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**Ovariectomy in female rats increases pro-inflammatory cytokine expression in whole
hippocampal tissue and isolated microglia**

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Abstract

Estrogen's role in neuroinflammation has been increasingly investigated in recent years, giving rise to evidence of its anti-inflammatory properties. Depletion of estrogen from female rats by ovariectomy had detrimental effects on immune functioning, including an increase in mRNA expression of pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 in whole hippocampal tissue and an increase in expression of IL-1 β and TNF- α in isolated microglia cells following treatment with lipopolysaccharide (LPS). However, estradiol administration following ovariectomy reversed this effect. Ovariectomy did not produce deficits in fear conditioning response, regardless of LPS or saline administration. The precise mechanisms by which estrogen exerts its anti-inflammatory actions is unknown. Additionally, the specific cell types responsible for mediating estrogen's effects are unknown, though these results suggest a role for microglia and possibly another neuronal cell type. Uncovering estrogen's precise role in inflammation may allow for novel treatment of inflammatory or sexually dimorphic conditions with currently limited treatment options.

Introduction

In recent years, interest in glial cells has grown as research has unveiled their great importance in various neuronal functions. Microglia, the immune cells of the central nervous system (CNS), play a critical role not only in immune function, but also in general nutritive support and synaptic pruning and plasticity (Paolicelli et. al., 2011). The ability of microglial cells to survey and adapt to their environment is what allows them to sense and phagocytose foreign materials, as well as unnecessary synaptic connections or dying neurons. Interestingly, several characteristics of microglia behavior are thought to be sex-dependent, including

microglial colonization and reactivity. For example, males have more microglia than females in early postnatal development (P3-P4). In contrast, females show greater microglia density in select brain regions through adolescence and adulthood (Schwarz et. al, 2012). Additionally, protein expression of the antigen-presenting peptide major histocompatibility complex II (MHC II) is significantly reduced in rat adult male microglia as compared to adult female microglia, suggesting a more activated microglia phenotype in females (Unpublished findings, Fonken et. al, 2017).

A number of neurodegenerative diseases are sexually dimorphic in prevalence and linked with inflammatory responses. For example, Parkinson's disease (PD), which is twice as likely to develop in males than in females, is thought to be at least partially mediated by microglial activity (Villa et. al, 2016). Additionally, the role of estrogen in inflammation is being considered because neurodegenerative diseases typically occur in aged individuals, and post-menopausal women produce less estrogen. Thus, it is conceivable that estrogen produces neuroprotective effects prior to menopause, which would attenuate the progression of neurodegenerative diseases up to this point. Indeed, several interactions between microglia and estrogen have been investigated, suggesting a possible role for estrogen inhibition of neuroinflammatory responses, for example the finding that 17β estradiol attenuated morphological changes in microglia induced by LPS (Vegeto et. al., 2000). Because estrogen is a steroid hormone, its actions influence the transcription of various genes, including certain pro-inflammatory cytokines. However, its specific actions at microglial cells versus neuronal cells and potential interactions between these two cell populations remains unclear.

Although estrogen's precise role in neurodegeneration is contested (Villa et. al, 2016), there is growing evidence that its presence is correlated with anti-inflammatory activity. For

example, expression of the neuroinflammatory gene Mac-1 is increased by ovariectomy (OVX) in female rats, but returns to normal levels following chronic administration of estrogens (Savari et. al, 2011). Furthermore, estrogen has been implicated in studies of multiple neuroinflammatory conditions, including stroke (Elzer et. al., 2010), multiple sclerosis (MS)(Luchetti et. al., 2014), Alzheimer's disease (AD)(Vegeto et. al., 2006), and PD (Murray et. al., 2003), all of which also have sexually dimorphic pathologies and/or pervasiveness.

Although the actions of estrogen are widespread, affecting processing related to psychiatric and neurodegenerative disorders (Villa et. al., 2016), chronic pain (Rghava et. al., 2017), aging (Zarate et. al., 2017), and others, this study's focus is on the effect of estrogen depletion and replacement on microglia-mediated release of pro-inflammatory cytokines and on the hippocampus-dependent task of contextual fear conditioning. Because several lines of research have linked changes in estrogen with changes in microglia functioning (Benedek et. al., 2016, Vegeto et. al., 2000, Zhao et. al., 2016), the effects of estrogen on the release of pro-inflammatory cytokines from microglial cells makes a good starting point for understanding the full scope of estrogen's anti-inflammatory actions. Additionally, a comparison of whole neural cell populations to isolated microglia cells could provide some insight as to the extent by which microglia are mediating these anti-inflammatory properties, and the possibility of a modulating role for some other neural cell type such as neurons or astrocytes.

Materials and Methods

Animals

Female Sprague-Dawley rats from Envigo laboratories were used, with an average mass of 230 g. They were housed in pairs at a temperature of $22 \pm 1^\circ \text{C}$, kept on a 12h:12h light:dark

cycle and had access to food and water *ad libidum*. All procedures were approved by and in accordance with the University of Colorado Boulder Institutional Animal Care and Use Committee.

Ovariectomy

Animals were anesthetized with 2% isoflurane and the surgical site was shaved and disinfected. A single 3cm incision down the posterior midline (posterior to the 13th rib) was made through the dermis using a sterile scalpel. Separate incisions in the right and left muscle layer were made directly above the corresponding ovary. Once the ovaries were identified, they were clamped with forceps to mitigate bleeding and ovaries were removed at the level of the fallopian tubes using surgical scissors. The fallopian tubes were then replaced and the muscle layers sutured using absorbable sutures (4-0 Webgut, Patterson Veterinary). The dermal layer was then stapled and animals were allowed to recover from anesthesia in a heated recovery box. Staples were removed 7-14 days following surgery. Sham surgeries were performed identically with the exception that the ovaries were identified but not removed.

Estradiol injections

Animals randomly assigned to the OVX+E condition were given an intraperitoneal injection of 10mg/kg estradiol dissolved in sesame oil every 4 days following ovariectomy until tissue collection. Animals in the OVX and SHAM condition received vehicle injections of sesame oil on the same schedule.

LPS injections

10µg/kg LPS was administered intraperitoneally. Control groups were given a vehicle injection of 0.9% saline.

Fear conditioning

A standard fear conditioning paradigm was used in which animals were placed in a chamber for two minutes and then they were exposed to a 5 second tone and a co-terminating 2 sec 0.6mA foot shock. 48 hours following the training session, animals were scored for freezing behavior in the conditioned context for 5 minutes and 4 minutes in a control novel context (2 minutes pre-tone, 2 minutes post-tone). Freezing was operationally defined as no detectable movement other than respiration. The chambers (conditioned context) consisted of Igloo ice chests (54 cm x 30 cm x 27 cm; L x W x H) with a speaker and a 24 V DC lightbulb inside the top surface, as well as a removable stainless steel foot-shock delivery system on the bottom surface. The novel context consisted of a round wire rat cage.

Hippocampal dissection

Animals were given a fatal dose of sodium pentobarbital (Fatal-plus; 150 mg/kg IP in 0.7 mL sterile saline) and transcardially perfused with 0.01 M phosphate buffer saline (PBS) solution. Whole brains were then removed, put on ice and dissected for hippocampi. The tissue was flash frozen in liquid nitrogen and stored at -80° C until processing.

Microglia isolation

Prior to tissue collection, glass homogenizers were labeled, filled with 3mL of 0.2% glucose buffer diluted in 1xPBS and placed on ice. Animals were administered a lethal dose of sodium pentobarbital and transcardially perfused with 0.01M PBS solution. Brains were then rapidly removed, put on ice and dissected for hippocampi. Hippocampi were then placed in a glass homogenizers and slowly homogenized avoiding formation of bubbles. Using sterile, aerosolized pipette tips, 3mL of homogenate were passed through a 70µm filter into 50mL

conicles. The homogenizers were then rinsed with 0.2% glucose buffer and remaining homogenate was passed through the filter. The filtrate was then spun at 1000 g for 10 minutes at room temperature in 5mL tubes. Room temperature isotonic Percoll solutions were made using 10xPBS at 100%, 70%, and 40% to create a Percoll density gradient. Supernatant from each tissue sample was then decanted and the cell pellets were suspended in 2mL of 70% Percoll and pipetted gently to mix without creating bubbles. 2mL of 40% Percoll was then gently pipetted onto the 70% Percoll + cells mixture, followed by 1mL of 1xPBS. Samples were then spun at 1200g for 45 minutes at room temperature. Following centrifugation, the white myelin layer was removed from each sample and microglial layers were extracted from the 40/70 interface and transferred to new 5mL tubes. Each tube was filled to the top line with 1xDPBS and inverted. Samples were then centrifuged at 1200g at room temperature for 10 minutes. Supernatant from each sample was then decanted and tubes were placed on ice. Cells were resuspended in 200 μ L media (Millipore filtered 1x DMEM + 10% FBS). Microglia were counted from each sample using a hemocytometer at 10X in a 4x4 quadrant, excluding dead cells which had been stained with trypan blue. Cells were plated with media (0ng/mL LPS), 10ng/mL LPS, or 100ng/mL LPS and incubated at 37°C for 2 hours. Plates were then spun at 1000g for 10minutes at 4°C to cease the LPS reaction. The supernatant was then decanted and 100 μ L cold 1xPBS was added to each well. Samples were then spun again for 10 minutes at 4°C. A cell lysis protocol was then followed (Invitrogen) as previous described in Frank et. al, 2006. Samples were reverse transcribed for cDNA synthesis using a cells direct kit according to the manufacturer's instructions and analyzed using qPCR (described below).

RNA extraction

1mL of trizol was added to each tissue sample and then homogenized on ice for 20 seconds. After sitting for 5 minutes at room temperature, 200 μ L of chloroform was added and the samples shaken vigorously for 15 seconds. Once the samples began to separate (~3 minutes), they were spun at 12,000g for 15 minutes at 4° C. The supernatant was then decanted into 1.5 mL tubes and mixed with an equal amount of 100% isopropanol (~500 μ L). The samples were then incubated at room temperature for 10 minutes and then centrifuged at 12,000g at 4° C for 10 minutes. The supernatant was then decanted and 1mL 75% EtOH made with nuclease-free water was added. The samples were then centrifuged at 7,500g for five minutes, or until a pellet was visible, and the supernatant decanted once more. The samples then air-dried for approximately 45 minutes and were then re-suspended in 40 μ L milipore water. All samples were then analyzed for RNA concentration using a Thermo Scientific (Waltham, Massachusetts) NanoDrop One spectrophotometer.

cDNA synthesis

RNA was reverse transcribed into cDNA according to the SuperScript II First Strand Synthesis System (Invitrogen). RNA samples and nuclease-free water were combined in their respective calculated amounts to achieve equal RNA concentrations for each sample. 1 μ L of random primers and 1 μ L of dNTP mix (master mix 1) were added to each sample and then incubated at 65°C for 5 minutes. Samples were then quick chilled on ice and subsequently centrifuged for 3-5 seconds using a tabletop Mini Vortexer from Fisher Scientific to displace any bubbles. Then 4 μ L 5X first strand buffer, 2 μ L 0.1 DTT, and 1 μ L of RNase OUT (master mix 2) were added to each sample before incubating at 25°C for 2 minutes in a Bio-Rad iCycler thermocycler. 1 μ L of SuperScript II was then added to each sample with samples kept in the iCycler. Each sample was then incubated according to a standard cDNA synthesis protocol: 25°C

for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. Samples were subsequently transferred to the -80 °C for storage.

Quantitative real-time PCR

Each well was filled with 13µL SYBR, 1µL forward primer, 1µL reverse primer, and 10µL nuclease-free water, then 1µL of vortexed cDNA with each sample run in duplicate. The plates were then spun at room temperature at 1000rpm for 1 minute. Samples were then run in a Bio-Rad iQ5 thermocycler according to the “3 step 57” protocol (denaturation at 98°C, annealing at 57°C, and extension at 72°C). The primers used in this experiment are as follows: β -actin (Forward: 5'-TTCCTTCCTGGGTATGGAAT-3'; Reverse: 5'-AAGGAAGGACCCATACCTTA-3'), IL1- β (Forward: 5'-CCTTGTGCAAGTGTCTGAAG-3'; Reverse: 5'-GGAACACGTTACAGACTTC-3'); IL6 (Forward: 5'-AGAAAAGAGTTGTGCAATGGCA-3'; Reverse: 5'-TCTTTTCTCAACACGTTACCGT-3'), TNF- α (Forward: 5'-CAAGGAGGAGAAGTTCCCA-3'; Reverse: 5'-GTTCCCTCCTCTTCAAGGGT-3').

Statistical analysis

All data are expressed as mean \pm SEM and were analyzed using two-way ANOVA or multiple t-tests with Prism 7 (GraphPad software, Inc.). An $\alpha=0.05$ was used to establish statistical significance.

Results

Expression of pro-inflammatory cytokines in whole hippocampal tissue

The mRNA of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α were expressed at greater levels in whole hippocampal tissue of female rats that underwent ovariectomy and LPS administration (IL-1 β interaction of OVX and LPS: $F_{2,42} = 4.053$, $p < 0.05$; IL-1 β LPS: $F_{1,42} = 22.19$, $p < 0.05$; IL-1 β OVX: $F_{2,42} = 1.622$, $p > 0.05$; TNF- α interaction of OVX and LPS: $F_{2,42} = 3.726$, $p < 0.05$; TNF- α LPS: $F_{1,42} = 5.449$, $p < 0.05$; TNF- α OVX: $F_{2,24} = 0.3469$, $p > 0.05$; IL-6 interaction of OVX and LPS: $F_{2,42} = 4.083$, $p < 0.05$; IL-6 LPS: $F_{1,42} = 29.35$, $p < 0.05$; IL-6 OVX: $F_{2,42} = 2.357$, $p > 0.05$). Ovariectomized rats treated with estradiol and LPS were not different from sham or saline controls.

Figure 1

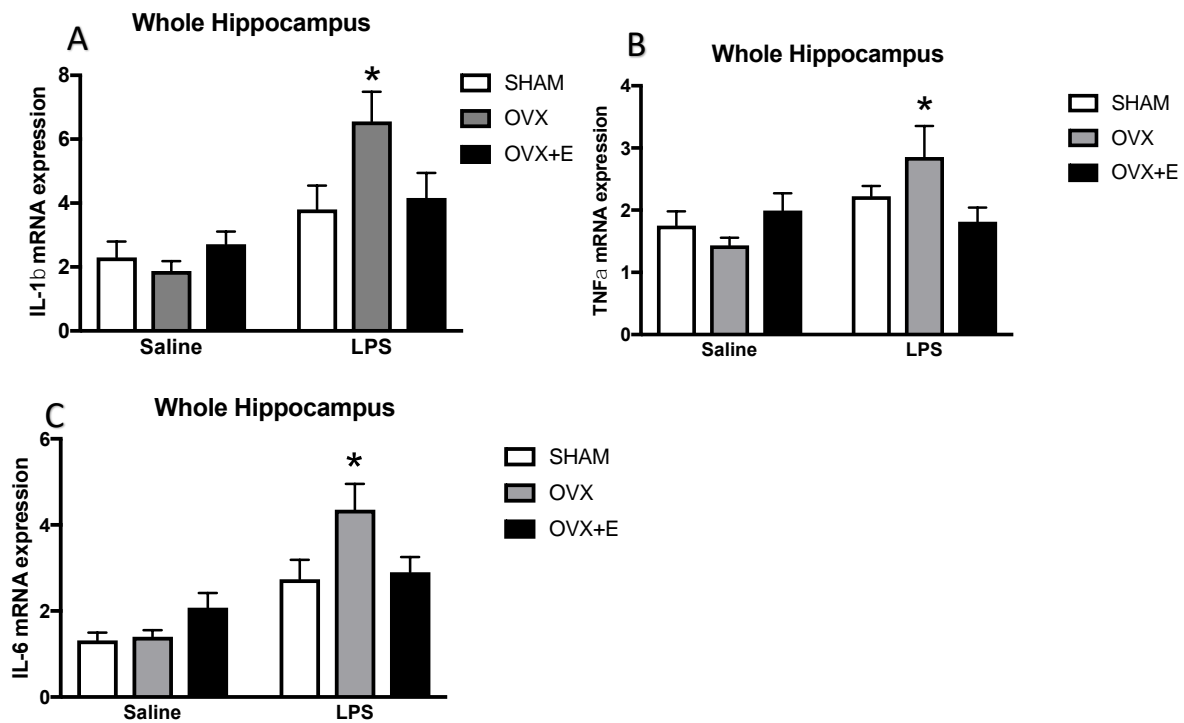


Figure 1 a. Whole hippocampal expression of pro-inflammatory cytokine IL-1 β was increased in OVX rats following LPS administration ($p < 0.05$). No difference in SHAM and OVX+E conditions was observed. Figure 1b. Whole hippocampal expression of pro-inflammatory cytokine TNF- α was increased in OVX rats following LPS administration ($p < 0.05$). No difference in SHAM and OVX+E conditions was observed. Figure 1c. Whole hippocampal expression of pro-inflammatory cytokine IL-6 was increased in OVX rats following LPS administration ($p < 0.05$). No difference in SHAM and OVX+E conditions was observed.

Expression of pro-inflammatory cytokines in isolated microglia

The mRNA of pro-inflammatory cytokines IL-1 β and TNF- α were expressed at greater levels in LPS treated microglia isolated from the hippocampus of rats that underwent ovariectomy compared to sham and OVX+E rats. IL-1 β and TNF- α mRNA expression was only increased in microglia treated with the 100 ng/mL LPS dose, and not 1ng/mL or 10ng/mL. IL-6 mRNA expression was not different in ovariectomized rats compared to sham animals (IL-1 β interaction of OVX and dose: $F_{6,48}=1.964$, $p < 0.05$; IL-1 β OVX: $F_{2,48} = 5.771$, $p<0.05$; IL-1 β dose: $F_{3,48} = 20.72$; TNF- α interaction of OVX and dose: $F_{6,44}=1.807$, $p < 0.05$; TNF- α OVX: $F_{2,44} = 3.893$, $p<0.05$; TNF- α dose: $F_{3,44}=26.05$, $p<0.05$; IL-6 interaction of OVX and dose: $F_{6,48}=1.545$, $p=0.1841$; IL-6 OVX: $F_{2,48}=2.43$, $p>0.05$; IL-6 dose: $F_{3,48}=32.79$, $p<0.05$).

Figure 2

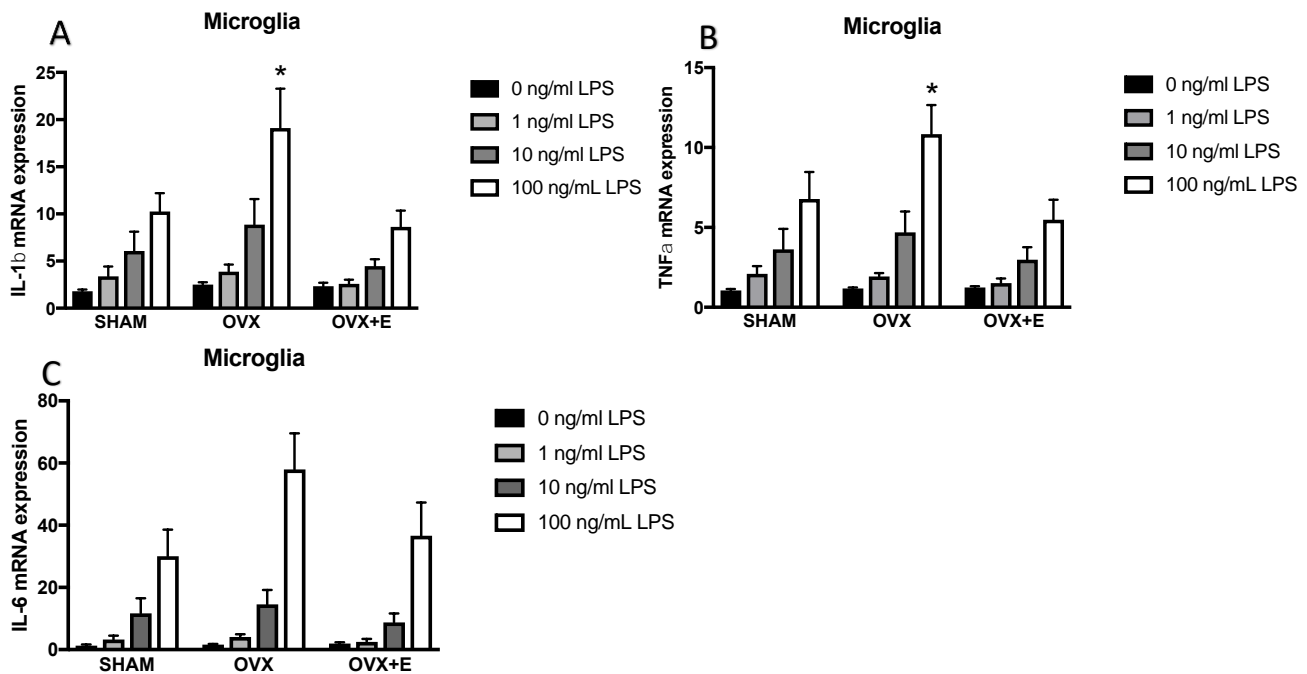


Figure 2 a. Microglial expression of pro-inflammatory cytokine $IL-1\beta$ was increased in OVX rats following LPS administration ($p < 0.05$). No difference in SHAM and OVX+E conditions was observed. Figure 2b. Microglial expression of pro-inflammatory cytokine $TNF-\alpha$ was increased in OVX rats following LPS administration ($p < 0.05$). No difference in SHAM and OVX+E conditions was observed. Figure 2c. No difference in $IL-6$ expression was observed in OVX, OVX+E, and SHAM conditions ($p=0.1841$).

Fear conditioning

Ovariectomized rats showed no difference in fear conditioning response (% freezing) compared to sham and OVX+E animals, regardless of LPS or saline administration (fear conditioning interaction of OVX and LPS: $F_{2,29}=0.211$, $p=0.8109$; fear conditioning LPS: $F_{1,29} = 3.055$, $p>0.05$; fear conditioning OVX: $F_{2,29}=2.934$, $p>0.05$).

Figure 3

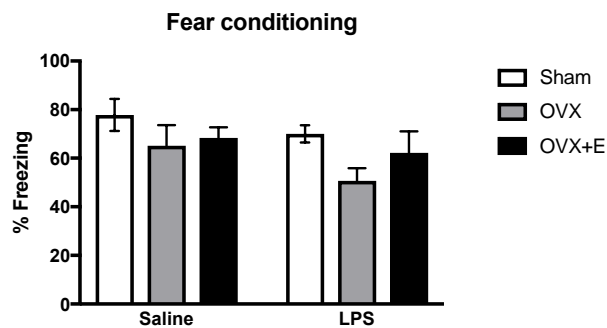


Figure 3. Fear conditioning response expressed as percent freezing was not affected by OVX or LPS conditions ($p>0.05$).

Body mass

Animals showed no difference in body mass prior to surgery, averaging approximately 215g across conditions. By day 21, the OVX group increased to an average of 250g, OVX+E to an average of 230g, and SHAM to an average of 220g.

Figure 4

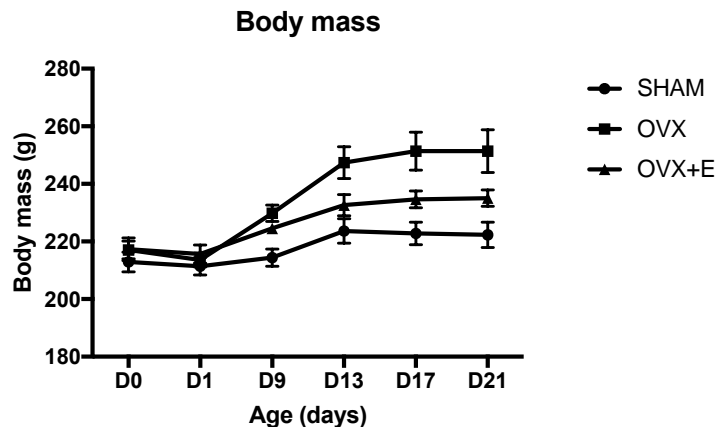


Figure 4. OVX, but not SHAM caused weight gain in rats, which was abrogated by estradiol replacement (OVX+E).

Discussion

These results, in combination with other research, suggest a multi-faceted role for estrogen in neuroinflammation. Specifically, they suggest that through a mechanism not yet fully elucidated, estrogen acts on microglia and/or neurons to influence mRNA expression of certain pro-inflammatory cytokines. The fact that expression of IL-1 β and TNF- α is altered by estrogen in microglia alone, in addition to tissue containing microglia, neurons, astrocytes, and other cell types, suggests that microglia may contribute to this anti-inflammatory response in the presence of estrogen. However, the fact that all three cytokines that were tested (IL-1 β , TNF- α , and IL-6) were reduced by estrogen in whole hippocampal tissue suggests a modulatory role for some cell type present in the whole tissue other than microglia. Because the results of fear conditioning from this study showed no significant differences between groups, it can be inferred that estrogen deficiency in combination with LPS treatment in female rats does not affect the cognitive processes required for fear conditioning. However, effects on other cognitive tasks could be investigated.

To discover if microglia are the sole mediators of estrogen-induced anti-inflammation, microglia-deficient cell populations could be challenged with LPS and tested for inflammatory markers. In order to investigate the role of other glial cells, such as astrocytes, in estrogen-dependent anti-inflammatory mechanisms, an astrocyte isolation method could be used in place of or in conjunction with the microglia isolation protocol from this experiment (with similar treatment groups). Astrocyte and microglia cell cultures could then be treated with estrogen and LPS and compared to estrogen-deficient cultures in cytokine expression, or other neuroinflammatory measures. However, this leaves the question of estrogen receptor specificity unanswered. In order to understand both cell type and receptor specificity in mediating estrogen's anti-inflammatory effects, a method called Drugs Acutely Restricted by Tethering (DART) could be used. DART uses a bacterially derived enzyme called HaloTag to recruit and tether drugs to a specific receptor, while being exclusively expressed in a desired cell type. This can include specific glial cells, or distinct varieties of neurons. Additionally, it has relatively high temporal resolution (seconds to minutes), allowing it to be used in vivo during behavioral experimentation (Shields et al., 2017).

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