Stressor re-exposure effects on behaviour and expression of pro-inflammatory cytokines in male and female mice.

Stephanie Hudson

A Thesis submitted to

The Faculty of Graduate and Postdoctoral Affairs

In partial fulfillment of the requirements for the degree of:

Master of Science

In Neuroscience

Carleton University

Ottawa, Canada

Abstract:

The neurochemical effects of stressful events involve a range of adaptive responses to environmental challenges. However, repeated stressors may result in the development of psychopathologies such as anxiety and depression. The immune signaling molecule, pro-inflammatory cytokines, has been linked to the development of such illnesses. Interestingly, distinct differences in stressor responsiveness exist between the sexes, alongside a much higher rate of affective disorders in females. In the present experiments we examined if repeated exposure to stressors would impact male and female CD-1 mice differently with respect to behaviour as well as cytokine expression. Repeatedly stressed males demonstrated impulsive behaviour along with sensitized IL-1β expression. In follow-up experiments the role of 17β-estradiol was examined, with stressed males that were exposed to estrogen showing reduced TNF-α expression while similarly treated females had a drastic elevation in IL-6. The current results demonstrate the differences in the stressor response system between the sexes.

Acknowledgements:

I would like to express my gratitude to Dr. Hymie Anisman for his guidance through my Master's level work, as well as everyone in Dr. Anisman's laboratory for their assistance. I would also like to thank the faculty, staff and students within the Carleton University Neuroscience program, as well as members of the Ottawa Society for Neuroscience, for helping to create such a collaborative and inclusive atmosphere.

This work would not have been possible without the tireless support of my wonderful parents, or my friends who made it their mission to keep my spirits high.

This work is dedicated to my grandmother Patricia Blochert, a scientist who inspired my passion for learning.

This research was supported by the Canadian Institutes of Health Research (CIHR).

Table of Contents

List of Figures

1. Introduction:

Stressful events, through their impact on central neurochemical processes, might enhance an organism's ability to cope successfully with environmental challenges. Biological responses, such as elevated hypothalamic-pituitary-adrenal (HPA) functioning and monoamine activity in frontal cortical regions, are generally adaptive when activated in response to an acute stressor, but chronic or repeated exposure to unpredictable stressors may lead adaptive processes becoming overloaded and could result in the development of affective disorders (Anisman et al., 2008; McEwen, 2000).

Considerable evidence has indicated that immunological processes mediate the stress-depression relationship (Anisman et al., 2008; Paykel, 2001). Specifically, exposure to a stressful event can influence the functioning of cytokines, which serve as signaling molecules of the immune system (Audet et al., 2011; Dantzer et al., 2008; Maes et al., 1995) and which also appear in brain, likely coming from microglia (Rivest, 2009). Under some conditions, the cytokine response is highly adaptive and may enhance normal cognitive functioning (Leonard, 2010; Penkowa et al., 2003). However, persistent or excessive cytokine elevations may give rise to neuroendocrine and neurotransmitter variations resulting in a reduced level of neurogenesis, which can promote an affective disturbance such as depression (Anisman et al., 2003; Anisman & Merali 2002; Herbert & Cohen, 1993).

Consistent with the view that cytokine variations elicited by stressors lead to depression (Anisman et al., 2008), affective illnesses are frequently associated with heightened levels of circulating cytokines, such as IL-1β, IL-6 and TNF-α (Anisman et al., 2008, Howren et al., 2009; Jaremka et al., 2013). High concentrations of cytokines

have also been reported in healthy patients exposed to stressors (Frommberger et al., 1997) and it seems that stressors can act synergistically with cytokines in stimulating depressive-like behaviors (Gibb et al., 2008).

Stressful experience can also lead to the sensitization of biological processes so that greater elevations are engendered upon future stressor exposure, thus increasing vulnerability to depressive disorders (Frank et al., 2007; Gibb et al., 2011; Johnson et al., 2003). In this respect, rodents that have been repeatedly stressed exhibit exaggerated cytokine release within regions of the brain responsible for higher-order processing, such as the prefrontal cortex (PFC) and hippocampus (Anisman et al., 2003; Belda et al., 2008; Jedema et al., 1999; Nisenbaum & Abercrombie 1993). For example, adult male mice that were repeatedly exposed to social stressors demonstrated elevated expression of TNF-α and IL-1β (Audet et al., 2011).

The cascade of central and peripheral changes elicited upon stressor re-exposure may also have pronounced behavioral ramifications (Anisman et al., 2008; Hayley et al., 2003). According to the "stress sensitization" hypothesis it is thought that with each episode of depression, the level of a stressor needed to trigger a recurring episode is progressively smaller (Post, 2010). Individuals who experienced greater exposure to adversity and stressful situations, and have a history of anxiety and depressive disorders, displayed increased severity of depression following low levels of episodic stress (Espejo et al., 2007).

Although stressful experiences, cytokine reactivity and affective disorders are highly associated (Anisman et al., 2008; Bertini et al., 2013; Gibb et al., 2009), most studies that only included male animals and neglected to explore the relations evident in

females. However, it has been observed that ovariectomy increased anxiety and depressive behaviours, whereas the administration of estrogen reversed these effects (Bowman et al., 2002; Estrada-Camerena et al., 2003). Furthermore, rats that had received estrogen treatment prior to stressor exposure demonstrated decreased anxiety (Frye & Walf, 2004; Frye & Wawrzycki, 2003). The paucity of data concerning stressor effects in females that had existed for years is surprising given that it was long known that in humans, females are more likely to develop depressive disorders (Earls, 1987; Kessler et al., 1993). This is the case for both typical and atypical depressive illness, although the gender difference is greater in the latter instance, often being accompanied by elevated anxiety (Gorman, 2006; Seeman, 1997). As well, basal cortisol levels have been correlated with the efficacy of antidepressant treatment for men, but not for women, suggesting the existence of HPA axis related sex differences in depression (Binder et al., 2009). Paralleling the dimorphisms evident with respect to other biological systems, cytokine elevations that are associated with depression have also been reported to be more pronounced in women than in men (Amin et al., 2005).

Despite the greater vulnerability toward developing anxiety or depressive disorders among females (Baca-Garcia et al., 2000; Bloch et al., 2000; Breslau et al., 1995; Kessler et al., 1994), whether and how estrogen contributes to this susceptibility is still uncertain. In this regard, it might be that both progesterone and androgens (or their metabolites) that occur alongside the estrogen changes are involved in the evolution of depression (Rocha et al., 2005). In essence, estrogen has a wide range of adaptive effects on the brain, including therapeutic potential in diminishing affective disorders (Walf et al., 2004).

Alongside the evidence that estrogen may be a source of anxiolytic and antidepressant effects, it was also found to be involved in HPA axis modulation and physiological stressor response (Duka et al., 2000; De Leo et al., 1998). In ovariectomized mice that had received acute estrogen administration, stress-induced corticosterone levels were elevated following relatively high or repeated estrogen doses (McCormick et al., 2002; Walf & Frye 2005). However, acutely restrained rats that received moderate doses of estrogen (5 or 10 μg) exhibited diminished anxiety and depressant-like behavioral effects, as well as lowered corticosterone levels, while higher or lower amounts of estrogen did not promote these changes. Evidently, a nonlinear dose-response relationship exists between estrogen and the therapeutic effects it has been hypothesized to exert (Walf & Frye, 2006). As elevated cytokine response following exposure to stressors has been implicated in the development of negative affective disorders, estrogen may also attenuate stress response by modulating cytokine release (Asai et al., 2001). Following TNF-α release by macrophages in rats, exposure to estradiol reduced the proliferation of the cytokine release (Chao et al., 1995). Likewise, estradiol administrations was able to reduce the release of IL-1β, IL-6 and TNF-α that were ordinarily elevated following lipopolysaccharide stimulation (Rogers & Eastell, 2001).

The role that sex differences play in the relationship between stress, immune reactivity and affective disorders has not been thoroughly examined, despite strong evidence of sexual dimorphism in relation to behavioral and neurochemical responses to stressors (Darnall & Suarez, 2009). Moreover, the role of estrogen in the inter-relations between stress-immune functioning and affective disorders remains to be determined.

The present investigation was undertaken to determine whether (a) exposure to an acute stressor differentially influences behavioral and brain cytokine responses in males and females, (b) re-exposure to a stressor will further modulate the behavioral and immune responses in males and females, and (c) estrogen treatment of male mice would alter the effects of stressors on behavioral and pro-inflammatory cytokine levels.

In Experiment 1 we assessed whether acute exposure to a restraint stressor affected the performance of male and female mice in an elevated Plus-Maze test in order to evaluate anxiety-related behaviors 90 minutes following stress exposure. We also investigated whether this effect varied on the basis of previous stressor experience, and whether this outcome differed between the sexes. In Experiment 2 we applied the same protocol to assess plasma corticosterone levels as well as different pro-inflammatory cytokine patterns (IL-1β, IL-6 and TNF-α, and their respective receptors) in the prefrontal cortex and hippocampus. In Experiment 3 we investigated whether seven days of oral estrogen treatment prior to an acute stressor would alter performance in an elevated Plus-Maze as a measure of anxiety behaviour in male and female mice. Finally, in Experiment 4, this same protocol was again applied, and we evaluated plasma corticosterone levels and pro-inflammatory cytokine levels (IL-1β, IL-6 and TNF-α and receptors).

2.0 Materials and Methods:

2.1 Experimental Animals

Male mice and female CD-1 mice were used as experimental subjects. The mice were purchased from Charles River Canada, located in St. Constant, Quebec. Upon arrival, mice were approximately 45 days old and were allowed two weeks to become

acclimatized to the laboratory, which also ensured they would be at least 70 days old (adult age) when the experiment began. They were housed individually in standard sized (27 x 21 x 14 cm) polypropylene cages and were exposed to 12 hour light-dark cycles (from 0700-1900 h), with the temperature and humidity kept constant (22°C and 63% respectively). The animals had free access to food and water. The study met the guidelines laid out by the Canadian Council on Animal Care and approved by the Carleton University Animal Care Committee.

2.2 Experiment 1: Effects of acute and re-exposure to stressors on Plus-Maze behaviour.

For the initial experiment, CD-1 male and female mice were exposed (or not) to a variable stressor regimen for three consecutive days (days 70-72). The mice were then not disturbed for 6 weeks. At this point mice were randomly assigned to either a stressor or no stressor treatment (N=8-10 per group). Mice were then exposed to a behavioral paradigm beginning 90 minutes after stress exposure. The elevated Plus-Maze consists of two open arms and two arms enclosed by high walls. The open arms are placed perpendicularly to the closed arms, so the four arms intersect to form a "plus" sign. The closed arms represent a secure location for the mice, while the open arms give mice the opportunity to explore. It is hypothesized that anxious mice would spend less time in the open arm region of the Plus-Maze. The behavior of the mice within this maze is assessed over a five minute period by the latency of time before entering the open arms of the maze, the number of entries into the open arms and closed arms of the maze, as well as the time spent in the open and closed arms.

2.3 Experiment 2: Effects of acute and re-exposure to stressors on plasma corticosterone and brain pro-inflammatory cytokine mRNA expression.

A second set of CD-1 male and female mice underwent an identical procedure to that of Experiment 1, with the exception that 90 minutes following the stressor exposure mice were sacrificed by rapid decapitation. Trunk blood was collected to measure corticosterone. Brains were collected and dissected to inspect mRNA expression of IL-1β, IL-6, TNF-α and their respective receptors, IL-1 receptor (IL-1R), IL-6 receptor (IL-6R) and TNF-α (TNF- α R) receptor in the prefrontal cortex (PFC) and the hippocampus, brain regions associated with cytokine activation and stress regulation (Anisman et al., 2003; Hayley et al., 2003).

2.4 Experiment 3: Effects of oral 17β-estradiol exposure and an acute stressor on Plus-Maze behaviour.

In a follow-up experiment, male and female CD-1 mice were randomly assigned to estrogen, vehicle or a no treatment control group as well as a stressor or control group (N=6-8 per group). For two days mice were exposed to a food supplement regimen of Nutella and sesame oil, to allow them to become familiar with the oral supplement. Mice's weights (in grams) were recorded on the first and final day of the experiment. For the subsequent seven days mice in the estrogen group were given oral doses of 17β-estradiol at 1.5 micrograms a day that was mixed into the vehicle of Nutella and sesame oil, at an overall weight of .06 grams of daily. Mice exposed to the vehicle condition were given a mixture of Nutella and sesame oil .06 grams daily as part of their diet for these same seven days. On test day, females were vaginally lavaged and cells were spread

onto a microscopic slide. Cells were stained with Crystal Violet, covered and examined under a light microscope in order to determine estrus cycle phase. Pro-estrus cells are irregular in shape and have small nuclei and are found in organized clumps, while estrus cells are non-nucleated. It was determined that female mice being exposed to the estrogen supplement were in the pro-estrous phase while control and vehicle mice were in the estrus phase. Mice in the control group were given no food supplement. After seven days of estrogen or vehicle exposure mice were exposed to an acute restraint stressor of 15 minutes in length (or no stressor) and were tested in the Plus-Maze 90 minutes after stressor exposure. Mice's weights (in grams) were recorded on the first and final day of the experiment. Again, females cycles were examined on test day and it was determined that female mice being exposed to estrogen were in then in the pro-estrus phase of the cycle, whereas the control and vehicle mice were in the estrus stage.

2.5 Experiment 4: Effects of oral 17β-estradiol exposure and an acute stressor on plasma corticosterone and brain pro-inflammatory cytokine mRNA expression.

In the fourth experiment, CD-1 male and female mice underwent an identical procedure to that of Experiment 3, however, 90 minutes following the stressor exposure mice were sacrificed by rapid decapitation. Trunk blood was collected to measure corticosterone. Brains were collected and dissected to inspect mRNA expression of IL-1β, IL-6, TNF-α and their respective receptors, IL-1β receptor (IL-1R), IL-6 receptor (IL-6R) and TNF-α (TNF- αR) receptor in the prefrontal cortex (PFC) and the hippocampus.

2.6 Experiment 5: *Effects of seven day oral 17β-estradiol exposure on weight gain.*

In the final experiment, the weights of CD-1 male and female mice in Experiment 3 and 4 were recorded (in grams) on the first day of vehicle exposure as well as on the final day (the seventh day) of estrogen exposure.

2.7 Corticosterone Determination

Corticosterone was determined using a commercial radioimmunoassay RIA kit (ICN Biomedicals, CA). For each experiment, corticosterone levels were determined, in duplicate, in a single run to avoid inter-assay variability, and the intra-assay variability was less than 8%.

2.8 Reverse Transcription-Quantitative Polymerase Chain Reaction Analysis.

Brain tissue punches were homogenized using Trizol and total brain RNA was isolated according to the manufacturer's instructions (Invitrogen, Burlington, ON, Canada). The total RNA was then reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) and the resulting cDNA aliquots were analyzed in simultaneous quantitative polymerase chain reactions (qPCR). SYBR green detection was used according to the manufacturer's protocol (Bio-Rad, CA, USA) and a MyiQ2 RT PCR Detection System (Bio-Rad, CA, USA) collected the data. All designed PCR primer pairs generated amplicons between 100 and 200 bp. Amplicon identity was verified by restriction analysis. Primer efficiency was measured from the slope relation between absolute copy number of RNA quantity and the cycle threshold (Ct) using the Bio-Rad IQ5 version 2.0 software (Bio-Rad, CA, USA). All primer pairs had a minimum of 90%

efficiency. House-keeping genes used were Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), one of the most frequently used and stably expressed reference gene (Boda et al., 2009; Gutkowska et al., 2009) and Synaptophysin which was previously found to be stable in brain samples of stressed mice (Anisman, et al., 2008; Gibb, et al., 2009). Thus, the expression of each gene of interest within the PFC was normalized by subtracting the averaged C_t of Gapdh and Synaptophysin from the gene of interest C_t (ΔC_t). The $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008) was then used to convert ΔC_t values to mRNA fold changes relative to the no variable stressor- no re-exposure to stressor control group (calibrator). Primer sequences used for qPCR were as follows: Gapdh, forward: GGT CGGTGTGAACGGATTTG, reverse: TGCCGTGAGTGGAGTCATACTG; Synaptophysin, forward: GGACGTGGTGAATCAGCTGG, reverse: GGCGAAGATGGCAAAGACC; IL-1β, forward: TGTCTGAAGCAGCTATGGCAAC, reverse: CTGCCTGAAGCTCTTGTTGATG; IL-6, forward: ACGGCCTTCCCTACTTCACA , reverse: TGCCATTGCACAACTCTTTTCTC; TNF-α, forward: CTCAGCCTCTTCTCATTCCTGC, reverse: GGCCATAGAACTGATGAGAGGG; IL-1 receptor, forward: ACT CAC CAT AAG TGC GGA GTG C, reverse: CAC TGA CTT CTC AGG GCC TTT G; IL-6 receptor, forward: CTCTCCAACCACGAAGGCTG, reverse: TGCAACGCACAGTGACACTATG; TNF-α receptor, forward: CAGAACACCGTGTGTAACTGCC, reverse: GGGTTTGTGACATTTGCAAGC.

2.9 Data Analyses

For Experiments 1 and 2 the behavioral data, corticosterone levels as well as fold changes for cytokines IL-1β, IL-6, TNF-α and their respective receptors, in the PFC and hippocampus, were analyzed through a 2 (sex: male and female) x 2 (initial stressor: three day variable stressor and no three day variable stressor) x 2 (re-exposure treatment: control and restraint) between-group analysis of variance (ANOVA). For Experiments 3 and 4 the same outcomes were measured within the PFC and hippocampus through a 2 (sex: male and female) x 3 (control, vehicle or estrogen) x 2 (control or restraint) between group ANOVA. For Experiment 5, the final weight (measured on test day) and overall weight change were measured through a 2 (sex: male and female) x 3 (control, vehicle or estrogen) group ANOVA. Follow-up comparisons were performed using t tests with a Bonferroni correction to maintain the α level at 0.05. Several samples were lost over the course of the study, thus the degrees of freedom differed across the outcome measures. All tests were done with SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

3.0 Results

3.1 Experiment 1: Effects of acute and re-exposure to stressors on Plus-Maze behaviour.

The latency to first enter the open arms of the Plus-Maze varied as a function of the Sex x Initial Variable Stressor interaction, $F(1, 67) = 6.54$ $p < .05$. Follow-up tests indicated that among males, exposure to the three day variable stressor significantly reduced the latency to enter an open arm, compared to the males who were not exposed to the Initial Variable Stressor ($p < .0005$). In contrast, as depicted in Figure 1A, the stressor had no effect on the latency to enter the open arm among females.

Exposure to the stressors did not provoke any significant effects on time spent in the open arms of the maze (Figure 1B), but the number of entries to the open arms varied as a function of the interaction between Sex x Acute Stressor exposure, $F(1, 67)=5.3$, $p < .05$. Follow up tests indicated that female mice who experienced the acute stressor exhibited elevated entries to the open arms relative to males that also experienced the 15 minute restraint stressor on the test day ($p < .05$) (see Figure 1C). Entries to the open arms were also found to be elevated overall in animals that experienced the three day long initial stressor, $F(1, 67) = 6.282$, $p < .005$.

Time spent within the closed arms of the maze did not significantly vary by Sex, the Initial Variable Stressor, the Acute Stressor or any of the interaction involving these variables (Figure 1D). However, the number of entries to closed arms of the maze (Figure 1E) were greater in males than in females irrespective of the stressor condition, $F(1, 69) = 8.82$, $p < .005$.

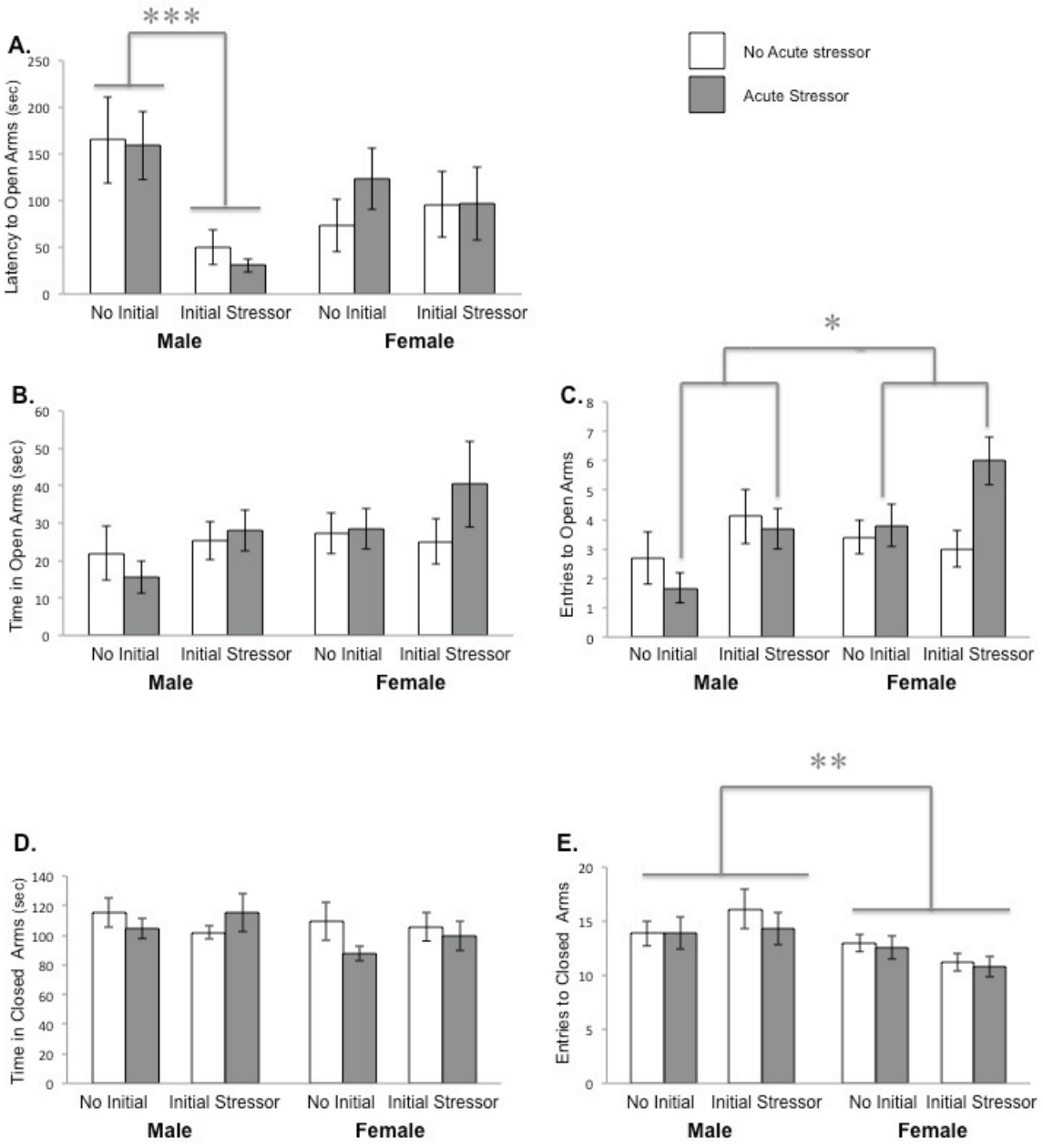

Figure 1 – Effects of the initial stressor and re-exposure treatment on adult male and female behaviour in an elevated Plus-Maze. (A) Mean (±SEM) latency to open arms, (B) mean (±SEM) time spent in open arms, (C) mean (±SEM) number of entries to open arms, (D) mean (±SEM) time spent in closed arms, and (E) mean (±SEM) number of entries to closed arms. Females that experienced the Acute Stressor relative to acutely stressed males, *$p < 0.05$. Males relative to females, **$p < 0.005$.
Male mice that experienced the Initial Variable Stressor relative to those that did not, ***$p < 0.0005$.

3.2 Experiment 2: Effects of acute and re-exposure to stressors on plasma corticosterone levels.

Levels of plasma corticosterone for the different stressor groups differed as a function of an interaction between the Initial Variable Stressor x Gender, $F(1, 70) = 4.50$, $p < .05$ (Figure 2A). Follow-up tests indicated that female mice that experienced the three day Initial Variable Stressor had higher levels of circulating plasma corticosterone than male mice that also experienced an stressor regimen ($p < .0005$), regardless of experiencing the subsequent Acute Stressor.

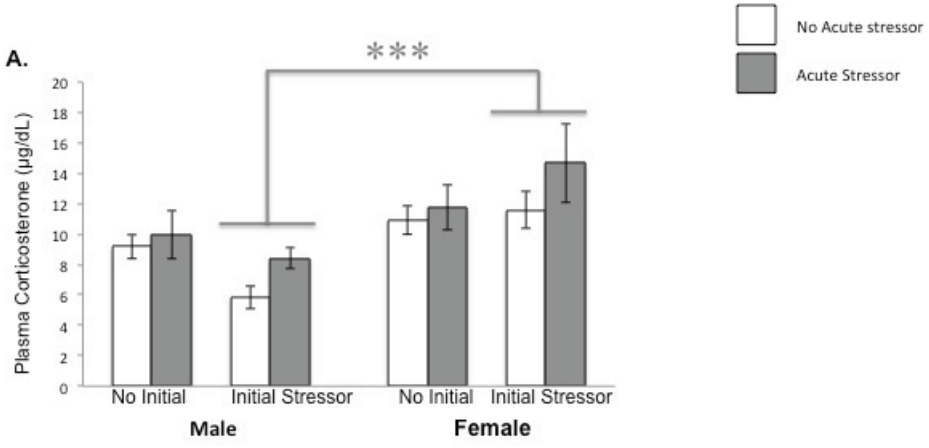

Figure 2 - Mean (±SEM) concentrations on plasma corticosterone levels in response to a three day variable stressor and subsequent acute stressor exposure (restraint). Female mice that were initially stressed demonstrated higher levels of corticosterone relative to male mice who experienced the same stressor paradigm, ***$p < .0005$.

3.3 Experiment 2: Effects of acute and re-exposure to stressors on prefrontal cortex pro-inflammatory cytokine mRNA expression.

IL-1β and IL-1R

The mRNA expression of the IL-1β within the PFC (Figure 3A) varied as a function of the interaction between Sex, the Initial Variable Stressor and the Acute Stressor treatment, $F(1, 63) = 6.07$, $p < .05$. Follow up tests indicated that the observed increase in IL-1β levels was attributable to a marked rise of this cytokine's mRNA expression in male mice that had been exposed to both the initial three day stressor and the subsequent acute stressor session, relative to mice that had been stressed initially ($p < .005$) (see Figure 3A). The re-exposed male mice also showed a significant elevation in IL-1β expression above male mice that were only acutely stressed, though this significance was lost in Bonferroni corrections ($p = .019$). This same effect was not apparent in females. The expression of the cytokine receptor, IL-1R (Figure 3B) increased among mice that had been exposed to the Acute Stressor, $F(1, 66) = 28.70$, $p < .005$, irrespective of the sex or the initial stressor treatment.

TNF-α and TNF-αR

The expression of TNF-α in the PFC varied as an interaction between the Sex and the Initial Variable Stressor conditions, $F(1, 65) = 6.35$ $p < .05$. Follow up tests indicated that exposure to the Initial Variable Stressor increased TNF-α mRNA expression in males compared with females who experienced the same stressor regimen ($p < 0.005$) (Figure 4A). Tests also revealed that there was an overall was elevation among the mice exposed to the Acute Stressor relative to those that did not experience this 15 minute period of

restraint, $F(1, 65) = 8.05$, $p < .05$. No significant changes were found in TNF-αR as a result of either the three day or the acute stressor conditions (Figure 4B).

IL-6 and IL-6R

Relative to females, the male mice had higher levels of both IL-6, $F(1, 59) = 10.14$, $p < .005$ and IL-6R, $F(1, 65) = 19.89$, $p < .005$ (Figures 5A and 5B). Mice that experienced the Acute Stressor also exhibited increased levels of IL-6, $F(1, 63) = 4.04$, $p < .05$. and IL-6R, $F(1, 65) = 8.81$, $p < .005$ when compared with mice that did not experience the 15 minute restraint stressor. However, as seen in Figure 3, in the case of IL-6 expression, the magnitude of these effects was actually very small, amounting to less than a 0.5 fold change.

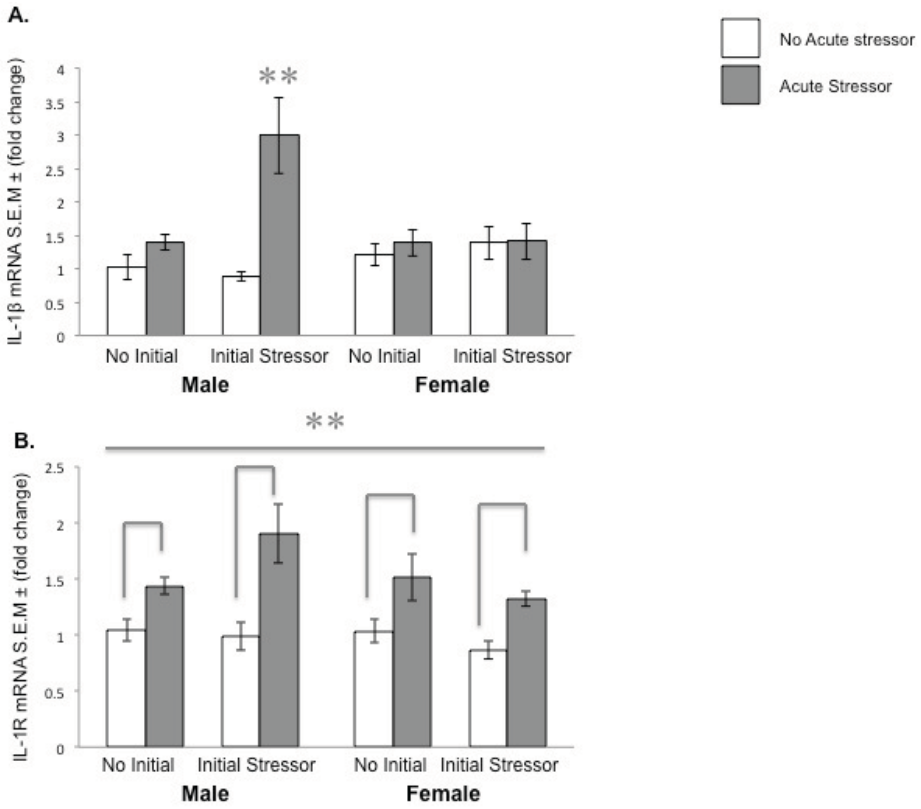

Figure 3 – Prefrontal cortex (PFC) mRNA expression (mean fold changes ±SEM) in response to exposure to a three day variable stressor and subsequent acute stressor exposure (restraint) on (A) IL-1β (B) IL-1R levels. In Panel A, male mice that experienced the initial variable stressor and then re-exposed to the acute stressor exhibited greater IL-1β expression than mice that had initially been stressed, but not re-exposed to a stressor, **$p<0.005$. In Panel B, IL-1R expression was elevated among mice that received the acute stressor relative to those mice that had not received this treatment, **$p < 0.005$.

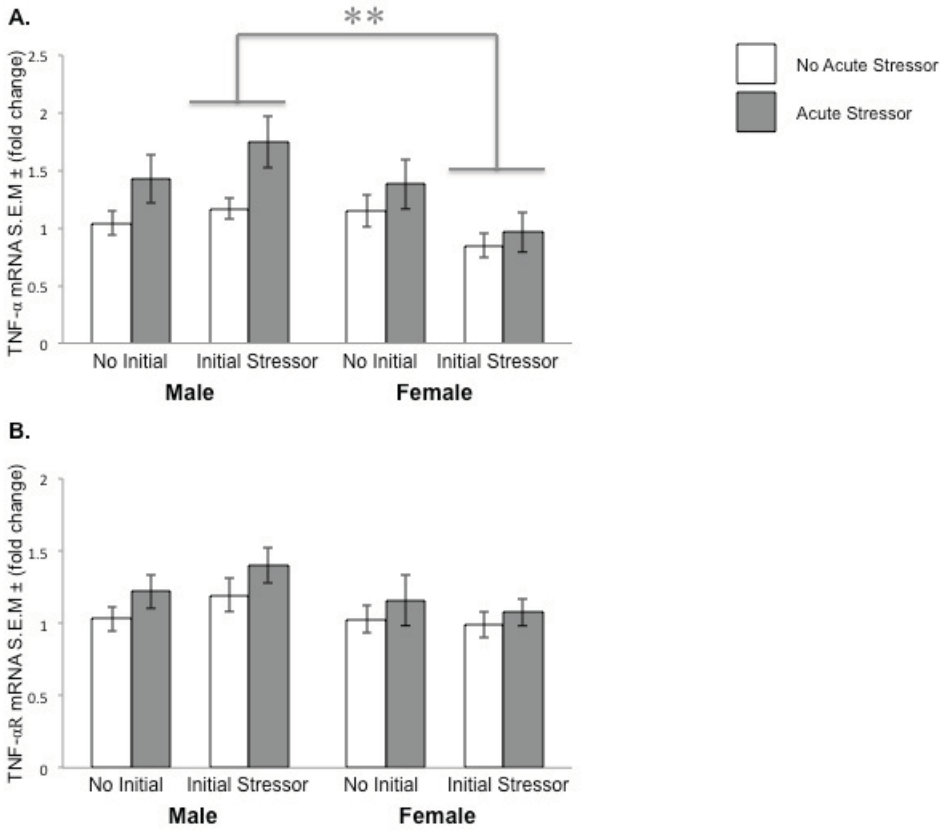

Figure 4 - Prefrontal cortex (PFC) mRNA expression (mean fold changes ±SEM) in response to a three day variable stressor and subsequent stressor (restraint) exposure on (A) TNF-α and (B) TNF-αR mRNA expression. Males that were exposed to the Initial Stressor exhibited higher TNF-α expression than identically stressed female mice, **$p<0.005$.

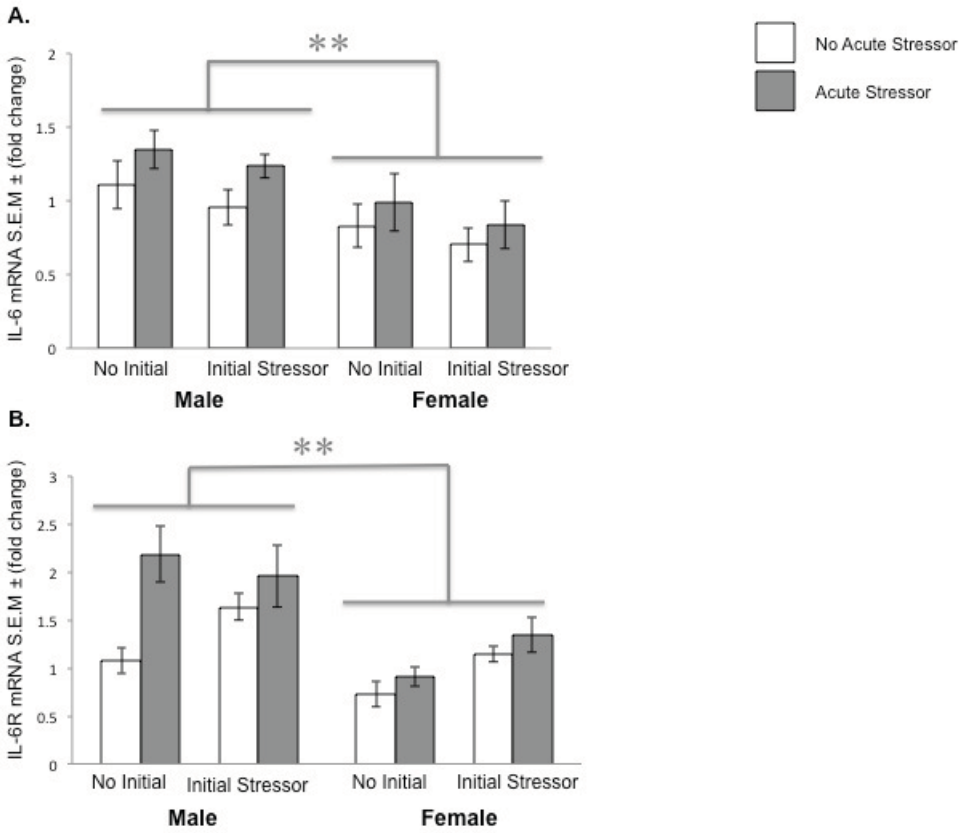

Figure 5 – Prefrontal cortex (PFC) mRNA expression (mean fold changes ±SEM) in response to a three day variable stressor and subsequent stressor exposure (restraint) on (A) IL-6 and (B) IL-6R mRNA expression. Males exhibited higher expression of IL-6 and IL-6R than did females, irrespective of the treatment condition, **$p < .005$.

3.4 Experiment 2: Effects of acute and re-exposure to stressors on hippocampal pro-inflammatory cytokine mRNA expression.

IL-1β and IL-1R

The mRNA expression of the IL-1β (Figure 6A) within the hippocampus varied as a function of interaction between Sex, the Initial Variable Stressor and the Acute Stressor, $F(1, 58) = 6.64$, $p < .05$. Follow up tests indicated that IL-1β levels were increased significantly in males who were exposed to both the Initial Variable Stressor and the Acute Stressor when compared with males who were only exposed to the initial three day stressor and when compared with males only exposed to the test day acute stressor (p's $< .05$) (see Figure 6A). Follow up tests also demonstrated that unstressed females and acutely stressed females demonstrated elevated IL-1β expression compared to their identically stressed male counterparts (p's $< .005$).

The IL-1R expression was a function of an interaction between Sex and the Acute Stressor treatment (restraint), $F(1, 59) = 10.34$, $p < .005$. The follow-up tests indicated that female mice that had not been acutely stressed had higher levels of IL-1R than males in the same experimental condition ($p < .005$) (Figure 6B). Tests also indicated that IL-1R was elevated in mice that experienced the three day Initial Variable Stressor $F(1, 59) = 6.06$, $p < .05$

TNF-α and TNF-αR

As seen in Figure 7A, TNF-α expression in the hippocampus of mice varied as an interaction between the Sex x the Acute Stressor, $F(1, 58) = 11.98$, $p < .005$. Follow up tests revealed higher levels of TNF-α among female mice that had not been given the acute test day stressor relative to their male counterparts ($p < .0005$) (Figure 7A). The

mRNA expression for TNF-αR also varied as a function of Sex x Initial Variable Stressor, $F(1, 62) = 8.51$, $p = .005$, and the follow-up tests revealed again that females that were not exposed to the Initial Variable Stressor demonstrated elevated levels of TNF-αR compared to males which were also not exposed to this initial stressor ($p < .0005$) (Figure 7B).

IL-6 and IL-6R

The mRNA expression of both IL-6 (Figure 8A) and IL-6R (Figure 8B) within the hippocampus, was elevated in female mice relative to males, $F(1, 56) = 13.86$, $p < .0005$ and, $F(1, 61)=15.91$, $p < .0005$.

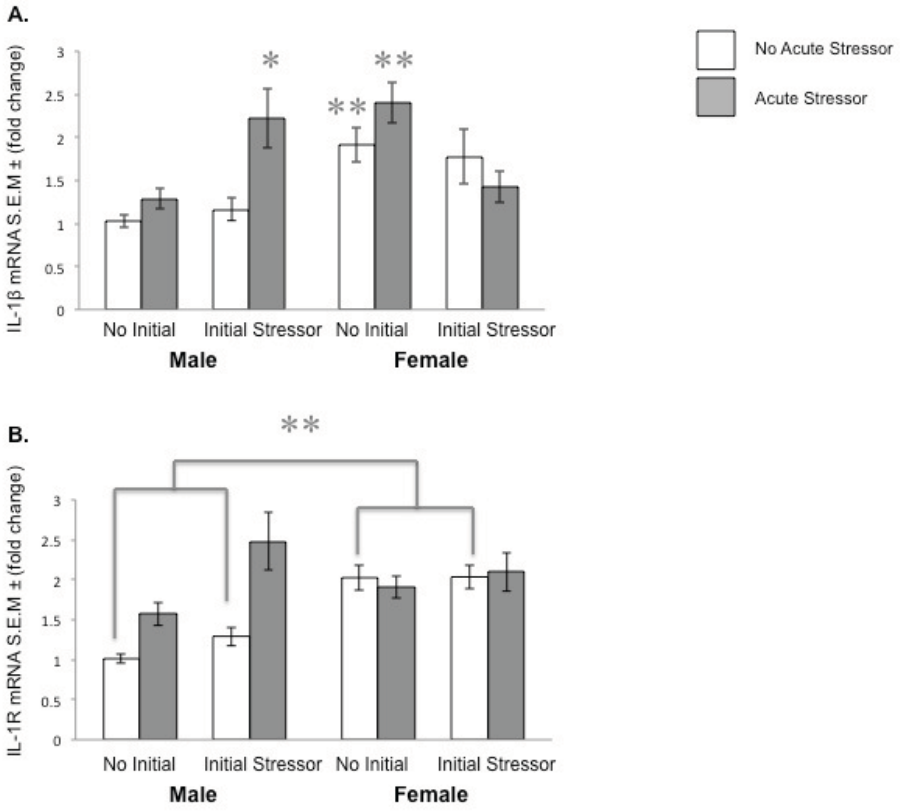

Figure 6 – Hippocampal mRNA expression (mean fold changes ±SEM) in response to a three day variable stressor and subsequent stressor exposure (restraint) on (A) IL-1β and (B) IL-1R mRNA expression. In Panel A, male mice that experienced both the initial three day stressor and the subsequent acute stressor expressed greater IL-1β than those male mice that received only the three day or the acute stressor treatment, *$p < .05$. Females that had not initially been stressed exhibited higher IL-1β mRNA expression than did similarly treated male mice, **$p < 0.005$. In Panel B, IL-1R was elevated in female mice that were not acutely stressed relative to similarly non-stressed male mice, **$p<.005$.

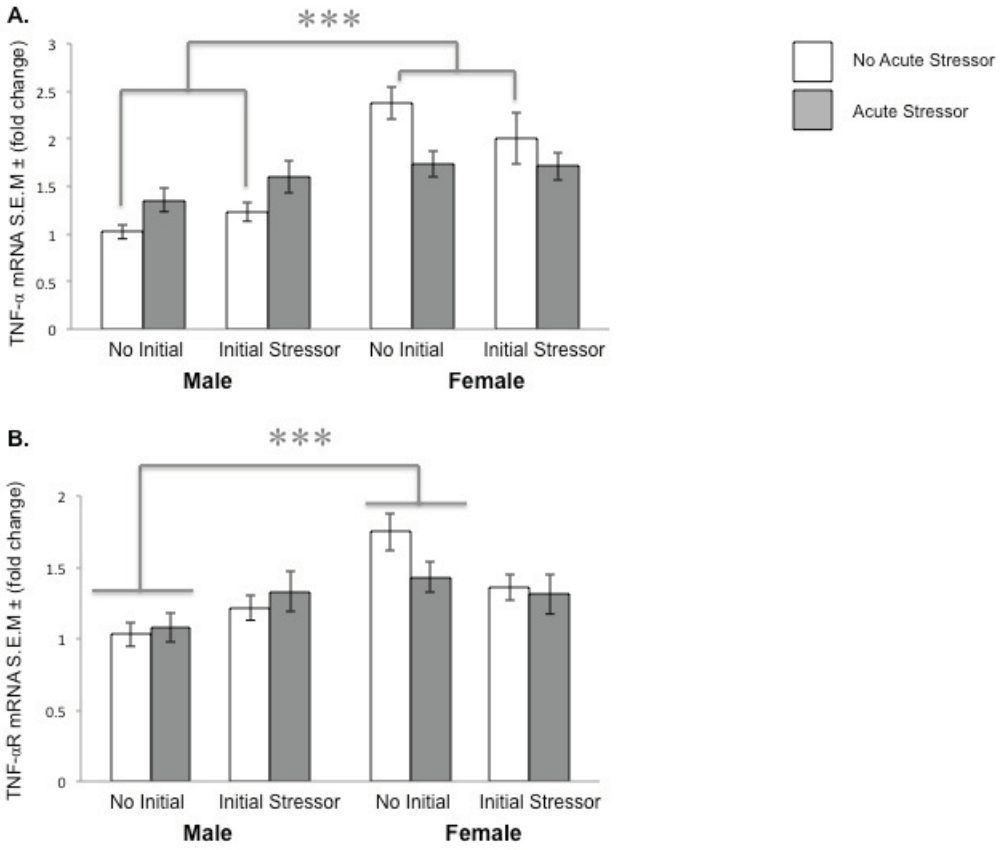

Figure 7 - Hippocampal mRNA expression (mean fold changes ±SEM) in response to a three day variable stressor and subsequent stressor exposure (restraint) on (A) TNF-α and (B) TNF-αR mRNA expression. In Panel A, females that were not acutely stressed exhibited higher TNF-α expression relative to similarly non-stressed males,***$p < .0005$. In Panel B females that did not experience the Initial Variable Stressor exhibited higher TNF-αR expression relative to similarly non-stressed males, ***$p < .0005$.

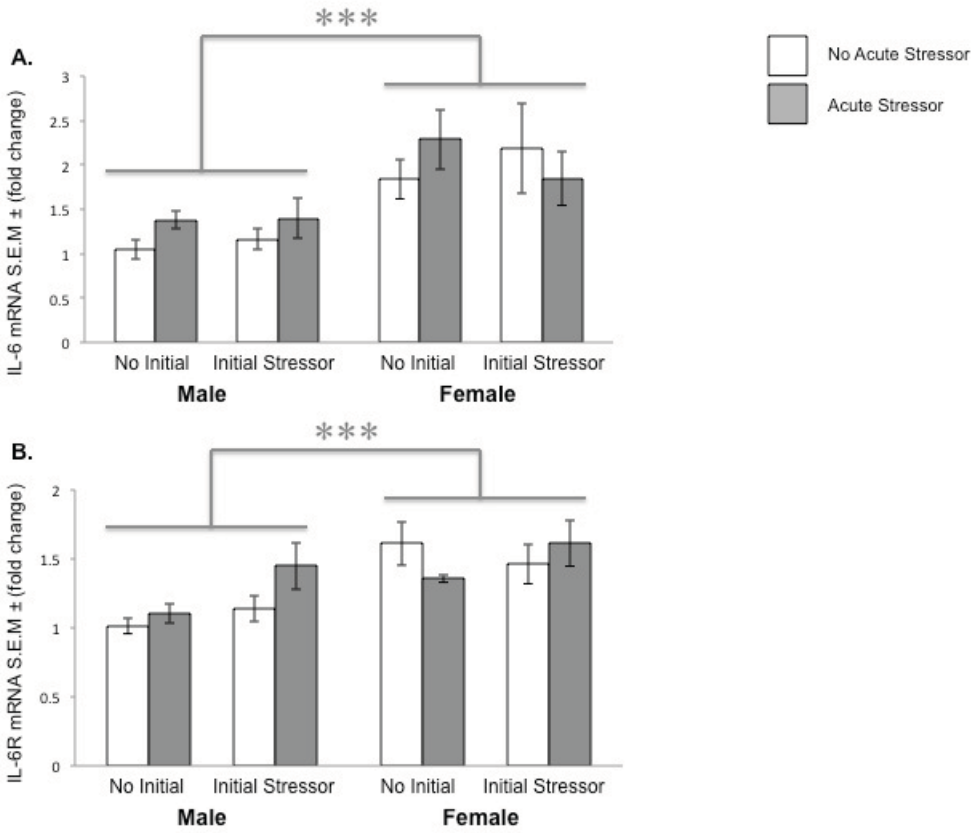

Figure 8 - Hippocampal mRNA expression (mean fold changes ±SEM) following exposure to a three day variable stressor and subsequent stressor exposure (restraint) on (A) IL-6 and (B) IL-6R mRNA expression. Females exhibited higher expression of both IL-6 and IL-6R relative to males, irrespective of the stressor treatments, ***$p < .0005$.

3.5 Experiment 3: : Effects of oral 17β-estradiol exposure and an acute stressor on Plus-Maze behaviour.

Behaviour within the elevated Plus-Maze varied with the Hormone treatment. The latency of time before first entering the open arms of the maze (Figure 9A) was found to be significantly affected by this measure, $F(2, 83) = 3.95$, $p < .05$. Bonferroni corrected follow-up t tests revealed that the mice in the Vehicle ($p=.063$) and Estrogen ($p=.046$) treatment conditions exhibited shorter latencies enter the open arms than did the control mice that did not receive a food supplement. A similar finding was demonstrated for number of entries to the open arms of the maze (Figure 9C), with Hormone treatment again significantly influencing this measure, $F(2, 82) = 8.35$, $p < .001$. Follow-up tests indicated that both the Vehicle ($p < .005$) and the Estrogen ($p < .005$) treatments influenced entries to the open arms, regardless of Stressor exposure or Sex.

No significant effects were found for either time spent in the open arms of the maze (Figure 9B) or number of entries to the closed arms of the maze (Figure 9E), but the time spent within the closed arms of the maze (Figure 9D) was again found to be influenced by the Hormone treatment group mice were placed in, $F(2, 84) = 5.69$, $p < .01$. Once again, the follow-up tests indicated that mice that were given the vehicle or estrogen treatment (p's $< .005$) spent significantly less time in the closed arms of the maze than mice that were not given a food supplement, regardless of Stressor exposure or Sex.

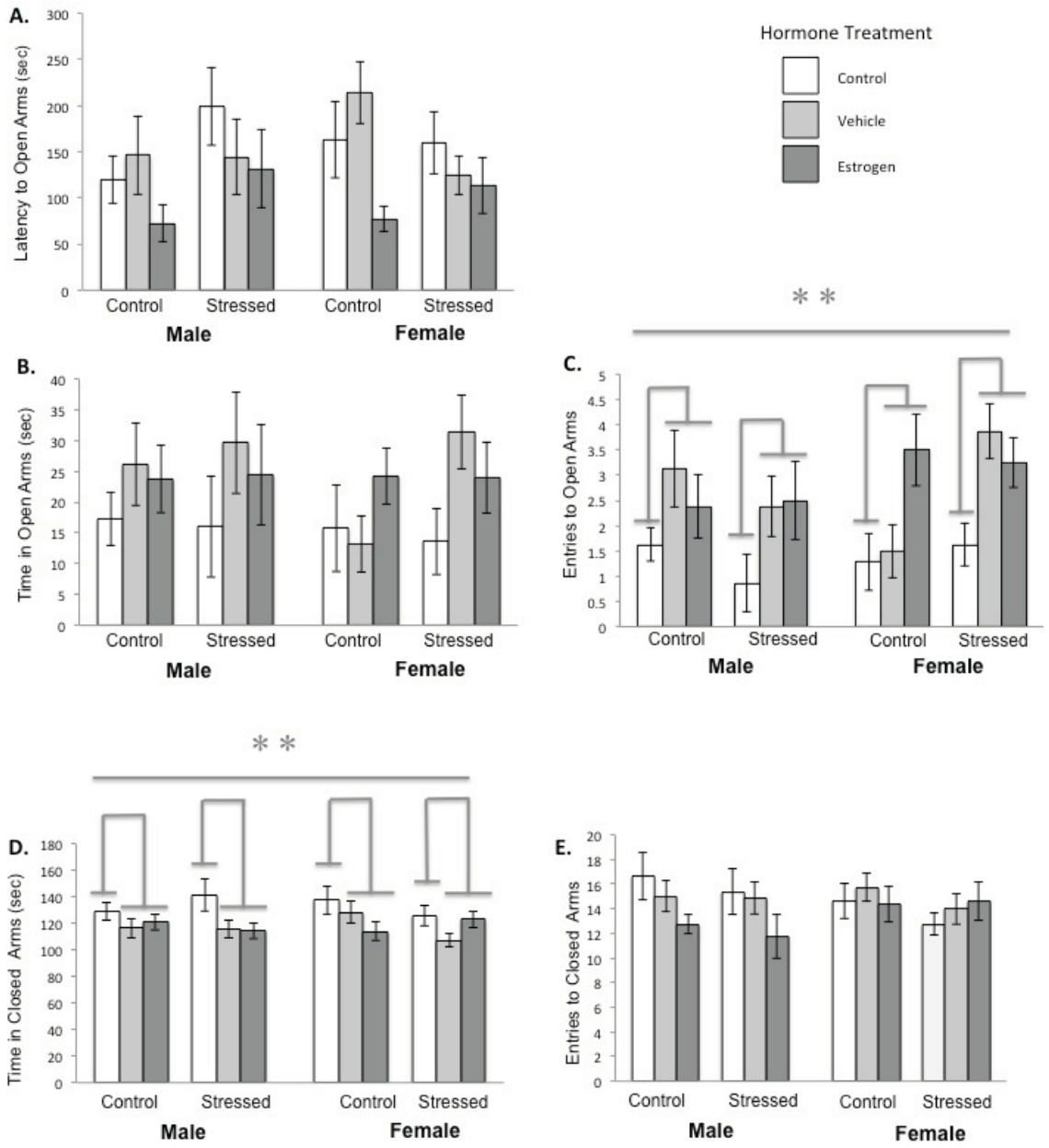

Figure 9 – Elevated Plus-Maze behaviour in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day. (A) Mean (±SEM) latency to open arms, (B) mean (±SEM) time spent in open arms, (C) mean (±SEM) number of entries to open arms, (D) mean (±SEM) time spent in closed arms, and (E) Mean (±SEM) number of entries to closed arms. Mice exposed to the vehicle or the estrogen supplement demonstrated higher levels when compared with control mice, **p's < .005.

3.6 Experiment 4: Effects of oral 17β-estradiol exposure and an acute stressor on plasma corticosterone levels.

Plasma corticosterone levels differed as an interaction between Stressor x Hormone treatment, $F(2, 76)=3.50$, $p < .05$ (Figure 10A). Bonferroni corrected t-tests revealed that stressed mice that received the estrogen treatment differed significantly from stressed mice that had received the vehicle supplement, across both sexes (p's $< .05$) (Figure 10A). As it had been predicted that males and females would be differentially affected by the stressor and estrogen treatments, separate analyses were conducted for each gender. Statistical significance was found primarily in the male mice through an interaction between stress and hormone exposure, $F(2, 41) = 8.68$, $p < .005$. Follow-up tests revealed that stressed males given estrogen had corticosterone levels significantly elevated above stressed males given the vehicle and unstressed male mice treated with estrogen (p's $< .05$) and stressed control males ($p < .005$). When females were analyzed independently of males, the analyses did not reveal significant effects for either Hormone or Stressor treatment.

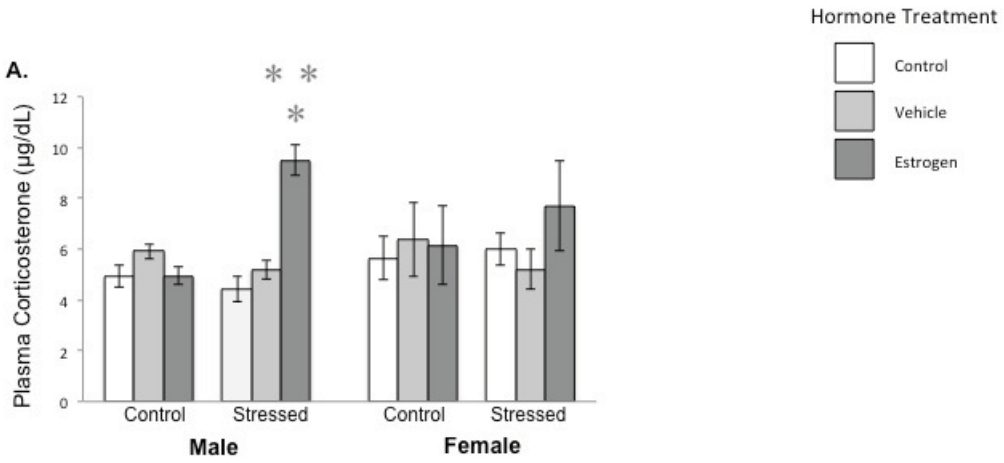

Figure 10- Mean (±SEM) concentrations of plasma corticosterone in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day. Stressed male mice that received the estrogen supplement had elevated corticosterone when compared to stressed male mice that received the vehicle treatment and unstressed male mice given estrogen, *$p < .05$. Stressed male mice that received the estrogen supplement had elevated corticosterone when compared to stressed male mice that received the control treatment, **$p < .005$.

3.7 Experiment 4: Effects of oral 17β-estradiol exposure and an acute stressor on prefrontal cortex pro-inflammatory cytokine mRNA expression.

IL-1β and IL-1R

The mRNA expression of IL-1β was affected as an interaction between the Sex and Stressor, $F(1, 69) = 5.39$, $p < .05$ (Figure 11A). Follow up tests demonstrated that although the stressor did not influence the cytokine's expression in either male or female mice, the male mice that encountered the acute stressor had significantly higher IL-1β expression than female mice, $p < .005$. The cytokine receptor, IL-1R (Figure 11B) was elevated in mice that were exposed to the 15 minute restraint session compared with mice (across both sexes and hormone treatments) that did not experience a stressor, $F(1, 69) = 5.86$, $p < .05$.

TNF-α and TNF-αR

A three way interaction between Sex, Hormone and the Stressor condition influenced TNF-α mRNA expression, $F(2, 74) = 3.76$, $p < .05$ (Figure 12A). Follow up tests revealed that male mice who experienced both the seven day estrogen regimen, along with an acute incident of a restraint stressor, demonstrated significantly lowered levels of TNF-α mRNA expression, when compared with male mice who experienced identical stressor exposure, but were fed a vehicle supplement that did not contain estrogen ($p < .05$). It also appeared that stressed male mice that were given the vehicle supplement demonstrated significantly higher TNF-α levels than unstressed male mice that were given the vehicle ($p = .005$) (Figure 12A). Finally, the non-stressed male mice that were fed the vehicle supplement approached significance ($p=.02$) in lowering mRNA expression of TNF-α when compared with male controls. The receptor of TNF-α, (Figure

12B) however, was elevated in male relative to female mice, $F(1, 78) = 21.86$, $p < .0005$ regardless of hormone or stressor condition.

IL-6 and IL-6R

The expression of IL-6 mRNA varied with the Stressor x Hormone treatment x Sex interaction (Figure 13A), $F(2, 71)= 4.04$ $p < .05$. It appeared that female mice exposed to a seven day estrogen regimen, as well as those given an acute restraint stressor on test day, demonstrate significantly elevated IL-6 expression when compared with females who were similarly stressed, but were given a vehicle supplement that did not contain 17β-estradiol ($p < .05$). Stressed females given no treatment also demonstrated reduced IL-6 expression compared with estrogen-treated stressed females but the statistical significance but was lost with Bonferroni corrections ($p = .03$). For the receptor, IL-6R (Figure 13B) mRNA expression was higher in male than female mice, $F(2, 71) = 6.32$, $p < .05$, but did not vary with the other experimental treatments.

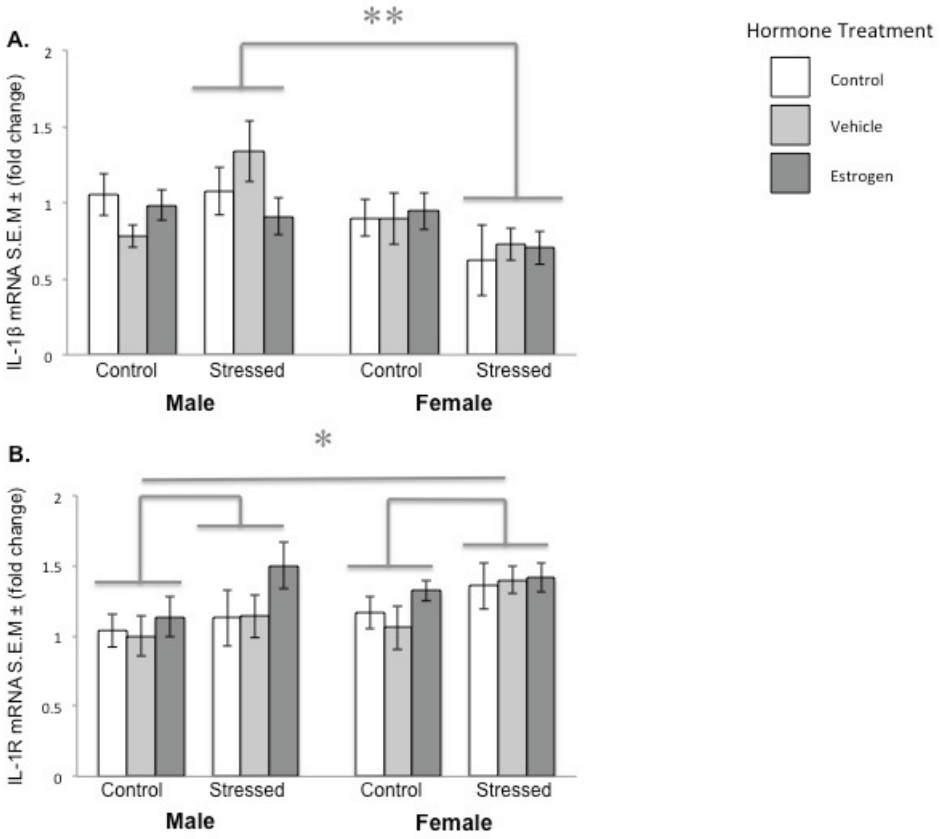

Figure 11 - Prefrontal cortex (PFC) mRNA expression (mean fold changes ±SEM) in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day on (A) IL-1β and (B) IL-1R expression. Mice that experienced the 15 minute restraint stressor had higher expression of IL-1R relative to mice that did not, *$p < .05$. Stressed male mice had higher expression of IL-1β than stressed female mice, **$p<.005$.

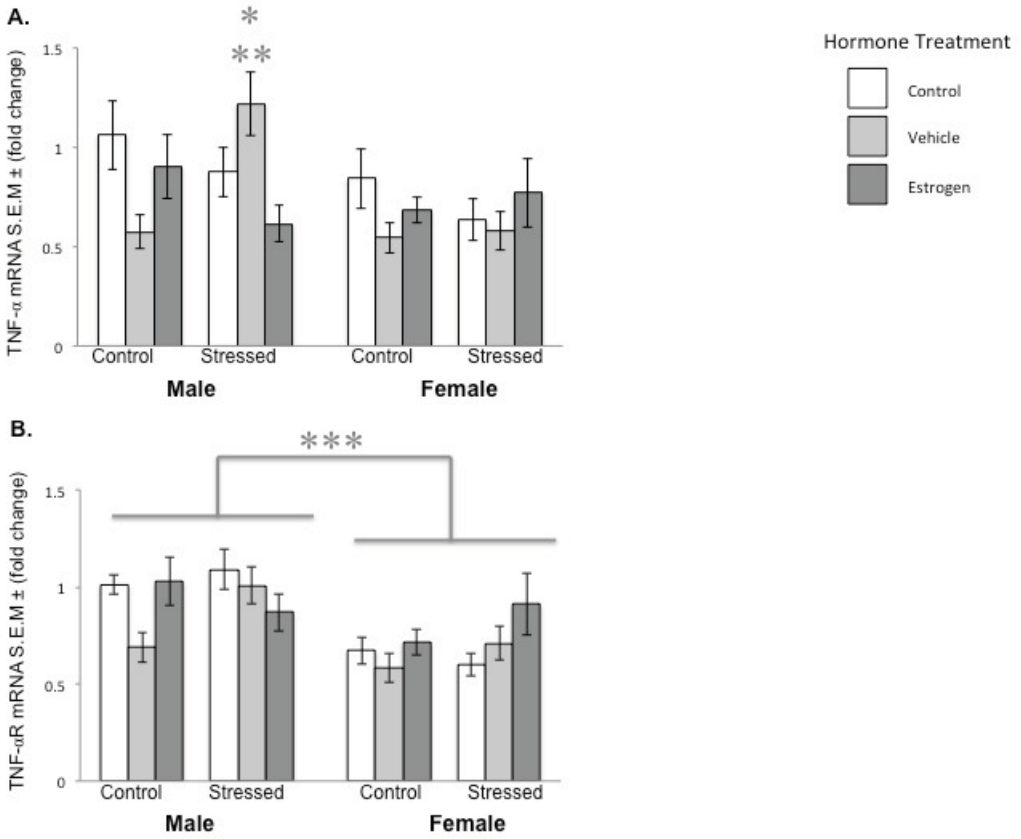

Figure 12 - Prefrontal cortex (PFC) mRNA expression (mean fold changes ±SEM) in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day on (A) TNF-α and (B) TNF- αR expression. Stressed male mice that received the vehicle treatment had higher expression of TNF-α than identically treated females or stressed males exposed to estrogen, *$p < .05$. Stressed males that received the vehicle treatment had higher expression of TNF-α than unstressed males given the vehicle, **$p = .005$. Males had higher levels of TNF- αR than females, irrespective of treatment or stressor, ***$p < .0005$.

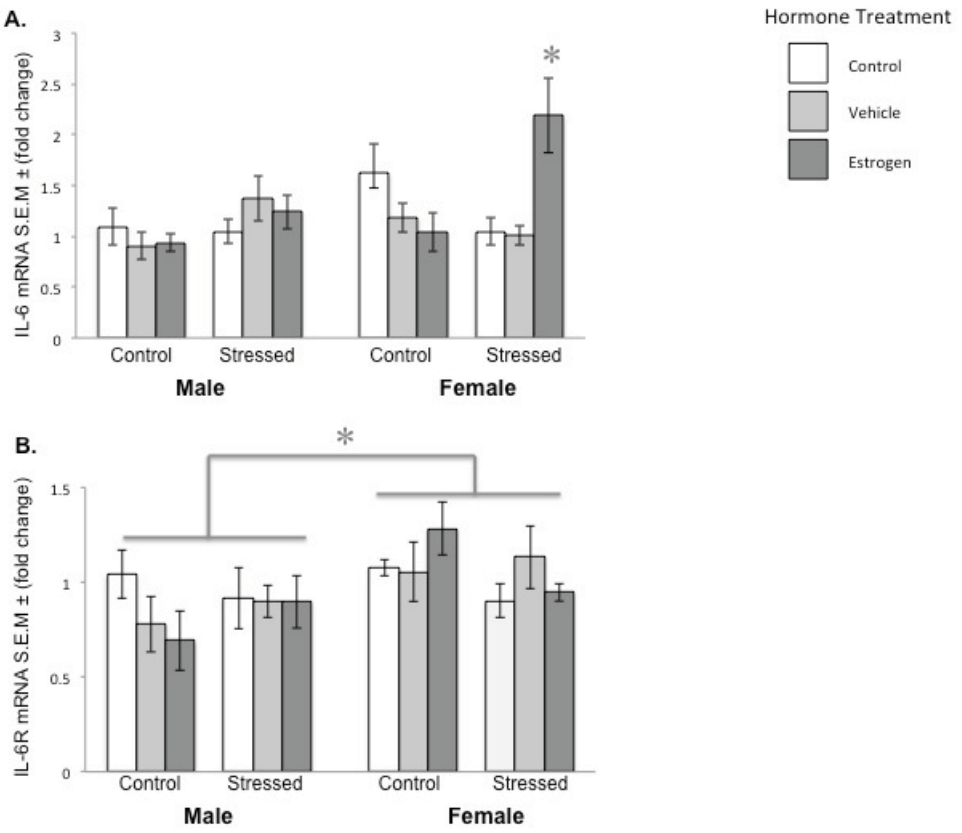

Figure 13 – Prefrontal cortex (PFC) mRNA expression (mean fold changes ±SEM) in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day (A) IL-6 and (B) IL-6R expression. In Panel A, stressed females that were exposed to estrogen demonstrated elevated IL-6 expression compared with stressed females given the vehicle, *$p < .05$. In Panel B, females demonstrated elevated expression compared with males, *$p < .05$.

3.8 Experiment 4: Effects of oral 17β-estradiol exposure and an acute stressor on hippocampal pro-inflammatory cytokine mRNA expression.

IL-1β and IL-1R

The expression of IL-1β varied as an interaction between the Sex and Stressor conditions, $F(1, 74) = 5.79$, $p < .05$ (Figure 14A). Follow-up tests indicated that male mice that did not experience the restraint stressor demonstrated reduced IL-1β expression compared with similarly unstressed female mice ($p < .005$). Owing to modest changes brought about by the stressor, the sex difference that was otherwise apparent was absent following the stressor treatment. The expression of IL-1R was also elevated in female mice, $F(1, 75) = 32.44$, $p < .0005$ (Figure 14B), irrespective of the stressor or estrogen treatments.

TNF-α and TNF-αR

No significant effects in TNF-α or its receptor expression occurred as a result of the Stressor or Hormone conditions, or between the sexes (Figures 15A and 15B).

IL-6 and IL-6R

Variation of IL-6 mRNA expression resulted as an interaction between the Sex x Hormone conditions, $F(2, 74) = 3.98$, $p < .05$ (Figure 16A). Follow-up tests demonstrated that female mice that had not experienced either of the food supplement conditions (vehicle or estrogen) demonstrated significantly higher IL-6 mRNA expression than did mice that received the vehicle treatment, regardless of the stressor condition ($p < .05$) (Figure 16A). Control female mice also exhibited somewhat elevated IL-6 expression relative to female mice that received the estrogen treatment, though the significance of this effect was lost with Bonferroni corrections ($p = .032$). IL-6R did not vary

significantly as a function of any of the Stressor, Hormone or Sex conditions (Figure 17B).

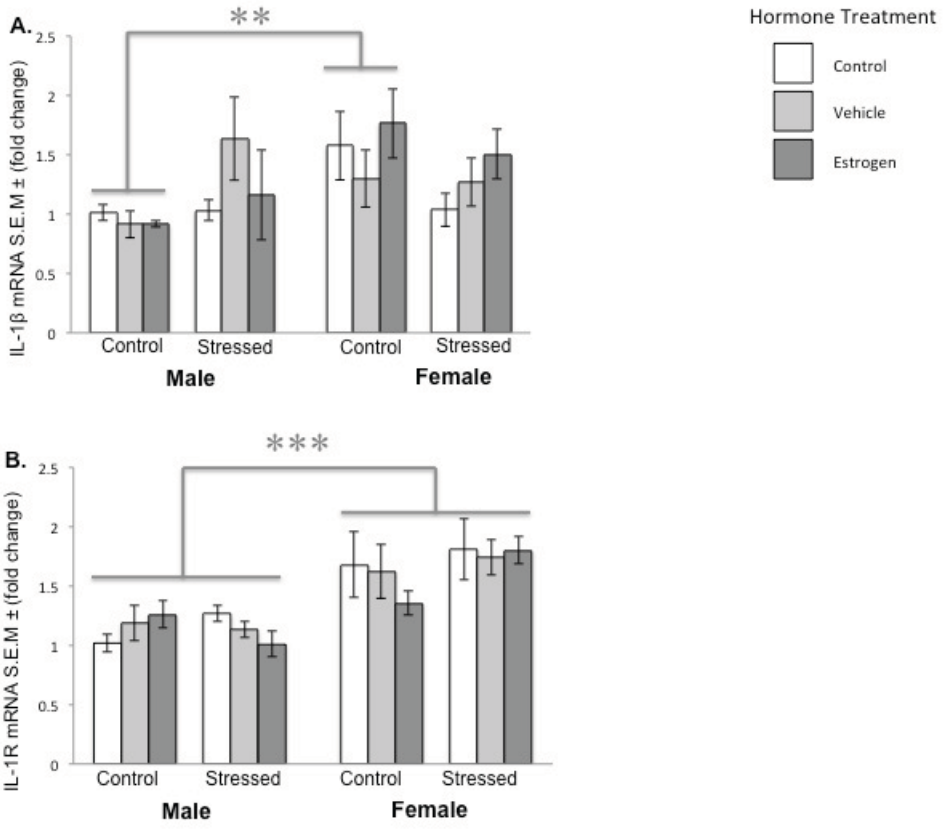

Figure 14 – Hippocampal mRNA expression (mean fold changes ±SEM) in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day on (A) IL-1β and (B) IL-1R expression. Unstressed females had higher expression of IL-1β than unstressed males, regardless of hormone treatment, **p<.005. Females had higher expression of IL-1R than males, regardless of stressor or treatment, ***p < .0005.

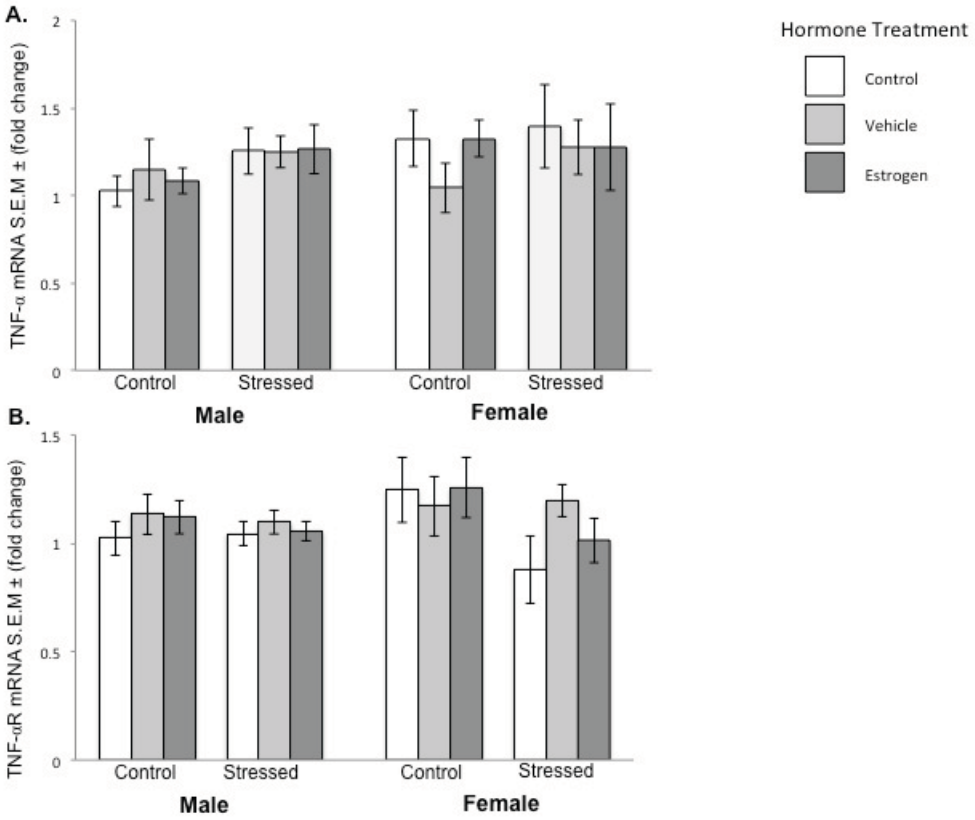

Figure 15 . Hippocampal mRNA expression (mean fold changes ±SEM) in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day on (A) TNF-α and (B) TNF- αR expression.

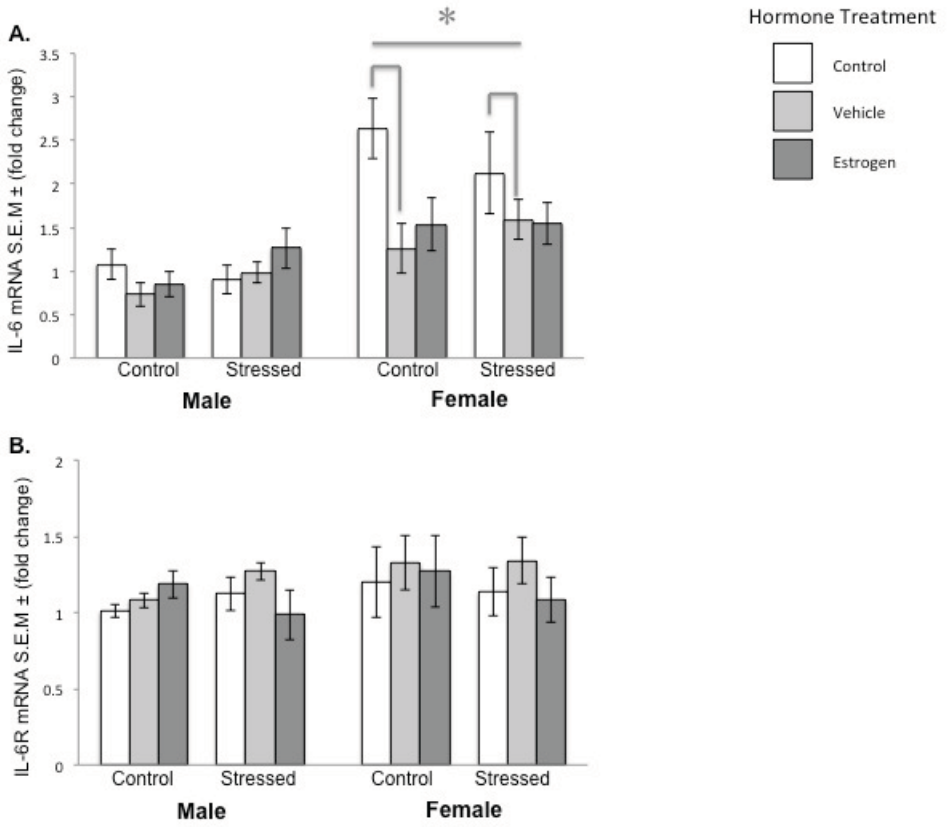

Figure 16 – Hippocampal mRNA expression (mean fold changes ±SEM) in response to a week long regimen of 17β-estradiol and a subsequent acute 15 minute restraint on test day on (A) IL-6 and (B) IL-6R expression. Control females had elevated IL-6 expression relative to vehicle exposed female mice, regardless of stressor, *$p < .05$.

3.9 Experiment 5: Effects of exposure to oral administration of 17β-estradiol on weight.

The weights of animals prior to the initiation of the treatments did not vary between groups. In contrast, weight after 7 days of treatment varied as a function of the Sex x Hormone interaction, $F(2, 185) = 6.76$, $p < .005$ (Figure 17A). Follow-up tests demonstrated that male mice that received the seven day estrogen treatment had significantly lower weights than male mice that received either the vehicle or no treatment (p's $< .05$). Female mice did not demonstrate a weight change in relation to any hormone treatment or food supplement given. A separate analysis that evaluated weight change over the seven day period paralleled these effects, $F(2, 185) = 11.94$, $p < .0005$. The Bonferroni corrected follow up tests demonstrated that male mice that received estrogen had reduced weight gain than males that received the vehicle or no treatment (p's $< .005$) (Figure 17B).

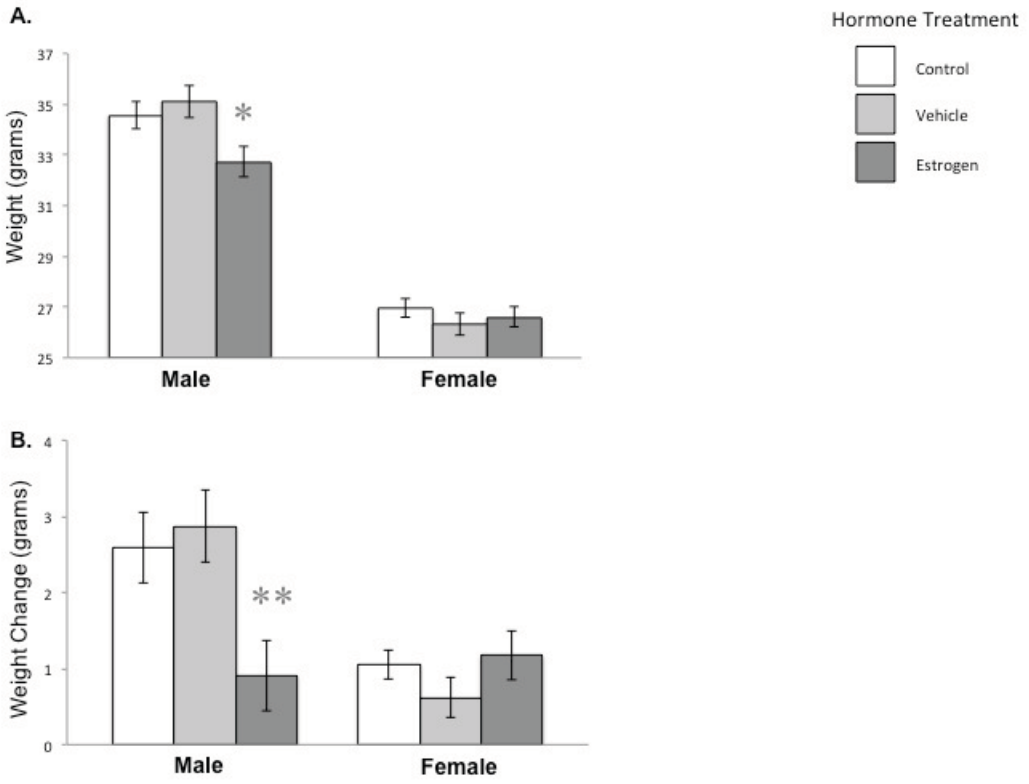

Figure 17 – Mean (±SEM) mouse weights (grams) following a week long regimen of estrogen or vehicle treatment and at corresponding times in controls (A). Change in weight over the course of estrogen or vehicle exposure, or at corresponding times in controls (B). Male mice that received estrogen had reduced weight relative to control and vehicle mice on test day,*$p < .05$. Male mice that received estrogen had reduced weight change relative to control and vehicle mice on test day, **$p < .005$.

4.0 Discussion

Stressful events and activation of the inflammatory immune system may influence behavioural and neurochemical responses to challenges that are encountered some time later (Anisman et al. 2008), and might thus contribute to the development of depression and anxiety disorders (Dantzer et al., 2008). Given the different prevalence rates of depressive disorders in men and women, in the current investigation we assessed whether this sexual dimorphism would be evident with respect to stressor-provoked cytokine mRNA expression within the PFC and hippocampus, both of which have been linked to anxiety and depressive illnesses.

As previously observed (Barna et al., 2003; Doremus-Fitzwater et al., 2009; Palanza, 2001), sex-specific behavioural patterns were evident in response to stressors. In this regard, just as early-life maternal separation resulted in males later showing faster latencies to enter an open arm of an elevated "T" maze (Slotten et al., 2006), in the present study a three day stressor regimen administered to adult mice elicited similar effects upon Plus-Maze testing six weeks later. This outcome was apparent irrespective of the treatment administered on the test day, indicating that the initial stressor experience was responsible for the protracted behavioral changes observed.

As the open arms of the Plus-Maze are relatively threatening, longer latencies to enter the open arms and decreased entries into these arms have typically been considered as an index of anxiety. Yet, the latency to enter an open arm can also be influenced by impulsivity or arousal elicited by experimental treatments. For instance, stressors applied to juvenile animals elicited markedly reduced latencies to enter an open arm and increased the number of entries into these arms, likely reflecting elevated arousal and

impulsivity. However, upon retesting 2 weeks later, once the initial arousal/impulsivity had diminished, the behavior of these animals reverted to the profile ordinarily expected in association with anxiety largely comprising immobility and avoidance of the open arms (Jacobson-Pick et al., 2012). The findings of the present study mice, in contrast, were indicative of arousal at impulsivity when animals were first tested in the Plus-Maze 6-weeks after the initial stressor experience. Unlike the procedure used by Jacobson-Pick, mice were tested in the maze for the first time at this point, and hence prior habituation to the maze was precluded. Thus, it is uncertain whether the different findings stemmed from the age of the animals upon initial testing (juvenile period vs adults) or whether anxiety and impulsivity varied as a function of whether mice had had a previous experience in the test situation. The reduced time spent exploring the closed arms of the maze in the present study might reflect impulsivity or risk-taking behaviour in the male mice that experienced the three day variable stressor (Jacobson-Pick et al., 2012).

In contrast to the behavior of male mice, the stressed females did not differ from the non-stressed controls with respect to the latency to enter an open arm. The mechanisms responsible for these sex dimorphisms are uncertain, but it may be significant that sex differences related to impulsiveness following a stressor experience has also been demonstrated in humans. For instance, men's decision making was faster and riskier in comparison to that evident in non-stressed men's or similarly stressed women (Lighthall et al., 2009, 2011; Preston et al. 2007, van den Bos et al., 2009).

The behavioural profile associated with an acute stressor applied 90 min prior to the Plus-Maze test yielded outcomes that could be distinguished from those associated with the stressor regimen applied 6-weeks earlier. Although acutely stressed males

entered the open arms more quickly than non-stressed males, this did not occur in females. However, acutely stressed females made more entries to the open arms than did similarly stressed males, and when female mice had been exposed to the stressor on both occasions, the number of entries to the open arms was especially elevated. Precisely this pattern has also been seen in female rats that responded to stressors, exhibiting increased exploration of high-risk regions (Aguilar et al., 2003; Cavigelli et al., 2011). These findings compliment the hypothesis that males and females may have adapted to respond differently to stressors in order to meet unique sex-specific environmental challenges (Palanza, 2001; Taylor et al., 2000). The impulsiveness demonstrated among males, reflected by faster entries to the open arms of the Plus-Maze, may represent the classic "fight-or-flight" response, where males benefit by responding quickly to a threatening situation. However, this response may not benefit females who must protect and care for offspring. A more cautious attempt at subsequently exploring surroundings may allow the female to safely care for her offspring, and allow her to be aware of the dangers that present themselves. This relatively blunted stressor response may be present in females of reproductive age, and potentially exacerbated if rodents were pregnant or lactating (Deschamps et al., 2003; Neumann et al., 2001; Windle et al., 1997).

Although the observable behavioural effects of stressors are more apparent in male rodents (such as impulsivity), females are found to higher baseline levels of the adrenal hormone corticosterone (Atkinson & Waddell, 1997; Dalla et al., 2005; Iwasaki-Sekino et al., 2009) and show greater elevations in levels following stress (Aoki et al., 2010). Indeed, in Experiment 2 female mice that experienced the three day stressor demonstrated comparatively higher levels of plasma corticosterone than males. Although

it only approached significance, male mice that experienced this multi-day stressor actually demonstrated a reduction in corticosterone output compared with their same sex controls. It is possible that corticosterone levels were elevated in male mice, but had been reduced in the 90 minutes following stressor termination, a pattern that has been previously demonstrated in male rats (McCormick et al., 1998). Following multiple days of stressors, male rodents also exhibit habituated (reduced) corticosterone response (Bhatnagar et al., 2002; Cole et al., 2000) whereas females do not demonstrate this same habituation (Bhatnagar et al., 2005), suggesting a sex difference in terms of HPA axis functioning.

It is intriguing that although stressed female rodents show a greater adrenal response to stressor, these effects are not translated into observable anxiety behaviour (Brotto et al., 2000; Dalla et al., 2005, Roman & Arborelius; 2009). It may be that corticosterone has less of an influence on anxiety in females overall, as demonstrated by studies where adrenalectomized females' behaviour did not differ from that of sham-operated control females, with or without corticosterone replacement (Kokras et al., 2012; Walf &Frye, 2005). This differed from studies that found adrenalectomized male mice showed reduced anxiety levels when compared with their same-sex sham-operated controls. As the two sexes were not equally affected by the manipulations of corticosterone production, it is possible that mechanisms of stress response differ between the sexes, in that females' stress responses are less dependent on circulating corticosterone levels (Kokras et al., 2012).

Numerous sex-dependent differences have been reported in response to stressors (e.g., Mitsushima et al., 2006; Stark et al., 2006). For instance, male mice have generally

been found to be particularly responsive to physiological stressors, such as restraint or foot-shock, and often display heightened neuronal activation within the PFC, reflected by increased *c-fos* expression, whereas comparable changes are not observed in females (Bland et al. 2005; Cavus & Duman, 2003; Galea et al., 1997; Molteni et al., 2001). However, sex differences regarding cytokine levels or mRNA expression following stressor treatments have scarcely been explored, although repeated restraint has been found to increase IL-1β within the prefrontal cortex of male rats (Gądek-Michalska et al., 2011) and to our knowledge, no previous studies have assessed if repeated stressor exposure will elicit similar pro-inflammatory cytokine variations in female rodents.

Although different neuronal processes may be activated by specific stressors (Franklin et al, 2012; Merali et al 2004), the present findings indicated that the effects of stressors on cytokine expression are reminiscent of the effects of stressors on neuronal processes. In the current study, sex differences were observed in regard to baseline brain cytokine expression as well as in response to stressors. The baseline levels of the various proinflammatory cytokines within the PFC were similar for male and female mice, whereas in the hippocampus cytokine mRNA expression was consistently higher in females than in males. Following stressor exposure cytokine expression was increased in both the PFC and the hippocampus of mice repeatedly stressed 6 weeks early, and this outcome was greater in males than in females, whereas cytokine expression was not increased in response to an acute stressor administered 90 min earlier, although IL-1R expression was elevated in response to the acute stressor in both strains. Of particular interest was that a particularly marked elevation of IL-1β expression occurred within the PFC of males that had been stressed on both occasions. Unlike IL-1β, expression of IL-6

and TNF-α in the PFC, unlike that of IL-1β, was not subject to a sensitization effect, as the re-exposure treatment did not have any effects beyond that elicited by the acute stressor itself.

In most respects, the cytokine profile in the hippocampus was comparable to that evident within the PFC, in that re-exposure to a stressor among males significantly increased IL-1β levels relative to males that were not re-exposed to the stressor. Once again, a comparable increase of IL-1β was not apparent following stressor-re-exposure among females. A similar sensitization of IL-1β expression has previously been reported in the hippocampus of male adolescent rats challenged with lipopolysaccharide as adults (Pyter et al., 2013) and, as in the present investigation, a sensitized response was found not to occur in females.

Although stressors did not significantly influence cytokine or receptor mRNA expression in female mice as it did in males, higher levels of baseline expression of each the cytokines and their receptors was observed within the hippocampus of females. Inasmuch as elevated cytokines have been implicated in the evolution of depression, it is possible that the high expression of inflammatory cytokines in the hippocampus of females might dispose them to depressive disorders, although admittedly, males and females differ on multiple dimensions and this simple association is insufficient to make this causal conclusion.

The differences between the sexes are interesting from yet another perspective. Specifically, it has been reported that the hippocampus in females is less susceptible to stressor-induced morphological changes than in males (Lin et al., 2009; Rantamäki et al., 2006). In this regard, stressors were found to produce atrophy of dendrites within

hippocampal neurons in males, but not in females (Conrad et al., 1999 and 2003; Galea et al., 1997). Likewise, behavioural studies indicated that females responded to stressors with unchanged or superior performance on tests of cognitive functioning, such as object recognition, whereas in males, performance in cognitive tasks more often declines (Bowman et al., 2009; Cohen &Yehuda et al., 2011; Galea et al., 1997). Given that high levels of inflammatory cytokines may impair memory consolidation among depressed individuals (Kelley, 2003; Song, 2002), it is possible that the sensitized increase of IL-1β mRNA expression exhibited by the males re-exposed to a stressor contributes to deficits of hippocampal functioning.

It is uncertain why the stressor re-exposure treatment did not elicit the elevated cytokine expression in females. This said, however, it has been suggested that although females might develop certain illnesses, such as depression, PTSD and autoimmune disorders more frequently than males, females might actually be more resilient to the impact of stressors (Bowman et al., 2002, 2009, Cohen & Yehuda, 2011; Conrad et al., 2003; Luine et al., 1994). It has been suggested that stress response systems in females were designed to be relatively resistant to immediate stressor-induced changes (Bourke et al., 2012) given that they might need to protect their young and to conserve their energy and resources. In contrast, males evolved to respond more readily to immediate environmental stressors, such threats from aggressors or competing for food (Taylor et al., 2000). From this perspective, the limited cytokine changes in females might be a reflection of this resilience, although this would need to be confirmed in relation to estrogen variations, given that this hormone has been tied to female resilience in other stressor contexts (Wei et al., 2013). The apparent increase in vulnerability towards

affective disorders in females may be due, in greater part, to the impact of psychosocial factors or differences in the willingness to report affective disturbances, rather than changes of cytokine functioning.

Given the distinct sex differences in regards to behaviour, HPA axis activity and cytokine profiles following stressor exposure, we chose to investigate the role of estrogen in these processes. In response to stressors estrogen seems to modulate both behavioural and physiological responses in female rodents and humans (Amin et al., 2005; Bodo & Rissman, 2006; Darnall & Suarez, 2009; Ter Horst et al., 2009). In ovariectomized female rodents that were administered estrogen to induce levels similar to those found in reproductively normal females, a reduction of anxiety and depressive behaviour was seen (Estrada-Camerena et al., 2003; Frye et al., 2004).

Given that the therapeutic properties of estrogen have been previously demonstrated on ovariectomized mice, we assessed whether exogenous estrogen administration would influence cytokine expression among intact female mice, as well as males. There has been a call for researchers to explore the effects of estrogen on reproductively intact animals (Clipperton-Allen et al., 2010; Segarra et al., 2014) as the effects on ovariectomized rodents who are treated with estrogen have been established, including the reducing effect it has on pro-inflammatory cytokine expression (Ma et al., 2007; Matejuk et al., 2001). As well, ovariectomized rodents are hormonally compromised and do not represent the natural state of the animal (as removing the ovaries also impairs multiple functions) and thus the meaningfulness of these studies is uncertain.

An interesting effect of estrogen exposure observed in the present investigation

was that female mice that received the 17β-estradiol treatment were in the pro-estrus phase of the cycle on test day, while all other female mice in the study remained in the estrus phase. It is common for virgin, female mice that are housed in the same room to remain in the estrus phase for long periods of time (Goldman et al., 2007) and it is possible that exposure to the estrogen may have produced a prolonged phase of pro-estrus for them as well. Unfortunately, the procedure for vaginal cytology is stressful for the mice, and in an effort to maintain similar conditions across both sexes, female mice were only examined for phase of cycle once (on test day), and thus it is not known whether they were cycling normally.

The administration of the seven day estrogen regimen and an acute stressor produced no behavioural sex differences. It may be that the 15 minute acute restraint stressor was not sufficiently strong to produce distinct anxiety behaviour. In the previous behavioural investigation, male mice that only experienced one acute restraint stressor did not exhibit reduced exploration prior to entering the open arms of the maze, as did mice that experienced multiple days of stress. Behavioural sex differences influenced by estrogen and stress may also have been masked by the use of the vehicle, Nutella hazelnut cream. The oral method of estrogen administration was chosen as previous research had demonstrated it produces steady levels of serum concentration of estrogen, while injections and pellets or capsules produce supraphysiological concentrations that quickly diminished (Isaksson et al., 2011). However, a downside to this method may be that the vehicle influenced behaviour in ways that masked both the estrogen and stressor induced effects. Indeed, this was reflected by the overall increase in the number of entries to the open arms of the maze, irrespective of sex and stressor condition, in mice groups

that received either the week long vehicle or estrogen treatment.

Although no behavioural differences between the sexes were found as a result of estrogen exposure, the estrogen treatment elicited reduced weight gain in male mice relative to animals that had been treated otherwise. It has previously been demonstrated that estrogen reduces caloric intake of male mice and increases insulin sensitivity, thus leading to reduced weight gain (Dubuc 1985, Takeda et al., 2003). Furthermore, among ovariectomized females that were supplemented with estrogen, weight reduction was observed (Bailey & Ahmed-Sorour., 1980; Louet et al., 2004), whereas intact females supplemented with exogenous estrogen and fed a normal diet weight remained stagnant (Bryzgalova et al., 2008). The results of the present investigation are consistent with these earlier findings. If nothing else, the reduced weight gain in estrogen supplemented males suggests that oral hormone administration was sufficient to promote effects like those elicited by systemic administration, although this doesn't necessarily imply comparable effects within the brain.

Along with diminishing weight gain in male mice, the application of estrogen in this experiment increased corticosterone as has been observed by others (Dayas et al., 2000; McCormick et al., 2002; Walf & Frye, 2005). In Experiment 4, both male and female mice that were exposed to the week long estrogen treatment and acute stressor on test day, demonstrated levels of corticosterone that were elevated above those of similarly stressed mice that were given the vehicle treatment. This compliments previous findings of elevated plasma corticosterone levels in response to an acute stressor in female rats that were treated with estrogen relative to that seen in stressed animals that had not received the estrogen treatment (Handa et al., 1994; Lunga & Herbert, 2004) The precise

sites and mechanisms responsible for estrogen induced glucocorticoid elevation are uncertain, though it has been demonstrated that this effect occurs as a result of increased adrenal sensitivity (Figueiredo et al., 2007).

The interactive effects of estrogen and the stressor was not limited to glucocorticoid activity, as mRNA expression of pro-inflammatory cytokines within the PFC and hippocampus were also affected by these treatments. Estrogen has typically been found to induce an anti-inflammatory effect, including reducing circulating levels of pro-inflammatory cytokines (Ghisletti et al., 2005) although opposite effects have been elicited with longer estrogen exposure (Soucy et al., 2005). The findings of the present study revealed that estrogen can influence brain cytokine expression, and such an outcome was sex-dependent.

Coinciding with the results of Experiment 2, stressed males' IL-1β mRNA expression within the PFC exceeded that of similarly treated females. Estrogen did not influence IL-1β expression, nor that of its receptor IL-1R. However, the mRNA expression of TNF-α within the PFC was significantly reduced in male mice that were exposed to the estrogen regimen and stressed on test day. This effect compliments previous findings, where exposure to 17β-estradiol was found to diminish TNF-α expression both in plasma and within the brain of injured male rats (Gaston et al., 2009; Özveri et al., 2001). It is interesting that the vehicle given to male mice interacted with the stressor condition, and resulted in elevated TNF- α expression. It is possible that this was an effect of the acute stressor interacting with elevated glucose levels from the high-sugar containing vehicle, to produce an elevated inflammatory response (Kiecolt-Glaser, 2010, O'Keefe, et al., 2008). While the precise factors that contributed to the notable

elevation of TNF-α in this group is uncertain, these levels were significantly reduced in the comparable group that received the estrogen treatment.

Females, in contrast, did not demonstrate significant changes in expression of either IL-1β or TNF- α within the PFC in response to the acute stressor or the estrogen treatment. However, in the case of IL-6, an interaction existed between the acute stressor and the estrogen treatment in that a marked elevation of mRNA expression was apparent among female mice, whereas this did not occur in males. Reports on the effects of 17β-estradiol on cytokine production have varied, with the anti-inflammatory properties of estrogen typically being held to be responsible for reducing production of central and peripheral cytokines (Ghisletti et al., 2005; Vegeto et al., 2001). However, more recent studies have revealed differences in the effects of short-term *in vitro* and long-term *in vivo* estrogen exposure, with the latter producing an increase of cytokine expression in females that experience a lipopolysaccharide challenge (Calippe et al., 2008; Soucy et al., 2005). However, when ovariectomized females were treated with physiological levels of estrogen, pro-inflammatory cytokine production following an immune challenge was reduced (Ma et al., 2007). In the present study females were reproductively intact, and received moderate levels of exogenous estrogen, potentially elevating their 17β-estradiol levels above a normal range. Given that abnormally high levels of estrogen may exacerbate IL-6 production (Li et al., 1993), cytokine changes may be the result of a nonlinear relationship between estrogen and pro-inflammatory cytokine expression.

The baseline mRNA expression of cytokines in the hippocampus echoed our findings from Experiment 2, where control female mice showed notably higher levels of pro-inflammatory cytokine expression than did male control animals. Exposure to

estrogen did not elicit any effects within the hippocampus and stressed females that were exposed to estrogen did not exhibit heightened IL-6, as they did within the PFC. Given the resistance of the female hippocampus to stressor induced changes (Lin et al., 2009) the difference in IL-6 expression between these two brain regions within the female mice may similarly be a reflection of this hippocampal resilience to stressor effects.

In general, decreased pro-inflammatory cytokine expression was not induced by the stressor and estrogen treatments in female mice, whereas TNF-α was reduced in the PFC of stressed males following hormone exposure. Given that males have lower levels of circulating estrogen, it may be that the exogenous exposure was able to dampen the cytokine response. However, the estrogen given to the female mice (who already have naturally higher levels of estrogen circulating) may have resulted in a ceiling effect, so that stressor-induced changes were not elicited, or as in the case of IL-6 expression in the PFC, an exaggerated response was elicited. This post hoc reasoning is highly speculative, of course, and need to be confirmed in further studies.

4.1 Limitations and Conclusion

The current investigation had several limitations. First, the estrus cycle was not tracked in Experiments 1 or 2. It is possible that phase of cycle may have influenced female Plus-Maze behavior or cytokine expression following stressor exposure. Also, the precise levels of circulating 17β-estradiol were not determined in any of the experiments. Thus, we could only infer the effectiveness of the estrogen treatments in Experiments 3 and 4 based on established side-effects of estrogen exposure in males (excessive corticosterone response and weight loss) and that female mice given estrogen were found to be pro-

estrus (heightened estrogen) on test day. As well, the behavioral results from Experiment 3 precluded the hyper-arousal effects the vehicle induced, which may have masked Plus-Maze behavioral differences induced by the stressors or estrogen treatment. Finally, our investigation primarily examined mRNA levels in the brain, which might not necessarily represent cytokine protein synthesis.

Stressor responsiveness has been found to differ between the sexes in a number of measures in both human and animal models (Darnall & Suarez., 2009). The studies presented here demonstrated that male and female CD-1 mice showed behavioral differences following exposure to stressors, as well as in relation to pro-inflammatory cytokine expression within the PFC and hippocampus, with males being more reactive upon stressor re-exposure. The role that estrogen plays in these differences is still unclear, though a nonlinear relationship may exist between estrogen and the stressor-immune response. Given the vast differences between the sexes, including those presented, it is essential to be mindful of these differences when exploring both etiology and therapeutic treatment for depressive and anxiety disorders.

References

Aguilar, R., Gil, L., Gray, J. A., Driscoll, P., Flint, J., Dawson, G. R., Tobeña, A. (2003). Fearfulness and sex in F2 Roman rats: males display more fear though both sexes share the same fearfulness traits. *Physiology & Behavior*, *78*(4–5), 723–732. doi:10.1016/S0031-9384(03)00043-X

Amin, Z., Canli, T., & Epperson, C. N. (2005). Effect of estrogen-serotonin interactions on mood and cognition. *Behavioral and cognitive neuroscience reviews*, *4*(1), 43–58. doi:10.1177/1534582305277152

Anisman, H., & Merali, Z. (2002). Cytokines, stress, and depressive illness. *Brain, behavior, and immunity*, *16*(5), 513–524.

Anisman, H., Merali, Z., & Hayley, S. (2003). Sensitization associated with stressors and cytokine treatments. *Brain, behavior, and immunity*, *17*(2), 86–93.

Anisman, H., Gibb, J., & Hayley, S. (2008). Influence of continuous infusion of interleukin-1beta on depression-related processes in mice: corticosterone, circulating cytokines, brain monoamines, and cytokine mRNA expression. *Psychopharmacology*, *199*(2), 231–244. doi:10.1007/s00213-008-1166-z

Aoki, M., Shimozuru, M., Kikusui, T., Takeuchi, Y., & Mori, Y. (2010). Sex differences in behavioral and corticosterone responses to mild stressors in ICR mice are altered by ovariectomy in peripubertal period. *Zoological Science*, *27*(10), 783–789. doi:10.2108/zsj.27.783

Asai, K., Hiki, N., Mimura, Y., Ogawa, T., Unou, K., & Kaminishi, M. (2001). Gender differences in cytokine secretion by human peripheral blood mononuclear cells: role of

estrogen in modulating LPS-induced cytokine secretion in an ex vivo septic model. *Shock (Augusta, Ga.)*, *16*(5), 340–343.

Atkinson, H. C., & Waddell, B. J. (1997). Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. *Endocrinology*, *138*(9), 3842–3848. doi:10.1210/endo.138.9.5395

Audet, M.-C., Jacobson-Pick, S., Wann, B. P., & Anisman, H. (2011). Social defeat promotes specific cytokine variations within the prefrontal cortex upon subsequent aggressive or endotoxin challenges. *Brain, behavior, and immunity*, *25*(6), 1197–1205. doi:10.1016/j.bbi.2011.03.010

Baca-García, E., Díaz-Sastre, C., de Leon, J., & Saiz-Ruiz, J. (2000). The relationship between menstrual cycle phases and suicide attempts. *Psychosomatic medicine*, *62*(1), 50–60.

Bailey, C. J., & Ahmed-Sorour, H. (1980). Role of ovarian hormones in the long-term control of glucose homeostasis. *Diabetologia*, *19*(5), 475–481. doi:10.1007/BF00281829.

Barna, I., Bálint, E., Baranyi, J., Bakos, N., Makara, G. B., & Haller, J. (2003). Gender-specific effect of maternal deprivation on anxiety and corticotropin-releasing hormone mRNA expression in rats. *Brain Research Bulletin*, *62*(2), 85–91. doi:10.1016/S0361-9230(03)00216-8

Belda, X., Fuentes, S., Nadal, R., & Armario, A. (2008). A single exposure to immobilization causes long-lasting pituitary-adrenal and behavioral sensitization to mild stressors. *Hormones and behavior*, *54*(5), 654–661. doi:10.1016/j.yhbeh.2008.07.003

Bertini, M., Conti, C. M., & Fulcheri, M. (2013). Psychoneuroimmunology and health psychology: inflammation and protective factors. *Journal of biological regulators and homeostatic agents*, *27*(3), 637–645.

Bhatnagar, S., Huber, R., Nowak, N., & Trotter, P. (2002). Lesions of the posterior paraventricular thalamus block habituation of hypothalamic-pituitary-adrenal responses to repeated restraint. *Journal of Neuroendocrinology*, *14*(5), 403–410.

Bhatnagar, S., Lee, T. M., & Vining, C. (2005). Prenatal stress differentially affects habituation of corticosterone responses to repeated stress in adult male and female rats. *Hormones and Behavior*, *47*(4), 430–438. doi:10.1016/j.yhbeh.2004.11.019

Binder, E. B., Künzel, H. E., Nickel, T., Kern, N., Pfennig, A., Majer, M., Holsboer, F. (2009). HPA-axis regulation at in-patient admission is associated with antidepressant therapy outcome in male but not in female depressed patients. *Psychoneuroendocrinology*, *34*(1), 99–109. doi:10.1016/j.psyneuen.2008.08.018

Bland, S. T., Schmid, M. J., Der-Avakian, A., Watkins, L. R., Spencer, R. L., & Maier, S. F. (2005). Expression of c-fos and BDNF mRNA in subregions of the prefrontal cortex of male and female rats after acute uncontrollable stress. *Brain Research*, *1051*(1–2), 90–99. doi:10.1016/j.brainres.2005.05.065

Bloch, M., Schmidt, P. J., Danaceau, M., Murphy, J., Nieman, L., & Rubinow, D. R. (2000). Effects of gonadal steroids in women with a history of postpartum depression. *The American journal of psychiatry*, *157*(6), 924–930.

Boda, E., Pini, A., Hoxha, E., Parolisi, R., & Tempia, F. (2009). Selection of reference genes for quantitative real-time RT-PCR studies in mouse brain. *Journal of Molecular Neuroscience: MN*, *37*(3), 238–253. doi:10.1007/s12031-008-9128-9

Bodo, C., & Rissman, E. F. (2006). New roles for estrogen receptor beta in behavior and neuroendocrinology. *Frontiers in Neuroendocrinology*, *27*(2), 217–232. doi:10.1016/j.yfrne.2006.02.004

Bourke, C. H., Harrell, C. S., & Neigh, G. N. (2012). Stress-induced sex differences: adaptations mediated by the glucocorticoid receptor. *Hormones and Behavior*, *62*(3), 210–218. doi:10.1016/j.yhbeh.2012.02.024

Bowman, R. E., Ferguson, D., & Luine, V. N. (2002). Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. *Neuroscience*, *113*(2), 401–410.

Bowman, R. E., Micik, R., Gautreaux, C., Fernandez, L., & Luine, V. N. (2009). Sex-dependent changes in anxiety, memory, and monoamines following one week of stress. *Physiology & Behavior*, *97*(1), 21–29. doi:10.1016/j.physbeh.2009.01.012

Brotto, L. A., Barr, A. M., & Gorzalka, B. B. (2000). Sex differences in forced-swim and open-field test behaviours after chronic administration of melatonin. *European Journal of Pharmacology*, *402*(1–2), 87–93. doi:10.1016/S0014-2999(00)00491-X

Bryzgalova, G., Lundholm, L., Portwood, N., Gustafsson, J.-Å., Khan, A., Efendic, S., & Dahlman-Wright, K. (2008). Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *American Journal of Physiology - Endocrinology and Metabolism*, *295*(4), E904–E912. doi:10.1152/ajpendo.90248.2008

Breslau, N., Schultz, L., & Peterson, E. (1995). Sex differences in depression: a role for preexisting anxiety. *Psychiatry research*, *58*(1), 1–12.

Calippe, B., Douin-Echinard, V., Laffargue, M., Laurell, H., Rana-Poussine, V., Pipy, B., Gourdy, P. (2008). Chronic Estradiol Administration In Vivo Promotes the Proinflammatory Response of Macrophages to TLR4 Activation: Involvement of the Phosphatidylinositol 3-Kinase Pathway. *The Journal of Immunology*, *180*(12), 7980–7988.

Cavigelli, S. A., Michael, K. C., West, S. G., & Klein, L. C. (2011). Behavioral responses to physical vs. social novelty in male and female laboratory rats. *Behavioural Processes*, *88*(1), 56–59. doi:10.1016/j.beproc.2011.06.006

Cavus, I., & Duman, R. S. (2003). Influence of estradiol, stress, and 5-HT2A agonist treatment on Brain-Derived Neurotrophic Factor expression in female rats. *Biological Psychiatry*, *54*(1), 59–69. doi:10.1016/S0006-3223(03)00236-1

Chao, T. C., Van Alten, P. J., Greager, J. A., & Walter, R. J. (1995). Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cellular immunology*, *160*(1), 43–49.

Clipperton Allen, A. E., Cragg, C. L., Wood, A. J., Pfaff, D. W., & Choleris, E. (2010). Agonistic behavior in males and females: Effects of an estrogen receptor beta agonist in gonadectomized and gonadally intact mice. *Psychoneuroendocrinology*, *35*(7), 1008–1022. doi:10.1016/j.psyneuen.2010.01.002

Cohen, H., & Yehuda, R. (2011). Gender differences in animal models of posttraumatic stress disorder. *Disease Markers*, *30*(2), 141–150. doi:10.3233/DMA-2011-0778

Cole, M. A., Kalman, B. A., Pace, T. W., Topczewski, F., Lowrey, M. J., & Spencer, R. L. (2000). Selective blockade of the mineralocorticoid receptor impairs hypothalamic-pituitary-adrenal axis expression of habituation. *Journal of Neuroendocrinology*, *12*(10), 1034–1042.

Conrad, C. D., Magariños, A. M., LeDoux, J. E., & McEwen, B. S. (1999). Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. *Behavioral Neuroscience*, *113*(5), 902–913. doi:10.1037/0735-7044.113.5.902

Conrad, C. D., Grote, K. A., Hobbs, R. J., & Ferayorni, A. (2003). Sex differences in spatial and non-spatial Y-maze performance after chronic stress. *Neurobiology of Learning and Memory*, *79*(1), 32–40. doi:10.1016/S1074-7427(02)00018-7

Dalla, C., Antoniou, K., Drossopoulou, G., Xagoraris, M., Kokras, N., Sfikakis, A., & Papadopoulou-Daifoti, Z. (2005). Chronic mild stress impact: Are females more vulnerable? *Neuroscience*, *135*(3), 703–714. doi:10.1016/j.neuroscience.2005.06.068

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., & Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature reviews. Neuroscience*, *9*(1), 46–56. doi:10.1038/nrn2297

Darnall, B. D., & Suarez, E. C. (2009). Sex and gender in psychoneuroimmunology research: past, present and future. *Brain, behavior, and immunity*, *23*(5), 595–604. doi:10.1016/j.bbi.2009.02.019

Dayas, C. V., Xu, Y., Buller, K. M., & Day, T. A. (2000). Effects of chronic oestrogen replacement on stress-induced activation of hypothalamic-pituitary-adrenal axis control pathways. *Journal of Neuroendocrinology*, *12*(8), 784–794.

De Leo, V., la Marca, A., Talluri, B., D'Antona, D., & Morgante, G. (1998). Hypothalamo-pituitary-adrenal axis and adrenal function before and after ovariectomy in premenopausal women. *European journal of endocrinology / European Federation of Endocrine Societies*, *138*(4), 430–435.

Deschamps, S., Woodside, B., & Walker, C.-D. (2003). Pups presence eliminates the stress hyporesponsiveness of early lactating females to a psychological stress representing a threat to the pups. *Journal of Neuroendocrinology*, *15*(5), 486–497.

Doremus-Fitzwater, T. L., Varlinskaya, E. I., & Spear, L. P. (2009). Social and non-social anxiety in adolescent and adult rats after repeated restraint. *Physiology & Behavior*, *97*(3-4), 484–494. doi:10.1016/j.physbeh.2009.03.025

Dubuc, P. U. (1985). Effects of estrogen on food intake, body weight, and temperature of male and female obese mice. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *180*(3), 468–473.

Duka, T., Tasker, R., & McGowan, J. F. (2000). The effects of 3-week estrogen hormone replacement on cognition in elderly healthy females. *Psychopharmacology*, *149*(2), 129–139.

Earls, F. (1987). Sex differences in psychiatric disorders: origins and developmental influences. *Psychiatric developments*, *5*(1), 1–23.

Espejo, E. P., Hammen, C. L., Connolly, N. P., Brennan, P. A., Najman, J. M., & Bor, W. (2007). Stress Sensitization and Adolescent Depressive Severity as a Function of Childhood Adversity: A Link to Anxiety Disorders. *Journal of Abnormal Child Psychology*, *35*(2), 287–299. doi:10.1007/s10802-006-9090-3

Estrada-Camarena, E., Fernández-Guasti, A., & López-Rubalcava, C. (2003). Antidepressant-like effect of different estrogenic compounds in the forced swimming test. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*, *28*(5), 830–838. doi:10.1038/sj.npp.1300097

Figueiredo, H. F., Ulrich-Lai, Y. M., Choi, D. C., & Herman, J. P. (2007). Estrogen potentiates adrenocortical responses to stress in female rats. *American Journal of*

Physiology - Endocrinology and Metabolism, *292*(4), E1173–E1182. doi:10.1152/ajpendo.00102.2006

Frank, M. G., Baratta, M. V., Sprunger, D. B., Watkins, L. R., & Maier, S. F. (2007). Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. *Brain, behavior, and immunity*, *21*(1), 47–59. doi:10.1016/j.bbi.2006.03.005

Franklin, T. B., Saab, B. J., & Mansuy, I. M. (2012). Neural mechanisms of stress resilience and vulnerability. *Neuron*, *75*(5), 747–761. doi:10.1016/j.neuron.2012.08.016

Frommberger, U. H., Bauer, J., Haselbauer, P., Fräulin, A., Riemann, D., & Berger, M. (1997). Interleukin-6-(IL-6) plasma levels in depression and schizophrenia: comparison between the acute state and after remission. *European Archives of Psychiatry and Clinical Neuroscience*, *247*(4), 228–233. doi:10.1007/BF02900219

Frye, C. A., & Wawrzycki, J. (2003). Effect of prenatal stress and gonadal hormone condition on depressive behaviors of female and male rats. *Hormones and behavior*, *44*(4), 319–326.

Frye, C. A., & Walf, A. A. (2004). Estrogen and/or progesterone administered systemically or to the amygdala can have anxiety-, fear-, and pain-reducing effects in ovariectomized rats. *Behavioral neuroscience*, *118*(2), 306–313. doi:10.1037/0735-7044.118.2.306

Gądek-Michalska, A., Tadeusz, J., Rachwalska, P., Spyrka, J., & Bugajski, J. (2011). Effect of prior stress on interleukin-1β and HPA axis responses to acute stress. *Pharmacological Reports: PR*, *63*(6), 1393–1403.

Galea, L. A., McEwen, B., Tanapat, P., Deak, T., Spencer, R., & Dhabhar, F. (1997). Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. *Neuroscience*, *81*(3), 689–697. doi:10.1016/S0306-4522(97)00233-9

Gaston, J. W., Maass, D. L., James W. Simpkins, Idris, A. H., Minei, J. P., & Wigginton, J. G. (2009). Estrogen treatment following severe burn injury reduces brain inflammation and apoptotic signaling. *Journal of Neuroinflammation*, *6*(1), 30. doi:10.1186/1742-2094-6-30

Ghisletti, S., Meda, C., Maggi, A., & Vegeto, E. (2005). 17β-Estradiol Inhibits Inflammatory Gene Expression by Controlling NF-B Intracellular Localization. *Molecular and Cellular Biology*, *25*(8), 2957–2968. doi:10.1128/MCB.25.8.2957-2968.2005

Gibb, J., Hayley, S., Gandhi, R., Poulter, M. O., & Anisman, H. (2008). Synergistic and additive actions of a psychosocial stressor and endotoxin challenge: Circulating and brain cytokines, plasma corticosterone and behavioral changes in mice. *Brain, Behavior, and Immunity*, *22*(4), 573–589. doi:10.1016/j.bbi.2007.12.001

Gibb, J., Audet, M.-C., Hayley, S., & Anisman, H. (2009). Neurochemical and behavioral responses to inflammatory immune stressors. *Frontiers in bioscience (Scholar edition)*, *1*, 275–295.

Gibb, J., Hayley, S., Poulter, M. O., & Anisman, H. (2011). Effects of stressors and immune activating agents on peripheral and central cytokines in mouse strains that differ in stressor responsivity. *Brain, behavior, and immunity*, *25*(3), 468–482. doi:10.1016/j.bbi.2010.11.008

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects*

Research. Part B, Developmental and Reproductive Toxicology, *80*(2), 84–97. doi:10.1002/bdrb.20106

Gorman, J. M. (2006). Gender differences in depression and response to psychotropic medication. *Gender Medicine*, *3*(2), 93–109. doi:10.1016/S1550-8579(06)80199-3

Gutkowska, J., Broderick, T. L., Bogdan, D., Wang, D., Lavoie, J.-M., & Jankowski, M. (2009). Downregulation of oxytocin and natriuretic peptides in diabetes: possible implications in cardiomyopathy. *The Journal of Physiology*, *587*(Pt 19), 4725–4736. doi:10.1113/jphysiol.2009.176461

Handa, R. J., Nunley, K. M., Lorens, S. A., Louie, J. P., McGivern, R. F., & Bollnow, M. R. (1994). Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors. *Physiology & Behavior*, *55*(1), 117–124. doi:10.1016/0031-9384(94)90018-3

Hayley, S., Merali, Z., & Anisman, H. (2003). Stress and Cytokine-elicited Neuroendocrine and Neurotransmitter Sensitization: Implications for Depressive Illness. *Stress: The International Journal on the Biology of Stress*, *6*(1), 19–32. doi:10.1080/1025389031000091167

Herbert, T. B., & Cohen, S. (1993). Depression and immunity: a meta-analytic review. *Psychological bulletin*, *113*(3), 472–486.

Howren, M. B., Lamkin, D. M., & Suls, J. (2009). Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. *Psychosomatic medicine*, *71*(2), 171–186. doi:10.1097/PSY.0b013e3181907c1b

Isaksson, I.-M., Theodorsson, A., Theodorsson, E., & Strom, J. O. (2011). Methods for 17β-oestradiol administration to rats. *Scandinavian Journal of Clinical and Laboratory Investigation*, *71*(7), 583–592. doi:10.3109/00365513.2011.596944

Iwasaki-Sekino, A., Mano-Otagiri, A., Ohata, H., Yamauchi, N., & Shibasaki, T. (2009). Gender differences in corticotropin and corticosterone secretion and corticotropin-releasing factor mRNA expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala in response to footshock stress or psychological stress in rats. *Psychoneuroendocrinology*, *34*(2), 226–237. doi:10.1016/j.psyneuen.2008.09.003

Jacobson-Pick, S., Audet, MC., McQuaid, RJ., Kalvapalle, R., Anisman, H. (2012). Stressor exposure of male and female juvenile mice influences later responses to stressors: modulation of GABAA receptor subunit mRNA expression. *Neuroscience(215): 114-26.* doi: 10.1016/j.neuroscience.2012.04.038

Jaremka, L. M., Fagundes, C. P., Glaser, R., Bennett, J. M., Malarkey, W. B., & Kiecolt-Glaser, J. K. (2013). Loneliness predicts pain, depression, and fatigue: understanding the role of immune dysregulation. *Psychoneuroendocrinology*, *38*(8), 1310–1317. doi:10.1016/j.psyneuen.2012.11.016

Jedema, H. P., Sved, A. F., Zigmond, M. J., & Finlay, J. M. (1999). Sensitization of norepinephrine release in medial prefrontal cortex: effect of different chronic stress protocols. *Brain research*, *830*(2), 211–217.

Johnson, J. D., O'Connor, K. A., Hansen, M. K., Watkins, L. R., & Maier, S. F. (2003). Effects of prior stress on LPS-induced cytokine and sickness responses. *American journal*

of physiology. Regulatory, integrative and comparative physiology, *284*(2), R422–432. doi:10.1152/ajpregu.00230.2002

Kelley, K. W., Bluthé, R.-M., Dantzer, R., Zhou, J.-H., Shen, W.-H., Johnson, R. W., & Broussard, S. R. (2003). Cytokine-induced sickness behavior. *Brain, Behavior, and Immunity*, *17*(1, Supplement), 112–118. doi:10.1016/S0889-1591(02)00077-6.

Kessler, R. C., McGonagle, K. A., Swartz, M., Blazer, D. G., & Nelson, C. B. (1993). Sex and depression in the National Comorbidity Survey I: Lifetime prevalence, chronicity and recurrence. *Journal of Affective Disorders*, *29*(2–3), 85–96. doi:10.1016/0165-0327(93)90026-G

Kessler, R C, McGonagle, K. A., Zhao, S., Nelson, C. B., Hughes, M., Eshleman, S., Kendler, K. S. (1994). Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Archives of general psychiatry*, *51*(1), 8–19.

Kiecolt-Glaser, J. K. (2010). Stress, Food, and Inflammation: Psychoneuroimmunology and Nutrition at the Cutting Edge. *Psychosomatic Medicine*, *72*(4), 365–369. doi:10.1097/PSY.0b013e3181dbf489

Kokras, N., Dalla, C., Sideris, A. C., Dendi, A., Mikail, H. G., Antoniou, K., & Papadopoulou-Daifoti, Z. (2012). Behavioral sexual dimorphism in models of anxiety and depression due to changes in HPA axis activity. *Neuropharmacology*, *62*(1), 436–445. doi:10.1016/j.neuropharm.2011.08.025

Leonard, B. E. (2010). The concept of depression as a dysfunction of the immune system. *Current immunology reviews*, *6*(3), 205–212. doi:10.2174/157339510791823835

Li, Z. G., Danis, V. A., & Brooks, P. M. (1993). Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro. *Clinical and Experimental Rheumatology*, *11*(2), 157–162.

Lin, Y., Horst, G. J. T., Wichmann, R., Bakker, P., Liu, A., Li, X., & Westenbroek, C. (2009). Sex Differences in the Effects of Acute and Chronic Stress and Recovery after Long-Term Stress on Stress-Related Brain Regions of Rats. *Cerebral Cortex*, *19*(9), 1978–1989. doi:10.1093/cercor/bhn225

Lighthall, N. R., Mather, M., & Gorlick, M. A. (2009). Acute Stress Increases Sex Differences in Risk Seeking in the Balloon Analogue Risk Task. *PLoS ONE*, *4*(7), e6002. doi:10.1371/journal.pone.0006002

Lighthall, N. R., Sakaki, M., Vasunilashorn, S., Nga, L., Somayajula, S., Chen, E. Y., Mather, M. (2011). Gender differences in reward-related decision processing under stress. *Social Cognitive and Affective Neuroscience*, nsr026. doi:10.1093/scan/nsr026

Louet, J.-F., LeMay, C., & Mauvais-Jarvis, F. (2004). Antidiabetic actions of estrogen: insight from human and genetic mouse models. *Current Atherosclerosis Reports*, *6*(3), 180–185.

Luine, V., Villegas, M., Martinez, C., & McEwen, B. S. (1994). Repeated stress causes reversible impairments of spatial memory performance. *Brain Research*, *639*(1), 167–170. doi:10.1016/0006-8993(94)91778-7

Lunga, P., & Herbert, J. (2004). 17beta-Oestradiol Modulates Glucocorticoid, Neural and Behavioural Adaptations to Repeated Restraint Stress in Female Rats. *Journal of Neuroendocrinology*, *16*(9), 776–785. doi:10.1111/j.1365-2826.2004.01234.x

Ma, L. J., Guzmán, E. A., DeGuzman, A., Muller, H. K., Walker, A. M., & Owen, L. B. (2007). Local cytokine levels associated with delayed-type hypersensitivity responses:

modulation by gender, ovariectomy, and estrogen replacement. *The Journal of Endocrinology*, *193*(2), 291–297. doi:10.1677/JOE-06-0024

Maes, M., Vandoolaeghe, E., Ranjan, R., Bosmans, E., Bergmans, R., & Desnyder, R. (1995). Increased serum interleukin-1-receptor-antagonist concentrations in major depression. *Journal of affective disorders*, *36*(1-2), 29–36.

Matejuk, A., Adlard, K., Zamora, A., Silverman, M., Vandenbark, A. A., & Offner, H. (2001). 17β-estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis. *Journal of Neuroscience Research*, *65*(6), 529–542. doi:10.1002/jnr.1183

McCormick, C. M., Kehoe, P., & Kovacs, S. (1998). Corticosterone release in response to repeated, short episodes of neonatal isolation : evidence of sensitization. *International Journal of Developmental Neuroscience*, *16*(3–4), 175–185. doi:10.1016/S0736-5748(98)00026-4

McCormick, C. M., Linkroum, W., Sallinen, B. J., & Miller, N. W. (2002). Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. *Stress (Amsterdam, Netherlands)*, *5*(4), 235–247. doi:10.1080/1025389021000061165

McEwen, B. S. (2000). The neurobiology of stress: from serendipity to clinical relevance. *Brain Research*, *886*(1–2), 172–189. doi:10.1016/S0006-8993(00)02950-4

Merali, Z., Khan, S., Michaud, D. S., Shippy, S. A., & Anisman, H. (2004). Does amygdaloid corticotropin-releasing hormone (CRH) mediate anxiety-like behaviors? Dissociation of anxiogenic effects and CRH release. *European Journal of Neuroscience*, *20*(1), 229–239. doi:10.1111/j.1460-9568.2004.03468.x

Mitsushima, D., Yamada, K., Takase, K., Funabashi, T., & Kimura, F. (2006). Sex differences in the basolateral amygdala: the extracellular levels of serotonin and dopamine, and their responses to restraint stress in rats. *European Journal of Neuroscience*, *24*(11), 3245–3254. doi:10.1111/j.1460-9568.2006.05214.x

Molteni, R., Lipska, B. K., Weinberger, D. R., Racagni, G., & Riva, M. A. (2001). Developmental and stress-related changes of neurotrophic factor gene expression in an animal model of schizophrenia. *Molecular Psychiatry*, *6*(3), 285–292. doi:10.1038/sj.mp.4000865

Neumann, I. D., Toschi, N., Ohl, F., Torner, L., & Krömer, S. A. (2001). Maternal defense as an emotional stressor in female rats: correlation of neuroendocrine and behavioural parameters and involvement of brain oxytocin. *The European Journal of Neuroscience*, *13*(5), 1016–1024.

Nisenbaum, L. K., & Abercrombie, E. D. (1993). Presynaptic alterations associated with enhancement of evoked release and synthesis of norepinephrine in hippocampus of chronically cold-stressed rats. *Brain research*, *608*(2), 280–287.

O'Keefe, J. H., Gheewala, N. M., & O'Keefe, J. O. (2008). Dietary strategies for improving post-prandial glucose, lipids, inflammation, and cardiovascular health. *Journal of the American College of Cardiology*, *51*(3), 249–255. doi:10.1016/j.jacc.2007.10.016

Özveri, E. S., Bozkurt, A., Haklar, G., Çetinel, Ş., Arbak, S., Yeğen, C., & Yeğen, B. Ç. (2001). Estrogens ameliorate remote organ inflammation induced by burn injury in rats. *Inflammation Research*, *50*(12), 585–591. doi:10.1007/PL00000238

Palanza, P. (2001). Animal models of anxiety and depression: how are females different? *Neuroscience & Biobehavioral Reviews*, *25*(3), 219–233. doi:10.1016/S0149-7634(01)00010-0

Paykel, E. S. (2001). Stress and affective disorders in humans. *Seminars in clinical neuropsychiatry*, *6*(1), 4–11.

Penkowa, M., Camats, J., Hadberg, H., Quintana, A., Rojas, S., Giralt, M., Hidalgo, J. (2003). Astrocyte-targeted expression of interleukin-6 protects the central nervous system during neuroglial degeneration induced by 6-aminonicotinamide. *Journal of Neuroscience Research*, *73*(4), 481–496. doi:10.1002/jnr.10681

Post, R. M. (2010). Mechanisms of illness progression in the recurrent affective disorders. *Neurotoxicity research*, *18*(3-4), 256–271. doi:10.1007/s12640-010-9182-2

Pyter, L. M., Kelly, S. D., Harrell, C. S., & Neigh, G. N. (2013). Sex differences in the effects of adolescent stress on adult brain inflammatory markers in rats. *Brain, Behavior, and Immunity*, *30*, 88–94. doi:10.1016/j.bbi.2013.01.075

Preston, S. D., Buchanan, T. W., Stansfield, R. B., & Bechara, A. (2007). Effects of anticipatory stress on decision making in a gambling task. *Behavioral Neuroscience*, *121*(2), 257–263. doi:10.1037/0735-7044.121.2.257

Rantamäki, T., Knuuttila, J. E. A., Hokkanen, M.-E., & Castrén, E. (2006). The effects of acute and long-term lithium treatments on trkB neurotrophin receptor activation in the mouse hippocampus and anterior cingulate cortex. *Neuropharmacology*, *50*(4), 421–427. doi:10.1016/j.neuropharm.2005.10.001

Rivest, S. (2009). Regulation of innate immune responses in the brain. *Nature Reviews Immunology*, *9*(6), 429–439. doi:10.1038/nri2565

Rocha, B. A., Fleischer, R., Schaeffer, J. M., Rohrer, S. P., & Hickey, G. J. (2005). 17β-Estradiol-induced antidepressant-like effect in the Forced Swim Test is absent in estrogen receptor-β knockout (BERKO) mice. *Psychopharmacology*, *179*(3), 637–643. doi:10.1007/s00213-004-2078-1

Rogers, A., & Eastell, R. (2001). The effect of 17beta-estradiol on production of cytokines in cultures of peripheral blood. *Bone*, *29*(1), 30–34.

Roman, E., & Arborelius, L. (2009). Male but not female Wistar rats show increased anxiety-like behaviour in response to bright light in the defensive withdrawal test. *Behavioural Brain Research*, *202*(2), 303–307. doi:10.1016/j.bbr.2009.04.019

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*(6), 1101–1108. doi:10.1038/nprot.2008.73

Seeman, M. V. (1997). Psychopathology in Women and Men: Focus on Female Hormones. *American Journal of Psychiatry*, *154*(12), 1641–1647.

Segarra, A. C., Torres-Díaz, Y. M., Silva, R. D., Puig-Ramos, A., Menéndez-Delmestre, R., Rivera-Bermúdez, J. G., Agosto-Rivera, J. L. (2014). Estrogen receptors mediate estradiol's effect on sensitization and CPP to cocaine in female rats: Role of contextual cues. *Hormones and Behavior*, *65*(2), 77–87. doi:10.1016/j.yhbeh.2013.12.007

Slotten, H. A., Kalinichev, M., Hagan, J. J., Marsden, C. A., & Fone, K. C. F. (2006). Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-dependent effects. *Brain Research*, *1097*(1), 123–132. doi:10.1016/j.brainres.2006.04.066

Song, C. (2002). The effect of thymectomy and IL-1 on memory: Implications for the relationship between immunity and depression. *Brain, Behavior, and Immunity*, *16*(5), 557–568. doi:10.1016/S0889-1591(02)00012-0

Soucy, G., Boivin, G., Labrie, F., & Rivest, S. (2005). Estradiol Is Required for a Proper Immune Response to Bacterial and Viral Pathogens in the Female Brain. *The Journal of Immunology*, *174*(10), 6391–6398.

Stark, R., Wolf, O. T., Tabbert, K., Kagerer, S., Zimmermann, M., Kirsch, P.,Vaitl, D. (2006). Influence of the stress hormone cortisol on fear conditioning in humans: Evidence for sex differences in the response of the prefrontal cortex. *NeuroImage*, *32*(3), 1290–1298. doi:10.1016/j.neuroimage.2006.05.046

Takeda, K., Toda, K., Saibara, T., Nakagawa, M., Saika, K., Onishi, T., Shizuta, Y. (2003). Progressive development of insulin resistance phenotype in male mice with complete aromatase (CYP19) deficiency. *The Journal of Endocrinology*, *176*(2), 237–246.

Taylor, S. E., Klein, L. C., Lewis, B. P., Gruenewald, T. L., R, A., & Updegraff, J. A. (2000). Biobehavioral responses to stress in females: Tend-and-befriend, not fight-or-flight. *Psychological Review*, *107*(3), 411–429. doi:10.1037/0033-295X.107.3.411

Ter Horst, G. J., Wichmann, R., Gerrits, M., Westenbroek, C., & Lin, Y. (2009). Sex differences in stress responses: Focus on ovarian hormones. *Physiology & Behavior*, *97*(2), 239–249. doi:10.1016/j.physbeh.2009.02.036

van den Bos, R., Harteveld, M., & Stoop, H. (2009). Stress and decision-making in humans: Performance is related to cortisol reactivity, albeit differently in men and women. *Psychoneuroendocrinology*, *34*(10), 1449–1458. doi:10.1016/j.psyneuen.2009.04.016

Vegeto, E., Bonincontro, C., Pollio, G., Sala, A., Viappiani, S., Nardi, F., Maggi, A. (2001). Estrogen Prevents the Lipopolysaccharide-Induced Inflammatory Response in Microglia. *The Journal of Neuroscience*, *21*(6), 1809–1818.

Walf, A. A., Rhodes, M. E., & Frye, C. A. (2004). Antidepressant effects of ERβ-selective estrogen receptor modulators in the forced swim test. *Pharmacology, Biochemistry and Behavior*, *78*(3), 523–529. doi:10.1016/j.pbb.2004.03.023

Walf, A. A., & Frye, C. A. (2005). ERβ-Selective Estrogen Receptor Modulators Produce Antianxiety Behavior when Administered Systemically to Ovariectomized Rats. *Neuropsychopharmacology*, *30*(9), 1598–1609. doi:10.1038/sj.npp.1300713

Walf, A. A., & Frye, C. A. (2006). A Review and Update of Mechanisms of Estrogen in the Hippocampus and Amygdala for Anxiety and Depression Behavior. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*, *31*(6), 1097–1111. doi:10.1038/sj.npp.1301067

Wei, J., Yuen, E. Y., Liu, W., Li, X., Zhong, P., Karatsoreos, I. N., Yan, Z. (2013). Estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and cognition. *Molecular Psychiatry*. doi:10.1038/mp.2013.83

Windle, R. J., Wood, S., Shanks, N., Perks, P., Conde, G. L., da Costa, A. P., Lightman, S. L. (1997). Endocrine and behavioural responses to noise stress: comparison of virgin and lactating female rats during non-disrupted maternal activity. *Journal of Neuroendocrinology*, *9*(6), 407–414.