that they may respond to different stimuli and be recruited differentially. For instance CCR2 is only expressed on inflammatory monocyte thus only the pro-inflammatory phenotype will respond to MCP-1. Assessing the effects of melanocortins on the composition of leukocyte recruitment could make an interesting progression of this project.

7.0. General discussion

7.1. Summary of important results

Throughout this project a murine model of global cerebral I/R has been used to investigate the actions of the melanocortins on leukocyte recruitment in the brain. This BCCAo model was characterised by a rapid I/R induced recruitment of leukocytes in the pial microcirculation, which escalated as the length of reperfusion was extended. Neutrophil depleting experiments revealed the recruited cells consisted largely or even entirely of neutrophils. Coinciding with the enhanced neutrophil recruitment at 2 h, was an increase in circulating TNF-α and an activation of brain resident immune competent microglia.

Melanocortin treatments have been shown to inhibit leukocyte recruitment in a number of different models [127, 140, 151], however the effect of these peptides on the leukocyte recruitment cascade have until now not been investigated in the unique microcirculation found in the brain. During the course of this project the physiological roles and pharmacological potential of the MRS has been investigated in an inflammatory context relevant to stroke.

Treatments with the endogenous non-selective melanocortin agonist α-MSH revealed potent inhibitory actions on both rolling and adhesion of I/R induced leukocytes. These effects appeared to be via actions on vascular or circulating elements as no effect on microglial activation was observed, as would have been expected from previous *in vitro* studies [269].

Pharmacological investigations identified both MC_1 and MC_3 to suppress cerebral leukocyte recruitment. MC_1 was found to be most important at reducing early leukocyte recruitment events however by 2 h of reperfusion MC_3 mediated anti-inflammatory signalling appeared to predominate.

In addition to inhibitory actions on leukocyte recruitment melanocortin treatments reduced the expression of the pro-inflammatory cytokines TNF-α and MCP-1. However,

indicative of the immuno-modulatory role of the melanocortins, treatments did not result in an indiscriminate inhibition of all cytokines but instead both α-MSH and [D-TRP^{8}]-γ-MSH were found to induce the anti-inflammatory cytokine IL-10 and increase expression of the pleiotropic cytokine IL-6.

The observed switch in MC receptor importance was however unlikely to be due to up regulation of the receptor, with qRT-PCR experiments revealing no change in receptor transcript. Instead, considering early reductions in leukocyte recruitment were rapid and likely to be transcriptionally independent, and that by 2 h there was evidence of classical melanocortin signalling and NF-κB inhibition, it is likely that apparent changes in receptor importance are due to the activation of different signalling pathways being activated

Investigations into the physiological role of these receptors in regulating leukocyte recruitment, using receptor mutant and KO mice, were found to support the pharmacological findings. An enhanced induction of early leukocyte rolling and TNF-α expression following I/R in the MC_1 mutant e/e mouse suggested an early anti-inflammatory role for MC_1. On the other hand $MC_3^{-/-}$ mice displayed no phenotype at this early stage. However with an extended period of reperfusion the inflammatory phenotype of the e/e mouse had subsided to levels similar to WT suggesting that compensatory mechanisms prevail, which according pharmacological studies is likely to be MC_3 mediated.

Experiments using an *in vitro* model of neutrophil recruitment showed that α-MSH mediated reduction in leukocyte-endothelial cell interactions was achievable in human cells. Furthermore these effects resulted in a significant reduction in neutrophil diapedesis, a parameter that could not be measured in the murine model. Despite a wealth of evidence on the effects of melanocortin treatments on endothelial adhesion molecules, these *in vitro* investigations revealed neutrophils to be the effector cells of α-MSH effects on leukocyte recruitment at the time point tested. Further assessment of the effects of melanocortin treatments on neutrophil function produced the novel finding that MC_1 activation enabled the inhibition of neutrophil chemotaxis toward the cellular injury chemotactic signal fMLP.

This work demonstrates that melanocortin receptor activation can effectively ameliorate pathological I/R induced leukocyte recruitment in the unique restrictive environment provided by the cerebral microcirculation. While this work highlights important roles for MC_1 and MC_3 in mediating these effects, crucially these studies have also revealed that the melanocortin reporter subtype important for providing anti-inflammatory protection not only varies with the physiological environment and pathological status (as shown by others) but also varies with the temporal progression of pathology.

7.2. Developing melanocortin drugs for stroke

While existing preclinical data is overwhelmingly positive, limitations do still exist. One potential problem of targeting these receptors is that long term stimulation of the receptors has still not been adequately investigated. Initial studies have shown however that continual activation of MC_4 results in de-sensitisation and an internalization of the receptor [333]. and while the present investigations have shown no change in blood mRNA levels of MC_1 and MC_3, studies by Montero-Melendez *et al.* have shown MC_1 MC_3 and MC_5 mRNA expression to be reduced in isolated macrophages following treatment with high doses of the melanocortin agonist AP-214 compared [287].

Slight cardiovascular side effects have also been previously observed with long term administration of Melanotan II in obese rats, raising arterial blood pressure and heart rate [334] which could limit the use of MC targeted drugs in chronic disorders or for prophylactic use. These effects have however been shown to be limited to MC_4. In obese humans treatment with the highly selective MC_4 agonist LY2112688, caused increases in blood pressure and heart rate [335]. It may be that such effects are limited to obese patients as no studies yet performed in healthy individuals have demonstrated elevated blood pressure. Since high blood pressure poses a strong risk factor for stroke, cardiovascular side effects could seriously limit the use of MC_4 targeted drugs for the treatment of stroke.

On the other hand, very high doses (up to 2 mg/kg daily for 12 weeks) of the non-selective melanocortin agonist NDP-α-MSH (relative affinity MC_1>MC_3>MC_4>MC_5) have shown no toxic side effects in both mice and rats. Dosing trials have also been performed for NDP-α-MSH in humans. Trialling the peptide for therapeutic tanning,

investigators have found 0.08 mg/kg/day for 10 days to produce minimal side effects, with short-lived flushing of the face, in approximately 40 % of subjects and nausea in less than 10 % [336]. Subsequent dose escalation studies showed up to 0.16 mg/kg/day to be well tolerated [336]. The does used in the present study to inhibit cerebral leukocyte recruitment equate to approximately 0.4 mg/kg and as such it is feasible that these levels could be achieved in man with minimal side effects. Furthermore considering the low BBB permeability of melanocortin peptides and the seemingly peripheral effects described in the present study, treatments are unlikely to reach MC_4 expressing cells in the CNS and thus minimising cardiovascular side effects.

The inhibition of NF-κB regulated transcription is a key element to the broad protective actions of the melanocortins. Recently the proteasome inhibitor MLN519 has entered clinical trials for the treatment of stroke. This drug has a similar action to the melanocortins in that it inhibits IkB degradation and has been shown to be greatly effective at reducing inflammation and providing neuroprotection up to 10 hours following cerebral I/R injury [337]. The outcome of these clinical trials may give an indication of feasibility of targeting NF-kB mediated transcription for the treatment of stroke and possibly forecast the success of melanocortin targeted therapy.

Another widely reported mechanism by which the melanocortins can act is via the C terminal KPV region. This sequence has been shown to provide potent anti-inflammatory actions and the efficacy of this tri-peptide has also been demonstrated in the present studies. Due to structural similarities with IL-1β and observations of IL-1R binding mounting evidence suggests the terminal α-MSH region acts as an IL-1R antagonist. The IL-1R antagonist Anakinra (brand name Kineret) is currently used for the treatment of rheumatoid arthritis and in experimental stroke has been shown to reduce neutrophil infiltration and decrease infarct size by 50 %, even in co-morbid animals and when treatment was delayed until 3 h post stroke [338]. The IL-1R antagonist has even undergone phase II clinical trials, showing the drug to be safe and well tolerated in acute stroke, with initial evidence also showing improved pathophysiology and clinical outcome [116].

Thus in addition to highly promising preclinical data, initial clinical toxicological studies show melanocortin drugs to be well tolerated and with a number of drugs having similar

mechanisms of action showing promise in clinical trials, the existing picture seems highly auspicious for the use of melanocortin based drugs to treat post stroke inflammatory responses.

While to my knowledge no clinical trials are underway for drugs which induce cleavage of adhesion molecules or inflammatory receptors (an additional mechanism by which the melanocortins act), the multitude of mechanisms by which the melanocortins control inflammation is likely to provide a potent and broadly acting anti-inflammatory response. Due to the robust nature of the host inflammatory response showing functional overlap and redundancy in inflammatory signalling, the multifaceted targeting of this response provided by the melanocortins, may offer great advantages over therapeutic strategies that target a single element of the inflammatory cascade.

7.3. Conclusions

Investigations into the importance of melanocortin receptor sub types in modulating post I/R leukocyte recruitment in the cerebral microcirculation have revealed potent inhibitory actions of MC agonists along with a novel temporal switch in the MC signalling. The present work allows one to postulate a new model for MC receptor signalling in modulating innate inflammatory responses:

MC_1 provides rapid protection against pathological leukocyte recruitment via mechanisms independent of NF-κB regulation, most likely acting via direct effects on neutrophils. MC_3 activation may also provide potent inhibitory actions on leukocyte recruitment however these actions are delayed, inhibiting NF-κB activation and providing a more prolonged effect than the immediate MC_1 response.

Figure 7.1 illustrates the key findings and the potential mechanisms put forward in this thesis.

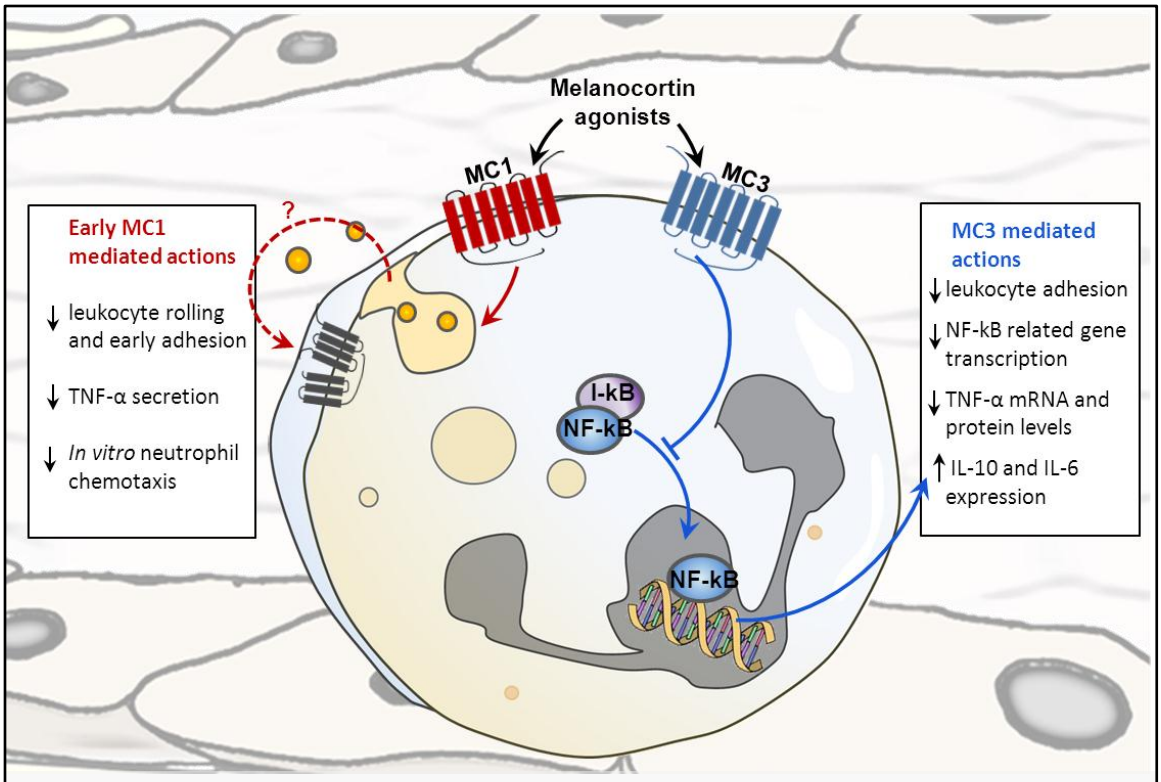

Figure 7.1. ***Proposed anti-inflammatory mechanisms in light of the present findings.*** Melanocortin agonists provide inhibitory actions on leukocyte recruitment following cerebral I/R. *In vitro* experiments suggest these effects are primarily due to actions on neutrophils. *In vivo* experiments revealed MC_1 to provide both endogenous and pharmacological protection against early leukocyte recruitment and TNF-α release. These actions are likely to be independent of transcription and may involve autocrine cleavage of cell surface receptors. *In vitro* studies also showed pharmacological activation of MC_1 to reduce neutrophils chemotactic ability. MC_3 mediated effects were found to be delayed compared to MC_1 actions but provided more prolonged inhibition of leukocyte recruitment, inhibiting the transcription of NF-κB related genes and enhancing the production of anti-inflammatory IL-10. These different mechanisums may also take place in different cell types. Proposed MC_1 mechanisms shown in red and MC_3 in blue. (P. Holloway, *un-published*)

In light of the novel finding showing a switch in receptor signalling, further investigations into the temporal importance of these receptors will need to be performed at later time points. With a greater understanding of the roles of these receptors, particularly MC_1 and MC_3, as identified in this study, the development of selective melanocortin ligands displaying appropriate pharmacokinetic profiles could thus pave the way for the production of powerful new anti-inflammatory drugs for the treatment of stroke.

Given the complexity of MC regulation along with the variable nature of stroke and relative difficulty in determining time of onset, an alternative approach which could prove fruitful, may be to activate multiple receptors in a non (or perhaps partially) selective manner. Indeed given that both MC_1 and MC_3 have demonstrated to initiate powerful anti-inflammatory signalling in the present study it may be that a non-selective approach would provide a more robust protection than targeting a single receptor. Indeed the only licenced melanocortin based therapy NDP-α-MSH (Afamelanotide, licenced in Italy for treatment of Erythropoietic protoporphyria) is a non-selective melanocortin agonist. Additionally the non-selective melanocortin agonist AP214 has shown great promise in preclinical studies in a number of diseases including lung inflammation, gouty arthritis, peritonitis and acute kidney injury [287]. Furthermore in 2011 Action Pharma announced positive results from phase IIb trials for AP214 in postoperative acute kidney injury. This compound has since attracted attention from global pharmaceutical companies and in 2012 was purchased by Abbott for $110 million for development to treat kidney injury. Given the results of the present investigations, the use of these super-potent non-selective agonists could provide novel and effective therapeutic interventions to abrogate damaging post stroke leukocyte recruitment.

While the most lucrative strategy for harnessing the MRS to provide anti-inflammatory protection in stroke remains unclear, the present study has provided convincing evidence that the melanocortin system offers a valuable pharmacological target to inhibit post stroke leukocyte recruitment.

Appendix

Effect size (δ) was calculated by the maximum likelihood estimator of Cohen's δ using preliminary data in which mice underwent BCCAo and 40 min of reperfusion with either saline (control) or dexamethasone (treated) being given i.p 30 min before BCCAo, level of adhesion was then measured as a parameter of anti-inflammatory effect.

Tables A1: Preliminary data used for power calculations

Adhesion

Group	$\bar{x}$	σ	*n*
1 (control)	292.54	17.29	3
2 (treated)	57.47	12.46	3

Calculating effect size using Cohen's δ: $$\delta = \frac{\bar{X}_1 - \bar{X}_2}{\sigma}$$

Where σ is defined as : $$\sigma = \sqrt{\frac{(n_1 - 1)\,\sigma_1^2 + (n_2 - 1)\,\sigma_2^2}{n_1 + n_2 - 2}}$$

Solving for σ: $$\sigma = \sqrt{\frac{(3 - 1)\,17.29^2 + (3 - 1)\,12.46^2}{3 + 3 - 2}} = 227.1$$

Therefore $$\delta = \frac{292.54 - 57.47}{227.1}$$

As such effect size = 1.04

Power calculations were then performed to determine group size with G* power 3 software (Heinrich Heine University of Dusseldorf) using a power of 0.8, an alpha value of 0.05 and the above effect size. Minimum group sizes to provide sufficient power to avoid a type II error was found to be 4.

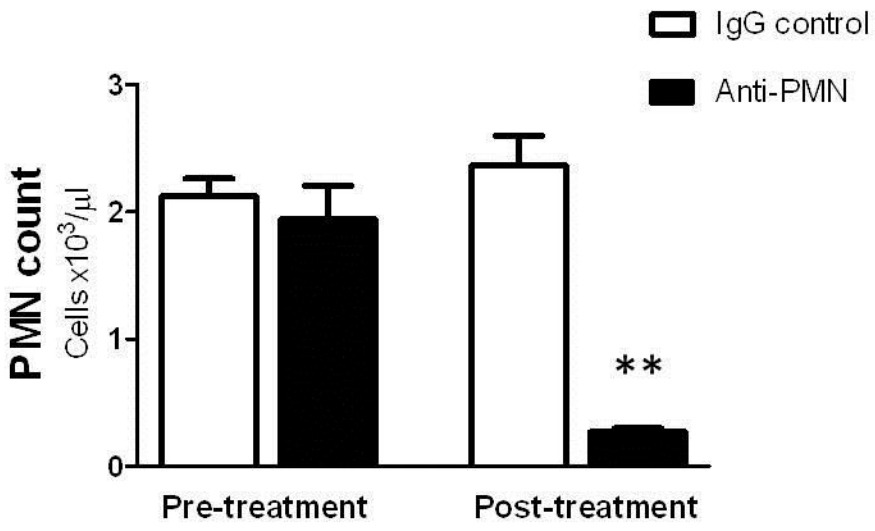

Figure A1. ***PMN depletion in mice***. C57BL/6 male mice were treated with either anti-mouse PMN serum or IgG matched serum (10ml/kg, i.p.) once daily for two consecutive days. Tail vein blood samples, for total white cell count and differential cell counts, were collected pre-treatment (before the first i.p injection) and post treatment following 2 days of treatment. Anti-PMN treatment reduced total PMN count by ~ 85 %. Values are mean ± SEM of 4 mice. ** denotes significant to Anti-PMN pre-treatment group P<0.001.

Table A2: WBC counts pre and post PMN depletion

Cell count		**Pre-treatment**		**Post-treatment**	
		IgG	Anti-PMN	IgG	Anti-PMN
Total WBC (Cells x 10^3/µl)		10.38 (±0.4)	10.15 (±0.5)	11.1 (±0.5)	9.51 (±0.7)
PMN (%)	**Neutrophils**	**18.88 (±1.5)**	**17.38 (±2.0)**	**19.38 (±1.6)**	**1.75 (±0.3)**
	Basophils	0.75 (±0.1)	0.63 (±0.2)	0.75 (±0.1)	0.38 (±0.1)
	Eosinophils	1.00 (±0.2)	1.00(±0.2)	1.13 (±0.3)	0.75 (±0.3)
Lymphocytes (%)		78.00 (±1.8)	79.5 (±2.3)	77.13 (±1.6)	94.13 (±0.4
Monocytes (%)		1.3 (± 0.2)	1.5 (±0.4)	1.63 (±0.2)	3.00 (±0.5)

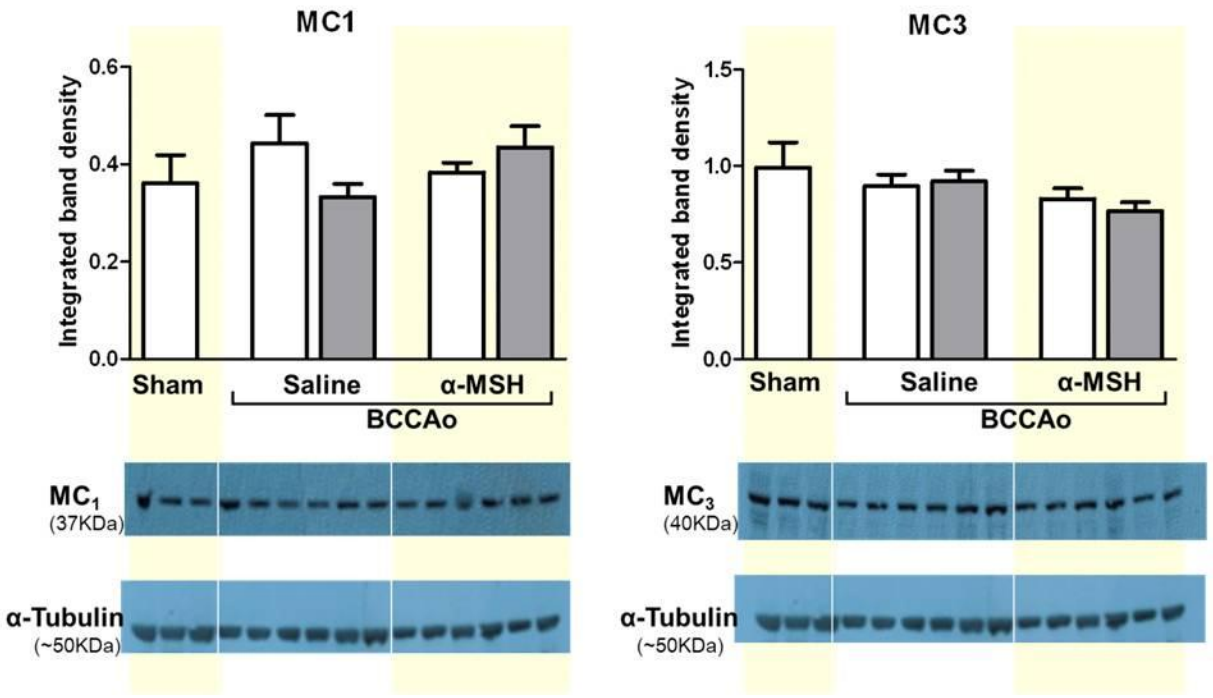

Figure A2. ***Western blot analysis of MC_1 and MC_3 expression in brain.*** Histograms and representative blots showing MC_1 and MC_3 expression from whole brain homogenates. C57BL/6 mice were subjected to sham or BCCAo surgery with 40 min (white bars) or 2 h (grey bars) of reperfusion and treated at the start of reperfusion with either saline or α-MSH. No significant variation in receptor expression was detected across all treatments. N=3 for all groups

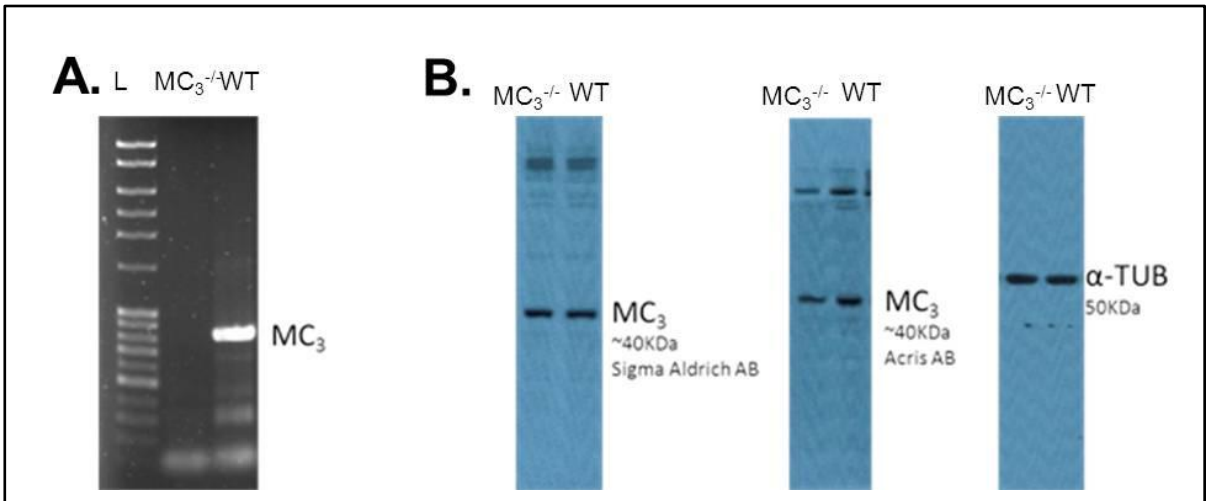

Figure A3. ***MC_3 antibodies display non-specific binding.*** (A) PCR analysis demonstrates the absence of MC_3 RNA transcript in samples from $MC_3^{-/-}$ mice. (B) Western blot analysis with two separate MC_3 antibodies produce bands at the correct height in brain homogenates from both wild type and $MC_3^{-/-}$ mice suggesting non-specific binding to other melanocortin receptors or unrelated proteins of a similar molecular weight.

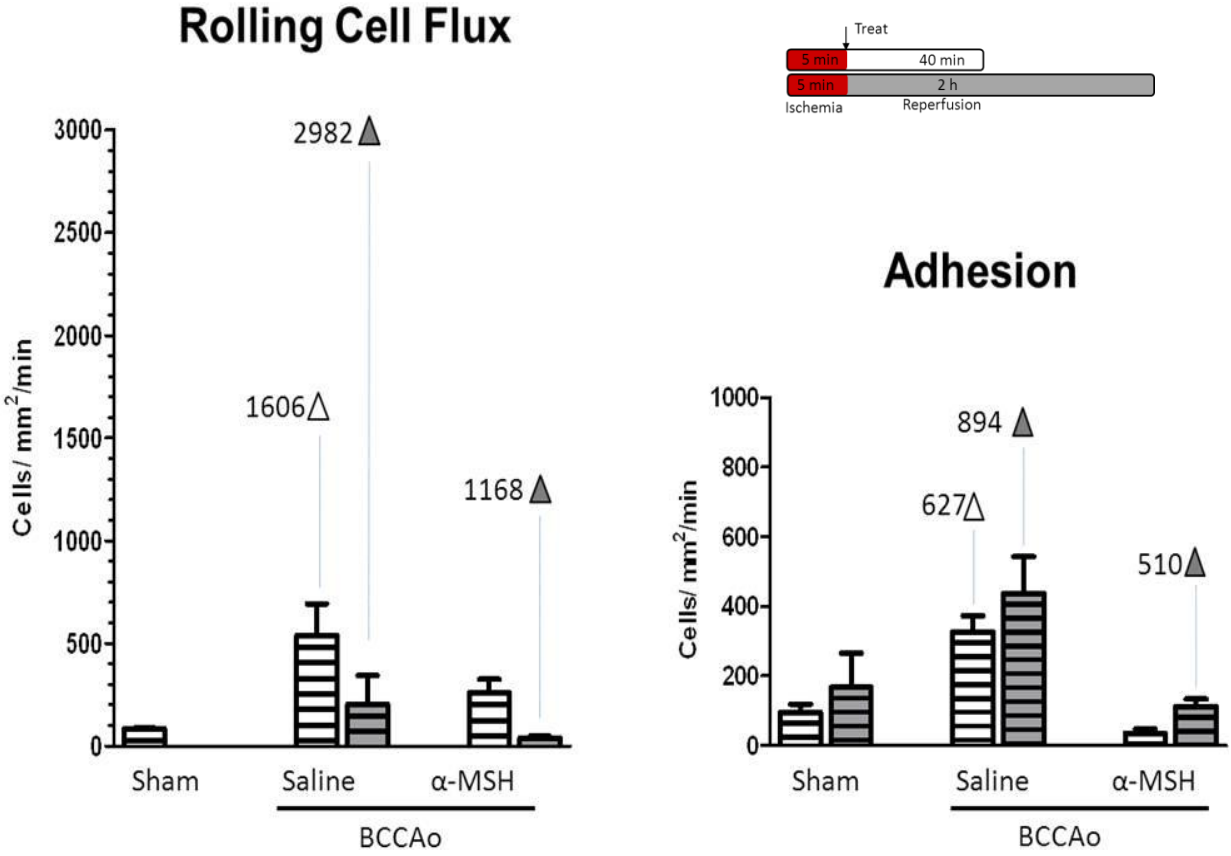

Figure A4. ***Enhanced leukocyte recruitment in e/e mice bearing wounds from fighting*** Triangles (with exact values given to the left) represent levels of leukocyte recruitment from individual mice that were found to have superficial wounds to the tail and back from fighting. Data from these mice was excluded from analysis during this thesis and while no statistical analysis could be performed due to n = 1 in each group, leukocyte endothelial interactions in these mice is clearly highly elevated when compared to otherwise un-injured animals of the same strain (as shown by the white and grey bars). The levels of leukocyte rolling and adhesion detailed here for both 40 min and 2 h saline treated BCCAo animals was far greater than levels observed in any individual mouse (of any strain or treatment group) across the whole of this project. No notable changes in WBC rolling velocity were observed in these mice (data not shown).

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