Published in final edited form as:

Horm Behav. 2007 March ; 51(3): 335–345.

Acute pretreatment with estradiol protects against CA1 cell loss and spatial learning impairments resulting from transient global ischemia

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Abstract

Estradiol can act to protect against hippocampal damage resulting from transient global ischemia, but little is known about the functional consequences of such neuroprotection. The present study examines whether acute estradiol administered prior to the induction of transient global ischemia protects against hippocampal cell death and deficits in performance on a spatial learning task. Ovariectomized female rats were primed with estradiol benzoate or oil vehicle 48 and 24 hrs prior to experiencing one of three durations of 4-vessel occlusion (0, 5, or 10 min). Performance on the cued and hidden platform versions of the Morris water maze was assessed one week following ischemia. On the cued platform task, neither hormone treatment nor ischemia significantly influenced acquisition. When tested on the hidden platform task, however, oil-treated rats exhibited impairments in spatial learning after either 5 or 10 min of ischemia while estradiol-treated rats showed no impairments after 5 min of ischemia and only mild impairments after 10 min of ischemia. Immediately following behavioral testing, rats were perfused and survival of CA1 pyramidal cells was assessed. Ischemia was associated with the loss of CA1 pyramidal cells but rats that received estradiol prior to ischemia showed less severe damage. Furthermore, the extent of cell loss was correlated with degree of spatial bias expressed on a probe trial following hidden platform training. These findings indicate that acute exposure to estradiol prior to ischemia is both neuroprotective and functionally protective.

Keywords

estrogen; ischemia; cardiac arrest; hippocampus; neuroprotection; spatial learning; water maze

Acute pretreatment with estradiol protects against CA1 cell loss and spatial learning impairments resulting from transient global ischemia

Transient global ischemia leads to the dramatic loss of neurons in the CA1 region of the hippocampus in humans (Horn and Schlote, 1992; Tanabe et al., 1999; Taraszewska et al., 2002), non-human primates (Zola-Morgan et al., 1992), and rodents (Pulsinelli et al., 1982). This neuronal damage can be accompanied by severe impairments in cognition. For example, human survivors of ischemic strokes typically exhibit disruptions in performance on tasks requiring attention, learning and memory (Elwan et al., 1994; Volpe et al., 1986; Zola-Morgan

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et al., 1986). Animal models of transient global ischemia, including 4-vessel occlusion in rats, also cause significant impairments in performance on a variety of learning and memory tasks (see Block, 1999).

Sex differences in the consequences of ischemia have been described in both the human and animal literatures with females suffering less severe ischemia-induced cognitive deficits and neuronal damage than males (Alkayed et al., 1998; Roof and Hall, 2000; Zhang et al., 1998). These findings suggest that ovarian steroids, particularly estradiol, may play an important protective role. When administered before the onset of focal ischemia, estradiol significantly reduces the volume of the resultant infarct in both male (Toung et al., 1998) and female (Dubal et al., 1998) rats. Similarly, when administered prior to the onset of transient global ischemia, estradiol significantly protects against the characteristic cell death that occurs in the pyramidal cell layer of the CA1 region of the hippocampus (Gulinello et al., 2006; He et al., 2002; Jover et al., 2002; Miller et al., 2005; Shughrue and Merchenthaler, 2003).

The extent to which ischemia-induced cell loss is related to impairments in cognition and the degree to which estradiol-mediated neuroprotection translates into preservation of behavioral function are not well understood. Transient global ischemia results in severe impairments in performance on hippocampally-dependent spatial learning tasks such as the Morris water maze (Block and Schwarz, 1997, 1998; Hagan and Beaughard, 1990; Jaspers et al., 1990; Nelson et al., 1997; Nunn et al., 1994; Olsen et al., 1994; Wright et al., 1996). Ischemia also leads to deficits in working memory as assessed with object and place recognition tasks (Gulinello et al., 2006; Mumby et al., 1996; Plamondon et al., 2006; Wood et al., 1993). While some studies suggest that the magnitude of hippocampal damage resulting from transient global ischemia is correlated with performance on learning and memory tasks (e.g., Kiyota et al., 1991; Olsen et al., 1994), others have reported no correlation between these neural and behavioral measures (Kondo et al., 1997; Nunn et al., 1994). In a study exploring the protective effects of estradiol, Kondo, Suzuki, and Sakuma (1997) reported that the administration of estradiol prior to transient global ischemia protects against impairments in learning on a water maze task but does not significantly alter the ischemia-induced cell loss in CA1. Similarly, Gulinello and colleagues (2006) reported that chronic estradiol administration before and after 10-min of transient global ischemia protects against impairments in visual working memory and spatial memory but does not protect against CA1 cell loss. When administered directly to the brain following ischemia, a pharmacological dose of estradiol protects against both cognitive deficits and CA1 cell loss, but these measures are not significantly correlated. Together, these studies indicate that chronic estradiol administered before transient global ischemia can significantly influence the cognitive and neuronal outcome though the nature of the relationship between estradiol's protective effects on brain and its effects on behavior remains unclear. While a pharmacological dose of estradiol administered immediately after ischemia appears to protect both brain and behavior, it is not known whether more physiologically meaningful doses of estradiol administered acutely before ischemia protect against either cell loss or impairments in learning and memory.

In the present study, we examine the effects of acute estradiol administered prior to transient global ischemia on hippocampal cell loss and performance on a spatial learning task. Ovariectomized female rats were primed with estradiol or vehicle 48 and 24 hrs prior to either 0, 5, or 10 min of global ischemia. Because maximal CA1 cell loss is apparent seven days following ischemia, rats were tested on a cued platform version of the Morris water maze (MWM) 6 days after surgery followed 24 hrs later by testing on the hippocampally-dependent hidden platform version of the WMM. Immediately after behavioral testing, rats were sacrificed in order to examine the relationship between performance on the spatial learning task and hippocampal damage.

Materials & Methods

Animals

The 72 female Sprague-Dawley CD strain rats (Charles River Laboratories, Kingston, NY) used in this study were housed in groups of two or three in tub cages (48 cm x 27 cm x 20 cm) in a colony room maintained on a 12:12 light:dark cycle with lights on at 6 am. Rats had free access to food (Mouse/Rat Diet 7012, Harlan Teklad, Madison, WI) and water. All procedures were conducted in accordance with NIH guidelines and were approved by the Williams College Institutional Animal Care and Use Committee. A timeline of experimental events is presented in Fig. 1.

Surgical Procedures & Hormone Administration

At 33 days of age, rats were ovariectomized under isoflurane anesthesia (2% in oxygen) via small bilateral incisions in the dorsal flanks. Following the procedure, antibiotic ointment was applied to the wound and buprenorphine hydrochloride (0.05 mg/kg, subcutaneously) was administered as an analgesic. Following ovariectomy, rats were assigned to one of two hormone treatments (estradiol or oil) as well as one of three surgical conditions (0, 5, or 10 min of ischemia).

Six days after the ovariectomies, rats were injected subcutaneously with either 10 µg of estradiol benzoate (Steraloids, Inc., Newport, RI) suspended in 100 µl of sesame oil (Sigma, St. Louis, MO) or 100 µl of the oil vehicle alone. Twenty-four hours later, immediately after the first stage of the 4-vessel occlusion procedure (described below), they received a second injection. In order to avoid manipulations that may further alter cardiovascular function during ischemia, we did not draw blood to quantify serum estradiol levels at the time of ischemia. However, this dose of hormone administration has previously been shown to result in pharmacological levels of serum estradiol within hours of each injection with circulating levels falling into the high physiological range when measured 24 hrs after the second injection (Woolley and McEwen, 1993). As such, it is likely that serum estradiol levels in our estradiol-primed rats, though initially very high, were in the high physiological range during the ischemic and sham operations.

Transient global ischemia was induced via a two-stage 4-vessel occlusion procedure (4-VO; based on Pulsinelli and Brierley, 1979) that began seven days following ovariectomy. During the first stage of the procedure, rats were anesthetized with isoflurane (2% in oxygen) and positioned in a stereotaxic apparatus. A midline occipital-suboccipital incision was made and the vertebral arteries were permanently occluded by insertion of a cautery probe into the alar foramina of the first vertebra. This procedure prevents collateral blood flow to the forebrain during the subsequent occlusion of the common carotid arteries. Following occlusion of the vertebral arteries, the wound was closed with surgical staples, the rat was repositioned on its back and a midline incision was made at the base of the neck. The common carotid arteries were isolated and surgical suture was loosely looped around the arteries to facilitate isolation during the second stage of the procedure. The incision site was then closed with surgical staples and buprenorphine was administered (0.05 mg/kg, subcutaneously). Approximately 24 hrs later, the rats in the 5- and 10-min ischemia conditions were lightly anesthetized and atraumatic arterial clamps were applied to the common carotid arteries to occlude blood flow. At this point, the rats were removed from the anesthesia and the loss of the righting reflex was monitored throughout the period of occlusion. A rectal temperature probe was inserted and a core body temperature of 37 °C was maintained with a heat lamp. After either 5 or 10 min, the arterial clamps were removed to allow reperfusion and the wound was closed with surgical staples. Rats in the 0-min ischemia condition, were divided between non-operated controls and sham-operated controls that experienced cauterization of the vertebral arteries and isolation of

the carotid arteries. During stage 2, these sham-operated controls were lightly anesthetized, but the clamps were not applied to the carotid arteries. There were no statistical differences between non-operated and sham-operated controls on any measures so they were combined into one group for all other analyses.

Behavioral Testing

The Morris water maze (MWM) consisted of a white circular plastic tank (diameter = 165 cm; height = 46 cm) positioned in a large testing room with a variety of salient extramaze cues. The tank was filled to a height of 36 cm with water maintained at 24–26 °C. The water was made opaque by the addition of non-toxic white paint (Handy Art Tempera, Rock Paint Dist. Corp., Milton, WI). A circular escape platform (diameter = 10 cm; height 35 cm) was constructed of white plastic. A video camera suspended above the pool was connected to a video tracking system (HVS 2020 Plus, HVS Image, Hampton, UK) that recorded the swimming pattern including the length of the swimpath on each trial.

Testing on the Morris water maze (MWM) task was conducted over three consecutive days beginning 5 days following the 4-VO. On the first day, rats received platform pretraining. On the second day, rats were trained on a cued platform version of the maze (cMWM) in which a visually salient platform was repositioned for every trial. On the third day, rats were trained on a hidden platform version of the maze (hMWM) in which a submerged platform was positioned in the center of one of the pool quadrants throughout all trials. This time course of testing was selected to insure that hidden platform testing occurred during the period of maximal cell loss and before the appearance of any new CA1 neurons (Bendel et al., 2005).

Platform Pretraining—Five days following occlusion, rats received platform pretraining on the water maze task. During pretraining, a tall insert (diameter = 75 cm; height = 100 cm) was placed in the pool, the platform was positioned in the center of this arena, and a salient visual cue was positioned above the platform. This visual cue consisted of a cylinder (diameter = 5.0 cm; height = 7.6 cm) covered with a black and white checkerboard pattern. The cue was attached to the platform with a thin rod, such that the bottom of the cue was positioned 7.6 cm above the center of the platform. During pretraining, rats were initially placed on the platform and allowed to stay there for 10 s. They were then placed in the pool at the edge of the platform with their front paws touching it and were allowed to climb out of the water onto the platform and stand for 10 s. This was repeated three additional times. Finally, they were placed at the edge of the insert and allowed to swim to the cued platform and climb onto it. Animals that failed to locate the platform within 30 s were manually guided to it.

Cued platform Morris water maze (cMWM) training—One day following platform pretraining, the pool insert was removed and rats performed 12 trials on the cMWM task. Prior to each trial, the cued platform was repositioned in a novel pool location. On each trial, the rat was gently placed into the pool facing the wall at one of four possible starting locations (N, E, S, W) and was allowed to swim until climbing onto the cued platform or until 60 s had elapsed at which point it was guided to the platform. The rat was allowed to stand on the platform for 15 s after which it was removed from the maze, gently dried with a towel, and placed in a holding cage with a dry towel for a 2 min intertrial interval (ITI). Following the 12th trial, the rat was returned to its home cage.

Hidden platform Morris water maze (hMWM) training—One day following cMWM training, the cue was removed from the platform and each rat performed 12 trials on the hMWM task followed by a probe trial. For each rat, the platform was consistently placed in one of 4 locations within the pool. These locations were positioned 33 cm from the edge of the pool in the center of one of the four pool quadrants. On each of the 12 hMWM trials, the rat was placed

in the pool at one of the four possible starting locations (N, E, S, W) and was allowed to swim until climbing onto the escape platform or until 60 s had elapsed at which point it was guided to the escape platform. After 15 s on the platform, the rat was removed from the maze, gently dried with a towel, and placed in a holding cage with a dry towel for a 2 min ITI.

Following the 12 training trials, the escape platform was removed from the maze and each rat performed one probe trial. For the probe trial, the rat was placed in the pool at a position directly opposite the previous platform location and was allowed to swim for 60 s after which it was removed from the pool, gently dried with a towel, and returned to its home cage.

Histology

Immediately following behavioral testing, rats were deeply anesthetized with a cocktail of ketamine (26 mg/kg), xylazine (5 mg/kg), and acepromazine (0.9 mg/kg). Once anesthetized, blood was collected via cardiac puncture for subsequent assessment of serum estradiol levels (see below). The rats were then perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed and a block approximately 5 mm thick (1.0 mm to 6.0 mm posterior to bregma, Paxinos and Watson, 2004) was postfixed in 4% paraformaldehyde for 2 hrs followed by cryoprotection in 30% sucrose in 0.1 M PB. After the tissue blocks sank in 30% sucrose, they were placed in a plastic mold, surrounded by TBS cutting medium, and quickly frozen in -70°C isopentane. Tissue blocks were stored at -80°C until sectioning. Twenty micron thick sections were cut through the dorsal hippocampus and mounted onto slides. After drying overnight, sections were stained with toluidine blue for subsequent assessment of cell survival.

A brief period of 4-VO results in delayed, selective death of CA1 pyramidal neurons that is maximal approximately 7 days following ischemia (see Zukin et al., 2004) and prior research using stereological techniques has confirmed that 4-VO does not significantly influence the volume of the CA1 (Miller et al., 2005). As such, we restricted assessment of CA1 cell loss to hippocampal sections at a level approximately 3.3 mm posterior to bregma. Digital images were captured through a 20X objective using a Zeiss AxioCam mounted on a Zeiss Axioskop. These images were opened in Zeiss AxioVision (v. 4.2) and the numbers of surviving cells in $400\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ sections of the stratum pyramidale of the left and right CA1 were counted. Cells exhibiting blebbing of the plasma membrane characteristic of necrotic cell death as well as cells exhibiting shrinkage of the cytoplasm characteristic of apoptotic cell death were excluded from these counts. Cell counts are expressed as the mean number of surviving cells per $400\text{ }\mu\text{m}$ averaged across the left and right CA1 based on 2–3 sections per animal.

Estradiol Radioimmunoassay

Blood collected prior to perfusion was allowed to clot for approximately 45 min and was then centrifuged at 3000 rpm for 10 min. The serum was then transferred to clean cryotubes and stored at -20°C until analyzed using a commercially available double-antibody radioimmunoassay kit (KE2D5, Diagnostic Products Corp., Los Angeles, CA). The reported analytical sensitivity of this kit is 1.4 pg/ml. To enhance the sensitivity, an aliquot of the lowest calibrator provided with the kit (5 pg/ml) was diluted to produce an additional calibrator of 2.5 pg/ml. When sufficient sample was available, the assay was run in duplicate and the mean coefficient of variation among the duplicate samples was 5.94%.

Statistical Analyses

The distance traveled on each trial was recorded for each rat and collapsed into three-trial blocks. Performance on the cMWM task and performance on the hMWM task were similarly analyzed using 3-factor ANOVAs with hormone treatment and surgical treatment as between-subjects factors and trial blocks analyzed as a linear trend. In addition, performance on the final

block of hMWM trials was analyzed with a 2-factor ANOVA with hormone treatment and surgical treatment as between-subjects factors. Finally, the percentage of time during the probe trial that the rats spent in the target quadrant was analyzed with a 2-factor ANOVA. Post-hoc tests were conducted using Fishers LSD test where appropriate. Cell counts were analyzed using a 2-factor ANOVA with Fishers LSD post-hoc tests. In addition, Spearman's rank-order correlation coefficients (ρ) were computed to examine the relationships between cell survival and spatial learning (rate of acquisition as measured by the linear trend contrast value and the percentage of time in the target quadrant during the probe). Serum estradiol levels at the time of sacrifice were analyzed with ANOVA with hormone treatment and surgical treatment as between-subjects factors.

Results

One rat assigned to the 0-min (sham) surgical condition died during Stage 1 of the procedure. In addition, 6 rats (4 estradiol primed, 2 oil primed) assigned to the 5-min ischemia condition died during surgery as did 6 rats (4 estradiol primed, 2 oil primed) assigned to the 10-min ischemia condition. While more animals primed with estradiol died during the ischemia procedures, the probability of surviving surgery was statistically independent of hormone treatment ($p > .05$).

Behavioral Testing

cMWM Performance—The distances traveled on the 12 cued platform training trials were collapsed into four blocks of three trials for each rat (Fig. 2). These data were then analyzed using a 3-factor ANOVA with hormone treatment (oil, estradiol) and surgical treatment (0, 5, 10 min of ischemia) as between-subjects factors and trial blocks analyzed as a linear trend. The linear trend of trial blocks was statistically significant, $F(1, 53) = 111.31, p < .001$. There were no other statistically significant main effects or interactions ($ps > .05$). These results indicate that rats swam more directly to the cued platform as training progressed and that neither estradiol nor ischemia significantly influenced that learning.

hMWM Performance—The distances traveled on the 12 hidden platform training trials were collapsed into four blocks of three trials for each rat (Fig. 3) and these data were analyzed in a manner similar to that described for the cued platform training. A 3-factor ANOVA with hormone treatment (oil, estradiol) and surgical treatment (0, 5, 10 min of ischemia) as between-subjects factors and trial block analyzed as a linear trend yielded a significant linear trend across trial blocks, $F(1, 53) = 47.00, p < .001$, confirming that swim distance declined across training trials. The main effect of hormone treatment was significant, $F(1, 53) = 6.12, p < .05$, with rats that received estradiol priming swimming shorter distances, on average, than rats primed with oil. The main effect of surgery approached statistical significance, $F(2, 53) = 2.72, p = .08$, with rats that received 5 and 10 min of ischemia swimming longer distances, on average, than animals in the 0 min control condition. The interaction between the linear trend across trial blocks and hormone treatment was significant, $F(1, 53) = 5.59, p < .05$, with estradiol-primed rats exhibiting a steeper acquisition function than oil-primed rats. The interaction between the linear trend across trial blocks and surgical condition was also significant, $F(2, 53) = 5.01, p < .05$, with rats that experienced 0 min of ischemia exhibiting significantly steeper acquisition functions than rats in the 5-min condition ($p < .05$) and marginally steeper acquisition functions than rats in the 10-min condition ($p < .10$). The interaction between hormone treatment and surgical condition was statistically significant, $F(2, 53) = 3.96, p < .05$. Post-hoc testing confirmed that only among rats that experienced 5 or 10 min of ischemia was there a difference, across all trials, in the performance of estradiol- and oil-primed rats ($ps .05$). The 3-way interaction among the linear trend across trial blocks, hormone treatment, and surgical treatment approached statistical significance, $F(2, 53) = 2.29, p = .11$, suggesting that

differences in the rates of acquisition between estradiol and oil treated rats were not the same across the different ischemia conditions (Fig. 4). Pairwise comparisons confirmed that the linear trend across trial blocks differed between estradiol and oil treated rats only for rats that experienced 5 min of ischemia ($p < .05$). Furthermore, there were no differences in the rate of acquisition among estradiol treated rats. Among oil-primed rats, those that experienced 5-min of ischemia exhibited significantly worse acquisition than those that experienced 0-min of ischemia while the 10-min of ischemia group did not differ from the 0- or 5-min groups. To assess performance at the end of training, the last block of trials was separately analyzed with a 2-factor ANOVA with hormone treatment and surgical condition as between-subjects factors. This analysis revealed main effects of both hormone, $F(1, 53) = 10.43$, $p < .01$, and surgical treatment, $F(2, 53) = 9.63$, $p < .001$. In addition, the interaction between hormone treatment and surgical condition was significant, $F(2, 53) = 7.34$, $p < .01$. Post-hoc testing confirmed that the mean pathlength for the final block of trials differed between estradiol- and oil-primed rats that experienced either 5 or 10 min of ischemia ($ps < .05$), but not for those that experienced 0 min of ischemia ($p > .05$). Furthermore, performance on the final block of trials did not differ among rats that were primed with estradiol, regardless of surgical condition ($ps > .05$); in contrast, oil-primed rats that experienced 0 min of ischemia swam significantly shorter distances on the final block of trials than those that experienced either 5 or 10 min of ischemia ($ps < .05$).

Because improvements across training in performance on the hMWM task can be due to the adoption of non-spatial strategies that are not hippocampally-dependent (e.g., circular swimming patterns a fixed distance from the pool edge), a probe trial was administered immediately following the final training trial. Performance on this trial was measured by the percentage of time spent in the quadrant of the pool that previously contained the hidden platform (Fig. 5). These data were analyzed with a 2-factor ANOVA with hormone treatment and surgical condition as between-subjects factors. The main effect of surgical condition was statistically significant, $F(2, 53) = 5.76$, $p < .01$, while the effect of hormone treatment was marginally significant, $F(1, 53) = 3.17$, $p = .08$. The interaction between hormone treatment and surgical condition was significant, $F(2, 53) = 7.10$, $p < .01$. Post-hoc testing confirmed that the difference in probe trial performance between estradiol- and oil-primed rats was significant only for those that experienced 5-min of ischemia ($p < .05$). Furthermore, probe trial performance did not differ among rats that were primed with estradiol, regardless of surgical condition ($ps > .05$); however, oil-primed rats that experienced 0-min of ischemia exhibited a significantly stronger bias for the target quadrant than did those that experienced either 5 or 10 min of ischemia ($ps < .05$).

Histology

Ischemia markedly reduced the number of surviving cells in the CA1 region of the hippocampus and estradiol-priming largely prevented this cell loss (Figs. 6 & 7). A 2-factor ANOVA with hormone treatment and surgical condition as between-subjects factors revealed main effects for both hormone treatment, $F(1, 53) = 10.74$, $p < .01$, and surgical condition, $F(2, 53) = 18.45$, $p < .001$. The interaction between hormone treatment and surgical condition was also statistically significant, $F(2, 53) = 3.16$, $p = .05$. Post-hoc tests confirmed that rats that received estradiol priming exhibited more CA1 cells than oil-primed rats. Furthermore, the magnitude of cell loss was related to the duration of ischemia with 5 min of ischemia causing significant cell loss and 10 min of ischemia causing more cell loss than 5 min. When compared within each surgical condition, cell survival among estradiol- and oil-primed rats did not differ after experiencing 0 min of ischemia, though estradiol-priming did significantly improve cell survival relative to oil-priming among rats that experienced either 5 or 10 min of ischemia ($ps < .05$).

Brain-Behavior Relationship

To determine whether the magnitude of cell loss is related to performance on the hMWM task, the relationships among cell counts, rate of acquisition as reflected by the linear trend across trial blocks (higher value reflects steeper rate of acquisition), and the percent of time spent in the target quadrant on the probe trial (greater percentages indicative of greater use of a spatial strategy) were examined (Table 1 and Fig. 8). Spearman's correlation coefficients were first computed based on all rats tested; however, because inclusion of sham animals can inflate the degree of correlation (see Nunn et al., 1994), the correlations were also computed including only rats that experienced 5 or 10 min of ischemia. When all rats were included in the analyses, the correlation between cell count and the rate of acquisition approached significance ($\rho = .23$, $p = .08$) and the correlation between cell count and target quadrant bias on the probe trial was significant ($\rho = .57$, $p < .01$). Among only the ischemic rats, there was no significant relationship between cell count and acquisition ($\rho = .02$, $p = .90$) though the significant relationship between cell count and use of a spatial strategy did persist ($\rho = .62$, $p < .01$).

Estradiol Radioimmunoassay

The levels of estradiol in serum collected seven days following the final priming administration were analyzed with a 2-factor ANOVA with hormone treatment and surgical condition and between-subjects factors. This analysis revealed no significant main effects or interactions ($ps > .05$). Serum levels of estradiol in rats that were previously primed with estradiol were 13.86 pg/ml ($SEM = 1.53$) while levels in rats previously primed with oil were 11.02 pg/ml ($SEM = 1.38$).

Discussion

The findings presented here demonstrate that acute estradiol administered to ovariectomized rats 48 and 24 hrs prior to transient global ischemia is both neuroprotective and preserves spatial learning ability. Ischemia did not significantly affect performance on the cued platform version of the task, confirming that all the rats could see the local visual cue and could learn to swim directly to it. In contrast, both acquisition and probe trial performance on the hippocampally-dependent hidden platform version of the MWM task were significantly disrupted by both 5 and 10 min of transient global ischemia; acute priming with estradiol before ischemia protected against these impairments. Both 5 and 10 min of ischemia led to significant duration dependent cell death among CA1 pyramidal neurons but priming with estradiol reduced the magnitude of this damage. In contrast to recent work examining the functional consequences of estradiol-mediated neuroprotection (Gulinello et al., 2006), the relationship between performance on the spatial learning task and hippocampal damage was significant. Even when only rats that experienced ischemia were included in the analysis, the positive relationship between cell number and spatial bias for the target quadrant was significant. These findings indicate that increases in hippocampal damage are associated with impairments in both acquisition on the hMWM task as well as successful use of a spatial strategy and that preischemic administration of estradiol limits the adverse neuroanatomical and functional effects of ischemia.

Studies examining the effects of transient global ischemia on spatial learning using the MWM task have yielded inconsistent results (for review, see Block, 1999). For example, several studies report that the most pronounced differences in water maze performance between ischemic and control rats occur at the beginning of training and that the differences between ischemics and controls decline as training progresses (Block et al., 1995; Block and Schwarz, 1998; Green et al., 1995; Hagan and Beaughard, 1990; Olsen et al., 1994). In contrast, several studies including ours report that the performance of ischemic rats and controls is comparable at the start of water maze training but diverges across training as ischemic rats exhibit less improvement over repeated trials (Nunn et al., 1994). The source of such disparate patterns of

acquisition is unclear, though a variety of factors may contribute including differences in the degree of ischemia and associated cell loss, the recovery period between the ischemia surgery and the onset of behavioral testing, and the training protocol employed. Rats in our study performed 12 consecutive trials on a single day rather than the more typical spacing of blocks of training trials across several days (e.g., Block and Schwarz, 1998; Nunn et al., 1994). Spaced training and massed training on the MWM lead to differences in the retention of spatial learning (Spreng et al., 2002) and it is possible that this condensed training protocol is a more sensitive test of ischemia-related impairments in spatial learning. While studies vary in the reported patterns of performance of ischemic rats across training trials on the MWM task, they consistently report that ischemic rats exhibit significantly weaker spatial biases on probe trials that follow training (Block and Schwarz, 1997, 1998; Jaspers et al., 1990; Nelson et al., 1997; Nunn et al., 1994; Wright et al., 1996). Our data confirm this pattern of results with ischemic animals demonstrating a significantly weaker spatial bias on the probe trial.

Our data indicate that acute estradiol treatment before ischemia protects against the deficits in acquisition on the hMWM task that occur following either 5 or 10 min of transient global ischemia. In addition, estradiol priming before 5 min of ischemia prevents the impairment in probe trial performance exhibited by oil-primed animals though it does not protect against deficits seen in rats that experienced 10 min of ischemia. These findings contribute to a small, but growing literature reporting protective effects of estradiol on cognitive functions following ischemia. Using gerbils, Kondo, Suzuki, and Sakuma (1997) showed that treatment with high levels of estradiol via silastic capsules beginning one week prior to ischemia prevents the deficit in water maze acquisition that is apparent in untreated controls. Gulinello and colleagues (2006) recently reported that both chronic pretreatment with physiologic levels of estradiol as well as acute administration of high levels estradiol directly to the brain immediately following ischemia yield significant protection against impairments in visual and spatial working memory assessed with an object recognition task and an object placement task. Most recently, 15 days of estradiol treatment prior to ischemia has been shown to yield long lasting protection from ischemia-induced deficits in performance on an object recognition task and deficits in reference memory assessed with a radial arm maze (Plamondon et al., 2006). Unlike these studies which utilized either multiple weeks of estradiol prior to ischemia (Gulinello et al., 2006; Kondo et al., 1997; Plamondon et al., 2006) or one high pharmacological dose administered directly to the brain (Gulinello et al., 2006), we administered two acute injections of estradiol 48 and 24 hrs prior to ischemia. This pattern of hormone administration likely resulted in circulating levels of estradiol that were in the high physiological range at the time of ischemia (Woolley and McEwen, 1993). Such acute injections, however, do cause pharmacological levels of circulating estradiol within hours of each injection and it is possible that the pharmacological levels of estradiol achieved shortly after the injections are responsible for the behavioral and histological findings reported here. Whether similar evidence of neuroprotection and preservation of spatial learning ability is achieved by alternate forms of acute estradiol replacement that result in more stable, physiological levels of estradiol (e.g., time release pellets) has not been examined.

Our results also corroborate the findings of many studies that have reported estradiol-mediated neuroprotection in animal models of both focal ischemia (Dubal et al., 1998; Dubal and Wise, 2001; Rusa et al., 1999) and global ischemia (Bagetta et al., 2004; Jover et al., 2002; Shughrue and Merchenthaler, 2003; Sudo et al., 1997). Studies of the neuroprotective effects of estradiol using transient global ischemia have typically administered the hormone for multiple weeks prior to the ischemic insult (Gulinello et al., 2006; Jover et al., 2002; Kondo et al., 1997; Miller et al., 2005; Plamondon et al., 2006; Wang et al., 1999). For example, implanting pellets that continuously secrete estradiol for two weeks before and one week after ischemia yields significant protection of CA1 neurons (Jover et al., 2002; Miller et al., 2005). In these studies, neuroprotection was evident when animals were sacrificed 7 days after ischemia, when

estradiol levels continued to be elevated, but not when sacrificed 9 days after ischemia, after estradiol levels had fallen considerably (Gulinello et al., 2006). Based on these findings, it is possible that elevated serum estradiol levels must be maintained following ischemia in order to protect cells. Alternatively, estradiol may simply postpone the time course of cell death such that it is not evident after 7 days but is apparent after 9 days (see Corbett and Nurse, 1998). Neither of these hypotheses is supported by a recent study reporting that administration of estradiol for 15 days prior to ischemia, but not after, yields neuroprotection assessed six months later (Plamondon et al., 2006). Our data also suggest that estradiol levels need not be continuously elevated in order for neuroprotection to be maintained for at least 7 days as levels of estradiol in serum collected at that point in time did not differ between rats that had received preischemic priming with estradiol and those that received oil. Our assessment of cell survival is based on toluidine blue staining of hippocampal sections. While this methodology is widely used (De Butte et al., 2006; Gulinello et al., 2006; Jover et al., 2002; Miller et al., 2005), it is possible that cells identified as surviving are, in fact, beginning a period of delayed neuronal death. As such, we do not yet know whether the neuroprotective effects of acute estradiol administered prior to ischemia reported here are long-lasting.

In our study, the number of surviving CA1 neurons was significantly correlated with the expression of a spatial bias on the probe trial suggesting that these neurons may be critically involved in spatial learning and that damage to these cells via ischemia may underlie the impairments in spatial learning (Auer et al., 1989). Somewhat unexpectedly, the correlation between cell counts and the rate of acquisition across training as indicated by the linear trend was not statistically significant ($p = .08$). These patterns persisted even when only ischemic rats were included in the analysis. These findings may be due to the adoption of a non-spatial strategy by rats with extensive CA1 damage. In fact, the linear improvement across trials among oil-treated rats that experienced 10 min of ischemia did not differ from that of oil-treated rats that experienced 0 min of ischemia; on the probe trial, however, oil-treated rats that experienced 10 min of ischemia exhibited no bias for the target quadrant. Together, these findings suggest that severe cell loss associated with longer periods of ischemia may result in the adoption of a non-spatial strategy on the hMWM task. We are currently evaluating this hypothesis by implementing multiple probe trials at points throughout the training period (Flinn et al., 2005). While our data suggest that ischemia-induced deficits in spatial learning are related to hippocampal damage, several studies have reported little relationship between neuroanatomical damage and performance on learning and memory tasks (Gulinello et al., 2006; Kondo et al., 1997; Nunn et al., 1994). Other, however, have reported significant correlations between cell loss and performance on learning and memory tasks (Block and Schwarz, 1997, 1998; Kiyota et al., 1991; Nelson et al., 1997; Olsen et al., 1994). Several factors may contribute to these discrepancies. First, the use of learning tasks for which a non-spatial strategy can lead to improvements in performance may weaken the apparent correlation between cell loss and performance (Olsen et al., 1994). For example, rats with extensive cell loss may adopt alternative strategies to decrease pathlength across training on the water maze task and such use of a non-spatial strategy could diminish the magnitude of the correlation between performance and cell loss. Second, the ventral hippocampus is less vulnerable to ischemia than the dorsal hippocampus (Auer et al., 1989) and the inclusion of this region in the histological examination may result in a decrease in the size of the correlation. In the present study, we included only the dorsal hippocampus in our histological assessment and note a significant correlation between cell counts and spatial learning as reflected in the spatial bias demonstrated on the probe trial both when 0 min controls are included and when they are excluded. This finding suggests that increases in the degree of ischemia-induced CA1 cell loss lead to decrements in spatial learning and that animals with extensive cell loss may adopt alternative (non-spatial) strategies to decrease pathlength as training progresses.

Estradiol has previously been shown to significantly modulate performance on a variety of spatial learning and memory tasks (e.g., Daniel et al., 2006; Gibbs, 1999; Luine et al., 2003; Luine et al., 1998; Sandstrom and Williams, 2001, 2004; Walf et al., 2006). While estradiol significantly influenced performance on the hMWM task among ischemic rats, it did not influence performance of rats that did not experience ischemia. Some studies using the water maze have reported deleterious effects of high estradiol levels (Frye, 1995; Warren and Juraska, 1997; though see Berry et al., 1997). Behavioral testing in the present study occurred one week following hormone treatment. As such, it is likely that no differences in performance were evident because serum estradiol levels in estradiol-primed rats had returned to baseline by that point in time.

Several mechanisms have been identified through which estradiol may exert its protective effects on neurons and cognition. Estradiol may act centrally on the classic estrogen receptors, ER α and ER β , both of which act as ligand-activated transcription factors (see Toran-Allerand, 2004) and are expressed in hippocampal neurons (Milner et al., 2005; Milner et al., 2001; Shughrue et al., 1997). Both of these receptors have been shown to play significant roles in estradiol-mediated neuroprotection (Carswell et al., 2004; Dubal et al., 2001; Miller et al., 2005). In addition, estradiol has vasodilating effects 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). However, even when blood flow is controlled, estradiol yields protective effects on transient global ischemia induced cell death (Wang et al., 1999), highlighting an important perfusion-independent mechanism for neuroprotection. In addition to these receptor mediated actions, estradiol also has antioxidant effects that may also contribute to its neuroprotective properties (Behl et al., 1997; Prokai et al., 2003). It is not clear which of these mechanisms may underlie the protective effects of acute preischemic estradiol demonstrated in the present study.

The present findings demonstrate that estradiol protects against both CA1 cell loss and deficits in spatial learning when administered prior to transient global ischemia in young female rats. At the time of ischemia, however, the rats in our study were only 41 days of age. Estradiol has previously been shown to protect against ischemia-induced hippocampal damage in rats around this age (Gulinello et al., 2006; Miller et al., 2005). While adult female rats also experience significant neuroanatomical and behavioral protection from pre-ischemic estradiol (Plamondon et al., 2006), recent data suggest that middle-aged rodents may not benefit from estradiol administered before and after ischemia (De Butte et al., 2006). These findings suggest that the protective effects of estradiol may only be apparent in young animals. In fact, findings from the Women's Health Initiative studies on hormone replacement therapy indicate that postmenopausal women receiving either estrogens alone or estrogens plus progestins were at increased risk for cardiovascular diseases including stroke relative to women receiving placebo (Anderson et al., 2004; Rossouw et al., 2002). Together, these findings highlight the need to carefully examine the conditions under which and the mechanisms through which estradiol may impact cardiovascular function and response to ischemic events.

In conclusion, our findings demonstrate that acute estradiol administered prior to transient global ischemia limits the degree of CA1 cell loss and protects against impairments in spatial learning. Furthermore, the degree of spatial learning impairment resulting from ischemia is related to the magnitude of hippocampal cell loss. These findings contribute to a growing literature examining the effects of estradiol on neuronal and behavioral outcome following global ischemia. Further research examining both the periods before and after ischemia during which the brain is susceptible to the protective effects of estradiol, the stability of these effects over time, and the relationship between age and effect of estradiol may significantly influence the development of hormonally based strategies for the prevention of adverse neurobiological and cognitive effects of ischemia.

Acknowledgements

Thanks to Erika Williams, Lauren Williamson, and Sarah Chuzi for assistance with histology 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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Fig 1.

Time course of experimental manipulations and behavioral testing. Rats were ovariectomized at 33 days of age and received two priming injections of estradiol benzoate at 39 and 40 days of age. The first stage of the 4-VO procedure was performed at 40 days of age with clamping of the carotid arteries at age 41 days. Behavioral training began 5 days following surgery and consisted of platform pretraining, cMWM training, and hMWM training. Rats were sacrificed immediately following hMWM training on day 48.

Fig 2.

Neither estradiol nor ischemia affects acquisition on the cMWM task. All three surgical conditions (0, 5, and 10 min of ischemia) demonstrated significant improvements across training with no effect of hormone treatment. Data are reported as the mean distances traveled per trial in blocks of three trials ($\pm$ SEM).

Fig 3.

Estradiol administered 48 and 24 hrs prior to ischemia limits the ischemia-induced impairment in performance on the hMWM task. Among rats that experienced 0 min of ischemia (left panel), estradiol had no effect on acquisition. Among rats that experienced 5 min of ischemia (middle panel), pre-ischemic estradiol prevented the significant impairment in acquisition evident in rats that received oil. Among rats that experienced 10 min of ischemia (right panel), pre-ischemic estradiol maintained acquisition while priming with oil resulted in a slight impairment in acquisition. See text for a complete description of the statistical analyses. Data are reported as the mean distances traveled per trial in blocks of three trials ($\pm$ SEM). Significant differences between oil-primed and estradiol-primed groups are indicated by * ($p < .05$).

Fig 4.

Estradiol protects against impairments in hMWM acquisition resulting from mild ischemia (5 min). The rate of acquisition as reflected by the linear trend across trial blocks (higher values indicative of steeper slope) was significantly lower among oil-primed rats that experienced 5 min of ischemia. Pre-ischemic estradiol maintained the rate of acquisition among rats that experienced 5 min of ischemia. The rate of acquisition for rats that experienced 10 min of ischemia was not significantly affected, regardless of whether they received priming with oil or estradiol. Data are reported as the mean linear trend contrast value (higher values reflect steeper acquisition function) based on the mean distances traveled per trial in blocks of three trials ($\pm$ SEM). Significant differences are indicated by * ($p < .05$).

Fig 5.

Estradiol priming protects against the ischemia-induced decrease in the bias for the target quadrant during the probe trial. Both 5 and 10 min of ischemia caused a reduction in the spatial bias exhibited on the probe trial but estradiol priming prevented this decline for animals that experienced 5 min of ischemia. Data are reported as the mean percentage of the probe trial spent in the target quadrant ($\pm$ SEM). Significant differences are indicated by * ($p < .05$).

Fig 6.

Pre-ischemic estradiol prevents cell loss resulting from transient global ischemia.

Ovariectomized rats were primed with oil or estradiol 48 and 24 hrs prior to either 0, 5, or 10 min of 4-VO. Rats were then behaviorally tested and sacrificed 7 days following ischemia. Twenty micron thick sections were stained with toluidine blue and surviving neurons were counted in a 400 x 100 μm area of the hippocampal CA1 (boxed in lower magnification images a–c, g–i). Higher magnification images (d–f, j–l) depict boxed areas. Scale bars: lower magnification (a–c, g–i), 500 μm ; higher magnification (d–f, j–l), 100 μm .

Fig 7.

Acute estradiol administered prior to ischemia increases cell survival among CA1 pyramidal neurons. Cell survival declined as the duration of ischemia increased, but cell survival was enhanced by estradiol priming for animals that experienced either 5 or 10 min of ischemia. Data are reported as the mean number of cells per 400 μm segment of CA1 ($\pm$ SEM). Significant differences are indicated by * ($p < .05$).

Fig 8.

Survival among CA1 pyramidal cells is positively correlated with the use of a spatial strategy. The rank-ordered data for cell counts per 400 μm is plotted as a function of the rank-ordered data for the percentage of the probe trial spent in the target quadrant. This relationship was significant when all animals were included in the analysis (solid line: $\rho = .57, p < .01$) as well as when only those animals that experienced ischemia were included in the analysis (dashed line: $\rho = .62, p < .01$).

Table 1

Spearman's rank order correlations between CA1 cell survival and performance on the hMWM task. Cell counts were correlated with the rate of acquisition as measured by the linear improvement in pathlength across trial blocks (linear trend contrast value with higher values reflect steeper rates of acquisition), and with the degree of target quadrant preference on the probe trial. Separate correlations were computed based on the data from all rats and on data only from rats in the 5 and 10 min ischemia conditions. Significant correlations indicated by * ($p < .01$)

	CA1 Cell Counts	
	All Rats (0, 5, & 10 min) N = 59	Ischemics Only (5 & 10 min) N = 36
Rate of Acquisition (linear trend contrast value)	$\rho = .23$	$\rho = .02$
% Time in Target Quadrant	$\rho = .57 *$	$\rho = .62 *$