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The Role of ER-Alpha and the Ovaries in the Enduring Altered Behavioral Response to Pubertal Immune Stress

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**THE ROLE OF ER-ALPHA AND THE OVARIES IN THE ENDURING ALTERED
BEHAVIORAL RESPONSE TO PUBERTAL IMMUNE STRESS**

A Thesis Presented

By

BETHANY ANN RAPPLEYEA

Submitted to the Graduate School of the
University of Massachusetts-Amherst in partial fulfillment
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Neuroscience and Behavior Program

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ABSTRACT

THE ROLE OF ER-ALPHA AND THE OVARIES IN THE ENDURING ALTERED BEHAVIORAL RESPONSE TO PUBERTAL IMMUNE STRESS

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Peripubertal immune stress alters adult responsiveness to estradiol (E₂) and progesterone (P). When female mice are injected with the bacterial endotoxin lipopolysaccharide (LPS) at six weeks of age, or during pubertal development, they display a decrease in response to ovarian hormones. In contrast, females ovariectomized prior to peripubertal immune stress display typical levels of sexual behavior following sequential injections of E₂ and P in adulthood. Additionally, intact females exposed to peripubertal immune stress display a decrease in estrogen receptor alpha (ER- α)-immunoreactive (ir) cells in the medial preoptic area (MPOA) and ventromedial nucleus of the hypothalamus (VMH) in adulthood. However, ER- α has not been studied in mice that have been ovariectomized prior to receiving LPS. The objective of the present study is two-fold: to replicate the finding that ovariectomy prior to pubertal development prevents the deleterious effects of LPS administration, and to examine the status of ER- α in areas of the brain important to sex behavior. We predicted that mice ovariectomized after LPS injection would display fewer ER- α -ir cells and a decreased responsiveness to ovarian hormones than saline controls and those mice ovariectomized prior to LPS injection. To test this, female mice were ovariectomized or sham-operated prior to LPS treatment. Then, at six weeks of age, all mice were injected with saline or LPS. Following that, sham-operated mice were ovariectomized

and ovariectomized mice were sham-operated. Mice were primed weekly with E₂ and P, and sex behavior testing occurred once a week for 5 weeks. After the final behavior test, all mice were euthanized, their brains removed, and stained for ER- α via immunocytochemistry. Results revealed a large variability in hormone responsiveness. However, animals that received peripubertal LPS, but still had their ovaries, had significantly lower sexual receptivity when compared to animals that were ovariectomized prior to the pubertal period and given LPS. Further, there were no differences between groups in ER- α -ir numbers. External environmental stressors, such as animal housing and vibrations and noise from nearby construction, may have caused some of the results found here, which are inconsistent with previous findings.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
LIST OF FIGURES.....	vii
CHAPTER	
1. BACKGROUND AND SIGNIFICANCE.....	1
Specific Aims.....	6
Methods.....	7
Animals.....	8
Ovariectomy.....	8
Sham-Operations.....	9
LPS Treatment.....	9
Sickness Behavior Quantification.....	10
Sexual Receptivity Testing.....	10
Tissue Collection.....	11
ER-alpha Immunocytochemistry.....	11
Tissue Mounting.....	12
Image Analysis.....	12
Statistical Analysis.....	13
Results.....	14
Sickness Behavior Following LPS Injection.....	14
Body Weight Change Following LPS or Saline Treatment.....	15
Sex Behavior.....	16
Sex Behavior: LPS Groups Only.....	17
Number of ER- α -ir Cells.....	18
2. DISCUSSION.....	22
REFERENCES.....	29

LIST OF FIGURES

Figure	Page
1. Selection of Best-Matched Sections.....	13
2. Sickness Behavior.....	15
3. Percent Body Weight Change Post-Injection.....	16
4. Sex Behavior.....	17
5. Sex Behavior: LPS Groups Only.....	18
6. Arcuate.....	19
7. MPOA.....	19
8. VMH.....	20
9. AVPV.....	21

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Puberty is a critical developmental period during which an organism transitions from a non-reproductive, juvenile state to a reproductive, adult state (Sisk & Foster, 2004). Changes that occur during the pubertal period fall into multiple categories, including physical, neural, and behavioral changes. Because of the sensitive nature of brain reorganization (and the behavioral changes that follow), recent studies have focused on the pubertal period as a critical developmental stage that, when disrupted, may lead to permanent modifications in the brain and in behavior.

Many of these neural and behavioral changes are driven by hormonal fluctuations. Specifically, pubertal development in female mice occurs due to two separate surges in estradiol. The first surge causes the opening of the vagina, which is typically cited as the first day of pubertal development, and the start of the period during which the animal is sensitive to external stressors (Rodriguez, Araki, Khatib, Martinou & Vassalli, 1997). In the CD1 strain of mouse, VO occurs at approximately 30 days of age (Ismail & Blaustein, unpublished results). Then, gonadotropin releasing hormone (GnRH) secretion from neurons in the brain intensifies (Sisk & Zehr, 2005). This amplification in the secretion of GnRH goes on to stimulate the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Sisk & Zehr, 2005). Together, FSH and LH lead to the second surge in estradiol. This second surge in estradiol generally occurs between 4 to 8 weeks of age, depending on the strain of mouse, and marks the end of both pubertal development and the start of reproductive maturation, as indicated by ovulation (Sisk & Zehr, 2005; Nelson, Karelus, Felicio & Johnson, 1990).

This cascade of hormonal events leads to changes in behavioral responsiveness to steroid hormones. Female rats have been shown to increase in sensitivity to ovarian hormone administration with age (Sodersten 1975). Further, female guinea pigs administered ovarian hormones prior to the pubertal period do not display adult levels of responsiveness, as measured by lordosis quotient (Olster & Blaustein, 1989). This suggests that the brain requires the above-mentioned cascade to become sensitive to sex steroid hormones and produce adult behaviors.

The effects of sex steroid hormones during the pubertal developmental period are not limited to outward physical and behavioral changes. Permanent modifications also occur in the brain (Sisk & Foster, 2004). For example, sex steroids lead to the sexual differentiation of the locus coeruleus, which is responsible for some behavioral responses to stress and the release of norepinephrine (Pendergast, Tuesta, & Bethea 2008; Berridge & Waterhouse 2003; Luque, de Blas, Segovia, & Guillamón, 1992). Additionally, the primary visual cortex in female rats has approximately 19% less neurons than that of males, and this change is driven by the secretion of ovarian hormones (Sodhi, Nuñez, & Juraska, 2002). However, it should be noted that some pubertal brain reorganization occurs independently of sex steroid hormones. For example, in female rats, the anteroventral periventricular (AVPV) nucleus grows during the pubertal period, and ovariectomy prior to the pubertal period has no effect on this growth (Davis, Shryne, & Gorski, 1996).

This extensive reorganization of the brain during the pubertal period is vulnerable to certain external stressors, as indicated by enduring behavioral disruptions following these stressors. For example, peripubertal male golden hamsters subjected to forced

submission display an increase in aggressive behaviors, whereas forced submission in adults increases avoidant behaviors (Wommack, Salinas, Melloni, & Delville 2004). In addition, peripubertal female rats exposed to chronic stress will express a greater sensitization to nicotine in adulthood. Specifically, females, but not males, that are subjected to stress during the pubertal period display increased locomotor activity following a nicotine injection in adulthood (McCormick, Robarts, Gleason, & Kelsey 2004).

The stress of shipping mice during the pubertal period also modifies brain organization and has lasting effects on behavior. During shipping, mice experience various and unpredictable potential stressors. These potential stressors include disruption of circadian rhythms, food deprivation, exposure to unfamiliar odors, wide temperature fluctuations, potential pathogens, and others (Blaustein & Ismail 2013). When ovary-intact female mice are shipped during the pubertal period, numerous deleterious effects are observed well into adulthood. Specifically, if female mice are shipped during pubertal development and then are ovariectomized and hormone-primed as adults, a decrease in responsiveness to ovarian hormones is observed. This decrease in responsiveness is seen in both inbred C57Bl/6 mice and outbred CD1 strains, which illustrates that shipping during the pubertal period has effects on multiple strains of mice (Laroche, Gasbarro, Herman, & Blaustein 2009b; Ismail, Garas, & Blaustein, 2011). However, if mice are shipped prior to or following 6 weeks of age, or the pubertal developmental period, this decrease in responsiveness to ovarian hormones is not observed. The deleterious effects that peripubertal shipping has on responsiveness to ovarian hormones in adulthood underscores the pubertal period as a time of critical development of behavioral systems

that respond to ovarian hormones (Sisk & Foster 2004; Laroche *et al.*, 2009b; Ismail *et al.*, 2011).

Injection of lipopolysaccharide (LPS), a bacterial endotoxin found in the cell wall of gram negative bacteria, is the only other peripubertal stressor known besides shipping that decreases adult female behavioral responsiveness to estradiol and progesterone (Laroche, Gasbarro, Herman, & Blaustein 2009a; Olesen, Ismail, Merchasin, Blaustein, 2011; Ismail, Garas, & Blaustein 2011; Ismail, Kumlin & Blaustein, 2012). Systemically injected LPS is known to disrupt both the physiology and behavior of mice. For example, female mice injected with LPS during the pubertal period and then given sequential injections of estradiol and progesterone in adulthood 48 and 6 hours before behavior testing, respectively, or are implanted with a silastic capsule containing estradiol at the time of ovariectomy display a decrease in body weight, an increase in sickness behavior, an increase in depression-like behavior, an increase anxiety-like behavior, a decrease in sexual receptivity, and a disruption in cognition in adulthood (Ismail & Blaustein 2013, Ismail *et al.*, 2012, Laroche *et al.*, 2009a, Olesen *et al.*, 2009). Mice administered LPS prior to and following the pubertal period do not display these injurious effects (Ismail & Blaustein 2013, Ismail *et al.*, 2012, Laroche *et al.*, 2009a, Olesen *et al.*, 2009).

Although female mice must receive an LPS injection at approximately six weeks of age to experience the most profound decrease in responsiveness to ovarian hormones in adulthood, age of injection does not alter other short-term effects. For instance, CD1 female mice given LPS at eight weeks of age display similar levels of sickness behavior and loss of body weight when compared to mice given LPS at eight weeks of age (Ismail,

Garas & Blaustein, 2011). Additionally, corticosterone levels are known to rise following other stressors applied during the pubertal period, but the decrease in responsiveness to ovarian hormones is not observed (Laroche *et al.*, 2009). Therefore, body weight loss, levels of sickness scored by outward physical symptoms, and corticosterone increases are most likely not the cause of this decrease in responsiveness to ovarian hormones following pubertal LPS.

The mechanism that underlies this decrease in adult sex behavior following pubertal LPS is unknown, but estrogen receptors (ERs) may be involved. ERs are essential for the expression of female sex behavior. ER- α knockout mice display almost no sexual receptivity, and site-specific knockout of ER- α eliminates both proceptive and receptive behaviors, and increases rejection behaviors in female mice. (Rissman, Wersinger, Taylor & Lubhan, 1997; Musatov, Chen, Pfaff, Kaplitt, Ogawa 2006). Further, in the female mouse brain, ER- α is found in areas such as the AVPV, VMN, MPOA, and Arc (Shughrue, Lane & Merchenthaler, 1997). The number of ER- α decreases in the MPOA, VMN and Arc following shipping at six weeks compared with mice shipped at eight weeks (Ismail *et al.*, 2011). Similarly, female mice given pubertal LPS display decreased expression of ER- α in the MPOA and VMN, although expression is not affected in the Arc (Ismail & Blaustein, unpublished observation).

The deleterious effects of peripubertal LPS treatment on ER- α is most likely linked to the increase in estradiol that occurs during the pubertal period. Generally, estradiol is considered a neuroprotective agent, but, in the presence of LPS, estradiol is permissive and allows activation of microglia (Soucy, Boivin, Labrie, & Rivest, 2005). Thus, inflammation occurs. Further, estradiol is secreted by the ovaries, and removal of

the ovaries prior to pubertal development prevents the alterations of adult behavioral responsiveness to injected ovarian hormones following pubertal LPS treatment (Rappleyea, Ismail, & Blaustein, unpublished observation). Although decreases in ER- α have been well documented in mice with their ovaries at the time of peripubertal immune stress, the status of ER- α in females ovariectomized prior to peripubertal stress remains unknown.

In the current study, we examined the effects of LPS on ER- α number in ovary-intact and ovariectomized, six-week old female mice. Because LPS has long-term effects on behavioral responses to ovarian hormones, we predicted that mice administered LPS before ovariectomy would display decrease of ER- α in the VMH, MPOA, and Arc, but that ovariectomy prior to administering LPS would prevent these changes in ER- α . This would suggest that the presence of ovarian hormones during pubertal immune stress alters both number of ER- α and adult sex behavior. If the number of ER- α -immunoreactive (ir) cells is not modified by the combinatorial effects of pubertal LPS treatment and ovariectomy, then some other cellular mechanism may be responsible for the observed decrease in responsiveness to ovarian hormones.

Specific Aims

Recent work has shown that removing the ovaries prior to pubertal development eliminates the deleterious effects of peripubertal LPS. However, in recent experiments completed in our laboratory, the initial, well-replicated finding that LPS causes long-term changes in behavioral response to ovarian hormones has not been consistent. This is believed to be due to environmental perturbations, such as nearby construction (Dallman, Akana, Bell, Bhatnagar, Choi, Chu, Gomez, Laugero, Soriano Viau 1999; Shepherd,

Helliwell, Mace, Morgan, Patel, Kellett 2004; Raff, Bruder, Cullinan, Ziegler, Cohen 2011). In order to proceed, it was first necessary to replicate previous work and test the neuronal mechanism underlying the effect. Therefore, the aims of this thesis are:

1. **To determine the effects of peripubertal LPS and the ovaries on responsiveness to ovarian hormones in adulthood.**

Although previous work in this lab has shown that removing the ovaries prior to the pubertal period prevents the deleterious long-term effects of LPS on responsiveness to ovarian hormones, recent changes in the laboratory have resulted in inconsistent results than seen previously. Therefore, replicating these effects is important for furthering the work.

2. **To determine the effects of LPS and the ovaries on number of ER- α -ir cells in areas of the brain involved in hormonal regulation of female sexual behavior.**

ER- α decreases in adulthood following pubertal LPS administration. However, the status of ER- α in animals that have their ovaries removed prior to the pubertal period and given LPS during pubertal development is unknown. This study will uncover if ovarian secretions are responsible for the decrease in numbers of ER- α -ir in the VMH, MPOA, and Arc following a peripubertal immune stressor.

Methods

Briefly, 32 female mice arrived at our animal facility at 3 weeks of age. Following a two-week acclimation period, half of the mice were ovariectomized, and the other half were sham-operated. After a one-week recovery period, at six weeks of age, half of the ovariectomized and half of the sham-operated mice received LPS injections, and the other half of each group received saline injections. At seven weeks of age, the previously sham-operated mice were ovariectomized, and the previously ovariectomized mice were sham-operated. Mice recovered for one week, and then were given sequential injections

of estradiol and progesterone prior to sexual behavior testing once a week for 5 weeks. Important to note is that mice ovariectomized prior to LPS injection did not have a natural hormone environment throughout the pubertal period; in fact, females ovariectomized prior to LPS injection did not receive hormone injections until eight weeks of age, or when full reproductive maturation was reached. Following the last behavioral test, all mice were euthanized, brains removed and cut into 30 μ m sections. Then brain, tissue was stained via immunocytochemistry. Best-matched sections for the Arc, MPOA, VMH, and AVPV were used for imaging, and were identified using the using *The Mouse Brain Atlas in Stereotaxic Coordinates* (Franklin & Paxinos, 1997).

Animals

Thirty-two, 3-week old female CD1 mice were purchased from Charles River Laboratory (Kingston, NY). The animals were kept in a temperature-controlled colony room (~72°F) with a reversed 14 hours of light: 10 hours of dark cycle (lights off at 11am). All mice were housed in groups of four per cage, and given *ad libitum* access to dry rodent chow (Teklad 2014, phytoestrogen-reduced diet, Harlan Laboratories, Madison, WI) and water.

Ovariectomy

Half of the mice were ovariectomized, and the other half sham-operated at five weeks of age. Mice were treated with LPS or saline at six weeks of age. Following a week of recovery after LPS or saline administration, previously ovariectomized mice were sham-operated, and previously sham-operated mice were ovariectomized. The mice were anesthetized with isoflurane (3% at 1/min). Once anesthetized, hair was clipped

from the ventral surface of the left abdominal side. An incision was made on left side of the abdomen, cutting through both skin and muscle. Uterine horns were tied, ovaries removed, and bleeding stopped by cauterization. Absorbable sutures were used to close the incision in the muscle wall, and wound clips were used to close the skin incision. Mice were then placed in a recovery chamber with a heating pad, and monitored until fully conscious and mobile. Following this recovery of motor function, the females were returned to their home cages. As described by Laroche *et al.*, 2009, each cage was given a water bottle with 3% Children's Tylenol (160 mg acetaminophen/mL) for 48 hours following surgery. Each animal was monitored for any signs of post-surgery distress for seven days following the procedures.

Sham-Operations

Sham-operated mice underwent the same procedure as ovariectomized mice with the following exception: instead of ovary removal, only some abdominal fatty tissue was displaced. The ovaries were not manipulated. All animals were given one week to recover from surgery before testing continued.

Lipopolysaccharide (LPS) and Saline Treatment

At six weeks of age, 16 mice were injected intraperitoneally with LPS (1.5 mg/kg body weight), and the other 16 were injected with sterile saline. The LPS was obtained from *Escherichia coli* serotype O26:B6; no. L3755 (Sigma Chemical Co., St. Louis, MO), and dissolved at 0.1mg/ml in sterile saline. Each cage of 4 mice was randomly assigned to either the LPS or saline group, with the constraint that half of the LPS and saline groups were ovariectomized, and the other half sham-operated at the time of treatment.

Sickness Behavior Quantification

Sickness behavior was quantified in two ways, similar to that described by Gibb, Hayley, Gandhi, Poulter & Anisman (2008). First, sickness behavior was scored at four time points: 30 minutes, four hours, 24 hours, and 48 hours following LPS or saline injection. Experimenters blind to treatment groups examined mice individually for number of sickness behaviors displayed, including: ptosis (drooping/swollen eyelids), piloerection (raised fur), lethargy (slow movement), and huddling (legs tucked beneath body, unmoving, and likely in close proximity with another animal). A score of zero to four was assigned to each animal based upon the number of behaviors observed. Changes in body weight were also recorded at five time points: 24, 48, 72, 96, and 120 hours following LPS or saline treatment.

Sexual Receptivity Testing

At eight weeks old, two weeks after LPS or saline treatment, testing for sexual receptivity began. To induce sexual receptivity, all mice were injected subcutaneously (sc) with two μg of estradiol benzoate, and 48 hours later with an sc injection of 100 μg of progesterone. Five hours following the progesterone injection, the hormonally primed females were placed in a plexiglass enclosure with a sexually experienced male (Laroche *et al.*, 2009a, Laroche *et al.*, 2009b). Each female received up to 20 mounts from a male. A mount was counted when the forepaws of the male made contact with the hindquarters of the female, in such a position that intromission would be possible. Intromissions were also scored, and males were allowed a maximum of 10 thrusts per mount. In the case of

an ejaculation, the test was terminated for both the involved male and female. All sex behavior testing took place during the dark phase, in a dark room lit with dim red light.

Sexual receptivity of the female was calculated by the lordosis quotient (LQ): (number of intromissions/number of mounts) X 100. Testing occurred once a week for five weeks, and the experimenter scoring sex behavior was blind to surgery type and to treatment.

Tissue Collection

Mice were deeply anesthetized via intraperitoneal injections of pentobarbital (200 mg/kg) prior to decapitation. Following careful removal, brains were immersion-fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 hours. Then, brains were moved to fresh tubes containing PBS (pH 7.2) and 30% sucrose. Following that, all brains were flash-frozen with dry ice and stored at -80 °C until sectioning. All brains were cut into 30 µm sections with a cryostat, in a one-in-four series, and then stored in cryoprotectant at -20 °C until immunostained for ER- α .

ER- α Immunocytochemistry

Cohorts containing animals from each treatment group were processed in the same tray. Briefly, sections from one series were rinsed in 0.5M Tris-buffered saline (TBS) and then in 0.5M gel TBS, containing 10% gelatin, 10% sodium azide, and 10% Triton X. Sections were then incubated in a blocking buffer containing 10% bovine serum albumin (BSA), 20% normal goat serum (NGS), and 3.5% hydrogen peroxidase. Sections were then incubated in a rabbit ER- α antibody (Millipore, Temecula, CA, US)

for 48 h at 4°C. After incubation in primary antibody, sections were rinsed in gel TBS, and then transferred to 1.5 mL tubes until mounted.

Tissue Mounting

Following immunocytochemistry, sections were carefully removed from the 1.5 mL tubes and placed into a Petri dish containing TBS. Sections were serially arranged, and then mounted in order on 2% gelatin-coated glass slides. Completed slides were allowed to dry for 3 days, rehydrated and serially dehydrated in distilled water and ethanol, and then coverslipped with polymount.

Image Analysis

Slides were anatomically matched and numerically coded so that the investigator conducting the analysis was blind to the experimental groups. Sections were analyzed from a one-in-four series (adjacent sections separated by 90 μm) containing the MPOA, VMH, and Arc. In addition, the AVPV was used as a control. One best-matched brain section was used in the analysis, thus six contours, or sectors per bilateral nucleus were counted and an average of ER- α labeled was derived. An animal was excluded from analysis for a particular region if a best-matched section could not be obtained. The MPOA, VMH, Arc, and AVPV were identified using *The Mouse Brain Atlas in Stereotaxic Coordinates* (Franklin & Paxinos, 1997): AVPV (plate 31), MPOA (plate 32), VMN and Arc (plate 43) (Figure 1).

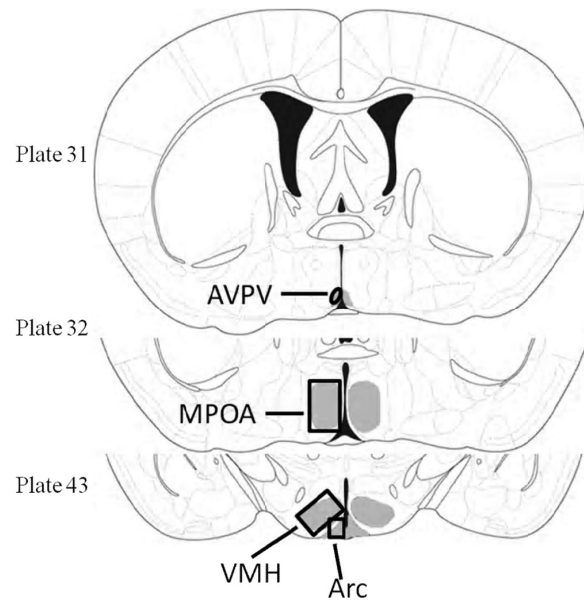


Figure 1. Selection of Best-Matched Sections. Selection of best-matched sections was based on plates 31, 32, and 43 from *The Mouse Brain Atlas in Stereotaxic Coordinates*, from Franklin and Paxinos 1997.

Statistical Analysis

Sickness behavior following saline or LPS treatment was analyzed using a three-way repeated measures analysis of variance (ANOVA) with time points as a within-subjects factor and treatment (saline or LPS) and surgery type (ovariectomy or sham operation prior to pubertal development) as between-subjects factors. Differences in sickness behavior between mice that received LPS and mice that receive saline were detected using pairwise comparisons.

Changes in body weight following saline or LPS treatment was analyzed in a similar manner with time of measurement as the within-subjects factor and treatment and surgery type as the between-subjects factors. Pairwise comparisons were used to examine differences in body weight change between mice given saline or LPS treatment.

A three-way repeated measures ANOVA was used to assess statistically significant differences in sexual receptivity. Sexual behavior trials were the within-subjects factor, and between-subjects factors were treatment and surgery type.

Differences in ER- α -ir numbers were analyzed using a two-way ANOVA. The between subjects factors was treatment and type of surgery. Pairwise comparisons were used to determine if LPS treatment altered the number of ER- α -immunoreactive cells.

Results

Sickness Behavior Following LPS or Saline Treatment

A three-way, repeated measures ANOVA showed main effects of time ($F_{3, 84} = 138.81, p < .001$) and treatment ($F_{1, 28} = 13.99, p < .001$) on sickness behavior. In addition, there were significant time x treatment ($F_{1, 28} = 11.32, p < .001$), and time x surgery ($F_{3, 84} = 2.505, p < .05$) interactions. Post hoc comparisons using the Tukey HSD comparisons showed that LPS-treated mice displayed significantly more sickness behavior than saline-treated ones at 4 hours ($p < .001$), and 24 hours ($p < .001$) following treatment. Further, pairwise comparisons revealed significant differences between saline and LPS groups ($p < .001$). (Figure 2)

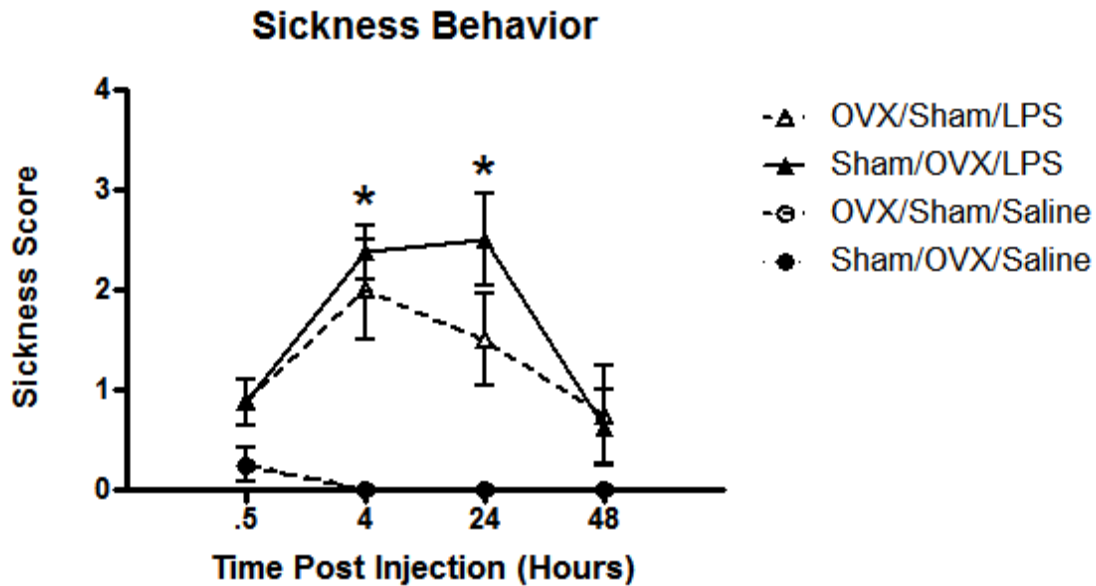


Figure 2. Mean sickness behavior scores of female mice injected at six weeks old with LPS or saline.

Body Weight Change Following LPS or Saline Treatment

A three-way, repeated measures ANOVA showed main effects of time ($F_{3, 84} = 5.45, p < .001$), and treatment ($F_{1, 28} = 179.49, p < .001$) on body weight change. In addition, there was a significant time x treatment interaction ($F_{1, 28} = 7.47, p < .001$), but not a time x surgery ($F_{3, 84} = .685, p = .56$) interaction. Post hoc comparisons using the Tukey HSD showed that LPS-treated mice lost significantly more weight when compared to saline-treated ones at 24 hours ($p < .001$), and 48 hours ($p < .001$) following treatment (indicated by *). Animals that were ovariectomized prior to saline treatment were heavier than any other group at 24 ($p < .001$), 48 ($p < .001$), 72 ($p < .001$), 96 ($p < .05$), and 120 ($p < .05$) hours after injection (indicated by #). Further, pairwise comparisons revealed significant differences between saline and LPS groups ($p < .001$) (Figure 3).

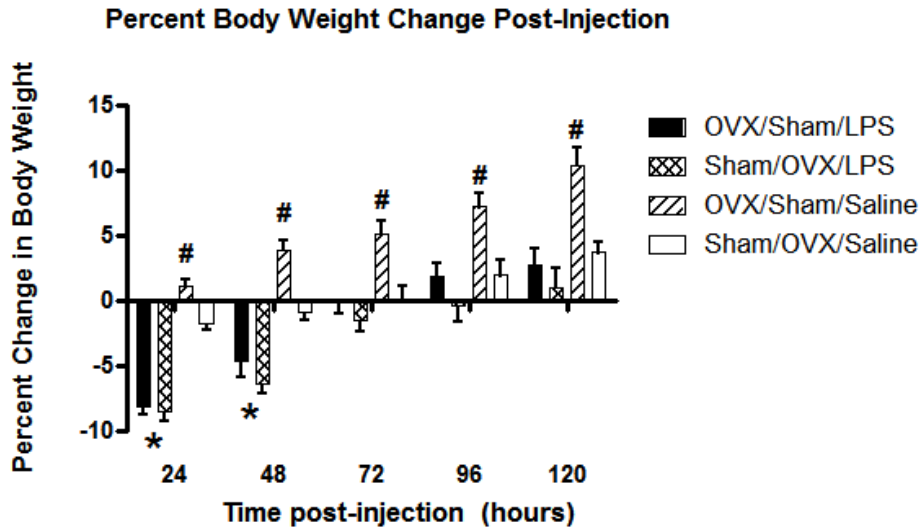


Figure 3. Mean percent body weight change (in grams) from 1 hour prior to LPS or saline treatment of female mice injected at six weeks old with LPS or saline.

Sex Behavior

A three-way, repeated measures ANOVA revealed a main effect of test session week ($F_{3, 84} = 17.503$, $p < .001$), but not of treatment ($F_{1, 28} = .028$, $p = .868$), nor of surgery ($F_{1, 28} = .940$, $p = .340$). There was not a significant treatment x surgery interaction ($F_{1, 28} = 6.15$, $p = .019$) nor a test x treatment x surgery interaction ($F_{3, 84} = .611$, $p = .655$), suggesting that test session week, treatment (peripubertal LPS or saline administration), surgery type (ovariectomy prior to or following peripubertal stress) together did not vary lordosis quotient (LQ) across groups. Pairwise comparisons revealed no differences between saline and LPS groups ($p = .868$) (Figure 4).

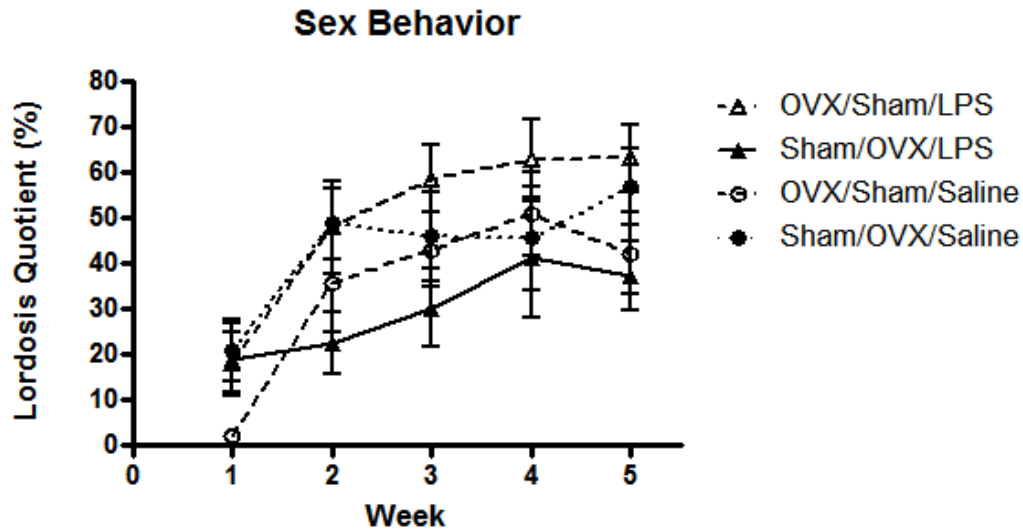


Figure 4. Mean lordosis quotient of female mice ovariectomized prior to or following pubertal LPS or saline injections at six weeks old.

Sex Behavior: LPS Groups Only

A two-way, repeated measures ANOVA revealed main effects of test session week ($F_{3, 84} = 8.272, p < .001$) and of surgery type ($F_{1, 28} = 5.120, p < .05$), indicating that LQ increased over session number, and that surgery type of the group (ovariectomy prior to or following peripubertal LPS injection) significantly changed LQ. However, there was not a significant week x surgery interaction ($F_{1, 28} = 1.544, p = .202$). Post hoc tests of between-subjects effects revealed no significant differences at week 1 ($p = .953$), week 2 ($p = .058$), or week 4 ($p = .198$). Significant differences were observed at weeks 3 ($p < .05$) and 5 ($p < .05$) (Figure 5).

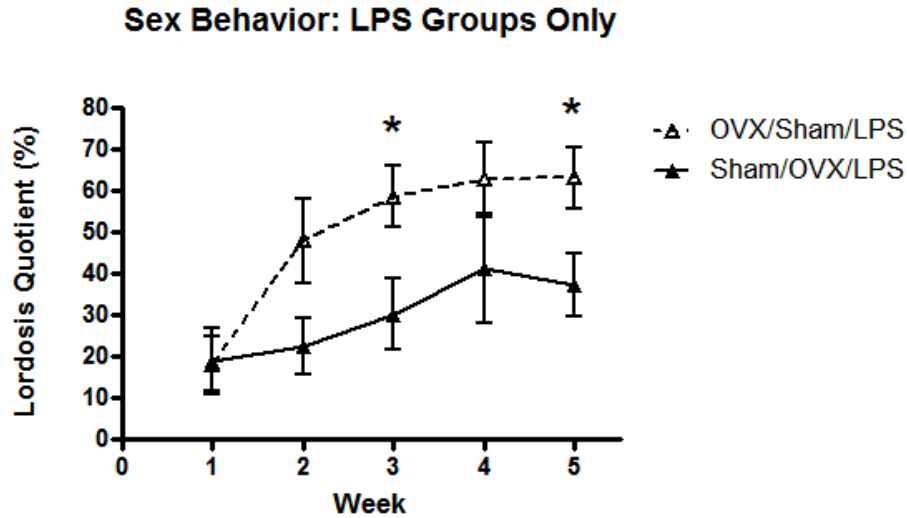


Figure 5. Mean lordosis quotient of female mice ovariectomized prior to or following pubertal LPS injections only at six weeks old.

Number of ER- α -ir Cells

In the Arc, a two-way, repeated measures ANOVA revealed no main effects of treatment ($F_{1,28} = .792, p = .382$), or surgery ($F_{1,28} = .514, p = .480$) in the number of ER- α -ir cells. There was not a significant treatment x surgery interaction ($F_{1,28} = .030, p < .864$), which indicates that treatment (peripubertal LPS or saline administration) and surgery type (ovariectomy prior to or following peripubertal stress) together did not affect number of ER- α -ir cells. Further, pairwise comparisons revealed no differences between saline and LPS groups ($p = .382$) (Figure 6).

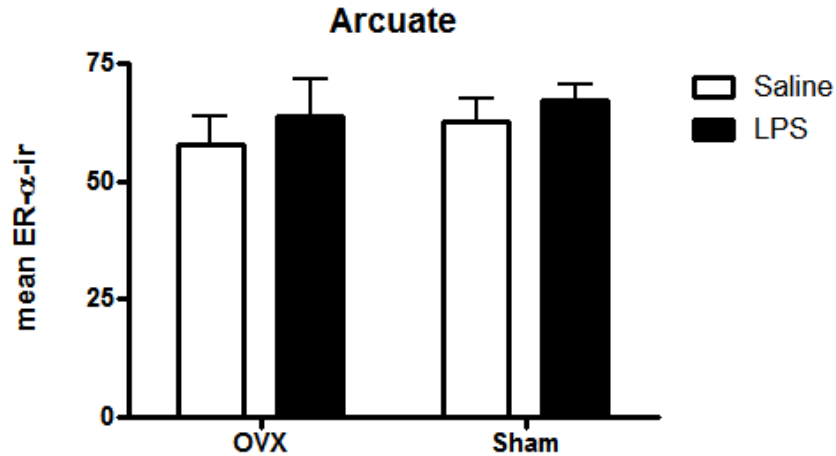


Figure 6. Number of ER- α -ir cells (Mean \pm SEM) in the Arcuate of female mice injected at six weeks old with LPS or saline.

In the MPOA, a two-way repeated measures ANOVA revealed no main effects of treatment ($F_{1,28} = .323, p = .574$) nor of surgery ($F_{1,28} = .044, p = .835$), which indicates that treatment (peripubertal LPS or saline administration) and surgery type (ovariectomy prior to or following peripubertal stress) did not affect number of ER- α -ir cells. There was not a significant treatment \times surgery interaction ($F_{1,28} = .035, p < .854$). Further, pairwise comparisons revealed no differences between saline and LPS groups ($p = .574$) (Figure 7).

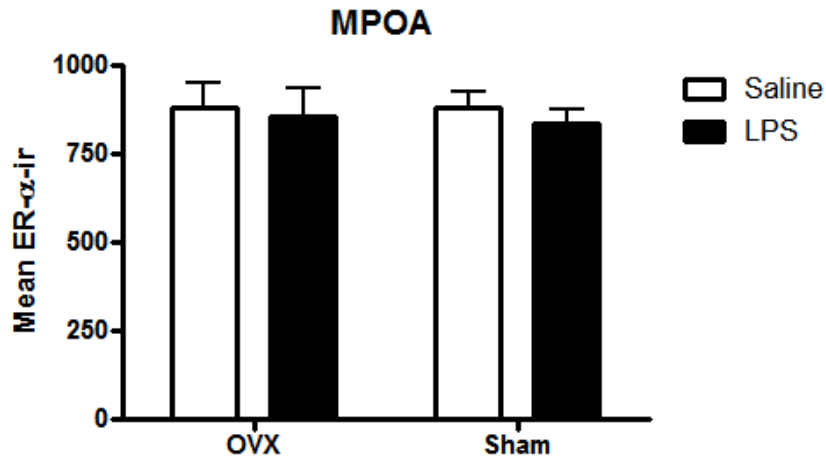


Figure 7. Number of ER- α -ir cells (Mean \pm SEM) in the MPOA of female mice injected at six weeks old with LPS or saline.

In the VMH, a two-way repeated measures ANOVA revealed no main effects of treatment ($F_{1,28} = .883, p = .356$) nor of surgery ($F_{1,28} = .288, p = .596$) which indicates that treatment (peripubertal LPS or saline administration) and surgery type (ovariectomy prior to or following peripubertal stress) did not affect number of ER- α -ir cells. There was not a significant treatment x surgery interaction ($F_{1,28} = 1.52, p < .228$) Further, pairwise comparisons revealed no differences between saline and LPS groups ($p = .356$) (Figure 8).

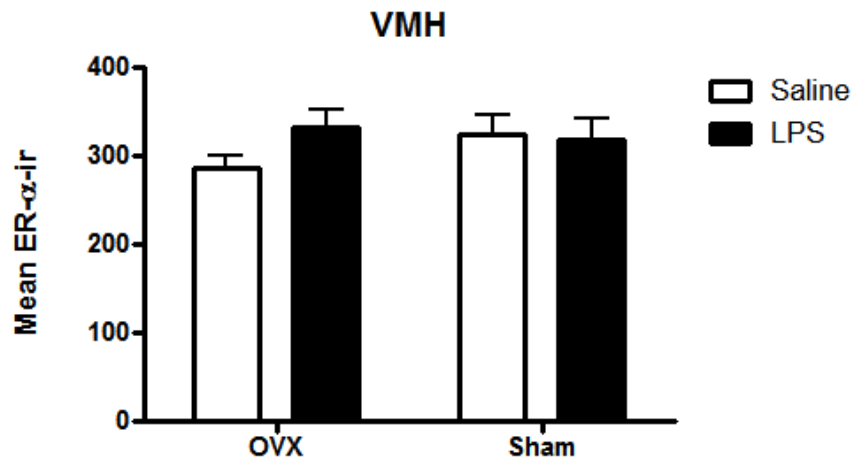


Figure 8. Number of ER- α -ir cells (Mean \pm SEM) in the VMH of female mice injected at six weeks old with LPS or saline.

In the AVPV, a two-way repeated measures ANOVA revealed no main effects of treatment ($F_{1,28} = .062, p = .805$) nor of surgery ($F_{1,28} = .035, p = .854$), which illustrates that treatment (peripubertal LPS or saline administration) and surgery type (ovariectomy prior to or following peripubertal stress) did not affect number of ER- α -ir cells. There was not a significant treatment x surgery interaction ($F_{1,28} = .079, p < .781$) Further, pairwise comparisons revealed no differences between saline and LPS groups ($p = .805$) (Figure 9).

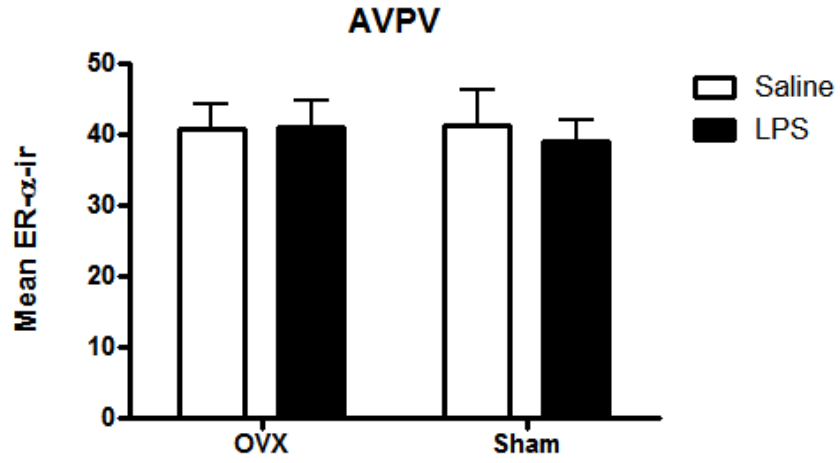


Figure 9. Number of ER- α -ir cells (Mean \pm SEM) in the AVPV of female mice injected at six weeks old with LPS or saline with or without ovaries.

CHAPTER 2

DISCUSSION

The aim of this thesis was two fold: to examine and replicate the effects of ovary removal prior to an immune challenge during pubertal development and to examine the effects of pre-pubertal ovary removal on ER- α -ir number in the Arc, MPOA, and VMH. We hypothesized that mice ovariectomized after peripubertal LPS treatment would show a decrease in adult sexual receptivity, as has been observed previously (Laroche *et al.*, 2009a; Ismail *et al.*, 2011). Based on the results of an earlier experiment (Rappleyea *et al.*, unpublished observation), we expected that mice ovariectomized prior to pubertal immune challenge would not display a decrease in responsiveness to ovarian hormones, as indicated by sexual receptivity. Further, we hypothesized that ovary-intact female mice exposed to LPS during pubertal development would have a decrease in number of ER- α -ir cells in the Arc, VMH, and MPOA. The results of this study partially supported our hypothesis that the long-term effect of LPS is dependent on the presence of the ovaries; mice ovariectomized prior to immune challenge administered during pubertal development did not display loss of behavioral responsiveness to estradiol and progesterone when compared only to other mice that received pubertal LPS but were ovary-intact. Further, our hypothesis that number of ER- α -ir cells would decrease following pubertal LPS injection in animals that were ovary-intact, but unaltered in mice ovariectomized prior to pubertal development, was not supported by the data. All animals displayed numbers of ER- α -ir cells in the Arc, AVPV, MPOA, and VMH that were not significantly different, regardless of surgery or immune challenge treatment.

Mice ovariectomized prior to pubertal immune challenge showed levels of sickness behavior that were not significantly different to those females ovariectomized after the peripubertal period. Both LPS groups displayed significantly more sickness behaviors than saline-treated animals at 4 hours and 24 hours following LPS injection.

Both groups of female mice administered LPS lost more body weight than the control animals given saline at 24 and 48 hours post-LPS injection. The amount of body weight lost did not differ between those females ovariectomized prior to and those ovariectomized after pubertal immune challenge. However, the females that were ovariectomized prior to pubertal development and then given saline during pubertal development weighed significantly more than all other groups at 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours post-injection. Weight gain following ovariectomy has been well documented; thus, this finding is unsurprising (Eckel 2011; Witte, Resuehr, Chandler, Mehle, Overton 2010).

In the two LPS groups, animals that were ovary-intact at the time of LPS injection displayed a decrease in responsiveness to ovarian hormones at week 3 and week 5 of behavioral testing. However, when the animals that were both ovary-intact and ovariectomized at the time of saline injection are included in analysis, differences between groups disappear. Importantly, in ovariectomized animals that received peripubertal LPS, the lack of decrease of behavioral responsiveness to ovarian hormones is not due to differences in body weight loss or sickness behavior between groups that received LPS.

In all of the neuroanatomical areas examined (Arc, MPOA, VMH, and AVPV) there were no significant effects of LPS or time of ovariectomy on the number of ER- α -ir

cells, indicating a failure of replication of previous results that showed peripubertal LPS treatment leads to a decrease in ER- α -ir cells (Ismail & Blaustein, unpublished observation). The control area, the AVPV, which increases in size during the pubertal period regardless of ovarian hormone status, also displayed no differences in the number of ER- α -ir cells between groups (Davis *et al.*, 1996). This failure to replicate effects on numbers of ER- α -ir cells and the variable behavioral results may be due, in part, to environmental perturbations during the experiment. Further, the possibility remains that the decrease in responsiveness to ovarian hormones following peripubertal LPS administration is not due to a decrease in numbers of ER- α -ir cells in the Arc, MPOA, or VMH. The decrease in responsiveness observed might be due to disruptions in another pathway that regulates female adult responsiveness to ovarian hormones.

Female mice display a relatively profound change in responsiveness to estradiol and progesterone following a pubertal immune challenge when compared to male mice or other rodents. Specifically, male mice shipped at six weeks of age display a significant decrease in number of mounts during sexual behavior testing, but do not exhibit any decrease in other sexual behaviors, such as mount latency and latency to ejaculate (Laroche *et al.*, 2009a). Further, female rats do not display a decrease in behavioral responsiveness to estradiol and progesterone following peripubertal LPS treatment (King & Blaustein, unpublished observation).

This disparity in sensitivity to peripubertal immune stress when comparing mice to rats may be due to length of pubertal development. For instance, female rats begin estrous cycling shortly after vaginal opening (Parker & Mahesh, 1976). Female mice, on the other hand, display a delay that lasts approximately 4 to 8 weeks, depending on the

strain, between the time of vaginal opening and the start of estrous cycling (Nelson *et al.*, 1990). This period during which the hormonal milieu is changing may be crucial for the observed disruptive effect that peripubertal LPS treatment has on adult responsiveness to ovarian hormones.

In previous work, disruption of adult behavior and a decrease in ER- α was observed after an immune or shipping stress during the pubertal period. Shipping or immune challenge in C57Bl/6 mice during the pubertal period led to a decrease in responsiveness to ovarian hormones in adulthood, which was revealed by a decrease in sexual receptivity (Laroche *et al.*, 2009a &b). Additionally, CD1 mice, a strain of mouse which has a slightly delayed pubertal period, subjected to peripubertal shipping or LPS displayed a decrease in responsiveness to ovarian hormones in adulthood (Ismail *et al.*, 2011; Ismail & Blaustein, unpublished observations). Further, peripubertal LPS reverses the anti-depressive and anxiolytic effects of estradiol, and disrupts the effects of estradiol on cognitive behaviors (Ismail & Blaustein 2013, Ismail *et al.*, 2012, Laroche *et al.*, 2009a, Olesen *et al.*, 2009). In the case of sexual receptivity, ovariectomy prior to the LPS injection prevented the decrease in responsiveness to ovarian hormones in adulthood (Rappleyea, Ismail, & Blaustein, unpublished observation).

Estradiol binds to ER- α in the Arc, MPOA, and VMH. These areas regulate many of the behaviors that change in ovarian hormone responsiveness following peripubertal LPS, such as anxiety-like and female sex behaviors (Choleris, Gustafsson, Korach, Muglia, Pfaff, & Ogawa, 2003; Choleris, Ogawa, Kavaliers, Gustafsson, Korach, Muglia, & Pfaff 2006; Ogawa, Eng, Taylor, Lubahn, Korach, & Pfaff, 1998). Therefore, a decrease in ER- α is thought to be part of the molecular mechanism behind this disruption

in responsiveness to ovarian hormones following peripubertal stress (Ismail *et al.*, 2011; Ismail & Blaustein, unpublished observation).

Based on earlier studies, a decrease in responsiveness to ovarian hormones in animals that received LPS during the pubertal period and a decrease in ER- α in areas important to the expression of sexual receptivity was expected (Ismail *et al.*, 2011; Ismail & Blaustein, unpublished observation). Additionally, ovariectomy prior to the peripubertal stressor was predicted to prevent all of these effects of the immune challenge (Rappleyea *et al.*, unpublished observations). These predictions were not supported by the data. Unfortunately, a number of suboptimal circumstances in the lab may have led to these results. Specifically, nearby construction work and changes in animal housing may have resulted in unpredictable stressors throughout the experiment.

Nearby construction caused unexpected vibrations and increases in noise levels within our animal housing facility. The stressors associated with construction are known to affect the physiology and behavior of laboratory animals. For example, rats exposed to a loud noise displayed not only an increase in ACTH and corticosterone levels, but also activation in several brain regions believed to be linked to hypothalamo-pituitary-adrenocortical axis activation (Burow, Heidi, & Campeau 2005). Further, nearby construction work has a number of deleterious effects, such as delaying the emergence of a phenotype in the 5HT2C knockout mouse and decreasing food intake in control animals (Dallman *et al.*, 1999). Raff, Bruder, Cullinan, Ziegler & Cohen (2011) explored the effects of construction by measuring hormones related to stress in rats before, during, and after a nearby construction project. Although vibrations and noise levels were so low that the researchers could not detect them, ACTH and corticosterone levels almost doubled in

the rats tested during construction (Raff *et al.*, 2011). The possibility of these confounding stressors may have led to the variability in sexual receptivity, as chronic, increased levels of stress hormones have been shown to reduce sexual receptivity in mammals (Papargiris, Rivalland, Hemsworth, Morrissey, Tilbrook, 2011).

Animal housing is another stressor that may have contributed to the variable data seen here. Previously, mice in this lab were housed in shoebox style, wire-top cages. Following a parvovirus outbreak and subsequent sterilization of the lab, passively ventilated isolator lids were required by the university veterinarian. Many studies have shown the harmful effects of these static, covered cages. Cages with isolator lids but without active ventilation have poor moisture exchange, an increase in humidity, an increase in ammonia levels, and an increase in carbon dioxide levels (Lipman 1999; Corning & Lipman 1991). Increases in carbon dioxide inhalation have been shown to be proinflammatory, and may lead to inflammation of the lungs (Abolhassani, Guais, Chaumet-Riffaud, Sasco, & Schwartz 2009). Further, rats inoculated intranasally with *M. pulmonis* and then exposed to ammonia developed more severe symptoms of murine respiratory mycoplasmosis (Broderson, Lindsey, Crawford 1976). The possible development of a more aggressive immune reaction is particularly confounding in a study such as the one described here, which examines the effect of an immune stressor.

Future studies should not only work to prevent these types of confounds in stress research, but also in numerous areas of scientific research. The effects of uncontrolled stress can change brain organization, which, in turn, affects behavioral outputs. For example, studies involved in learning and memory may be negatively impacted by uncontrolled stressful variables. Stress leads to a rise in corticosterone levels, which

decreases density of cells in areas important for memory, such as the dentate gyrus of the hippocampus (Cameron & Gould 1994).

In this study, we failed to replicate the previous findings that peripubertal LPS administration leads to a decrease in adult responsiveness to ovarian hormones and a decrease in ER- α . However, ovariectomy prior to peripubertal LPS treatment did prevent this decrease in responsiveness to ovarian hormones, but only if the saline controls were removed from analysis. The failure of these experiments is most likely due to the confounding, uncontrolled factors explored above. Future research in the lab should focus on replicating previously observed effects by minimizing external variables and returning to earlier animal housing procedures, such as using regular shoebox style cages with wire tops.

Importantly, this work has many implications for women's healthcare. Although findings from research in female mice may not be generalized to women, it is the first step towards understanding and possibly treating or preventing the harmful effects of prolonged peripubertal stress.

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