SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

Adult male C57/BL6 mice (23-30g; Charles River, Wilmington, Mass) were maintained on a 14:10 light/dark cycle in a temperature and humidity-controlled vivarium. All mice were allowed *ad libitum* access to food and water. Experimental mice were housed either individually (socially isolated) or with an ovariectomized female (pair housed) for a period of 1 week prior to surgery and throughout the reperfusion period. All mice were randomly assigned to housing, surgery and drug conditions. A sample size of six-eight animals per group was determined sufficient for achieving statistical significance based on power calculations. The study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals and under protocols approved by the institutional animal care and use committee.

Surgery

Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). The mice were anesthetized with 1.5% halothane in oxygen-enriched air provided though a face mask. Body temperature was maintained at 37 ± 0.5°C through the use of a homeothermic blanket system. Briefly, unilateral right MCAO was achieved by insertion of a 6-0 nylon monofilament into the internal carotid artery to a point 6mm beyond the internal carotid-pterygopalatine artery bifurcation. Once secured, the wound was sutured and the animal was allowed to awaken from anesthesia. After 60 minutes of occlusion, the animal was re-anesthetized and reperfusion was initiated by removal of the filament. For SHAM surgery, the internal carotid artery was exposed, but not disturbed, all other aspects of the surgery remained the same. Sixty minutes following MCAO surgery, a neurological score was assigned to each animal as previously described [1] .

Intracerebroventricular Cannulation and Drug Administration

Mice were implanted with an Alzet minipump (Model 1002, Durect, Cupertino, CA) connected via tubing to an ICV cannula (2.75mm projection, Plastics One, Roanoke, VA) implanted into left lateral ventricle. Briefly, the mice were anesthetized with 1-1.5% isofluorane in oxygen-enriched air and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). An incision was made along midline to locate bregma. The cannula was positioned at +0.02 posterior and -0.95 lateral of bregma, and lowered 2.75 mm. The pumps delivered aCSF, OT (Bachem Biosciences Inc, King of Prussia, PA), or a selective oxytocin antagonist [(OTA); desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT, generously donated by Dr. Maurice Manning, The University of Toledo] at a rate of 0.25µL/hour. Socially housed mice were treated with the vehicle, artificial cerebrospinal fluid, (aCSF), 50ng OTA, 500ng of OTA delivered daily (dose reflects amount delivered across a 24-hour period for the duration of the experiment). Socially isolated mice were treated with aCSF, 2ng OT, 20ng of OT, or a cocktail of 20ng OT with 50ng OTA delivered daily. The doses for both manipulations were chosen based on previous research demonstrating their efficacy[2,3] . Drug infusion was initiated 1 week prior to MCAO or SHAM surgery, and continued until tissue collection after 24 or 72 hours of reperfusion. Correct cannula placement was confirmed through cresyl violet staining.

Histochemistry

Immediately following cervical dislocation and decapitation, fresh brains were removed, sectioned into five 2-mm-thick coronal sections and incubated for 15-minutes with 2,3,5-triphenyltetrazolium (TTC) at 37°C, which stains live mitochondria. Slices were post-fixed with 10% buffered formalin for 3-5 days before image analysis, at which point the slices were photographed and infarct area throughout the cerebrum was analyzed using Inquiry software (Loats Associates, Inc. Westminster, MD). Infarct size was determined as a percentage of the contralateral hemisphere after

correcting for edema, using the following formula: [1-(total ipsilateral hemisphere- infarct)]/total contralateral hemisphere] X100. Mice with an infarct size of 5% or smaller, and a neurological score of 0 or 1 were excluded from further analysis because it suggests that the occlusion was not effective.

The TTC-stained sections were stored in 10% formalin for an additional 15 days. The sections were then embedded in paraffin blocks and further sectioned on a microtome at 5μm and mounted on slides. The product of the TTC stain, red formazan, was dissolved during the embedding process allowing the tissue to be used for additional stains[4]. Importantly, a recent study indicated that protein analysis is not compromised by TTC staining. Serial sections were used for two stains. Slides were deparafinized, rinsed in distilled water, quenched in H_2O_2, and then blocked with goat serum. Slides were then incubated for 24 hours at room temperature with antibodies to GFAP (1:500, Dako, Carpinteria, CA) in phosphate buffered saline containing 0.3% Triton-X and goat serum. Slides were then rinsed and incubated with anti-rabbit secondary antibody (1:500, Vector Labs, Burlingame, CA) for 2 hours prior to visualization with DAB (Vector Labs, Burlingame, CA). Slides were photographed and the glial scar area was outlined using ImageJ (NIH) according to the morphology of the GFAP-positive cells which clearly delineated the infarct from undamaged tissue.

The procedure for microglia analysis was similar, except tissue was blocked with bovine albumin serum and incubated for 4 hours in biotynilated isolectin B4, a lectin from Griffonia (Bandeiraea) simplicifolia (1:75, Vector Labs, Burlingame, CA). Images were digitized and proportional stained areas were assessed using ImageJ (NIH). Briefly, fixed size rectangular boxes were superimposed over the images and the proportion of stained area within the defined region was recorded.

Determination of Serum Corticosterone and Interleukin-6 Concentrations

Trunk blood samples were collected immediately following rapid cervical dislocation and decapitation. The samples were centrifuged at 6,000 rpm for 30 minutes at 4°C; sera were collected, aliquoted, and stored at -80°C until assayed. Corticosterone concentrations were determined in serum collected in experiment 1 (72 hours of reperfusion) by using an I^{125} corticosterone kit (MP Biomedical, Solon, OH). The standard curve was run in triplicate and samples were run in duplicate.

An additional aliquot of serum samples was collected in experiment 2 (24 hours of reperfusion) and diluted 1:5. IL-6 expression was assayed using a sandwich ELISA kit (BD Biosciences, San Jose, CA) according to manufacturer's protocol. The standard curve and samples were run in duplicate. All samples within an experiment were run in a single assay.

Real-time PCR

RT-PCR was conducted in a separate cohort of mice after 24 hours of reperfusion. Bilateral samples were dissected from the cortex and striatum, and total RNA was extracted using a homogenizer (Ultra-Turrax T8, IKA Works, Wilmington, NC) and an RNeasy Mini Kit (Qiagen, Valencia CA) according to manufacturer's protocol. Extracted RNA was suspended in 30μL of RNase-free water and RNA concentration was determined by a spectrophotometer (NanoDrop ND-1000, Wilmington, DE). The following inventoried primers and probes (Applied Biosystems, Foster City, CA) were used: OT, IL-6, CD11b (a pattern recognition complement receptor protein expressed on macrophage-lineage cells) and glial fibrillary acidic protein (GFAP; an intermediate filament protein that is up-regulated in astrocytes following injury). A TaqMan 18S rRNA primer and probe set (labeled with VIC dye: Applied Biosystems, Foster City, CA) were used as a control gene for relative quantification. Amplification was performed on an ABI 7000 Sequencing Detection System by using Taqman Universal PCR master mix. The universal

two-step RT-PCR cycling conditions used were: 50°C for 2min, 95°C for 10min followed by 40 cycles of 95°C for 15sec and 60°C for 1min.

To determine OTR mRNA expression in neurons, anti-NeuN clone A60 (Millipore, Billerica, MA) was labeled using DSB-X Biotin Protein Labeling Kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Neurons from whole brain homogenates were enriched using Dynabeads FlowComp Flexi Kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA). RT-PCR on the isolated neurons was conducted as described above using inventoried primers and probes for OTR (Applied Biosystems, Foster City, CA).

Oxidative stress assays

Following MCAO, whole hemispheres were homogenized in cold 20mM Tris-buffered saline and centrifuged at 8500 x g at 4°C for 10 minutes. Supernatants were collected, aliquoted, and stored at -80°C for determination of antioxidant enzyme activity. GPx activity was measured using a commercial kit (Calbiochem, San Diego, CA) according to manufacturer's protocol. All samples were run in duplicate in a single assay. One unit of GPx is defined as the amount of enzyme that causes the oxidation of 1.0 nmol of NADPH per minute. Data are presented as unit per mg protein, as measured by the Bio-Rad protein assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA).

Oxidative stress was measured as a ratio of reduced (GSH) to oxidized (GSSG) glutathione, using a commercial kit (Oxford Biomedical Research, Oxford, MI). Glutathione peroxide reduces hydrogen peroxide and lipid hydroperoxides to water and oxygen, during which time reduced glutathione becomes oxidized glutathione. Exposure to oxidative stress decreases the GSH/GSSG ratio due to increasing accumulation of GSSG, thus the GSH/GSSG ratio is a common and useful indicator of oxidative stress . Samples were prepared according to assay instructions with slight modifications. Briefly, 200µL

of tissue homogenates were diluted with 200µL of cold assay buffer and 5% metaphosphoric acid, centrifuged and new supernatants collected. The GSSG sample was further added to 15µL of 2-vinylpyridine prior to the assay. All samples were run in duplicate in a single assay.

Cell isolation, microglial isolation and flow cytometry

Brain tissue was obtained at indicated experimental time points immediately following euthanasia. Single-cell suspensions were obtained by passage through cell strainers. At least 10^6 cells/sample were re-suspended in staining wash buffer. Cell surface Fc receptors were blocked by incubation with anti-CD16/32 antibody (eBioscience, San Diego, CA) and then washed. All antibody incubations were performed on ice in the absence of light. The cells were incubated with antibodies to CD11b (eBioscience, San Diego, CA, 1:200), NeuN (a neuronal marker, Millipore, Billerica, MA, 1:100), GFAP (Santa Cruz, Santa Cruz, CA, 1:66) and oxytocin receptor (OTR: rabbit anti-OTR, Abcam, Cambridge, MA, 1:100) for 30minutes. The OTR antibody is specific to oxytocin receptors. The cells were further incubated with AlexaFluor 647 conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA, 1:500) secondary antibody for 30 minutes. The gating strategy is shown in Figure S2.

Microglial cultures were prepared from brain tissue isolated from socially isolated mice immediately following euthanasia. Single cell suspensions were re-suspended in 70% Percoll. A density gradient was set up as follows: 70%, 50% and 0% Percoll. The gradient was centrifuged for 45 minutes at 1200g. The middle interface between 70% and 50% consisting of enriched microglia was removed, washed. Microglia were incubated for 2 hours in the presence or absence of OT and OTA, followed by a 22 hour LPS challenge (1 µg/mL, serotype 0127:B8, Sigma Aldrich, St. Louis, MO). The following conditions were compared; 1) control: no stimulation or treatment, 2) LPS treatment only, 3) LPS + 0.1 µM OT, 4) LPS + 1µM OT and 5) LPS + 1µM OT + 1 µM OTA. Microglia activation was determined by

MHC class II expression (1:500; eBioscience, San Diego, CA). First, cell surface Fc receptors were blocked by incubation with anti-CD16/32 antibody and then microglia activation was determined by MHC ClassII expression. All antibody incubations were performed on ice in the absence of light. Flow cytometry data was acquired using a BD LSRII instrument (Davis Heart and Lung Flow Core Facility at OSU) and analyzed using FlowJo software (TreeStar, OR). For any given marker, all of the analysis gates were identical in size and position for all groups.

Data Analysis

All data were assessed by individuals unaware of experimental condition assignments. Infarct size, histology and circulating corticosterone and IL-6 results were analyzed as a two-way ANOVA (housing X drug). At no point did SHAM operated mice differ significantly across housing condition or drug treatment ($P>0.05$), so SHAM data were collapsed where appropriate. Significant ANOVA results were followed by a Tukey HSD post hoc test. OT RT-PCR data were analyzed using an independent samples t-test. All other RT-PCR data were expressed as a ratio of ipsilateral to contralateral hemisphere gene expression and were analyzed via a nonparametric Mann-Whitney U test. Oxidative stress data were analyzed as a four-level one-way ANOVA (factor was group). OTR RT-PCR and flow cytometry data were analyzed using students t-test, with alpha adjusted to 0.025 for LPS data to account for the possibility of Type I error. All other treatment groups were considered statistically different at $P \leq 0.05$.

Supplemental Results

Circulating corticosterone concentrations measured after 72 hours of reperfusion ($F_{1,78}$ = 46.147, *P* = 0.0001) were elevated in MCAO relative to SHAM groups. A Tukey post-hoc analysis revealed that only one MCAO group, socially housed aCSF-treated mice, did not differ from SHAM mice on circulating corticosterone concentrations (*P* > 0.05). Further, among socially housed groups, treatment with 50ng OTA further increased circulating corticosterone relative to the aCSF group (*P* = 0.013) (Table S1).

Serum concentrations of IL-6 protein, measured after 24 hours of reperfusion, varied significantly among the groups ($F_{3,33}$ = 3.191, *P* = 0.038). A Tukey post-hoc analysis revealed that there were no differences by housing condition among aCSF-treated groups; however, among socially isolated groups, OT treatment reduced circulating IL-6 relative to aCSF treatment (*P* = 0.023). OTA treatment did not influence circulating IL-6 in socially housed mice (*P* > 0.05) (Table S1).

There were no group differences in body mass (*P* > 0.05) or neurological score among MCAO mice (*P* > 0.05) in these experiments. During surgery, body temperature was elevated in mice undergoing MCAO relative to SHAM surgery ($F_{7,119}$ =32.754, *P* = 0.001), however, there were no differences in body temperature among MCAO groups (*P* > 0.05).

Social housing and OT influence glial expression

Microglia and astrocytes play a critical role in the progression of neuronal damage following an ischemic event . A strong induction of CD11b and GFAP mRNA was evident in the ischemic hemisphere after MCAO. CD11b mRNA gene expression differed across groups; both social housing (*U* = 2.0, *P* = 0.017) and OT treatment (*U* = 5.0, *P* = 0.028) reduced post-ischemic striatal CD11b mRNA expression relative to social isolation (Figure S1-A). Additionally, CD11b mRNA expression was increased in socially housed mice treated with OTA (*U* = 1.0, *P* = 0.011). Furthermore, GFAP mRNA gene expression differed across groups. Again both social housing (*U* = 6.0, *P* = 0.05) and OT treatment (*U*= 6.0, *P* = 0.05) reduced

post-ischemic striatal GFAP mRNA expression relative to social isolation, however, there was no effect of OTA treatment on GFAP mRNA expression (Figure S1-A; $P > 0.05$). There were no group differences in cortical GFAP mRNA expression (all $P > 0.05$) (Figure S1-B).

Protein expression of microglia and astrocytes was confirmed histologically in the ischemic hemisphere. The same set of tissue that was assessed for TTC staining was analyzed for microglial labeling using Griffonia simplicifolia I isolectin-B4. As expected, MCAO induced microglial expression of isolectin-B4 in the ischemic hemisphere; however, among socially isolated mice, cortical microglial expression was suppressed by OT treatment ($F_{1,18} = 4.811$, $P = 0.043$). There was no drug effect on microglial expression among socially housed mice ($P > 0.05$) and there were no significant effects of housing or drug treatment in the striatum ($P > 0.05$). MCAO also induced astrocytic expression (that is, GFAP-positive staining) and produced glial scarring. The glial scar area was reduced by OT treatment of socially isolated mice ($F_{1,16} = 5.930$, $P = 0.026$). There was no drug effect on glial scar area among socially housed mice ($P > 0.05$; Figure S1-C-F). Inconsistency between mRNA and protein expression is likely the consequence of a lag in measurement time (24 hours post-MCAO for PCR vs. 72 hours post-MCAO for histology) as well as the use of different markers (i.e. microglia: CD11b for PCR and isolectin B4 for histology) for analysis.

Supplemental References

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Table S1. Circulating corticosterone and interleukin-6 concentrations.

Group	Corticosterone (ng/mL)	Interleukin-6 (pg/mL)
SHAM	62.67 (16.63)	----------
Isolated + aCSF	295.97 (132.26)*	454.70 (336.20)
Isolated + 2ng OT	470.94 (263.23)*	----------
Isolated + 20ng OT	344.60 (248.90)*	179.35 (146.32)†
Social + aCSF	193.89 (238.12)	327.44 (163.93)
Social + 0.05µg OTA	582.83 (177.11)*#	283.70 (146.12)
Social + 0.5µg OTA	395.64 (240.30)*	----------

Shown are circulating corticosterone concentrations, mean (SD)

* = significantly different from SHAM ($P < 0.05$)

† = significantly different from Social + aCSF ($P < 0.05$)

Figure S1. Gene and protein expression of glial markers following MCAO. (A-B) Striatal and cortical mRNA gene expression of CD11b and GFAP is significantly altered by social housing and OT treatment. Data are expressed as a ratio of ischemic to non-ischemic hemisphere. An asterisk (*) indicates a statistically significant difference from indicated groups, and a pound sign (#) indicates a statistically significant difference from socially isolated aCSF-treated mice ($P < 0.05$). (C-F) Representative images of cortical microglial activation in socially isolated (C) aCSF and (D) OT-treated mice, and GFAP-positive glial scars of (E) aCSF and (F) OT-treated mice.

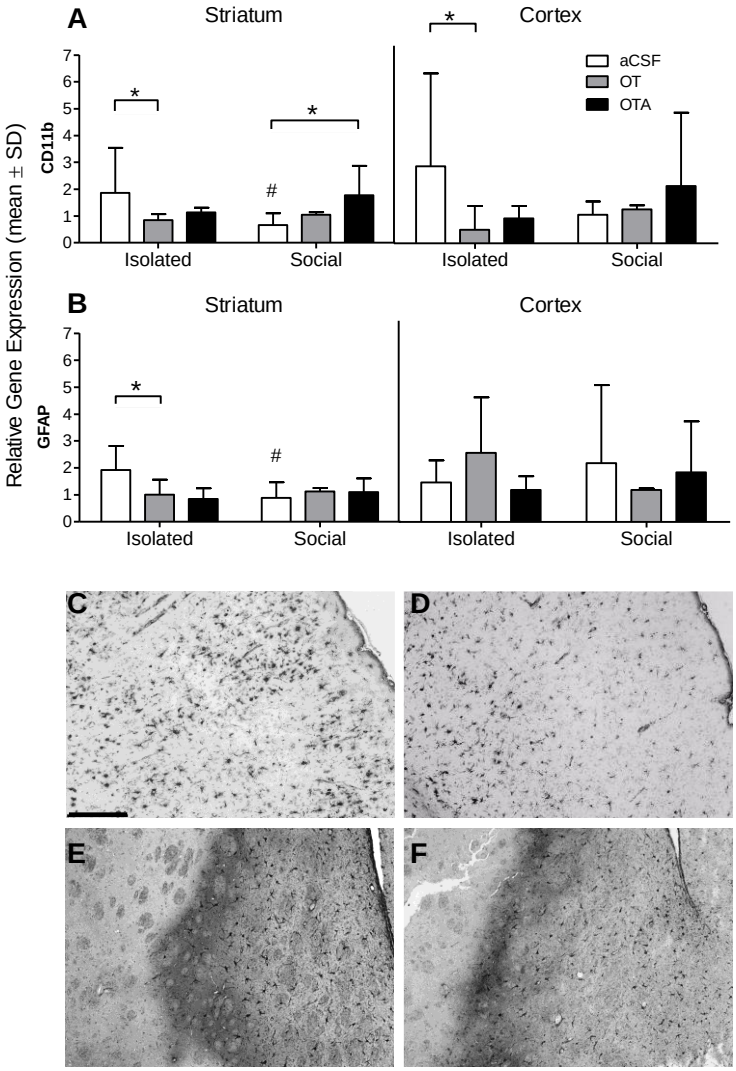

Figure S2. Representative dot blots. OTR expression was measured on total NeuN-positive neurons, CD11b-positive microglia and GFAP-positive astrocytes as gated above. Isotype control values were subtracted from OTR values prior to creating graph.