Oxytocin Mediates Social Neuroprotection After Cerebral Ischemia

Kate Karelin, PhD; Kathleen A. Stuller, PhD; Brant Jarrett, MS; Ning Zhang, MD; Jackie Wells, BS; Greg J. Norman, PhD; A. Courtney DeVries, PhD

Background and Purpose—The reduced incidence, morbidity, and mortality of stroke among humans with strong social support have been well-documented; however, the mechanisms underlying these socially mediated phenomena remain unknown, but may involve oxytocin (OT), a hormone that modulates some aspects of social behavior in humans and other animals.

Methods—In the present study, adult male mice were socially isolated (housed individually) or socially paired (housed with an ovariectomized female); social pairing increased hypothalamic OT gene expression. To determine whether a causal relationship exists between increased OT and improved stroke outcome, mice were treated with exogenous OT or OT receptor antagonist beginning 1 week before induction of experimental stroke via middle cerebral artery occlusion.

Results—Relative to social isolation, social housing attenuated infarct size, neuroinflammation, and oxidative stress following experimental stroke; the neuroprotective effect of social housing was eliminated by receptor antagonist treatment. In contrast, administration of OT to socially isolated mice reproduced the neuroprotection conferred by social housing. We further report evidence for a direct suppressive action of OT on cultured microglia, which is a key instigator in the development of neuroinflammation after cerebral ischemia.

Conclusions—These findings support the hypothesis that OT mediates the neuroprotective effect of social interaction on stroke outcome. (Stroke. 2011;42:3606-3611.)

Key Words: oxytocin ■ cerebral infarct ■ social interaction ■ neuroinflammation ■ oxidative stress

See related article, page 3329.

Social factors have a profound influence on disease outcome.1 The diverse negative health effects of social isolation and lack of social support have been reported for rheumatoid arthritis,2 renal disease,3 and cancer.4 The influence of social interactions on disease outcome is particularly well-described in the context of vascular disease, including cerebrovascular and cardiovascular disorders5; this is an effect that has been replicated in animal models of global6 and focal cerebral ischemia.7,8 Social interaction reduces neuronal damage caused by cerebral ischemia among both male and female mice, regardless of the number of other mice in the cage.6–8 Furthermore, physical contact is an important component of the neuroprotective effect of social interaction.9 One likely mediator of psychosocial influences on disease outcomes is oxytocin (OT), a nonapeptide produced in the paraventricular and supraoptic nuclei of the hypothalamus, which is released during physical contact. OT is both induced by and facilitates social behaviors10; exogenous administration of OT potentiates social behaviors,11 and central blockade of OT signaling disrupts social memory, parental behaviors, and pair-bonding.12 Indeed, exogenous OT protects against several of the physiological and behavioral consequences of social isolation, including autonomic dysfunction and depression,13 stress-induced hypothalamic-pituitary-adrenal axis activation, and anxiety-like behavior.14 In addition to social buffering, recent data indicate that OT also may have anti-inflammatory and antioxidant properties. Exogenous OT administration alleviates tissue damage in a variety of animal models of injury.15–18 Further, coadministration of an oxytocin receptor antagonist (OTA) blocks the cardioprotective effects of OT during cardiac ischemia in rats.16 The protective actions of OT in these models may be associated with decreased levels of circulating proinflammatory cytokines15,19 and decreased neutrophil infiltration to the site of injury.15,17,19

Taken together, the role of endogenous OT as a mediator of social behaviors and the protective role of OT in the pathophysiology of several diseases makes it a compelling candidate as the mediator of social influences on disease outcome. To our knowledge, no studies have directly assessed the role of OT in mediating social influences on any of the major causes of human morbidity and mortality. The present study
was designed to investigate the effects of pharmacological manipulation of OT in mediating the influences of social interaction on stroke outcome. Specifically, we examined the effects of OT treatment and social housing conditions on histological, neuroendocrine, inflammatory, and antioxidant measures following cerebral ischemia.

Methods and Materials

All procedures were approved by the Ohio State University and were conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and under protocols approved by the Institutional Animal Care and Use Committee. The experiments were conducted in accordance with recommendations from the Stroke Therapy Academic Industry Roundtable (STAIR20; specific details provided in supplemental material).

In brief, adult male C57/BL6 mice (23–30g; Charles River) were randomly assigned to an experimental group and housed either individually (socially isolated) or with an ovariectomized female (pair-housed) for a period of 1 week before surgery and throughout the reperfusion period. Alzet minipumps (Model 1002, Durect) were connected via tubing to an intracerebroventricular cannula (2.75 mm projection, Plastics One) implanted into the left lateral ventricle for constant infusion of OT, OTA, or the vehicle (artificial cerebrospinal fluid [aCSF]). Mice underwent middle cerebral artery occlusion (MCAO) or SHAM surgery; brains and sera were harvested 24 or 72 hours after reperfusion. Tissue samples were assessed for damage and glial activation using standard histological and gene expression assays; the individual collecting the data and performing the assays was blinded to group assignment. Oxidative stress and antioxidant enzyme activity were assessed in serum using commercial glutathione peroxidase (Calbiochem) and reduced/oxidized glutathione (Ox- ford Biomedical Research) kits.

Whole brain homogenates were harvested from additional cohorts of paired and isolated mice that did not undergo MCAO. To determine cell type-specific patterns of oxytocin receptor (OTR) expression, isolated cells were incubated with antibodies to CD11b, NeuN, glial fibrillary acidic protein, and OTR. Finally, to confirm an interaction on stroke outcome. Specifically, we examined the manipulation of OT in mediating the influences of social housing conditions. OT treatment of isolated mice did not reduce circulating corticosterone relative to aCSF, indicating that the neuroprotective effects of the high dose of OT are likely independent of circulating glucocorticoids. (Online Only Results and Supplemental Table S1, http://stroke.ahajournals.org).

Results

Social Housing Condition and OT Influence Infarct Size

To examine a modulatory role of OT in this model, mice were socially housed or isolated and hypothalami were extracted 1 week later. RT-PCR confirmed an increase in OT mRNA in socially housed mice (t23=2.230; P<0.05; Figure 1). To determine whether housing-induced increase in OT gene expression contributes to ischemic outcome, socially housed or isolated mice underwent MCAO or SHAM surgery, and were assessed for neuronal damage (infarct size) after 72 hours of reperfusion. Housing condition significantly influenced infarct size (Figure 2). Among vehicle-treated (aCSF) groups, social housing decreased infarct size relative to social isolation (F1,14=7.598; P<0.016). To determine whether the neuroprotective effect of social housing is mediated by endogenous OT, socially housed mice were treated with OTA. Daily central treatment of socially housed mice with either 50 ng or 500 ng of OTA increased infarct size (F1,20=13.914; P<0.001) relative to aCSF. Similarly, daily OT treatment of socially isolated mice dose-dependently reduced infarct size relative to aCSF-treated mice (F4,30=3.417; P<0.020). The high-dose (20 ng/d), but not the low-dose treatment (2 ng/d), reduced infarct size relative to aCSF among socially isolated mice (P<0.045). Moreover, coinfusion of OTA with the effective OT dose eliminated the neuroprotection conferred by OT treatment (P<0.009 relative to aCSF), indicating a receptor-mediated effect of OT treatment. Importantly, treatment of isolated mice with OTA alone did not significantly alter infarct volume relative to aCSF (P<0.996), indicating that OTA is not neurotoxic.

Because stroke is itself a potent stressor, and social isolation exacerbates stress-induced glucocorticoid release,21 circulating corticosterone concentrations were assessed in all drug and housing conditions. OT treatment of isolated mice did not reduce circulating corticosterone relative to aCSF, indicating that the neuroprotective effects of the high dose of OT are likely independent of circulating glucocorticoids. (Online Only Results and Supplemental Table S1, http://stroke.ahajournals.org).

Social Housing and OT Influence Neuroinflammation

Focal cerebral ischemia triggers a marked neuroinflammatory response, particularly in the cortical and striatal regions of the ischemic hemisphere. Central interleukin-6 (IL-6) is neuroprotective in ischemia, and we recently reported a role for IL-6 as a mediator of the neuroprotection conferred by social housing. Among aCSF-treated groups, pair-housing increased striatal IL-6 mRNA (Mann-Whitney U=6.0; P=0.032) relative to social isolation, reaffirming the relationship between central IL-6 and neuroprotection after stroke.22 In keeping with this pattern, IL-6 mRNA expression was reduced in socially housed mice treated with OTA (U=3.0; P=0.05). OT was administered to socially housed mice; however, although OT treatment resulted in increased expression of IL-6 mRNA relative to OTA treatment (U=1.0; P=0.014), we did not observe an additive effect of social housing and OT treatment. Conversely, treatment of socially isolated mice with OT increased IL-6 mRNA expression relative to aCSF (U=7.0; P<0.05; Figure 3). In addition, OTA administration to socially isolated mice did not alter IL-6 mRNA expression relative to aCSF treatment, indicating that endogenous OT signaling is low in isolated mice and not further antagonized with the 50 ng dose of OTA, and central administration of this dose of OTA does not

Results

Social Housing Condition and OT Influence Infarct Size

To examine a modulatory role of OT in this model, mice were socially housed or isolated and hypothalami were extracted 1 week later. RT-PCR confirmed an increase in OT mRNA in socially housed mice (t23=2.230; P=0.036; Figure 1). To determine whether housing-induced increase in OT gene expression contributes to ischemic outcome, socially housed or isolated mice underwent MCAO or SHAM surgery, and were assessed for neuronal damage (infarct size) after 72 hours of reperfusion. Housing condition significantly influenced infarct size (Figure 2). Among vehicle-treated (aCSF) groups, social housing decreased infarct size relative to social isolation (F1,14=7.598; P=0.016). To determine whether the neuroprotective effect of social housing is mediated by endogenous OT, socially housed mice were treated with OTA. Daily central treatment of socially housed mice with either 50 ng or 500 ng of OTA increased infarct size (F1,20=13.914; P=0.001) relative to aCSF. Similarly, daily OT treatment of socially isolated mice dose-dependently reduced infarct size relative to aCSF-treated mice (F4,30=3.417; P<0.020). The high-dose (20 ng/d), but not the low-dose treatment (2 ng/d), reduced infarct size relative to aCSF among socially isolated mice (P=0.045). Moreover, coinfusion of OTA with the effective OT dose eliminated the neuroprotection conferred by OT treatment (P<0.009 relative to aCSF), indicating a receptor-mediated effect of OT treatment. Importantly, treatment of isolated mice with OTA alone did not significantly alter infarct volume relative to aCSF (P<0.996), indicating that OTA is not neurotoxic.

Because stroke is itself a potent stressor, and social isolation exacerbates stress-induced glucocorticoid release, circulating corticosterone concentrations were assessed in all drug and housing conditions. OT treatment of isolated mice did not reduce circulating corticosterone relative to aCSF, indicating that the neuroprotective effects of the high dose of OT are likely independent of circulating glucocorticoids. (Online Only Results and Supplemental Table S1, http://stroke.ahajournals.org).

Social Housing and OT Influence Neuroinflammation

Focal cerebral ischemia triggers a marked neuroinflammatory response, particularly in the cortical and striatal regions of the ischemic hemisphere. Central interleukin-6 (IL-6) is neuroprotective in ischemia, and we recently reported a role for IL-6 as a mediator of the neuroprotection conferred by social housing. Among aCSF-treated groups, pair-housing increased striatal IL-6 mRNA (Mann-Whitney U=6.0; P=0.032) relative to social isolation, reaffirming the relationship between central IL-6 and neuroprotection after stroke. In keeping with this pattern, IL-6 mRNA expression was reduced in socially housed mice treated with OTA (U=3.0; P=0.05). OT was administered to socially housed mice; however, although OT treatment resulted in increased expression of IL-6 mRNA relative to OTA treatment (U=1.0; P=0.014), we did not observe an additive effect of social housing and OT treatment. Conversely, treatment of socially isolated mice with OT increased IL-6 mRNA expression relative to aCSF (U=7.0; P<0.05; Figure 3). In addition, OTA administration to socially isolated mice did not alter IL-6 mRNA expression relative to aCSF treatment, indicating that endogenous OT signaling is low in isolated mice and not further antagonized with the 50 ng dose of OTA, and central administration of this dose of OTA does not
independently influence the neuroinflammatory response to cerebral ischemia. Additional PCR and histological gliotic data are included in supplemental materials and Figure 1.

**Post-MCAO Antioxidant Capacity and Oxidative Stress**

Several studies have established that OT has antioxidant properties; OT scavenges peroxinitrite, inhibits lipid peroxidation, and attenuates nicotinamide adenine dinucleotide phosphate-dependent superoxide activity.23–25 Brain antioxidant levels (glutathione peroxidase) were increased in socially housed (F1,13 = 7.816; P = 0.016) and OT-treated mice (F1,12 = 9.715; P = 0.009) relative to those in aCSF-treated socially isolated mice (Figure 4A). Oxidative stress was attenuated by social housing (F1,11 = 4.660; P = 0.048) as well as by OT treatment (F1,11 = 5.066; P = 0.048) relative to aCSF-treated isolated mice. Among socially housed mice, OTA treatment significantly increased oxidative stress (F1,11 = 4.722; P = 0.045; Figure 4B). Thus, modulation of postischemic oxidative stress is a second mechanism through which OT may be mediating social neuroprotection.

**Evidence for Glial and Neuronal Oxytocin Receptor Expression**

The ability of OT and social interaction to modulate the pathophysiological response to cerebral ischemia likely indicates endogenous changes in OTR expression as a result of social experiences and the presence of OTR on cell populations that mediate neuroinflammation. OTR mRNA and protein expression were assessed on neuronal and glial cell populations of socially housed and isolated mice using RT-PCR and flow cytometry, respectively. As previously reported, both neuronal (NeuN-positive) and astroglial (glial fibrillary acidic protein-positive) cells express OTR.26 Social housing increased neuronal OTR mRNA (t10 = 2.047; P = 0.05) (Figure 5A) and protein (t9 = 2.200; P = 0.05; Figure 5B) expression relative to isolation. In addition, approximately 16% of CD11b-positive cells express OTR, regardless of housing condition (Figure 5B).

**OT Modulates Microglial Reactivity**

To determine whether microglial OTR play a regulatory role during microglial activation, enriched microglia were incubated with or without OT (0.1 or 1 μmol/L), then were stimulated with 1 μg/mL LPS. Activated microglia act as antigen-presenting cells and upregulate MHC class II expression; thus, MHC class II expression was used as a measure of microglial reactivity. As expected, primary microglial cultures stimulated with LPS increased expression of MHC class II relative to the nonstimulated control (Figure 6; t12 = 9.715; P = 0.0001). OT dose-dependently attenuated LPS-stimulated MHC class II expression relative to LPS alone (t12 = 2.578; P = 0.024). Taken together, these in vitro data indicate the potential for a direct role of OT in inhibiting microglial reactivity.

**Discussion**

The influence of social interaction on disease outcome suggests an endogenous signaling pathway that links the

---

**Figure 2.** Social housing condition and oxytocin influence infarct size. Social housing reduces infarct size relative to isolation (aCSF: social n = 8; isolated n = 8); A, However, daily treatment of socially housed mice with OTA (50 ng n = 8 and 500 ng n = 9) eliminates the neuroprotective effect of social housing on infarct size. B, Daily treatment of socially isolated mice with 20 ng (n = 11) OT (but not 2 ng, n = 8) reduces infarct size. OTA infusion alone (n = 6) or with OT (n = 6) does not affect infarct size. Representative triphenyltetrazolium chloride photomicrographs are shown above each group. *Statistically different from the socially isolated aCSF-treated mice (P < 0.05).

**Figure 3.** Relative gene expression of interleukin-6 following MCAO. Striatal IL-6 mRNA is elevated in socially housed (aCSF n = 6) and OT-treated mice (n = 7) relative to social isolation (aCSF n = 7). OT treatment increased IL-6 expression relative to OTA in socially housed mice, whereas OTA treatment did not influence IL-6 expression in isolated mice. *Indicates a statistically significant difference between indicated groups (P < 0.05). Data are expressed as a ratio of ischemic to nonischemic hemisphere. # Significantly different from socially isolated aCSF-treated mice (P < 0.05).
IL-6 mRNA expression in socially housed and OT-treated anti-inflammatory effect on cultured microglia. Following 1 week of social interaction, and OT exerts a direct action, OT and OTR expression are altered in healthy animals socially housing blocks the protection conferred by social interaction or chronic central administration of OT leads to a reduction in ischemic damage, as well as to measures of neuroinflammation and oxidative stress. A role for OT is supported by the findings that: chronic OTR antagonism during social housing blocks the protection conferred by social interaction, OT and OTR expression are altered in healthy animals following 1 week of social interaction, and OT exerts a direct anti-inflammatory effect on cultured microglia.

Data in the current study indicate an increase in central IL-6 mRNA expression in socially housed and OT-treated mice. In addition, serum concentrations of IL-6 in the same cohort of mice were reduced in OT-treated mice (online only Results and Supplemental Table S1). This is consistent with an anti-inflammatory IL-6 profile, as central IL-6 is neuroprotective in ischemia22; however, contrary to its central actions, peripheral IL-6 promotes acute-phase protein induction and is thus indicative of a proinflammatory state.27 Whereas these data may reflect a reduction in neuronal injury in the socially housed or OT-treated mice, we have recently reported that a single treatment with an IL-6 neutralizing antibody before ischemia both increases infarct size and eliminates the social-buffering effect.8 There also is a direct reciprocal relationship between OT and cytokine signaling both in vivo and in vitro.15,17,19,24,28 The OTR gene contains response elements for inflammatory mediators, including nuclear factor-IL-6, as well as nuclear factor-κB, acute-phase response factor, and binding sites for activator protein-1.28,29 Upregulation of proinflammatory cytokines, such as IL-6 or interleukin-1β, modulates both OT and OTR gene transcription.28,30 In addition, OT attenuates LPS-stimulated IL-6 secretion from cultured peripheral macrophages and endothelial cells.24 Additional support for a potent anti-inflammatory component of OT-mediated neuroprotection comes from the discovery that OT attenuates gliosis in vivo and microglial activation in vitro. In the current study, social housing and OT treatment attenuated CD11b and glial fibrillary acidic protein expression in vivo, whereas OTA treatment increased CD11b expression similar to levels observed in socially isolated mice (Supplemental Figure S1). CD11b-positive microglia contribute substantially to ischemic injury, indeed treatment that inhibits microgliosis, reduces infarct size, and attenuates proinflammatory cytokine production.31 The identification of OTR expression on CD11b-positive cells in the brain provides a mechanistic link that may further explain attenuation of neuroinflammation by OT. To determine whether OT could be acting directly on microglia via the OTR receptor, microglia were enriched and incubated in the presence of OT; indeed, OT attenuated LPS-induced MHC class II expression in cultured microglia. These data are consistent with a recent publication demonstrating that OT reduces LPS-stimulated IL-6 and superoxide production in cultured macrophages and endothelial cells.24 Given the role of microglia in the production of proinflammatory cytokines and reactive oxygen species following cerebral ischemia, these data indicate that the antioxidant and anti-inflammatory effects of OT may be driven by OT signaling directly on resident microglia and invading macrophages.

**Figure 4.** Antioxidant enzyme activity and oxidative stress levels. A, GPx activity is increased in OT-treated (n=6) and socially housed (n=6) mice relative to social isolation (n=7). B, Likewise, relative to social isolation, oxidative stress is reduced following OT treatment or social housing, but is significantly elevated in socially housed mice following OTA treatment (n=7). *Statistically significant difference between indicated groups (P<0.05). #Indicates a statistically significant difference from socially isolated aCSF-treated mice (P<0.05).

**Figure 5.** Oxytocin receptor mRNA and gene expression. A, Expression of OTR mRNA in enriched neurons is increased in socially housed (n=12) relative to isolated (n=9) mice. B, Percent OTR expression in socially housed and isolated mice is shown on neuronal (NeuN-positive), astroglial (glial fibrillary acidic protein-positive), and microglial (CD11b-positive) cell populations assessed using flow cytometry, n=5–6 per group. *Indicates a statistically significant social condition difference (P<0.05).
Oxidative stress, marked by production of free radicals, antioxidant depletion, and lipid peroxidation, occurs rapidly after the onset of ischemia and is a detrimental consequence of the return of blood flow to the affected brain area during reperfusion. In the current study, both social housing and exogenous OT treatment increased antioxidant activity (glutathione peroxidase) and attenuated oxidative stress. It remains to be determined whether the reduction in oxidative stress in the current study is a function of reduced neuronal damage in OT-treated and socially housed mice, or whether it is a contributing factor to the developing ischemic injury. However, OT has been shown to act as a free-radical scavenger and to reduce lipid peroxidation. In addition, the antioxidant capacity of OT has also been reported in models of renal and hepatic ischemia/reperfusion injury. Importantly, the reduction of oxidative damage in these studies was accompanied by an attenuation of proinflammatory cytokines. Thus, it is likely that the neuroprotective and anti-inflammatory effects of OT in cerebral ischemia are in part mediated by its antioxidant properties.

Taken together, these data indicate a regulatory role for OT as the mechanism by which social interaction influences ischemia pathophysiology. Of note, OT is part of a tightly regulated neuroendocrine system that is both affected by and can exert influence on cerebral ischemia outcome. For example, OT regulates (and is regulated by) hypothalamic-pituitary-adrenal axis activity. Indeed, OT is released in response to physical and psychological stressors, and evokes a potent anxiolytic effect. Given the highly stressful nature of cerebral ischemia, hypothalamic-pituitary-adrenal axis hormones (ie, corticosterone, adrenal corticotropin hormone, and corticotropin-releasing factor) cannot be discounted as playing a potentially critical role in modulating the effects of OT on measures of ischemia outcome. In addition, hypothalamic release of OT is typically concurrent with release of the structurally related neuropeptide vasopressin. Vasopressin is a potent vasodilator and regulator of brain osmolarity, and thus plays a pivotal role in cerebral ischemia outcome. Importantly, OTRs have a low affinity for vasopressin, indicating this neuropeptide as another potential contributing factor to social neuroprotection during cerebral ischemia.

Overall, the results from this study support the growing body of evidence that social interaction and support improve pathological and functional measures of stroke outcome. On nearly all measures assessed in the current study, the neuroprotection conferred by social housing can be mimicked in...
socially isolated mice via chronic central infusion of OT. These data further extend recent research on the neuroinflammatory and antioxidant properties of OT, and emphasize that OT is uniquely suited to integrate psychosocial stimuli with pathophysiological responses to tissue injury. Taken together, these data support a role for OT as a mediating factor of social modulation of health outcomes.

Acknowledgements

We thank Megan Cochran for technical support, Zach Weil for critiquing this manuscript, and Dr. Maurice Manning for his generous contribution of the selective oxytocin antagonist used in these experiments.

Sources of Funding

This work was supported by grants from the American Heart Association (EJA to A.C.D. and predoctoral fellowship to K.K.), NINDS Core Grant P30 NS045758 (to A.C.D.), NINDS RO1NS40267–05 (to A.C.D.), and NHLBI RO1HL080249–01 (to A.C.D.).

Disclosures

None.

References

Oxytocin Mediates Social Neuroprotection After Cerebral Ischemia
Kate Karelina, Kathleen A. Stuller, Brant Jarrett, Ning Zhang, Jackie Wells, Greg J. Norman and A. Courtney DeVries

Stroke. 2011;42:3606-3611; originally published online September 29, 2011;
doi: 10.1161/STROKEAHA.111.628008
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/42/12/3606

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2011/10/02/STROKEAHA.111.628008.DC1
http://stroke.ahajournals.org/content/suppl/2012/08/21/STROKEAHA.111.628008.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
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-ptyerygopalatine 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$_2$)$_3$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: \[ 1 - \frac{\text{total ipsilateral hemisphere - infarct}}{\text{total contralateral hemisphere}} \] 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 5\( \mu \)m 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 deparaffinized, rinsed in distilled water, quenched in \( \text{H}_2\text{O}_2 \), 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 10⁶ 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 30 minutes. 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


Table S1. Circulating corticosterone and interleukin-6 concentrations.

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

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.
オキシトシンは脳虚血後の社会的神経保護作用を媒介する
Oxytocin Mediates Social Neuroprotection After Cerebral Ischemia

Kate Karelina, PhD1; Kathleen A. Stuller, PhD1; Brant Jarrett, MS1; Ning Zhang, MD1; Jackie Wells, BS3; Greg J. Norman, PhD1,2; A. Courtney DeVries, PhD1

1 Departments of Neuroscience, 2 Psychology, and 3 Institute of Behavioral Medicine Research, The Ohio State University, Columbus, OH.

Abstract

社会的孤立と比較して、社会的飼育により実験的脳虚血中頸動脈の閉塞の大きさ、神経炎症、および酸化ストレスが低減された。社会的飼育による神経保護作用は受容体拮抗薬の投与により認められなくなった。反対に、社会的に孤立させたマウスにOTを投与したとき、社会的飼育で得られた神経保護作用が再現された。ミクロエリアは脳虚血後の神経炎症の主要因子であり、我々は養殖ミクロエリアに対するOTの直接的抑制作用に関するデータについても報告する。

結果：社会的飼育と比較して、社会的飼育により実験的脳虚血中頸動脈の閉塞の大きさ、神経炎症、および酸化ストレスが低減された。社会的飼育による神経保護作用は受容体拮抗薬の投与により認められなくなった。反対に、社会的に孤立させたマウスにOTを投与したとき、社会的飼育で得られた神経保護作用が再現された。ミクロエリアは脳虚血後の神経炎症の主要因子であり、我々は養殖ミクロエリアに対するOTの直接的抑制作用に関するデータについても報告する。

結論：本研究の結果は、脳虚血中頸動脈に関して、OTが社会的交流による神経保護作用を媒介するという仮説を支持する。

图1 つがいにしたマウスと孤立させたマウスにおけるオキシトシンmRNA遺伝子発現量。オキシトシンmRNAは、社会的飼育状態と比べ、1週間つがい飼育後に上昇した（12匹/群）。*社会的飼育マウスとの統計学的な差（p < 0,05）。

图2 社会的飼育状態およびオキシトシン（OT）は梗塞の大きさに影響を及ぼす。社会的飼育では、孤立飼育に比して梗塞の大きさが縮小した（acSF：社会的8匹、孤立8匹）。A、社会的飼育マウスにOTAを連日投与したとき（50 ng：8匹および500 ng：9匹）、社会的飼育による梗塞の大きさへの神経保護作用が認められなくなかった。B、社会的飼育マウスにOT 20 ngを連日投与したとき（11匹）、梗塞の大きさは縮小した（しかし、2 ngでは縮小なし、8匹）。OTAの単独注（6匹）またはOTとの併用投与（6匹）は、梗塞の大きさに影響しない。塩化トリフェニレチルトリゾリウムにて染色した各群の顕微鏡写真の例を示した。*社会的に孤立させacSFを投与したマウスとの統計学的な差（p < 0,05）。OTA：オキシトシン受容体拮抗薬。acSF：人工脳脊髄液。