Differential effects of estrogen in the injured forebrain of young adult and reproductive senescent animals

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Abstract

Estrogen attenuates neural damage resulting from a variety of experimental injury models in adult female rats. To determine whether estrogens neuroprotective actions are age-specific, the present study compared the effects of estrogen on young adult and reproductive senescent animals subject to excitotoxic injury to the forebrain. NMDA was injected bilaterally into the olfactory bulbs of estrogen and placebo-replaced young adult and reproductive senescent animals. Lysates of the olfactory bulb and its basal forebrain afferent, the horizontal limb of the diagonal band of Broca (hlDBB), harvested 24 h later were analyzed for expression of IL-1β, IL-10, and nerve growth factor (NGF). NMDA injections resulted in local activation of microglia and an increase in IL-1β. Estrogen replacement decreased IL-1β expression in young adult females, but paradoxically enhanced its expression in reproductive senescent females. Furthermore, bulb injury increased IL-1β production in the hlDBB of reproductive senescent animals although estrogen replacement was able to suppress lesion-induced expression of this cytokine. In both, the olfactory bulb and hlDBB, constitutive expression of the anti-inflammatory cytokine IL-10 was significantly higher while that of NGF was almost 50% lower in senescent animals as compared to young adults, indicating that aging preferentially altered Th2-type secretions. The present findings are consistent with our earlier observations that estrogen does not exert trophic effects in the aging forebrain and supports the hypothesis that estrogen treatment to reproductive senescent females may exacerbate neural injury.

1. Introduction

The gonadal hormone estrogen affects several neural groups, including the forebrain cholinergic system, and regulates the expression of key enzymes, cytoskeletal proteins and growth factors. Many of these events are critical during neural development [27,45,53,72,79,80], and several of them continue to occur in the adult brain as well. Besides its actions on forebrain growth factor expression [68–70] and the basal forebrain cholinergic system [21,42,44,54,67,71], estrogen also attenuates tissue damage resulting from experimental injury models such as cerebral ischemia [16,64], septo-hippocampal transection [57] and excitotoxic lesions [71]. Collectively, this evidence supports the hypothesis that estrogen replacement may be neuroprotective in forebrain degenerative disease.

Age-related changes in hormone responsiveness, however, may decrease estrogens ability to attenuate tissue damage in the forebrain. Estrogen-mediated LH surges are blunted in middle-aged rats [87] and aging affects the density of alpha-1 adrenergic receptors and their diurnal rhythms in specific hypothalamic nuclei [85]. In some cases specific genes such as GAP-43 mRNA [66], neurotensin mRNA [20] and ChAT mRNA [65] remain sensitive to estrogen in both young and aging animals, while in other cases such as trkA, the NGF specific tyrosine kinase receptor, the gene is equally sensitive to estrogen in the young and old animals in the nucleus basalis region but not in the horizontal limb [65]. Using a model of hormonal aging, i.e. reproductive senescence, our recent data shows that estrogen has paradoxical effects on the young adult and reproductive senescent female rat that can be classified into two patterns. Genes were either refractory to estrogen treatment, as in the case of trkA and trkB which estrogen typically increases in the olfactory bulb of young adults, or responded to estrogen in a manner diametrically opposite to that of young adults, as in the case of BDNF and p75 [35]. The present study therefore investigated whether

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estrogen would be neuroprotective to the aging brain as has been shown for the young adult animal.

The olfactory bulb-hlDBB circuit, where age-related regulation of neurotrophins were observed, is prototypical of other estrogen-sensitive forebrain cholinergic pathways radiating from the basal forebrain to targets such as the hippocampus and cortex. Efferent neurons in these circuits synthesize neurotrophins that are retrogradely transported to their basal forebrain afferents and neurons in these circuits are vulnerable to Alzheimer's disease (AD)-related lesions. In view of emerging evidence that inflammation may be a critical component of neurodegenerative disease, the present studies investigated whether estrogen replacement would alter the inflammatory phenotype resulting from excitotoxic lesions of the olfactory bulb, and whether this regulation would differ in young adult and reproductive senescent animals. To assess the inflammatory response following excitotoxic lesions, we measured expression of IL-1β, a key pro-inflammatory cytokine, as well as IL-10, a cytokine typically produced by the Th2 class of helper T cells, which inhibit systemic inflammatory reactions. Our data reveal that estrogen replacement decreases the proinflammatory phenotype, derived as a ratio of IL-1β to IL-10, at the primary lesion site in young adult animals while exacerbating it in older animals. Furthermore, bulb lesions alter cytokine expression in the hlDBB afferent in old but not young animals, indicating that age may accelerate transneuronal toxicity in forebrain circuits.

2. Methods

2.1. Animals

All rats were purchased from Harlan Laboratories (IN) as young adults (∼250 g, 4 months; $n = 58$). Reproductive senescent animals (13–16 months; 325–410 g) were used after they were retired from a breeding program (Alcohol and Brain Research Laboratory, J.R. West TAMUSHSC). These animals were used solely for breeding purposes. Retired breeders used in this study met previously established criteria [35] for reproductive senescence, which included 4–5 successful prior pregnancies followed by two consecutive reproductive failures. Vaginal smears from these animals also indicated irregular estrous cycles characterized by constant estrus. All animals were maintained in an AALAC-approved facility on a 12-h light:12-h dark cycle with lights on at 06:00 h and with food and water available ad libitum. All procedures were in accordance with NIH and institutional guidelines governing animal welfare.

2.2. Surgical techniques

2.2.1. Ovariectomies

Animals were anesthetized with ketamine (87 mg/kg)/xylazine (13 mg/kg) and bilateral ovariectomies were performed using a dorsal midline incision inferior to the palpated rib cage and kidneys [34–36,71]. Ovaries and surrounding tissue were removed and 60-day time-release 17-β estradiol pellets (1.0 mg) or placebo pellets (Innovative Research, FL) were inserted subcutaneously (s.c.) prior to closing the incision. These pellets have been used extensively in our work and have resulted in physiological levels of plasma estradiol for periods of 3, 4, and 6 weeks [35,71].

2.2.2. Stereotaxic surgeries

After 3 weeks of estrogen or placebo treatment, all animals were anesthetized with ketamine (87 mg/kg)/xylazine (13 mg/kg) and placed in a rodent stereotaxic apparatus. Skin and cranial fascia were resected and the skull exposed. Two small craniotomies were made in all lesion group rats to expose the olfactory bulbs at the following coordinates: 7.6 mm anterior to bregma and 1.0 mm lateral to the sagittal suture. The tip of a Hamilton syringe needle was briefly lowered to a depth of 3.4 mm, and immediately raised by 0.2 μm to create a trough. A total of 2 μl (1 μl per side) of 50 nM NMDA was injected at a rate of 0.2 μl/30 s. The needle was raised slowly, craniotomies filled with gel-foam, and scalp sutured with wound clips. Surgical controls were anesthetized, restrained in the stereotaxic apparatus and their scalp and cranial fascia resected and reclipped. Other reports [1], and our preliminary studies, indicated that at 1-day post injury (dpi) a saline injection is not an appropriate control since the injection itself can result in injury. Our pilot data indicates that at 1 and 3 dpi, the vehicle solution (PBS) also increases IL-1β. However, at 1 dpi, IL-1β expression in the NMDA group is no different from the PBS group, but at 3 dpi, the NMDA group is clearly different (higher) from the PBS group. Since the present studies were conducted at 1 dpi, this paradigm best resembles a stab wound injury model, hence the most appropriate control is a sham injection. NMDA injections were used in this case to allow for comparisons across other planned experiments, which will monitor changes in inflammation over a period of time. Hence four groups each of young adult and reproductive senescent animals were prepared: OS: placebo-replaced, sham lesioned, OL: placebo-replaced, NMDA lesioned, ES: estrogen replaced, sham lesioned, EL: estrogen replaced, NMDA lesioned.

Animals were sacrificed by rapid decapitation and trunk blood was collected for estimation of estradiol content by radioimmunoassay (Diagnostic Systems Laboratories, TX). Olfactory bulbs and hlDBB were rapidly removed and stored at −80 °C. Proteins were isolated using previously established procedures [34,35,71] and total protein concentrations were determined using the BCA protein assay kit (Pierce, IL).

2.2.3. Enzyme-linked immunosorbent assay (ELISA)

Commercial kits for IL-10 (Biosource International, Inc., CA), IL-1β (R&D Systems, MN), and NGF (Promega, IL) were used to determine cytokine/neurotrophin expression in tissue lysates, using procedures recommended by the

manufacturers. Briefly, standards, controls, samples and a biotinylated secondary antibody were pipetted into 96-well plates pre-coated with antibodies specific for rat IL-10 and IL-1β, and incubated at RT for 2 h (IL-1β, IL-10). For NGF, microplates were coated with anti-NGF pAB overnight and later incubated with standards, controls and samples (6 h at RT) followed by a biotinylated detection antibody (overnight at 4 °C). Following washes, plates were sequentially incubated with streptavidin peroxidase (IL-10: 30 min, IL-1β: 2 h, NGF: 2.5 h) and substrate solution for 10 min (NGF) or 30 min (all others). Plates were read at 450 nm in a microplate reader (Bio-Tek, VT). Standard curves were established from optical densities of wells containing known dilutions of standard, using KC3 software (Bio-Tek, VT), and sample measurements were interpolated from standard curves.

2.2.4. Lectin histochemistry

An additional set of ovariectomized young-adult animals ($n = 12$) were prepared with either placebo or estrogen-containing pellets. Animals were either sham-lesioned or NMDA-lesioned as before. Twenty-four hours later, animals were anesthetized with 0.6 ml pentobarbital solution (50 μg/ml pentobarbital, 10% ethanol, 40% propylene glycol) and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were removed from the cranial vault, post-fixed in 4% paraformaldehyde (2 h). In order to minimize batch artifacts in the staining process, brains were shipped to NeuroScience Associates (Knoxville, TN) for embedding and sectioning using their proprietary Multi-Brain Technology™. Upon receipt of rat brains at Neuro-Science Associates, they were treated with 20% glycerol and 2% dimethylsulfoxide to prevent freeze artifacts, and then embedded as a group in a gelatin matrix. Forty micron freeze-cut, coronal sections through the entire olfactory bulbs were obtained with an AO 860 sliding microtome and collected in a 4 × 6 array of containers filled with 10% phosphate-buffered formalin. After 24 h, sections were rinsed and transferred into Antigen Preserve solution (50% PBS, pH 7.0, 50% ethylene glycol, 1% PVP) for storage and shipping. Sections through the olfactory bulb were subject to lectin histochemistry, which identifies microglia. Sections were rinsed in PBS and sequentially incubated with 3% hydrogen peroxide (30 min RT) followed by 5 μg/ml lectin (B. Simplicifolia conjugated with HRP, Sigma, WI) solution (4 h, RT). After rinses, lectin-stained cells were identified by enzyme-catalyzed color reaction. Sections were processed through alcohols and xylenes and coverslipped with permount.

2.2.5. Statistical analysis

For each cytokine, it was of interest to determine its regulation by age, lesion and estrogen treatment and the interactions between these factors. Hence group differences were analyzed by a 3-way ANOVA for age, estrogen treatment and lesion as independent variables, using a statistical package (SPSS). Group differences were considered significant when $P \leq 0.05$. In the case of the inflammatory phenotype (ratio of IL-1β/IL-10), it was of interest to determine if estrogen treatment would alter the lesion-induced phenotype. Since constitutive age-related differences in the cytokines would provide a confounding factor and increase the probability of spurious significant differences, this ratio was analyzed separately for each age by 2-way ANOVA with lesion and estrogen treatment as independent variables. Thus, four such ANOVA's were performed, each of two regions (olfactory bulb, hlDBB) and reproductive age (young adult, reproductive senescent).

3. Results

3.1. Confirmation of estrogen treatment

Ovariectomized animals replaced with an estrogen-containing pellet (E2) had an average plasma estradiol level of 57.64 ± 6.19 pg/ml in young adult and 81.6 ± 12.76 for the reproductive senescent animals, typical of hormonal levels seen at proestrus. The average weight gain as a result of estrogen treatment was -10.5 g ($+5.68$) in the reproductive senescent group and -0.75 g ($+3.82$) in the young adult group. In contrast, ovariectomized, placebo-replaced animals (OVX) had low estradiol levels in both young adult (9.96 ± 0.61 pg/ml) and reproductive senescent (8.48 ± 0.43 pg/ml) animals, with a correspondingly large weight gain (59.36 ± 7.87 g for young adults and 37.45 ± 5.73 g for reproductive senescent animals).

3.2. Lectin histochemistry

Microglia were detected by lectin histochemistry using B. simplicifolia conjugated to HRP. In sham-lesioned animals, lectin staining was restricted to the subependymal layer of the olfactory bulb (Fig. 1A). Fine, ramified microglia were also present, but poorly seen, in sham-lesioned animals (Fig. 1B). In lesioned animals, lectin-stained profiles were profusely present (Fig. 1C). Both ameboid and ramified microglia were visible, the former located close to the injury site (white arrows), while the latter were seen at the injury site and as far laterally as the external plexiform layer (black arrows). Ramified microglia in the lesioned animals had short, densely stained branches (Fig. 1C) with round bodies. No staining was seen in sections that were processed without lectin (Fig. 1D).

3.3. Cytokine expression

3.3.1. IL-1β

3.3.1.1. Olfactory bulb. There was a significant main effect of age ($F_{1,34} = 107.78$, $P < 0.05$) and lesion ($F_{1,34} = 35.705$, $P < 0.05$) on local IL-1β expression 24 h
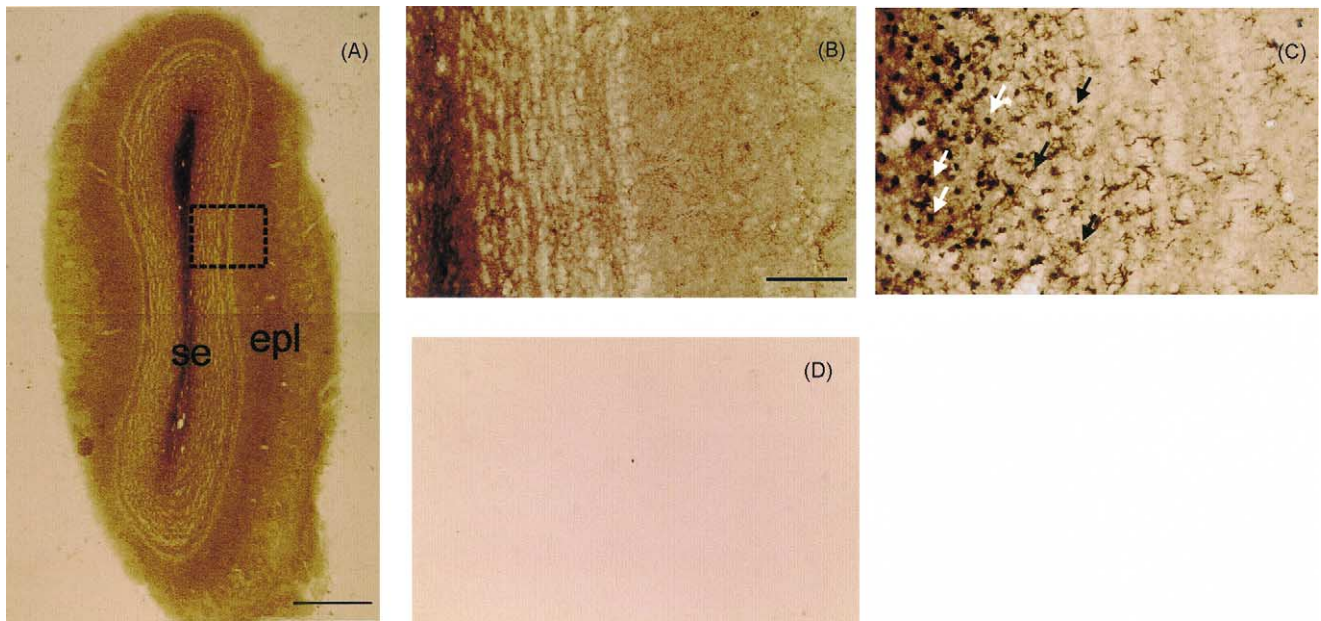
Fig. 1. Microglial expression in the olfactory bulb. Microglia were detected using lectin histochemistry. In the uninjured (sham) animal, lectin staining was mainly seen in the subependymal layer (A). At higher magnifications, fine ramified microglial profiles are faintly detected in other bulb layers (B). The hatched region in A indicates location of photomicrographs in B–D. In NMDA-lesioned animals, activated microglia were profusely detected near the injury site (C). Ameboid microglia were seen in close proximity to the injection site (white arrows), while ramified microglia with thick, densely stained processes and round bodies could be seen as far laterally as the external plexiform layer (black arrows). No staining was seen in sections processed without lectin (D). Key—se: subependymal layer; epl: external plexiform layer. Bar in A = 450 μm, B–D = 125 μm.

after the lesion (Fig. 2), indicating that both lesion and advanced age increased IL-1β expression. However, there was also a significant age × lesion interaction ($F_{1,34} = 27.36$, $P < 0.05$) that suggests that age-related increases in IL-1β expression was mainly due to the fact that the older animals had a heightened response to lesion as compared to young adults. While there was no main effect of estrogen treatment, there was significant age × lesion × estrogen interaction effect ($F_{1,34} = 6.429$, $P < 0.05$), indicating that estrogen treatment modulated the expression of this cytokine differently in young adult and reproductive senescent animals following lesion. As shown in the histogram (Fig. 2A), estrogen attenuated lesion-induced increases in IL-1β in young adults but exacerbated production of this cytokine in the reproductive senescent animal.

3.3.1.2. hlDBB. A similar age × lesion × estrogen interaction ($F_{1,34} = 5.18$, $P < 0.05$) was seen in the hlDBB. As shown in the histogram (Fig. 2B), there was a dramatic increase in IL-1β in the placebo-replaced reproductive senes-
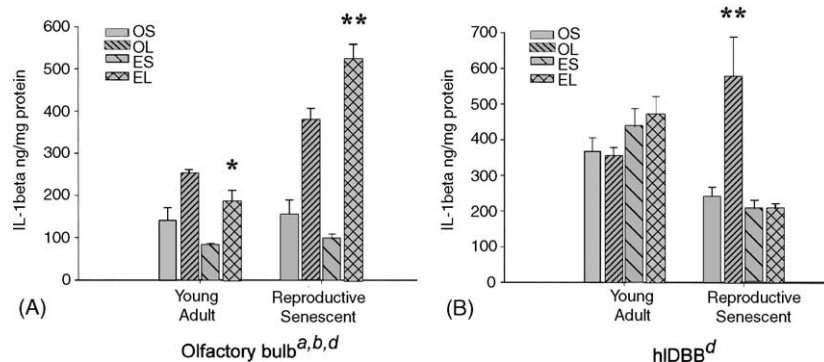

Fig. 2. IL-1β expression in the olfactory bulb-hlDBB. (A) In all cases, olfactory bulb lesions elevate IL-1β expression as compared to shams. Chronic estrogen treatment blunts IL-1β in YA animals (OL > EL, *$P < 0.05$), and paradoxically elevates this cytokine in RS animals (OL > EL, **$P < 0.05$). (B) Olfactory bulb lesions did not affect IL-1β expression in the young adult hlDBB. However, it resulted in a lesion-induced increase in IL-1β in the hlDBB of reproductive senescent animals. Unlike the olfactory bulb, estrogen treatment completely attenuated this rise in IL-1β in the hlDBB of senescent animals. YA: young adults; RS: reproductive senescent animals; OS: placebo-replaced ovariectomized animals/sham lesion; OL: placebo-replaced ovariectomized animals/NMDA lesion; ES: estrogen replaced ovariectomized animals/sham lesion; EL: estrogen replaced ovariectomized animals/NMDA lesion; [a]: main effect of age; [b]: main effect of lesion; [d]: interaction effect of age × lesion × estrogen treatment.
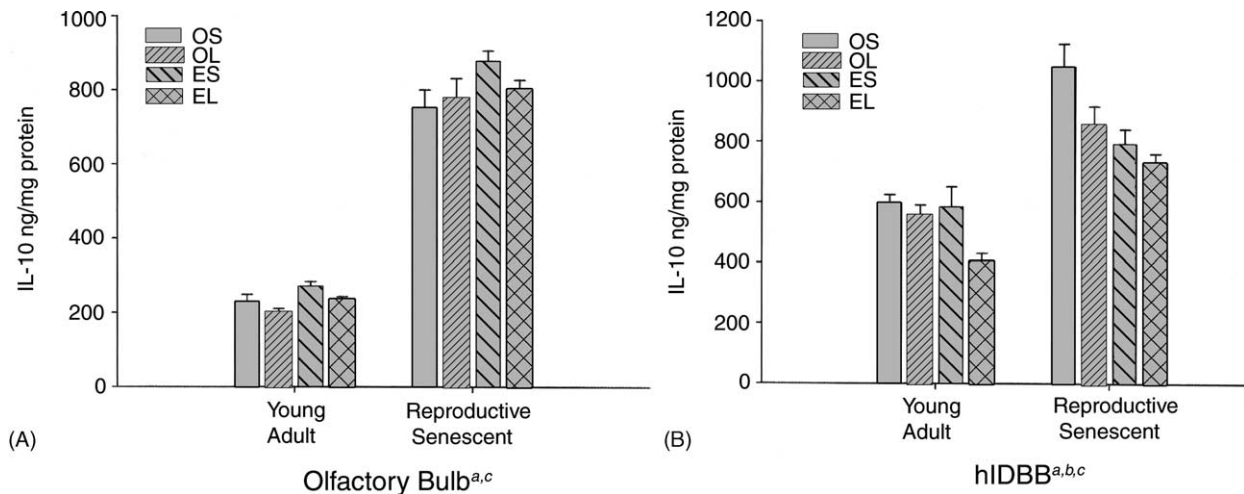
Fig. 3. Lesion-induced changes in IL-10 expression. (A) Lesion did not affect IL-10 expression in the olfactory bulb, however, there was a main effect of age on expression of this cytokine. Reproductive senescent animals had significantly greater constitutive expression of this cytokine than young adults. There was a modest increase in IL-10 due to estrogen at both ages. (B) In the hlDBB, as in the olfactory bulb, reproductive senescent animals had a higher constitutive expression of this cytokine as compared to young adults. There was a modest decline in IL-10 in the hlDBB due to olfactory bulb lesions and due to estrogen treatment. YA: young adults; RS: reproductive senescent animals; OS: placebo-replaced ovariectomized animals/sham lesion; OL: placebo-replaced ovariectomized animals/NMDA lesion; ES: estrogen replaced ovariectomized animals/sham lesion; EL: estrogen replaced ovariectomized animals/NMDA lesion; [a]: main effect of age; [b]: main effect of lesion; [c]: main effect of estrogen treatment.

cent animals that was not seen in the young adults, and this was effectively quenched by estrogen-treatment.

3.3.2. IL-10

There was a main effect of age ($F_{1,34} = 650.34$, $P < 0.05$) and estrogen treatment ($F_{1,34} = 6.44$, $P < 0.05$) on IL-10 expression in the olfactory bulb, but no lesion effect ($F_{1,34} = 1.750$ $P > 0.05$). As seen in the histogram (Fig. 3A), reproductive senescent animals had markedly greater IL-10 expression than young adult animals. There was a small but statistically significant main effect of estrogen, suggesting that irrespective of age and lesion, estrogen produced a modest increase in IL-10 expression.

3.3.2.1. hlDBB.
In the hlDBB, there were significant main effects of lesion ($F_{1,34} = 8.99$, $P < 0.05$), age ($F_{1,34} = 99.53$, $P < 0.05$) and estrogen treatment ($F_{1,34} = 15.76$, $P < 0.05$), although their actions were not uniform. As seen in the histogram (Fig. 3B), lesion led to a small decrease in IL-10 expression as did estrogen treatment. However, reproductive senescent animals had a significantly higher expression of IL-10, as in the olfactory bulb. Unlike IL-1β expression there were no significant interaction effects between the variables for IL-10 suggesting that outside of the constitutive difference in IL-10 expression, estrogen and lesions had uniform effects on this cytokine at both age groups.

3.4. Inflammation phenotype

To assess the effect of estrogen, the inflammatory profile, i.e. the ratio of pro- (IL-1β) and anti- (IL-10) inflammatory cytokine, was calculated separately for every animal

as in [58]. As expected, NMDA injections increased the inflammatory phenotype in the olfactory bulb, in both young adult ($F_{1,18} = 37.845$, $P < 0.05$) and reproductive senescent animals ($F_{1,16} = 189.595$, $P < 0.05$) (Fig. 4A and B). Estrogen-treatment ($F_{1,18} = 18.546$, $P < 0.05$) reduced the inflammatory phenotype in injured young adult females as compared to placebo-replaced lesioned animals. Estrogen treatment per se did not significantly alter the inflammatory phenotype in reproductive senescent animals ($F_{1,16} = 2.196$, $P > 0.05$). However, there was a significant interaction between lesion and estrogen treatment at this age group ($F_{1,16} = 19.304$, $P < 0.05$), indicating that estrogen significantly exacerbated lesion-induced increases in the inflammatory phenotype (Fig. 4B). In the hlDBB, there was no overall effect of lesion on the inflammatory phenotype in young adult animals ($F_{3,18} = 2.621$, $P > 0.05$, Fig. 4C). In the reproductive senescent animals, however, there was a main effect of lesion on the inflammatory phenotype in the hlDBB ($F_{3,16} = 7.930$, $P < 0.05$). Bulb lesions increased the inflammatory phenotype in hlDBB ($F_{1,16} = 13.210$, $P < 0.05$), however, the inflammatory phenotype of the hlDBB of bulb lesioned animals was reduced in the group treated with estrogen ($F_{1,16} = 7.38$, $P < 0.05$; Fig. 4D).

3.5. NGF expression

In the case of NGF, there was a main effect of age ($F_{1,34} = 88.219$, $P < 0.05$), estrogen treatment ($F_{1,34} = 5.35$, $P < 0.05$) and lesion ($F_{1,34} = 8.997$, $P < 0.05$) on growth factor expression. As seen in the histogram (Fig. 5), NGF expression, irrespective of lesion or hormone treatment, was lower in the reproductive senescent animals, as
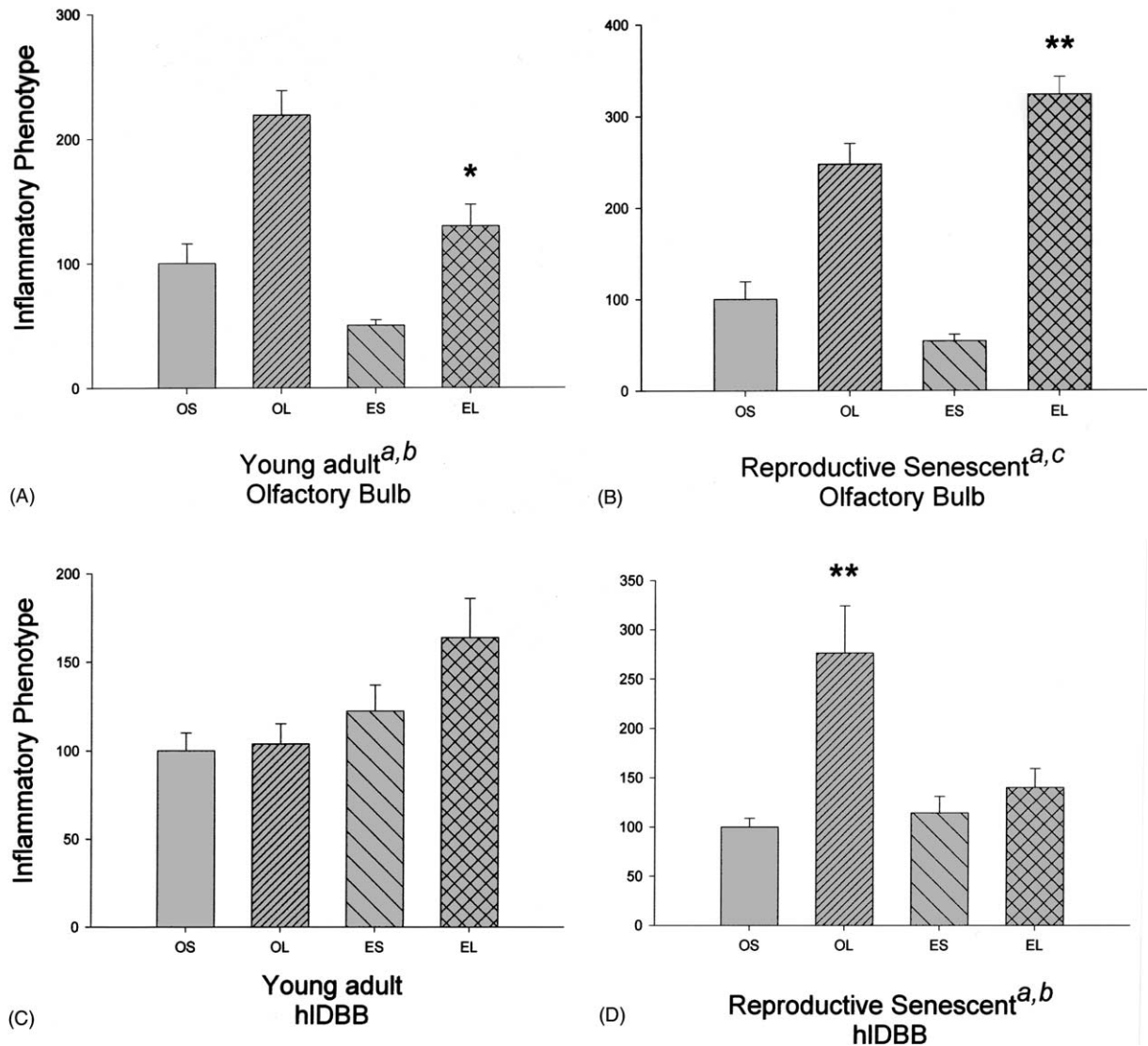
Fig. 4. Inflammatory phenotype: the ratio of IL-1β (pro-inflammatory) and IL-10 (anti-inflammatory) was calculated for each animal. As expected, the inflammatory phenotype was greater in animals that received an NMDA injection as compared to sham injections. Estrogen treatment decreased the pro-inflammatory phenotype at the lesion site in young adult animals (A; * P < 0.05) while increasing it in reproductive senescent animals (B; ** P < 0.05). In the hlDBB, there was no significant change in the difference in the inflammatory phenotype of any group in the young adult (C). However, bulb lesions resulted in a significant increase in the inflammatory potential in the hlDBB of reproductive senescent animals, which was attenuated by estrogen treatment (D). [a]: Main effect of lesion; [b]: main effect of estrogen treatment; [c]: interaction effect. Groups—OS: placebo-replaced ovariectomized animals/sham lesion; OL: placebo-replaced ovariectomized animals/NMDA lesion; ES: estrogen replaced ovariectomized animals/sham lesion; EL: estrogen replaced ovariectomized animals/NMDA lesion.

compared to young adults. Estrogen and lesion independently increased the expression of this growth factor. Note that, as in the case of IL-10, there were no interaction effects among the three factors, suggesting that there may be less age-related variations in response to hormone or lesion on Th2 type secretions in this model.

4. Discussion

Infectious or traumatic injury to the brain results in microglial activation, and the consequent release of inflam-matory cytokines, such as the interleukins, initiates leuko-cyte recruitment. Inflammation, which is a primary com-ponent of the immune response, has both degenerative and reparative aspects. Prolonged neural inflammation, however, can lead to "bystander damage" of healthy neurons. Re-cent evidence indicates that inflammation may contribute to the etiology of Alzheimer's disease (for reviews see [2,7]) and long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) is highly correlated with a decreased risk for AD [47]. In the present study olfactory bulb lesions produced microglial activation at 1 dpi as shown by lectin-stained profiles at the lesion site and increased local expression of
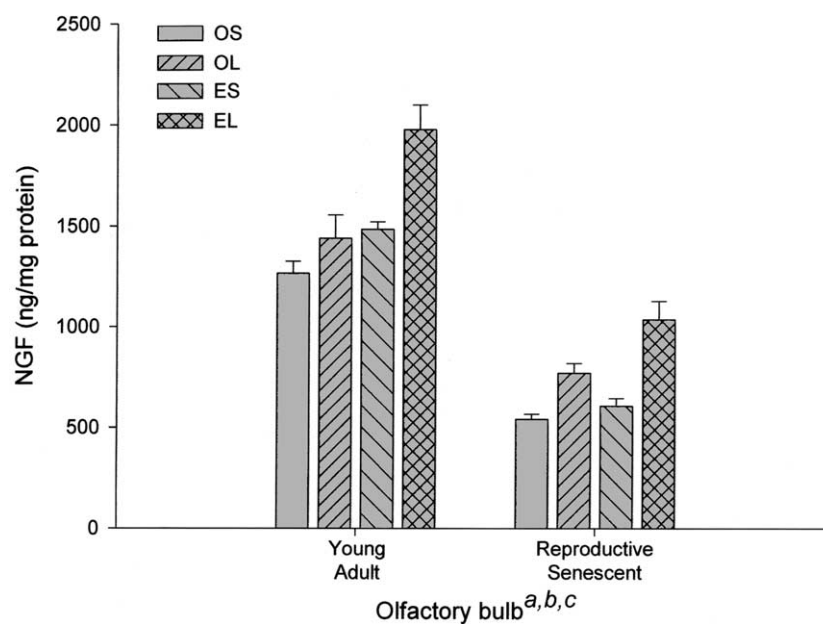
Fig. 5. NGF expression in the lesioned olfactory bulb. NMDA lesions increased local NGF expression in young adult and reproductive senescent animals. However, reproductive senescent females had significantly lower bulbar NGF as compared to young adults. Furthermore, although estrogen treatment increased NGF expression, there was a significant lesion by estrogen effect, indicating that estrogen enhanced lesion-induced increases in NGF expression. Groups—OS: placebo-replaced ovariectomized animals/sham lesion; OL: placebo-replaced ovariectomized animals/NMDA lesion; ES: estrogen replaced ovariectomized animals/sham lesion; EL: estrogen replaced ovariectomized animals/NMDA lesion; [a]: main effect of age; [b]: main effect of lesion; [c]: main effect of estrogen treatment.

active IL-1β. Estrogen reduced IL-1β expression and reduced the inflammatory phenotype in young adult animals but not reproductive senescent females, suggesting that while estrogen may have anti-inflammatory actions in vivo, these actions may be age-related.

Rapid estrogen-induced decreases in IL-1β may provide a mechanism by which the hormone may attenuate neural injury in experimental models such as wound injury [57,71] and ischemia [16,33,64]. Pro-inflammatory interleukins have been implicated in the pathogenesis of AD, due to their expression in AD brains [24–26] and ability to induce expression of beta-amyloid and other plaque-related proteins [10,22]. While important for cellular repair, interleukins also stimulate astrocyte-derived S100 proteins [63] that result in dystrophic neuritis. Additionally, high levels of IL-1β impair consolidation of hippocampal-dependent learning [55]. By increasing IL-1β in lesioned reproductive senescent animals, estrogen may therefore exacerbate the magnitude of the subsequent immune response. In view of the increased possibility of neuronal death that a prolonged or enhanced inflammatory state may cause, these data suggest that estrogen replacement to reproductive senescent populations may be deleterious.

The present data are consistent with our previous findings that, unlike young adults, estrogen does not have a trophic effect on the reproductive senescent forebrain [35]. In young adult rats, estrogen increases neurotrophin and trk receptor expression in the olfactory bulb and its basal forebrain afferent hlDBB, while a similar estrogen regimen to reproductive senescent females does not [35]. However, in ischemic injury

models, estrogen pretreatment effectively decreases infarct size in middle aged/reproductive senescent animals [3,18]. The discrepancy between these studies and the present data may be related to the type of injury, the specific criteria for reproductive senescence and the dose and length of estrogen replacement. On the other hand, the present data are consistent with other reports of age-related differences in the effectiveness of anti-inflammatory treatment. For example, while TGFβ-1 decreases LPS-induced production of nitric oxide in young microglia, it is less effective with aged microglia [59]. Similarly, while the NSAID NO-fluriboprofen effectively decreased LPS-induced microglial activation in young adult animals, it paradoxically increased microglial activation in aged animals [29]. Epidemiological findings further underscore the hypothesis that estrogen replacement may not be as effective to older populations. Prospective studies correlating estrogen use and the incidence of AD indicate that estrogen replacement at menopause reduces the risk for this disease [75], while large-scale clinical trials with female AD patients indicated that long-term estrogen replacement to this older population does not ameliorate measures of cognitive decline [30,50] and actually worsens scores on some performance measures [50].

Several reports indicate that aged animals may have a chronic inflammatory condition. LPS injections that routinely activate microglia in young adult females have little effect on older females mainly due to the increased basal expression of microglia in aging females [29]. Basal expression of cytokines such as IL-1β, TNF-α and IL-10 is increased in aged [88] and aging-accelerated [77] animals,

as are wound-induced increases in these cytokines [41]. In the present study, the age-related differences in IL-1β expression were mainly due to the heightened response of aged animals to lesion. On the other hand, the basal expression of IL-10 in reproductive senescent animals was 3–4-fold higher than in the young adult groups. Preferential increase in constitutive [32] and wound-induced IL-10 expression has been reported in aged rats [38] and rhesus monkeys [46]. IL-10, which is produced by Th2 type cells, usually acts as a check on the pro-inflammatory cytokines, reducing microglial and monocyte-derived IL-1β expression [74] and arresting TNF-α action by preventing the degradation of I-κB and the nuclear translocation of NF-κB [62]. Since IL-10 expression is thought to be a delayed response to injury following pro-inflammatory signals, high expression of this cytokine is often interpreted as evidence of an existing inflammatory condition.

A key difference in the lesion response of young adult and senescent animals centers on the vulnerability of afferent neurons. Bulb lesions do not significantly alter inflammatory phenotype in the hlDBB of young adult animals, but produce a strong pro-inflammatory response in reproductive senescent animals. Interestingly, this response is restricted to placebo-replaced animals and is not seen in estrogen-treated animals. These data indicates an important variation between the two ages, primarily, that reproductive senescent animals are more sensitive to transneuronal effects of injury than the young adults. Secondly, the fact that estrogen attenuates these transneuronal effects in reproductive senescent animals signals the presence of regional variations in estrogen responsiveness. Peripherally, estrogens actions as an anti-inflammatory are known to be tissue specific. For example, estrogen reduces endotoxin-induced cellular infiltration and interleukin expression in the anterior chamber of the eye [48], decreases the extent of tissue damage in carrageenan-induced pleurisy in the lungs [12] and adjuvant-induced arthritis [5,84]. However, estrogen stimulates edema [76], increases vascular permeability and influx of macrophages in the uterus [13,37,39,56] and promotes inflammation in the prostate gland [51]. In the nervous system, estrogen enhances mast cell secretions [78] that degrade specific myelin proteins [14], although it reduces cytotoxic products of activated microglia and reduces their phagocytic activity [9,15,82,83].

Although the mechanisms underlying these age-related variations in estrogen's anti-inflammatory properties are not known, we have previously shown that ER-α is abnormally expressed in the olfactory bulb of senescent animals coupled with a loss of steroid receptor coactivator (SRC)-1. Of the two known forms of the estrogen receptor, ER-α [23] and ER-β [40,49,81], only ER-α is dramatically increased following neural injury [17]. Recent studies on the ER-α knockout mouse present conflicting evidence about the role of ER-α$^{-/-}$ in injury. Wild type mice were no different from ER-α$^{-/-}$ animals in the extent of infarct following ischemia, suggesting that the loss of ER-α did not increase

vulnerability to the injury [60,73]. However, unlike the wild type or the ER-β$^{-/-}$, estrogen replacement did not reduce infarct size in ovariectomized ER-α$^{-/-}$ mice [19], suggesting that the presence of ER-α mediates the neuroprotective response. We propose that an optimal dose of ER-α may be necessary for estrogen dependent neuroprotection and that abnormal increases in this receptor may decrease estrogens effectiveness as a trophic factor.

Unlike cytokines, estrogens regulation of NGF at the injury site is similar in the lesioned young adult and reproductive senescent animals. NGF is a potent neural growth factor with an expanding role in immune function. NGF rapidly increases following a variety of injurious stimuli [11,43,86], and may prevent toxic effects of other inflammatory cytokines [28]. While NGF is secreted by activated astrocytes stimulated by the Th2 cytokine IL-10 [8], recent evidence indicates that T lymphocytes of the Th2 phenotype may themselves synthesize and secrete NGF [4,6]. NGF acting via the p75 receptor has been shown to suppress immune responses by inhibiting the induction of major histocompatibility complex (MHC) class II proteins [52]. Our recent work indicates that high local availability of NGF in estrogen-treated animals activates JNK2 [36], a MAP kinase involved with regeneration and repair [31,61]. In the present study, estrogen treatment increases lesion-induced expression of NGF at either age group, however, estrogen only decreases IL-1β in the younger animals. While this may be related to the substantial reduction in NGF levels seen in the aging animals, it may be the differential regulation of NGF receptors, rather than NGF itself, that is a better predictor of estrogens effects on IL-1β.

In conclusion, estrogen pretreatment was effective in reducing the proinflammatory potential in young adult animals but exacerbates the immune response in reproductive senescent animals. While these data indicate that estrogen replacement to reproductive senescent animals may be deleterious, it remains to be determined whether estrogen intervention in females with declining reproductive capability is beneficial. Furthermore, as investigations focus on the molecular mechanisms of estrogens anti-inflammatory actions, these senescence-related differences in the response to estrogen will provide a useful tool for discriminating events that suppress or activate the immune response.

Acknowledgments

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