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

Paul M. Holloway, Pascal F. Durrenberger, Marjan Trutschl, Urska Cvek, Dianne Cooper, A. Wayne Orr, Mauro Perretti, Stephen J. Getting, Felicity N.E. Gavins

Objective—Neutrophil recruitment is a key process in the pathogenesis of stroke, and may provide a valuable therapeutic target. Targeting the melanocortin (MC) receptors 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 MC peptides on cerebral neutrophil recruitment, demonstrating important yet discrete roles for both MC₁ and MC₃.

Approach and Results—Using intravital microscopy, in 2 distinct murine models of cerebral ischemia-reperfusion (I/R) injury, we have investigated MC control for neutrophil recruitment. After global I/R, pharmacological treatments suppressed pathophysiological neutrophil recruitment. MC₁ selective treatment rapidly inhibited neutrophil recruitment while a nonselective MC agonist provided protection even when coadministered with an MC₃ antagonist, suggesting the importance of early MC₁ signaling. However, by 2-hour reperfusion, MC₁-mediated effects were reduced, and MC₃ anti-inflammatory circuits predominated. Mice bearing a nonfunctional MC₁ displayed a transient exacerbation of neutrophil recruitment after global I/R, which diminished by 2 hours. However importantly, enhanced inflammatory responses in both MC₁ mutant and MC₃−/− mice resulted in increased infarct size and poor functional outcome after focal I/R. Furthermore, we used 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 MC control for neutrophil recruitment in the unique pathophysiological context of cerebral I/R, while also demonstrating the potential therapeutic value of targeting multiple MCs in developing effective therapeutics. (Arterioscler Thromb Vasc Biol. 2015;35:1936-1944. DOI: 10.1161/ATVBAHA.115.305348.)

Key Words: inflammation ■ leukocyte ■ melanocortins ■ neutrophil ■ stroke

Inflammation plays a central role in cerebral ischemia-reperfusion (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 after 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 (MC₁–₅) and the endogenous agonists, adrenocorticotrophic hormone, α, β, and γ melanocyte-stimulating hormones (MSHs) make up the MC receptor system.² During the past 15 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 many 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 a source of MCs suggesting that the MC receptor system may provide a self-limiting anti-inflammatory loop, serving to promote inflammatory resolution.³ Such pleiotropic anti-inflammatory
**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BCCAo</td>
<td>bilateral common carotid artery occlusion</td>
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<tr>
<td>e/e</td>
<td>MCe, mutant recessive yellow e/e</td>
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<tr>
<td>I/R</td>
<td>ischemia-reperfusion</td>
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<tr>
<td>MC</td>
<td>melanocortin</td>
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<tr>
<td>MC3−/−</td>
<td>melanocortin receptor 3 null</td>
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<td>MSH</td>
<td>melanocyte-stimulating hormone</td>
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Actions make these receptors a promising therapeutic candidate to address aberrant inflammation in stroke.\(^6\)

Of the 5 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).\(^3\) However, the exact anti-inflammatory role of MC subtypes remains unclear, and may vary with the pathophysiological environment.

In this study, we use 2 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 MCs 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.

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**Results**

**α-MSH Abrogates Neutrophil Recruitment After Cerebral I/R**

To investigate leukocyte recruitment in the cerebral microvasculature, global ischemia was induced, followed by 40-minute reperfusion or 2-hour reperfusion and leukocyte to endothelial cell interactions in pial vessels were assessed by intravital microscopy (Figure 1). Sham surgery, produced little to no leukocyte recruitment, however, I/R caused significant leukocyte rolling (191.0±31.49 cells/mm\(^2\) per minute) and adherence (282.3±49.4 cells/mm\(^2\) per minute), with 2-hour reperfusion resulting in a further significant increase in adhesion to 555.2±85.3 cells/mm\(^2\) per minute (Figure 1C; Movies I and II in the online-only Data Supplement). Correlating with the observed effects on leukocyte recruitment was an enhanced serum soluble E-selectin by 2-hour reperfusion, as detected by ELISA, and a trend toward an increase in the number of intracellular adhesion molecule-1 (ICAM-1)– and vascular cellular adhesion molecule-1 (VCAM-1)–positive vessels detected in the brain. No effect was observed with respect to soluble P-selectin (Figure II in the online-only Data Supplement). α-MSH (10 µg IP), a nonselective MC agonist, given at the start of reperfusion strongly inhibited leukocyte recruitment at 40 minutes, reducing rolling by 80% and adhesion by 68%, to levels comparable with sham animals. Furthermore, these protective effects remained highly significant even after 2 hours of reperfusion (Movie III in the online-only Data Supplement). In line with the reduced leukocyte recruitment at 2 hours, α-MSH was also found to reduce levels of soluble E-selectin and a modest reduction in vessel ICAM-1 and VCAM-1 expression.

Finally, to ascertain the role of neutrophils, some mice were depleted of neutrophils before I/R. After I/R, neutropenic

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**Figure 1.** Bilateral common carotid artery occlusion (BCCAo) induces leukocyte recruitment, which is abrogated by α-melanocyte-stimulating hormone (MSH) treatment. A, Representative intravitral microscopy video stills show interactions of leukocytes (white arrow) on the cerebral vessel wall, with clearly observable increases after BCCAo. Images taken on an Olympus BW61WI microscope, magnification ×40. 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 30 s or longer (Adhesion: cells/mm\(^2\) per minute). Values represent mean±SEM; n=6 mice/group. *Significance to sham "P<0.01, **P<0.001. †Significance to saline-treated BCCAo group P<0.01, †† P<0.001. $Significant increase to 40-minute reperfusion group, P<0.05 is considered significant.

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mice displayed significant reductions of leukocyte rolling (81%) and adhesion (76%), consistent with the majority or all cells observed being neutrophils (Figure I in the online-only Data Supplement).

**Effect of α-MSH on NF-κB–Related Cytokine and mRNA Expression**

The effect of cerebral I/R on serum cytokines was investigated using multicytokine analysis (Figure IIIA–IIIC in the online-only Data Supplement). Expression of interleukin (IL)-12p70, interferon (IFN)-γ, and MCP-1 (monocyte chemoattractant protein) remained below the reliable detection range across all treatments (Data not shown). I/R induced a significant increase in serum tumor necrosis factor (TNF)-α by 2 hours, which was abolished by α-MSH treatment. The anti-inflammatory cytokine IL-10 showed a trend toward an increase at 2-hour reperfusion (to 34.7 pg/mL), but significantly increased (88.7 pg/mL) after α-MSH treatment. Levels of the pleotropic cytokine IL-6 remained unchanged after I/R, however, treatment with α-MSH was found to result in a significant upregulation of IL-6 by 2 hours. Considering IL-6 signaling via STAT3 has been shown to reduce neutrophil recruitment, we also investigated levels of tyrosine 705 phosphorylated STAT3 in leukocyte nuclear fractions by Western blot, finding a slight enhancement of STAT3 in α-MSH–treated animals at 2 hours (Figure IV in the online-only Data Supplement).

To investigate whether this influence over serum cytokines could be because of NF-κB inhibition, mRNA levels of the NF-κB regulatory protein IkB (which closely corresponds to NF-κB activation) were assessed in both blood and brain using quantitative reverse transcription polymerase chain reaction (Figure IVA and IVB in the online-only Data Supplement). IkB levels were not significantly elevated 40 minutes after I/R, however by 2 hours, IkB was significantly increased in the blood, and this was suppressed by α-MSH treatment. Suppression of NF-κB activation in leukocytes at 2-hour reperfusion was further confirmed by Western blot analysis of serine 536 phosphorylated NF-κB p65 in leukocyte nuclear fractions.

**Effect of MC1 and MC3 Agonists on Neutrophil Recruitment and Circulating Cytokines**

The relative contribution of MC subtypes on I/R-induced neutrophil recruitment was investigated using more selective MC agonists. Activation of MC1 by BMS-470539 provided a potent inhibition of bilateral common carotid artery occlusion (BCCAo)–induced neutrophil rolling and adhesion at 40-minute reperfusion (Figure 2A and 2B), however, treatment with [DTRP]-γ-MSH, which has a high affinity for MC3, only inhibited cell adhesion at 40-minute reperfusion.
yet by 2 hours this effect was stronger, with neutrophil rolling also being significantly reduced. Furthermore, [DTRP]-γ-MSH significantly reduced BCCAo-induced TNF-α release by 2 hours, while also elevating serum levels of the pleiotropic cytokine IL-6 and the anti-inflammatory IL-10 (Figure 2C–2E).

**Pharmacological Investigations Using the MC₃/₄ Antagonist SHU9119**

BMS-470539 is almost entirely selective for MC₁, while [DTRP]-γ-MSH may activate other MCs than MC₃. To further examine the roles of specific MC subtypes, the MC₃/₄ antagonist SHU9119 was coadministered with either α-MSH or [DTRP]-γ-MSH (Figure 3C and 3D), revealing that MC₃/₄ antagonism caused no increase in rolling or adhesion at 40-minute reperfusion versus α-MSH or [DTRP]-γ-MSH alone. In fact SHU9119 administered alone, or in conjunction with α-MSH, reduced neutrophil rolling. However, by 2 hours, coadministration of SHU9119 blunted the α-MSH–induced reductions in rolling and adhesion, and prevented the protective effects of [DTRP]-γ-MSH.

**MC Receptor Expression**

To determine whether the delayed importance of MC₃ was because of change in receptor expression antibody-based investigations into receptor expression were undertaken. However, initial analysis of MC₁ and MC₃ protein expression using Western blotting revealed antibody binding in MC₃−/− mice, using both the Sigma Aldrich (M4937) and Acris (AP10124PU-N) MC₃ antibodies, despite polymerase chain reaction confirmation of the MC₃−/− (Figure V in the online-only Data Supplement). This suggests that both antibodies tested display nonspecific binding to protein at a similar molecular weight to MC₃, as has been previously described. Therefore, quantitative reverse transcription polymerase chain reaction was used to quantify MC expression at the mRNA level. MC₃ was detected in both blood and brain, however, BCCAo induced no changes at either 40 minutes or 2 hours after BCCAo (Figure VI in the online-only Data Supplement).

**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) MC₁ mutant mice displayed enhanced (nearly 3×) neutrophil rolling at 40 minutes after I/R versus wild-type (WT; Figure 4A) accompanied by elevated serum TNF-α (Figure 4C). In the absence of a functional MC₁, 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. MC₃ null mice showed no significant differences in neutrophil recruitment, or in their ability to respond to α-MSH at 40 minutes. This is consistent with the predominant anti-inflammatory role of MC₁ at early reperfusion. In agreement with the diminished role of MC₁ observed in pharmacological studies at 2 hours, 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).

**Figure 3.** Effects of MC₃/₄ antagonism on the actions of α-melanocyte-stimulating hormone (MSH) and [D-TRP]-γ-MSH. Leukocyte recruitment in the cerebral microcirculation was quantified in terms of: rolling cell flux and adhesion (cells/mm² per minute), after BCCAo and 40-minute reperfusion or 2-hour reperfusion. Affect of the MC₃/₄ antagonist SHU9119 when administered 10 µg intraperitonially 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]-γ-MSH alone (10 µg) or in combination with SHU9119 (10 µg). α-MSH 40 minutes cotreatment group n=6 mice/group all other cotreatment 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. †Statistical significance to saline-treated BCCAo group P<0.01. ††P<0.001.
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 (ie, the middle cerebral artery occlusion model). We chose this model because of the fact that focal ischemia accounts for ≈80% of ischemic stroke.

Figure 6 demonstrates that at 24 hours post ischemia while e/e mice showed elevated adhesion, MC3−/− 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 MC Effects on Neutrophil Functioning
The MC receptor system displays many disparities between humans and rodents and many MC agonists and antagonists have different selectivity in MCs from different species.9,11 To assess the effectiveness of MC treatments on human cells, we used the neutrophil flow chamber model and chemotaxis assay to investigate neutrophil inflammatory function (Figure VII in the online-only Data Supplement). In the flow chamber, treating neutrophils with just 10 µg/mL of α-MSH (a comparable dose to in vivo studies) resulted in significant reductions in capture (54% reduction), adherence (68%), and transmigration (67%) versus saline (Figure VIIA in the online-only Data Supplement).
Treating human umbilical vein endothelial cells (HUVECs) with α-MSH (≤100 µg/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, pretreatment with 10 µg/mL α-MSH or [DTRP8]-γ-MSH failed to inhibit neutrophil migration toward fMLP, however, BMS-470539 significantly reduced this response, suppressing the number of migrated cells by ≈75% (Figure VIIB in the online-only Data Supplement), suggesting MC1 to play a specific role in inhibiting neutrophil chemotaxis.

Discussion

This study provides, for the first time, evidence that MC treatments can effectively inhibit neutrophil recruitment in the unique microenvironment of the cerebral microvasculature. Using both selective ligands and MC mutant mice, we have gained an insight into MCs involved in modulating neutrophil recruitment in 2 separate models of cerebral I/R, finding both MC1 and MC3 to display important inhibitory roles. In addition, MC treatment was effective in modulating human neutrophil inflammatory functioning, and may represent a novel treatment to stem poststroke inflammation.

Both ischemic models used (BCCAo and middle cerebral artery occlusion) here resulted in a pronounced increase in neutrophil rolling and adhesion compared with sham, as has previously been observed.12,13 Nonselective 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 MC influence for leukocyte adhesion. Although anti–ICAM-1 antibody Enlimomab failed clinical trials for stroke (possibly because of inflammatory side effects to the mouse monoclonal antibody) strategies inhibiting CAMs have shown great promise preclinically. Soluble P- and E-selectin are early markers of endothelial activation. Although levels of soluble P-selectin were found only to produce a trend toward an increase after BCCAo, which was unchanged by treatment, soluble E-selectin was increased by 2 hours post ischemia. Elevated levels of soluble E-selectin have been demonstrated in human stroke and during sepsis.14 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.15,16 α-MSH treatment significantly reduced BCCAo-induced soluble E-selectin, probably 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 preclinical and clinical evidence demonstrating blockade of IL-1β signaling to be beneficial in stroke.17 MSH treatment also simultaneously enhanced anti-inflammatory IL-10 by 2-hour reperfusion. The ability of MCs to suppress proinflammatory responses while enhancing anti-inflammatory signals suggests that these receptors form an endogenous proresolving system. In humans, α-MSH concentrations increases in myocardial infarction and infection18 and higher MC levels correlate with better outcome in patients with stroke.19 Thus, given the results from this study the MC receptor system may prove a valuable therapeutic target for the treatment of stroke.

Although others have demonstrated a contribution of either MC1 or MC3 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 MC1-mediated effects predominate in early protection, while MC3 actions are more delayed and induce proresolving 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 MC1 agonist BMS-470539, significantly inhibited early neutrophil rolling and adhesion, while activation of MC3 using [D-TRP8]-γ-MSH only reduced adhesion. However, by 2 hours after BCCAo, despite a robust pharmacodynamic...
half-life of $\approx 8$ hours,19 BMS-470539 lost its inhibitory actions on rolling and the reduction of adhesion was diminished slightly. However at 2 hours, [D-TRP$^\gamma$]-γ-MSH anti-inflammatory actions were enhanced. Thus suggesting MC$_3$-mediated effects become more prominent at later time points. SHU9119 (MC$_{3\alpha}$ antagonist) was used to further explore the role of MC subtypes. By 40-minute reperfusion, SHU9119 enhanced, rather than inhibited, α-MSH and [D-TRP$^\gamma$]-γ-MSH effects. Furthermore, administered alone, SHU9119 reduced neutrophil adhesion at 40 minutes, possibly because of its solid report agonist actions at MC$_{3\alpha}$, further supporting the predominance of MC$_3$-mediated effects at early stages of reperfusion. By 2-hour reperfusion, SHU9119 lost these anti-inflammatory effects, and cotreatments with SHU9119 inhibited the protective effects of α-MSH and [D-TRP$^\gamma$]-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 after 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 before such upregulation occurs. The shift in receptor importance may instead reflect activation of distinct mechanisms of action 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 30 minutes after TNF-α stimulation, followed by gene transcription 30 minutes later,4 NF-κB inhibition is unlikely to mediate the rapid effects on neutrophil recruitment at 40 minutes. Indeed, IκB transcript levels were not significantly increased at 40 minutes, yet by 2-hours blood IκB elevated 25-fold and nuclear levels of phosphorylated 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.

MC treatments inhibited neutrophil recruitment at 40 minutes 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,20 and α-MSH can act via MC$_3$ to shed cell surface CD14 on macrophages and IL-8 receptors on neutrophils independently of mRNA changes. Although the understanding of NF-κB--independent MC effects is still in its infancy, these mechanisms may fit well with the early efficacy of the absence of MC$_3$ treatments in suppressing neutrophil recruitment.

Differences in cytokine regulation between MC$_3$-targeted activation and MC$_3$-targeted nonspecific activation further support the hypothesis that MC$_3$ and MC$_3$ act via distinct mechanisms. Both α-MSH and [D-TRP$^\gamma$]-γ-MSH significantly reduced BCCAo-induced TNF-α while 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 humans23 while the anti-inflammatory cytokine IL-10 has been shown to be protective in several models of cerebral I/R.24,25 BMS-470539 treatments, however, had no effect on these NF-κB--controlled cytokines further supporting a distinct mechanism of action for the MC$_3$-mediated early inhibition of neutrophil recruitment, while MC$_3$ may be more prominent in initiating proresolving effects. Conflicting reports have been made as the effect of MC treatments on IL-6 levels, however, in the current investigations both α-MSH and [D-TRP$^\gamma$]-γ-MSH increased this pleiotropic cytokine at 2 hours, despite a decrease in NF-κB levels. As such the observed IL-6 release may be a result of elevated release of presynthesized IL-6. α-MSH has previously been shown to influence IL-6 levels via MC$_3$,26 thus, explaining why IL-6 induction was not observed in BMS-470539–treated animals. Although IL-6 is an endogenous pyrogen with chemotactic activity, IL-6 knockout mice show no protection after experimental stroke.27 Indeed IL-6 signaling with STAT3, has been shown to limit the inflammatory recruitment of neutrophils,28 that given that IL-10 may also activate STAT3 and levels were increased after α-MSH treatment such findings seem to be consistent with the present results.

Furthermore, it has been demonstrated that NF-κB signaling may induce expression of IL-6 and IL-10 in cells with a high level of STAT3 phosphorylation.29 In line with such observations, we found nuclear extracts from α-MSH-treated BCCAo animals to show a trend toward an elevation of phosphorylated STAT3 protein.

Our investigations using MC mutant mice, in 2 independent models of stroke, further support a temporal difference in MC$_3$ and MC$_3$ actions. Recessive yellow e/e mice displayed a transiently enhanced neutrophil rolling and elevated TNF-α at the 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 upregulation of other MCs may mask the anti-inflammatory role of this receptor in MC$_3^{−/−}$. Montero-Melendez et al20 have previously shown that while in macrophages isolated from WT and e/e zymosan challenge induced no change in MC$_3$, MC$_3$, or MC$_4$ expression, in MC$_3^{−/−}$ inflammatory challenge led to marked gene activation for MC$_3$, and MC$_3$, perhaps indicating compensatory regulation of other MC in the absence of MC$_3$. However, in the focal stroke model, at 24-hours 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 with WT, perhaps as a result enhanced rolling before 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$_3^{−/−}$. As such, the observed enhanced leukocyte recruitment is unlikely to be because of an initially high leukocyte count in these animals. As observed in BCCAo animals both receptors seem to be of importance with MC$_3$ anti-inflammatory circuits predominating at later time points.
Previously, Leoni et al\textsuperscript{1} demonstrated that the MC\textsubscript{1},−/− mouse displays higher levels of leukocyte adhesion and emigration in the mesenteric microcirculation after I/R, while the response of the e/e mouse was not significantly different from WT. Yet in the same model, the MC\textsubscript{3} agonist BMS-470539 was effective in reducing leukocyte recruitment (an effect which was absent in e/e mice).\textsuperscript{3} 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 2 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 with WT, further illustrating that both receptors are important pharmacological targets.

Our studies have further demonstrated that melanocortin peptides may affect neutrophil inflammatory functioning in human cells. As the MC receptor system displays several disparities in organization and function between humans and rodents. Such as differences in potency and selectivity of MC 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\textsubscript{3} but not MC\textsubscript{1}, is expressed on neutrophils supports the concept of an MC\textsubscript{3}-specific effect. These effects were rapid, occurring within 30 minutes after treatments, again a timeframe incompatible with transcriptional mechanisms. MC\textsubscript{3}-specific direct effects on neutrophils were further illustrated by the ability of MC\textsubscript{3}-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\textsubscript{3} on neutrophil recruitment.

Taken together, novel experimental data presented here highlight a role for use of MC-based treatments as potential therapeutics for stroke, and potentially other neurovascular diseases. This work also demonstrates important roles for both MC\textsubscript{1} and MC\textsubscript{3}, showing MC\textsubscript{1} effects to provide rapid inhibition of leukocyte recruitment via mechanisms independent of NF-κB regulation, while MC\textsubscript{3} actions seem 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 1 MC alone. Indeed, it may be telling that currently the most promising MC compounds (NDP-α-MSH and AP214) are both nonselective MC agonists. Further investigations into later time points of treatment and in animals with comorbidities will help to reveal the full potential of this promising therapeutic target for stroke.

Acknowledgments

P.M. Holloway performed, designed, and analyzed experiments and wrote the article. P.F. Durrenberger performed experiments, D. Cooper performed some flow chamber experiments, M. Perretti provided scientific input and provided the e/e and MC\textsuperscript{3}−/− animals, S.J. Getting and F.N.E. Gavins designed and analyzed the experiments and wrote the article. We also thank Monika Dowweko (University of Westminster, United Kingdom) for genotyping the MC\textsuperscript{−/−} mouse, Dr Lucy Norling (William Harvey Research Institute, United Kingdom) for her help with the flow chamber model and Jonette Green (LSUHSC-S) for help with the immunofluorescent staining. Marjan Trutschl and Urska Cvek provided input on the statistical analysis and techniques.

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Disclosures

None.

References

6. Holloway PM, Smith HK, Renshaw D, Flower RJ, Getting SJ, Gavins FN. Targeting the melanocortin receptor system for anti-stroke therapy. 
9. Joseph CG, Yao H, Scott JW, Sorenson NB, Marnane RN, Mountjoy KG, Haskell-Luevano C. γ₂ Melanocyte stimulation hormone (γ₂-MSH) truncation studies results in the cautionary note that γ₂-MSH is not selective for the mouse MCSR over the mouse MCSR. 
11. Hruby VJ, Lu D, Sharma SD, Castrucci AL, Kesterson RA, al-Obeidi FA, Hadley ME, Cone RD. Cyclic lactam alpha-melanotropin analogues of Ac-Nle4-cyclo(Aps5, D-Phe7,Lys10) alpha-melanocyte-stimulating hormone-(4-10)-NH2 with bulky aromatic amino acids at position 7 show high agonist potency and selectivity at specific melanocortin receptors. 


**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.
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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$^9$]-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 10 µg in 100 µl 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 °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 cryoprotected 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°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 ∆∆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. Horseradish 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 (2.5x10⁶ cells/ml) in µ-Slide VI0.4 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 (1x10⁷ 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/cm² 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 (4x10⁶ cells/ml) were incubated for 10min at 37.5°C, 5% CO₂ either with RPMI or α-MSH, BMS-470539 or [DTRP³]-γ-MSH (10µg/ml) in RPMI. fMLP 10⁻⁶M 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% CO₂). 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-Lilliefors 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.

References
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 mm² (termed rolling cell flux) and (C) those cells stationary for 30sec or longer (termed adhesion: cells/mm²/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 40 min and 2 h 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 2 h, 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 2 h. n=3 for all groups. * denotes significance to sham, † shows significance to 2 h 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 phosphorylated 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 IκB 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. **MC₃ antibodies show binding in tissue from mice lacking the MC₃ transcript.** (A) PCR analysis demonstrates the absence of MC₃ RNA transcript in samples from MC₃⁻/⁻ mice. (B) Western blot analysis with two separate MC₃ antibodies produce bands at the correct height in brain homogenates from both wild type and MC₃⁻/⁻ 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 MC1, MC3, MC4, and MC5. 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 (1x10⁶ 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 (4x10⁶ cells/ml) were pre-treated 10min with 10µg/ml α-MSH, BMS-470539 or [D-TRP⁸]-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.