Both MC1 and MC3 Receptors Provide Protection From Cerebral Ischemia-Reperfusion-Induced Neutrophil Recruitment

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Both MC_1 and MC_3 Receptors Provide Protection from Cerebral Ischemia Reperfusion Induced Neutrophil Recruitment

Running title: Melanocortins deter cerebral leukocyte recruitment

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Abstract

Objective

Neutrophil recruitment is a key process in the pathogenesis of stroke, and may provide a valuable therapeutic target. Targeting the melanocortin receptors (MC) has previously shown to inhibit leukocyte recruitment in peripheral inflammation, however it is not known whether treatments are effective in the unique cerebral microvascular environment. Here, we provide novel research highlighting the effects of the melanocortin peptides on cerebral neutrophil recruitment, demonstrating important yet discrete roles for both MC_1 and MC_3.

Approach and Results

Using intravital microscopy, in two distinct murine models of cerebral ischemia-reperfusion (I/R) injury we have investigated melanocortin control over neutrophil recruitment. Following global I/R, pharmacological treatments suppressed pathological neutrophil recruitment. MC_1 selective treatment rapidly inhibited neutrophil recruitment while a non-selective MC agonist provided protection even when co-administered with an $MC_{3/4}$ antagonist, suggesting the importance of early MC_1 signaling. However by 2h reperfusion, MC_1 mediated effects were reduced, and MC_3 anti-inflammatory circuits predominated. Mice bearing a non-functional MC_1 displayed a transient exacerbation of neutrophil recruitment following global I/R, which diminished by 2h. However importantly, enhanced inflammatory responses in both MC_1 mutant and $MC_3^{-/-}$ mice resulted in increased infarct size and poor functional outcome following focal I/R. Furthermore we utilized an *in vitro* model of leukocyte recruitment to demonstrate these anti-inflammatory actions are also effective in human cells.

Conclusions

These studies reveal for the first time melanocortin control over neutrophil recruitment in the unique pathophysiological context of cerebral I/R, whilst also demonstrating the potential therapeutic value of targeting multiple MCs in developing effective therapeutics.

Abbreviations

BCCAo - Bilateral common carotid artery occlusion, EC - Endothelial cell, e/e - MC1 mutant recessive yellow e/e, I/R - Ischemia-reperfusion, IVM - Intravital microscopy, L-E - Leukocyte - endothelial cell, MC - Melanocortin Receptor, $MC_3^{-/-}$ - Melanocortin receptor 3 null, MCAo – Middle cerebral artery occlusion, MSH - Melanocyte stimulating hormone

Introduction

Inflammation plays a central role in cerebral I/R injury. Infiltrating neutrophils contribute to a highly neurotoxic milieu as illustrated by the reduced infarct size and improved functional outcome in models of cerebral I/R following depletion of circulating neutrophils[1]. Anti-inflammatory strategies focused on inhibiting neutrophil recruitment by blocking adhesion molecules have however thus far proven ineffective in clinical trials[1]. The most probable limiting factor to such therapies is that the inflammatory response is a robust system, propagated by diverse pathways, and as such, cannot be effectively subdued by neutralizing a single component. Thus, harnessing endogenous mechanisms for the resolution of inflammation, which impact multiple elements of the inflammatory response, may provide a fruitful strategy.

Five G-protein coupled melanocortin receptors ($MC_{1\text{-}5}$) and the endogenous agonists, adrenocorticotrophic hormone, α, β and γ melanocyte-stimulating hormones (MSH) make up the melanocortin receptor system [2]. Over the last fifteen years, research by our team has been pivotal in helping to unravel the biological effects of peptides within this system demonstrating robust anti-inflammatory actions in a number of inflammatory situations including gouty, rheumatoid, osteoarthritis as well as in cardiovascular and I/R models [3-5]. These actions are proposed to be mediated primarily through inhibition of NF-κB. Furthermore, leukocytes are both a target for, and source of, melanocortins suggesting that the melanocortin receptor system may provide a self-limiting anti-inflammatory loop, serving to promote inflammatory resolution[2]. Such pleiotropic anti-inflammatory actions make these receptors a promising therapeutic candidate to address aberrant inflammation in stroke[6].

Of the five MCs identified, anti-inflammatory actions have been attributed primarily to MC_1 and MC_3[2]. These receptors have both been shown to be expressed at varying levels in the brain and also on endothelial cells and immune cells (MC_1 expression on neutrophils; monocytes and macrophages; dendritic cells; natural killer cells and B lymphocytes. MC_3 expression on monocytes; macrophages and B lymphocytes)[2]. However, the exact anti-inflammatory role of MC subtypes remains unclear, and may vary with the pathophysiological environment.

In this study, we utilize two distinct murine models of cerebral I/R to evaluate the dynamic recruitment of neutrophils in the cerebral microcirculation. Using both pharmacological and genetic approaches, we have demonstrated potent inhibitory actions of the melanocortins on cerebral leukocyte trafficking, and gained an insight into the relative importance of MC subtypes in mediating these effects.

Materials and Methods

Materials and Methods are available in the online-only data supplement.

Results

α-MSH abrogates neutrophil recruitment following cerebral I/R

To investigate leukocyte recruitment in the cerebral microvasculature, global ischemia was induced, followed by 40min or 2h reperfusion and leukocyte to endothelial cell (L-E) interactions in pial vessels were assessed by IVM (Figure 1). Sham surgery, produced little to no leukocyte recruitment, however I/R caused significant leukocyte rolling (191.0±31.49cells/mm^2/min) and adherence (282.3±49.4cells/mm^2/min), with 2h reperfusion resulting in a further significant increase in adhesion to 555.2±85.3cells/mm^2/min (Figure 1C, supplemental video I and II). Correlating with the observed effects on leukocyte recruitment was an enhanced serum soluble E-selectin by 2h reperfusion, as detected by ELISA, and a trend toward an increase in the number of ICAM-1 and VCAM-1 positive vessels detected in the brain. No effect was observed with respect to soluble P-selectin (supplemental figure II). α-MSH (10μg i.p.), a non-selective MC agonist, given at the start of reperfusion strongly inhibited leukocyte recruitment at 40min, reducing rolling by 80% and adhesion by 68%, to levels comparable to sham animals. Furthermore these protective effects remained highly significant even after 2h of reperfusion (supplemental video III). In line with the reduced leukocyte recruitment at 2h, α-MSH was also found to reduce levels of soluble E-selectin and a modest reduction in vessel ICAM-1and VCAM-1 expression.

Finally, in order to ascertain the role of neutrophils, some mice were depleted of neutrophils prior to I/R. Following I/R, neutropenic mice displayed significant reductions of leukocyte rolling (81%) and adhesion (76%), consistent with the majority or all cells observed being neutrophils (Supplemental Figure I).

Effect of α-MSH on NF-κB related cytokine and mRNA expression

The effect of cerebral I/R on serum cytokines was investigated using multi-cytokine analysis (Supplemental Figure IIIA-C). Expression of IL-12p70, IFN-γ and MCP-1 remained below the reliable detection range across all treatments (Data not shown). I/R induced a significant increase in serum TNF-α by 2h, which was abolished by α-MSH treatment. The anti-inflammatory cytokine IL-10 showed a trend toward an increase at 2h reperfusion (to 34.7pg/ml), but significantly increased (88.7pg/ml) following α-MSH treatment. Levels of the pleotropic cytokine IL-6 remained unchanged following I/R, however treatment with α-MSH was found to result in a significant up-regulation of IL-6 by 2h. Considering IL-6 signaling via STAT3 has been shown to reduce neutrophil recruitment[27], we also investigated levels of tyrosine 705 phosphorylated STAT-3 in leukocyte nuclear fractions by western blot, finding a slight enhancement of STAT3 in α-MSH treated animals at 2h (supplemental figure IV).

To investigate whether this influence over serum cytokines could be due to NF-κB inhibition, mRNA levels of the NF-κB regulatory protein IκB (which closely corresponds to NF-κB activation[7]) were assessed in both blood and brain using qRT-PCR (Supplemental Figure IVA and B). IκB levels were not significantly elevated 40min following I/R, however by 2h, IκB was significantly increased in the blood, and this was suppressed by α-MSH treatment. Suppression of NF-κB activation in leukocytes at 2h reperfusion was further confirmed by western blot analysis of serine 536 phophorylated NF-kB p65 in leukocyte nuclear fractions.

Effect of MC_1 and MC_3 agonists on neutrophil recruitment and circulating cytokines

The relative contribution of MC subtypes on IR induced neutrophil recruitment was investigated using more selective MC agonists. Activation of MC_1 by BMS-470539 provided

a potent inhibition of BCCAo induced neutrophil rolling and adhesion at 40min reperfusion (Figure 2A and B) however, became less effective by 2h, with only the level of adhesion being significant vs. vehicle. On the other hand, treatment with [DTRP^8]-γ-MSH, which has a high affinity for MC_3, only inhibited cell adhesion at 40min reperfusion, yet by 2h this effect was stronger, with neutrophil rolling also being significantly reduced. Furthermore [DTRP^8]-γ-MSH significantly reduced BCCAo induced TNF-α release by 2h whilst also elevating serum levels of the pleiotropic cytokine IL-6 and the anti-inflammatory IL-10 (Figure 2C-E).

Pharmacological investigations using the $MC_{3/4}$ antagonist SHU9119

BMS-470539 is almost entirely selective for MC_1, whilst [DTRP^8]-γ-MSH may activate other MCs than MC_3[8]. To further examine the roles of specific MC subtypes, the $MC_{3/4}$ antagonist SHU9119 was co-administered with either α-MSH or [DTRP^8]-γ-MSH (Figure 3C and D), revealing that $MC_{3/4}$ antagonism caused no increase in rolling or adhesion at 40min reperfusion vs. α-MSH or [DTRP^8]-γ-MSH alone. In fact SHU9119 administered alone, or in conjunction with α-MSH, reduced neutrophil rolling. However by 2h, co-administration of SHU9119 blunted the α-MSH induced reductions in rolling and adhesion, and prevented the protective effects of [DTRP^8]-γ-MSH.

Melanocortin receptor expression

To determine whether the delayed importance of MC_3 was due to change in receptor expression antibody based investigations into receptor expression were undertaken. However initial analysis of MC_1 and MC_3 protein expression using western blotting revealed antibody binding in $MC_3^{-/-}$ mice, using both the Sigma Aldrich (M4937) and Acris (AP10124PU-N) MC_3 antibodies, despite PCR confirmation of the $MC_3^{-/-}$ (Supplemental figure V). This suggests that both antibodies tested display non-specific binding to protein at a similar molecular weight to MC_3, as has been previously described[9]. Therefore, qRT-PCR was used to quantify MC expression at the mRNA level. $MC_{1,3,4\&5,}$ was detected in both blood and brain, however BCCAo induced no changes at either 40min or 2h following BCCAo (Supplemental Figure VI).

Physiological effects of MC1 and 3 in cerebral I/R-induced inflammation

We tested whether the physiological effects of receptor deficiency would support our findings from pharmacological treatments. Recessive yellow (e/e) MC_1 mutant mice displayed enhanced (nearly 3 times) neutrophil rolling at 40min following I/R vs. WT (Figure 4A) accompanied by elevated serum TNF-α (Figure 4C). In the absence of a functional MC_1, α-MSH reduction of rolling was also hampered at this time point, however e/e mice displayed no derangements in cell adhesion and were able to respond to α-MSH treatment. MC_3 null mice showed no significant differences in neutrophil recruitment, or in their ability to respond to α-MSH at 40min. This is consistent with the predominat anti-inflammatory role of MC_1 at early reperfusion. In agreement with the diminished role of MC_1 observed in pharmacological studies at 2h, at this time point the inflammatory phenotype was not maintained, with TNF-α levels and neutrophil rolling and adhesion being comparable to WT (Figure 5).

Physiological role in focal stroke model

Stroke is highly variable in its severity and magnitude. The global model of cerebral I/R, represents human stroke conditions caused by atherosclerotic degeneration of the common carotid arteries and respiratory or cardiac arrest. To investigate whether the MC anti-inflammatory effects we were observing were specific to global I/R, we also undertook investigations using a focal stroke model (i.e. the middle cerebral artery occlusion (MCAo) model). We chose this model due to the fact that focal ischemia accounts for ~80% of ischemic stroke. Figure 6 demonstrates that at 24h post ischemia while e/e mice showed elevated adhesion, $MC_3^{-/-}$ displayed a more severe inflammatory phenotype with significantly

enhanced rolling and adhesion. Importantly, the increased neutrophil recruitment observed, translated into elevated infarct volume and poor functional outcome (detected through neurological scoring) in animals with deficits in either receptor, suggesting both receptors to be potential pharmacological targets.

In vitro investigations of melanocortin effects on neutrophil functioning

The melanocortin receptor system displays a number of disparities between humans and rodents and a number of MC agonists and antagonists have different selectivity in MCs from different species[8,10]. To assess the effectiveness of melanocortin treatments on human cells, we utilized the neutrophil flow chamber model and chemotaxis assay to investigate neutrophil inflammatory function (Supplemental Figure VII). In the flow chamber, treating neutrophils with just 10μg/ml of α-MSH (a comparable dose to *in vivo* studies) resulted in significant reductions in capture (54% reduction) adherence (68%) and transmigration (67%) vs. saline (Supplemental Figure VIIA). Treating HUVECs with α-MSH (≤100μg/ml), however failed to induce significant reductions in neutrophil recruitment (data not shown), suggesting neutrophils to be the effector cells of MC actions in this context. In the chemotaxis assay, pre-treatment with 10μg/ml of α-MSH or [DTRP^8]-γ-MSH failed to inhibit neutrophil migration towards fMLP, however BMS-470539 significantly reduced this response, suppressing the number of migrated cells by ~75% (Supplemental Figure VIIB), suggesting MC_1 to play a specific role in inhibiting neutrophil chemotaxis.

Discussion

The present study provides, for the first time, evidence that melanocortin treatments can effectively inhibit neutrophil recruitment in the unique microenvironment of the cerebral microvasculature. Utilizing both selective ligands and MC mutant mice, we have gained an insight into MCs involved in modulating neutrophil recruitment in two separate models of cerebral I/R, finding both MC_1 and MC_3 to display important inhibitory roles. In addition, melanocortin treatment was effective in modulating human neutrophil inflammatory functioning, and may represent a novel treatment to stem post-stroke inflammation.

Both ischemic models used (BCCAo and MCAo) here resulted in a pronounced increase in neutrophil rolling and adhesion compared to sham, as has previously been observed[11,12]. Non-selective treatment with α-MSH caused an abrogation of BCCAo-induced neutrophil rolling and adhesion, congruent with a trend toward a reduction in ICAM-1 and VCAM-1 expression in the cerebral vascular. Considering these effects were not significant, other adhesion molecules and integrin activation state may also play a role in the melanocortin influence over leukocyte adhesion. While anti-ICAM-1 antibody Enlimomab failed clinical trials for stroke (possibly due to inflammatory side effects to the mouse monoclonal antibody) strategies inhibiting CAMs have shown great promise pre-clinically. Soluble P and E-selectin are early markers of endothelial activation. While levels of soluble P selectin were found only to produce a trend toward an increase following BCCAo which was unchanged by treatment, soluble E-selectin was increased by 2h post ischemia. Elevated levels of soluble E-selectin have been demonstrated in human stroke and during sepsis[13]. *In vitro* studies have also shown that endothelial cells stimulated with IL-1β, TNF-α, endotoxin or serum deprivation and TNF-α release E-selectin into the culture supernatant[14,15]. α-MSH treatment significantly reduced BCCAo induced soluble E-selectin, likely a reflection of reduced endothelial cell activation, consistent with the reduced levels of IL-1β and TNF-α observed.

Treatment also reduced serum TNF-α and IL-1β. IL-1β in particular plays a pivotal role in propagating inflammatory responses and is an established pathological factor in cerebrovascular disease, with significant pre-clinical and clinical evidence demonstrating blockade of IL-1β signaling to be beneficial in stroke[16]. MSH treatment also simultaneously enhanced anti-inflammatory IL-10 by 2h reperfusion. The ability of MCs to suppress pro-inflammatory responses whilst enhancing anti-inflammatory signals suggests that these

receptors form an endogenous pro-resolving system. In humans α-MSH concentrations increases in myocardial infarction and infection[2] and higher melanocortin levels correlate with better outcome in stroke patients.[17] Thus, given the results from the present study the melanocortin receptor system may prove a valuable therapeutic target for the treatment of stroke.

While others have demonstrated a contribution of either MC_1 or MC_3 in providing anti-inflammatory protection in different systemic inflammatory models, we have, for the first time, identified protective roles for both receptors in the cerebral-microvasculature. In particular, we have identified that MC_1 mediated effects predominate in early protection, while MC_3 actions are more delayed and induce pro-resolving factors. This study reveals an additional layer of complexity in MC inflammatory modulation, emphasizing the importance of drug treatment directed at both receptors.

Pharmacological investigations in the BCCAo model revealed the MC_1 agonist BMS-470539, significantly inhibited early neutrophil rolling and adhesion, while activation of MC_3 using [D-TRP^8]-γ-MSH only reduced adhesion. However by 2h following BCCAo, despite a robust pharmacodynamic half-life of ~8 h[18], BMS-470539 lost its inhibitory actions on rolling and the reduction of adhesion was diminished slightly. However at 2h, [D-TRP^8]-γ-MSH anti-inflammatory actions were enhanced. Thus suggesting MC_3 mediated effects become more prominent at later time points. SHU9119 ($MC_{3/4}$ antagonist) was used to further explore the role of MC subtypes. By 40min reperfusion, SHU9119 enhanced, rather than inhibited, α-MSH and [D-TRP^8]-γ-MSH effects. Furthermore, administered alone, SHU9119 reduced neutrophil adhesion at 40min, possibly due to its seldom reported agonist actions at $MC_{1/5}$[10], further supporting the predominance of MC_1 mediated effects at early stages of reperfusion. By 2h reperfusion SHU9119 lost these anti-inflammatory effects, and co-treatments with SHU9119 inhibited the protective effects of α-MSH and [D-TRP^8]-γ-MSH, illustrating role for MC_3 in mediating delayed effects on neutrophil adhesion.

Despite the apparent change in MC engagement, no change in MC RNA expression was detected following BCCAo. Whether this is also reflected in protein expression is difficult to discern given the lack of specificity of the antibodies. Even if the surface expression of MC_1 or MC_3 is increased following stroke, α-MSH and [D-TRP^8]-γ-MSH have short half-lives and therefore are most likely exerting their effects before such up-regulation occurs. The Shift in receptor importance may instead reflect activation of distinct mechanisms of action and/or effector cells. Neutrophils express MC_1, while MC_3 expression is limited to endothelial cells and macrophages/monocytes. Rapid inhibitory actions exerted by MC_1 may be through direct interactions with neutrophils, while the more prolonged MC_3 actions are likely via actions on other effector cells. Inhibition of NF-κB is a key element of the protective MC actions[2]. Given NF-κB DNA binding occurs only after 30min following TNF-α stimulation, followed by gene transcription 30min later[7], NF-κB inhibition is unlikely to mediate the rapid effects on neutrophil recruitment at 40min. Indeed IκB transcript levels were not significantly increased at 40min, yet by 2h blood IκB elevated 25-fold and nuclear levels of phosphorlyated NF-κB protein were enhanced, which was inhibited by α-MSH. Given our finding of the delayed effect of MC_3 targeted treatment on neutrophil this later effect by α-MSH on IκB may be mediated predominantly via MC_3 signaling.

Melanocortin treatments inhibited neutrophil recruitment at 40min after BCCAo, a timeframe incompatible with transcriptional changes. Such a phenomenon has previously been observed in other models; in zymosan stimulated macrophages AP214 reduced IL-1β and TNF-α release with no effect on mRNA levels[19], and α-MSH can act via MC_1 to shed cell surface CD14 on macrophages[20] and IL-8 receptors on neutrophils[21] independently of mRNA changes. While the understanding of NF-κB independent MC effects is still in its infancy,

these mechanisms may fit well with the early efficacy of MC_1 treatments in suppressing neutrophil recruitment.

Differences in cytokine regulation between MC_1 targeted activation and MC_3 targeted/non-specific activation further support the hypothesis that MC_1 and MC_3 act via distinct mechanisms. Both α-MSH and [D-TRP^8]-γ-MSH significantly reduced BCCAo induced TNF-α whilst enhancing anti-inflammatory IL-10. TNF-α has multiple roles in stroke pathology and early increases in blood TNF have been shown to correlate with stroke severity in humans[22] while the anti-inflammatory cytokine IL-10 has been shown to be protective in a number of models of cerebral I/R[23,24]. BMS-470539 treatments however had no effect on these NF-κB controlled cytokines further supporting a distinct mechanism of action for the MC_1 mediated early inhibition of neutrophil recruitment, while MC_3 may be more prominent in initiating pro-resolving effects. Conflicting reports have been made as the effect of melanocortin treatments on IL-6 levels, however in the current investigations both α-MSH and [D-TRP^8]-γ-MSH increased this pleiotropic cytokine at 2h, despite a decrease in NF-κB levels. As such the observed IL-6 release may be as a result of elevated release of pre-synthesized IL-6. α-MSH has previously been shown to influence IL-6 levels via MC_3[25], thus, explaining why IL-6 induction was not observed in BMS-470539 treated animals. While IL-6 is an endogenous pyrogen with chemotactic activity, IL-6 KO mice show no protection following experimental stroke [26]. Indeed IL-6 signaling via STAT3, has been shown to limit the inflammatory recruitment of neutrophils[27], given that IL-10 may also activate STAT3 and levels were increased following α-MSH treatment such findings appear to be consistent with the present results. Furthermore it has been demonstrated that NF-κB signalling may induce expression of IL-6 and IL-10 in cells with a high level of STAT3 phosphorylation [28] In line with such observations we found nuclear extracts from α-MSH treated BCCAo animals to show a trend toward an elevation of phosphorylated STAT-3 protein.

Our investigations using MC mutant mice, in two independent models of stroke, further support a temporal difference in MC_1 and MC_3 actions. Recessive yellow e/e mice displayed a transiently enhanced neutrophil rolling and elevated TNF-α at the very onset of the BCCAo induced inflammatory response, while $MC_3^{-/-}$ mice showed no inflammatory phenotype at this early time point. It is possible that compensatory up regulation of other MCs may mask the anti-inflammatory role of this receptor in $MC_3^{-/-}$. Montero-Melendez *et al.,* have previously shown that while in macrophages isolated from wild type and e/e 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 [19] perhaps indicating compensatory regulation of other MC in the absence of MC_3. However in the focal stroke model, at 24h $MC_3^{-/-}$ animals displayed enhanced rolling and adhesion further supporting a delayed role of this receptor, while e/e animals only showed elevated adhesion compared to WT, perhaps as a result enhanced rolling prior to the observation period. Previous reports and unpublished data from our laboratory have shown no significant differences in the basal circulating levels of leukocytes between WT, e/e and $MC_3^{-/-}$ [3]. As such the observed enhanced leukocyte recruitment is unlikely to be due to an initially high leukocyte count in these animals. As observed in BCCAo animals both receptors appear to be of importance with MC_3 anti-inflammatory circuits predominating at later time points.

Previously, Leoni *et al.,* demonstrated that the $MC_3^{-/-}$ mouse displays higher levels of leukocyte adhesion and emigration in the mesenteric microcirculation following I/R, while the response of the e/e mouse was not significantly different from WT [3]. Yet in the same model the MC_1 agonist BMS-470539 was effective in reducing leukocyte recruitment (an effect which was absent in e/e mice)[4]. These results led the authors to postulate that the receptors show different physiological roles but that both may be harnessed pharmacologically. The present findings demonstrate a physiological role for both receptors in the cerebral microcirculation. Indeed the observed of temporally distinct roles of these two receptors may

help to explain such apparently conflicting findings across studies investigating the anti-inflammatory actions of these receptors. Crucially, our study demonstrates that the absence of signaling from either receptor resulted in enhanced infarct size and worse functional outcome compared to WT, further illustrating that both receptors are important pharmacological targets.

Our studies have further demonstrated that melancortin peptides may effect neutrophil inflammatory functioning in human cells. As the melanocortin receptor system displays a number of disparities in organization and function between humans and rodents. Such as differences in potency and selectivity of melanocortin ligands, and receptor expression between species, we also investigated MC roles in human neutrophil functioning. The facts that α-MSH treatment was found to suppress neutrophil recruitment to HUVECs via actions on neutrophils, and that MC_1, but not MC_3, is expressed on neutrophils supports the concept of an MC_1 specific effect. These effects were rapid, occurring within 30min following treatments, again a timeframe incompatible with transcriptional mechanisms. MC_1 specific direct effects on neutrophils were further illustrated by the ability of MC_1 targeted treatments to significantly reduce neutrophil chemotaxis (performed in the absence of other cell types). Further investigation however, will need to be made to establish the effector cell type of the delayed, but potent, effects of MC_3 on neutrophil recruitment.

Taken together, novel experimental data presented here highlights a role for utilization of melanocortin based treatments as potential therapeutics for stroke, and potentially other neurovascular diseases. This work also demonstrates important roles for both MC_1 and MC_3, showing MC_1 effects to provide rapid inhibition of leukocyte recruitment via mechanisms independent of NF-κB regulation, while MC_3 actions appear more robust at later time points. Given the complexity of MC regulation along with the variable nature of stroke, strategies targeting multiple MCs in a non-(or perhaps partially) selective manner may be more fruitful in providing robust protection, rather than targeting one MC alone. Indeed, it may be telling that currently the most promising melanocortin compounds (NDP-α-MSH and AP214) are both non-selective MC agonists. Further investigations into later time points of treatment and in animals with co-morbidities will help to reveal the full potential of this promising therapeutic target for stroke.

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Significance

Stroke is a leading cause of mortality and morbidity worldwide. Following the initial ischemic brain damage an ensuing inflammatory response may exacerbate injury. Neutrophils are key arbitrators of this damaging response and inhibiting neutrophil recruitment in cerebral ischemia reperfusion injury may provide therapeutic benefit. We have previously demonstrated that melanocortin treatments can reduce leukocyte recruitment in peripheral tissues, but these actions have yet to be demonstrated in the unique microcirculation of the brain. Here in, we show that melanocortins reduce post ischemic leukocyte recruitment in the brain, and that these effects are mediated by the distinct actions of both melanocortin receptors 1 and 3. Therefore, targeting these receptors provides a novel therapeutic strategy for treating stroke, and other cerebrovascular diseases.

Figure Legends

Figure 1. BCCAo induces leukocyte recruitment, which is abrogated by α-MSH treatment. A) representative IVM video stills show interactions of leukocytes (white arrow) on the cerebral vessel wall, with clearly observable increases following BCCAo. Images taken on an Olympus BW61WI microscope, magnification x 40. B-C) Leukocyte recruitment in the cerebral microcirculation was quantified in terms of: (B) number of cells rolling along the vessel wall per mm^2 (termed rolling cell flux); and C) those cells stationary for 30sec or longer (Adhesion: cells/mm^2/min). Values represent mean ± SEM. n=6 mice/group. * denotes significance to sham *P<0.01,**P<0.001. † = significance to saline treated BCCAo group P<0.01, †† P<0.001. ‡ = significant increase to 40min reperfusion group. P<0.05 is considered significant

Figure 2. The effect of pharmacologically selective treatments on L-E interactions and circulating cytokines. Leukocyte recruitment in the cerebral microcirculation was quantified in terms of: A) rolling cell flux and B) adhesion (cells/mm^2/min), at either 40min or 2h following 5min BCCAo. Treatments were given i.p. at the start of reperfusion using either 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. n=6 mice/group. * denotes significance to sham P<0.01,**P<0.001, † denotes significance to saline treated BCCAo group P<0.01, †† P<0.001. Serum levels of circulating cytokines C)TNF-α, D) IL-6 and E) IL-10 were assessed using a cytometric bead array, n=3 mice/group and performed in duplicate.

Figure 3. Effects of $MC_{3/4}$ antagonism on the actions of α-MSH and [D-TRP^8]-γ-MSH. Leukocyte recruitment in the cerebral microcirculation was quantified in terms of: rolling cell flux and adhesion (cells/mm^2/min), following BCCAo and 40min or 2h reperfusion. Affect of the $MC_{3/4}$ antagonist SHU9119 when administered 10μg i.p at the start of reperfusion on A) leukocyte rolling cell flux and B) leukocyte adhesion. Leukocyte rolling C) and adhesion D) was also assessed in mice treated with either α-MSH or [D-TRP^8]-γ-MSH alone (10μg) or in combination with SHU9119 (10μg). α-MSH 40min co-treatment group n=6 mice/group all other co-treatment groups n=4. Comparisons were made to singular treatment groups from Figure 2. 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

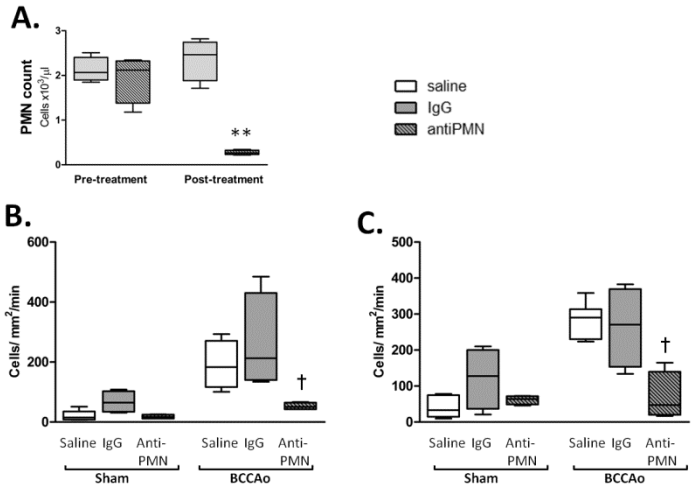
Figure 4. Mice bearing a non-functional MC_1 display acutely enhanced L-E interactions and TNF-α levels following I/R. Wild type C57BL/6 (white bars), MC_1 mutant e/e (lined bars) and $MC_3^{-/-}$ (checked bars) mice were subjected to sham or BCCAo surgery with 40min reperfusion and treatment with either 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 and B) adhesion (cells/mm^2/min). WT groups n=6 mice/group, e/e and $MC_3^{-/-}$ sham and $MC_3^{-/-}$ α-MSH groups n=3 all other groups n=4 mice/group. C) ELISA measurements of serum TNF-α levels n=3 mice/group and performed in duplicate. 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.

Figure 5. The e/e mouse inflammatory phenotype is diminished by 2h. WT (C57BL/6) (white bars) and e/e (lined bars) mice were subjected to sham or 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: in terms of: A) rolling cell flux and B) leukocyte adhesion (cells/mm^2/min). n = 4 mice/group for e/e saline group, and n=3 mice/group for sham and α-MSH e/e groups, comparisons were made to WT groups as in figure 1 (n=6 mice/group). 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. C) ELISA measurements of serum TNF-α in WT and e/e mice. * denotes significance to sham groups $P<0.01$, † denotes significance to BCCAo $P<0.05$.

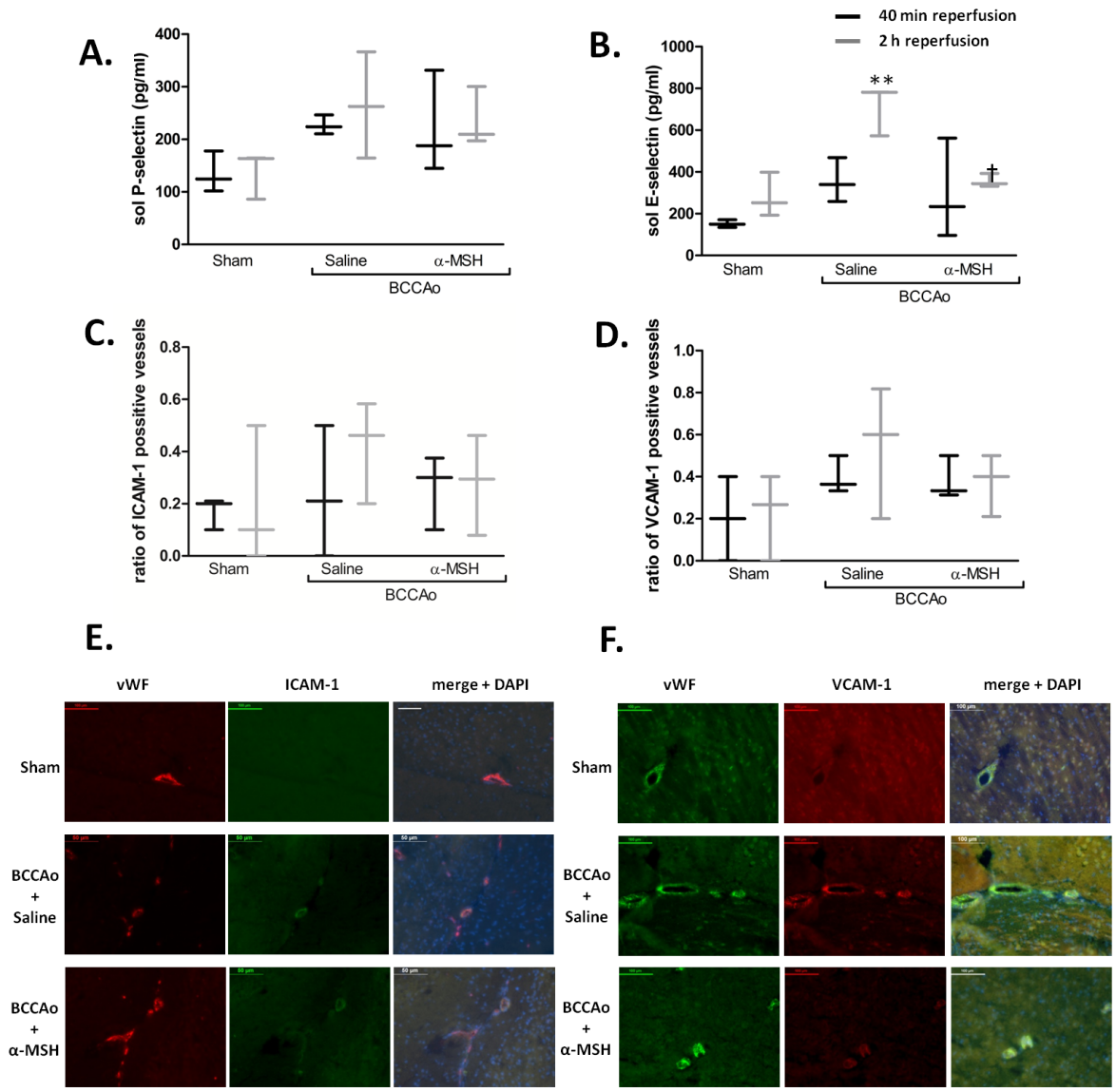
Figure 6. Physiological role of MC_1 and MC_3 in focal cerebral I/R. (A and B) Leukocyte recruitment in the cerebral microcirculation was quantified following MCAo and 24h reperfusion in terms of: rolling cell flux and leukocyte adhesion (cells/mm^2/min). C) Infarct volume and D) Neurological score were also assessed. Values represent mean ± SEM. n=4-6 mice/group. * denotes significance to corresponding sham group, +denotes significance to WT MCAo group. # denotes significance to WT e/e group. $P<0.05$ is considered significant.

SUPPLEMENTAL MATERIAL

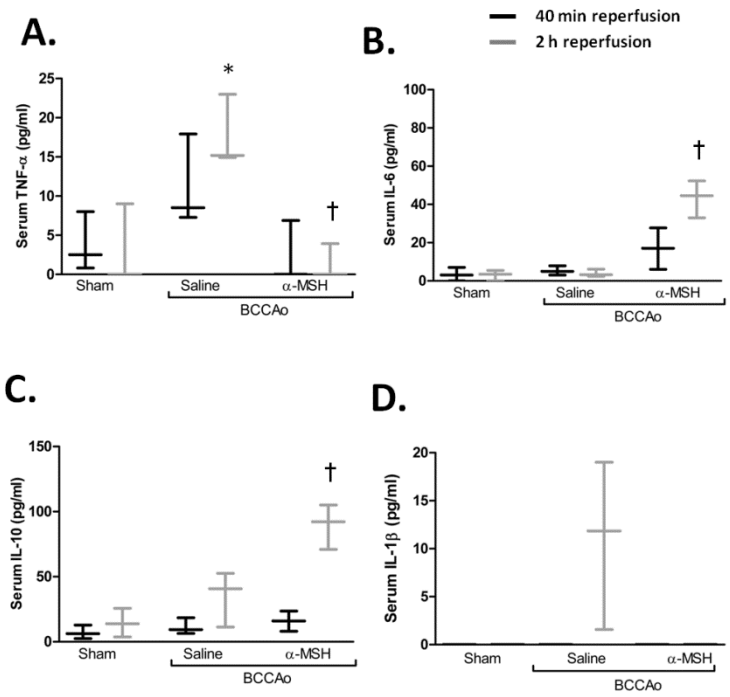
Supplemental Figures and Figure Legends



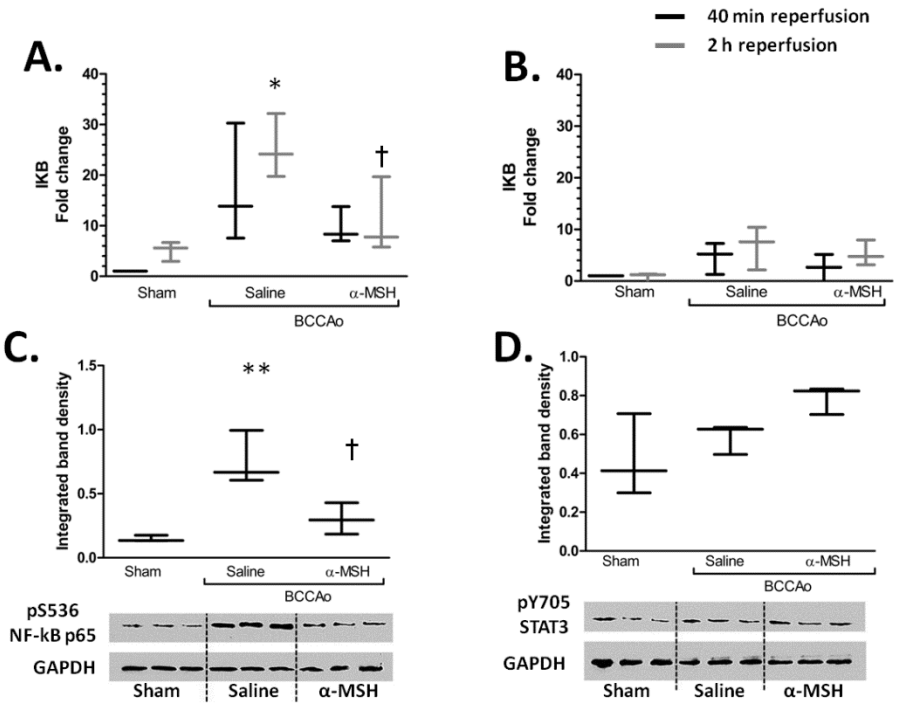
Supplemental Figure I. Effects of PMN depletion on cerebral I/R induced leukocyte recruitment. (A) C57BL/6 mice were treated with either anti-mouse PMN serum or IgG matched serum (10ml/kg, i.p.) once daily for two consecutive days. Tail vein blood samples, for total white cell count and differential cell counts, were collected pre-treatment and post treatment. Anti-PMN treatment reduced total PMN count by ~85%. ** denotes significant to Anti-PMN pre-treatment group P<0.001. (B-C) Following sham or BCCAo and 40min reperfusion, IVM was used to assess leukocyte recruitment in the cerebral microcirculation in terms of: (B) number of cells rolling along the vessel wall per mm^2 (termed rolling cell flux) and (C) those cells stationary for 30sec or longer (termed adhesion: cells/mm^2/min). Depleting mice of neutrophils was found to significantly inhibit the number of observed leukocytes interacting with venules of the cerebral microcirculation. † denotes statistical significance to IgG group P<0.01. Values represent mean ± SEM. All IgG and anti-PMN treated groups n=4 mice/group.



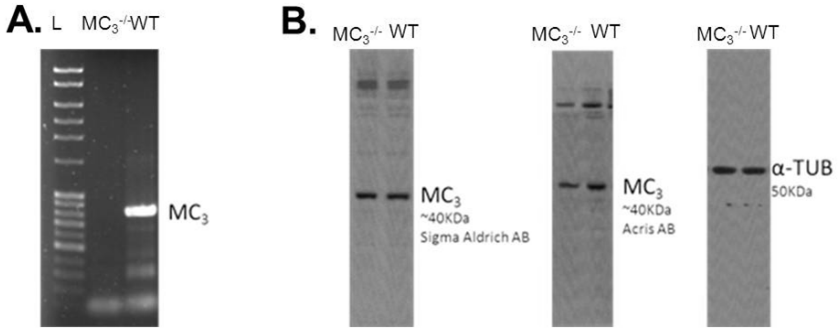
Supplemental Figure II. Modulation of adhesion molecule expression. (A and B) levels of circulating soluble P and E selectin were measured in serum samples showing enhanced P-selectin levels by 2h reperfusion which were suppressed by α-MSH treatment. (C and D) the cerebral microcirculation was stained for ICAM-1 and VCAM-1 adhesion molecules, BCCAo was found induce a trend toward an increase in the number of ICAM-1 and VCAM-1 positive vessels compared to sham with α-MSH treatment showing a modest trend toward a reduction. (E and F) Representative x20 micrographs of cerebral vessels stained for vWF (red) and ICAM-1 (green) or vWF (Green) and VCAM-1(Red).



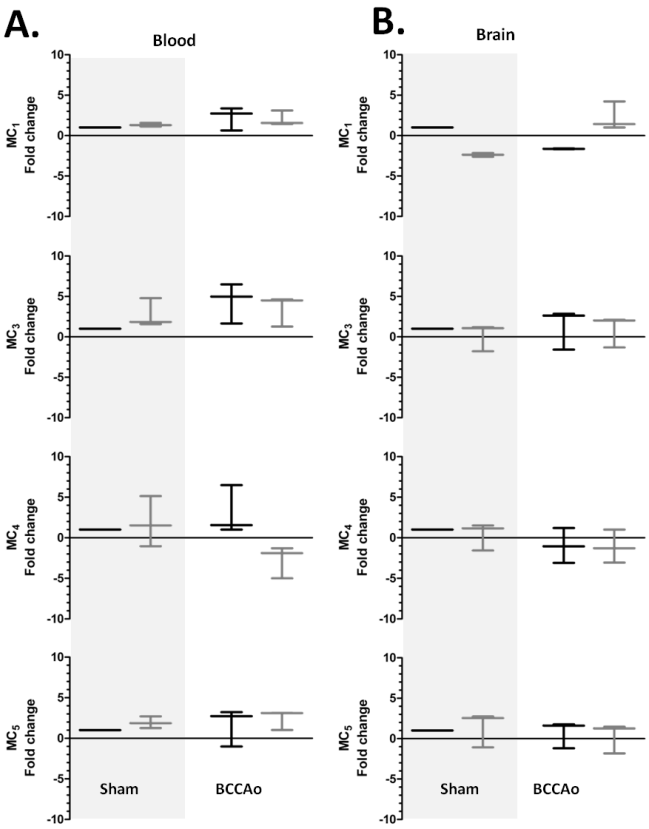
Supplemental Figure III. Cytokine response to BCCAo and α-MSH treatment. (A-C) CBA was used to quantify circulating serum cytokine levels (IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70) and ELISA used to detect IL-1β levels following BCCAo and MSH treatment at both 40min and 2h reperfusion. Expression of IL-12p70, IFN-γ and MCP-1 remained below the reliable detection range across all treatments (Data not shown). However BCCAo induced significant increases in TNF-α and IL-1β by 2h, which was abrogated by α-MSH treatment. Treatment also induced significant increases in the pleiotropic cytokine IL-6 and the anti-inflammatory IL-10 at 2h. n=3 for all groups. * denotes significance to sham, † shows significance to 2h BCCAo saline treated group $P<0.01$.



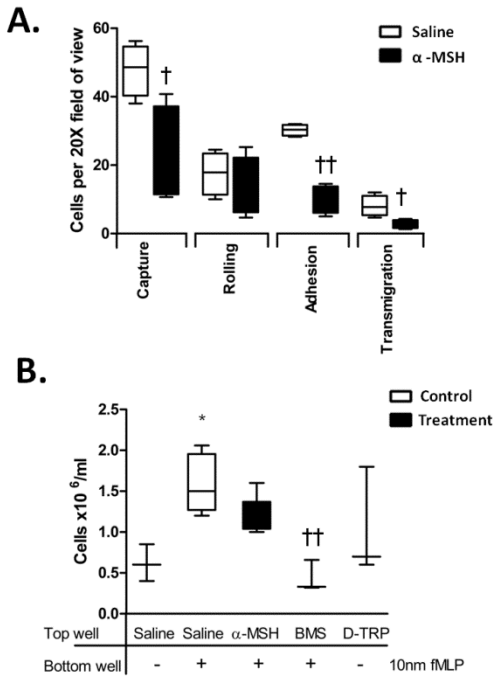
Supplemental Figure IV. NF-κB and STAT-3 activation. NF-κB activation was assessed by quantifying mRNA levels of IκB using qRT-PCR in (A) blood and (B) brain. To further investigate the NF-κB activation in blood, leukocyte protein levels of the serine 536 phospholyated form of NF-κB in nuclear fractions were detected using western blot (C and D). NF-κB activation as detected by protein level at 2h reflected IkB RNA levels (C). Considering IL-10 and IL-6 can activate protective STAT-3 pathways the level of STAT-3 activation in leukocytes was also assessed using western blot at 2h (D). n=3 for all groups. * denotes significance to sham, † shows significance to 2h BCCAo saline treated group $P<0.01$.



Supplemental Figure V. MC_3 antibodies show binding in tissue from mice lacking the MC_3 transcript. (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



Supplemental Figure VI. BCCAo does not alter melanocortin receptor mRNA expression. Total blood RNA was isolated from whole blood (A) or brain (B) of C57BL/6 mice subjected to Sham surgery or BCCAo with reperfusion times of 40min or 2h. qRT-PCR used to quantify the mRNA levels of MC_1 MC_3 MC_4 and MC_5. Values represent mean ± SEM of fold change in expression compared to 40min sham operated control. n=3 for all groups, no significant changes were detected.



Supplemental Figure VII. Melanocortin peptides directly affect human neutrophil functioning. (A) Human neutrophils ($1x10^6$ cells/ml) were perfused over TNF-α stimulated (10ng/ml, 4h) confluent HUVEC monolayers. Neutrophils were pre-treated with saline or 10μg/ml of α-MSH. Leukocyte recruitment was quantified in terms of Capture (all cells interacting with the monolayer), number of rolling cells, adherent cells (cells stationary for 10sec or longer) and number of cells transmigrating across the HUVEC monolayer, n=4 for all groups. (B) Levels of neutrophil chemotaxis found to migrate across a porous membrane toward the chemoattractant FMLP (10nM). Neutrophils ($4x10^6$ cells/ml) were pre-treated 10min with 10μg/ml α-MSH, BMS-470539 or [D-TRP^8]-γ-MSH then applied to the membrane and incubated for 1.5h before assessing neutrophil migration. Control fMLP and α-MSH groups n=5, all other groups n=3. * denotes significance to un-stimulated group $P<0.05$. **= $P<0.001$. † denotes significance to saline treated group, $P < 0.05$, †† = $P< 0.001$

Supplemental Video legends

Supplemental video I

Intravital video clip of a representative pial brain vessel of a sham treated C57BL/6 mouse. Demonstrating a single adherent leukocyte within the vessel.

Supplemental video II

Intravital video clip of a representative pial brain vessel from a BCCAo 2h reperfusion saline treated C57BL/6 mouse demonstrating rolling and adhesion of leukocytes within the vessel

Materials and Methods

Animals

All Housing conditions and experimental procedures were in strict accordance with Home Office Regulations (Scientific Procedures Act, 1986) and the European Directive 2010/63/EU. All animals were used at 18-30g body weight. Male C57BL/6 mice were supplied by Charles River (Kent, UK), recessive yellow (e/e) mice are the result of a natural mutation causing a single nucleotide deletion at position 549 and were supplied by Prof. Mauro Perretti at the William Harvey Research Institute, London. The $MC_3^{-/-}$ colony, backcrossed for six generations onto a homogenous C57Bl6 background(1), were kindly donated by Dr. H. Chen (Merck Research Laboratories, NJ, USA) and bred at the William Harvey Research Institute, London. Animals were maintained on a 12h/12h light/dark cycle with an ambient temperature between 21-23°C with access to standard chow pellet diet and water *ad libitum*.

Drug treatments

The pan receptor agonist α-MSH (Asc-189, Ascent Scientific, Cambridge, UK) the MC_3 partially selective agonist [$DTRP^8$]-γ-MSH (4272, Tocris, Bristol, UK), the MC_1 selective agonist BMS-470539 (4053, Tocris, Bristol, UK) and the $MC_{3/4}$ antagonist SHU9119 (3420, Tocris, Bristol, UK) were utilized in this study. Drug treatments were all administered at the start of reperfusion by intraperitoneal (i.p) injection at 10µg in 100µl saline, with the exception of BMS-470539, which was given at 9.4mg/Kg. Drug dose was chosen with regards to optimal doses in the literature(2-6). Treatments were given at the start of reperfusion.

Global Stroke Model: bilateral common carotid artery occlusion (BCCAo)

Following anaesthesia (100mg/kg pentobarbital i.p, Merial, Essex, UK), the animals temperature was monitored using a rectal thermometer and maintained throughout the procedure as close to 37.5 ^{o}C as possible using a heat mat. The common carotid arteries, exposed and occluded using aneurysm clips(7). Following 5min of occlusion clips were removed to allow for reperfusion. Sham animals omitted BCCAo.

Focal Cerebral I/R Model: Middle Cerebral Artery Occlusion (MCAO)

Animals were anaesthetized with. ketamine (150 mg/kg i.p.; Fort Dodge Animal Health, Southampton, UK) and xylazine (7.5 mg/kg i.p.; Bayer Healthcare, Newbury, UK) and body temperature maintained as above. The middle cerebral artery (MCA) was occluded using the intraluminal filament method (8), using a 60min occlusion period followed by a period of 24h reperfusion. Sham-operated mice were subject to the same surgical procedures omitting MCA occlusion.

Intravital Microscopy (IVM)

A crainotomy was performed and rhodamine-6G (100µl, 0.02% in saline) injected through a jugular vein cannula to fluorescently label circulating leukocytes. The cerebral microcirculation visualized using intravital microscopy (IVM) as described previously(8). Off-line analysis of leukocyte-endothelium interactions yielded: ‘rolling cell flux’ (number of cells passing a fixed point/minute) and ‘adhesion’ (cells remaining stationary for 30sec or longer) and calculated as cells/mm^2 of vessel/min as described previously(8).

Infarct Volume

After reperfusion (24h), MCAo mice were sacrificed, brains removed and placed into 4°C PBS for 15min. 2mm coronal sections were cut with a tissue cutter, and sections stained with 2% 2,3,5-triphenyltetrazolium chloride in phosphate buffer for 15min followed by fixation in 10% formaldehyde. Sections were photographed and the infarct area quantified in digitized images using NIH Image Software version 1.57.

Neurological score

The functional consequences of cerebral 24h I/R injury were evaluated using a five-point neurological deficit score: 0=no deficit; 1=failure to extend right paw; 2=circling to the right; 3=falling to the right; and 4=unable to walk spontaneously (8).

Neutrophil depletion
Anti-mouse PMN serum or serum matched control (AIA31140 and AIS403, Gentaur, London, UK) diluted 1:10 in saline and used at 10ml/kg was administered (i.p) daily for two days prior to BCCAo surgery. PMN depletion was confirmed 48h following injection by total white blood cells count in Turk's solution. WBC differential counts were obtained from blood smears stained with Wright-Giemsa. Both counts were compared with samples taken prior to treatment and in all cases PMNs were found to be depleted by >85%

Immunofluorescent staining
Experimental animals were perfused with saline followed by 4% paraformaldehyde and brains cyroprotected in 20% sucrose for 18h before freezing in isopentane and embedded in OCT (Sakura). 18µm coronal cryostat sections taken between 1mm and 3mm posterior to the bregma and mounted on charged microscope slides before being stored at -20 °C. Sections were hydrated in PBS for 5min, before performing heat mediated antigen retrieval with Vector Laboratories' Antigen Unmasking Solution for 10min. Blocking was then performed in PBS +1% BSA and 10% serum for 2h. Sections were rinsed in TBST before adding the primary antibodies Rat anti-ICAM-1 (ab119871, Abcam) and Rabbit anti-vWF (A0082, Dako); or Rabbit anti VCAM-1(SC-8304, Santa Cruz) and Sheep anti -vWF (ab11713, Abcam) overnight at 4^{o}C. Sections were incubated with secondary antibodies (Alexa Fluor 546 Donkey Anti-Rabbit IgG, 1:200) for 2h at room temperature. Sections were counterstained with DAPI and imaged using a Nikon Eclipse inverted microscope with Nikon Elements acquisition software. Number of vessels between 10-100µm in diameter found to be I-CAM or V-CAM positive across 5 x20 fields of view per section were expressed as a ratio of total number of vessels observed (vWF positive).

Quantitative Real-Time PCR
Quantitative Real-Time PCR (qRT-PCR) was employed to measure the expression of MCs_{1-5}, TNF-α and IκB-α mRNA in both brain and blood samples from experimental mice. Total RNA was isolated from whole brain and blood using an RNeasy RNA purification kits (Qiagen).

qRT- PCR was performed using QuantiFast SYBR green one-step RT-PCR kit (Qiagen) with QuantiTect primer assays, containing Quiagen forward/reverse primers for MC_1 (QT00305011), MC_3 (QT00264404), MC_4 (QT00280861) and MC_5 (QT01166494) and IκB (QT00134421). A panel of reference genes were tested for coefficient of variance and TATAA-box binding protein (QT00198443) was found to be the most suitable, and was thus as an internal control. Reactions containing 50ng of template RNA were run in triplicate using an MX3000P real time cycler (Stratagene) Fluorescence data collection was performed during the annealing/extension phase. Dissociation curves, performed at the end of each reaction confirmed the presence of a single PCR product. Relative quantification of gene expression was determined using the $\Delta\Delta$CT method as previously described[8].

ELISA
Serum from blood samples taken by cardiac puncture were stored at -80°C until use. Quantikine® Colorimetric TNF-α, IL-1β, soluble E-selectin and soluble P-selectin ELISA kits (R&D Systems, Oxfordshire, UK) were used for the analysis of serum proteins according to the manufactures guidelines.

Cytometric Bead Array
Quantitative detection of IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 was performed on mouse serum samples using a BD Cytometric Bead Array (CBA) Mouse Inflammation Kit

(552364, BD Biosciences, Oxfordshire, UK), and assessed using a dual laser BD FACSCalibur, with BD CellQuest pro software for acquisition.

Western blot
Western blots were performed on whole brain homogenates or leukocyte nuclear fractions obtained using active motif nuclear extract kit. Rabbit anti mouse MC3 antibodies were used to detect brain MC3 expression (Sigma Aldrich M4937 and Acris AP10124PU-N). For investigation of transcription factor activation in leukocytes rabbit anti-phospho S536 NF-κB p65 (ab86299, Abcam) antibody was used to detect NF-κB activation, whilst rabbit anti phospho Y705 STAT-3 antibody was used to detect STAT activation. Horse radish peroxidase conjugated anti-rabbit antibody was used as the secondary and protein expression determined by densitometry using Image J software and normalized to α-tubulin or GAPDH expression.

Human neutrophil isolation
Due to differences in MC biology between humans and rodents investigations were also undertaken using human neutrophils. Blood collection and leukocyte isolation was performed with ethical approval from the National Health Service Research Ethical Committee (2009,04/Q0401/40). Samples were taken from healthy individuals, with no history of recent acute or chronic illness, and neutrophils were isolated using a density gradient, as described previously(9).

Flow chamber leukocyte recruitment assay
For investigations into human neutrophil recruitment human umbilical vein endothelial cells (HUVECs) were seeded ($2.5x10^6$cells/ml) in μ-Slide $VI^{0.4}$ flow chamber slides (IBIDI, Munich, Germany) and grown to confluence before stimulating with TNF-α (10ng/mL) for 4h prior to flow. HUVECs were treated with α-MSH (1-100μg/ml) or vehicle 2h prior to flow. Isolated neutrophils ($1x10^7$cells/ml) were either pre-treated with vehicle or 10μg/ml α-MSH 30min before flow. Neutrophils were perfused over HUVECS at a shear stress of 1dyne/cm^2 and number of rolling, firmly adherent (cells stationary for ≥10sec) and transmigrated cells (cells changing from phase bright to dark) recorded as previously described(9)

Neutrophil chemotaxis assay
Chemotaxis assays were performed using neuroprobe 96-well disposable plates (Neuro Probe Inc.). Neutrophils ($4x10^6$ cells/ml) were incubated for 10min at 37.5°C, 5% CO_2 either with RPMI or α-MSH, BMS-470539 or [$DTRP^8$]-γ-MSH (10μg/ml) in RPMI. fMLP 10^{-6}M in RPMI + 0.1% BSA was used as a chemo-attractant and placed in the bottom wells. The top chamber was filled with 25μL of neutrophils. Plates were incubated for 1.5h (37.5°C, 5% CO_2). The number of migrated cells were then counted on a hemocytometer using Turk's solution.

Statistics
Data are expressed as mean +/- SEM. Results from intravital microscopy experiments were confirmed to follow a normal distribution using Kolmogorov-Smirnov test of normality with Dallal-Wilkinson-Lilliefor corrected P Value. Data that passed the normality assumption was analyzed using Student's t-test or ANOVA with Bonferroni post-tests, which were performed using GraphPad Prism5 software. Data that failed the normality assumption were analyzed using the non-parametric Mann-Whitney U test. Data are shown as mean values ± standard error of the mean (SEM). Differences were considered statistically significant at a value of $P<0.05$.

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