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Estrogen- and experience- dependent Fos expression in locus coeruleus in adult female mice following exposure to pup calls

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Abstract

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A key aspect to studying maternal behavior is understanding the mechanisms governing a mother's heightened response to infant sensory cues when compared to non-mothers. Previous literature has focused on the motivational mechanisms behind this enhanced response, however sensory changes following parturition may contribute as well. In part, increased detection of infant cues by mothers evidently results from neural plasticity within a number of sensory systems following parturition. Two of the main factors that may contribute to heightened response to infant cues in mothers include experience with offspring and changes in the female's hormonal environment. Immediately before parturition, plasma estradiol levels reach a peak in mothers and may play a role not only in eliciting maternal behavior, but also in the processing of salient auditory stimuli. Estradiol may serve to prime neuromodulatory systems that facilitate brain plasticity including in the auditory system. This concept is of particular interest, as norepinephrine has been shown to play a role in both maternal behavior and acoustic sensitivity to salient stimuli. Here, we examine the effects of estradiol and maternal experience on Fos expression in the locus coeruleus, the sole source of norepinephrine to the forebrain, in adult virgin ovariectomized *CBA/CaJ* female mice following exposure to pup calls. We examined these effects using cohorts of animals with manipulated levels of maternal experience and estradiol levels. Following exposure to a pup call stimulus, we measured Fos protein expression in the locus coeruleus. Our results suggest that animals with elevated levels of estradiol and

maternal experience have greater Fos expression in the locus coeruleus following exposure to pup calls when compared with animals lacking estradiol but with maternal experience. Additionally, animals with maternal experience but lacking estradiol have less Fos expression in the locus coeruleus following exposure to pup calls when compared with animals lacking estradiol and without maternal experience. This study suggests that elevated levels of estradiol in animals with pup experience enhances the processing of salient infant auditory cues. Additionally, the findings suggest that pup calls may be salient, perhaps for different reasons, for both animals with pup experience as well as those without pup experience.

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Introduction

Maternal behavior arises following parturition and can be influenced by a number of different factors. Previous literature has focused on studying motherhood in the context of the motivational mechanisms governing maternal behavior. However, motherhood requires learning to provide care in response to infant cues and changes in how these cues are perceived by mothers could also support increased maternal responsiveness (Banerjee and Liu, 2013).

Evidence from a number of sensory systems suggests that neural plasticity occurs within those systems in the maternal context, resulting in increased detection of infant cues in mothers. Neural plasticity in ewes occurs in the olfactory bulb immediately following parturition and enables individual recognition of lambs (Kendrick et al., 1997). In addition, somatosensory cortical representation of tactile inputs involved in maternal behavior expands in mother rats (Xerri et al, 1994). This increased representation following parturition in mothers may be a consequence of maternal behavior. Finally, there is a growing body of evidence suggesting plasticity for infant cues in the auditory domain as well.

Across species, infants produce characteristic vocalizations that are particularly behaviorally relevant to mothers. Overall the neural detection and discrimination of infant vocalizations seems to significantly improve in mothers compared with virgin females. Mouse pups produce characteristic vocalizations near 65 kHz that occur in bouts separated by ~180 ms (Liu et al., 2003). These vocalizations are referred to as isolation calls and are emitted by pups when they are removed from the nest or exposed to temperature stress. These ultrasonic vocalizations often cause a mother to search for and retrieve the calling pup (Haack et al., 1983). A growing body of evidence suggests that there is increased detection of infant pup vocalizations

in the maternal auditory cortex. (Lin et al., 2013; Liu and Schreiner, 2007; Liu et al., 2006; Galindo-Leon et al., 2009). Plasticity in the auditory cortex is believed to enhance maternal detection of vocalizations (Galindo-Leon et al., 2009). These sensory changes, along with established changes in maternal motivation, may explain mothers' increased behavioral sensitivity to pup vocalizations. However, the mechanisms that enable maternal sensory plasticity are not well understood.

Molecular mechanisms can enable neural plasticity and these mechanisms are often examined through studying the expression of immediate early genes (IEGs). IEGs are genes whose transcription is rapidly induced by extracellular stimuli. In neurons, transcription and translation of IEGs often follows membrane depolarization or action potential firing, which results from receptor activation and an increase in intracellular calcium levels (Sheng and Greenberg, 1990). Thus, IEG induction is frequently used as a marker of neural activity. IEGs can both directly and indirectly influence cellular function. In terms of directly affecting cellular function, some IEGs can encode for "effector" proteins such as Arc, which is involved in mediating cytoskeletal changes underlying neuronal plasticity (Lyford et al., 1995). Other IEGs indirectly influence cellular function by encoding for proteins that serve as transcriptionally activated transcription factors, meaning that IEG induction leads to the transcription of transcription factors that can only regulate gene expression once they are translated (Hughes and Dragunow, 1995). Once these IEGs are translated, their products are thought to activate late-response genes that result in further gene activity. Overall, IEGs play an important role in the neuronal signal transduction pathway from receptor to genetic response. Here, we use expression of *cfos,* an IEG, as a marker of neuronal response. *Cfos* is one of the most widely studied IEGs. Like many others, *cfos* encodes transcription factors that target many other genes

(Cirelli and Tononi, 2000). *Cfos* induction is generally associated with membrane depolarization and changes in the temporal firing pattern of a neuron (Cirelli and Tononi, 2000). Note, however, that it is currently not known whether *cfos* induction necessarily indicates action potential firing (Cirelli and Tononi, 2000). Therefore, the terms "*cfos* induction" or "Fos expression" is used in this study to indicate cellular genomic activity as well as neuronal response without necessarily indicating spiking activity. In this study we use Fos expression to study the effects of estrogen on the processing of pup isolation calls.

A mother's hormonal state directly following parturition plays a role in her enhanced behavioral response to pups. Plasma levels of estradiol (E2), a form of estrogen, peak in mothers immediately before birth (Barkley et al., 1979) and this change in hormone levels immediately before parturition suggests that E2 may be involved in maternal care. E2 in pup-sensitized virgin female mice has been shown to facilitate retrieval behavior (Koch and Ehret, 1989), which is a characteristic maternal behavior in which mothers retrieve their pups and return them to the nest upon hearing their ultrasonic calls (Ehret and Haack, 1982). This finding provides a basis for further studying estrogen's role in acoustic recognition of infant cues. This phenomenon may be related to E2's known role in the processing of salient acoustic stimuli. In non-human models, estrogen has been found to play a role in the processing of behaviorally relevant stimuli, particularly in the auditory forebrain. In female white-throated sparrows, for instance, playback of conspecific song elicited an increase in expression of ZENK, an IEG, in the auditory forebrain of E2-treated birds when compared to ZENK expression in the auditory forebrain of E2-treated birds played frequency-matched tones. In contrast, there was no difference in auditory forebrain ZENK-expression following conspecific song and frequency-matched tones in songbirds with low levels of plasma E2 (Maney et al., 2006). The IEG expression the auditory forebrain

following exposure to salient stimuli indicates that E2 may play a role in the auditory processing of behaviorally relevant stimuli.

The effects of estradiol on auditory processing of salient stimuli may occur by its direct influence on the auditory system. Systemic injection of E2 in adult male Australian zebra finches results in an increase in local E2 levels in the songbird analog of the auditory cortex. In addition, administration of a pharmacological inhibitor of aromatase, the enzyme that converts androgens into estrogen, results in a local decrease of E2 levels (Remage-Healey et al., 2008). These findings suggest that E2 can act locally on the auditory system. Furthermore, upon hearing male song, adult males showed an acute increase in E2 levels. This finding leads to the understanding that not only is E2 concentrated in the auditory cortex, but it also may be involved in the processing of behaviorally relevant auditory stimuli. Additional studies support this finding in showing that acute inhibition of estrogen production in the songbird analog of the auditory cortex suppresses male songbird behavioral response to the bird's own song (Remage-Healey et al., 2010). Taken together these findings suggest that acute regulation of E2 in the auditory system may be involved in the processing of social stimuli.

Other lines of research have indicated that another possible mechanism underlying the sensory influences on maternal behavior is driven by estrogen's influence on neuromodulatory systems (Miranda and Liu, 2009). Neuromodulatory neurotransmitters involved in the arousal system, including acetylcholine, norepinephrine, and histamine, facilitate sensory cortical plasticity (Gu, 2002). Norepinephrine, specifically, has been shown to play a critical role in maternal behavior. Mothers lacking the gene for dopamine beta-hydroxylase, the enzyme responsible for converting dopamine into norepinephrine, appear to lack maternal responsiveness, to the extent that their pups die shortly after birth (Thomas and Palmiter, 1997).

The most prominent source of norepinephrine in the brain comes from the brainstem structure locus coeruleus (LC). The LC is heavily involved in modulating neuronal circuits required in alert waking states and extensively innervates sensory networks, including the central auditory system. One essential role of the LC-noradrenergic system is to modulate the processing of salient sensory information through acting on sensory, attentional, and memory processes (Berridge and Waterhouse, 2003). LC neurons fire in a tonic mode, during normal waking, as well as a phasic mode, when presented with an arousing or salient stimulus (Carter et al., 2010). Although it is currently unknown if c*fos* induction in the LC is associated with tonic or phasic firing, studies suggest that Fos expression in the cerebral cortex is associated with the presentation of salient stimuli, that normally elicit phasic firing in the LC (Anokhil et al, 1995; Zhu et al, 1995, Radulovic et al, 1998). In addition, Fos can target the gene for tyrosine hydroxylase, suggesting its involvement in TH synthesis and possibly the production of norepinephrine (Gizang-Ginsberg and Ziff, 1990). Thus, increased *cfos* expression may indicate an increase in norepinephrine production and release in noradrenergic neurons in response to arousing stimuli. The framework for this study is that hearing a salient stimulus arouses an animal by an increase in LC phasic firing which can be observed by an increase in Fos expression in the LC when compared to expression during tonic firing.

The noradrenergic neurons of the LC have been shown to play a role in the processing of salient acoustic stimuli. Adult female zebra finches exposed to conspecific male songs showed a greater number of catecholaminergic cells expressing the immediate early gene ZENK in the LC when compared with silence-exposed females (Lynch et al., 2012). This result suggests that the noradrenergic cells of the LC may be involved in an animal's behavioral or arousal response to conspecific song. As previously discussed, norepinephrine, specifically arising from the

noradrenergic neurons of the LC, and E2 have been shown to be involved in the processing of both presumably salient stimuli and maternal behavior.

E2 may modulate the activity of noradrenergic LC neurons and this modulation may play a role in the processing of salient stimuli. In rats LC neurons have been shown to concentrate E2 (Heritage et al., 1980) as well as express mRNA for estrogen receptors (Shughrue et al., 1997). These findings suggest that estrogen may be able to act directly on the cells in the LC and influence their neuronal activity. Additionally, E2 affects the production norepinephrine in the LC. Tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) are two enzymes required for norepinephrine biosynthesis. TH is the rate-limiting enzyme in the production of norepinephrine and therefore the abundance of this enzyme in the LC can be used as an indication of norepinephrine production (Spector et al., 1965; Levitt et al., 1965). There is an E2 dose-dependent increase in TH and DBH mRNA expression in the rat LC, indicating that that estrogen may modulate norepinephrine production in LC neurons (Serova et al, 2002). Further, there is evidence that E2's influence on the activity of the LC may aid in the processing of acoustic stimuli. In adult female white-throated sparrows, E2 has been shown to modulate catecholaminergic innervation of the songbird analog of the auditory cortex (Matragrano et al., 2011). Treating nonbreeding female songbirds with E2 to increase plasma levels to those typical of the breeding season leads to an increase in catecholaminergic fiber density in areas of the auditory system involved in processing of song. This finding suggests that E2 not only modulates catecholaminergic projections to the auditory system and therefore may be involved in the processing of acoustic stimuli.

Maternal processing of salient infant cues may not only be influenced by hormonal changes brought about by motherhood, but may also be influenced by the experiential changes

following birth. Ovariectomized virgin female mice with five days of maternal experience show significantly more maternal behavior, quantified by pup retrieval, when compared with ovariectomized virgin females without maternal experience. These findings suggest that maternal experience, even in the absence of ovarian hormones, leads to increased maternal behavior. However, ovariectomized virgin female mice with both pup-experience and experimentally elevated levels of E2 show the highest percent of retrievals (Koch and Ehret, 1989). The interaction between maternal hormones and behavioral experience may enhance maternal response to acoustic pup stimuli.

Here, we investigated the effect of hormonal state and pup experience on Fos expression in the LC following exposure to a pup call stimulus. Because E2 plays a role in maternal behavior and the processing of salient stimuli, we are interested in studying the role of E2 on the arousal response to a pup call stimulus. In order to study this effect, we treated adult female mice with E2 or vehicle implants. Additionally, as previously discussed, maternal experience in virgin female mice leads to an increase in maternal behavior. Due to this finding, we are interested in observing the role that maternal experience has in influencing the arousal response to a pup call. In order to study the effect of pup experience on the LC response to pup isolation calls, half of the animals in our study were given pup experience while the other half were not given any pup experience. Our predictions for this experiment is two-fold. We predict that E2 will increase the number of noradrenergic LC neurons expressing Fos in response to an acoustic pup-call stimulus. We predict that this increased Fos expression may be due to the established link between E2 and recognition of stimulus. In addition, we predict that upon hearing pup calls, females with maternal experience, perhaps due to increased arousal upon hearing a behaviorally

relevant stimulus, will have more noradrenergic LC neurons expressing Fos when compared to those females without maternal experience.

Materials and Methods

Animals

All procedures were approved by the Emory University Institutional Animal Care and Use Committee. Experiments were performed on adult virgin female *CBA/CaJ* mice. Animals were weaned at 21 days and were placed in single-sex housing with two to four animals per cage under a reverse-light cycle (14 hours of light/ 10 hours of dark) and had access to food and water *ad libitum*. Females were between 12-14 weeks of age at the time of pup-call sound exposure.

Hormonal Manipulation

At 10.6 ± 0.57 weeks postnatal, animals were ovariectomized and implanted with a subcutaneous capsule (2 mm Silastic tubing, sealed with silicone aquarium sealant) containing either sesame oil (n = 22) or E2 benzoate (50 µl at 3 mg/ml; n = 19) dissolved in sesame oil. The E2 implant concentration was chosen because two weeks after implantation, animals treated with this dose have plasma E2 levels similar to those in mothers immediately before parturition. Prior to implantation, all capsules were soaked in 0.9% saline solution and sterilized using hydrogen gas sterilization for 29 minutes. After the surgery animals were individually housed.

Experiential Manipulation

Vehicle and E2-implanted animals were assigned to one of two experiential groups: naive or co-carer (half of each experiential group had an E2 implant and half had a vehicle implant) (Figure 1). Naïve animals were not given any experience with pups. At 10 ± 4 days after the ovariectomy and implantation surgery, each co-carer animal was removed from her cage and

placed in a cage with a mother due to give birth in 5 days. The co-carers were removed five or six days after the dam gave birth and were isolated in a new cage overnight prior to sound exposure the following day (Figure 2).

Stimulus Recording

The pup call stimulus was a 10 minute recording of natural CBA/CaJ pup isolation calls. The pup isolation calls were induced by removing a pup from its nest and placing it in an empty cage. One-minute samples from recordings of 10 different pups were combined into a 10 minute audio file and high-pass filtered above 25 kHz to attenuate low frequency noise. The background noise stimulus consisted of 10 minute-long segments from the pup isolation recordings that were clipped to exclude any pup vocalizations.

Stimulus Presentation

On the following day (pups at postnatal day six or seven) the co-carer and naïve mice were placed in separate sound attenuated chambers equipped with a speaker. All experimentation took place during the dark phase of the light cycle and occurred in red light. After an acclimation period of 4 hours, each animal was either played a 10 minute pup call stimulus ("stimulus-exposed group"; n= 23) or a 10 minute background noise stimulus ("background-exposed group"; n= 18). A bat detector was used to ensure the successful playback of the pup isolation calls. All animals remained in their respective sound-attenuating chambers for 90 minutes following the onset of the stimulus to allow for immediate early gene induction and expression. Following the 80 minutes of silence, animals were euthanized using carbon dioxide and transcardially perfused with KPBS and 4% paraformaldehyde.

Immunohistochemistry

Brains were removed from the skulls and post-fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose, and stored at 4°C until 50-µm coronal floating sections containing the LC were cut, and then sections were stored in cryoprotectant at -20°C. Double-label immunohistochemistry using both Fos antibody and TH antibody was conducted on every other section. The double-label procedure was carried out as follows: free-floating sections were washed in PBS, incubated for 15 minutes in 0.1% sodium borohydride, washed again in PBS, and rinsed for 30 minutes in 30% H_2O_2. Sections were then washed in PBST (PBS with 0.3% Triton-X 100) and blocked using 20% normal goat serum (NGS) in PBST for 1 hour. Sections were then transferred to Fos primary antibody (polyclonal *fos* antibody raised in rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. No. sc-52) at a dilution of 1:2,000 in PBSTN (0.3% triton, 2% NGS) and stored at 4°C for 2 days. After 2 days of incubation with Fos antibody, sections were rinsed in PBSTN and incubated for 1 h in biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA; cat. No.BA-1000) diluted to 1:250 in PBSTN. Sections were then washed in PBST and incubated in an ABC solution diluted to 1:200 (Vector Laboratories, Burlingame, CA, USA; cat. No. PK-6100) for 1 h. Sections were then washed in PBS, rinsed in acetate buffer, and protein expression was visualized using nickel-enhanced diaminobenzidine. Sections were then washed in acetate buffer and PB before being rinsed in 30% H2O2 for 20 minutes. Sections were then rinsed in PBTN and transferred to TH antibody (Immunostar, Hudson, WI, USA; cat. No. 22941) at a dilution of 1:1,000 in PBTN and stored at 4°C for 2 days. After 2 days of incubation with TH antibody, sections were rinsed in PBTN and incubated for 1 h in biotinylated goat anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA; cat. NO. BA-9200) diluted to

1:250 in PBTN. Sections were then washed in PBT and incubated in an ABC solution for 1 h. Following ABC incubation, sections were washed in PB and protein expression was visualized using diaminobenzidine. Sections were then washed and stored in PB. Sections were then mounted and coverslipped.

Quantification

Using the 40x objective of a (Zeiss Axioplan) light microscope, Fos-expressing TH-ir cells were quantified in the LC by an observer blind to treatment condition. TH staining clearly delineated the LC (Figure 3a) at approximately 5.34 mm to 5.80 mm caudal to Bregma. TH staining was concentrated in cell bodies (Figure 3b) and Fos staining was concentrated in cell nuclei (Figure 3c). TH-ir cells expressing Fos were identified as a TH-ir stained cell body surrounding a Fos-positive stained nucleus (Figure 3d). Protein expression in 7 ± 2 sections was quantified per animal starting with the most rostral section containing the LC. Using a camera connected to the microscope, images of the LC were taken with a 40x objective in the imaging program, MagniFire. The density of TH-staining precluded the quantification of individual TH-stained cell bodies, therefore the area covered by TH-immunoreactivity (TH-ir) cells in each section was quantified using NIH Image J. For each section the TH staining was manually color thresholded to include all TH-expressing cells. Using the color threshold, the area of TH-ir was measured in square pixels (Figure 4). Using a conversion factor of 9193024 square pixels to one square millimeter at this magnification, the area was converted into square millimeters. The number of Fos-positive TH-ir cells, which were clearly discernible as niDAB-labeled nuclei, was expressed as a proportion of this area.

Statistical Analysis

In order to test for effects of experiential and hormonal state on TH-ir area in the LC, the average area of TH-ir per section in each animal was calculated. The average area of TH-ir per section across the four animal groups (Vehicle-implanted naïve animals, E2-implanted naïve animals, vehicle-implanted co-carer animals, and E2-implanted co-carer animals) was analyzed by 2 x 2 ANOVA with hormone treatment and experiential treatment (co-care or naïve) as factors. The average of the area of TH-ir per section in each animal was quantified as opposed to the sum of the area of TH-ir in each animal because for each animal only half of the sections in the LC were quantified and due to technical discrepancies, the sections quantified for each animal were not necessarily at the same locations in the LC. To ensure that Fos expression was uniform across the LC and justify the quantification of Fos expression as the number of TH-ir cells expressing Fos per square millimeter of TH-ir, we tested for a rostro-caudal effect of Fos expression. The average number of TH-ir cells expressing Fos per square millimeter of TH-ir in the rostral half of the LC and the caudal half of the LC for each animal was calculated. A rostro-caudal effect was analyzed by five 2 x 2 ANOVAs with the following as factors: hormone treatment and rostral/caudal location (in pup-call stimulus exposed animals), experiential treatment and rostral/caudal location (in pup-call stimulus exposed animals), hormone treatment and rostral/caudal location (in background noise stimulus exposed animals), experiential treatment and rostral/caudal location (in background noise stimulus exposed animals), and stimulus treatment (pup-call stimulus or background noise stimulus) and rostral/caudal location. A t-test was used to compare the number of TH-ir cells expressing Fos per square millimeter of TH-ir in background-noise stimulus exposed animals and pup-call stimulus exposed animals. For each type of auditory stimulus (pup isolation call exposure and background-noise exposure) the number of TH-ir cells expressing Fos per square millimeter of TH-ir was analyzed by 2x2

ANOVA with hormone treatment and experiential treatment as factors. Where an interaction was significant, planned comparison t-tests were performed to test for an effect of hormonal treatment within each experiential treatment group and vice versa.

Results

Effects of E2 treatment and pup experience on TH-ir area

Animals were implanted with either an E2 implant or a sesame oil vehicle implant and assigned to either be given pup experience (co-carers) or to remain pup-naïve (naives). While our primary interest was in the proportion of TH-ir cells that underwent *cfos* induction following acoustic stimulation, we wanted to first assess whether any of our manipulations impacted the area of TH-ir cells in the LC. The effects of hormone treatment and experiential treatment on TH-ir area in the LC are plotted in Figure 5**.** A 2 x 2 ANOVA revealed no significant main effect of either hormone treatment ($F_{1,34}$=2.79, p=0.104) or experiential treatment ($F_{1,34}$=0.26, p=0.6137) on the TH-ir area in the LC per section. In addition, there was no significant interaction between the two factors ($F_{1,34}$=0, p=0.9933). Therefore, we were unable to detect an effect of E2 treatment or experiential treatment on the area of TH-ir in the LC across animals.

Effects of E2 treatment and pup experience on baseline LC Fos expression

After being assigned to one of four hormonal/experiential treatment groups (Vehicle-implanted naïve animals, E2-implanted naïve animals, vehicle-implanted co-carer animals, E2-implanted co-carer animals) animals were assigned to be exposed to either a background noise stimulus or a pup-call stimulus. There was no rostro-caudal main effect of TH-ir cells expressing Fos per square millimeter of TH-ir in the LC (Table 1). In addition, there was no significant interaction between position (rostral or caudal) and any of the three between-subject factors

(Table 2). This finding suggests that Fos expression in TH-ir cells per square millimeter of TH-ir was uniform across the LC. This uniformity justifies our metric of TH-ir cells expressing Fos per square millimeter of TH-ir per section for each animal. In the animals exposed to the background-noise stimulus here was no significant main effect of either hormone treatment ($F_{1,14}$=0.11, p=0.7413) or experiential treatment ($F_{1,14}$=0.07, p=0.7987) on the number TH-ir cells expressing Fos per square millimeter of TH-ir in the LC (Figure 6). In addition, there was no significant interaction between the two factors ($F_{1,14}$=1.33, p=0.2682). These results suggest that regardless of hormonal and experiential treatment, the baseline fos-expression in the TH-ir cells of the LC were similar across groups.

Effects of E2 treatment and pup experience on LC Fos expression following stimulus exposure

In addition to the finding that Fos-expression across groups in response to the background noise stimulus was similar, we also found a significant increase in TH-ir cells expressing Fos per square millimeter of TH-ir in the LC of animals exposed to a pup-call stimulus when compared to animals exposed to a silence stimulus (p=0.0001). When taken together, these two results allowed us to justifiably compare the number of TH-ir cells expressing Fos per mm^2 of TH-ir in LC following pup call exposure between treatment groups without normalizing data to baseline levels of Fos expression. The effects of hormone treatment and experiential treatment on the number of TH-ir cells expressing Fos per mm^2 of TH-ir in LC following pup call stimulus exposure are plotted in Figure 7. A 2 x 2 ANOVA revealed no main effect of hormonal treatment ($F_{1,19}$=0.0242, p=0.8780) or experiential treatment ($F_{1,19}$=0.3486, p=0.5618) on the number TH-ir cells expressing Fos per mm^2 of TH-ir in LC following pup call stimulus exposure. However the interaction between factors was significant ($F_{1,19}$=6.6898, p=0.0181). A planned comparison t-test did not show an effect of hormone treatment on the

number of TH-ir cells expressing Fos per square millimeter of TH-ir in the LC in naïve animals ($p= 0.218$). In addition, a t-test did not show an effect of experiential treatment on the number of TH-ir cells expressing Fos per square millimeter of TH-ir in the LC in E2-treated animals ($p= 0.2550$). A t-test showed an effect of experiential treatment on the number of TH-ir cells expressing Fos per square millimeter of TH-ir in the LC in vehicle-treated animals ($p= 0.0457$). A t-test showed an effect of hormone treatment on the number of TH-ir cells expressing Fos per square millimeter of TH-ir in the LC of co-carer animals ($p= 0.02$). Whereas baseline responses to background noise did not differ with hormonal manipulation or pup experience, *cfos* responses to the pup call stimulus, which was relevant to co-carer groups, were modulated by experience and hormonal condition.

Discussion

In this study we show that both E2 and maternal experience affect Fos expression in the noradrenergic LC neurons in response to infant vocalizations. We found that in the LC, Fos expression between treatment groups exposed to the background noise stimulus is similar. However, our findings suggest that pup-call stimulus-induced *cfos* responses in the LC are dependent on the hormonal treatment and maternal experience of an animal.

We found a significant increase in Fos expression of E2-treated co-carer animals when compared with vehicle-treated co-carer animals. These females, to whom the pup call stimulus is relevant, show greater Fos expression in the LC when E2 is present. Consistent with our hypothesis, this finding suggests that elevated E2 in co-carer animals leads to an increase in the neuronal response of LC neurons following exposure to pup calls. It is possible that in the E2 implanted co-carer animals, E2 acts to maintain the salience of the pup isolation calls and facilitates firing of LC neurons in the phasic mode. If, as previously stated, Fos expression is

associated with states in which LC neurons are firing in the phasic mode, Fos expression can be used as an indicator of a burst in neural activity in the LC.

Contradictory to our original hypothesis, E2 did not modulate the naïve response to the pup call stimulus. While the difference in Fos expression between E2-implanted naïve animals and vehicle-implanted naïve animals was not significant, graphical representation (Figure 7) suggests that naïve animals exhibited a trend that appeared opposite to that seen in co-carers. Specifically, E2 appeared to blunt the *cfos* response to pup calls in naïve animals when compared to vehicle-treated animals. This apparent disparity, although not statistically significant, may be caused by different pathways leading to fos induction in the naïve and co-carer groups. In addition, experiential treatment did seem to modulate the Fos expression in vehicle-implanted animals, leading to the finding that vehicle implanted co-carer animals had significantly less Fos expression than vehicle-implanted naïve animals. Although LC response to the pup call stimulus in co-carers may be due to its behavioral relevance resulting from their prior experience with it, the naïve LC may be activated by presentation of this unexpected stimulus. Indeed, the LC is known to be activated by both salient (Sara and Segal, 1991) and unexpected stimuli (Dayan and Yu, 2006). E2 may not appear to lead to an elevated level of Fos expression in naïve animals, whereas it does in co-carer animals, if the pathways projecting to the LC involved in processing unexpected versus salient stimuli are different, and are modulated differently by E2. However, whether unexpected and salient stimuli indeed activate different processing streams upstream of the LC, and why E2 would oppositely affect LC response in these two scenarios is unknown. The differences in the effect of E2 on LC response in naïve animals and co-coarers need to further studied by increasing the sample size. Explanations behind any differences in averages must be interpreted with caution because the only significant differences in Fos expression between

groups was found between E2-implanted co-carer animals and vehicle-implanted co-carer animals as well as vehicle-implanted naïve animals and vehicle-implanted co-carer animals. It is interesting to note, however, that pup call exposure in E2-implanted co-carers results in a *cfos* response that seems similar in magnitude to that seen in vehicle-treated naïve animals. Thus, even while the pup call stimulus may not be unexpected in the co-carer group, it leads to a neural response that suggests the same salience as an unexpected stimulus in those animals with an E2 implant. Therefore, E2 may play a role in maintaining the salience of important stimuli after the repetitive presentation of the salient stimulus in the environment could lead to habituation.

Once the *cfos* gene is transcribed and translated it may serve as a transcription factor for other genes. Once such gene, as previously stated, is the gene responsible for producing tyrosine hydroxylase (Gizang-Ginsberg and Ziff, 1990; Icard-Liepkalns et al., 1992). The increased expression of Fos protein in the LC may imply the increased production of TH, and therefore norepinephrine. Rat LC neurons have been shown to fire upon presentation of salient stimuli (Sara and Segal, 1991). Taken together, increased cell firing as well as a potential increase in norepinephrine production in the LC following the presentation of salient stimuli may lead to enhanced norepinephrine release from the LC. Enhanced norepinephrine release in the auditory cortex has been associated with altering frequency selectivity in auditory cortical neurons. Pharmacological enhancement of the noradrenergic system, leading to an increase in extracellular norepinephrine, increases frequency selectivity in the rat auditory cortex (Edeline, 1995). In addition, paired norepinephrine administration with tones has led to frequency-selective modifications in tuning curves (Manunta and Edeline, 2004). Together these findings suggest that a possible increased production of norepinephrine in E2 implanted co-carers may lead to an increase in frequency selectivity in the auditory cortex. Due to prior exposure to pups,

auditory cortical neurons in these animals may be selectively tuned to frequencies associated with pup calls. It is possible that E2 acts to prime noradrenergic projections to the auditory cortex leading to frequency selectivity for familiar stimuli.

If elevated levels of E2 in co-carer animals do indeed lead to an increased recognition of salient stimuli, in part due to increased release of NE in response to behaviorally relevant sounds, the histological results of this study are in agreement with the findings of previous behavioral studies. Others have shown an increase in maternal behavior following exposure to salient acoustic stimuli in mothers when compared to virgins with 5 days of pup experience (Geissler et al., 2013). In our study, co-carer animals implanted with E2 have a hormonal and experiential state similar to mothers, so the effects we observed may mirror those that occur in mothers during the natural course of parturition and pup rearing. Additionally, the experiential state is of our vehicle-implanted co-carer animals is identical to virgins with 5 days of pup exposure and, like virgin females, they presumably have lower levels of plasma E2 than mothers. When taking into this study, our E2-implanted co-carer group may show an increase in maternal behavior following exposure to salient infant vocalizations when compared with our vehicle-implanted co-carer group. Our results showed that in response to pup calls, co-carer animals with elevated levels of E2 showed increased Fos expression in the LC when compared to vehicle-implanted co-carer animals. This increased neuronal response in the LC of E2-implanted co-carer animals may have led to increased recognition of pup calls. An improved recognition of acoustic infant cues would seemingly result in an increase in maternal behavior.

Additional Analysis

In order to make the findings of this study more robust a few additional analyses need to be conducted. Although the 2 x 2 ANOVA analyzing the effect of hormonal and experiential

treatment on Fos expression following pup-call stimulus exposure yielded a significant interaction, Fos expression differed only between co-carer vehicle-implanted and co-carer E2-implanted animals as well as vehicle-implanted naïve animals and vehicle-implanted co-carer animals. Graphical representation of this data showed non-significant trends between other treatment groups. In order to further analyze these trends the sample size in all treatment groups needs to be increased. In addition, behavioral data has been collected in the form of videos of about half of the animals during the ten minute sound stimulation. These videos can be scored to note movement patterns of animals during the stimulus exposure. An increase in movement may indicate an increase in arousal or agitation. We hypothesize that those animal groups with an increase in Fos expression following the pup-call stimulus exposure will also have an increase in movement during the stimulus presentation when compared to animals with low levels of Fos expression. Lastly, serum samples were taken from a number of animals and these samples need to be analyzed in order to confirm that those animals with E2 implants do indeed have significantly greater levels of E2 than vehicle-implanted animals.

Future Studies

In order to elucidate the role of E2 in maternal processing of salient acoustic stimuli, we suggest a number of future experiments. The current experiment should be repeated with the addition of a group of animals that are exposed to a neutral tone. Results of this experiment will help determine whether the Fos activation observed in this experiment was due to the salience of the conspecific stimulus or simply due to the presentation of any arousing auditory stimulus. Co-carer animals are given experience caring for pups and through this experience they also become accustomed to hearing pup calls. Future studies should further explore the degree to which Fos expression in the LC following pup-call stimulus exposure is due to the novel context of the

stimulus. The results of this study would elucidate whether the increased Fos expression in co-carers following pup call exposure was due to the familiarity with the stimulus (or pup-calls in general) or the previous maternal experience. Lastly, future studies should focus on Fos expression in the auditory cortex. In addition to quantifying Fos expression in the auditory cortex, such future studies should immunohistologically stain for DBH expression in order to test whether neural activity in the auditory cortex is occurring in those neurons synaptically connected to noradrenergic projections. The results of this future study would be important in understanding whether neural activity in the LC following pup call exposure affects auditory cortical processing of salient stimuli.

Table 1.

Rostro-caudal (rostral/caudal location) main effect on number of TH-ir cells expressing Fos per square millimeter of TH-ir in the LC. Factor 1 and Factor 2 are the two factors, as defined in the text, which were compared in a 2 X 2 ANOVA.

	Factor 1	Factor 2	F statistic	P-value
	Stimulus treatment	Rostral/caudal location	$F_{1,64}$=0.7146	0.4011
Pup-call Exposed animals	Hormonal treatment	Rostral/caudal location	$F_{1,30}$=0.4954	0.4870
	Experiential treatment	Rostral/caudal location	$F_{1,30}$=1.3139	0.2608
Background-noise Exposed animals	Hormonal treatment	Rostral/caudal location	$F_{1,30}$=0.2332	0.6326
	Experiential treatment	Rostral/caudal location	$F_{1,30}$=0.1460	0.7050

Table 2.

Rostral/caudal location interaction with the three between-subject factors (stimulus treatment, hormonal treatment, and experiential treatment). Factor 1 and Factor 2 are the two factors, as defined in the text, which were compared in a 2 X 2 ANOVA.

	Factor 1	Factor 2	F statistic	P-value
	Stimulus treatment	Rostral/caudal location	$F_{1,64}$=0.1433	0.7062
Pup-call Exposure	Hormonal treatment	Rostral/caudal location	$F_{1,30}$=0.8869	0.3538
	Experiential treatment	Rostral/caudal location	$F_{1,30}$=0.0301	0.8635
Background-noise Exposure	Hormonal treatment	Rostral/caudal location	$F_{1,30}$=1.9431	0.1736
	Experiential treatment	Rostral/caudal location	$F_{1,30}$=0.0115	0.9155

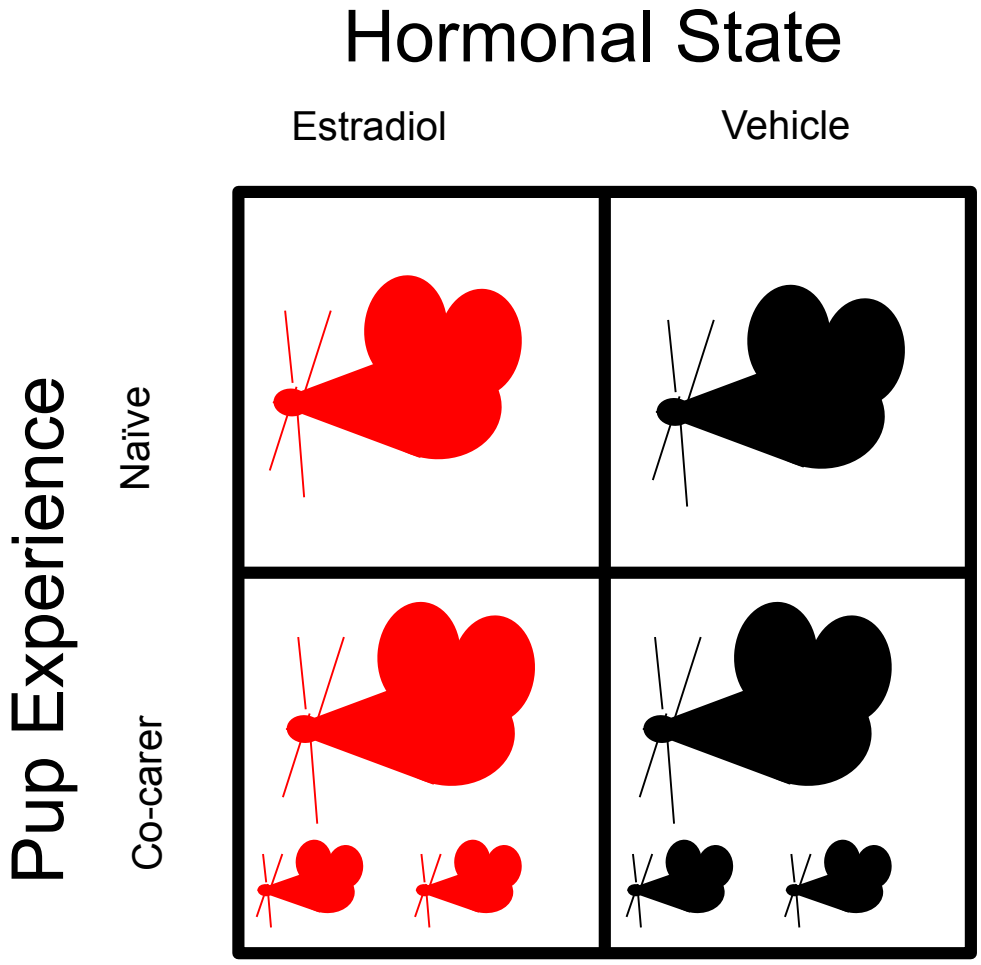

Figure 1.

Schematic of the four experimental groups used in this study. Columns represent hormonal treatment (red= E2 implant, black= vehicle implant). Rows represent pup experience.

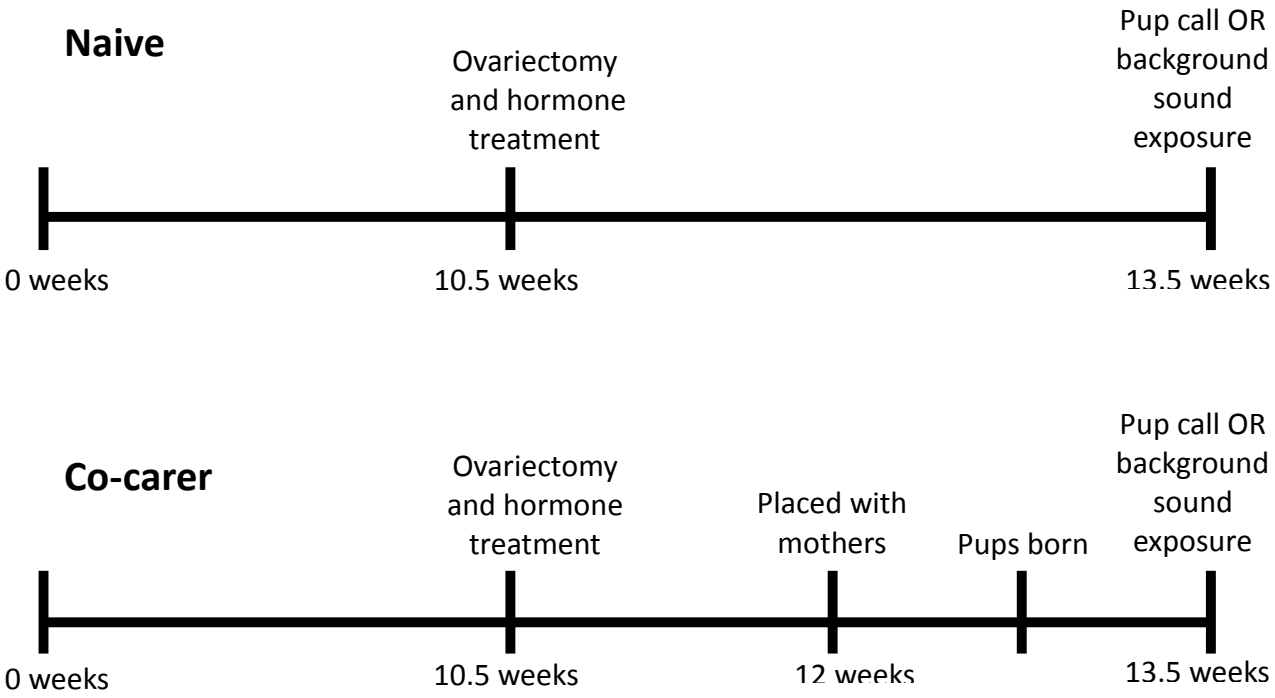

Figure 2.

Timeline of hormonal, experiential, and sound stimulus treatments of animals.

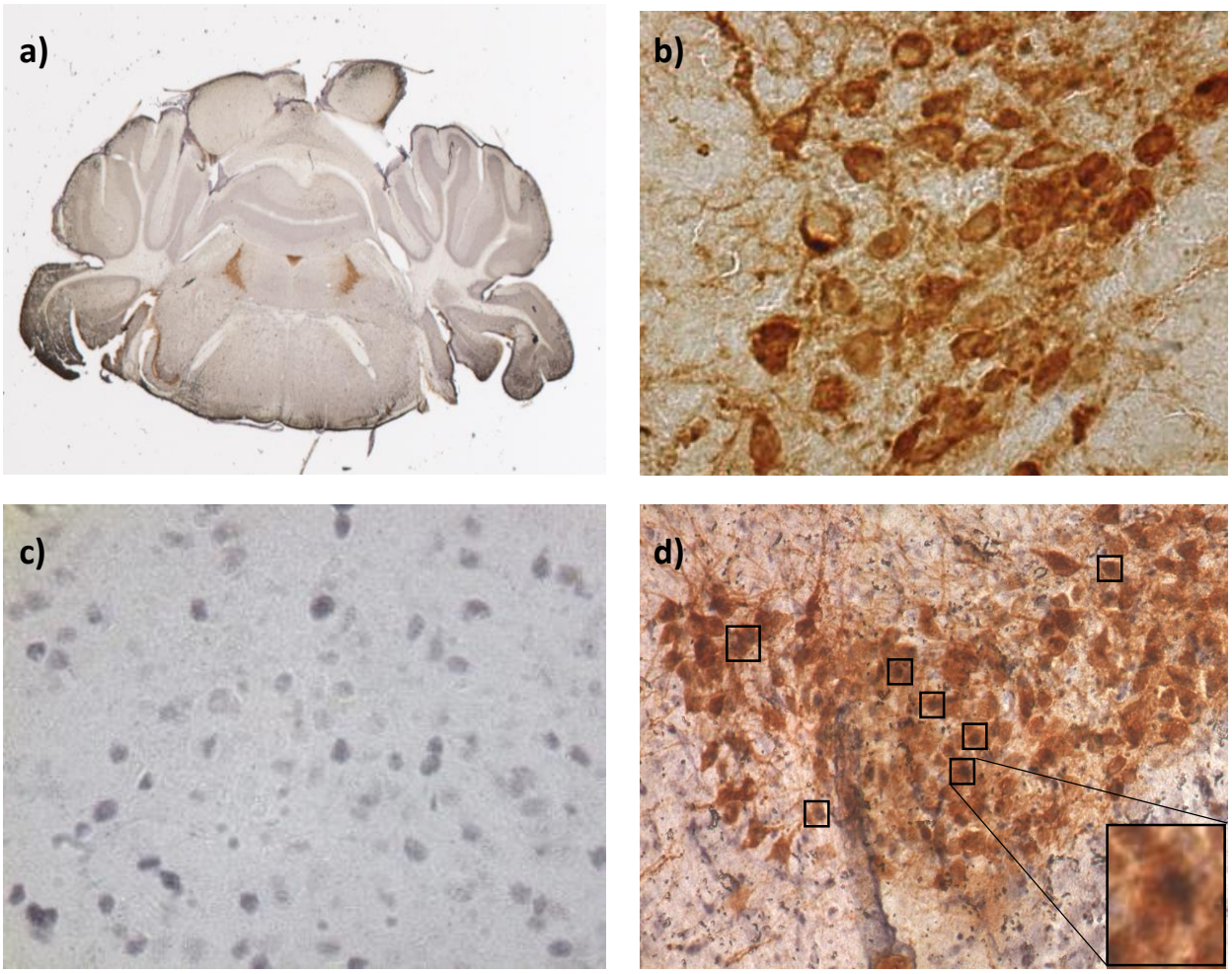

Figure 3 (a) Whole-section image showing defined TH staining in the LC, (b) TH staining in the cell bodies of LC neurons, (c) Fos staining in the nuclei of neurons, (d) TH-Fos costained cells in the LC (boxes denote TH-Fos costained cells).

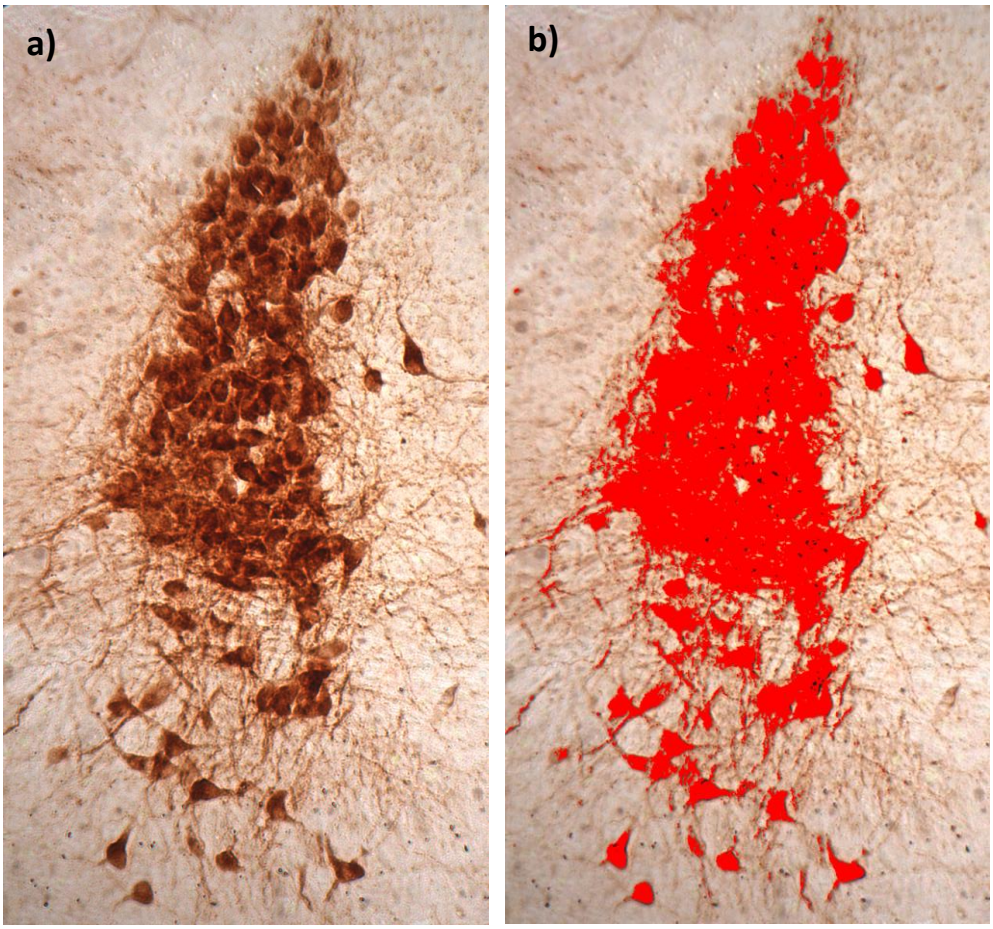

Figure 4 (a) TH stained cells in the LC, (b) Color thresholded image used to measure the area of TH-ir in the LC.

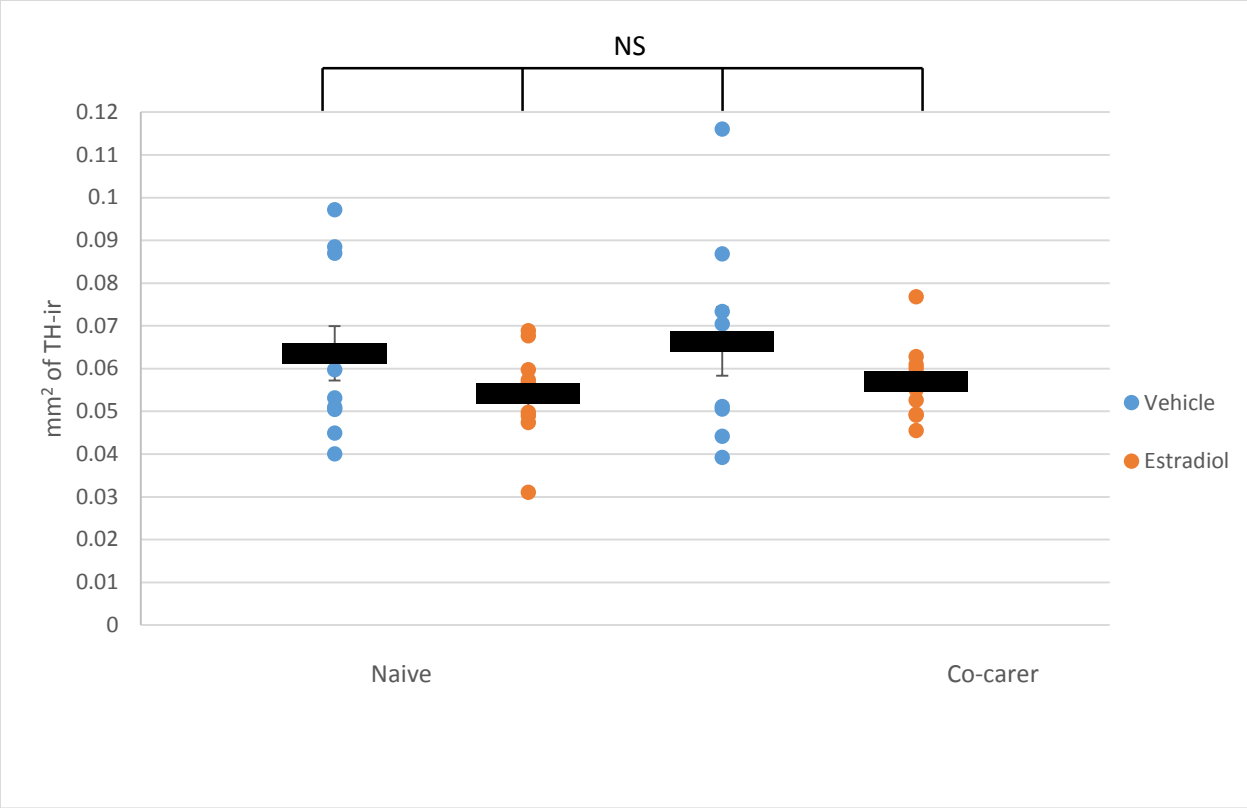

Figure 5.

Side-by-side dot plot of the area covered by TH-ir per section for each hormonal and experiential treatment. Each point represents the average area of TH-ir per section for an individual animal. Black bars represent the average for each group. There was no significant difference in the area covered by TH-ir per section between groups (2 x 2 ANOVA, main effect of hormone treatment $F_{1,34}$=2.79, p=0.104, main effect of experiential treatment $F_{1,34}$=0.26, p=0.6137, interaction between the two factors $F_{1,34}$=0, p=0.9933).

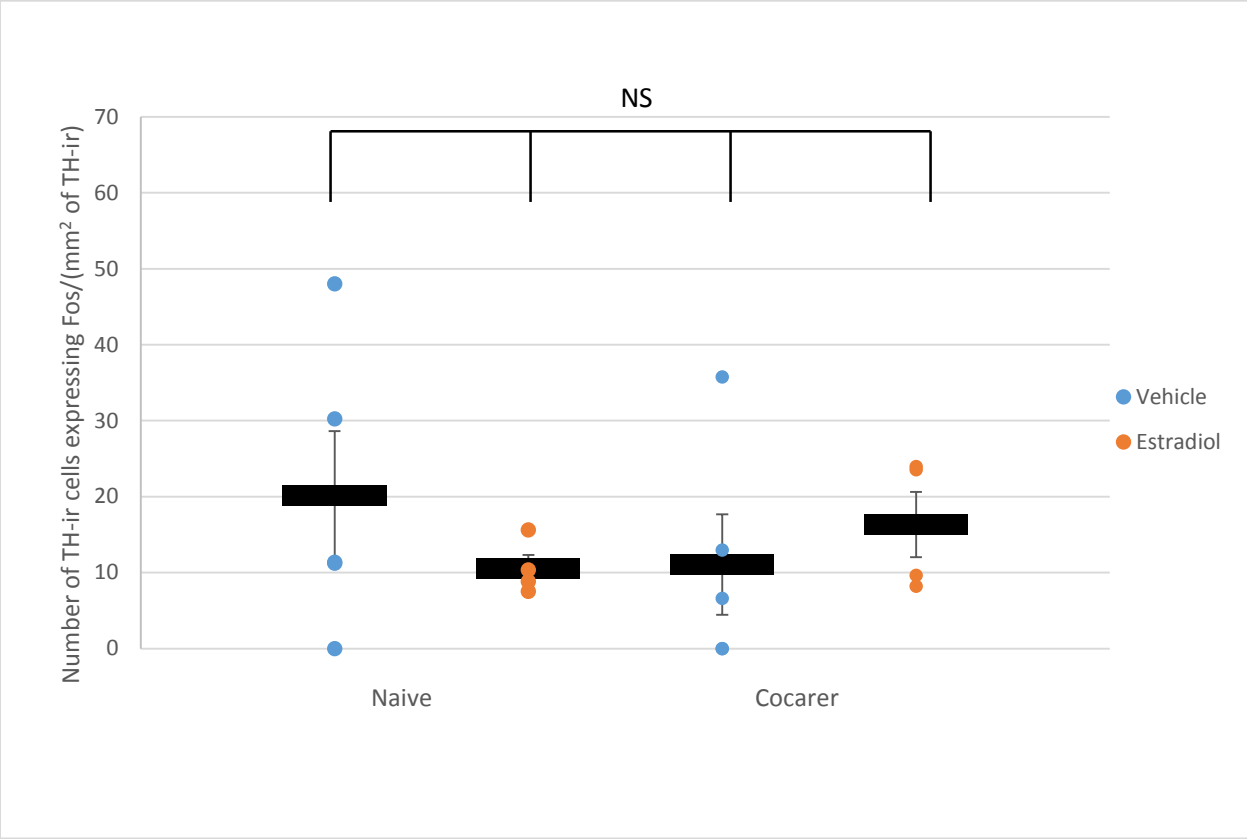

Figure 6.

Side-by-side dot plot of the number of TH-ir cells expressing Fos per square millimeter of TH-ir for each hormonal and experiential treatment. Each point represents the average number of TH-ir cells expressing Fos per square millimeter of TH-ir for an individual animal (Vehicle-implanted naïve animas n= 5, E2-implanted naïve animals n= 4, vehicle-implanted co-carer animals n= 4, E2-implanted co-carer animals). Black bars represent the average for each group. There was no effect of hormone treatment or experiential treatment on TH-ir cells expressing Fos per square millimeter of TH-ir (2 x 2 ANOVA, main effect of hormone treatment $F_{1,14}$=0.11, p=0.7413, main effect of experiential treatment $F_{1,14}$=0.07, p=0.7987, interaction between two factors $F_{1,14}$=1.33, p=0.2682).

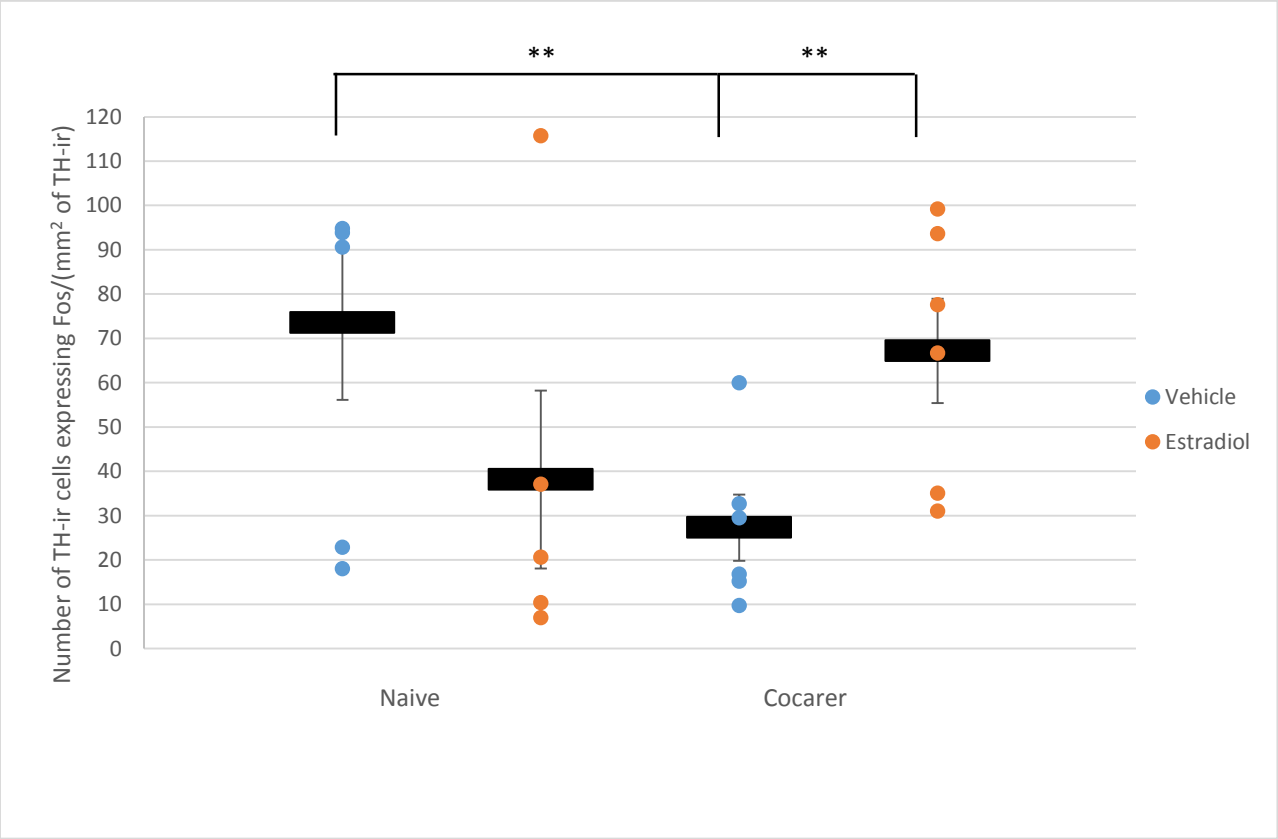

Figure 7.

Side-by-side dot plot of the number of TH-ir cells expressing Fos per square millimeter of TH-ir for each hormonal and experiential treatment. Each point represents the average number of TH-ir cells expressing Fos per square millimeter of TH-ir for an individual animal (Vechicle-implanted naïve animals n= 6, E2-implanted naïve animals n= 5, vehicle-implanted co-carer animals n =6, E2-implanted co-carer animals n=6). Black bars represent the average for each group. There is no significant main effect of hormonal treatment or experiential treatment on the number of TH-ir cells expressing Fos per square millimeter (2 x 2 ANOVA, main effect of hormone treatment $F_{1,19}$=0.0242, p=0.8789, main effect of experiential treatment $F_{1,19}$=0.3486, p=0.5618). There interaction between the two factors was significant ($F_{1,19}$=6.6898, p=0.0181). There was a significant increase in the number of TH-ir cells expressing Fos per square millimeter of TH-ir in E2-implanted co-carer animals when compared to vehicle-implanted co-carer animals (p=0.02). There was a significant increase in the number of TH-ir cells expressing Fos per square millimeter of TH-ir in vehicle-implanted co-carer animals when compared to vehicle-implanted naïve animals (p= 0.0457).

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