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Social Interaction Modulates the Neuroinflammatory Response to Global Cerebral Ischemia in Male Mice

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Abstract

Social isolation is a risk factor for cardiovascular and cerebrovascular diseases, although the underlying mechanisms remain underspecified. Considering the potential of microglia to become sensitized by stressors and their role in neuroinflammation, we hypothesized that social isolation primes microglia, resulting in an exaggerated neuroimmune response to experimental cerebral ischemia. First, major histocompatibility complex II (MHC II) gene expression, an indicator of microglial priming, was compared between mice that were socially isolated or pair-housed. MHC II increased in the hippocampus and cortex of socially isolated mice, which is suggestive of isolation-induced microglial priming. In experiment 2, isolated and pair-housed mice underwent ~8 minutes of global cerebral ischemia. Hippocampal mRNA expression of tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6) was significantly increased among both isolated and pair-housed ischemia groups relative to sham controls. Hippocampal expression of interleukin 1 beta (IL-1 β) and cortical TNF- α , IL-1 β and IL-6, were significantly increased 24-hours post ischemia in isolated mice, but not pair-housed mice, relative to controls. Ischemia-induced increases in microglial cell body area and percent area fraction of ionized calcium binding adaptor molecule 1 (Iba-1) positive staining were also observed in isolated, but not pair-housed mice, relative to controls. For experiment 3, brain sections from socially isolated and pair-housed mice underwent 15 minutes of oxygen glucose deprivation (OGD), an *ex vivo* model of cerebral ischemia. IL-6 gene expression was significantly elevated following OGD only in hippocampi from mice that had been socially isolated, indicating that isolation prior to ischemia is sufficient to modulate the neuroinflammatory response. Together, these data suggest microglial priming as a possible mechanism underlying the detrimental effects of social isolation on cerebral ischemia outcome.

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Keywords

Social isolation; Global cerebral ischemia; Neuroinflammation

1. Introduction

Social isolation presents as a risk factor for cardiovascular and cerebrovascular diseases (Mookadam and Arthur, 2004; Brainin and Dachenhausen, 2013; Holt-Lunstad and Smith, 2016), and is associated with increased all-cause mortality (House, et al. 1988; Rebelin and Uchino, 2008). In contrast, social support is associated with a positive impact on health and disease outcome (DeVries, et al. 2007; Karelina and DeVries, 2011). Although loneliness cannot be assessed in non-human animals, the physiological changes triggered by isolation in social species mirror those experienced by humans encountering loneliness (Hawkley and Capitanio, 2014). At times, the health benefits of social interaction are attributed to improved health behaviors, but the ability to recapitulate the effects of social behavior on health outcomes in non-human animals suggests that this explanation is not sufficient (Karelina and DeVries, 2011). Indeed, there is growing evidence that social modulation of the immune system may explain social influences on a wide range of experimental and clinical settings.

Studies from a range of species, including humans, indicate that social environment can modify risk and outcome to cerebral ischemia (Glass, et al. 1993; Reeves, et al. 2008; Karelina and DeVries, 2011; Brainin and Dachenhausen, 2013). Specifically, rodent experiments have demonstrated that the negative effects of social isolation on cerebral ischemia include an increased neuroinflammatory response, greater neuronal death, and impaired functional recovery (Craft, et al. 2005; Weil, et al. 2008; Norman, et al. 2010a; Venna, et al. 2012; Karelina, et al. 2011). These detrimental effects are likely linked to increased ischemia-induced activation of microglia among socially isolated mice relative to socially integrated mice (Weil, et al. 2008; Karelina, et al. 2011). There is ample evidence that a wide range of stressors, including social stress, can prime microglia to respond in an exaggerated and prolonged manner to a subsequent inflammatory stimulus (Wohleb, et al. 2011; Frank, et al. 2012). Thus, microglial sensitization may underlie the exacerbating effects of social isolation of cerebral ischemia. Microglia perform important homeostatic functions in the brain (Davalos, et al. 2005; Kim and deVilles, 2005), but rapidly become activated in response to neuronal injury, including cerebral ischemia (Danon and Dalton, 2003; Iadecola and Anrather, 2011). Activated microglia can secrete pro-inflammatory cytokines and produce reactive oxygen species (Hanish, 2002; Block, et al. 2007; Iadecola and Anrather, 2011). Specifically, following cerebral ischemia, gene expression of tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), and nuclear factor kappa b (Nf κ b) are significantly upregulated, indicating a pro-inflammatory response (Schneider, et al. 1999; Stephenson, et al. 2000; Norman, et al. 2010a; Doll, et al. 2014). Generally, increased post-ischemic neuroinflammation is associated with increased cell death and worse neurological outcome (Wang and Shuaib, 2002; Weil, et al. 2008; Neigh, et al. 2009; Norman, et al. 2010a; Stuller, et al. 2012; Doll, et al. 2014; Wang, et al. 2015).

The present work utilizes both *in vivo* and *ex vivo* models of global cerebral ischemia, to examine social modulation of the neuroinflammatory response to ischemia. Cardiac arrest is an *in vivo* model of global cerebral ischemia, in which heart stoppage eliminates blood flow throughout the body, leading to global ischemia (Kofler, et al. 2004; Neigh, et al. 2004; Noppens, et al. 2005; Hutchens, et al. 2014; Khodanovish and Kisel, 2015). In this model, body temperature is lowered to protect the peripheral tissues while the brain is maintained at typical body temperature, thus limiting the direct effects of ischemia cell death to the brain (Kofler, et al. 2004; Neigh, et al. 2004). The neurological damage that evolves following cardiopulmonary resuscitation (CPR) results from both the direct effects of cerebral ischemia and reperfusion, as well as secondary effects of the associated physiological alterations, such as disruption of the blood brain barrier. On the other hand, oxygen-glucose deprivation (OGD) is an *ex vivo* model of global cerebral ischemia (Whittingham, et al. 1984; Tasca, et al. 2015). Following dissection and sectioning of the brain, control samples are incubated in artificial cerebrospinal fluid (aCSF) while the ischemic samples are incubated in similar conditions minus the glucose and oxygen (Xie, et al. 2008; Tasca, et al. 2015). An advantage of the *ex vivo* models of cerebral ischemia is the availability of tissue from the same animal for both the control and ischemic group, minimizing individual variability and animal usage. Nonetheless, it limits the investigation to that occurring in the isolated brain, eliminating the possible influence of other physiological systems. Thus, any differences in OGD outcome in the current studies are attributed to the pre-ischemic state of the brain tissue.

In the current studies, male mice were housed in social isolation or with an ovariectomized female beginning one week prior to the induction of cerebral ischemia and then through the reperfusion period; similar housing manipulations have been used to recapitulate the effects of social environment on a wide array of health conditions (Weil, et al. 2008; Norman, et al. 2010b; Karelina, et al. 2011). To date, all studies identifying social interaction as neuro-protective against ischemic damage were completed using *in vivo* models (Craft, et al. 2005; Weil, et al. 2008; Norman, et al. 2010a; Venna, et al. 2012; Venna, et al. 2014; Verma, et al. 2014). With the *ex vivo* OGD model one can determine whether the social environment prior to an ischemic event is sufficient to influence the post-ischemic inflammatory response. If so, this will further support the hypothesis that upon social isolation animals undergo physiological changes that can prime their immune responses, and that a poor social environment may convey disease vulnerability.

2. Results

2.1 Experiment 1

Adult male mice were socially isolated or pair-housed with an ovariectomized female for a week. Following tissue collection, the hippocampus and pre-frontal cortex were dissected and assayed to examine gene expression.

2.1.1 Gene expression in the hippocampus and cortex following isolation—

Analysis of the hippocampal and cortical mRNA expression of MHC II and Nfkb1 suggests that seven days of social isolation sensitizes but does not activate the immune system. There

is a statistically significant increase in the hippocampal (Figure 1A; $t_{19}=3.163$, $p<0.05$) and cortical (Figure 1B; $t_{19}=3.960$, $p<0.05$) mRNA expression of MHC II among isolated mice relative to paired mice. In contrast, gene expression of $\text{Nf}\kappa\text{b1}$ in the hippocampus (Figure 1C; $t_{19}=0.2838$, $p>0.05$) and cortex (Figure 1D; $t_{21}=0.2592$, $p>0.05$) does not vary in response to the social environment.

2.2 Experiment 2

Adult male mice were socially isolated or pair-housed for a week, and then received a sham control or cardiac arrest/cardiopulmonary resuscitation (CA/CPR) procedure. Twenty-four hours later, tissue was collected for either gene expression analysis or histological analysis of microglia.

2.2.1 Hippocampal gene expression of pro-inflammatory cytokines after

CA/CPR—Analysis of the hippocampal mRNA expression of pro-inflammatory cytokines $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 , indicates that CA/CPR elicits neuroinflammation, some components of which are modified among pair-housed mice. There is a statistically significant difference among the treatment groups in hippocampal mRNA expression of $\text{TNF-}\alpha$ (Figure 2A; $F(2,33)=15.83$, $p<0.05$). Specifically, the sham group expresses significantly lower $\text{TNF-}\alpha$ mRNA, than both the paired CA/CPR and the isolated CA/CPR groups ($p<0.05$ for both). Hippocampal mRNA expression of $\text{IL-1}\beta$ also differs significantly among experimental groups (Figure 2B; $F(2,33)=6.723$, $p<0.05$). Specifically, the sham group has significantly lower $\text{IL-1}\beta$ mRNA expression within the hippocampus relative to the isolated CA/CPR group ($p<0.05$), whereas $\text{IL-1}\beta$ mRNA expression does not differ between the sham group and the paired CA/CPR group ($p>0.05$). The pattern of hippocampal IL-6 mRNA expression is similar to $\text{TNF-}\alpha$; there is a statistically significant difference among the experimental groups (Figure 2C; $F(2,32)=11.24$, $p<0.05$), with the sham group expressing significantly lower IL-6 mRNA than both the paired CA/CPR ($p<0.05$) and the isolated CA/CPR ($p<0.05$) groups.

2.2.2 Cortical gene expression of pro-inflammatory cytokines after CA/CPR—

In the cortex, increased expression of pro-inflammatory cytokines $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 , indicates that CA/CPR elicits neuroinflammation, a response that is exacerbated in isolated mice relative to those that are pair-housed. There is a statistically significant difference among the treatment groups in the gene expression of $\text{TNF-}\alpha$ (Figure 3A; $F(2,31)=10.98$, $p<0.05$). Specifically, the socially isolated CA/CPR group expresses $\text{TNF-}\alpha$ mRNA at a significantly higher level than both the sham controls and pair-housed CA/CPR group ($p<0.05$ for both). Cortical mRNA expression of $\text{IL-1}\beta$ also differs statistically among the experimental groups (Figure 3B; $F(2,32)=15.55$, $p<0.05$). The sham group exhibits significantly lower mRNA expression of $\text{IL-1}\beta$ than the CA/CPR groups ($p<0.05$ for both), and the paired CA/CPR group has lower $\text{IL-1}\beta$ mRNA expression than the isolated CA/CPR group ($p<0.05$). IL-6 mRNA expression also differs significantly among the treatment groups (Figure 3C; $F(2,30)=12.39$, $p<0.05$). Specifically, the sham group expresses significantly less IL-6 mRNA within the cortex relative to the isolated CA/CPR group ($p<0.05$), but neither group differs from the paired CA/CPR group ($p>0.05$ for both).

2.2.3 Histological analysis of hippocampal microglia following CA/CPR—

Analysis of the microglia cell body area and the Iba-1 proportional area within the hippocampus suggests that CA/CPR elicits microglial activation, occurring at a greater extent in socially isolated mice. When activated, the cell body area of microglia increases. In the current study, there is a significant treatment effect on cell body area (Figure 4A; $F(2,44)=3.672$, $p<0.05$). Specifically, isolated mice that receive the CA/CPR procedure exhibit increased microglia cell body area relative to sham controls ($p<0.05$), however, the paired CA/CPR group is not significantly different than either the sham controls or the isolated CA/CPR group ($p>0.05$ for both). Likewise, Iba-1 staining proportional area in the whole hippocampus differs among groups (Figure 4B; $F(2,43)=7.262$, $p<0.05$). For this measure, the isolated CA/CPR mice display increased Iba-1 percent area fraction relative to sham controls ($p<0.05$) while the paired CA/CPR group does not differ significantly from either the sham or isolated CA/CPR groups ($p>0.05$ for both).

2.3 Experiment 3

Young adult male mice were socially isolated or pair-housed for a week. At the time of tissue collection, brain slices were sectioned and exposed to either aCSF or OGD conditions for 15 min. Upon completion of the *ex vivo* reperfusion, hippocampal tissue was dissected and assayed for gene expression.

2.3.1 Hippocampal expression of pro-inflammatory cytokines after OGD—

Ischemia induced by OGD elicits an inflammatory response, characterized by an increase in the expression of pro-inflammatory cytokine genes, a response that is exaggerated in the tissue from mice that were previously isolated. In the current study there is a time by group interaction in the gene expression of pro-inflammatory cytokine TNF- α (Figure 5A; $F(6,72)=4.587$, $p<0.05$) and a main effect driven by the time variable ($F(3,72)=14.23$, $p<0.05$). Following 6 hours of reperfusion, both the socially paired and isolated OGD groups expressed significantly higher TNF- α gene expression than the aCSF controls ($p<0.05$ for both). When examining hippocampal mRNA expression of IL-1 β there were no main effects of time or experimental group (Figure 5B; $F(3,63)=1.980$, $p>0.05$ and $F(2,21)=0.5671$, $p>0.05$ respectively), and no interaction between these variables ($F(6,63)=0.9234$, $p>0.05$). The mRNA gene expression of IL-6 displayed a time main effect (Figure 5C; $F(3,81)=6.444$, $p<0.05$). Specifically, IL-6 expression is elevated in the isolated OGD group relative to the aCSF controls at 6 and 8 hours of reperfusion, while the pair-housed group remained comparable to both the isolated OGD and aCSF groups at all evaluated time points.

Discussion

3.1 Indication of isolation-induced microglial priming

Environmental stressors can sensitize microglia (Wohleb, et al. 2011; Frank, et al. 2012), thus this experiment examined whether social environment alters inflammatory markers in the brain. The Nf κ b signaling pathway activates pro-inflammatory cytokine production and release (Lawrence, 2009), thereby serving as an indicator of an inflammatory environment. As expected, hippocampal and cortical gene expression of Nf κ b1 displayed no variation in response to the mice housing conditions; however, gene expression of MHC II, a marker of

microglial sensitization (Norden and Godbout, 2013), exhibited an isolation-induced increase among males in the hippocampus and cortex. Together, these data suggest that male mice are susceptible to isolation-induced microglial priming.

3.2 Social isolation exacerbates the neuroinflammatory response to cerebral ischemia

Neuroinflammation is the first-line response to cerebral ischemia (Stoll, et al. 1998; Iadecola and Anarther, 2011). The innate immune response following an ischemic event is characterized by activation of glial cells and the release of pro-inflammatory mediators, both of which contribute to secondary injury (Yenari, et al. 2010; Iadecola and Anarther, 2011; Graeber, et al. 2011; Xiang, et al. 2016). In the present study, hippocampal gene expression of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6, were all increased following *in vivo* global ischemia (Figure 2). Neither TNF- α nor IL-6 exhibited a housing effect, however, IL-1 β expression was significantly upregulated in the isolated but not the pair-housed animals that received the cardiac arrest procedure relative to sham controls. Amelioration of the ischemia-induced inflammatory response by social interaction at 24 hours post-ischemia has been previously reported (Weil, et al. 2009; Norman, et al. 2010). In C3H/e mice, hippocampal gene expression of TNF- α was increased following cardiac arrest only among mice that were socially isolated (Weil, et al. 2009). Similarly, in c57/bl6 mice, gene expression of TNF- α and IL-1 β was elevated following cerebral ischemia, with the ischemia-induced increase of pro-inflammatory cytokines being significantly greater among isolated animals relative to their pair-housed counterparts (Norman, et al. 2010). Despite experimental differences between the studies, such as mouse strain and the length of the isolation period, the influence of the social environment upon the inflammatory response to cerebral ischemia was consistently replicated.

Global cerebral ischemia research primarily focuses on the physiological consequences within the hippocampus, the brain region most vulnerable to ischemic injury (Smith, et al. 1984; Ginsberg, et al. 1989; Schmidt-Kastner, 2015). Nonetheless, cortical damage has also been identified following cerebral ischemia (Pulsinelli, et al. 1982; Block, 1999). Thus, to further investigate the inflammatory response to cerebral ischemia, we measured gene expression of pro-inflammatory cytokines within the cortex (Figure 3). Comparable to the hippocampus, cortical gene expression of TNF- α , IL-1 β and IL-6, was significantly elevated following ischemia among socially isolated mice. However, within this brain region the post-ischemic expression of TNF- α and IL-1 β was significantly reduced among pair-housed mice, relative to socially isolated mice, thereby suggesting social modulation of the inflammatory response. Selective vulnerability of the hippocampus to global cerebral ischemia might underlie regional variation between the brain regions examined (Smith, et al. 1984; Ginsberg, et al. 1989); with severe inflammation within the hippocampus potentially abolishing some of the beneficial effects of social housing.

The innate immune cells of the central nervous system, microglia, mediate the first phase of the inflammatory response to cerebral ischemia by secreting pro-inflammatory cytokines and other inflammatory mediators (Yenari, et al. 2010; Ritzel, et al. 2015). To expand on the social modulation of microglial reactivity to cerebral ischemia, we stained brain slices with ionized calcium-binding adaptor 1 (Iba-1; Figure 4). Myeloid cells, microglia and

macrophages, are often labeled with constitutive marker Iba-1 to obtain information regarding their morphological and activation state (Kettenmann, et al. 2011; Korzhevskii and Kirik, 2015). The retraction of processes and an increase in cell body area are characteristic of microglial activation (Kettenmann, et al. 2011; Beynon and Walker, 2012). As expected, microglia in the brains of mice that experienced ischemic conditions displayed increased cell body area and Iba-1 positive staining within the hippocampus. Cell body area was significantly greater in microglia from the isolated cardiac arrest group relative to sham controls, while the cell body area measures of microglia from the paired cardiac arrest group were not significantly different from either the sham or the isolated CA/CPR groups. When examining Iba-1 proportional area within the whole hippocampus, once again, only the isolated cardiac arrest group displayed significantly greater Iba-1 expression than the sham controls. Together with previous work reporting an ischemic-induced increase in macrophage-1 antigen (MAC-1) staining in the hippocampus of isolated mice relative to their pair-housed counterparts and the sham controls (Weil, et al. 2009), the data presented here (Figure 4) indicate that social isolation can exacerbate ischemia-induced neuroinflammation throughout microglial activation. Administration of minocycline, a tetracycline antibiotic that can reduce microglial activation to a neurological insult (Zemke and Majid, 2004; Fan, et al. 2007), attenuates behavioral deficits, inflammation, and neurodegeneration following global cerebral ischemia (Neigh, et al. 2009; Wang, et al. 2015).

In vivo animal studies have identified social isolation as detrimental to outcome following cerebral ischemia (Craft, et al. 2005; Weil, et al. 2008; Norman, et al. 2010; Venna, et al. 2012). The OGD *ex vivo* model of global cerebral ischemia allowed the examination of whether social environment prior to ischemic injury alters the brain in a way to predispose it to increased ischemic damage. Following *ex vivo* ischemia, there was a delayed increase in TNF- α and IL-6 mRNA expression (Figure 5). Specifically, in this OGD study, after a reperfusion period of 2 or 4 hours, there were no significant differences in hippocampal gene expression of pro-inflammatory cytokines TNF- α , IL-1 β or IL-6, between the control and ischemic samples. Nonetheless, after 6 hours of reperfusion, hippocampal gene expression of TNF- α was increased by ischemic conditions in tissue from both paired and isolated mice. Additionally, hippocampal gene expression of IL-6 at the 6 and 8 hour time points was significantly elevated among the isolated ischemic group relative to controls, while the paired ischemic group was not significantly different from either the isolated ischemic group or the control group. These data demonstrate that social environment has an impact on the brain which modulates the inflammatory response to ischemic injury.

The *ex vivo* and *in vivo* models of cerebral ischemia elicited an inflammatory response, which was attenuated among pair-housed animals. The initial inflammatory response following brain injury is mediated by microglia (Rogove, et al. 2002; McKimmie, et al. 2006; Graeber and Streit, 2010). These cells give rise to inflammation that ultimately exacerbates neuronal death (Fernandes, et al. 2014). In the *ex vivo* model of ischemia, as expected, OGD conditions elicited increases in TNF- α and IL-6 mRNA expression, suggesting hippocampal inflammation. The IL-6 response differed by housing condition; IL-6 mRNA was elevated among tissue collected from socially isolated mice but not pair-housed mice. Similarly, following CA/CPR, the ischemia-induced increase in hippocampal

IL-1 β and cortical TNF- α , IL-1 β and IL-6 were attenuated by social interaction. During the OGD *ex vivo* model, brain slices experience ischemia for 15 minutes, whereas in the cardiac arrest model blood flow is stopped for ~8 minutes. Prolonged periods of ischemia contribute to extended damage and post-infarct dysfunction (Kalogeris, et al. 2012); nevertheless, OGD requires longer time periods to produce cell injury (Kurian and Pemaih, 2014). Reperfusion time, the dynamic phase following ischemia in which cell death can be either controlled or extended (Zhao, et al. 2000), also differed between our models. Despite experimental variation between the *ex vivo* OGD model and the *in vivo* CA/CPR model, that can potentially influence the results, social modulation of the neuroinflammatory response to cerebral ischemia was evident in both.

3.3 Conclusions

Together, the data support social modulation of neuroinflammation. Following a week of the housing manipulation, hippocampal and cortical gene expression of MHC II was increased among isolated male mice relative to their pair-housed counterparts. The elevation in MHC II expression by social isolation serves as the first indication of isolation-induced microglial priming. As expected from a sensitized immune system, the ischemia-induced increase of several pro-inflammatory cytokines was greater among the isolated animals, in both *in vivo* and *ex vivo* models of global cerebral ischemic injury. An exacerbated inflammatory response was further supported by morphological changes indicating greater microglial activation in the isolated CA/CPR group relative to the pair-housed counterparts.

In previous studies examining the effects of social environment on cerebral ischemia outcome (Craft, et al. 2005; Weil, et al. 2008; Norman, et al. 2010; Venna, et al. 2012), it was not possible to determine whether social isolation primed the brain in a way that made it more sensitive to ischemia. The *ex vivo* OGD model allowed us to determine that isolation prior to ischemic onset is sufficient to sensitize the subsequent immune response to ischemia. An exaggerated ischemia-induced inflammatory response in tissue from animals that were previously isolated supports that social isolation can alter brain physiology and consequently prime microglia to respond in an exaggerated manner.

4. Experimental Procedure

These studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and under protocols approved by the Ohio State Institutional Animal Care and Use Committee. All data were collected by individuals who were uninformed of experimental assignment.

4.1 Animals

Young adult c57/bl6 experimental male mice (49–56 days of age, Charles River, Wilmington, MA) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved vivarium with a 14:10 light/dark cycle. Upon arriving to our facility, all animals were provided *ad libitum* access to food and filtered tap water, as well as cotton nesting material. Animals were singly housed in cages that were

situated in ventilated racks with controlled temperature and humidity, and allowed one week to habituate prior to experimental assignment.

4.2 Experimental Designs

4.2.1 Experiment 1: Social influences on healthy mice—Adult (~8 week old) c57/bl6 male mice remained socially isolated (n=12) or were pair-housed with an ovariectomized female (n=12). On day 7, the brains were collected for further analysis.

4.2.2 Experiment 2: *In vivo* global cerebral ischemia—Adult (~8 week old) c57/bl6 male mice remained socially isolated (n=20) or were pair-housed with an ovariectomized female (n=20) beginning one week prior to CA/CPR or the sham procedure and continuing through tissue collection. Mice received a cardiac arrest/cardiopulmonary resuscitation (CA/CPR) (n=10/per group) or sham (n=10/per group) procedure, and at 24 hours post-ischemia brains were collected for gene expression analysis. A second cohort (N=48, 12 per group) was completed, and brain tissue was used for histological analysis.

4.2.3 Experiment 3: *Ex vivo* global cerebral ischemia—Adult (~8 week old) c57/bl6 male mice remained socially isolated (n=8) or were pair-housed (n=8) for a week prior to tissue collection. Following euthanasia, brains were dissected and sectioned using a vibratome. Brain slices were habituated for 1 hour in artificial cerebrospinal fluid (aCSF) with bubbling oxygen, and then exposed to oxygen-glucose deprivation (OGD) or control conditions for 15 minutes. Tissue was transferred back to aCSF and collected after 2, 4, 6 or 8 hours of reperfusion for gene expression analysis. From every animal, 8 brain slices were collected; 4 per treatment condition (control or OGD) and 1 per time point.

4.3 Ovariectomy

Ovariectomized adult stimulus females were used for pair housing. The mice were anesthetized with isoflurane (induced at ~3% and maintained at ~1.5%) and both ovaries were removed under sterile surgery. Buprenorphine (0.05 mg/kg; SC) was administered to provide analgesia. The incisions were fully healed prior to pairing, and to minimize animal usage, the same stimulus females were used in all three experiments described in this study.

4.4 Housing manipulation

At day 0, the experimental mice were either paired with an ovariectomized female or kept in social isolation. Ovariectomized females were used as the pairing stimulus to minimize the aggressive and territorial behavior often exhibited by male mice (Van Loo, et al. 2003) and because the ovariectomy procedure eliminates the influence of the female estrous cycle over male physiology and behavior (Ingersoll and Weinhold, 1987).

4.5 Global Cerebral Ischemia

4.5.1 Cardiac arrest/Cardiopulmonary resuscitation—Cardiac arrest/cardiopulmonary resuscitation (CA/CPR) was used to induce ~8 minutes of global cerebral ischemia *in vivo*. Mice were anesthetized with 3% isoflurane in air and then maintained on 1.5% isoflurane during the procedure. CA/CPR was induced as previously described (Neigh, et al. 2004), but with minor modifications. A temperature probe was inserted in the

temporalis muscle on the left side of the head, and the temporalis muscle temperature was used as an index of brain temperature. A second temperature probe was placed into the rectum, and feedback was provided from a heating blanket (Harvard Apparatus, Holliston, MA) to maintain body temperature. A cannula (Fine Science, Foster City, CA) was inserted into the right femoral artery and connected to a blood pressure transducer (Columbus Instruments, Columbus, OH). A PE 10 catheter was placed into the right jugular vein for drug administration. An intubation tube was connected to a ventilator (Columbus Instruments, Columbus, OH) and mice were ventilated with a tidal volume of 120 μ L and a respiratory rate of 160 breaths per minutes. Following all the preparatory steps, mice were allowed to stabilize for 10 minutes, during which blood pressure and temperature were recorded. At the end of the acclimation period, body temperature was decreased to 27°C by circulating cold water through a coil system underneath the animal and placement of an alcohol patch over the ventrum in order to prevent damage to peripheral organs (Kofler, et al. 2004). To induce cardiac arrest, potassium chloride (KCl, 50 μ L, 0.5 M, 4°C) was injected via the jugular catheter and the mice were detached from the ventilator. Slow re-warming of the body via a thermal blanket began when the body temperature reached 27°C after approximately 4 minutes of the procedure. At 7.5 minutes into the arrest period, the mouse was reattached to the ventilator and ventilated with 100% oxygen with a tidal volume of 150 μ L and a respiratory rate of 190 breaths per minutes. At 8 minutes following the KCL injection, cardiopulmonary resuscitation was initiated via an injection of epinephrine (EPI, 16 μ g in 0.6 cc saline, 37°C) into the jugular vein catheter and followed by chest compressions. Mice were maintained on 100% oxygen for 25 minutes following return of spontaneous circulation and then extubated, followed by the removal of catheters, skin suturing, and buprenorphine (0.05 mg/kg; SC) administration. Once the mice had recovered, they were returned to their normal housing conditions until tissue collection. The procedure described above was similar for CA/CPR and sham animals, except that the control animals received isotonic saline instead of KCL or epinephrine, and did not experience chest compressions.

4.5.2 Oxygen-glucose deprivation—Oxygen-glucose deprivation was used to induce ischemic conditions *ex vivo*. Briefly, mice were administered an intraperitoneal injection of 0.5 mL/100 g of 8% chloral hydrate and once unresponsive to stimuli they were decapitated for brain dissection. The brains were then sectioned in ice-cold (4°C) oxygenated (95% O₂/5% CO₂) aCSF with reduced Ca²⁺ and increased Mg²⁺ (in mM: 125 NaCl, 3.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 0.1 CaCl₂ and 10 Glucose) at a pH of 7.3–7.4, into eight 300 μ m coronal slices. To minimize possible effects of regional variability, the assignment of the slices was counterbalanced across groups (both time and treatment). Following a 1 hour recovery period in oxygenated artificial cerebrospinal fluid (aCSF), four slices were exposed to OGD for 8 minutes whereas the remaining four slices were kept in aCSF. The OGD solution contained (in Mm) 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 3.5 KCl, 2 CaCl₂ and 1 MgCl₂, and was bubbled with 95% N₂/5% CO₂ (Xie, et al. 2008). Upon completion of the OGD, tissue experienced *ex vivo* reperfusion for 2, 4, 6 or 8 hours, and then the hippocampal section of the slices were collected for gene expression analysis.

4.6 Gene expression

Messenger RNA (mRNA) from the samples was extracted using the Rneasy microkit or minikit (Qiagen, Germany). The minikit was used for mRNA extractions of the hippocampal and cortical samples from the *in vivo* studies (experiments 1 and 2), whereas the microkit was used for the mRNA extraction of hippocampus samples from the *ex vivo* study (experiment 3). Extractions were performed according to the manufacturer's instructions. mRNA concentrations were determined using a spectrophotometer (NanoDrop, Thermo Fischer Scientific Inc.). Complementary DNA (cDNA) was synthesized using M-MLV reverse transcription and diluted 1:10 for subsequent qPCR. The qPCR reaction was ran using endogenous control eukaryotic 18S, probes from Applied Biosystems (Myh2, Mm01332564_m1; Nfkb1, Mm00476361_m1; Tnfa, Mm00443260_g1; Il1b, Mm00434228_m1; Il6, Mm00446190_m1), and Taqman Universal Master Mix (all from Life Technologies), on an ABI 7000 Sequencing Detection System. Relative gene expression was calculated using a relative standard curve, and normalized to the 18S signal. Fold change relative to the sham group was determined.

4.7 Histology

4.7.1 Perfusion—For perfusions, mice were deeply anesthetized with a 200 μ L injection of a 20 mg/mL pentobarbital solution, then once non-responsive they were transcardially perfused with ice cold 4% paraformaldehyde (PFA) in 0.1M PBS.

4.7.2 Cryoprotection—Following fixation of the tissue with 4% PFA, brains were incubated overnight for cyroprotection with 30% sucrose in 0.1M PBS. Then, the tissue was frozen and stored in -80°C until sectioning.

4.7.3 Sectioning—Coronal brain slices were sectioned at 20 μ m using a cryostat and mounted on Superfrost Plus Slides (Fisher Scientific, USA).

4.7.4 Staining—For microglial staining, slides were stained for Iba-1. First, slides were incubated in a Tris-EDTA solution for 20 minutes in a steamer for antigen retrieval, and rinsed with 0.1M PBS. Then, slides were incubated for 24 hours with a rabbit anti Iba-1 antibody (catalogue # 019-19741; Wako Chemicals, Japan). After rinses with 0.1M PBS slides were incubated for 2 hours with an Alexa Flour 488 donkey anti-rabbit antibody (Jackson Immuno Research, USA), rinsed, dried, and then coverslipped.

4.7.5 Imaging—Images were captured using a Nikon Eclipse E800 fluorescence microscope and analyzed using Image J software (Java, USA). An individual blinded to experimental assignment completed imaging and histological analysis. For microglia analysis, three sets of images were collected per mouse; one at 4X magnification to display the entire hippocampus, and another two at magnifications 10X and 40X focusing on the CA1 region of the hippocampus. Prior to analyzing, images were inverted for black and white visualization. In the 4X images, the hippocampus was manually traced to define the region of interest (ROI). Threshold for positive staining was determined for all images, and percent area above threshold was reported as the percent area fraction of Iba-1 (Strahan, et al. 2017). The 40X images were collected in z-stacks to allow for better visualization of

microglia cells for tracing using the Simple Neurite Tracer application from FIJI by Image J (Longair, et al. 2011; Karelina, et al. 2014; Li, et al. 2017). The cell body and processes of three unobstructed microglia within an image were traced and averaged for analysis of cell body area and total processes length.

4.8 Statistical analysis

For all data sets, the Grubbs test was used once to detect outliers within groups. Samples with a Z-score > 2 were removed prior to completing statistical analysis and graphing. When comparing two groups unpaired t-test was used to analyze the data (experiment 1), whereas one-way Analysis of Variance (ANOVA) was performed when comparing three groups (experiments 2). For experiment 3, a two-way repeated measures ANOVA was completed to compare groups across time. When an ANOVA was statistically significant ($p < 0.05$), multiple comparisons were calculated. The Tukey's method was used following the one-way ANOVA and simple effects based on time were determined following two-way repeated measures ANOVA. GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used for all statistical analysis and to create the graphs. Data are presented as mean $\pm$ SEM. Throughout the different parameters analyzed, the paired and single sham/aCSF groups did not differ statistically, and so the data were combined into one control group.

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Abbreviations

CA/CPR	cardiac arrest/cardiopulmonary resuscitation
OGD	oxygen-glucose deprivation
aCSF	artificial cerebrospinal fluid
rtPCR	real-time polymerase chain reaction
MHC II	major histocompatibility complex II
TNF-α	tumor necrosis factor alpha
IL-1β	interleukin 1 beta
IL-6	interleukin 6
Nfκb	nuclear factor kappa B
Iba-1	ionized calcium-binding adaptor 1

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Highlights

- Social isolation primes microglia, as indicated by the increased gene expression of major histocompatibility complex II (MHC II) in the hippocampus and the cortex of male mice.
- The neuroinflammatory response to global cerebral ischemia is significantly increased relative to sham treated mice only among the socially isolated mice; specifically, hippocampal IL-1 β gene expression, cortical TNF- α and IL-6 gene expression, microglial cell body area, and Iba-1 proportional area in the hippocampus, are significantly elevated in the isolated CA/CPR group relative to sham controls. In contrast, gene expression among mice in the pair-housed CA/CPR group is not elevated relative to sham controls.
- Social isolation prior to an ischemic event is sufficient to aggravate the inflammatory response to global cerebral ischemia. Following *ex vivo* cerebral ischemia, hippocampal IL-6 gene expression was significantly upregulated at 6 and 8 hours only in tissue from mice that had been socially isolated.

Figure 1.

Among male mice, one week of social isolation increases hippocampal (A) and cortical (B) expression of MHC II, but does not influence the expression of Nfκb1 in the hippocampus (C) or cortex (D). N=21–23. Data were collected using rtPCR and are presented as mean fold change $\pm$ SEM. An asterisk (*) indicates significant differences at $p < 0.05$.

Figure 2.

Cerebral ischemia induced by ~8 min of CA/CPR increases the mRNA expression of TNF- α (A), IL-1 β (B) and IL-6 (C) in the hippocampus of socially isolated mice relative to the sham control group. Among the pair housed mice, TNF- α (A) and IL-6 (C) mRNA expression were also elevated after CA/CPR, but their IL-1 β (B) mRNA expression remained comparable to both the sham control group and the socially isolated cardiac arrest group. N=35–36. Data were collected using rtPCR and are presented as mean fold change $\pm$ SEM. Groups that do not share the same letter are statistically different from one another, $p < 0.05$.

Figure 3.

Cerebral ischemia induced by ~8 min of CA/CPR increases cortical gene expression of pro-inflammatory cytokines TNF- α (A), IL-1 β (B) and IL-6 (C) in socially isolated mice relative to the sham control group. Pair housed mice exhibit TNF- α (A) and IL-6 (C) levels comparable to the control group, whereas IL-1 β (B) mRNA expression was significantly higher than the sham group and lower than the socially isolated cardiac arrest group. N=33–35. Data were collected using rtPCR and are presented as mean fold change $\pm$ SEM. Groups that do not share the same letter are statistically different from one another, $p < 0.05$.

Figure 4.

Cerebral ischemia induced by ~8 min of CA/CPR increases hippocampal microglial activation in socially isolated mice relative to the sham control group. Microglial cell body area (A) and hippocampal Iba-1 percent area fraction positive staining (B) are increased in the isolated CA/CPR group relative to the sham control group, whereas the pair-housed group remain comparable to both the sham group and the isolated CA/CPR group. Microglia cell body area was determined from cells in the CA1 region and the proportional area of Iba-1 staining was determined from within the whole hippocampus (C). Representative images are illustrated in panel D. N=47–46. Data were collected using immunohistochemistry staining with Iba-1 and are presented as mean $\pm$ SEM. Groups that do not share the same letter are statistically different from one another, $p < 0.05$. Scale bar in panel D = 500mm.

Figure 5.

Cerebral ischemia induced by 15 minutes of oxygen-glucose deprivation (OGD) increases the mRNA expression of TNF- α (A), and IL-6 (C) in the hippocampus. Following 6 hr of reperfusion, hippocampal tissue from brain slices that were exposed to ischemic conditions displayed increased TNF- α mRNA expression relative to aCSF controls, regardless of their previous social condition. In contrast, hippocampal gene expression of IL-6 at 6 and 8 hr of reperfusion was elevated only among the isolated OGD group relative to controls. N=24–30. Data were collected using rtPCR and are presented as mean fold change $\pm$ SEM. An asterisk (*) indicates significant differences at $p < 0.05$.