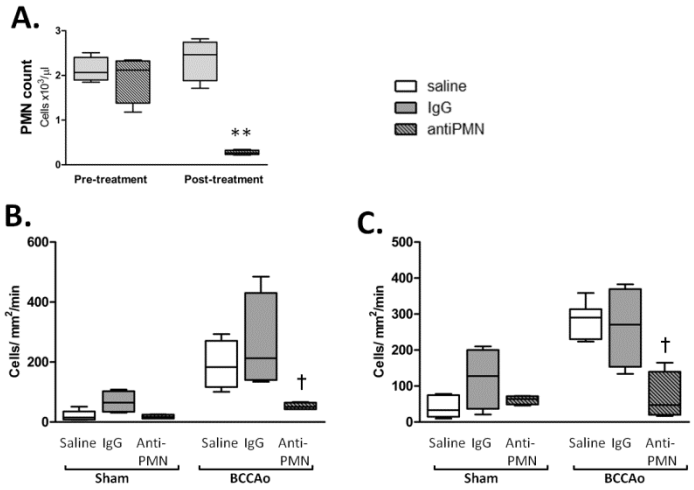
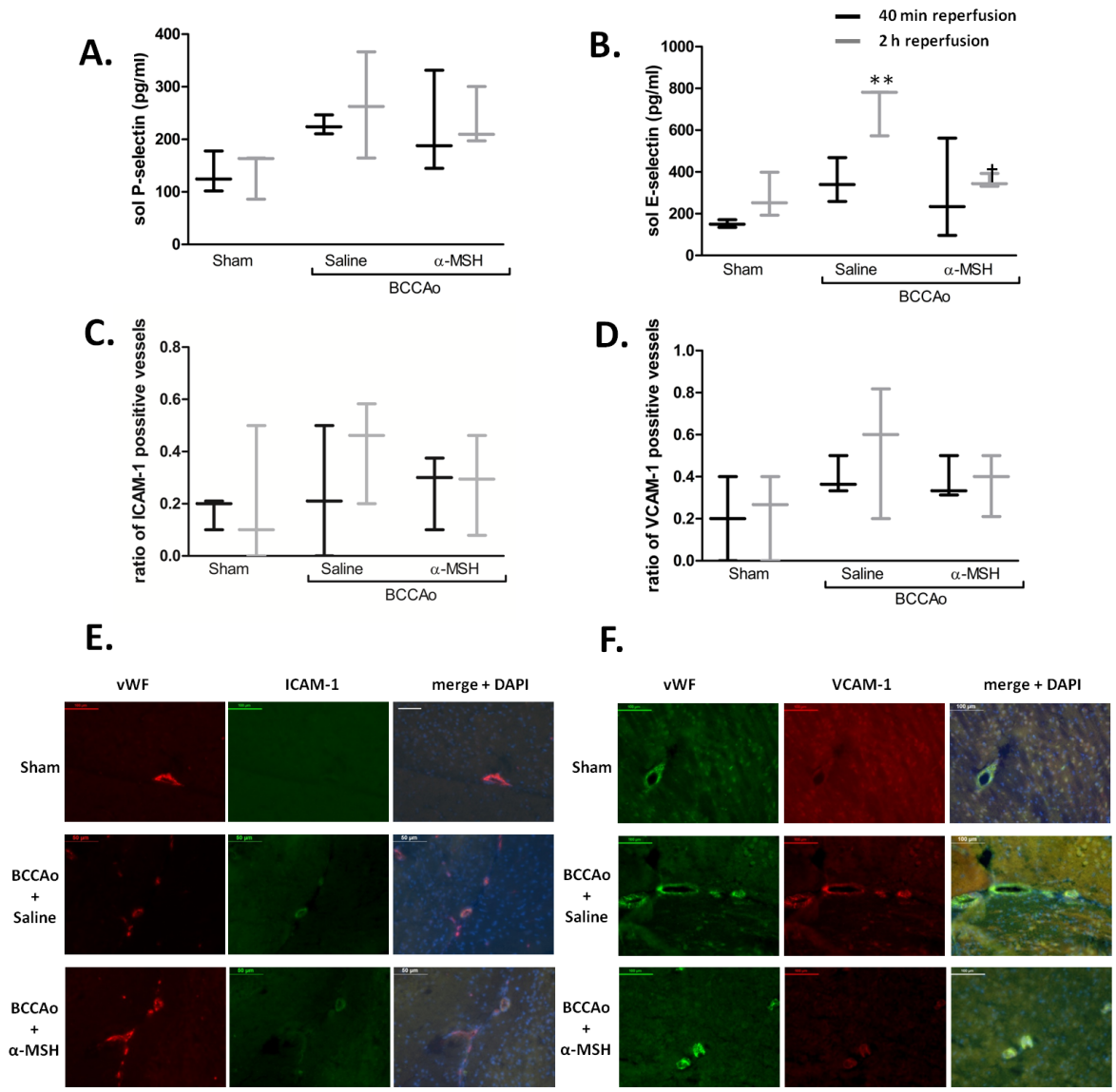
SUPPLEMENTAL MATERIAL

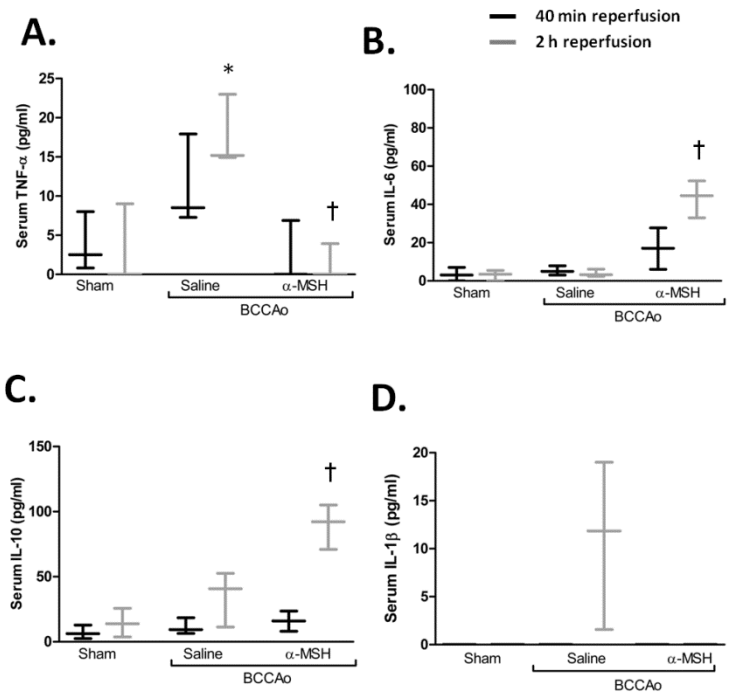
Supplemental Figures and Figure Legends



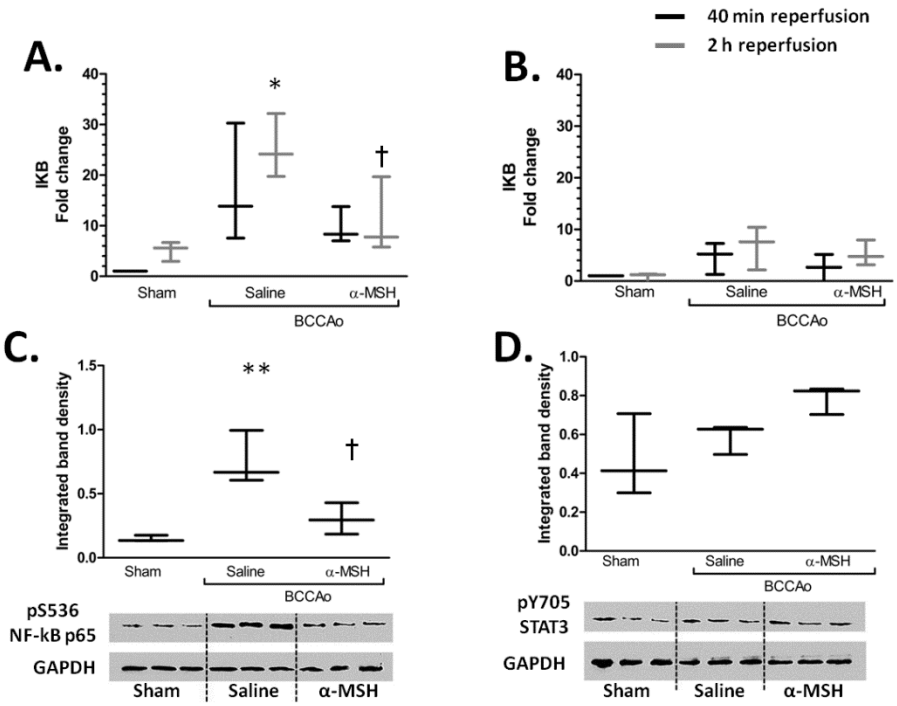
Supplemental Figure I. Effects of PMN depletion on cerebral I/R induced leukocyte recruitment. (A) C57BL/6 mice were treated with either anti-mouse PMN serum or IgG matched serum (10ml/kg, i.p.) once daily for two consecutive days. Tail vein blood samples, for total white cell count and differential 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.



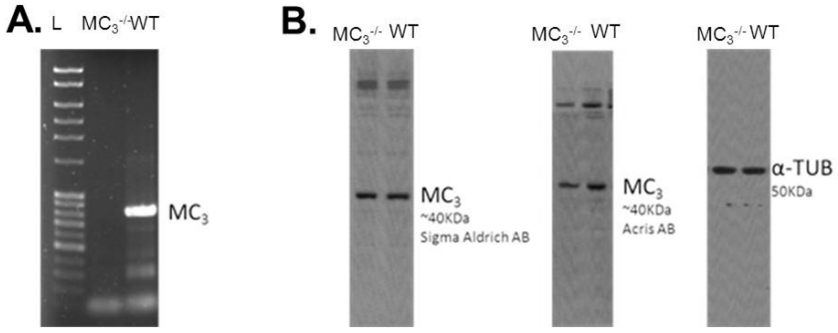
Supplemental Figure II. Modulation of adhesion molecule expression. (A and B) levels of circulating soluble P and E selectin were measured in serum samples showing enhanced P-selectin levels by 2h reperfusion which were suppressed by α-MSH treatment. (C and D) the cerebral microcirculation was stained 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).



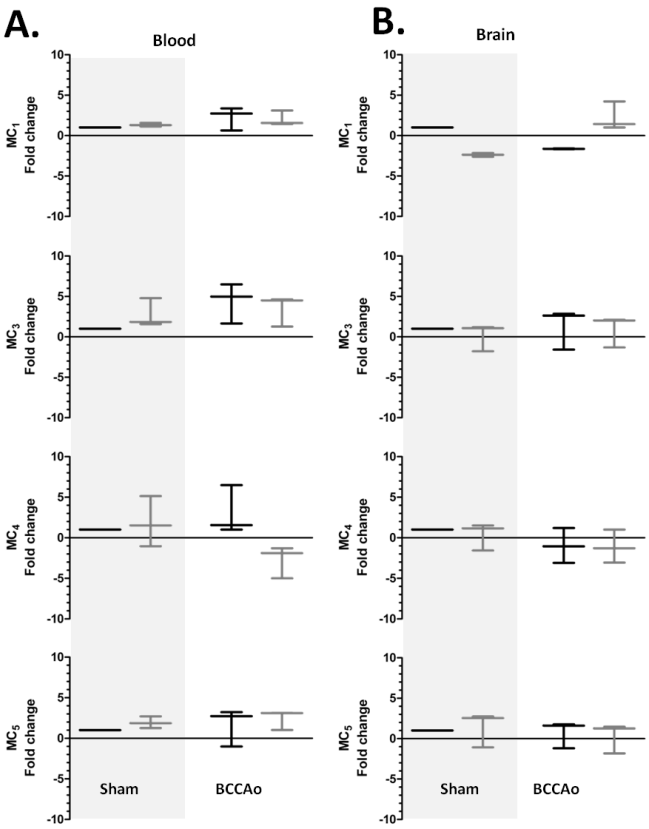
Supplemental Figure III. Cytokine response to BCCAo and α-MSH treatment. (A-C) CBA was used to quantify circulating serum cytokine levels (IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70) and ELISA used to detect IL-1β levels following BCCAo and MSH treatment at both 40min and 2h reperfusion. Expression 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 $P<0.01$.



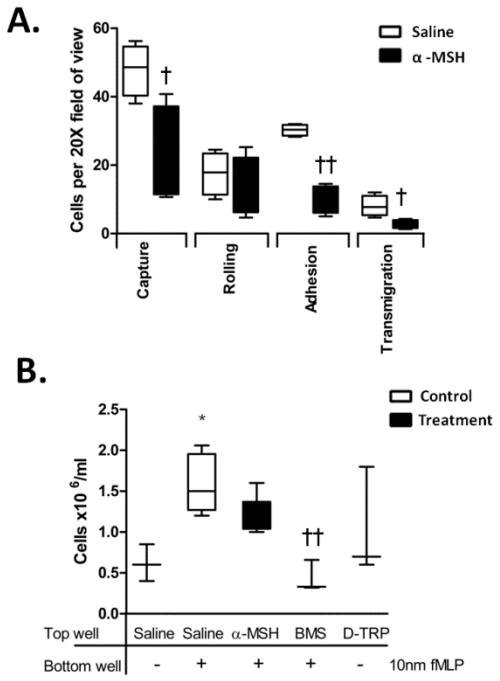
Supplemental Figure IV. NF-κB and STAT-3 activation. NF-κB activation was assessed by quantifying mRNA levels of IκB using qRT-PCR in (A) blood and (B) brain. To further investigate the NF-κB activation in blood, leukocyte protein levels of the serine 536 phospholyated form of NF-κB in nuclear fractions 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