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Long-Term Estrogen Therapy Worsens the Behavioral and Neuropathological Consequences of Chronic Brain Inflammation

L. K. Marriott, B. Hauss-Wegrzyniak, R. S. Benton, P. D. Vraniak, and G. L. Wenk
University of Arizona

Alzheimer's disease (AD) is accompanied by chronic neuroinflammation and occurs with greater incidence in postmenopausal women. The increased incidence may be delayed by estrogen replacement therapy (ERT). The authors investigated the interaction of chronic ERT and lipopolysaccharide (LPS)-induced neuroinflammation in the female rat. Ovariectomy did not impair water maze performance; however, addition of chronic ERT or neuroinflammation resulted in an impairment that became exacerbated by the simultaneous occurrence of both conditions. Chronic LPS activated microglia, which was not reduced by ERT. Intact females receiving LPS infusion were not impaired in the water maze and had significantly fewer activated microglia. Results suggest that chronic ERT in postmenopausal women may exacerbate the memory impairment induced by the chronic neuroinflammation associated with AD.

Inflammatory processes play an important role in the etiology and pathogenesis of Alzheimer's disease (AD; Akiyama et al., 2000). The neuritic plaques that are associated with this disease contain β-amyloid, as well as many activated glial cells that release inflammatory proteins (McGeer, Akiyama, Itagaki, & McGeer, 1989; Mrak, Sheng, & Griffin, 1995). The consequences of long-term exposure to inflammatory proteins may be neuronal degeneration (Banati, Gehrmann, Schubert, & Kreutzberg, 1993; Gonzalez-Scarano & Baltuch, 1999; McGeer & McGeer, 1998).

In previous studies, we used chronic infusion of lipopolysaccharide (LPS) into the fourth ventricle of male rats to produce an inflammatory response throughout the brain (Hauss-Wegrzyniak, Dobrzanski, Stoehr, & Wenk, 1998; Wenk & Hauss-Wegrzyniak, 2001). This model reproduced many components of the pathological, biochemical, and behavioral changes associated with AD (Wenk & Hauss-Wegrzyniak, 2001), including, but not limited to, the loss of hippocampal pyramidal neurons (Hauss-Wegrzyniak, Dobrzanski, et al., 1998). The greatest inflammatory response was found within the hippocampus–entorhinal cortex, cingulate gyrus, and nucleus basalis magnocellularis (NBM; Hauss-Wegrzyniak, Dobrzanski, et al., 1998; Wenk, Hauss-Wegrzyniak, & Willard, 2000); these regional neurobiological changes may underlie the observed impairment in learning and memory ability (Hauss-Wegrzyniak, Vraniak, & Wenk, 1999, 2000).

Estrogen deprivation has also been implicated in the pathogenesis of AD (Manly et al., 2000; Paganini-Hill & Henderson, 1994). Postmenopausal women demonstrate an increased incidence of AD (Gandy & Duff, 2000; Manly et al., 2000), and the level of serum estradiol is lower in women who develop AD (Manly et al., 2000). Epidemiological evidence suggests that postmenopausal estrogen replacement therapy (ERT) may reduce the risk or delay the onset of AD (Kawas et al., 1997; van Duijn, 1999; Yaffe, Sawaya, Lieberburg, & Grady, 1998). Because estrogen has many neuroprotective and neurotrophic proclivities (Goodman, Bruce, Cheng, & Mattson, 1996; Green & Simpkins, 2000; Wise, Dubal, Wilson, Rau, & Liu, 2001), its decline with menopause might leave the brain vulnerable to the toxic influence of elevated levels of β-amyloid or inflammatory proteins such as seen in AD (Brinton et al., 2000; Gandy & Duff, 2000; Jaffe, Toran-Allerand, Greengard, & Gandy, 1994; Li et al., 2000; Mattson, Robinson, & Guo, 1997; Paganini-Hill & Henderson, 1994; Silva, Mor, & Naftolin, 2001; Xu et al., 1998).

Estrogen's ability to attenuate the progress of AD (Duka, Tasker, & McGowan, 2000; Jacobs et al., 1998; Kawas et al., 1997) may be due to its ability to reduce the activation of microglia (Mor et al., 1999). Estrogen's ability to protect vulnerable neuronal populations within the AD brain may be due to the presence of estrogen receptors on neurons and glia (Mufson et al., 1999; Stone et al., 1997). A recent in vitro study demonstrated that 17β-estradiol could dose-dependently prevent the activation of primary cultures of rat microglia and reduce the expression of inflammatory proteins (Vegeto et al., 2001). Given the apparent neuroprotective actions of estrogen within the brain of female humans at risk for AD, the present study investigated estrogen's ability to influence the consequences of chronic widespread inflammation within the brain of female rats.

Method

Subjects

Forty virgin female F-344 rats (Harlan Sprague-Dawley breeders, Madison, WI), aged 3 months, were housed in triplicate in a temperature-controlled room (21 °C) with food and water available ad libitum. All rats were maintained on a 12-hr light–dark cycle with lights off at 10 a.m. and

L. K. Marriott, B. Hauss-Wegrzyniak, R. S. Benton, P. D. Vraniak, and G. L. Wenk, Arizona Research Laboratories, Division of Neural Systems, Memory, and Aging, University of Arizona.

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Correspondence concerning this article should be addressed to G. L. Wenk, Arizona Research Laboratories, Division of Neural Systems, Memory, and Aging, University of Arizona, 384 Life Sciences North Building, Tucson, Arizona 85724. E-mail: gary@nsma.arizona.edu

were allowed to adjust to their new environment for 1 week after arrival. The health of the rats was monitored regularly, and all rats were randomly assigned to one of six treatment groups (see below).

Surgery

Each rat received 0.3 ml atropine methylbromide (5 mg/ml ip) and Nembutal (50 mg/kg ip) and was placed in a stereotaxic instrument with the incisor bar set at 3.0 mm below the ear bars. An osmotic minipump (0.25 μl/hr, Model 2004; Alzet, Palo Alto, CA) containing either LPS (*E. coli*, serotype 055:B5, TCA extraction; 1.0 μg/μl; Sigma, St. Louis, MO) or artificial cerebrospinal fluid (CSF: 140.0 mM NaCl, 3.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM Na_2HPO_4; pH 7.4) was implanted into the dorsal abdomen and attached with Tygon tubing (0.02 in. [0.05 cm] i.d. $\times$ 0.06 in. [0.15 cm] o.d.) to a chronic indwelling cannula (28 gauge, Model 3280P; Plastics One, Roanoke, VA). The cannula was positioned stereotaxically –2.5 mm from lambda on the midline, so that the cannula tip extended 7.0 mm ventral from dura and into the fourth ventricle. Previous work established a dose–response curve for LPS infusions in male rats, identifying an optimal dose that would produce adequate levels of brain inflammation without producing systemic toxicity (Hauss-Wegrzyniak, Lukovic, Bigaud, & Stoeckel, 1998). The same optimal dose determined for male rats was selected for the female rats in the present study. Rats in the control group were infused with CSF. LPS was dissolved in CSF. A change in volume to the CSF space was discounted because the 0.25 μl/hr infusion rate contributed only to about 0.4% of the CSF volume produced by the rat each hour and was only 0.25% of the rat's total CSF volume. The Tygon tubing was prefilled with CSF for all rats and had a total volume of 12 μl; therefore, the LPS solution did not reach the fourth ventricle until approximately 2 days after surgery. This delay was introduced in order to prevent the development of the inflammatory reaction within the brain before the availability of estrogen from the silastic capsules (see below). A recent study suggested that the neuroprotective effects of estrogen are absent if the inflammatory reaction is already ongoing (Vegeto et al., 2001).

Experimental Design

In contrast to postmenopausal women, aged rats do not undergo an ovarian failure and experience a 2–3 month period of persistent estrus or diestrus before becoming acyclic (LeFevre & McClintock, 1988). Although aged female rats experience changes in gene expression, ovariectomized rats experience both the ovarian failure and the alterations in gene expression that more closely mimic the changes seen in postmenopausal women (for a review, see Rance & Abel, 2001). For these reasons, after implantation of the cannula, each rat was either bilaterally ovariectomized (OVX) or remained intact. All ovariectomized rats were implanted subcutaneously with 5 mm silastic capsules (0.058 in. [0.147 cm] i.d., 0.077 in. [0.195 cm] o.d.; Dow Corning, Midland, MI) containing estrogen (E2, 25% 17β-estradiol and 75% cholesterol, Sigma), or control capsules (oil, containing 100% cholesterol). Our preliminary radioimmunoassay studies showed that physiological levels of blood estrogen after capsule implantation were maintained at 30 and 5 pg/ml for estrogen and oil treatments, respectively. These capsules can maintain constant blood levels of 17β-estradiol for at least 2 months (Gibbs, 2000b) and were therefore exchanged for a new capsule containing the same treatment (E2 or oil) after 40 days to ensure continuous levels of estrogen for the duration of the experiment. The replacement of estrogen in a chronic, rather than cyclical, manner mimics the method of ERT commonly given to postmenopausal women (Ansbacher, 2001). The six treatment groups were as follows (minipump contents + hormonal status): CSF + OVX–oil (n = 7), CSF + OVX–E2 (n = 7), LPS + OVX–oil (n = 5), LPS + OVX–E2 (n = 8), CSF + Intact (n = 8), and LPS + Intact (n = 5).

For postoperative care, all rats were injected subcutaneously with 3 ml of 0.9% (wt/vol) sterile isotonic saline to prevent dehydration during recovery, and Betadine was applied to all incisions to minimize the risk of infection. The rats were monitored closely during recovery and kept in an incubator (29 °C, Ohio Chemical & Surgical Equipment, Madison, WI) until fully awake and active. All of the surgical procedures described above were performed during the same surgical episode. The rats were then housed in triplicate with other rats undergoing the same treatment in order to reduce the impact of housing on cycling in rats receiving different treatments (Stern & McClintock, 1998); however, all rats were housed in the same colony room for the duration of the experiment. Body weights were monitored, and nutritional supplements were provided as necessary during the infusion period.

Behavioral Testing

Vaginal smears were taken each morning, beginning at Day 49 and continuing for the duration of the study, in order to determine the cycle stage of intact rats, validate the ovariectomy procedure, and determine the effectiveness of ERT to the OVX rats. The osmotic minipumps ceased functioning by 42 days after surgery (according to calculations provided by the manufacturer). Behavioral testing for all rats began 56 days after surgery. Therefore, 2 weeks were allowed for the acute effects of the LPS infusion into the brain to dissipate. This delay was introduced because acute LPS-induced elevation of cytokines has been associated with increased drowsiness and sleep in rodents (Schiffelholz & Lancel, 2001).

All rats were tested in the morning (8 a.m.–12 p.m.) in the Morris water maze task, as previously described (Hauss-Wegrzyniak, Dobrzanski, et al., 1998). The water maze was located in the center of a room (2.30 m long $\times$ 2.50 m high $\times$ 2.75 m wide) that was surrounded by a circular black curtain with cardboard cutouts as distal cues. Proximal cues were leaned against the wall of the water maze and included a folding chair and metal board. The water maze was 185 cm in diameter, with white walls. The water was made opaque by adding white nontoxic paint and was maintained at 27 °C. The circular escape platform was 11.5 cm in diameter. For the spatial (hidden platform) version of the task, the platform was submerged 1 cm beneath the water surface and maintained in a constant location. Black capes were mounted on the rats to create a contrast between the rat and the opaque water. Rats were tracked with an overhead video camera connected to a VP114 tracking system (HVS Image, Buckingham, England), and the data were stored on a computer database for later analysis of the rat's path during trials.

Rats were trained over 4 consecutive mornings with three training blocks that consisted of two trials each (24 trials total). The rats did not receive any pretraining experience in the water pool. Training blocks were separated by 30 min to allow the rats to rest in the incubator (29 °C). On each trial, a rat was placed in the water, facing the wall, in one of seven random locations spaced evenly around the perimeter of the tank. After the rat found the escape platform, it remained there for 30 s. Rats that had not found the platform within 60 s were placed on the platform by the experimenter and allowed to remain there for 30 s. At the end of the 4th day (24 trials), the hidden platform was removed from the pool and the rat was given a probe trial, in which all rats were started from the same location and allowed to swim freely for 60 s. The visual acuity and swim ability of the rats were tested on a visible version of the task on Days 4 and 5. A visible platform was raised 2 cm above the water surface and moved randomly to one of four locations in the tank after each trial. Six visible-platform trials were performed each day. Path length data were analyzed by a two-way repeated measures analysis of variance (ANOVA); multiple planned, pairwise post hoc comparisons were performed with Tukey's tests.

Biochemical Studies

After completion of the behavioral testing, 20 rats were anesthetized with isofluorane and killed by decapitation; their brains were then removed

for dissection. A section of frontal sensorimotor cortex was isolated and stored (−70 °C) until assayed for choline acetyltransferase (ChAT) activity by the formation of [14C]-acetylcholine from [14C]-acetyl-coenzyme-A (New England Nuclear, Boston, MA) and choline according to the method of Fonnum (1969). The ChAT enzyme is specific for cholinergic cells; its decline in the cortex is used as a standard measure of cholinergic cell loss within the basal forebrain region (Wenk, Stoehr, Quintana, Mobley, & Wiley, 1994). Protein content was determined for each sample (Lowry, Rosenbrough, Farr, & Randall, 1951). All assays were performed in triplicate; results were analyzed by ANOVA.

Histological Studies

Twenty rats were anesthetized with isofluorane and transcardially perfused with ice-cold 0.9% (wt/vol) saline containing 51.0 mg/L heparin followed by perfusion with filtered 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). The brain was removed, postfixed for 1 hr in 4% paraformaldehyde/0.1 M sodium phosphate buffer before cryoprotection in 0.1 M sodium phosphate buffer containing increasing amounts of sucrose: 10%, 20%, and 30% (wt/vol) solutions at 4 °C for 24 hr each. Brains were then snap-frozen in isopentane on dry ice (−50 °C) and stored at −70 °C. Immediately before sectioning on a cryostat, the brains were allowed to warm to −20 °C before coronal sections (40 μm) were taken beginning at the anterior commissure. Sections containing the basal forebrain and hippocampus were placed in plastic wells containing 0.1 M phosphate buffered saline (PBS) before being transferred into a cryoprotective solution containing PBS, ethylene glycol, and glycerol (pH 7.4). The sections were stored in 1.5 ml Eppendorf centrifuge tubes (−40 °C) until used for immunohistochemistry. The sections were rinsed and pretreated in 0.3% (wt/vol) hydrogen peroxide solution before staining in order to block the endogenous peroxidase activity. Activated microglia were visualized by means of a monoclonal antibody (OX-6, 1/400 dilution; PharMingen, San Diego, CA) directed against the major histocompatibility complex, Class II (MHC II; Finsen, Jorgensen, & Zimmer, 1993; Flaris, Densmore, Molleston, & Hickey, 1993) antigens. The location of OX-6-positive cells was examined by light microscopy, and the cells were plotted with a camera lucida by an experimenter who was unaware of rats' group assignments. The number of OX-6-positive cells in the hippocampus, amygdala, lateral hypothalamus, zona incerta, central thalamic nucleus, cingulate gyrus/retrosplenial cortex, piriform/entorhinal cortex, and parafascicular cortex were counted, using multiple comparable regions from each rat in each group. These regions were chosen because they contained the highest concentration of activated microglia. Regions of brain outside of these areas contained comparatively few activated microglia. All sections from each brain were organized in order from rostral to caudal; landmarks were used to determine laterality for all sections. White matter and fiber tracts were drawn to serve as landmarks as brain sections were mapped onto Plates 20–24 of the atlas by Pellegrino, Pellegrino, and Cushman (1979). All attempts were made to remain consistent with the measurements used in the atlas, and all boundary measurements represent distances in real brain according to the atlas. The boundaries of the regions counted are shown in detail in Figure 1.

A horizontal line that connected the rhinal fissure bilaterally and a line that extended the boundaries of the external capsule determined the counting boundary of the amygdaloid complex. From the intersection of the rhinal fissure and external capsule, a 1.66-mm^2 box was drawn that extended medially and ventrally. The cingulate gyrus/retrosplenial cortex boundaries were 1.25 mm bilaterally from the longitudinal fissure and were bordered by the dorsal aspect of the brain and ventrally by the corpus callosum. The counting boundary of the lateral hypothalamus was determined by a vertical line 1.25 mm parallel to the third ventricle that extended dorsally 1.66 mm from the ventral aspect of the brain. A perpendicular line was drawn laterally for 1.25 mm, and another line descended ventrally to meet the optic tract. The dorsal borders of the optic tract and

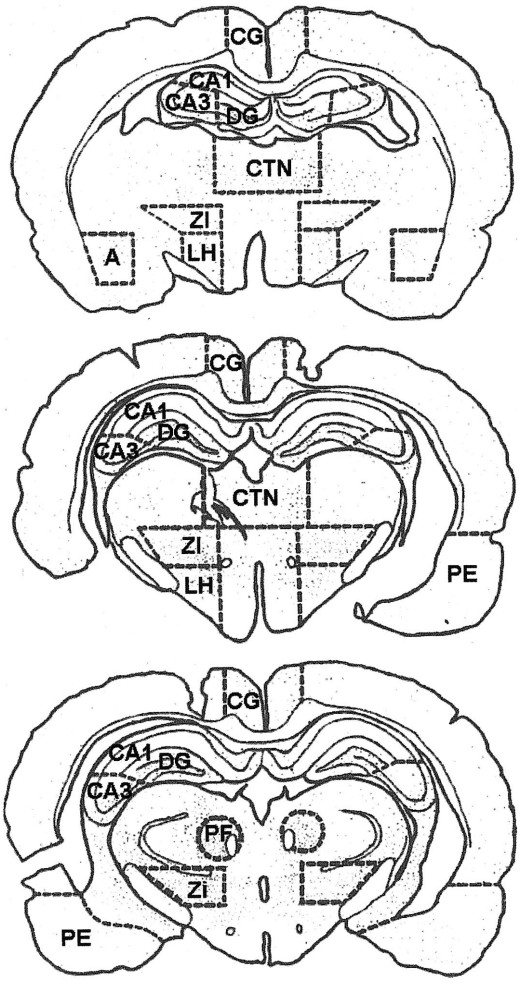


Figure 1. Camera lucida drawings of representative coronal sections showing the outline of brain regions in which the numbers of microglia were counted. The number of OX-6-positive cells in the cingulate gyrus/retrosplenial cortex (CG), hippocampus (including cornu ammonis [CA1, CA3] and dentate gyrus [DG]), central thalamic nucleus (CTN), zona incerta (ZI), amygdala (A), lateral hypothalamus (LH), piriform/entorhinal cortex (PE), and parafascicular cortex (PF) were counted by using four comparable regions from each rat from each group.

supraoptic decussation, as well as the ventral aspect of the brain, marked the ventral counting boundaries. The zona incerta was determined by extending, by 0.83 mm, the vertical line that was used to determine the medial aspect of the lateral hypothalamus. A perpendicular line was then drawn laterally 2.5 mm and connected at an acute angle to the dorsolateral corner of the lateral hypothalamic border. Although this approximate shape remained the same, variations in size reflected the rostrocaudal location of the section. The lateral boundary of the zona incerta was the dorsomedial border of the internal capsule, whereas the dorsal boundary of the lateral hypothalamus remained the ventral counting boundary. The entirety of the hippocampus served as the outer boundary of the hippocampus, excluding the fimbria. The subregions were then assigned to dentate gyrus, CA3, or CA1 as shown in Figure 1. The boundaries of the central thalamic nucleus were determined by drawing a horizontal line that extended 1.66 mm from the midline. From this lateral point, a perpendicular line was drawn 2.08 mm ventrally from the dorsal aspect of the thalamus. For the boundaries of the piriform cortex, the rhinal fissure was connected bilaterally and served

as the dorsal boundary. The lateral ventricles served as a medial border, and the outer aspects of the brain marked the edge of the lateral and ventral counting regions. Finally, a circle with a radius of 0.625 mm encompassed the parafascicular cortex containing the fasciculus retroflexus. The dorsal border of the circle began 1.25 mm ventrally from the dorsal edge of the thalamus; the medial border began 0.625 mm from the third ventricle. After all sections and microglia locations were drawn, each dot (representing three distinct activated microglia) was counted for each section by an experimenter who was unaware of rats' group assignments. The data were analyzed by ANOVA.

Vaginal smears were stained with hematoxylin (7.5 g/L, Sigma) and eosin (0.5% wt/vol in 90% ethanol, Sigma) solutions and staged by visual examination to confirm the phase of the estrous cycle. The lavages were examined under a light microscope to identify the type and proportion of cornified epithelial cells (estrus), nucleated epithelial cells (proestrus), or leukocytes (diestrus). Each daily smear was assigned to one of these three cytological stages by an experimenter who was unaware of rats' group assignments.

Results

All rats gained weight during LPS or CSF infusion. None of the rats was observed to have seizure activity or any other indication of toxicity or poor health. We have previously demonstrated that this infusion procedure does not produce fever in male rats (Hauss-Wegrzyniak, Dobrzanski, et al., 1998). OVX rats that received E2 treatment maintained a vaginal cytology of estrus for the duration of the experiment, whereas those that received the oil control treatment remained in diestrus. Intact rats had normal 4-day cycle fluctuations that continued during behavioral testing. On the 1st day of water maze testing, the CSF- and LPS-infused intact rats were in a variety of estrous states, that is, most rats were in estrus, and a few rats in each group were in either proestrus or diestrus.

Behavioral Studies

The results of analysis of path length to find the hidden platform for each group of rats are shown in Figure 2. Path length data are presented because they eliminate the effect of variations in the rats' swim speed, a factor that can be influenced by estrous phase (Frye, 1995; Healy, Braham, & Braithwaite, 1999). In the present investigation, velocity differed significantly across groups, $F(5, 959) = 26.1$, $p < .001$. A two-way repeated measures ANOVA of the path length data revealed an overall main effect of testing day, $F(3, 959) = 65.4$, $p < .001$, for all groups; an overall main effect of group, $F(5, 959) = 21.8$, $p < .001$; and a significant Group × Day interaction, $F(15, 959) = 1.99$, $p = .013$.

There was no difference ($p > .05$) in performance between intact rats infused with CSF and OVX rats infused with CSF. In contrast, the performance of OVX rats infused with CSF that were treated with chronic ERT was significantly impaired ($p = .001$) compared with that of OVX rats infused with CSF but not given constant ERT. There was no significant difference ($p > .05$) in performance between intact rats infused with LPS or intact rats infused with CSF.

Chronic infusion of LPS into the fourth ventricle of OVX female rats receiving oil significantly impaired ($p < .001$) performance compared with intact rats chronically infused with LPS. The chronic infusion of LPS into the fourth ventricle of OVX rats that were treated chronically with estrogen significantly impaired performance, as compared to LPS-infused rats treated chronically with oil ($p = .002$) or to CSF-infused rats treated chronically with estrogen ($p < .001$). All rats were able to discover the visible platform at the end of the behavioral testing. At the end of the hidden platform testing phase, all rats were given a probe trial, and the number of target crossings were determined. A one-way ANOVA of the probe data found a significant main effect of group, $F(5, 47) = 2.495$, $p < .046$, and the results paralleled those seen in the analysis spatial trial data.

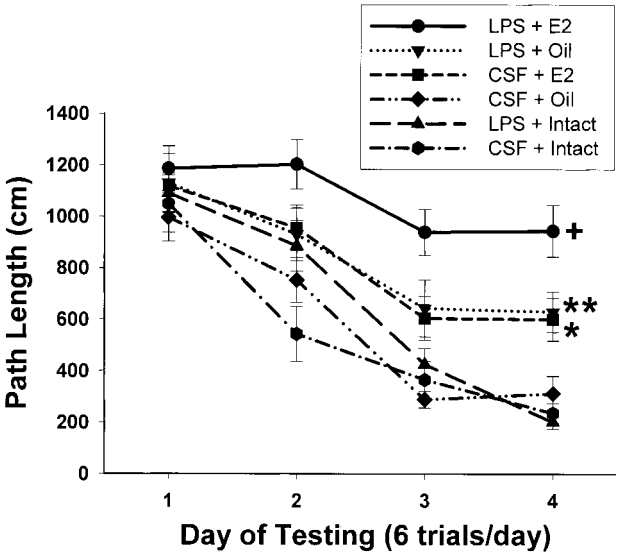


Figure 2. Performance of young female rats in the Morris water maze task. In the absence of ovaries, chronic neuroinflammation impaired performance (** $p = .002$) and chronic estrogen therapy impaired performance (* $p = .001$), as compared with intact rats or CSF + oil-treated rats. The combined presence of these two conditions produced a significantly greater impairment (+ $p < .001$) than either condition alone (i.e., as compared with the CSF + E2 and LPS + oil groups). Oil was 100% cholesterol as vehicle. CSF = cerebrospinal fluid infusion; LPS = lipopolysaccharide infusion; E2 = estrogen (a mixture of 25% 17β-estradiol and 75% cholesterol) treatment.

Histology

Intact rats infused with CSF or OVX rats infused with CSF and chronically treated with either oil or estrogen had only a small number of activated microglia scattered throughout their brain (see Figure 3). After chronic infusion of LPS into the fourth ventricle of intact or OVX rats, immunocytochemical staining for the MHC II (using the OX-6 antibody) found numerous, highly activated microglia distributed throughout the selected brain regions mentioned above. The activated microglia were characterized by a contraction of their highly ramified processes that appeared bushy in morphology (see Figure 4). The results of an investigation of the total number of OX-6-positive microglia within all eight regions outlined in Figure 1 are shown in Figure 5. An ANOVA found a significant main effect of group, $F(5, 20) = 34.1$, $p < .001$. Post hoc pairwise comparisons using a Tukey test found that the LPS infusion, into either intact ($p < .001$) or ovariectomized ($p < .001$) rats, significantly increased the number of activated microglia in these brain regions, as compared with all CSF-infused rats.

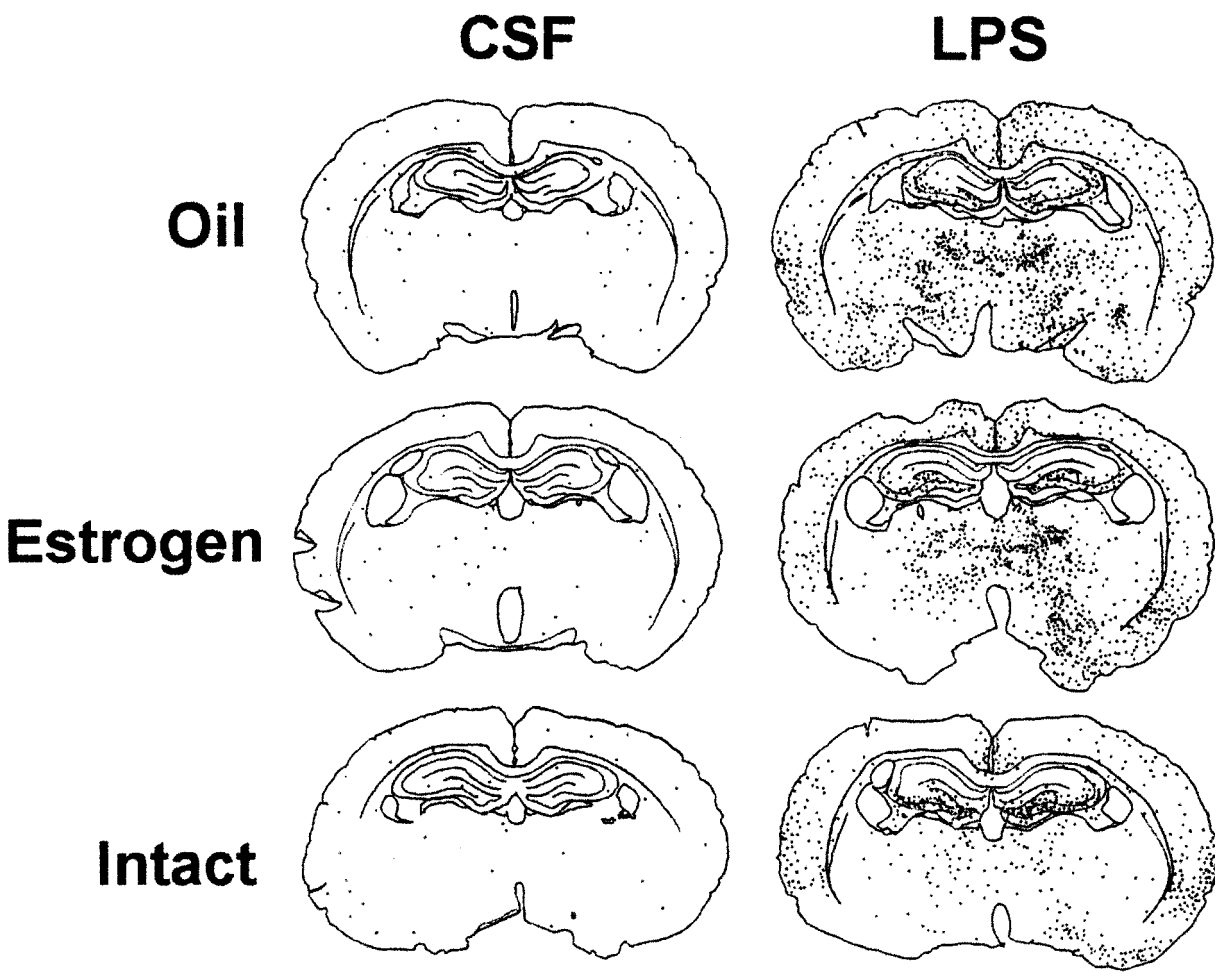


Figure 3. Camera lucida drawings of representative coronal sections showing brain inflammatory reaction in cerebrospinal fluid (CSF)- and lipopolysaccharide (LPS)-infused rats that were intact or ovariectomized and treated with estrogen (a mixture of 25% 17β-estradiol and 75% cholesterol) or oil (100% cholesterol as vehicle). Each dot represents three OX-6-positive, activated microglial cells. Only a few activated microglial cells are scattered throughout the brains of rats infused with CSF. Chronic LPS infusion significantly increased the number of activated microglial cells in all groups. See Figure 5 for quantification of microglial cell numbers.

However, LPS-infused intact rats had significantly fewer activated microglia as compared to LPS-infused, OVX rats given either estrogen ($p = .02$) or the oil vehicle ($p < .01$). Chronic ERT did not significantly increase the ability of the LPS infusion to activate additional microglia compared with oil treatment. Similar to the effects of these conditions upon behavior, chronic ERT or ovariectomy increased the responsiveness of resident microglia to the effects of chronic neuroinflammation. No significant correlations between any of the following variables were found: the number of activated microglia versus path length, blood level of estradiol versus path length, number of activated microglia versus blood level of estradiol.

Biochemistry

ChAT activity in the sensorimotor frontal neocortex of the CSF intact group was 47.8 nmol • hr^{-1} • mg $protein^{-1}$. An ANOVA found no significant alteration, $F(4, 16) = 1.09$, $p = .41$, in the level of this enzyme as a result of estrogen therapy or neuroinflammation in female rats (data not shown).

Discussion

The present study investigated the interaction of two conditions known to exist within the brains of female AD patients: the presence of chronic neuroinflammation and either estrogen deprivation or chronic ERT. It is worth noting that these two conditions are likely to precede the onset of symptoms associated with AD. In the present study, the administration of chronic LPS to young intact female rats resulted in no behavioral impairment and approximately half the number of activated microglia as compared with OVX rats receiving the same treatment. These results demonstrate a neuroprotective role of gonadal hormones, such as estrogen, and are consistent with findings from other laboratories reporting a neuroprotective role of estrogen in both in vivo and in

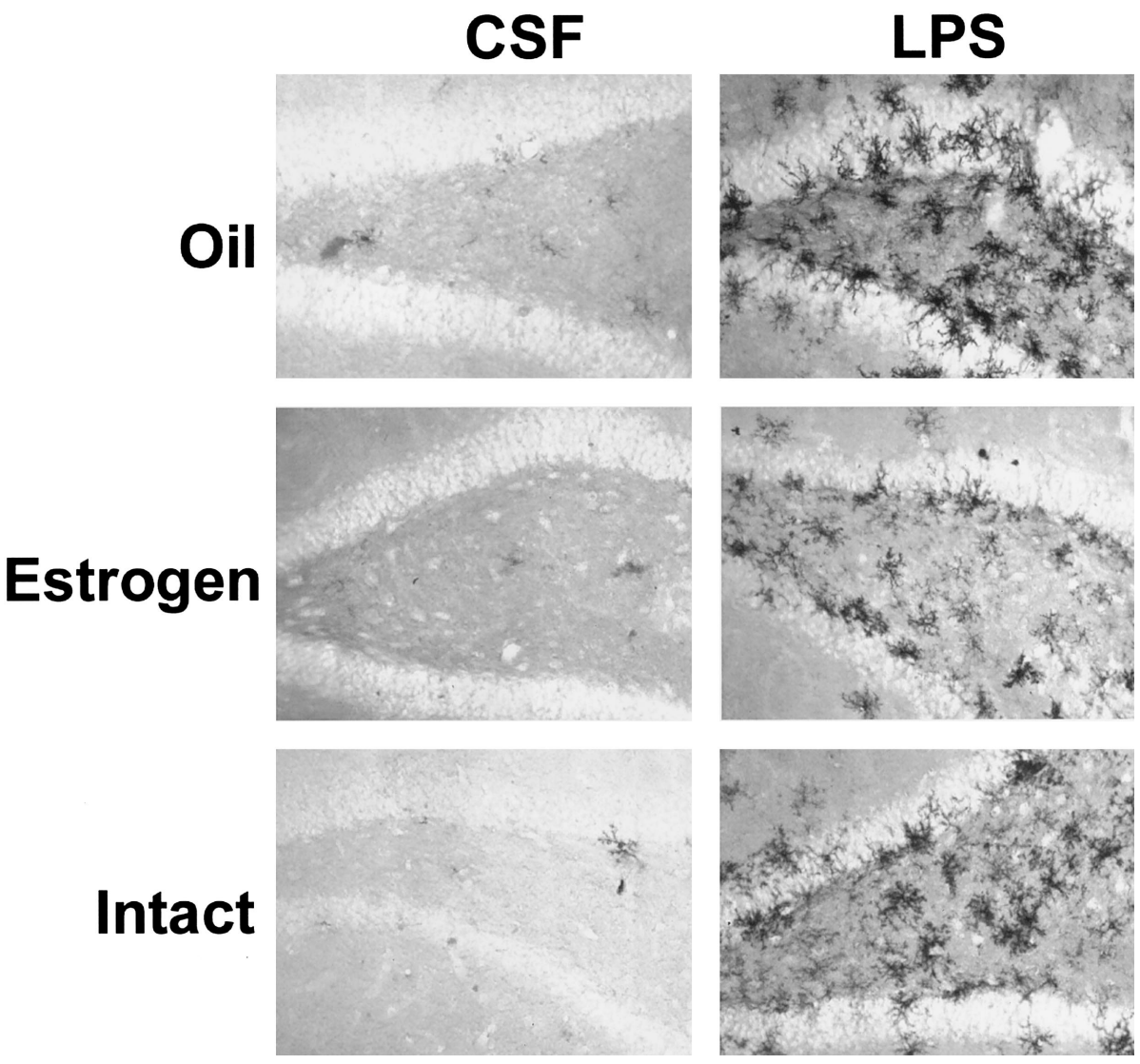


Figure 4. Reactive microglia (stained with OX-6 antibody) within the dentate gyrus of the hippocampus (×10 magnification). Only a few activated microglial cells are scattered throughout the brains of rats infused with cerebrospinal fluid (CSF), whereas chronic lipopolysaccharide (LPS) infusion significantly increased the number of activated microglial cells in all groups. The reactive microglia in the LPS-treated rats were characterized by a contraction of their highly ramified processes that appeared bushy in morphology. Estrogen was a mixture of 25% 17β-estradiol and 75% cholesterol; oil was 100% cholesterol as vehicle.

vitro preparations against a variety of brain insults (for review see Wise et al., 2001).

The interaction between estrogen and brain inflammation appears more complicated when estrogen is administered at constant low physiological doses for an extended period. In the present study, the removal of the ovaries was not sufficient to impair performance in the Morris water maze task. However, the introduction of either chronic ERT or chronic brain inflammation was sufficient to impair performance to a similar degree in OVX rats. Furthermore, the combined occurrence of both conditions, that is, chronic ERT and long-term brain inflammation in OVX rats, significantly worsened cognitive performance beyond that produced by either condition alone. These findings are significant because the conditions reproduced in this study are analogous to the situation of postmenopausal women who have existing brain inflammation caused by a neurodegenerative illness (Akiyama et al., 2000) or by head trauma (Rasmusson, Brandt, Martin, & Folstein, 1995) and then choose to undergo chronic ERT.

Our results suggest that chronic ERT in postmenopausal women may exacerbate the memory impairment induced by the presence of chronic neuroinflammation associated with AD. The results of a recent long-term (1-year), placebo-controlled study confirmed this prediction (Mulnard et al., 2000) and examined the effects of ERT on cognitive function in a large group of women with mild to moderate AD. The effects of ERT were initially beneficial, similar to previous reports using smaller groups of patients and a shorter

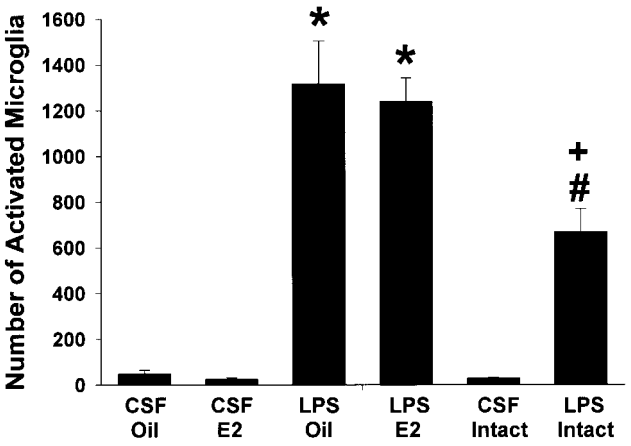


Figure 5. Semiquantitative determination of the number of OX-6-positive microglia in the hippocampus, amygdala, lateral hypothalamus, zona incerta, central thalamic nucleus, cingulate gyrus/retrosplenial cortex, piriform/entorhinal cortex, and parafascicular cortex was made by using four comparable regions from each rat from each group. The boundaries of the regions were defined with reference to an atlas of the rat brain (Pellegrino et al., 1979) and are shown in detail in Figure 1. Chronic lipopolysaccharide (LPS) activated microglia, which was not reduced by estrogen (E2; a mixture of 25% 17β-estradiol and 75% cholesterol) replacement therapy. Intact females receiving LPS infusion had significantly fewer activated microglia. Oil was 100% cholesterol as vehicle. CSF = cerebrospinal fluid infusion. Asterisks indicate a significant difference compared with all CSF-infused groups ($p < .001$); plus sign indicates a significant difference compared with the LPS intact group ($p < .001$); pound sign indicates a significant difference compared with LPS-infused ovariectomized rats ($p < .02$).

treatment duration (Asthana et al., 1999; Fillit et al., 1986; Honjo et al., 1989; Ohkura et al., 1994); however, the performance of women receiving chronic ERT ultimately declined more on the Clinical Dementia Rating Scale than that of women receiving placebo (Mulnard et al., 2000). Additional recent studies also concluded that ERT could not improve the cognitive abilities of women with mild to moderate AD (Henderson et al., 2000; Marder & Sano, 2000; Wang et al., 2000). When considered together, the results of these clinical trials suggest a pattern of beneficial effects on cognitive function after relatively short-term ERT; however, this beneficial effect is attenuated, and possibly reversed, after much longer treatment regimens. The designs of these clinical studies were criticized because they were conducted on postmenopausal women with advanced AD who may have been estrogen-deprived for decades (Toran-Allerand, 2000). Prolonged estrogen deprivation followed by long-term continuous ERT can down-regulate the number of estrogen receptors (Brown, Scherz, Hochberg, & MacLusky, 1996), which may limit the effectiveness of the ERT (Clark, MacLusky, Parsons, & Naftolin, 1981; Crespo, Cos, Fernandez-Viadero, & Gonzales, 1991; Simpkins et al., 1997). Although a comparison between humans and rodents must be made with caution, it is interesting that continuous long-term estrogen therapy immediately after ovariectomy in the present study parallels the detrimental cognitive effect seen in postmenopausal AD women who receive continuous, long-term ERT decades after the onset of menopause. Taken together, these findings suggest that estrogen may decrease the risk of AD but may not alter the progression of AD if the disease process has already begun (for a review, see Wise et al., 2001).

Cholinergic function decreases in patients with AD (Muir, 1997); a decline in forebrain cholinergic function might underlie the impaired performance in tasks that require spatial memory (Wenk, 1997). However, the behavioral impairment caused by chronic brain inflammation or long-term ERT observed in the present study does not appear to be due to impaired cholinergic function. The integrity of basal forebrain cholinergic neurons, as determined by the level of cortical ChAT activity, was unaffected after 5 weeks of chronic ERT. Recent studies have shown that the response of forebrain cholinergic neurons to ERT is significantly influenced by the duration of therapy (Gibbs, 2000a). Short term (i.e., up to 2 weeks) ERT in OVX rats increased ChAT activity in the cortex and hippocampus (Luine, 1985). Other measures of cholinergic neuronal activity, such as high-affinity choline uptake (Wenk, Hepler, & Olton, 1984), were also increased by short-term estrogen therapy (O'Malley, Hautamaki, Kelley, & Meyer, 1987). In contrast, OVX rats given daily estrogen for at least 4 weeks did not show increased ChAT activity in the frontal cortex or hippocampus; surprisingly, treatment for even longer periods of time were associated with a significant decline in high-affinity choline uptake within the hippocampus (Gibbs, 2000a). These rodent data might correspond to the condition observed in women with AD who did not show improved cognitive abilities after long-term ERT, despite short-term benefits. In addition, the fluctuations in cognitive ability might parallel the time-dependent fluctuations in cholinergic function. Once again, the reduced response of the female rats to the long-term ERT may be due to a down-regulation of estrogen receptors on these cholinergic neurons (Mufson et al., 1999).

The impact of estrous cycle must be considered in studies of estrogen's influence on cognitive abilities and the function of the hippocampus, as estrous cycle-induced differences have been reported on a number of spatial memory tasks that depend upon hippocampal function, although the effect tends to be variable, task dependent, and rather modest (Berry, McMahan, & Gallagher, 1997; Frye, 1995; Stackman, Blasberg, Langan, & Clark, 1997; Tropp & Markus, 2001; Warren & Juraska, 1997). Despite the alterations in synaptic function and cognition across the estrous cycle, the present study found no difference in the performance of intact females across the stages of the estrous cycle, regardless whether they received an infusion of LPS or CSF. Moreover, there was no correlation between performance on the water maze and estradiol blood levels, as determined by radioimmunoassay (data not shown). However, it is interesting to note that, at the beginning of testing, although most of the cycling females in the current study were in estrus—a state that is usually associated with the poorest performance in the water maze task (Frye, 1995)—these rats showed no deficit in performance, whereas OVX rats that received chronic estrogen and were in a state of constant estrus demonstrated a deficit in this task that could be exacerbated by the presence of chronic neuroinflammation. The increased vulnerability of these OVX rats to the effects of chronic estrogen and chronic neuroinflammation, as demonstrated by their impaired performance in the water maze task, may be due to the presence of chronic, rather than fluctuating, estrogen or to removal of the ovaries. The ovaries release many factors, including activin, inhibin, estrogen and progesterone; the combined presence of these

ovarian hormones in the intact females might underlie their unimpaired performance in the presence of chronic brain inflammation.

The absence of the ovaries clearly influenced the overall impact of chronic inflammation on the brain and behavior. Intact females given chronic infusion of LPS into the fourth ventricle were not impaired on a water maze task. This finding is in marked contrast to male rats that received the same treatment of LPS into the fourth ventricle. Earlier studies from our lab indicate that intact males given chronic LPS into the fourth ventricle are significantly impaired on the Morris water maze task (Hauss-Wegrzyniak, Dobrzanski, et al., 1998). The decreased vulnerability of intact females to the effects of LPS infusion may be due to the differential degree of the inflammatory response by brain microglia, as well as the differential pattern of the activated microglia. It is interesting that the reduced number of activated microglia in intact animals appears to be localized to extra-hippocampal regions, as there is no difference in the number of activated microglia seen in the dentate gyrus of the hippocampus between OVX and intact rats that received LPS (see Figure 4). In female rats, activated microglia were more widely distributed throughout the brain with the highest density in hippocampus, amygdala, lateral hypothalamus, zona incerta, central thalamic nucleus, cingulate gyrus/retrosplenial cortex, piriform/entorhinal cortex, and parafascicular cortex. In contrast, the highest density of microglia cell activation in male rats was focused within the hippocampus, amygdala, cingulate gyrus/retrosplenial cortex and piriform/entorhinal cortex (Hauss-Wegrzyniak, Willard, Pepeu, Del Soldato, & Wenk, 1999; Wenk et al., 2000). Moreover, studies from our lab have shown that males infused with LPS averaged over 500 activated microglia within their hippocampus (Hauss-Wegrzyniak, Willard, et al., 1999), whereas the hippocampus of intact LPS-infused females averaged only about 200 activated microglia (regional data not shown). It has been suggested that the number of activated microglia may influence the degree of memory impairment (Wenk & Hauss-Wegrzyniak, 2001), a finding that would support the differential impairment seen between intact male and female rats. However, our data from OVX female rats receiving chronic estrogen and brain inflammation indicate that despite a greater impairment in the water maze, these rats showed no more activated microglia than do OVX rats receiving chronic neuroinflammation alone. Moreover, intact female rats receiving LPS show no behavioral impairment despite the presence of activated microglia. Therefore, the influence of gender on the impact of chronic neuroinflammation remains to be determined.

The results of this study significantly contribute to our understanding of two different phenomena concerning the action of ERT on the female brain. These data support the findings of others showing a neuroprotective effect of fluctuating gonadal hormones, such as estrogen. Intact cycling female rats with LPS-induced chronic neuroinflammation had normal behavioral performance and a moderate inflammatory response, as demonstrated by a reduced number of activated microglia in the presence of LPS. Our results suggest that continuous long-term ERT in postmenopausal women with AD, or other diseases characterized by the presence of chronic neuroinflammation, may exacerbate their cognitive impairment. Our results are consistent with the hypothesis that either the presence of the ovaries or fluctuating levels of estrogen may be a critical factor in the protection of rats against the effects of chronic neuroinflammation. These findings suggest that a therapy designed to mimic the natural cycle of gonadal hormone fluctuation may provide a more effective therapy to slow the progression of AD in postmenopausal women.

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