Published in final edited form as:

Brain Res. 2012 March 14; 1443: 64–74. doi:10.1016/j.brainres.2012.01.014.

Estradiol Protects Against Hippocampal Damage and Impairments in Fear Conditioning Resulting from Transient Global Ischemia in Mice

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Abstract

Estradiol protects against hippocampal damage and some learning impairments resulting from transient global ischemia in rats. Here, we seek to validate a mouse model of transient global ischemia and evaluate the effects of estradiol on ischemia-induced hippocampal damage and behavioral impairments. Female C57Bl6/J mice were ovariectomized and implanted with estradiol- or oil-secreting capsules. One week later, mice experienced 15-min of 2-vessel occlusion (2-VO) or sham surgical procedures. Five days later, mice were exposed to a fear conditioning protocol in which a specific context and novel tone were paired with mild footshock. Twenty-four hours following conditioning, contextual fear was assessed by measuring freezing behavior in the conditioned context (in the absence of the tone). This was followed by assessment of cue fear by measuring freezing behavior to the conditioned tone presented in a new context. When tested in the conditioned context, oil-treated mice that experienced 2-VO exhibited a significant reduction in freezing behavior whereas estradiol-treated mice that experienced 2-VO showed no disruption in freezing behavior. Freezing behavior when presented with the conditioned tone was unaffected by either surgery or hormone treatment. These findings suggest that global ischemia causes impairments in performance on the hippocampally-dependent contextual fear task but not conditioned cue-based fear. Furthermore, estradiol prevented the ischemia-induced impairment in contextual fear conditioning. Fluoro-Jade (FJ) staining revealed neuronal degeneration throughout the dorsal hippocampus of mice that experienced 2-VO. Estradiol treatment reduced the number of FJ⁺ cells in CA1 and CA2, but not in CA3 or in the dentate gyrus. Together, these findings suggest that 15 min of global ischemia causes extensive hippocampal neurodegeneration and disrupts contextual fear conditioning processes in mice and that estradiol protects against these adverse effects.

Keywords

estradiol; ischemia; neuroprotection; fear conditioning; learning; memory

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1. Introduction

Ischemic events including stroke and cardiac arrest affect over 750,000 people in the United States each year, causing significant neural damage and associated cognitive impairment (Lloyd-Jones et al., 2009). Survivors of ischemic stroke typically exhibit impairment in performance on cognitive tasks requiring attention, learning, and memory (Elwan et al., 1994; Volpe and Petito, 1985; Volpe et al., 1986; Zola-Morgan et al., 1986). For example, cardiac arrest patients display anterograde amnesia and deficits in semantic memory that are characteristic of medial temporal lobe and hippocampal damage (Cummings et al., 1984; Di Paola et al., 2008; Rempel-Clower et al., 1996; Volpe and Petito, 1985). Post-mortem analysis of patients following cardiac arrest highlights the vulnerability of the hippocampus, striatum, and cortex (Fujioka et al., 2000; Fujioka et al., 2003; Kawahara et al., 2000; Taraszewska et al., 2002; Wijdicks et al., 2001). In fact, anoxic patients who, like cardiac arrest patients, experience oxygen deficits to the brain show severe atrophy of the hippocampus which correlates with performance on anterograde memory tests (Allen et al., 2006). Identification of effective neuroprotective strategies is a critical area of research and recent studies have sought to influence neural and behavioral outcome following ischemia through the use of a variety of putative neuroprotective agents including calcium channel blockers, glutamate antagonists, antioxidants, and hypothermia (for review, see Ginsberg, 2008). Recent studies have reported neuroprotective effects of the steroid hormone estradiol in rodent models of focal and global ischemia (Hoffman et al., 2006). In the current study, we explore the neural and behavioral consequences of estradiol treatment in a mouse model of transient global ischemia.

Rodent models of transient global ischemia typically result in extensive neuronal death (Jover et al., 2002; Kirino and Sano, 1984; Kirino et al., 1984; Nitatori et al., 1995). In rats, the pyramidal neurons of the CA1 region of the hippocampus are particularly vulnerable to temporary occlusion of blood flow to the forebrain, though other regions of the hippocampus as well as non-hippocampal regions can be affected by prolonged durations of ischemia (Pulsinelli et al., 1982). Given the vulnerability of the hippocampus, it is perhaps not surprising that impairments in learning and memory are among the most prominent behavioral consequences of transient global ischemia in rodent models. For example, performance on the hippocampally-dependent water maze task is impaired in ischemic rats (Block and Schwarz, 1997; Block and Schwarz, 1998; Hagan and Beaughard, 1990; Jaspers et al., 1990; Nelson et al., 1997; Netto et al., 1993; Nunn et al., 1994; Olsen et al., 1994a; Olsen et al., 1994b; Wright et al., 1996). Likewise, rats subjected to transient global ischemia via four-vessel occlusion exhibit impairments in contextual fear conditioning (Hamadate et al., 2010; Mori et al., 2001).

Rodent studies using both focal ischemia and transient global ischemia have recently described potential neuroprotective effects of the steroid hormone estradiol (Dubal et al., 1998; Dubal et al., 1999; Dubal and Wise, 2001; Dubal et al., 2001; Jover et al., 2002; Jover-Mengual et al., 2007; Miller et al., 2005; Plamondon et al., 2006; Rusa et al., 1999; Sandstrom and Rowan, 2007; Simpkins et al., 1997; Simpkins et al., 2004; Toung et al., 1998). Several recent studies have included behavioral measures confirming that treatments reducing the severity of hippocampal damage also positively impact performance on hippocampally-dependent tasks. For example, acute administration of estradiol prior to four-vessel occlusion (4-VO) reduces the severity of CA1 damage and reduces the severity of impairments in learning among ovariectomized female rats when tested on a hippocampally-dependent spatial learning task (Sandstrom and Rowan, 2007). Long-term treatment with continuous-release estradiol pellets similarly prevents ischemia-induced cell loss as well as deficits in visual spatial memory assessed with an object recognition task and spatial memory assessed with an object placement task (Gulinello et al., 2006). When tested on a

radial-arm maze task, rats treated with estradiol prior to global ischemia showed significantly greater hippocampal cell survival and exhibited less disruption in recognition and spatial memory (Plamondon et al., 2006). Together, these findings highlight the protective effects of estradiol with regard to global ischemia-induced hippocampal damage and impairments in cognition.

Little work has examined the effects of estradiol on neural and behavioral outcome following transient global ischemia in mice. This is unfortunate given the significant potential to probe the mechanisms underlying estradiol's effects through the use of genetically-modified mice. Using a two-vessel occlusion model (2-VO), estradiol was shown to reduce neuronal damage in CA1 of wild-type mice but not apolipoprotein E (apoE) deficient mice, suggesting that estradiol-mediated protection depends on apoE (Horsburgh et al., 2002). Similarly, using agonists specific to the alpha and beta subtypes of the estrogen receptor, Carswell and colleagues (2004) reported that estradiol acts through ER β to yield its protective effects. Unfortunately, neither of these studies examining estradiol-mediated neuroprotection using a mouse model of transient global ischemia included behavioral variables to determine whether the reduction in cell loss resulting from hormone treatment is associated with preservation of performance on hippocampally-dependent tasks.

Here, we examine how transient global ischemia and estradiol interact to influence hippocampal cell survival as well as behavior in mice. Female C57Bl/6J mice were ovariectomized and treated with estradiol-or vehicle-secreting capsules. After 2-vessel occlusion (2-VO) or sham surgical procedures, their performance on contextual-and cue-fear conditioning tasks was assessed as was their locomotor coordination. The extent of neuronal damage in the dorsal hippocampus resulting from 2-VO and the potential influence of estradiol were evaluated by examining hippocampal sections stained with Fluorojade, a marker of neuronal degeneration (Kundrotiene et al., 2004; Schmued and Hopkins, 2000).

2. Results

2.1 Mortality

No mice receiving sham surgical procedures died. Mortality associated with the 2-VO procedure was equally distributed across the animals treated with estradiol and those treated with oil with two animals dying during the 2-VO procedure in each treatment group.

2.2 Context and Cue Fear Conditioning

The percentage of the three-minute context test during which mice were immobile (i.e., freezing) was analyzed with a 2 (Hormone) $\times$ 2 (Surgery) ANOVA (Figure 2). While neither main effect was statistically significant ($ps > .05$), the interaction between Hormones and Surgery was significant, $F(1, 37) = 5.21, p < .05$. Analysis of the simple main effects confirmed that oil-treated mice that experienced 2-VO exhibited significantly less freezing in the conditioning context than did either oil-treated shams or estradiol-treated mice that experienced 2-VO ($ps < .05$).

The percentage of time spent immobile during the tone-on and tone-off periods of the cue test was analyzed with a mixed-model ANOVA with Hormone and Surgery as between-subjects factors and Tone (on, off) as a repeated measure (Figure 3). A significant main effect of Tone, $F(1, 37) = 32.39, p < .001$, confirmed that mice froze significantly more while the tone was on ($20.1\% \pm 2.1$) than while it was off ($10.5\% \pm 1.1\%$). No other main effects or interactions were statistically significant ($ps > .05$).

2.3 Locomotor Coordination

Latency to fall during the three rotarod tests was analyzed with a mixed model ANOVA with Hormone and Surgery as between-subjects factors and Trial as a repeated measure (Figure 4). A significant main effect of Trial, $F(2, 74) = 5.50, p < .01$, confirmed that performance improved over the course of the three rotarod tests. No other main effects or interactions were significant ($ps > .05$).

2.4 Histology

Neurodegeneration was quantified by the number of FJ⁺ cells in subregions of the dorsal hippocampal subregions (Figure 5). Given differences in the densities of neurons within these regions, each region was analyzed separately by counting FJ⁺ cells within counting boxes ($400 \mu\text{m} \times 100 \mu\text{m}$ for medial CA1, lateral CA1, and CA3; $200 \mu\text{m} \times 100 \mu\text{m}$ for CA2 and hilus of the DG) placed over the regions of interest. Due to the absence of FJ⁺ staining within the hippocampi of mice in the sham surgical condition, only mice from the 2-VO condition were compared. As indicated in Table 1, the brains of mice treated with estradiol exhibited significantly fewer FJ⁺ cells within the medial portion of the CA1 relative to those treated with oil, $t(17) = 2.56, p < .05$. Though trending toward a protective effect of estradiol, the difference within the lateral portion was not statistically significant, $t(17) = 1.80, p = .09$. Fewer FJ⁺ cells were also evident within the CA2 of estradiol-treated mice relative to oil-treated mice, $t(17) = 2.22, p < .05$. No differences were apparent within either the CA3 or DG regions, $ts(17) < 1.20, ps > .24$.

The percent of time immobile during the contextual fear test was not correlated with the degree of neurodegeneration in any of the hippocampal regions of mice in the 2-VO treatment conditions, nor was it correlated with the degree of damage summed across all regions of the hippocampus ($ps > .05$). Similarly, the percent of time immobile during the presentation of the conditioned tone was not correlated with the degree of neurodegeneration in any of the hippocampal regions, nor was it correlated with the degree of neurodegeneration summed across all regions of the hippocampus ($ps > .05$).

3. Discussion

The present findings demonstrate that 15 min of 2-VO causes extensive damage within the dorsal hippocampus in ovariectomized C57Bl/6J mice and that administration of estradiol beginning one week prior to ischemia reduces the severity of cell loss, specifically in the medial CA1 and CA2 subregions. Mice that experienced 2-VO exhibited significant reductions in freezing behavior during the test of contextual fear and estradiol eliminated this impairment. When behavior was assessed in a novel context, however, all treatment groups exhibited more immobility during the tone presentation than during the period when the tone was not presented. Neither hormone nor surgical condition impacted general coordination as assessed with the rotarod. Together, these findings suggest that transient global ischemia results in impairments in performance on a contextual fear conditioning task that depends, in part, on hippocampal function (Phillips and LeDoux, 1992) and that estradiol treatment protects against this cognitive impairment. These findings demonstrate that estradiol protects against neuronal damage and behavioral impairment in a mouse model of global ischemia and sets the stage for additional studies examining the mechanisms underlying these phenomena.

The present findings are consistent with behavioral studies showing that hippocampally-dependent memory is protected by treatment with estradiol prior to ischemia in rats (Gulinello et al., 2006; Plamondon et al., 2006; Sandstrom and Rowan, 2007) and in gerbils (Kondo et al., 1997). While these studies examined preservation of function on the Morris

water maze task, object recognition task, and object location task, the current study demonstrates preservation of function on the hippocampally-dependent contextual fear conditioning task in mice (Logue et al., 1997). Cue-based fear conditioning is less dependent on the hippocampus (see Maren, 2001) and, when tested in a novel context, all treatment groups in the present study showed greater freezing when the conditioned tone was on than when it was off and the groups did not differ from each other on this measure. These findings suggest that the hippocampal damage resulting from 2-VO particularly influences contextual (hippocampally-dependent) fear processes but not cue (hippocampally-independent) fear processes. Though mice did freeze more to the tone than to its absence during the cue-based fear conditioning test, it is notable that levels of freezing to the tone were somewhat lower than others using similar conditioning parameters have reported in the literature (e.g., Logue et al., 1997). This difference may be a consequence of differences in the testing protocol as we tested freezing to the tone on the same day as the context test. In addition, our test session protocol involved alternating periods of no-tone presentation with periods of tone presentation during the five-min test (rather than a continuous presentation of the tone throughout the test). Though the overall magnitude of freezing behavior was somewhat low, all treatment groups exhibited more freezing during the tone than when the tone was absent suggesting that behavioral deficits following global ischemia may be particularly evident when tested with hippocampally-dependent tasks.

It is notable that the correlation between the magnitude of ischemia-induced neurodegeneration in the CA1 and performance on the hippocampally-dependent contextual fear conditioning task were not significantly correlated. This finding stands in contrast to prior work in our lab that reported a correlation between CA1 damage and spatial memory in ischemic rats (Sandstrom and Rowan, 2007). Others, however, have reported little relationship between the extent of hippocampal damage and performance on a variety of learning and memory tasks thought to rely, in part, on the hippocampus (Gulinello et al., 2006; Kondo et al., 1997; Nunn et al., 1994). One factor that may contribute to these seemingly discrepant results is the role of the different hippocampal regions in modulating different behaviors (see Fanselow and Dong, 2010). Our study limited examination of damage to the dorsal hippocampus and it is possible that correlations between damage and behavior would emerge with a detailed parsing of the hippocampus along the dorsal-ventral axis. Additionally, the interval between ischemia and behavioral testing may also contribute to different degrees of relationship between damage and performance. To allow sufficient recovery prior to behavioral testing, mice in the current study were tested five to seven days following ischemia or sham surgical procedures. Though the numbers of FJ⁺ cells remain high after this interval following surgery, it is possible that it does not reflect the total amount of damage within these brain regions. That is, FJ staining may under-estimate the magnitude of damage as degenerating cells are phagocytosed. Preliminary studies in our lab indicate that the numbers of FJ⁺ cells peak 3–5 days following ischemia and though the decline when assessed at 7 days is not significant, it is possible that results in an underestimation of total damage resulting in the lack of a significant correlation between damage and behavior. Studies examining the time-course of neuronal and behavioral change associated with these manipulations will be important to understanding how ischemia and neuroprotective interventions impact cell survival and performance on learning and memory tasks in both the short-and long-term.

In the present study, ischemia (or sham surgery) was applied five days before conditioning and six days before testing. Deficits in contextual fear in our oil-treated ischemic mice suggests that neural damage resulting from global ischemia caused this cognitive impairment. Interestingly, studies employing neurotoxic lesions of the dorsal hippocampus have revealed that, while lesions performed *after* training yield significant deficits in contextual fear conditioning (Maren et al., 1997), neurotoxic lesions performed *before*

conditioning do not yield deficits in contextual conditioning (Cho et al., 1999; Gisquet-Verrier et al., 1999; Maren et al., 1997). In contrast, electrolytic lesions of the dorsal hippocampus prior to conditioning, however, have been shown to impair contextual fear (Maren and Fanselow, 1997; Phillips and LeDoux, 1994). Disruption of connections between the ventral subiculum and nucleus accumbens have been proposed as an explanation for the reason that electrolytic lesions disrupt encoding of contextual fear but neurotoxic lesions do not (Maren, 2001). The ischemia-induced damage to the dorsal hippocampus evident in our study obviously occurred prior to conditioning. This suggests that ischemia may have damaged connections running through the dorsal hippocampus or caused neuronal damage in other sites such as the subiculum that appear to be critical for acquisition of conditioned fear (Maren, 1999). Our study is limited in its focus on FJ⁺ cells in the dorsal hippocampus and it is certainly possible that ischemia-induced damage in other sites may contribute to the observed lack of correlation between neurodegeneration in the dorsal hippocampus and performance on the fear conditioning tasks.

In the current study, global ischemia in mice was induced by occlusion of the carotid arteries. When vascular clamps were applied prior to the cardiac infusion of ink, there was no evidence of flow to the forebrain (Figure 6). This finding, along with the extensive FJ staining within the hippocampus of oil-treated mice validates this surgical technique as a means of inducing transient global ischemia in mice. It is possible, however, that estradiol administration prior to ischemia in our experimental subjects caused increases in vasodilation that may have reduced the ability of 2-VO to effectively occlude blood flow. Estradiol acts as a vasodilator and can improve cerebral blood flow by stimulating endothelial nitric oxide synthase (eNOS) activity (Chen et al., 1999; He et al., 2002; Kim et al., 1999; McCullough et al., 2001; Pelligrino et al., 1998). Thus, pre-ischemic estradiol may protect against ischemia-induced brain damage by allowing more cerebral blood flow during clamping of the carotids. We think this mechanism is unlikely, however, as estradiol protects against global ischemia induced cell death in rats, even when controlling for blood flow (Wang et al., 1999). Future studies would benefit from the use of laser Doppler flowmetry to confirm uniform effects of vascular clamps on cerebral blood flow.

There are a variety of other mechanisms through which estradiol may act to minimize the neuroanatomical and behavioral deficits resulting from transient global ischemia. While we do not believe changes in vasculature result in differences in blood flow to the brain during the ischemic event, it is possible that these changes do significantly influence the response of forebrain neurons following reperfusion. Through its actions on endothelial cells, estradiol initiates a signaling cascade that results in the production of nitric oxide (NO), a potent vasodilator (McNeill et al., 1999). This effect may increase blood flow to/from vulnerable areas during a critical period immediately following reperfusion (Hurn and Macrae, 2000). Collectively, these effects could act to reduce the energy deficits experienced as a result of ischemia and to facilitate the elimination of toxic factors that result from the ischemic insult. In addition, estradiol exhibits a phenolic ring structure that confers it with antioxidant properties and, thus, the potential to reduce the impact of free radicals produced by transient global ischemia (Manthey and Behl, 2006; Sies, 1997; Singh et al., 2006). Furthermore, estradiol treatment has been shown to increase hippocampal expression of the anti-apoptosis protein bcl2 (Alkayed et al., 2001; Dubal et al., 1999) and down-regulates pro-apoptotic factors such as caspase 3 (Jover et al., 2002). By activating the PI3K-Akt signaling pathway as well as the ERK/MAPK pathway, estradiol may inhibit apoptosis signaling and promote growth factor signaling (Jover-Mengual et al., 2007). These diverse mechanisms may all contribute to the observed neuroprotective effects of estradiol in the context of transient global ischemia.

Recently, estradiol has been shown to exert some protective effects through binding to either ER α or ER β subtypes of the estrogen receptor within the brain. Intracerebroventricular administration of the general ER antagonist ICI 162,780 abolishes the protective effect of estradiol administered prior to global ischemia in rats (Miller et al., 2005). Peripheral administration of either the ER α -selective agonist propyl pyrazole triol (PPT) or the ER β -selective agonist WAY 200070-3 results in nearly complete protection against 4-VO-induced hippocampal damage in approximately half of treated rats (Miller et al., 2005). In contrast, the ER β agonist diarylpropionitrile (DPN), but not the ER α agonist PPT, protects against 2-VO-induced damage in mice (Carswell et al., 2004). Estradiol, ER α -specific agonists, and ER β -specific agonists have all been shown to up-regulate the expression of the anti-apoptotic marker Bcl-2 resulting in improved cell survival (Alkayed et al., 2001; Dubal et al., 1999; Schor et al., 1999; Zhao et al., 2004).

While pharmacologically targeting specific estrogen receptors is a valuable strategy to employ toward understanding the mechanisms through which estradiol exerts its protective effects, use of genetically-modified mice provides a particularly powerful, complementary approach. Using a focal ischemia model, Dubal and colleagues (2001; 2006) demonstrated that pre-ischemic treatment of both wild-type and ER knockout mice protected against the neuronal death normally associated with middle cerebral artery occlusion. In contrast, ER α knockout mice showed no protective effects of estradiol. These findings therefore suggest a critical role for ER α in this protective process. In contrast, when brain damage resulted from MPTP exposure, both ER α and ER β were shown to be involved in estradiol-mediated protection (Morissette et al., 2007). With the present demonstration of estradiol-mediated protection against the neuroanatomical and behavioral deficits resulting from transient global ischemia in mice, we are now positioned to use knockout mice and pharmacological manipulations to begin probing both the role of specific subtypes of estrogen receptors as well as particular signaling pathways on outcome following transient global ischemia.

While the primary focus of this study concerned the effect of estradiol on neural and behavioral outcome following ischemia, it is notable that we detected no significant effects of estradiol with regard to performance on the behavioral measures in mice that received sham surgeries. Others have failed to detect significant effects of estradiol on motor coordination in mice (Walf et al., 2009; Walf and Frye, 2010). However, estradiol has been shown to influence fear conditioning, though the nature of this relationship appears to depend on a variety of factors. For example, acute estradiol replacement to ovariectomized rats, while not influencing cue fear conditioning, has been shown to disrupt contextual fear conditioning (Hoffman et al., 2010). Similarly, Chang and colleagues (2009) similarly reported that ovariectomized female rats administered acute estradiol exhibit less freezing during a contextual fear test than those treated with vehicle. Recently, Barha and colleagues (2010) reported that a low dose of estradiol administered to ovariectomized rats prior to conditioning facilitates contextual fear conditioning but a high dose impairs conditioning. In the present study, mice were implanted with capsules releasing high physiological levels of estradiol mice (Dubal et al., 2001; Wise et al., 1981) beginning one week prior to 2-VO or sham surgery and capsules remained through the duration of the study. Little is known about how sustained elevations of estradiol impacts performance on hippocampally-dependent tasks, though our data would suggest that it does not significantly impact performance.

In conclusion, our findings demonstrate that estradiol-secreting capsules implanted one week prior to transient global ischemia reduce the severity of neurodegeneration evident in the CA1 and CA2 subfields of the dorsal hippocampus and protect against ischemia-induced deficits in contextual fear conditioning processes. These findings contribute to a growing literature on the protective effects of estradiol. Future studies using genetically modified mice will contribute to our understanding of the fundamental mechanisms through which

estradiol exerts these protective effects and may lead to the development of more specific therapeutic agents.

4. Experimental Procedures

A time-line of experimental procedures is depicted in Figure 1. All procedures were conducted in accordance with NIH guidelines and were approved by the Williams College Institutional Animal Care and Use Committee.

4.1 Subjects

Forty-five adult female C57Bl/6J mice derived from the in-house breeding of mice originally purchased from Jackson Laboratories (Bar Harbor, ME) were used for the behavioral study. Following weaning at 25 days of age, mice were ear-punched for identification and housed in same-sex groups with no more than 3 mice per cage (18.9 cm × 29.7 cm × 12.7 cm). Mice were maintained with free access to food (Mouse/Rat Diet 7012, Harlan Teklad, Madison, WI). Ten additional mice were used to examine the effect of 2-VO on cerebral perfusion (described below).

4.2 Surgical Procedures and Hormone Treatment

At approximately 4 months of age, mice were ovariectomized under isoflurane anesthesia (2% in oxygen) via small bilateral incisions in the dorsal flanks. Following removal of the ovaries, the incision site was closed with surgical staples and antibiotic ointment was applied. A silastic capsule (3.18 mm outside diameter, 1.57 mm inside diameter, Dow Corning, Midland, MI) was then implanted in the subcutaneous scapular space through a small incision along the nape of the neck. Capsules were filled with either 38.7 μ l (20.0 mm) of estradiol (180 μ g/ml in sesame oil) or sesame oil alone. Prior to implantation, capsules were placed in 70% ethanol for 4 h followed by sterile saline for 24 h. These capsule parameters have been shown to produce sustained serum levels of estradiol that fall in the physiological range for C57Bl/6J mice (Dubal et al., 2001; Wise et al., 1981). Furthermore, capsules of these dimensions have been shown to protect against neuronal damage resulting from focal ischemia in mice (Dubal and Wise, 2001; Dubal et al., 2001). Following implantation of the capsule, the incision was closed with a sterile surgical staple and buprenorphine (0.05 mg/kg, sc) was administered as an analgesic. Mice were monitored for 24 h before returning to the home colony.

One week following ovariectomy and capsule implantation, mice experienced 2-VO or sham surgical procedures. To reduce incidence of bowel impaction associated with the monitoring of core body temperature, mice were fasted for approximately four hours prior to surgery. Prior to 2-VO, mice were anesthetized with isoflurane (2% in oxygen) and a 1-cm midline incision was made on the ventral neck. The salivary glands were displaced and the sternomastoid muscles were retracted, exposing the common carotid arteries. Using fine-tipped forceps, the carotid arteries were isolated from the vagus nerve and associated connective tissues and segments of sterile suture were looped under the isolated arteries. Once both common carotid arteries were isolated, the looped sutures were pulled to expose the arteries and an atraumatic arterial clamp was applied to each of the arteries to occlude blood flow. After visually inspecting the preparation to insure that blood flow was occluded, the mouse was maintained for 15 min with continuous monitoring of core body temperature. Because hypothermia can act as a neuroprotectant (Yang et al., 1997), core body temperature was monitored with a rectal probe/thermometer and was maintained at 37.0 ± 0.5 °C using a heat lamp and/or alcohol swabs. After 15 min of occlusion, the clamps were removed and reperfusion was visually confirmed. The loops of surgical suture were removed and the skin was closed with sterile surgical staples. Mice were then administered

buprenorphine analgesic (0.05 mg/kg, sc) and were continuously monitored until they resumed movement, drinking, and grooming behavior. Sham surgical procedures were identical with the exception that the arterial clamps were not applied to the isolated carotid arteries.

Given variability across different mouse strains in their cerebral vasculature and susceptibility to global ischemia (Fujii et al., 1997; Wellons et al., 2000), it is critical to confirm that 2-VO significantly occludes blood flow to the forebrain. To do this, 10 additional mice were anesthetized with isoflurane and the common carotid arteries were isolated and looped with suture as described above. Immediately following isolation of the carotid arteries, the mice were deeply anesthetized with an intraperitoneal administration of ketamine (26 mg/kg), xylazine (5 mg/kg), and acepromazine (0.9 mg/kg) followed by cardiac perfusion with 0.9% saline. After the perfusion, four mice experienced cardiac infusion of 900 μ l of 17% India ink (Faber-Castell Co., Newark, NJ) in 3.5% bovine gelatin (Sigma-Aldrich, St. Louis, MO) dissolved in water warmed to 45 °C. The atraumatic arterial clamps were applied to the common carotid arteries of the remaining six mice prior to the infusion of inked gelatin. All mice exhibited successful ink infusion as evidenced by darkening of the liver. To assess whether application of the clamps to the carotid arteries occluded flow to the forebrain, mice were decapitated and the brains were removed for imaging.

Images of the dorsal and ventral brain surfaces as well as coronal cross-sections at the level of the dorsal hippocampus (~2.2 mm caudal to bregma) were collected using a digital camera (Nikon CoolPix 995) connected to a dissecting microscope with an eyepiece adaptor. As shown in Figure 6, the brains of mice that did not have clamps applied prior to ink infusion exhibited extensive filling of both the surface vasculature and throughout the cross-sections. In contrast, the brains of mice that had clamps applied prior to the ink infusion exhibited no evidence of ink in either the surface vasculature or the forebrain cross-sections. Importantly, these brains did show ink filling in the brainstem and cerebellum, regions of the brain supplied by the vertebrobasilar system and not by the common carotid arteries, indicating that ink was successfully infused but that blood flow to the forebrain was occluded by the arterial clamps. These findings confirm that the 2-VO protocol employed effectively blocks flow to the forebrain.

4.3 Context and Cue Fear Conditioning and Testing

4.3.1 Habituation—Four days following 2-VO or sham surgical procedures, mice were habituated to the two environments used in the context and cue fear conditioning procedures. The first environment consisted of a rectangular chamber (25 cm $\times$ 31 cm $\times$ 25 cm) with Plexiglas walls and a hinged Plexiglas lid. The floor consisted of stainless steel bars (0.32 cm diameter) positioned every 0.79 cm. A harness connected the floor to a shock generator (Med Associates, St. Albans, VT). One wall consisted of a mirror, allowing the entire context to be visible to a video camera positioned outside the clear Plexiglas wall opposite the mirror. To create a distinctive context, one side wall was black and the other side wall was white with a black circle. The second environment consisted of a circular arena with a diameter of 45 cm and with white plastic flooring. The walls of this arena were constructed of white plastic and measured 25 cm high. During habituation, mice spent 10 minutes in each of the two environments after which they were returned to the home cage in the colony.

4.3.2 Conditioning—Twenty-four hours following habituation, mice were conditioned in the rectangular chamber. The training session lasted a total of 8 min, during which mice received four pairings of a 30-s tone (2.8 kHz, 85 dB) followed immediately by a 2-s

footshock (0.5 mA). Tone onsets occurred after 1.5, 3.5, 5, and 7 min. Use of a variable inter-stimulus interval was intended to enhance tone-shock conditioning by eliminating time as an accurate predictor of shock onset. Immediately following the 8-min conditioning session, mice were returned to the home cage in the colony.

4.3.3 Testing—Twenty-four hours following conditioning, each mouse was placed in the training context and behavior was recorded for 3 min. Videos were subsequently scored by experimenters blind to the treatment conditions of the mice. Using observational coding software (ODLog, Macropod Software), the duration of freezing behavior was recorded. Freezing was operationally defined as the absence of any visible movement other than that which was required for respiration.

One hour after completion of the context test, mice were placed in the novel context for 5 minutes. At 2 min and 4 min, the 30-s tone was presented. Behavior was recorded throughout the trial and the percent of time spent freezing during the periods of tone presentation and the percent of time spent freezing during the trial when the tone was absent were recorded by experimenters blind to the treatment conditions of the mice.

4.4 Locomotor Coordination

One day following fear conditioning tests, motor coordination was assessed using a rotarod (IITC, Inc., Woodland Hills, CA). The rotarod consisted of 3.3 cm diameter cylinders positioned 20 cm above a Plexiglas floor. Each mouse was first placed on the stationary rod and allowed to habituate for 30 sec. Following habituation, mice were trained with a single 3-min trial with the rotation speed set at a constant 5 rpm. If a mouse fell off at any point during the training trial, it was immediately returned to the rod. After training, mice were given three test trials during which the rotation speed increased in a linear fashion from 1 rpm to a maximum speed of 30 rpm over the first 90 sec. Trials were terminated when the mouse fell from the cylinder. After each trial, the latency to fall and the total distance traveled were recorded.

4.5 Perfusion and Histology

Immediately following rotarod testing, mice were deeply anesthetized with an intraperitoneal administration of ketamine (26 mg/kg), xylazine (5 mg/kg), and acepromazine (0.9 mg/kg). The circulatory system was flushed with 0.9% saline followed by fixation with 10% neutral buffered formalin. Following perfusion, brains were extracted and post-fixed in formalin overnight. They were then transferred to a 30% sucrose solution for 24 h. After sinking in sucrose, brains were embedded in Tissue-Tek freezing medium and rapidly frozen in a slurry of dry ice and 2-methylbutane.

Twenty-micron thick coronal sections were cut through the dorsal hippocampus at a level within the range of coronal sections 73 to 76 of the Allen Mouse Brain Atlas (2011) at 20 μ m using a cryostat. Sections were collected onto Superfrost Plus slides. After drying, sections were stained using Fluorojade B (FJ). Slides were first soaked in 1% NaOH in 80% ethanol for 5 min. They were then soaked in 70% ethanol for 2 min followed by distilled water for 2 min. Slides were then soaked in 0.06% KMnO₄ for 5 min and rinsed in distilled water for 2 min. They were then incubated for 20 min in 0.0004% FJ in 0.1% acetic acid. After three 1-min rinses in distilled water, slides were dried on a 50 °C slide warmer for 5–10 min in the dark. After drying, they were soaked in xylene for 1 min and coverslipped using DPX mounting media.

Slides were imaged using a Nikon Eclipse 80i microscope equipped with a 10X objective and a QImaging Retiga 2000R camera. Three sections per mouse were imaged at a level

containing the dorsal hippocampus. FJ⁺ cells were counted in several regions of the dorsal hippocampus: the medial CA1, the lateral CA1, CA2, CA3, and the hilus of the dentate gyrus (DG). As indicated in Figure 5, a 400 $\mu\text{m} \times 100 \mu\text{m}$ counting box was positioned over the medial CA1, lateral CA1, and CA3, while a 200 $\mu\text{m} \times 100 \mu\text{m}$ counting box was positioned over CA2 and the hilus of the DG. FJ⁺ cell counts within each of these regions of interest were averaged across the three left hippocampal sections and the three right hippocampal sections. Counts were performed by experimenters blind to the treatment conditions of the mice.

4.6 Data Analysis

The percent of time spent freezing during testing in the conditioned context was analyzed using ANOVA with Hormone (Oil, Estradiol) and Surgery (2-VO, Sham) as between-subjects factors followed by simple-main effects analyses. Analysis of the percent time freezing during the cued test included Hormone and Surgery as between-subjects factors and the presence of the tone as a repeated measure (Tone Off, Tone On). Motor coordination on the rotorod was analyzed using a mixed-model ANOVA with Hormone and Surgery as between-subjects factors and Trial (1–3) as a repeated measure. Due to a lack of FJ⁺ cells in mice in the sham surgical condition (no sham mice had more than one FJ⁺ cell), only histological data from mice in the 2-VO condition were analyzed. Given differences in the densities of cells in the various subregions of the hippocampus, each region was separately analyzed with an independent groups *t*-test. Finally, to examine the relationship between hippocampal damage and freezing during the behavioral tests, correlations were computed between damage in each subregion of the hippocampus and immobility recorded during both the context and cue test sessions.

Acknowledgments

Thanks to Kylie Huckleberry for assistance with behavioral testing and to the animal care staff of Williams College. This work was supported by funds from the Essel Foundation and by NIH Grant NS052911 to NJS.

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Figure 1. Experimental Timeline

Female mice were ovariectomized and implanted with silastic capsules containing either estradiol suspended in sesame oil or vehicle alone one week prior to experiencing either 15 min of transient global ischemia or sham surgical procedures. Four days later, mice were habituated to the two environments used during fear conditioning. On the fifth post-surgical day, mice experienced context-cue fear conditioning followed 24 h later by assessment of freezing behavior in the conditioned context (in the absence of the cue) as well as in a novel context in the presence of the cue. On the following day, locomotor coordination was assessed with a rotorod followed immediately by perfusion for subsequent histological assessment.

Figure 2. Freezing During Context Test

Estradiol capsules prevented the ischemia-induced decrease in the percent of time spent immobile during testing in the conditioned context. (* $p < .05$ relative to Oil-Sham and Estradiol-2-VO).

Figure 3. Freezing During Cue Test

Neither hormone treatment nor surgical condition significantly influenced freezing during the cue test. Immobility during the periods when the tone was off are represented by black bars and immobility in response to the tone is represented by white bars. A main effect of tone indicates that mice were less mobile when the tone presentation than during when the tone was not presented. (* $p < .05$ relative to tone-off condition.)

Figure 4. Locomotor Coordination During Rotorod Test

All treatment groups exhibited comparable levels of performance on the rotorod, increasing the latency to fall across the three training trials.

Figure 6. FluoroJade staining of oil (top) and estradiol (bottom) treated mice that experienced 2-VO. Regions of interest are identified by white boxes. (Scale bars = 400 μ m)

Figure 6. Photographs of dorsal surface, ventral surface, and coronal section of brains from mice that were perfused with India ink in dilute gelatin when the carotid arteries were not clamped (Sham, left) and when the carotid arteries were clamped prior to the ink perfusion (2-VO, right). When clamps were applied prior to ink perfusion, only the cerebellum and brainstem were filled with ink indicating successful occlusion of the forebrain.

Table 1

Mean ($\pm$ SEM) number of FJ⁺ cells in hippocampal regions of ischemic mice treated with oil (n = 10) and estradiol (n = 9). FJ⁺ cells were counted in counting boxes measuring 400 μ m $\times$ 100 μ m (CA1, CA3) and 200 μ m $\times$ 100 μ m (CA2, DG). These values were converted to common units of cells/mm².

	Oil (cells/mm ²)	Estradiol (cells/mm ²)	<i>t</i> (17)	<i>p</i>
CA1 medial	639 $\pm$ 185	143 $\pm$ 81	2.56	0.02
CA1 lateral	182 $\pm$ 79	45 $\pm$ 16	1.80	0.09
CA2	1194 $\pm$ 26	596 $\pm$ 155	2.22	0.04
CA3	39 $\pm$ 26	9 $\pm$ 4	1.20	0.25
DG	399 $\pm$ 62	382 $\pm$ 62	0.20	0.85