## 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 $P<0.05$.

**References**

1. Chen AS, Marsh DJ, Trumbauer ME, *et al.* Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat. Genet.* 2000;26:97-102
2. Getting SJ, Christian HC, Lam CW, Gavins FN, Flower RJ, Schiöth HB, Perretti M. Redundancy of a Functional Melanocortin 1 Receptor in the Anti-inflammatory Actions of Melanocortin Peptides: Studies in the Recessive Yellow (e/e) Mouse Suggest an Important Role for Melanocortin 3 Receptor. *J. Immunol.* 2003;170:3323-3330.
3. Huang Q & Tatro JB. α-melanocyte stimulating hormone suppresses intracerebral tumor necrosis factor-α and interleukin-1β gene expression following transient cerebral ischemia in mice. *Neurosci. Lett.* 2002;334:186-190.
4. Kannengiesser K, Maaser C, Heidemann J, Luegering A, Ross M, Brzoska T, Bohm M, Luger TA, Domschke W, Kucharzik T. Melanocortin-derived tripeptide KPV has anti-inflammatory potential in murine models of inflammatory bowel disease. *Inflamm. Bowel Dis.* 2008;14:324-331.
5. Getting SJ, Lam CW, Leoni G, Gavins FN, Grieco P, Perretti M. [d-Trp8]-γ-Melanocyte-Stimulating Hormone Exhibits Anti-Inflammatory Efficacy in Mice Bearing a Nonfunctional MC1R (Recessive Yellow e/e Mouse). *Mol. Pharmacol.* 2006;70:1850-1855.
6. Getting SJ, Lam CW, Chen AS, Grieco P, & Perretti M Melanocortin 3 receptors control crystal-induced inflammation. *FASEB J.* 2006;20:2234-2241.
7. Fukuoka T, Hayashi T, Hirayama M, Maruyama H, & Tanahashi N. Cilostazol Inhibits Platelet–Endothelial Cell Interaction in Murine Microvessels after Transient Bilateral Common Carotid Artery Occlusion. *J. Stroke Cerebrovasc. Dis.* 2014;23:1056-1061.
8. Gavins FNE, Dalli J, Flower RJ, Granger DN, & Perretti M. Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation. *FASEB J.* 2007;21:1751-1758.
9. Norling LV, Sampaio ALF, Cooper D, & Perretti M. Inhibitory control of endothelial galectin-1 on in vitro and in vivo lymphocyte trafficking. *FASEB J.* 2008;22:682-690.