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Date

The Molecular Correlates of Auditory Cortical Plasticity from Social Auditory Experience

By

Amielle Moreno
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Neuroscience

_____
Robert C. Liu, PhD.
Advisor

_____
Brian Dias, PhD.
Committee Member

_____
Shannon Gourley, PhD.
Committee Member

_____
Mar Sanchez, PhD.
Committee Member

_____
Larry Young, PhD.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

The Molecular Correlates of Auditory Cortical Plasticity from Social Auditory Experience

By

Amielle Moreno
B.S., Cellular Molecular Biology University of Washington
B.S., Psychology University of Washington

Advisor: Robert C. Liu, Ph.D.

An abstract of
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Abstract

The Molecular Correlates of Auditory Cortical Plasticity from Social Auditory Experience
By Amielle Moreno

While infant cues are often assumed to innately possess behavioral significance to elicit maternal response in mothers, recent research highlights how cue processing is enhanced through plasticity events in the sensory cortex. Evidence from mice suggests experience caring for pups induces plasticity in the auditory cortex (AC), which improves pup-vocalization detection and processing. Neuromodulators such as norepinephrine and estrogens are associated with experience-dependent plasticity and social sound processing. Differences in sensorineural representations of novel versus familiar vocalizations and how experience encourages this transition, are still being explored at the molecular level. Here, I used ovariectomized and estradiol (E2) or blank implanted virgin female mice to delve into the behavior and AC molecular changes induced by pup-calls when novel, first paired with pup-caring, or familiar through experience. I addressed whether pup-calls engaged the AC and the noradrenergic locus coeruleus (LC) based on the subject's prior pup experience or E2 availability, using the cellular activity and plasticity marker, the immediate early gene *c-Fos*. Transcription of the memory-associated gene brain derived neurotrophic factor's *(Bdnf's)* was altered by the playback of pup-calls depending on social context. While E2 influenced the rate of maternal behavior at initial exposure, it did not influence the number of neurons expressing c-Fos (c-Fos-IR) in the AC or total AC *Bdnf* mRNA transcription. Social pup experience had a main effect decreasing AC c-Fos-IR in pup-familiar subjects, perhaps maintaining auditory representations of familiar vocalizations. Initial pup experience increased *Bdnf* mRNA transcription throughout the AC in an isoform specific manner. In the LC, E2 and pup experience interacted, increasing c-Fos-IR and locomotion during playback, consistent with the neuromodulatory center's activity reflecting both hormonal and experience-dependent influences on arousal. Experience and not hormones affected molecular response to social sounds in the AC. My data suggests that the engagement of sensory and neuromodulatory areas is situationally dependent and depends differentially on both internal physiology like hormones and the long lasting changes left by prior experience. This research expands our understanding of the molecular response to salient social sounds and is the first time *Bdnf* is associated with processing social stimuli in the AC.

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1,3,7-Trimethylxanthine, 1-(3,4-Dihydroxyphenyl)ethylamine), 5-Hydroxytryptamine, (+)-(S)-N-methyl-γ-(1-naphthyloxy)-2-thiophenepropylamine hydrochlorid, Ethanol, and 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin- 2-one.

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1. CHAPTER 1: INTRODUCTION TO MATERNAL AUDITORY PROCESSING AND AUDITORY CORTEX PLASTICITY

The world surrounds us with auditory information, some of which are the daily hum of inconsequential elements in our environment while other cues are vital for our successful navigation through life. Novel sounds in our environment engender curiosity, as we assess whether the stimulus is inert, rewarding, or harmful. Once the sound becomes familiar, our behavioral response will change to aid our survival. One of the most pressing questions in the study of sound processing is how *experience* with a cue changes our brain's response to it and what these novel or familiar responses look like at the site of auditory processing.

Because we are a social species, important auditory stimuli are often social in nature. Through auditory communication, the sender and receiver have the potential to gain beneficial outcomes, ultimately enhancing the chance of survival of oneself, one's social group, or one's genetics through inclusive fitness and offspring survival (Dawkins, 1989).

For mammalian species, the relevance of infant sounds changes when parenthood or care-taking begins, to encourage the care of the next generation. When first experienced, infant vocalizations are novel and do not necessarily initiate parental response in non-parents. But, the majority of mammalian mothers give birth to live offspring requiring parental care during infancy. In 95-97% of mammalian species, mothers or female groups feed, groom, protect, and interact socially with offspring (Clutton-Brock, 1989).

To facilitate social interaction, mothers across species undergo hormonal and neural changes that encourage social bonding with infants and familiarity with infant stimuli (Kinsley et al., 2011; Dulac et al., 2014).

With parental experience, infant cues gain more behavioral relevance and become familiar. The behaviors of dependent infants communicate their needs to experienced caregivers, while the parental response forms strong social bonds (Okabe et al., 2012; Kinsley et al., 2011). Once recognized, infant stimuli have the power to activate brain circuitry responsible for generating the care-giving crucial for successful parenting (Swain et al., 2007). Within a species, the transition to recognizing infant cues is influenced by the internal state of the organism such as the individual's sex, hormonal, and neuromodulatory state, and stage of development (Figure 1-1).

Parental response to infant vocalizations is common in many species, is often essential survival and thus useful for studying sound processing. Using a maternal animal model, we can address questions about the rate, mechanisms, and nature of plasticity in auditory processing areas of the brain such as the auditory cortex (AC). fMRI and electrophysiological response to infant vocalizations changes in a mother's AC with infant caring experience (Tasaka et al., 2018; Swain, 2011). Understanding what mechanisms are enacted at the molecular level to induce these changes can tell us how the transition of infant vocalizations from novel to familiar is accomplished. Several labs have recently used the mouse as an animal model to elucidate how behaviorally relevant

sounds such as infant vocalizations transition from being novel to familiar, over the time course of experience because of the robust and measurable change in an individual's behavior. Additionally, the maternal mouse paradigm allows for hormonal manipulation so one can examine how adult cortical plasticity is influenced by hormones and neuromodulators (Banerjee et al., 2013).

The questions addressed in this thesis involve the molecular underpinnings of AC plasticity, using a maternal mouse model of infant acoustic communication. I was interested in how hormones, specifically estradiol, and experience improves the processing of social cues, aiding in social response and behavior. I asked how the expression of the immediate early gene *c-Fos* changes in response to familiar or novel infant call exposure in the AC and the locus coeruleus, the primary site of norepinephrine production. I also designed a paradigm allowing me to examine the transcription of the memory-associated gene *Bdnf* after initial pup and pup-call experience.

Within this introductory chapter, I provide the background behind the studies I conducted. Specifically, I will begin with the role and operations of the AC during familiar and novel sound processing. To address the importance of recognizing new social vocalizations, I shift to describe the maternal experience, leading to how infant cues are meaningful and drive behavioral response that depends on the maternal physiological state. I then cover the molecular mechanisms of plasticity in the AC and

justify the use of the maternal mouse model of communication in my research. Once I lay the foundation of the maternal experience and the plasticity of the AC, I discuss the cortical plasticity associated with infant vocalization experience. The ways in which estrogens and norepinephrine affect auditory processing are covered. Finally, I explain current gaps in knowledge concerning how experience and estrogens enact molecular changes to induce auditory cortical plasticity, presenting an opportunity for discovery.

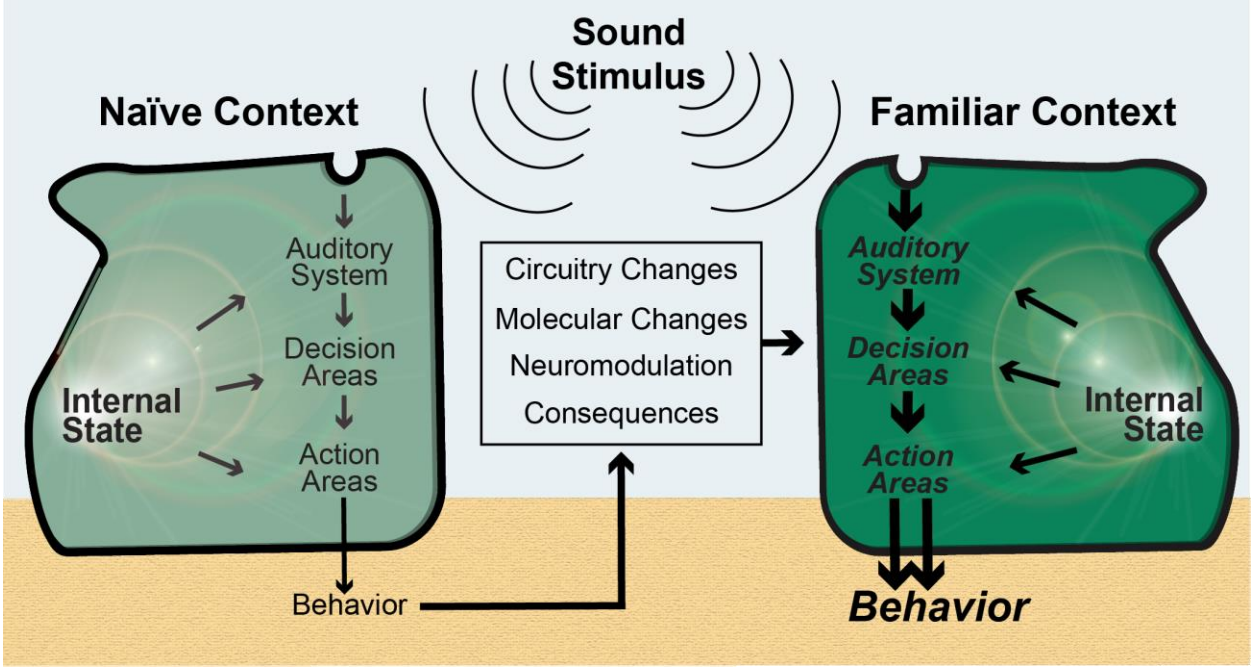

Figure 1-1 *Sound in a Naïve versus Familiar Context*

When a hearing organism is exposed to a sound stimulus, it is processed in its auditory system, creating a neural representation of the stimulus. This representation is

forwarded to decision processing areas and then to regions responsible for actions that generate behavior. This output is influenced by the organism's internal state, such as its brain circuitry, hormones, neuromodulators, and stage of development. When presented with a novel stimulus, the consequences of the actions made by the organism, whether positive, negative or neutral, are associated with internal changes in the neuromodulation, molecules, and circuitry of the brain. Distinct differences in sound processing have been found between organisms familiar or naive to sound experience. Ultimately, when the same sound is experienced again, the internal state and the changes made as a result of previous experience will influence the subsequent behavior displayed.

1.1 FAMILIAR VERSUS NOVEL SOUND PROCESSING

The auditory modality is a major source of stimuli experienced by hearing animals. Auditory stimuli are passed along the auditory peripheral and subcortical pathway, leading to the AC, which is generally associated with the conscious perception of sound. The AC is a principal site of auditory cue processing, with neurons here receiving thalamic input and forming a neural representation of the stimulus. AC neurons then transmit this refined stimulus information to downstream neurons.

Output from auditory processing areas travel to other parts of the brain involved with decision making, which guides an organisms' behaviors. When an important auditory cue is experienced for the first time, changes occur in the circuitry of the AC to aid future processing of that cue. When that familiar cue is presented again, AC activity helps individuals recognize the cue in its context, allowing an animal to respond quickly with an adaptive behavioral response (Ohl et al., 2005). A classic question within auditory research is how a sound, once familiar, generates a different representation in the AC compared to when it was novel (Wan et al., 2001; Mello et al., 1995; Ivanova et al., 2011).

Within the AC, the transition from novel to meaningful, can be aided by three types of cognitive operations; *detection*, *discrimination*, and *categorization* (Scheich et al., 2011; Bennur et al., 2013). To illustrate these distinct operations, imagine you have traveled to a conference in Nebraska, a place you have never been before. You are inside the hotel conference center for most of the day, and find a moment between sessions to sit down in a bustling lobby café. You are surrounded by the familiar clamor of an espresso machine, shoe heels hitting a marble floor, and people chatting, all which meld into a white noise. You focus your attention on returning e-mails.

Then, the air is pierced and you become cognitively aware of a droning sound. This sound, which you have never heard before, now has your attention. You *detect* this

sound because a neural representation of it is created by your AC, which recognizes it as a separate item from the other noises in your environment.

Once a sound is detected, it can be recognized by using the features of the sound to discriminate it from other similar stimuli. You begin to notice the various elements, or spectrotemporal features, of the sound including loudness, spatial location, and duration. It has a single source producing all of its components. It is complex, containing sweeps of artificial frequencies up and then back down in a rhythmic pace. The sound continues to drone. Compared to the other auditory cues in the lobby, this sound's features are unique. You *discriminate* the droning sound from the other sounds in your environment using its properties (Scheich et al., 2011). Attention to the features of the sound aid in its eventual transition from novel to familiar.

The droning sound can now be classified as containing elements that make it similar to other classes of stimuli, as defined by its acoustic properties. You can confidently say the sound is mechanically made and that it is novel to you. The persistence and strength would suggest it is an alarm sound, like a fire alarm, yet different. You have heard similar alarms in movies that feature war, military bases, and air raid horns. You become fearful. This type of sound is associated with imminent death from above; an instruction to take shelter. You have now *categorized* the sound as a member of a group with similar properties and meanings. While the details may vary, you can make predictions on the meaning of the novel sound thanks to the category you have placed it

within. While inside all day, you were oblivious to the large storm brewing outside and are hearing a tornado siren for the first time.

Because you were new to the North American Mid-West, a tornado siren was a novel sound. The consequences of your reaction, such as hurrying with the crowd to a safe stairwell, will cause subsequent physiological responses to the potential danger. With this experience, neuromodulatory reactions will change molecular production and brain circuitry and this once novel sound will never be processed by your AC the same way again (Figure 1-1). In this hypothetical story, the cognitive operations of sound detection, discrimination, and categorization were executed by your AC in order to enhance your chances of survival (Scheich et al., 2011; Ley et al., 2012).

Auditory neuroscientists have examined the transition from novel to familiar for the past 3 decades, slowly increasing the complexity of the target stimulus, background, prior experience, and physiological state. Relatively little has been done to explore this process in the context of social sounds, however. Attention and recognition of the auditory world is important, not only because of alarms as just described, but also because connections with others are often forged through auditory communication. We are now at a stage of research where we can study the neuromodulatory and molecular changes involved in brain plasticity in response to complex social sounds such as vocalizations (Figure 1-1). To understand the important dynamics of inter-species

communication, researchers can take advantage of ethological examples when communicating between conspecifics produces stereotyped behavior critical for survival.

1.2 THE MATERNAL EXPERIENCE

When their infant cries out, motivated parents jump to provide care, increasing the chance of their offspring's growth and survival. In mammals, the breadth of this transition differs across species and individuals. Rodent research has provided the most extensive insight into the behavior, endocrinology, and fundamental neural architecture of maternal behavior (Kohl et al., 2017a). In female adult rats, behavior changes from indifference to attraction towards infant stimuli. Born out of this research, *the onset-maintenance theory* of maternal behavior in rodents first ascribes the commencement of maternal behavior, before and at parturition, to a mother's hormonal milieu: a characterization of an individual's internal physiological state (Rosenblatt et al., 1981). Maternal hormones act on subcortical areas producing the onset of maternal behavior, and long-term changes to brain circuitry. The second component of the onset-maintenance theory attributes the maintenance of maternal responsiveness to the ability of infant stimuli to motivate behavior. Multimodal infant stimuli trigger the same subcortical maternal circuitry that underwent plasticity during onset. Sensory cues

produced by infants feature prominently in both aspects of this theory, yet little is known of the effect of hormones and experience on sensory areas processing infant cues.

During pregnancy and parturition, high hormone concentrations arise concurrently with the cues of newly born infants. Maternal hormones appear to encourage the rapid onset of care providing behavior, which is necessary for the immediate care of defenseless newborns. Female mammals in many mammalian species produce offspring along the altricial-precocial spectrum, which require thermo-regulation, nutrition through nursing, protection, and/or other forms of care for a period of time after birth (Numan et al., 2011). For non-mothers, as infant stimuli garnish the attention of an individual to the point where they are motivated to approach an infant, interact with them socially, and provide care, they are considered *sensitized* (Rosenblatt et al., 1995). Sensitization in the absence of maternal hormones happens slowly and is infant experience-dependent (Rosenblatt, 1967). While in some species both female and males can provide this infant care (Dulac et al., 2014), here I focus on maternal behavior and the recognition of infant vocalizations for reasons outlined below.

First, the mechanisms involved in parenting are related but not equal between males and females (Dulac et al., 2014). Fathers depend on similar underlying neurobiological circuitry and hormones as mothers to initiate parental care (Storey et al., 2016; Rilling et al., 2017), with the addition of the vasopressin system in rodents (Wang et al., 1994; Bester-Meredith et al., 2003). Fathers and mothers also both experience changes in the

concentration of hormones when they become infant caregivers (Rilling et al., 2017), however, the difference experienced by mothers is far more drastic than males. For example, when comparing the prolactin and cortisol levels of pregnant female-male human partners (*homo sapiens*) in response to infant stimuli prenatal and postnatally, female partners expressed changes nearly two to five fold that of their male partners (Storey et al., 2000). Additionally, the hormone testosterone is associated with decreased quality of male infant care (Weisman et al., 2014). While testosterone did decrease in new fathers (Gray et al., 2002), measurable levels of this hormone in postnatal fathers remained (Storey et al., 2000), potentially presenting interference with pro-parental behavior not experienced by mothers or non-mother females.

Second, maternal behavior is more prevalent than paternal behavior. Research using males in a parental sensitization paradigm would demand more animals, more time, and would not generalize to the majority of mammalian species. Behaviorally, only 5% of mammalian species see paternal care directly increasing the likelihood of infant survival (Clutton-Brock, 1989). In rodents, a clear disparity exists between males and females in their latency to display parental care. Male rats (*Rattus norvegicus*) and mice (*Mus musculus*) require more infant exposure before parental sensitization than females, averaging 10 days of experience to females' 4 days (Mayer et al., 1979; Kinsley et al., 1988). In terms of sensory recognition, Ehret et al. (1987) found male mice require more brood-caring experience than females in order to discriminate meaningful infant

vocalization sounds from irrelevant sounds. Males were also slower to learn retrieving behavior. Female subjects are more conducive for understanding how infant vocalizations drive behavior in sensitized mice.

Finally, females are generally understudied in the biological sciences (Blehar, 2003; Clayton et al., 2014; Beery et al., 2011). That, as well as evidence there are sex differences in auditory processing (Khaliq et al., 2003; Yoder et al., 2015), motivates the use of experimental designs that not only include but utilize the unique hormonal contributions of female biology to understand biological questions concerning parenthood. It was best to address my questions experimentally with the female sex, and thus I began my investigation of the molecular mechanisms enacted by experience and hormones associated with social auditory processing with female mice.

The call to action displayed by female caregivers in response to the sounds of an infant in distress is established through experience and enhanced by hormones. While a lot is known about the hormones, and subcortical neural circuitry involved with maternal response, fundamental questions remain concerning the plasticity that occurs in the sensory cortex during infant cue experience. But, before we can understand how infant stimuli contribute to maternal behavior, it is useful to understand the maternal physiological state in which these cues are processed, the subcortical circuitry that

receives infant stimuli input, and the infant cues that drive the behavioral response of caregivers.

1.2.1 The Maternal Physiological State

The neural and biological changes experienced by females during parturition are some of the most dramatic she will experience in her adult life (Bridges, 2015). The maternal physiological state is characterized by the extended and increased concentrations of estrogens, progesterone, cortisol, and prolactin hormones (Siegel et al., 1975; Rosenblatt et al., 1981; Bridges, 1984; Bridges et al., 1985; Nolten et al., 1981; Demey-Ponsart et al., 1982), with receptors for these hormones increasing throughout the brain (Numan, 2006; Numan et al., 2003; Koch et al., 1989b). This endocrine event facilitates fundamental and long-lasting re-wiring in the brains of new mothers and stimulates maternal behavior (Bridges, 2015; Kim et al., 2010; Kinsley et al., 2008; Hoekzema et al., 2017). As outlined in the onset-maintenance theory, hormones are powerful actors linked the commencement of maternal behavior at the time when the first infant stimuli is presented (Pryce et al., 1988; Maestripieri et al., 1998).

Studies examining the influence of hormones on parental behavior in non-human primates are mostly limited to correlational relationships within a small subset of species (Saltzman et al., 2011). To better understand how hormones alter the probability

of maternal behavior expression, with more precise measurement of endocrine function, classic experiments have used rats. Non-mother rats (nulliparous) can be experimentally ovariectomized and treated with maternal hormones such as the estrogen steroid estradiol or progesterone (Bridges, 1984; Doerr et al., 1981). Subjects with experienced high concentrations of estradiol and progesterone displayed a significant decrease in the latency of maternal behavior onset compared to the progesterone only or control group subjects. Other neurochemicals such as prolactin (Terkel et al., 1979b; Lucas et al., 1998; Bridges et al., 1985), and dopamine (Silva et al., 2001; Numan et al., 2009) influence maternal behavior as well, but the primary focus of my thesis is on estrogens and their influence on infant cue response.

The alpha form of the estrogen receptor (ERα) is necessary for the display of normal levels of maternal behavior by virgin female mice. When ERα knockout females were presented with pups in their home cage, they displayed significantly less maternal behavior and performed poorer at maternal tasks than wild-type or heterozygous littermates even when littermates were ovariectomized (Ogawa et al., 1998). The natural variation of ERα expression is significantly correlated with differences in licking and grooming behaviors in lactating rats (Champagne et al., 2003). Mother rats with low ERα expression in their medial pre-optic area, spent less time licking and grooming their own or foster pups, while high ERα expression mothers had elevated levels of licking and grooming.

The neuropeptide oxytocin is another moderating component of the maternal hormonal milieu. It is important for regulating the physiological responses of parturition, such as uterine contractions and milk ejection, as well as maternal and social behavior (Donaldson et al., 2008; Pedersen et al., 1979; Pedersen et al., 1982). Its receptors are expressed throughout the canonical maternal brain circuitry (Figure 1.2). Petersen et al (1994) found administration of oxytocin to ovariectomized and estradiol primed virgin rats induced the rapid onset of maternal behavior. Moreover, female mice knockouts for the oxytocin peptide display specific deficits in maternal behavior (Pedersen et al., 2006). Differences in the expression of oxytocin receptors were also linked to variations in maternal behavior (Francis et al., 2000). When mother rats expressed high levels of oxytocin receptors, they displayed higher than average licking and grooming behaviors (Champagne et al., 2001). These findings, as well as those by Champagne et al. (2003) on ERα expression variations, impressively linked molecular expression of a hormone receptor, either during development or initial pup experience to the display of maternal behavior.

Estrogens may act indirectly on maternal behavior through other hormonal peptides and molecules, like oxytocin. For example, oxytocin receptor mRNA and receptor binding affinity in the ventromedial hypothalamus and medial preoptic area (MPOA) tissue slices increased with estrogen benzoate treatment in male and female rats (Bale et

al., 1995; Caldwell et al., 1994). While oxytocin was able to induce maternal behavior, this only occurred in female rodents primed with estradiol and progesterone (Fahrbach et al., 1985; Fahrbach et al., 1984; Pedersen et al., 1982; Pedersen et al., 1979; Kendrick et al., 1987; Da Costa et al., 1996). Additionally, increases in estrogens and progesterone lead to increased expression of oxytocin receptor mRNA in various maternal circuitry and olfactory brain regions (Broad et al., 1999). However, other evidence suggests a weaker connection between estrogens and oxytocin. While the oxytocin gene has a putative estrogen response element adjacent to it, the induction of oxytocin via estrogens appears imperfect and might not serve a large role in vivo (Peter et al., 1990). Also, oxytocinergic cells have low levels of estrogen receptors (Burbach et al., 1995). Other neural signaling molecules such as glucocorticoids or thyroid hormone 1 may in part be responsible for oxytocin production as they upregulate oxytocin mRNA at parturition (Adan et al., 1993).

As described above, experimental delivery of hormones, especially estrogens, can recreate aspects of the maternal physiological state, including the rapid commencement of maternal behavior (Rosenblatt et al., 1998; Bridges, 1996; Stolzenberg et al., 2009; Maestripieri et al., 1998). However, hormones are not essential for sensitization, as gaining infant experience over time can lead to sensitization in the absence of gonadal hormones. Ovariectomized virgin female rats with enough exposure to pups eventually

display maternal care (Rosenblatt, 1967). Even aromatase knockout mice, incapable of synthesizing estrogens become sensitized with 4 days of experience (Stolzenberg et al., 2011). With similar underlying mechanisms and sites of action, dopamine systems might be capable of substituting for estradiol in the induction of maternal response (Numan et al., 2009; Stolzenberg et al., 2007). Thus, the role of hormones in maternal behavior onset is well established and should be considered in any studies where maternal behavior occurs, but, if unavailable, experience alone is able to bridge the gap creating experience-dependent sensitization. When present, maternal hormones as well as dopamine act on the quiescent subcortical circuitry, initiating plasticity and maternal behavior.

1.2.2 The Neural Circuitry of Motherhood

In communities of rhesus macaques, humans, and other primates pre-adult females will display interest in infant care, alloparenting before puberty and at higher rates than young males (Herman et al., 2003; Pryce, 1996; Lancaster, 1971; Maestripieri et al., 2002). In contrast, traditional laboratory rodents such as rats lack alloparental behavior (Kenkel et al., 2017). Nulliparous female rats are notorious for their initial aversion to infants and require extensive experience with infants (3-14 days) before their cues release a maternal response. Extensive work has been conducted in rodents to better

understand how the activation of subcortical regions called the canonical maternal circuit triggers the release of maternal behavior (Figure 1.2). As is often the case in biological sciences, our current knowledge on this circuitry is limited to rodent species. This circuitry will likely not always generalize to species with different parental behavior.

Information from sensory processing areas of rodents feed into the maternal circuit to initiate maternal behavior (Stolzenberg et al., 2019; Banerjee et al., 2013; Numan et al., 2010; Dulac et al., 2014). Brain regions in this circuit include, but are not limited to, the MPOA, the medial, central, basomedial, and basolateral amygdala (MeA, CeA, BMA, and BLA respectively), the paraventricular nucleus of the hypothalamus (PVN), the anterior hypothalamic nucleus (AHN), and ventromedial nucleus of the hypothalamus (VMN) of the hypothalamus, the ventral tegmental area (VTA), the nucleus accumbens (NAc), the ventral pallidum (VP), and the bed nucleus of the stria terminalis (BNST). Converging findings indicate that maternal brain circuitry is highly conserved across species, influenced by external environmental experience with infants and internal neuroendocrine mechanisms to initiate maternal response (Dulac et al., 2014; Saltzman et al., 2011; Pryce, 1992).

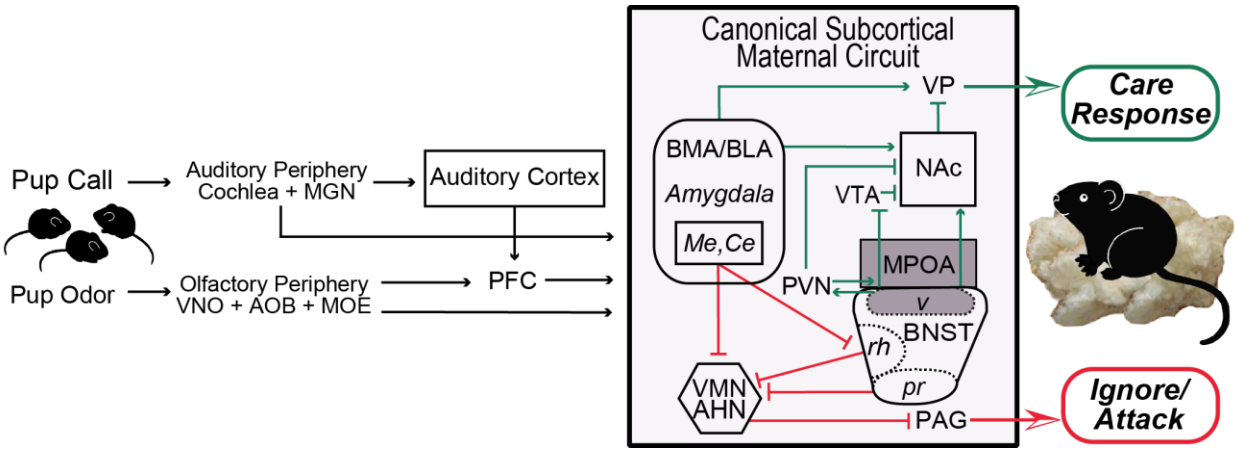

Figure 1-2 *Maternal Circuitry Schematic*

This diagram is a highly simplified representation of the tortuous maternal circuitry, the complete extent of which is not currently known. It depicts the neural pathways of pup olfactory and auditory cues projecting to the canonical maternal circuitry. Red lines indicate avoidance pathways and green lines indicate parental motivation pathways. The MPOA and vBNST are thought to contribute to similar pro-maternal circuitry and thus are depicted in a similar color with projections emanating from the border of the two areas. The MPOA and vBNST is believed to receive input and project output to the PVN. For simplicity, some connections are not depicted, such as MPOA and vBNST's inhibition of avoidance circuitry areas such as the BNSTrh, the AHN, VMN, and the PAG.

MGN, medial geniculate nucleus; VNO, vomeronasal organ; OB, olfactory bulb; MOE, main olfactory epithelium; PFC, prefrontal cortex; BMA/BLA, basomedial and basolateral amygdala; MeA, medial amygdala of the hypothalamus; CeA, central amygdala of the hypothalamus; VMN, ventromedial nucleus of the hypothalamus; ANH, anterior hypothalamic nucleus; PVN, paraventricular nucleus of the hypothalamus; MPOA, medial preoptic area; VTA, ventral tegmental area; NAc, nucleus accumbens; VP, ventral pallidum; BNST, bed nucleus of the stria terminalis, v- , ventral, -rh, rhombold , and -pr principle nucleus; PAG, periaqueductal gray matter. This figure was composed by the author with the aid of previously published review articles and schematics (Stolzenberg et al., 2019; Banerjee et al., 2013; Numan et al., 2010; Dulac et al., 2014).

The core of the maternal circuitry system is the MPOA of the hypothalamus, with lesions here irreparably disrupting parental behavior (Lee et al., 2000). It is composed of a heterogeneous collection of neurons, expressing high levels of hormone receptors such as estrogen, progesterone, prolactin, and oxytocin (Numan et al., 2003). Stimulation of MPOA with endocrine regimes mimicking pregnancy, including but not limited to high concentrations of estrogens and the rise and fall of progesterone, can initiate maternal behaviors in rodents and non-human primates (Rosenblatt et al., 1998; Numan, 2007). The role of the maternal circuitry is to mediate mother-infant

interactions after/during the hormonal changes associated with pregnancy, parturition and lactation.

In rats, the MPOA's actions include both inhibiting infant avoidance systems and activating infant approach systems have been studied extensively (Stolzenberg et al., 2019). Aggression toward or ignoring infants is due to a dedicated neural circuit; the avoidance system. This system includes the limbic region's MeA and CeA, the ANH, VMN, rhombold BNST (BNSTrh), principle nucleus of the BNST (BNSTpr), and PAG. When a nulliparous female rat is first presented with olfactory pup cues, the avoidance system is triggered and the subject finds them naturally aversive. Lesioning the amygdala or its major efferents in nulliparous rats caused them to display maternal approach, eliminating the avoidance displayed by those with proper amygdala functioning (Fleming et al., 1980).

In rodents, there are fundamental changes in the avoidance/approach maternal circuitry post parturition, which cement maternal behavior as the default response to infant stimuli (Lévy et al., 1995). The influx of hormones during late pregnancy and birth are believed to help trigger the onset of maternal behaviors; turning the avoidance system off, and switching the approach system on. Losing the hormones of parturition, means losing the immediate onset of maternal behaviors postpartum. For example, lesioning the PVN of pregnant female rats, a major site for oxytocin production, produced deficits in maternal care postpartum (Insel et al., 1989). However, the same

lesion made 4 days postpartum had no effect on the established maternal care displayed in mother rats. The combination of hormones and maternal experience at parturition leads to the long-lasting rewiring of the whole maternal circuitry for maternal response immediately and during future infant encounters (Bridges, 1975). The exact mechanisms and brain regions participating in this transition are still being explored.

Scientists are now identifying the MPOA neuronal populations responsible for various maternal behaviors. Within the heterogeneity of the MPOA, galanin-positive neurons are active during parenting behavior (Wu et al., 2014). Optogenetic activation of this subtype in male rats led to decreased rates of pup attacks and increased pup licking and grooming. Ablation of these neurons reduced the maternal behavior displayed by virgin females and pup-directed aggression emerged. MPOA galanin-positive neurons control discrete components of parental behavior including pup grooming via PAG disinhibition and inhibition of adult social interactions via the MeA (Kohl et al., 2018). Within the estrogen receptor alpha expressing subtype of MPOA neurons, the activity of neurons projecting to the VTA might drive pup retrieval behavior (Fang et al., 2018). Future research will surely identify other subpopulations that normally contribute to specific maternal behaviors.

The MPOA mediates offspring approach through activation of the mesolimbic dopamine system (Numan et al., 2009). In a pivotal paper by Fang et al. (2018),

researchers described the projections and behavioral role of estrogen receptor-expressing MPOA (MPOA-Esr1+) neurons. These neurons made up around a third of MPOA neurons and project to the VTA, one of the primary brain sites for dopamine production. Within the VTA, these projections did not target dopaminergic neurons but instead preferentially targeted and inhibited non-dopaminergic GABAergic neurons. Signaling this non-dopaminergic pool lead to the tonic inhibition of neighboring dopaminergic cells, driving VTA activity and maternal behavior. Around a third of MPOA-Ers1+ neurons displayed activity preceding infant retrieving behavior. Optogenetically shutting down these neuron's activity significantly decreased retrieval rates, while activation supported pup approach and retrieval. The authors concluded the activity of MPOA-Ers1+ cells are essential for maternal behavior. Studies such as this are beginning to identify the roots of behavior at the level of neural circuitry. A good deal is now known about the hypothalamic circuitry dealing with the onset of maternal behavior, but little about whether infant cue processing itself changes. This is especially relevant for the maintenance of maternal behavior, once initiated.

1.2.3 Behavioral Response to Infant Cues

At its most basic, parental behavior occurs when the tendency to withdraw from infant stimuli is less than the tendency to approach (Pryce, 1992). The detection and processing of offspring cues is the first critical step to parenting. Caregivers must come to recognize infant stimuli across sensory modalities. In primates, visual recognition of infants is common and activates cortico-limbic systems (Parr et al., 1999; Swain, 2011). Once maternal circuitry has been activated to facilitate maternal approach, it becomes the role of infant stimuli, such as the sound of an infant in distress, to motivate parental response (Rosenblatt et al., 1981). Because my thesis project will be conducted in mice, where olfaction and auditory cues are the major modalities of infant recognition and sufficient to activate maternal response, I will focus on those modalities at length.

1.2.3.1 Maternal Processing of Olfactory Cues from Infants

Much of the work on infant cue recognition examines how infant scent triggers avoidant or appetitive maternal response. There are major differences in the processing of infant scents across species. In some species, the actions of the olfactory system are essential for appropriate postpartum maternal behavior. For example, the olfactory bulb's (OB) of domestic sheep (*Ovis aries*) undergo extensive changes in synaptic circuitry upon parturition, thought to enhance the processing of infant olfactory cues

(Lévy et al., 2004). The lambs of sheep are precocial in their ambulatory ability, yet still require grooming and nutrition from their mothers. Damaging the main olfactory epithelium (MOE) of primiparous ewes decreased their maternal behavior compared to controls (Lévy et al., 1995). Additionally, removing the OB and thus olfactory sensitivity in mice, before parturition effectively eliminates maternal behavior (Gandelman et al., 1971). These findings intimate that disrupting the reception of infant scents can drastically affect the motivation of maternal care in mothers.

In new mother rats, there is a fundamental transformation in olfactory processing around the time of parturition, which prevents olfactory cues from triggering the avoidance pathway (Figure 1-2). In virgin female rats, the scent of infants can trigger avoidance and aversive responses. Infant olfactory cues feed into the avoidance pathway, from the VNO to the MeA, and BNST (likely the BNSTrh, and BNSTpr subregions) (Numan et al., 1994; Dulac et al., 2014). When lesioned, either at the main olfactory system or sectioned at the vomeronasal nerve, virgin rats display maternal behavior more rapidly (Fleming et al., 1979; Numan et al., 1994). Whether it is loving them or hating them, infant olfactory cues elicit a powerful behavioral response in female rats.

The increase in oxytocin during parturition might be responsible for changing the effect infant olfactory cues have on female rats. Oxytocin enhances olfactory processing and social recognition during initial social encounters, by activating the MeA and

increasing the signal-to-noise ratio of OB projection neurons (Ferguson et al., 2001; Oettl et al., 2016). Stimulation of the PVN, which is a key source for oxytocin release, leads to inhibition of OB mitral cells and excitation of OB granule cells (Yu et al., 1996). Both the MeA activation and the signal-to-noise ratio responses are blocked by infusing the OB with oxytocin antagonist. Thus, the large release of oxytocin during birth likely alters OB signaling in a way that prevents the triggering of the avoidance system.

Olfactory processing is important immediately post-birth to turn on the maternal response, and continues to be utilized but is less essential to maternal behavior after this time. For example, MOE damage in multiparous ewes does not decrease maternal behavior like it does for primiparous ewes (Lévy et al., 1995). Findings such as these highlight the importance of infant stimulus processing for the proper activation of the maternal response circuitry.

In order to provide maternal care to their own offspring, mothers across species form an imprint of their own infant's cues. Domestic sow (*Sus scrofa domestica*), goat (*Capra aegagrus hircus*), and human mothers can use olfaction alone to discriminate between their own and alien infants (Maletinska, 2002; Romeyer et al., 1994; Porter et al., 1983).

In some of the earliest rodent-pup retrieval studies, Beach et al. (1956b) examined how maternal lactating female rats discriminate their own versus alien offspring. A lack of the sense of touch via desensitization of snout and lips did not alter the ability of

females to discriminate between their own and foster pups, as measured by latency to retrieve foster pups. With the destruction of the OB, rates of their own versus foster pup retrievals were no longer significantly different. Researchers concluded the discrimination between their own versus alien offspring by mother rats required olfactory cues.

Ungulate infants are precocial in their mobility and are likely to attempt cross-fostering. Mothers of these species possess the ability to selectively bond with their offspring to ensure resources are provided to their newborns. Mother ewes became familiar with the unique scent of their lamb after postpartum lamb cleaning/licking, and could identify their offspring out of the herd within 1 to 2 hours (Kendrick et al., 1997). This depended on beta-noradrenergic receptors in the OB (Levy et al., 1990). Within 30 minutes postpartum, memory-associated molecular mechanisms including the increased transcription of *c-fos* and *zif-268*, were triggered in regions of the brain related to olfactory signal processing, such as the entorhinal cortex (Da Costa et al., 1997). Transcriptional changes might represent the early stages of cortical memory formation for an olfactory stimuli.

During and after parturition the olfactory system experiences changes in how infant odors induce neurotransmitter response. At parturition, multiparous ewes experienced the release of acetylcholine, noradrenaline, glutamate, GABA, and dopamine within the

OB in response to lamb scent not seen in primiparous ewes (Lévy et al., 1993; Keverne et al., 1993). Also, this change in neurotransmitter response was rapid. While not seen in primiparous ewes immediately, it was present within 6 hours post-parturition. Oxytocin in the OB of ewes may have facilitated the memory formation involved with individual lamb recognition via noradrenaline and acetylcholine release (Levy et al., 1995; Lévy et al., 1993). This type of rewiring of the OB and that of the entorhinal cortex has been argued to represent experience-dependent memory formation at the site of scent processing (Brennan et al., 1995; Da Costa et al., 1997).

1.2.3.2 Maternal Processing of Auditory Cues from Infants

Perhaps the most important social auditory cue for an animal to recognize is the sound of their infant. Auditory signals are a common modality by which infants gain a caregiver's attention. Mothers and sensitized caretakers will often approach the appetitive sound of infants (phonotaxis). For example, in domesticated pigs (*Sus scrofa domesticus*) postpartum sows displayed greater phonotaxis to vocalizing piglets, inevitably spending more time with them than silent piglets (Maletinska et al., 2002). With parental experience comes the ability to discriminate the sound of infants, categorize the different types of infant vocalizations, and in some species the ability to identify the sound of one's own offspring.

In many species, infants produce different types of vocalizations to indicate their various needs. For example, mouse pups made a variety of arousal-producing sounds that each have their own meaning to adult caretakers (Ehret, 2005). For instance, if in need of attention in the nest, infant mouse pups would produce a low-frequency wriggling call, which induces behavior in caregivers such as licking and grooming, posturing to expose nipples for suckling or nest building (Ehret et al., 1986). As an infant develops mobility, it becomes important to detect infant vocalizations from a distance. Unable to thermoregulate for themselves, mouse pups make a high pitched ultrasonic call in response to low temperatures, called an isolation-call (Okon, 1970; Branchi et al., 2001). In many laboratory mouse strains, infant vocalization rates peak around postnatal days 5-7, coinciding with when pup mobility first allows them to explore outside the nest (Elwood et al., 1982). These sounds arouse caretakers and provide a useful signal for search and retrieval of the distressed pup.

Some species possess the capacity to identify the vocalizations of their own infant in a crowded colony, or on a playground. Sows, sheep, goats, and various primates are all capable of recognizing the sound of their offspring's vocalizations (Illmann et al., 2002; Searby et al., 2003; Terrazas et al., 2003; Symmes et al., 1985; Hammerschmidt et al., 1998). In penguins, vocal identification occurs between parent and chick. For example, a cross fostering study found that Jackass penguins (*Spheniscus demersus*) are able to

identify the unfamiliar begging calls of foster chicks moved into their nest bowl (Seddon et al., 1993). The time of vocalization recognition of the caretakers, coincided with the beginning of chick exploration outside the nest.

Detection, discrimination, and categorization of auditory cues is a primary function of the AC. Within the human AC, there is a region strongly selective to human voices in the superior temporal sulcus/gyrus (STS) (Belin et al., 2000). The same region also displayed selective activity in mothers when they heard the sound of their own infant's cries (Swain et al., 2008). While the response to infant vocalizations by mothers might be innate, the recognition of infant vocalization categories or individuals requires parental experience, and plasticity within the AC. In other species, evidence is emerging that the detection and categorization of species-specific vocalizations is performed by regions homologous to the STS. Rhesus macaques (*macaca mulata*) possess a homologous region containing neurons which were selectively active to conspecific communication calls (Tian et al., 2001; Rauschecker et al., 2000). It has also suggested an area within the mouse auditory cortex displays neuronal selectivity for species-specific vocalizations (Issa et al., 2014; Tsukano et al., 2015). Investigating the existence of this functional subregion would require attention to stereotaxic location of plasticity events and could produce further support for the use of mice as experimental models for social auditory processing. The results in Chapter 2 potentially verified a region within the adult mouse AC selective to vocalization processing.

An open question within the auditory neuroscience community is how social auditory cues are processed. Infant vocalization processing presents an opportunity to examine the mechanisms of AC's processing of social sounds. The research within this thesis sought to understand how mouse pup vocalization encoding is achieved. Significant differences between the processing of familiar versus novel pup-calls were identified (Chapter 2) as well as the upregulation of a gene during initial pup exposure which might contribute to AC plasticity (Chapter 3).

1.2.3.3 Infant Cue Processing Across Modalities

Infant stimuli cross multiple modalities with some combinations being better at triggering maternal response than others. Female rats were subjected to combinations of sensory loss and tested on their ability to retrieve pups outside the nest (Beach et al., 1956a). Olfaction stood out as an important sense for pup retrieval. Rat subjects that were either anosmic and touch-insensitive, or anosmic and blind, displayed the greatest deficits in pup retrieval compared to the loss of any single sense or the combination of touch and blindness.

In a study using female rats, Smotherman et al. (1974) found olfaction was a necessary cue for rat-pup retrieval, but alone was not a strong enough directional cue for rat mothers to quickly localize pups. The combination of pup odors and ultrasonic vocalizations (USVs) was ideal for search behavior with the olfactory cues establishing the motivation to act maternally and the USVs guiding the approach direction. Lactating mother mice as well as female virgins with pup experience demonstrate olfactory-auditory sensory integration in the primary region of their AC (Cohen et al., 2011). Pup-odors appear to modulate and enhance the AC's pup USV detection and discrimination in both experienced mothers and virgin female caregivers, suggesting this modulation is experience-dependent. It should also be mentioned the potential role of lactation hormones was evident in the increased sensitivity of mothers to sounds, independent of an effect of odor, however.

Across species cues from infants can garnish attention from conspecific caregivers, sometimes elicit specific maternal care, and engender bonding through social interactions (Broad et al., 2006). Some sensory modalities appear to synergize with each other to improve parental responses, while others can stand alone. Infant signals are diverse and their recognition is the first and necessary step towards maternal response. Mothers across species possess the ability to form a memory of behaviorally important

sensory cues and via plasticity these memories are thought to be stored at the sites of processing.

1.3 PLASTICITY OF THE AUDITORY CORTEX

The vocalizations of infants mentioned in the previous section are processed in the AC of caretakers, forming neural representations that encourage detection. The AC is a highly organized sensory area designated for complex processing of sounds. It receives information about a stimulus experienced by the subject via ascending input from the medial geniculate nucleus of the auditory thalamus. The activity of the primary AC produces a neural code for this auditory stimulus and conveys the results of its processing back to the auditory thalamus, to secondary, tertiary AC areas, and across the corpus collosum to the AC of the opposite hemisphere.

In mice, the core AC contains the primary auditory field (AI) and the anterior auditory field (AAF) regions, and part of the ultrasonic field (UF) (Stiebler et al., 1997). Neurons in the core respond well to tones, with neighboring neurons appreciably co-tuned to similar frequencies (Guo et al., 2012). The responses of the neurons are arranged tonotopically along a ventral axes of the AI; with the best frequency (BF) eliciting maximum neuron activity, increasing from low, to middle, and then high (45 kHz). At the AI-AAF transition point, BFs decrease back down along the same axes. The

non-tonotopic UF features BFs above 40 kHz (Stiebler et al., 1997; Hofstetter et al., 1992). Calcium imaging of the upper cortical layers II/III suggest the core AC might have a more dorsoventral gradient than previously discerned (Issa et al., 2014). The non-core regions, which in primates is referred to as the belt region of the AC, include the secondary AC, the remaining parts of the UF, and the dorsoposterior field (Hackett, 2011). Non-core region neurons feature broader frequency tuning of individual neurons, with neighboring neurons possessing diverse BFs spanning as much as 2 to 3 octaves, and a dorsal-ventral frequency organization.

The field of auditory processing has long debated the necessity of the AC in sound recognition. AC lesion studies suggested AC damage did not prevent auditory-cued fear conditioning (LeDoux et al., 1984; Campeau et al., 1995). Instead, it appeared for a long time that efferents from the medial geniculate body of the thalamus projecting directly to the amygdala were primarily responsible for the learning and retention of auditory stimuli (Butler et al., 1957; LeDoux et al., 1985; LeDoux et al., 1986; Iwata et al., 1986; Jarrell et al., 1986). Other findings suggested the AC might play a role in discrimination between conditioned fear stimulus (CS) and the safe conditioned stimulus (Jarrell et al., 1987). For a period of scientific history, the necessity of the AC was not fully understood as it did not appear to be needed for tone discrimination.

Then, Romanski and LeDoux found auditory-cued fear conditioning can be mediated via multiple pathways, discovering both the established thalmo-amygdala and a thalmo-

cortico-amygdala projections were sufficient for inducing a conditioned response to a CS (Romanski et al., 1992; LeDoux, 2000). Experiments applying the GABA receptor agonist muscimol saw rapid and reversible inactivation of the AC. Working with this new tool revealed that previous lesion studies were confounded by the ability of the auditory system to reorganize and compensate for the tasks normally performed by the AC (Talwar et al., 2001). This result was later supported by other work distinguishing the thalmo-cortico-amygdala pathway as the principal route of auditory fear stimuli processing and memory (Boatman et al., 2006). The AC is now accepted as an important part of a system tasked with acquisition, learning, and expression of aversive and behaviorally relevant fear associated stimuli (LeDoux, 2000).

Research on auditory-cued fear learning indicated the AC was directly involved with the processing of basic pure tones (Talwar et al., 2001) and more spectrotemporally rich sounds (Letzkus et al., 2011; Weible et al., 2014). While an undeniably useful paradigm, one limitation of tone-shock fear acquisition work is a lack of ethological complexity, applying only to negative valence signals. Studying how the AC response to behaviorally relevant cues commonly experienced in natural setting tells us more about not only the abilities of the AC but how those abilities impact the daily lives of animals (Bennur et al., 2013). By studying the response of the AC to complex, natural sounds with social context, we now see that it is not simply a site of sound processing. It is a site of

integration between auditory stimuli, attention, decision making, and reward processing (Irvine, 2018).

The AC is argued to be the long-term site of auditory memory traces shaped by neural plasticity (Weinberger, 2004, 2007; Scheich et al., 2011). Here, I will use the definition offered by Dexter R.R. Irvine who stated *neural plasticity* is "dynamic changes in the structural and functional characteristics of neurons that occur in response to changes in the nature or significance of their input" (Irvine, 2007). Neural plasticity is a direct consequence of experiences with stimuli within one's environment and variations in physiological state. Evidence of experience-dependent plasticity in the AC is supported by PET and fMRI research in the human AC (Morris et al., 1998; Thiel et al., 2002). In the AC, plasticity as a result of one's environment can shape an individual's perspective of the acoustic world. This can occur during development (Kuhl et al., 1992; de Villers-Sidani et al., 2007) or adulthood (Norena et al., 2006; Diamond et al., 1986; Weinberger, 1995). The acoustic properties of stimuli associated with either appetitive or aversive outcomes are reflected in AC neuronal activity (Ohl et al., 2005; Brosch et al., 2011; David et al., 2012).

The AC displays many diverse types of plasticity when it acquires the significance of acoustic stimuli. AC neurons change their response characteristics, shifting their BF for a specific frequency in response to tone exposure (sound conditioning) (Gao et al., 2000), frequency discrimination tasks (perceptual discrimination) (Brown et al., 2004),

or shock-tone association (classic conditioning) (Bakin et al., 1990). In training paradigms, shifts in frequency selectivity can occur rapidly, within 10 minutes of training trials and persist for 24 hours or more (Edeline et al., 1993; Bakin et al., 1990). A subject's experiences can also cause AC neurons to decrease their response to non-target noise (Jones et al., 2013; Witte et al., 2005).

AC plasticity also occurs at the population level. One demonstration of this is how the topographical organization of the AC can change during adulthood in response to sensory experiences (Pienkowski et al., 2011; Schreiner et al., 2014). After perceptual and associative learning paradigms, the AC's tonotopic "map" can be reorganized (Polley et al., 2006; Bakin et al., 1990). For example, owl monkeys (*Aotus*) were trained in a frequency discrimination task and the representation of the AC's tonotopic map was assessed (Recanzone et al., 1993). Compared to a passive tone exposure control, task trained subjects demonstrated an expansion of the cortical area representing the target tone. The plasticity for the target tone significantly correlated with discrimination task performance. This type of map expansion with experience is not always demonstrated by the AC (Brown et al., 2004).

Another way the AC enhances familiar sound processing is with increased contrast between different groups of neurons. In response to a familiar sound, neurons in the primary AC tuned to that frequency can increase firing, while neighboring neurons outside the range of the sound demonstrate suppressed activity. Sound-evoked

suppression adjacent to sound-evoked firing creates contrast in neuron activity at the population level (Shepard et al., 2016; Fritz et al., 2003).

From representing tones or simple frequency modulations to complex social stimuli, AC neurons deftly process a variety of environmental sounds, refining how it handles important information. Examples thus far illustrate electrophysiological evidence of plasticity in neuronal response to familiar auditory cues. To address how this plasticity is established long-term, molecular experimentation is required. While determining the genes and proteins responsible, researchers have the opportunity to explore if social sound processing is handled with common or unique mechanisms. There is evidence of deficits in auditory processing which are specific to social information (Gervais et al., 2004; Boddaert et al., 2004; Klin, 1991; Green, 1996; Moore et al., 2009), which suggests unique mechanisms manipulate the processing of social auditory cues. By using models suited to control auditory exposure within a social context we can determine how experience with conspecific vocalizations change the molecular response of the AC.

1.4 Advantages of the Mouse Maternal Model for Studying Auditory Plasticity

The mouse maternal model of communication is an experimental paradigm that uses natural infant and adult mouse behaviors to elucidate how infant experience and hormones can change how adults process stimuli associated with pups. Young mouse pups are unable to thermoregulate their body temperature, and will produce USVs in response to decreased temperatures experienced outside of the nest (Okon, 1970). Parentally motivated adult mice display appetitive approach of pup calls (Smith, 1976; Geissler et al., 2004). During the act of retrieval, adults will approach pups, scruff the pup's neck, and carry them back to the nesting area. These behaviors present an opportunity for researchers to assess an adult mouse's auditory processing, sensitization, and recognition of communicative sounds (Hahn et al., 2005; Ehret, 2005).

The robust infant search and retrieval response of female mice makes them effective subjects for studying how infant associated cues affect neural responses and maternal behaviors. While other rodents, like rats, may take days if inexperienced with infant care, female mice display rapid onset of pup-retrieval behavior that is easy to induce, repeat, and replicate across individuals (Fleming et al., 1990; Ehret et al., 1987). Mentioned earlier, female mice require less brood-caring experience than males before sensitization and retrieval behavior (Mayer et al., 1979; Kinsley et al., 1988). They also

require less experience before the successful discrimination between meaningful infant vocalizations and irrelevant sounds (Ehret et al., 1987).

Because of these qualities, female mice are conducive for understanding how infant vocalizations gain "meaningfulness" and maintain maternal motivation; this addresses both the onset and maintenance components of the onset-maintenance theory. Approach behavior of female mice can generalize to the playback of pup-call audio recordings, or equivalent driving stimuli, such as tones modeled to the frequency and duration of pup-calls (Hahn et al., 2005; Ehret, 2005). The playback of pup-isolation calls alone provides enough directional orientation for pup-experienced female mice to navigate a Y-shaped maze towards the correct arm to retrieve pups, demonstrating the communicative significance of these vocalizations (Smotherman et al., 1974; Ehret, 1983).

Experiments from G. Ehret's lab demonstrated that certain acoustic features of pup call sounds were used for recognition– an ability that emerged with pup experience. They tested adult mouse phonotaxis towards either 50 kHz tones, bursting at a rate which mimicked pup-calls, or 20 kHz tones, which were outside pup vocalization range, to assess stimulus characteristics that could induce recognition and approach behavior (Ehret et al., 1987). Mothers consistently preferred to approach the 50 kHz tones, but virgin females did not show a preference until they possessed 5 days of pup experience. While pup-naïve females displayed maternal behavior, the recognition of model pup-

calls as behaviorally relevant must be learned through social experience with pups. Preferred approach by subjects towards USVs or model calls, over a neutral sound, indicated maternal and social sound learning had occurred. Two-alternative choice experiments can illustrate how factors such as experience caring for pups and hormones affect the recognition of a sound's communicative significance.

The maternal mouse model has been used to study how infant associated stimuli release maternal response and how this release could be facilitated by maternal hormones, including oxytocin and estrogens (Banerjee et al., 2013; Marlin et al., 2015; Lin et al., 2013). Experience with pup-care facilitated the ability of female mice to retrieve pups and prefer a 50 kHz over a 20 kHz pulsing tone, even in the absence of gonadal hormones (Koch et al., 1989a). Female reproductive hormones were necessary for spontaneous retrieving to occur in inexperienced females (Ehret et al., 1989). Estradiol treatment, but not prolactin or progesterone, reduced the period of contact with pups necessary for the preferred approach of pup-model calls (Ehret et al., 1989). The authors suggested the motivation to approach is induced by both hormones and experience, which increase the salience of pup-associated stimuli.

The maternal physiological state during pup experience might also act to stabilize USV recognition over long periods of time. Mother mice with 1 week of experience, and then ovariectomized, went on to retrieve significantly more than ovariectomized virgin females with 1 week of pup experience, when tested a month post-pup experience (Ehret

et al., 1989). As proposed by the onset-maintenance theory, maternal hormones support infant stimuli recognition if present during the time of infant experience (Rosenblatt et al., 1981). This suggests mothers possess long-lasting changes in how infant cues are processed.

Additionally, complex communication sounds might receive special processing by a subregion of the human AC. The human STS brain region is strongly selective for human vocalizations (Belin et al., 2000) and the cries of infants (Lorberbaum et al., 2002). Recent evidence suggests the existence of a vocalization sensitive region in the mouse AC (Issa et al., 2014; Tsukano et al., 2015), which would allow for new comparisons across species. When investigating pup-call processing in the AC of mice, close examination of this potential vocalization sensitive region should be made. The tonotopic location of protein expression was considered in the research presented in Chapter 2 with findings that suggest molecular mechanisms of plasticity occur within the AI-AAF transition region of the mouse AC with pup-call familiarity.

The maternal mouse model of vocal communication has and will continue to expanded our understanding of how the internal physiological state and its hormones influence maternal behavior. With a clear behavioral read-out of vocalization recognition and maternal sensitization, it allows experience dependent and hormonal influenced plasticity in the brain to be studied. Through the use of a maternal model of

infant communication, one may examine the molecular underpinnings of AC plasticity that drives enhanced social cue processing.

The research in this thesis builds upon knowledge gained from previous work using the maternal mouse model of communication, that indicated experience and estrogens both contribute to the behavioral recognition and meaningfulness of USVs in adult female mice. My experiments used this model to address questions regarding the effects of estradiol on vocalization processing and the molecular mechanisms of sensory cortical plasticity. Adult female mice in my studies were ovariectomized and estradiol or vehicle delivery systems were implanted. Their relationship with infants ranged from pup-naïve, recent 1 hour exposure to pups, or experience-sensitized over 5 days. The transcription and translation of memory-associated genes in their AC were judged to assess the state of sensory cortical plasticity in association with infant vocalizations.

1.5 Sensory Cortical Plasticity for Infant Vocalizations

A major activity in the daily lives of social species is the identification of and communication with other conspecifics. Perhaps the most fascinating nuance of the AC is its ability to decipher complex auditory stimuli with social relevance. It is not surprising that the highest site of auditory processing would play an integral role in the recognition of spectrotemporally complex vocalizations. Vocalizations can have negative

or positive valences (Morton, 1977) and the AC is equipped for emotional sound processing (LeDoux, 2000). When auditory communication with other conspecifics enhances survival, a vocalization's unique components drive neural AC responses which are selective for these features (Bennur et al., 2013; Ehret, 2005).

A number of studies have found significant differences in the AC's response to infant cues between mothers and those naïve to infant caring (Cohen et al., 2011; Liu et al., 2006; Galindo-Leon et al., 2009; Swain et al., 2008; Tasaka et al., 2018). In humans, a mother's AC experiences measurable changes associated with parenting and hormonal history. For example, vaginal delivery involves the positive feedback of oxytocin release to induce shorter and shorter durations between contractions. In response to the sounds of their own offspring crying, mothers that delivered vaginally express higher levels of cry-evoked BOLD signaling in the MPOA/Ventral BNST, caudate head, portions of the cingulate, the orbitofrontal cortex, and the STS compared to mothers who experience caesarian delivery (Swain et al., 2008). These findings suggest the physiological history of an individual affects the AC's response to the vocalizations of one's infant.

While complex language might be unique to humans, the emission of species-specific vocalizations is common. Using model organisms, it is possible to determine if their AC is capable of detecting whether an infant sound occurred, discriminating its features, and categorizing the type of vocalization. In the mouse maternal model of communication, the timing and magnitude of population and single-unit spiking in AC

demonstrated its ability to detect and discriminate between infant vocalizations (Liu et al., 2007). The AC neurons of anesthetized mother mice with characteristic frequencies (CF) close to the pup-call frequency range (40-90 kHz) showed earlier and narrower peaks in evoked firing rates compared to naïve females at the population and single-unit levels. Furthermore, the AC spiking in mother's conveyed significant discrimination and detection information over a larger time course and prevalence than naïve females. Hence, the AC of mothers demonstrated experience-dependent plasticity in neural spiking response thought to represent improved neural coding of pup-calls.

When experiencing spectrotemporally rich pup vocalizations, evidence suggests that the tonotopic map of the AC does not experience expansion but instead undergoes a form of plasticity called lateral band inhibitory plasticity. At the population and single-unit levels of the AC, awake, head-fixed mothers exhibited pup call-evoked inhibition of spiking earlier and the response was more stereotyped across trials than in naïve females (Galindo-Leon et al., 2009). This suppression occurred particularly in the bands of neurons whose BFs were lateral to the frequency of pup vocalizations (Shepard et al., 2016). The increased suppression at these lateral-band sites is hypothesized to enhance the signal-to-noise ratio and contrast of cortical activity in response to these socially relevant cues. The increased depth and duration of inhibition might be due to inhibitory neuron plasticity. In the AC of mother mice, inhibitory neurons that expressed parvalbumin shifted their BF closer to USV frequencies (Cohen et al., 2015). Inhibitory

mechanisms may play an important role in behaviorally relevant plasticity for vocalizations.

Further evidence of cortical response plasticity from experience with the behavioral relevance of pup vocalizations comes from molecular research. Molecular events are a driving force for remodeling synaptic connections and neuronal connectivity. In a study by Krishnan et al. (2017), they examined the enzymes, genes, and structures of neurons during pup-sensitization in virgin mice and found maternal experience triggered an increase in inhibitory neuron-related changes in the AC. An enzyme for GABA synthesis, GAD67, increased in the AC after 5 days of social pup experience. This study also used female mice heterozygous for the methly-CpG-binding protein 2 (*MeCP2*) gene in their AC. Heterozygous *MeCP2* females exhibited a significant increase in the inhibitory associated protein parvalbumin and the synapse structural proteins that form perineuronal nets (PNNs) (Krishnan et al., 2017). After 5 days of pup social experience, *MeCP2* expression in the AC was negatively correlated with the number of errors retrieving pups. When heterozygous females were treated with an enzyme that disintegrates PNNs before retrieval evaluation, social learning was boosted to wild-type animal levels, suggesting the PNNs were preventing structural changes necessary for AC plasticity. These findings suggest experience-sensitized mice display plasticity specific to inhibitory neurons in the AC and that this is detectable at a molecular level.

Evidence of pup-call categorization was reflected in the molecular transcription of an immediate early gene (IEG) in AC neurons. In response to neuronal activity, the mRNA of the IEG *Arc* (activity regulated cytoskeleton-associated gene) is summarily transcribed and transported from the nucleus, to the cytoplasm, and then to recently active synapses. In the AC of mice, the percentage of neurons with *Arc* mRNA in the nucleus or cytoplasm, depended on the time since recent sound exposure and previous sound experience (Ivanova et al., 2011). When the AC was assessed post-stimulus, nuclear localization of *Arc* mRNA was associated with those novel to the tone, while cytoplasmic localization of mRNA was associated with tone experienced mice. This suggests tone experience primed transcriptional mechanisms.

Next, Ivanova et al. (2017) used the maternal mouse model to judge the time course of *Arc* mRNA sub-cellular localization in AC neurons post-audio playback of pure-tone models with acoustic features akin to pup-calls (65 kHz tones), or natural pup USVs. The total percentage of *Arc*-positive neurons in core AC was not sensitive to the female subject's prior pup experience, but *Arc* mRNA's sub-cellular location within the nucleus or cytoplasm was. Within experienced caretakers, previous sound exposure may prime molecular machinery, leading to increased cytoplasmic *Arc* mRNA localization during re-experience (covered in more detail in Section 1.6.1).

Experience-dependent physiological changes are measurable in the AC's electrophysiological and molecular response to infant cues. Improved neural coding might be influenced by maternal physiological history and inhibitory mechanisms. As a vocalization transitions from novel to familiar, the molecular response of neurons in the AC shifts as well. We are beginning to understand what this transition looks like at a molecular level in the AC. Exploring what is modified, where, when, and why will allow us to better appreciate how the sensory cortex contributes to social behavior such as auditory communication.

1.6 Molecular Mechanisms of Learning and Memory in the Auditory Cortex

Whether it is improved perceptual performance (Bakin et al., 1996; Caras et al., 2017) or associative learning from experience and training (Leon et al., 2008; Galvan et al., 2002), plasticity of the adult AC improves its ability to detect behaviorally relevant sensory stimuli. Sustained electrophysiological changes of the AC after auditory experience is the readout of molecular machinery engaged at the genomic level and the resulting structural changes in neural connectivity. Neurotransmitter signaling between

neurons is translated post-synaptically into enzyme activation, transcription factor activity, IEG transcription, protein synthesis and transport, and epigenetic modifications, ending with the synaptic structural and functional changes that only few will go as far to say is "learning." As a result of experience-dependent plasticity, AC neurons display altered spike activity and, likely, a new, distinct profile of molecules and proteins. This molecular plasticity can alter the network of neurons activated by a learning experience, involved in the initial learning stage, *acquisition*, or the long-term storage of newly learned associations, *consolidation*. Next, I will review some more well studied examples of cellular and molecular mechanisms that inspired the direction and design of my research, emphasizing the steps between neural activity and the subsequent molecular cascades that are induced to change later neural responses.

1.6.1 RNA Translation and Localization

As we saw demonstrated with *Arc*, the transcription of IEGs can be different in auditory processing areas depending on the familiarity or novelty of the auditory stimuli (Ivanova et al., 2017). DNA microarray studies in songbirds have found conspecific song exposure leads to the change of expression of thousands of RNAs in songbirds (Dong et al., 2009). Discrete molecular profiles created at a genomic level accompany the acquisition of a novel song and its habituation. The molecular profile was examined in

the caudo-medial neostriatum (NCM), a region of the song bird forebrain analogous to the mammalian AC and sensitive to conspecific songs. The response of neurons within the NCM to novel sounds was dominated by transcription of genes involved in RNA processing and transcription, as well as a decrease in RNA for ion channel proteins. The profile of an individual songbird's NCM who had habituated to a song showed a decrease in the transcriptional characteristics of the novel profile, as well as changes in mitochondrial protein genes. The findings of Dong et al. (2009) suggest distinct molecular profiles for novel and habituation states in sound processing areas.

The IEG *Arc*'s transcription is tightly coupled to the encoding of behavioral information in neuronal circuits. It was the first gene whose mRNA was found localize to the dendritic spines after synaptic activity, allowing for local protein synthesis and synapse modifications (Link et al., 1995; Moga et al., 2004; Guzowski et al., 2005). As touched on earlier, using maternally experienced or naïve mice, Ivanova et al. (2017) examined the sub-cellular localization of *Arc* mRNA in AC neurons after pure-tone models of USV, or natural pup USVs. When *Arc* mRNA expression was assessed with fluorescent *in situ* hybridization, nuclear adjacent or "perinuclear" localization in the cytoplasm was more rapid and sustained in mothers post-stimulus, compared to the *Arc* mRNA in pup-naïve virgins. The USVs were also able to induce perinuclear localization in mothers earlier than the pure-tone models. The *Arc* mRNA localization of mother and

virgin female co-carers (virgin female mice housed with new mothers) was similar at 30 minutes post-USV exposure, suggesting experience alone lead to differences in localization. With *Arc*, the AC displayed sensitivity to a stimuli's category (familiar or novel) as well as its resemblance to previously experienced pup-calls (model or USV). The sensitivity of *Arc* to stimulus category, revealed through its mRNA localization, might aid in balancing the plasticity of new experiences with the stability necessary to preserve previously learned representations. The translation of the IEG *c-Fos* in the research of Chapter 2 could also be interpreted as maintaining this balance. Thus, the specific mRNA expressed and their location in AC neurons post infant experience might one day be enough to discern the status of a pup-call as novel or familiar.

1.6.2 Immediate Early Genes

IEGs are rapidly transcribed in response to neural stimulation, acting on cellular processes or as transcription factors (Farivar et al., 2004). They contribute to structural modifications at synapses directly or by increasing the transcription of other genes, often perpetuating the network activity that induced their own transcription. Once thought to merely identify recently firing neurons, research has now unveiled the various enterprises of IEGs spurred by experience-dependent learning. By identifying where in the brain IEGs are expressed after a sensory learning experience, and

measuring how many cells or how much their protein or mRNA is expressed, an image develops of the sites and magnitude of potential plasticity (Nikolaev et al., 1992). Between auditory brain regions, IEGs can differ in the type of stimulus that induces their transcription (Bailey et al., 2003), but when co-expressed within a cell, their mRNA have been known to co-localize (Velho et al., 2005). Here, I will summarize just a few of the more well-known examples of IEGs known to contribute to AC plasticity.

Zif-268/EGR1/NGFI-A/Krox-24 (or ZENK for protein, *Zenk* for gene) is a regulatory gene, encoding a transcription factor and closely related to induction of long-term potentiation (Mello et al., 1992; Link et al., 1995; Qian et al., 1993; Abraham et al., 1991; Williams et al., 1995; Jones et al., 2001). The rapid induction of *Zenk* is regulated by the extracellular signal-regulated kinase (ERK) which is an enzyme thought to be integral to memory (Sweatt, 2004; Bozon et al., 2003; Davis et al., 2003). It is theorized to directly relate to the formation of long-term auditory memories (Mello et al., 1995; Moorman et al., 2011).

Translation of the ZENK protein has provided insight into the differences between auditory stimulus processing during acquisition and consolidation. During tone-shock pairing acquisition in rats, ZENK expression is similar in the primary and secondary AC (Kwon et al., 2012). However, after 1 month (consolidation), the number of ZENK expressing neurons and the amount of ZENK translated in the secondary AC was significantly higher than in animals who recently learned or had never experienced the

tone-shock association. There were molecular distinctions between hearing versus hearing *and* remembering.

Zenk has been studied extensively within the songbird model of song perception, learning, and production. Originally used to map out songbird forebrain areas participating in conspecific vocalization processing (Mello et al., 1994; Mello, 2002; Mello et al., 1995), the components contributing to ZENK expression in the songbird forebrain are now appreciated as complex. Expression is affected by age (Jin et al., 1997; Stripling et al., 2001), sex, learning history, and the species of the subject (Bailey et al., 2003; Gobes et al., 2010). Beyond a binary response to song detection, *Zenk* mRNA induction in the NCM is directly related to how familiar an individual is with a song stimulus (Mello et al., 1995). In a display of molecular precision during sensory stimulus representation, the syllables of canary (*Serinus canaria*, Waterslager breed) songs create distinct patterns of ZENK in the NCM (Ribeiro et al., 1998). In addition, *Zenk*'s, as well as *Arc*'s, transcription varies depending on the discrimination of song quality, with higher quality of song syllables inducing more expression (Leitner et al., 2005). The expression in the zebra finch's (*Taeniopygia guttata*) NCM and caudomedial mesopallium (CMM) is significantly different depending on the social context of the call and the identity of the caller (Woolley et al., 2008).

A number of lessons can be taken from *Zenk* research. One lesson is when examining the transition of an auditory cue from novel to familiar using IEGs, such as that

conducted in Chapter 2, one should include the secondary AC for its potential role in emotional auditory memory storage (supported by other research (Sacco et al., 2010)). Another lesson is that ethologically relevant vocalizations engender distinct IEG expression in auditory regions. Also, the social context of a vocalization can yield specific molecular changes in the auditory system (explored further in Chapter 4).

One of the genes I used to understand both activity and plasticity associated with pup experience was *c-Fos*. *C-Fos* is a regulatory gene encoding a transcription factor, commonly used to label activated neurons but is also associated with memory formation (Sagar et al., 1988; Paylor et al., 1994). One example of its activity-related expression is that sensorineural hearing loss resulted in AC dormancy and a 90% decrease in the AC's expression of *c-Fos* (Tan et al., 2008). As an indicator of memory formation in songbirds, sound familiarity also affects c-Fos protein levels. When male zebra finches heard their tutor's song as adults, the expression of *c-Fos* mRNA in the NCM increased if more elements of the song were successfully learned by the subject (Bolhuis et al., 2000; Bolhuis et al., 2001). In the mouse AC, lentivirally delivered short strand RNA against *c-Fos*, blocked 40% of sound induced *c-Fos* translation and impaired sound discrimination learning (de Hoz et al., 2018). It did not, however, block the ability of the AC to passively habituate to repeated familiar tones. This research suggests *c-Fos* is specifically expressed in relation to behaviorally relevant sound plasticity and learning.

Additional findings suggested *c-Fos* might be connected to consolidation, as RNA interference animals showed evidence of weaker consolidation than controls.

As a stimulus is processed along the auditory pathway to higher cortical areas, the molecular responses of these regions is not identical. For example, after familiar pup vocalizations the expression of *c-Fos* in the female mouse secondary AC is higher than in the primary AC (Fichtel et al., 1999). It is possible the unique expression of IEG effectors produce differences between higher and lower order auditory areas (Horita et al., 2010). In this way, the same sound stimulus might enact different molecular responses in progressively higher order auditory processing areas. It is therefore crucial auditory researchers look for IEG in primary and secondary regions of the AC and not assume identical transcriptional phenotypes across the sensory cortex. This is another justification for including both primary and secondary AC locations in experimental paradigms (Chapter 2).

Interpreting *c-Fos* expression as a measure of neuronal activity oversimplifies its range of capabilities. In Chapter 2, *c-Fos* expression in response to familiar or novel pup-calls was assessed in both the AC and the locus coeruleus' norepinephrine producing neurons. Studies utilizing IEGs in this way allow us to make conclusions on the relationship between neuromodulatory regions and the AC as well as their differing roles in stimulus response.

1.6.3 Protein Synthesis

The connection between protein synthesis, and the formation of new memories was established by researchers as early as the 1970s. Using mice and aversion training, they linked the amnestic strength of a conditioned stimulus to the stimulus' severity (e.g. foot-shock voltage) (Quartermain et al., 1970; Flood et al., 1973). Preventing protein synthesis was effective in blocking the consolidation of long-term memories. Deficits in memory formation occurred after injecting protein synthesis inhibitors, such as anisomycin, at precise times post-training in rodents (Grecksch et al., 1980; Meiri et al., 1998), and birds (Tiunova et al., 1998). It was concluded there are 2 waves of gene induction and protein synthesis, possibly 3, associated with long-term memory formation: the first wave occurs immediately following training (0-1 hr), the second wave at 5-8 hours post-training, and the potential third wave at 15-18 hours (Stork et al., 1999). The 2 waves are apparent during the consolidation of auditory, taste (Tucker et al., 1976), and visual recognition (Tiunova et al., 1998).

Inhibiting protein synthesis in the AC of Mongolian gerbils (*Meriones unguiculatus*) prevented experience-dependent learning of frequency modulation (FM) discrimination (Kraus et al., 2002). Protein synthesis inhibitors were delivered to subjects at various stages of learning a FM discrimination task. In partially trained subjects, if the treatments were delivered to the AC immediately post-learning session, during the

"post-acquisition" phase, task improvement was hindered on subsequent sessions. The effects of a single injection could interfere with learning or the retrieval of long-term memories, lingering several days afterwards. Once consolidated, delivery of inhibitors before a discrimination session did not affect the ability to express established FM discrimination. The impaired long-term memory formation and/or retrieval but not short-term memory suggests that during the post-acquisition phase, modifications that are protein-synthesis dependent required for the stabilization of long-term memory at synaptic locations in the AC. The authors concluded a "protein-synthesis-dependent tract is produced that either sensitizes relevant local nodes (e.g., neurons, synapses) for future use or modifies them for later processes of consolidation" (Kraus et al., 2002).

The importance of protein synthesis in the formation of sensory memories is also apparent in a different form of memory – habituation – when one's response to a repeated, innocuous stimulus wanes. Hearing the same song repeatedly makes it grow dull, and perhaps songbirds experience this same phenomena with their songs. Chew et al. (1995) found in the NCM neurons of zebra finches, strong multi-unit activity to a conspecific song waned to 40% of its initial strength over 50 trials. This habituated level of activity was maintained even when other novel songs were interleaved with the playback of the habituated song. After a long enough time between playbacks (20 hours for conspecific songs and 4 hours for heterospecific songs), response to song playback

could return to original levels. Habituation rate provides a measure of "forgetfulness," with high neural firing rates associated with novel playback or extended time between playback. When protein synthesis inhibitor was delivered at 0.5 to 3 hours or 5 to 7 hours post-novel song habituation, the habituation rate was low, as if the song was forgotten or once again novel. These studies endorse the existence of mRNA and protein synthesis waves: critical time points for protein and RNA synthesis either during sound habituation or behavioral training. The research in this thesis was informed by previous work during experimental design to optimize the capture of maximum protein (Chapter 2) or mRNA (Chapter 3) expression.

1.6.4 Neurotrophic Factors

Quickly induced by long-term potentiation and experience-dependent learning paradigms, neurotrophic factors engage transcriptional machinery, such as transcription factors, promoting structural reorganization of neuronal circuitry (Stork et al., 1999). Neurotropic factors are major contributors to synaptic modulation and their transcription is neural activity dependent (Lu, 2003; Poo, 2001). Like other neurotrophins, the brain derived neurotrophic factor protein (BDNF) modulates synaptic formation in an activity-dependent manner (Lu, 2003) and is extensively involved with memory functions (Tyler et al., 2002).

The induction of the *Bdnf* gene begins with depolarization of the neuronal membrane and the subsequent influx of calcium. What follows is the phosphorylation of transcription factors, such as cAMP-response element (CRE) binding protein (CREB) causing its activation (Montminy, 1997). Phosphorylated CREB (pCREB) induces the transcription of the *Bdnf* gene (West et al., 2001). The resulting *Bdnf* mRNA, as well as that of its receptor, are localized somatodendritically, which insures protein synthesis upon neural stimulation is close to the synaptic site of action (Tongiorgi et al., 1997; Bramham et al., 2005). BDNF protein also stimulates pCREB, feeding back on the same system that influences its expression (Finkbeiner et al., 1997; Alonso et al., 2005).

BDNF expression occurs in brain regions experiencing plasticity and supports memory formation. Along with their decreased expression of *Bdnf* mRNA in the hippocampus and cortex, *Bdnf* knock-out mice display deficits when learning the Morris Water Maze (Linnarsson et al., 1997). Evidence of BDNF upregulation in response to experience extends to sensory cortical areas (Rocamora et al., 1996; Alonso et al., 2005; Nanda et al., 1998; Anomal et al., 2013). One example of *Bdnf* transcription in response to infant sensory learning was demonstrated in mother sheep. Increased *Bdnf* mRNA and its receptor, tyrosine receptor kinase (trk-B) mRNA, were found within the temporal, entorhinal, and pyriform cortices of mother sheep 4.5 hrs postpartum (Broad

et al., 2002). This suggested that plasticity in neural circuits are occurring in areas associated with olfactory and visual processing to aid in lamb recognition.

For how ubiquitous *Bdnf* studies are in the field of learning and neural plasticity, there is a paucity of studies examining BDNF in the AC during experience-dependent plasticity. In one of the few examples of studies on this subject, it was demonstrated in rats with normal hearing that BDNF protein is strongly localized in AC neuronal processes and postsynaptic compartments (Tan et al., 2008). After sensorineural hearing loss, BDNF expression density is reduced by up to 70%, suggesting a lack of peripheral stimulation from the down-stream auditory pathway lead to a reduction in BDNF expression. This could be rescued with a chronic 7-week regiment of electrical stimulation via cochlear implant.

The maternal mouse model of communication is an ideal paradigm to determine whether BDNF contributes to auditory plasticity. If AC *Bdnf* transcription increases in response to initial pup social and auditory experience, then BDNF likely participates in synaptic consolidation, strengthening the synaptic connections responsible for processing socially relevant cues. This could be one of the mechanisms responsible for the enhanced processing of familiar auditory cues. In Chapter 3, I used the maternal mouse model to determine whether initial experience with pups and their vocalizations lead to changes in this memory associated protein's expression. Changes in *Bdnf*

transcription as a result of pup auditory experience could be long lasting; preserving the neural trace holding the memory of infant auditory stimuli. Epigenetic modifications to *Bdnf* promoter regions are one way that memories are retained.

1.6.5 Epigenetic Regulation

When DNA or chromatin modifications occur within neurons in response to neural activity and neurotrophic signaling, the result is a change in the transcriptional phenotype of the neuron (Riccio, 2010). Epigenetic modifications are defined as non-permanent, stable, and heritable alterations to a genome, yielding a cellular phenotype without altering the DNA sequence (Berger et al., 2009). These modifications play a role in defining cell type, DNA repair, and transcription induction. It is believed these mechanisms have been co-opted by post-mitotic neurons to change the gene expression necessary for structural modifications, which support memory consolidation (Day et al., 2013).

Gene transcription can be regulated by post-translational modifications to histone proteins (Allfrey et al., 1964; Turner, 2005), which are responsible for organizing and compacting DNA strands. Various molecules termed epigenetic initiators, establish epigenetic modifications and/or maintain epigenetic states. One of the most prominent chromatin epigenetic modifications in learning and memory research is histone

acetylation (Levenson et al., 2004; Korzus et al., 2004). Acetylation and deacetylation of histones is managed by two epigenetic initiator enzymes; histone acetylation transferase (HAT) and histone deacetylase (HDAC). HATs transfer an acetylene group onto histone lysine residues, which can increase transcription by facilitating the binding of transcription factors to gene promoter regions (Sterner et al., 2000; Hassan et al., 2001). HDACs inhibit transcription by recruiting co-repressors and removing the acetylene groups, promoting tight histone-DNA compaction.

HDAC2 is highly associated with promoter regions of synaptic remodeling, plasticity, and memory associated genes such as *Bdnf* promoters I and II, *c-Fos, CamKIIa, Creb, Cbp,* and subunits of the NMDA receptor (Guan et al., 2009). In Guan et al. (2009) HDAC2's activity was linked to reduced synaptic number and impaired learning, while animals deficient in the enzyme displayed increased synapse number and hippocampal memory facilitation. Thus, regulating the activity of specific HDACs influences the formation of new memories.

Histone acetylation has also received attention in auditory experience-dependent plasticity as research begins to identify the subtle epigenetic effects of social vocalization familiarity. Epigenetic changes in subcortical maternal areas such as the MPOA facilitate the retrieval of isolated infants, a behavior triggered by auditory pup-calls (although, the vocalizations of the infants in this study were not accounted for) (Stolzenberg et al., 2012). In the AC, histone deacetylase activity can preserve changes in processing long-term. Four days after initial training in an auditory cue and water

reward learning paradigm, the treatment of rats with histone deacetylase 3 inhibitor (HDAC3-i) affects both behavior and primary AC plasticity (Bieszczad et al., 2015). Bieszczad et al. (2015) found highly specific expansion of the A1 to overrepresent the reward sound frequency precisely at the sound level (loudness) at which the cue was presented. Also, there was decreased frequency-tuning bandwidth to the reward frequency. This level of sensory detail was unique to HDAC3-i treatment, and the authors described it as "vivid memory" formation.

Phan et al. (2017) continued this line of research using HDAC3-i to enhance cortical remodeling to conspecific vocalizations. HDAC3-i treatment in the NCM of adult zebra finches was performed to determine whether this epigenetic initiator could enhance habituation memory. Songs were presented only 20 times; a repetition rate usually too low to induce significant habituation. They found treated birds showed song memory formation and increased expression of *Zenk,* specifically at a site associated with song-processing, suggesting prevention of HDAC activity can directly alter the regulation of *Zenk* during birdsong memory consolidation. Phan et al. (2017) identified one molecular/epigenetic mechanism responsible for regulating *Zenk* expression in the songbird auditory forebrain in response to songs. The research presented here lays some ground work towards epigenetic experiments within sensory regions using the maternal mouse model. With the findings presented in Chapter 3, additional research could examine the epigenetic state of the *Bdnf* promoter regions post-social auditory experience.

There are other indicators of memory formation that have not been covered in depth here, such as dendritic spine formation, synaptic structure, DNA methylation, and enzyme cascades. Unfortunately, the research on morphological changes to dendrites and synaptic structures in the AC is currently sparse, with a few examples suggesting that this line of research could be fruitful for measuring experience-dependent plasticity (Moczulska et al., 2013; Banerjee et al., 2017; Velho et al., 2008). Due to the large systems of molecular cascades, each with numerous players associated with activity-dependent plasticity of neurons, the subject of enzyme cascades will continue to be addressed as they relate to other mechanism (Figure 4.1 available for reference). The absence of other memory associated mechanisms is not to suggest their insignificance but that the direction of my thesis research did not rely on assessment of social auditory learning outside of molecular changes and maternal behavioral responses.

We have seen molecular responses take the form of gene transcription, mRNA translation and protein production. Some of these processes are most likely responsible for improved neural coding of social and behaviorally relevant auditory cues. Exactly which mechanisms are responsible is currently unknown. Cellular and molecular activity is influenced by the internal state of the animal, including the presence of hormones and neurotransmitters. In the next sections, I review two particular neurochemicals studied extensively within the maternal model and auditory plasticity.

1.6.6 Estrogen in Auditory Processing

Estrogens are ovarian sex-steroids whose hormonal subclass includes estrone, estradiol and estriol. They possess a fascinating array of roles in biological systems, from taking center stage during sexual maturation, reproduction and parturiton to encouraging and being produced in response to social interactions (Ervin et al., 2015; Sanchez-Andrade et al., 2011). Moreover, estrogens possess cognitive strengthening effects, promoting spinogenesis, epigenetic changes to *Bdnf* promoter regions, and enhancement of various forms of memory in the hippocampus (Frick, 2009; Fortress et al., 2014a). Given that parturuition involves the intial presentation of infants and their vocalizations, it is adaptive that estrogens and their cognitive enhancing effects are present during a time replete with new experiences. I will now address the evidence supporting estrogens enhancement of auditory processing of vocalizations, including those of infants.

Across many species, the processing of conspecific auditory cues can be profoundly modulated by estrogens (Sisneros et al., 2004; Maney et al., 2008; Kelley, 1980; Yoder et al., 2012b). For example, estrogens' role in processing speech sounds is evident in human populations with low estrogens. Women with anti-estrogen breast cancer medication, have had a hysterectomy, or are post-menopausal, experience cognitive deficits specific for verbal memory. Breast cancer treatments, which can include

aromatase inhibitors (preventing estrogen synthesis), or tamoxifen (an estrogen receptor antagonist) or both, cause impaired verbal processing speed and memory (Jenkins et al., 2004; Bender et al., 2006). Hysterectomized women experienced deficits in their immediate recall of a text read to them (Phillips et al., 1992). These same women have similar recall scores in other cognitive tasks compared to post-operative controls. This suggests verbal-specific memory issues. In menopausal women, estrogen-replacement therapy can improve the deficits specifically in verbal memory (Wroolie et al., 2011; Sherwin, 2005; Kampen et al., 1994).

Songbird research has provided insight into estrogen's ability to shape the perception of vocalizations (Maney et al., 2008; Remage-Healey et al., 2012). Treating the songbird's NCM with estradiol increases the response properties of NCM neurons (Remage-Healey et al., 2010). This induces downstream effects such as the enhancement of song selectivity by neurons in other auditory processing areas. Capable of deriving its own estrogens via the enzyme aromatase, the presentation of songs engenders estradiol production in the auditory forebrain of zebra finches (Remage-Healey et al., 2008). Inhibiting the production of neuro-derived estrogen in the songbird's NCM disrupts complex auditory processing of song stimuli and prohibits behavioral response (Yoder et al., 2012a). These studies layout estrogens' essential role in songbird vocal communication and the neurotransmitter-like influence of estrogens.

Another exemplar of estrogens' effects on social behavior comes from mammalian maternal studies. In the maternal context, fluctuating concentrations of estrogens, with their cognitive enhancing abilities, occur at the same time as new infant stimuli. During parturition an increase in estrogens is believed to permanently alter the behavior and the circuitry of a mother's brain (Numan, 2006; Love et al., 2005; Bridges, 2015; Macbeth et al., 2010; Kim et al., 2010; Lévy et al., 1993; Keverne et al., 1993). The maternal responses of new mothers to infants can be nearly immediate after birth, and so it is often assumed infant cues are not learned but innately motivate maternal response. But research on the sensory cortex, previously described here, highlights how infant experience leads to changes in the neural representations of infant vocalizations in a mother's AC (Liu et al., 2006; Fichtel et al., 1999). Research on the memory enhancing effects of estrogens (Fortress et al., 2014a), as well as its presence in high pre-partum concentrations suggests this neuromodulator might play a role in maternal infant cue representation plasticity (Banerjee et al., 2013; Miranda et al., 2009).

Exposure to estrogens in rodents hastens and facilitates the recognition of pup-calls over short periods of pup interactions, evident in a virgin female's rate of sensitization (Koch et al., 1989a). After 5 days of pup experience, ovariectomized and estradiol-treated females preferentially approach pup USVs while ovariectomized and vehicle-treated females need 21 days to consistently demonstrate this behavior. While

subcortical maternal circuitry is no doubt experiencing a transformation during this time, plasticity in the areas that supply sensory input to this circuitry might also feel estrogen's neuromodulatory effects. In the mouse, estrogen receptors are expressed all along the auditory pathway including the AC (Charitidi et al., 2010; Charitidi et al., 2009). Therefore, estrogens could be accelerating normal experience-dependent learning of pup-call meaning in sensory cortical areas.

Responses to infant vocalizations are also subject to changes in estrogen concentrations more subtle than those during parturition, such as those experienced during the estrus cycle. The probability that virgin female mice respond maternally to sound models of pup-calls increases during the period of their estrus cycle with high concentrations of estrogens: diestrus and proestrus (Ehret et al., 2009). The same calls during low estrogen metestrus, fail to elicit maternal behavior, suggesting higher concentrations of estrogens can broaden the stimuli recognized as behaviorally meaningful.

Estrogens may also affect long-term retention of social sensory information. Recent studies suggest maternal hormones can prolong recognition of pup USVs (Lin et al., 2013), highlighting the importance of the maternal hormone-sensory experience interactions. Enhanced long-term recognition is attributed to estrogens (Koch et al., 1989a; Ehret et al., 1989), yet the molecular mechanism that estrogens act through to

promote long-term social auditory perception is unknown. With a plethora of evidence of estrogen enhancing auditory processing the natural question to ask is *how*? For insight, we can turn to examples of how estrogen mechanistically enhances processing in other areas of the brain such as the hippocampus.

1.6.6.1 Molecular Mechanisms of Memory Enhancement by Estrogens

To understand how estrogens affect learning the meaning of vocalizations, we need to develop experiments that examine known memory-associated mechanisms found in other learning paradigms or brain regions (Kim et al., 2016; Phan et al., 2015; Frick, 2015; Luine et al., 2013; Zhao et al., 2010), and predict which mechanisms might be occurring at the sensory cortical level.

Memory-associated molecular candidates for the enhancement of auditory processing by estrogens include classical genomic actions, non-classical molecular cascade activation, and long-lasting epigenetic modifications (Frick et al., 2011; Marino et al., 2006; Sweatt, 2001; Abraham et al., 2004). Estrogens act via classical genomic actions, instigating the dimerization of nuclear estrogen receptors. This estrogen receptor-ligand complex then binds to gene sequences containing an estrogen response element (ERE). Genes with EREs in their promoter region include those for *c-Fos*

(Weisz et al., 1990), *Bdnf* (Sohrabji et al., 1995), and oxytocin (Mohr et al., 1991). Genomic actions have long latencies but induce long-term changes in neuron function.

Estrogens can also act on what has been termed non-genomic activity via membrane bound G-protein receptors, triggering a molecular cascade of kinase activity (Filardo et al., 2000). Membrane bound estrogen receptors have the potential to induce rapid behavioral and neuronal modifications, during non-classic estrogen actions. These membrane receptors are believed to be in close proximity to metabotropic glutamate receptors (such as 1a) and can activate the MAPK molecular cascade within an hour after an experience (Boulware et al., 2013; Laredo et al., 2014). Even though this pathway is called 'non-genomic,' it is widely known to be a misnomer, as activation leads to the transcription of genes that contain the cyclic adenosine monophosphate (cAMP) response element (CRE) (Zhou et al., 2005).

The MAPK/ERK pathway is co-opted by mature neurons to aid in synaptic plasticity and integrates cell signaling as a coincidence detector (Sweatt, 2001). This pathway leads to the activation of the CRE binding protein (CREB), which is both a transcription factor and a universal modulator of memory formation (Abraham et al., 2004; Zhou et al., 1996; Silva et al., 1998). Estradiol application to hippocampal neurons lead to an increase in the pCREB. However, when the enzyme that activates MAPK is inhibited, estradiol treatments failed to increase pCREB (Boulware et al., 2005). Therefore, estrogens trigger the MAPK pathway in hippocampal neurons, activating its

downstream mechanisms including pCREB via membrane localized receptors. Injections of estradiol intraperitoneally in mice or via intrahippocampal infusions boosted performance on novel object and object placement recognition tasks (Fernandez et al., 2008). In ovariectomized mice, the boost in memory provided by the treatment of either estradiol or an estrogen membrane receptor agonist was lost when the MAPK/ERK pathway was inhibited in the hippocampus. This provided evidence of one molecular pathway by which estrogens modulate hippocampal memory. Though limited, there is evidence within the AC linking pCREBs to auditory learning (Han et al., 2008) suggesting a gap in knowledge concerning estrogen's ability to activate this pathway to enhance auditory cortical plasticity.

The application of estrogens leads to memory-associated structural changes in neurons. In rats and mice, estradiol treatment enhanced object-placement and spatial episodic memory as well as spine density. First discovered in cultured hippocampal cells, the activation of membrane bound estrogen receptors and its downstream molecular cascade can lead to increases in spine density (Murphy et al., 1998; Woolley et al., 1993). When ovariectomized rats and mice were treated with estrogens, their CA1 neurons experienced an increase in the number of mushroom shaped spines and improved object placement and spatial episodic memory task performance (Li et al., 2004).

Estrogens can also induce the transcription of plasticity and memory-associated genes such as *Bdnf* through these kinase cascades (Swank et al., 2001), with pCREB directly binding to EREs (Sohrabji et al., 1995), or through long-lasting epigenetic modifications (Fortress et al., 2014b). Promoting epigenetic changes might explain how estrogens are capable of inducing stable changes in genetic expression (Zhao et al., 2010). Epigenetic modifications that regulate gene expression in the sensory cortex are known to promote long-lasting changes in neuronal activity (Swank et al., 2001; Silingardi et al., 2010). The long-lasting changes induced by maternity suggests estrogens might be a relevant player in auditory cortical plasticity.

There are multiple routes by which estrogen might aid in AC plasticity for the processing of social auditory stimuli. One promising avenue is that estrogens act to increase histone acetylation at promoter regions of *Bdnf* in the AC and hippocampus during maternal experience, as found during other learning experiences (Zhao et al., 2010; Frick et al., 2011). But before this research can be justified, a relationship needs to be established between *Bdnf* and auditory learning. In my thesis, I attempted to lay a groundwork for this avenue. In the experiments in Chapter 2 and 3, I examine whether systemic estradiol delivered to ovariectomized females affects the AC's c-Fos response to pup USVs, as well as the level of expression of *Bdnf* mRNA in response to pup-call stimuli.

The endocrinology of human females involves numerous neuropeptides and hormones whose activities fluctuate widely over time (Stricker et al., 2006). This makes studying females and estrogens a challenging area of research with the precise role of estrogens difficult to determine. In order to close the gap between female and male biological and biomedical research (Beery et al., 2011), a better understanding of the exact molecular actions of estrogens should be determined. The maternal mouse model provides an expedient method for understanding how estrogens effect auditory processing. Research on this subject can provide insight into how mothers rapidly experience auditory plasticity for the sound of their infants as well as how we might protect women from verbal cognitive decline with age.

1.6.7 Norepinephrine in Auditory Processing

Other neurochemicals may also be important for learning in the maternal context. Norepinephrine's (NE) actions are associated with sensory cortical plasticity (Shepard et al., 2015a; Edeline et al., 2011; Lynch et al., 2012; Ikeda et al., 2015; Martins et al., 2015) and maternal behavior (Thomas et al., 1997). By pairing tones with the stimulation of NE's endogenous site of production, the locus coeruleus (LC), the AC displays measurable changes in the processing of those tones which can last hours (Ikeda et al.,

2015; Martins et al., 2015). NE also plays a role in mediating social memories (Griffin et al., 1995; Pissonnier et al., 1985), raising the likelihood that arousing social sounds could engage the noradrenergic system to drive sensory cortical plasticity and facilitate behavioral responses.

In the context of infant cue presentation, NE-linked behavioral arousal and its ability to modulate sensory information processing could significantly promote maternal response to those cues (Berridge et al., 2003; Lévy et al., 1993). Measuring the activity of LC during the playback of novel or familiar pup-vocalizations would be telling as the LC may drive AC activity or demonstrate its own unique response based on the subject's previous history with those vocalizations. This was examined in Chapter 2.

In addition to a relationship with sensory processing, NE is closely associated with estrogens. NE and estrogens are enhanced or influenced by the presence of one another. Within the white-throated sparrow (*Zonotrichia albicollis*) forebrain, neurons with estrogen receptors or that produce aromatase responded to estradiol treatment with increased noradrenergic innervation (Matragrano et al., 2011; LeBlanc et al., 2007). Estradiol treatment also increased the expression of dopamine-Beta-hydroxylase (DBH) mRNA, the enzyme which produces NE in the LC (Serova et al., 2002). When delivered during development, estradiol administration increases NE in the robust nucleus of the arcopallium of the zebra finch auditory pathway (Wade et al., 2013). Additionally, there is a behavioral connection between the noradrenergic system and estradiol. Both were

capable of increasing song receptivity and the proclivity to produce seasonal mating calls (Wade et al., 2013). In an example of biologically conserved systems, estrogens, a reproductive hormone subclass, are linked to the activity of a system which encourages the advertisement of sexual responsiveness, the noradrenergic system.

Finally, NE is associated with maternal responsiveness. After parturition, noradrenergic projections from the LC released NE in the OBs of multiparous ewes in response to lamb scent (Lévy et al., 1993; Keverne et al., 1993). NE and acetylcholine release in the OB and entorhinal cortex of ewes are thought to facilitate the memory formation involved with individual lamb recognition via oxytocin (Levy et al., 1995; Lévy et al., 1993). Finally, the essential nature of NE is demonstrated in female mice that are heterozygous for DBH. When they become mothers they displayed low expression of maternal behaviors (Thomas et al., 1997). The LC is a major neuromodulatory area, related to maternal behavior, estrogens, auditory processing, and attention. In Chapter 2 of this thesis, I will address how a state of pup-call familiarity interacts with estradiol exposure in the LC of female mice, measured by c-Fos protein immunoreponsivity.

1.7 SIGNIFICANCE OF MY RESEARCH

It is my hypothesis that social experience and estrogens enact behavioral and molecular changes at the time of memory encoding and during future recognition of

social sounds. Using the maternal mouse model, I investigated this hypothesis by measuring changes in memory associated molecules in the female mouse's AC, with or without estradiol exposure before, during, or after they gained behavioral experience with pup-calls. The AC is an important part of a system tasked with auditory recognition and consolidation, projecting infant vocalization information to areas reaching the canonical maternal circuitry. By using the maternal mouse model of communication, we can better understand how this sensory cortical area experiences adult plasticity critical for infant survival.

Basic research to understand how experience, hormones, and other neuromodulators affect vocalization recognition in the AC and behavioral response will allow future researchers to develop focused therapeutics, improving the lives of individuals with deficits in auditory communication. Postmenopausal women can suffer from specific memory deficits for verbally conveyed information (Jenkins et al., 2004; Bender et al., 2006). Disorders such as autism, schizophrenia, and cochlear implant resolved deafness can impose deficits on conspecific vocalization recognition (Klin, 1991; Green, 1996; Moore et al., 2009; Gervais et al., 2004). Deficits in autistic and schizophrenic individuals can be specific for social auditory stimuli, suggesting that unique mechanisms underlie the encoding and recognition of social auditory information (Klin et al., 2009; Green, 1996). Older deaf individuals who receive cochlear implants require significantly more time to learn auditory speech than younger implant

receivers (Svirsky et al., 2000), generating a need for therapeutic enhancement of adult language learning. In fact, cochlear implants induce CREB activation and *Bdnf* expression in AC (Tan et al., 2008). Therefore, the therapeutic targeting of downstream effectors of estrogens has the potential to improve social auditory attention and language learning in deaf and low estrogen populations.

My central hypothesis is that processing behaviorally relevant sounds is modulated by an animal's experience and hormonal state, which can induce molecular and behavioral changes detectable at the time of memory encoding and memory recall. By examining likely molecular mechanisms of maternal experience-induced plasticity, and changes in behavioral response to pup auditory stimuli, this research was developed to determine how pup experience and estrogens affect AC neurons and behavior in the context of acoustic communication experience.

2 CHAPTER 2: FAMILIARITY WITH SOCIAL SOUNDS ALTERS C-FOS EXPRESSION IN AUDITORY CORTEX AND INTERACTS WITH ESTROGEN IN LOCUS COERULEUS

The following text was published in Hearing Research (Moreno et al., 2018). Amielle Moreno's contribution to this project specifically included but was not limited to: surgeries, running experiment day conditions and video recordings, auditory cortex brain sectioning, staining, and cell counts, final ANOVA and GLMM data analyses, contributed to the writing and review of each section of text, original and revision submission of text to publisher. Second first author, Ankita Gumaste, developed original locus coeruleus (LC) centered experimental design, sectioning, staining, and cell counting over half of the of LC sections and performing LC 'no playback' cell count analyses and original LC results. Geoff K. Adams designed and developed the GLMM with Amielle Moreno. Kelly K. Chong conducted the electrophysiology mapping of the AC to atlas work. Michael Nguyen processed and produced raw behavioral data while Amielle Moreno performed data analyses. Kathryn N. Shepard designed original LC experiment design with Ankita Gumaste. Experiments were conducted in the laboratory and with the guidance and editing support of Robert C. Liu.

2.2 *ABSTRACT*

When a social sound category initially gains behavioral significance to an animal, plasticity events presumably enhance the ability of that sound category's recognition in the future. In the context of learning natural social stimuli, neuromodulators such as norepinephrine and estrogen have been associated with experience-dependent plasticity and processing of newly salient social cues, yet continued plasticity once stimuli are familiar could disrupt the stability of sensorineural representations. Here we employed a maternal mouse model of natural sensory cortical plasticity for infant vocalizations to ask whether the engagement of the noradrenergic locus coeruleus (LC) by the playback of pup-calls is affected by either prior experience with the sounds or estrogen availability, using a well-studied cellular activity and plasticity marker, the immediate early gene *c-Fos*. We counted call-induced *c-Fos* immunoreactive (c-Fos-IR) cells in both LC and physiologically validated fields within the auditory cortex (AC) of estradiol or blank-implanted virgin female mice with either 0 or 5-days prior experience caring for vocalizing pups. Estradiol and pup experience interacted both in the induction of c-Fos-IR in the LC, as well as in behavioral measures of locomotion during playback, consistent with the neuromodulatory center's activity being an online reflection of both hormonal and experience-dependent influences on arousal. Throughout core AC, as well

as in a high frequency sub-region of AC and in secondary AC, a main effect of pup experience was to reduce call-induced c-Fos-IR, irrespective of estradiol availability. This is consistent with the hypothesis that sound familiarity leads to less *c-Fos*-mediated plasticity, and less disrupted sensory representations of a meaningful call category. Taken together, our data support the view that any coupling between these sensory and neuromodulatory areas is situationally dependent, and their engagement depends differentially on both internal state factors like hormones and external state factors like prior experience.

2.3 INTRODUCTION

Conspecific vocalizations are one of the most ethologically important auditory cues in an animal's environment. While evolutionary forces likely predispose sensory systems to respond innately to these signals (Garcia-Lazaro et al., 2015), recent research suggests sensorineural plasticity also plays a role in enhancing such responses (Shepard et al., 2015b) as individuals gain familiarity with a vocal category through experience. Learning the relevance of a vocal category can improve future recognition and behavioral response (Poremba et al., 2013; Tsunada et al., 2014). Plasticity events in auditory cortical and neuromodulatory areas are hypothesized to facilitate future processing to ensure robust behavioral response (Martins et al., 2015; Galindo-Leon et

al., 2009; Ivanova et al., 2017). However, driving new plasticity for increasingly salient sounds must also be balanced against the need to maintain established sensorineural representations. How sensory plasticity-related regions are engaged as a function of prior experience is still not fully understood.

Neuromodulators influence neural circuits that process ethologically relevant stimuli (Bargmann, 2012). One such neuromodulator is norepinephrine (NE), which is associated with behavioral arousal and facilitates sensory cortical plasticity (Berridge et al., 2003; Edeline et al., 2011; Lynch et al., 2012; Ikeda et al., 2015; Martins et al., 2015). NE also plays a role in mediating social memories (Griffin et al., 1995; Thomas et al., 1997; Marino et al., 2005; Pissonnier et al., 1985; Shepard et al., 2015a), raising the likelihood that arousing social sounds could engage the noradrenergic system to drive sensory cortical plasticity and facilitate behavioral responses.

Estradiol (E2), a steroidal hormone, is also strongly implicated in social behavior and plasticity. E2 is associated with improved performance on memory tasks like social recognition (Phan et al., 2011) and processing behaviorally relevant conspecific vocalizations (Maney et al., 2008; Remage-Healey et al., 2008; Remage-Healey et al., 2010). In mice, estrogen receptors are found along the auditory pathway (Charitidi et al., 2010), and E2 administration in pup-sensitized virgin females facilitates maternal approach behavior and recognition of model pup-calls (Koch et al., 1989a; Ehret et al.,

1984), though whether E2 acts on neuromodulatory or auditory areas to exert this influence is still unclear.

One hypothesis is E2 influences sensory cortex plasticity through actions on neuromodulatory systems (Miranda et al., 2009). Research in songbirds suggests noradrenergic neurons target sites with E2 receptors and express estrogen receptor mRNA (Heritage et al., 1980; Shughrue et al., 1997; Lynch et al., 2012). E2 administration modulates the concentration of NE in the zebra finch song system (Wade et al., 2013), the ewe ovine preoptic area (Goodman et al., 1995) and the mRNA of the enzyme that creates NE in the rat locus coeruleus (LC, (Serova et al., 2011)). Further, E2 plays a role in structural changes in catecholaminergic fibers within the songbird auditory forebrain (Matragrano et al., 2011; Appeltants et al., 2004). Hence, E2 could indirectly modulate the processing of salient auditory stimuli by influencing noradrenergic neurons in the LC.

These documented roles for both NE and E2 in social sensory processing and plasticity, as well as their relationship to one another, led us here to ask how these neurochemicals affect neural responses during playback of novel or familiar social auditory cues, using a maternal mouse model of social auditory learning. A growing body of literature suggests the detection of infant cues by mothers may be facilitated by maternal sensory plasticity in association with maternal hormones, including oxytocin and potentially E2 (Marlin et al., 2015; Banerjee et al., 2013). There is improved

detection, discrimination and categorization of infant pup vocalizations in the maternal auditory cortex (AC), persisting after maternal experience (Lin et al., 2013; Liu et al., 2007; Cohen et al., 2011; Galindo-Leon et al., 2009; Shepard et al., 2016). Here we asked whether E2 availability, manipulated systemically with subcutaneous implants, modulates how the noradrenergic LC responds when virgin ovariectomized females hear pup ultrasonic isolation vocalizations, either for the first time as adults (novel) or after social experience raising pups so that the call category is familiar. We measured the expression of the plasticity-associated immediate early gene (IEG) *c-Fos* (Dragunow et al., 1989a; Link et al., 1995; Montag-Sallaz et al., 1999) in the LC as well as the AC .

Our findings suggest E2 and familiarity with the social vocalizations affect c-Fos protein immunoresponsivity (c-Fos-IR) in neuromodulatory and auditory cortical processing regions in distinct ways. E2 and social experience interacted to drive both LC c-Fos-IR and locomotion measures, consistent with this neuromodulatory center playing a role in immediate behavioral responses to arousing stimuli. Meanwhile, the AC showed generally decreased c-Fos-IR in animals familiar with pup-calls, irrespective of E2 availability, consistent with a sensory cortical role in maintaining a more stable representation of stimuli that have gained behavioral relevance. Thus, after social sounds have become familiar, the genomic responses in LC and AC reflect

complementary roles these areas play, respectively, in these auditory cues' salience versus memory.

2.4 MATERIALS AND METHODS

2.4.1 Animals

The Emory University Institutional Animal Care and Use Committee approved all procedures involved in this study. Experiments were performed on adult virgin female CBA/CaJ mice. Animals were weaned at 21 days, placed in single-sex ALPHA-dri bedded housing with two to five 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.

Figure 1

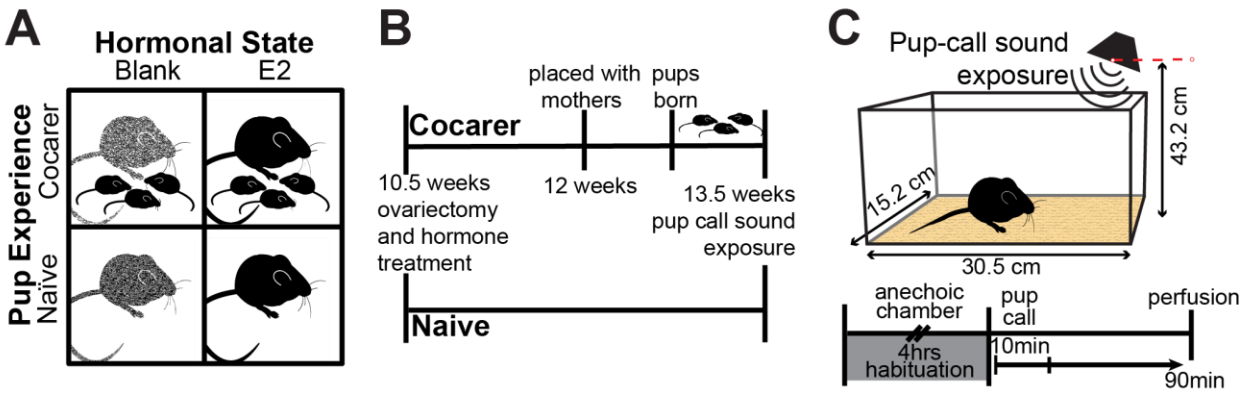

Figure 2-1 *Animal experimental conditions and timelines.*

(A) *Schematic of the four experimental groups used. Columns represent hormonal treatment (speckled = blank implant (B), solid = estradiol (E2) implant). Rows represent pup experience (top row = cocarers, bottom row = naive)* ***(B)*** *Timeline of experimental design* ***(C)*** *Sound exposure cage set up and timeline.*

2.4.2 Hormonal Manipulation

Animals in our 2x2 design (Figure 1A) were ovariectomized and randomly assigned to be implanted with a subcutaneous capsule (2 mm Silastic tubing, sealed with silicone aquarium sealant) containing either estradiol benzoate (E2) dissolved in sesame oil (50

μl at 3 mg/ml; n = 42), or a blank (B; n=43) control containing only sesame oil. Prior to implantation, all capsules were soaked in 0.9% saline solution and sterilized using hydrogen peroxide gas sterilization for 29 minutes (de Hoz et al., 2018).

On the day of experimentation, animals were an average of 19±4 days after hormone (or vehicle) implant surgery. The E2 implant concentration was chosen because two weeks after implantation, animals treated with this dose have plasma E2 levels comparable to mothers immediately before parturition (Barkley et al., 1979; McCormack et al., 1974; Miranda et al., 2014). Animals were housed individually after surgery for recovery. In 7 additional animals, ovariectomy and implantation of estradiol (n=3) and blank (n=4) capsules was performed and then the serum levels of estradiol in these animals tested 21 days later. Results confirmed that estradiol implanted animals had a significantly higher concentration of serum estradiol than the blank implanted animals ($p < 0.01$).

2.4.3 Pup Experience Manipulation

Blank and E2-implanted animals were assigned to one of two groups with different levels of pup experience: naive (N) or cocarer (CC). Naïve animals were singly housed

and not given any adult experience with pups. Cocarer animals were placed at around 12 weeks of age, after ovariectomy/implantation surgery and recovery, in a cage with a pregnant female littermate shortly before birth (Figure 1B). Cocarers spent 5-6 days caring for pups with the mother before being individually housed ahead of sound exposure the next day.

2.4.4 Stimulus Presentation

In mice, pups produce characteristic isolation calls when removed from the nest (Liu et al., 2003), which in turn elicit a maternal response from dams to find the vocalizing pups (Haack et al., 1983). On the day following separation into individual housing, each cocarer or naïve mouse in its home cage was placed in a sound-attenuating chamber (IAC Acoustics) equipped with a speaker (Figure 1C). All experimentation took place during the dark phase of the light cycle under red light. After an acclimation period of 4 hours (hr), we played a 10-minute (min) recording of natural ultrasonic *CBA/CaJ* pup isolation calls (n = 65 total animals, blank/naïve = 17, estradiol/naïve = 15, blank/cocarer = 16, estradiol/cocarer = 18) or a 10-min background noise recording (blank/naïve = 5, estradiol/naïve = 4, blank/cocarer = 5, estradiol/cocarer = 4). The pup isolation call recording consisted of concatenated one-minute bouts extracted from 10 different pups (Liu et al., 2003), sampled at 223 kS/s,

an average of 55 dB SPL with some calls reaching 95 dB SPL and high-pass filtered above 25 kHz to attenuate low frequency noise. The background noise stimulus consisted of 10-min-long segments from the pup isolation recordings, an average of 42 dB SPL that were also high-pass filtered above 25 kHz and clipped to exclude any pup vocalizations. An ultrasonic bat detector (Ultrasound Advice, MINI-3) was used to ensure the successful playback of the pup isolation calls. All animals remained in their respective sound-attenuating chambers for an additional 80 mins of silence following the 10-min stimulus to allow for immediate early gene (IEG) induction and expression (Chaudhuri et al., 2000). Animals were then euthanized using carbon dioxide and transcardially perfused with KPBS and 4% paraformaldehyde over the course of 10 mins.

As described in the Results, c-Fos-IR in response to the background noise was assayed for the LC, but not the AC. Research on immediate early genes from our laboratory showed that our type of background sound, while novel, does not elicit differences across pup experienced and inexperienced groups (Ivanova et al., 2017). Hence, here we focused in AC on comparing hormone and experience effects on the response to the *same* social sound stimulus.

2.4.5 Immunohistochemistry for Tyrosine Hydroxylase and c-Fos

Brains were removed from the skulls and post-fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose until no longer floating, and stored at 4C°. Brains were coronally sectioned at 50 microns (μm), and sections containing the LC or AC were stored in cryoprotectant at -20C°. Double-label immunohistochemistry using both c-Fos protein antibody (polyclonal c-Fos antibody raised in rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. No. sc-52) and tyrosine hydroxylase (TH) antibody (Immunostar, Hudson, WI, USA; cat. No. 22941) was conducted on every other LC section. Single labeling immunohistochemistry using c-Fos antibody was conducted on every other AC 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 mins in 3% 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 hr. Sections were then transferred to c-Fos primary antibody at a dilution of 1:2,000 in PBSTN (0.3% triton, 2% NGS) and stored at 4C° for 2 days with gentle agitation. After 2 days of incubation with c-Fos antibody, sections were rinsed in PBSTN and incubated for 1 hr 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 hr. Sections were washed in PBS, rinsed in acetate buffer, and protein expression was visualized using nickel-enhanced diaminobenzidine (niDAB). Multiple acetate buffer washes were complete before PB and then rinsing in 3% H_2O_2 for 20 mins. Sections were then rinsed in PBTN and transferred to TH antibody at a dilution of 1:1,000 in PBTN and stored at 4C° for 2 days. After 2 days of incubation with TH antibody, sections were rinsed in PBTN and incubated for 1 hr 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 hr. Following ABC incubation, sections were washed in PB and protein expression was visualized using diaminobenzidine. AC staining was conducted as described above omitting the nickel enhanced TH antibody staining steps. After staining, brain sections were washed and stored in PB, mounted and cover-slipped.

2.4.6 Electrophysiological Mapping

Four additional female mice, between 12-14 weeks of age, were used to determine the alignment between electrophysiological and anatomical atlas maps, which were used as the basis of identifying tissue sections for a AC staining. Auditory brainstem response (ABR) and cortical electrophysiology were performed following protocols described in previous publications (Shepard et al., 2015b). Briefly, peripheral hearing thresholds

were assessed via ABR for clicks and tone pips at 8, 16, 24, 32, 64 and 80 kHz (3 ms, 21 Hz repetition rate) to ensure animals were responsive to auditory cues. Frequency responses were next mapped electrophysiologically across the left hemisphere's AC. Multiunits were recorded across a craniotomy over the left AC using a 4 MΩ 3 × 1 tungsten matrix microelectrode (FHC) with 305 μm interelectrode spacing. The electrode array was driven into layer 4 (400 μm), and pure tones were played back (60 ms, seven intensities from 5 to 65 dB SPL, 30 frequencies log-spaced 2–32 kHz) in pseudorandom order, with five repetitions of each frequency–intensity combination. Stimuli were presented with a free-field speaker positioned 20 cm from and 45° anterolateral to the right ear.

To identify the spatial extent of core AC, consisting of primary auditory cortex (A1), anterior auditory field (AAF), and ultrasound field (UF), we looked for clear frequency tuning and a peristimulus time histogram (PSTH) peak <40 ms from sound onset, and in the case of UF, we looked for best frequencies (BF) >50kHz (Stiebler et al., 1997; Shepard et al., 2015b). Sites at the cusp of the A1-AAF best frequency gradient reversal point were denoted as "AAFA1." Weak or absent PSTH peaks, or latencies >40 ms indicated that a site was outside core AC, belonging instead to ventral secondary (A2) or dorsal posterior (DP) auditory fields. BFs were defined as the frequency that generated the highest average spike rate over all the intensities equal to or less than the threshold

intensity. Individual animals' BF maps were generated by Voronoi tessellation (Matlab: voronoin) of all recording sites for a given animal.

After electrophysiological mapping, brains were electrolytically lesioned at dorsal and ventral sites located in different rostral-caudal locations within AC (A365 World Precision Instruments, Inc. 2.5uA 5s), followed by perfusion with 4% paraformaldehyde for tissue fixation. The tissue was then preserved in 30% sucrose before being sectioned at 40 μm thickness on a microtome. Sections were stained in an alternating fashion with Nissl, DAB parvalbumin (PV, Swant PV235 Mouse monoclonal 1:4000), and DAB calbindin (CB, Sigma C9848 Mouse monoclonal 1:4000) repeating every 3 sections. Lesions were localized on the sections and matched to a cortical surface image and penetration map of the AC generated during electrophysiological experiments. Each section was aligned to the cortical surface image to scale based on the spacing of each PV-stained or CB-stained section (every 3rd section, each with 40um spacing, corresponding to a total of ~120 μm spacing between sections). Expected spacing of lesions based on the cortical surface image was confirmed in sections. Relative sizes of each physiologically determined auditory region (A1, AAF, AAFA1, A2, UF, DP, as determined by a combination of BF and response onset latency) on each section were estimated from the relative pixel conversion of the size of each region found on the corresponding cortical surface image.

Figure 2

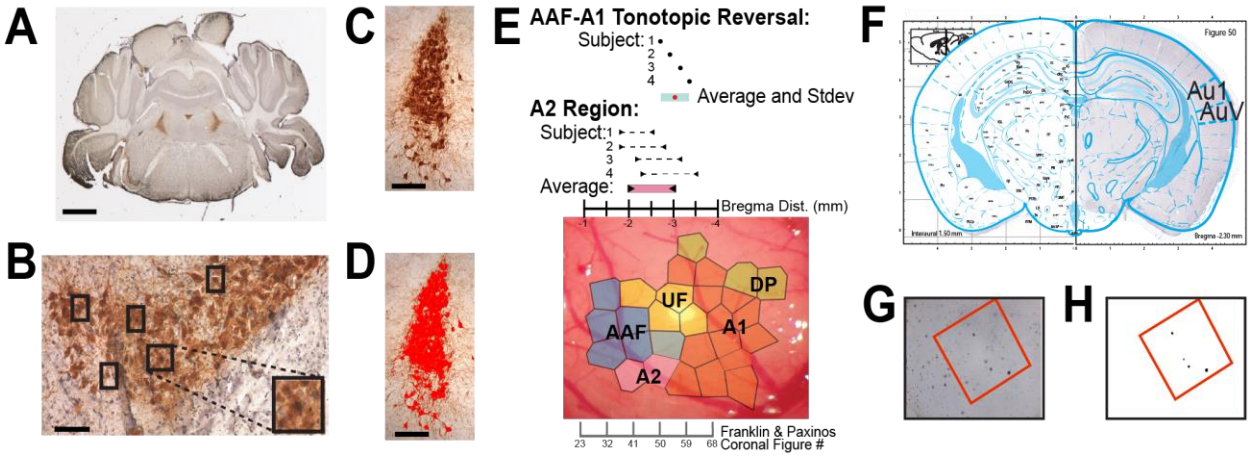

Figure 2-2 *TH/c-Fos immunohistochemistry, quantification and methodological steps for the cortical c-Fos-IR neuron counting in the AC.*

(A) *Whole-slice image of TH (brown) staining in the LC. Scale bar is 1mm.* ***(B)*** *Double stained LC where TH-IR brown staining localizes in cell bodies and c-Fos-IR stains some nuclei purple. Boxes indicate TH-IR/c-Fos-positive co-stained cells. Scale bar is 10 µm.* ***(C)*** *Before and* ***(D)*** *after color-thresholded image of TH stained cells used to measure area of LC.* ***(E)*** *Electrophysiological mapping and atlas alignment image over auditory cortex for mapping and auditory region assignments. Coronally sectioned brains were aligned to mouse atlas (Franklin & Paxinos, 3rd ED). Bregma*

coordinates of the brain section at which the AAF-A1 transition occurs over four individual animal subjects: Red: mean bregma location of transition (-3.035mm); Blue: Mean +/- Stdev (-3.3218~-2.7482mm); Black: subject animals (n=4). Bregma Coordinates of A2 – Average Rostral ~ Average Caudal Bregma (mm): Pink: rostral~caudal average (–2.03~–2.94mm), black arrows: individual animals (n=4). Voronoi tessellations overlaying example image of cortex delineate auditory cortical area assignments. ***(F)*** *Example of an immunohistologically stained section scanned at 4x and overlaid with the best matched atlas figure image (Franklin & Paxinos, 2008).* ***(G)*** *20x images with 200x200 µm square area at the center of the cortical area of interest selected for thresholding* ***(H)*** *Thresholded 200x200 µm square identifying neurons with c-Fos-IR.*

2.4.7 Quantification

Protein expression in the LC was quantified in 6 ± 2 (mean ± standard deviation) tissue sections per animal, spanning the full histological depth of the LC, verified by TH staining. TH staining clearly delineated the LC at approximately 5.34 mm to 5.80 mm caudal to Bregma (Figure 2A). Using a light microscope (Zeiss Axioplan) with an attached camera, images of the LC were taken with a 20x objective in the imaging program, MagnaFire. Using the 40x objective of the light microscope, c-Fos-expressing

TH-immunoreactive (TH-IR) cells were quantified by eye in the LC by an observer blind to treatment condition. TH and c-Fos costained cells were identified as a TH-IR stained cell body surrounding a purple c-Fos-positive stained nucleus (Figure 2B). The density of brown TH-staining of cell bodies precluded the quantification of individual TH-stained neurons, therefore the area covered by TH-IR cells in each section was manually color thresholded and measured to include all cells with TH-IR using NIH Image J (Figure 2C). The area of TH-IR was measured at 20x in square pixels and converted to square millimeter. The number of c-Fos-positive TH-IR cells, which were clearly discernible as niDAB-labeled nuclei, was expressed as a proportion of the thresholded LC area.

Coronal AC sections were identified at 2x using a slide imager (Meyer Instruments Path Scan Enabler IV slide imager). Both hemispheres of each section were matched to the best representation of its cortical position using The Mouse Brain atlas (Franklin et al., 2013)and overlaid with the matching atlas figure using Adobe Photoshop CS6 (Figure 2F). The Mouse Brain atlas presents sequential coronal sections as numbered figures, and the convenient asymmetry of the hemispheres in each figure allow for close matching between stained sections and hemisphere specific atlas images. Each altas hemisphere containing Au1 and/or AuV was assigned specific rostral-caudle positions on an ordinal scale. The hemispheres of each 2x stained section image were overlaid

with the best matching atlas figure. This aided in 20x magnification imaging of Au1 and AuV using a light microscope (Zeiss Axioplan).

The center of the cortical region of interest was identified and layers V/VI imaged using a light microscope with an attached camera and Magnafire software. Perpendicular and at least 50 μm distal from the floor of layer VI, a 200 x 200μm^2 square area was selected within an Atlas defined cortical brain region. To ensure unbiased stereological methodology, an observer blind to condition assigned the square location, with no regard to cell expression and conducted this analysis.

Layers V/VI were chosen for analysis because estrogen receptors have been primarily found in these deeper layers in mouse AC (Charitidi et al., 2010), and thus we anticipated the greatest effect of E2 may occur here. Moreover, layers V/VI of both the primary and secondary auditory cortices send output projections to subcortical and other cortical sites (Llano et al., 2008), making them particularly relevant for characterizing auditory cortical interactions with other brain areas.

ImageJ software was used to manually threshold our 200-μm^2 squares using the Yen filter, process for noise smaller than 9 pixels and count remaining objects to determine number of c-Fos-IR neurons. Imperfections such as small bubbles or debris within the count area prompted counting by eye. AC counts were converted to densities in cell/ mm^2 to facilitate consistent statistical analyses of both LC and AC.

Auditory cortical sections for cell quantification were chosen using our electrophysiological boundaries as well as The Mouse Brain atlas (Franklin et al., 2013). Stained sections were matched to the best figure and hemisphere of the atlas (See Section 2.7). The dorsal and ventral boundaries of both the primary and secondary auditory cortical regions were determined using the atlas. The rostral and caudal extent of AAF to AI used were those of the atlas' Au1, i.e. from bregma -2.2mm to -3.6mm or atlas figure 49 to 61, similar to previous publications (Tsukano et al., 2015). We found the mean bregma coordinates of the physiological AAF-AI transition occurred at -3.035mm with a standard deviation of -3.3218~-2.7482mm (Figure 2E), comparable to previous publications that also used bregma positions within this range (Llano et al., 2008; Tsukano et al., 2015). The averaged rostral to caudal bregma coordinates of the physiologically determined A2 extended between -2.03mm to 2.94mm, or the left hemisphere of atlas figure 53 to the left hemisphere of figure 58 in The Mouse Brain atlas. This alignment of the physiological auditory cortical fields to the atlas thus specified the best sections to designate as the AC core, AI-AAF high frequency transition area (AC-hf) and secondary (A2) auditory regions for c-Fos-IR cell counts. We use the AC core, AC-hf and A2 terminology when describing c-Fos-IR results.

2.4.8 Behavior Analyses

A subset of video recorded from the 10-min playback of pup-calls was subjected to behavioral analyses. Video tracking was performed with TopScan (CleverSys Inc). Video preprocessing involved image distortion correction and calibration of arena to the 171.45mm length of the cage. A text file was generated containing positional data captured at 30 frames per second; this was the input for MATLAB functions written to analyze movement. For each video, average velocity was determined by creating a vector with frame-by-frame pixel displacement of the animal's center-of-mass. To account for errors in motion tracking, a 20th order one dimensional median filter was applied to the vector. Quadrant crossings were counted in MATLAB, based on the XY coordinates of the middle of the cage. Percentage of time spent stationary was determined by evaluating the distance travelled for each second. If the distance was within a radius of 1mm, it was considered to be random image jitter, and that one second period was marked as stationary. While using awake and freely moving animals can make it difficult to unambiguously assess attention to or arousal by the stimulus, we used movement during sound playback as a proxy.

2.4.9 Statistical Analysis

Statistical analyses were performed in JMP data analysis software, unless otherwise stated. For each of the 3 brain regions and 3 behavior measures, the Huber Robust Fit Outlier application was used to identify cell count per section and behavioral outliers. Of the 423 LC, 354 AC core and 345 A2 section cell count and area measurements, 13, 2, and 3 were omitted from data analyses respectively. The LC outliers contained counts from all 4 groups (6 E/CC, 2 B/CC, 3 E/N, and 2 B/N). In the behavioral analysis, 1 animal was identified as an outlier with the Huber robust fit test for Average Velocity and Quadrant Crossing. Video of this outlier's behavior showed perpetual circular running motion throughout the 10-min pup-call playback. This animal was not sacrificed for c-Fos-IR and based on its outlier status and unusual behavior, it was removed from all behavior analyses.

Two-way ANOVA was conducted for each brain area. To calculate the average LC c-Fos-IR per animal, first the sum of all an animal's LC section counts was divided by the sum of the animal's calculated LC area. Second, this was multiplied by the pixel to mm conversion to produce an average LC-IR per animal. This method did not overly represent sections with high cell counts and small areas. Chi-squared test was used to compare the LC c-Fos-IR of animals, which experienced playback or background stimuli. AC core and A2 averages per animal were calculated by averaging all an animal's cell counts per 200 μm^2 and converted to c-Fos-IR per mm^2. Tukey's post-hoc

analysis was used, unless the Levene test indicated a significant difference in variance across groups, in which case Steel-Dwass was run instead.

To determine how our experimental conditions influence neural activity, we applied a Generalized Linear Mixed Model and considered that c-Fos-IR would depend on brain region, treatment group as well as nuisance parameters associated with an individual animal. Our statistical model treats our c-Fos-IR count observations as (quasi-) Poisson distributed, with rate parameter given by:

$$n \sim \text{Poisson}(\lambda)$$

$$\log\lambda_{rai} = \beta_{r0} + x_{ha}\beta_{rh} + x_{ea}\beta_{re} + x_{ha}x_{ea}\beta_{r,h\times e} + \log A_i$$

where x is the treatment value (0 or 1), β is effect magnitude, A is sample area, and the indices are r for brain region, a for animal, h for hormone, e for experience, and i for section ID. Unlike the two-way ANOVA analyses, no average per animal values were used here, and instead individual section counts and areas were labeled by their animal's ID. Using a quasi-Poisson distribution and mixed modeling, the covariance due to samples coming from the same animal was directly estimated, strengthening the statistical power compared to per-slice pooling or per-animal total pooling analysis. The

cross-region covariance was determined by plotting the per-animal random effects for two regions.

2.5 RESULTS

We sought to determine whether the way in which the neurochemical E2 modulates the noradrenergic LC and the AC when listening to conspecific vocalizations is affected by prior experience learning the social relevance of the sounds. Adult female mice aged 10.6±0.83 weeks (mean ± standard deviation) were ovariectomized and assigned to 1 of 4 animal groups. We used a 2x2 factor experimental design to investigate how pup care experience (N vs. CC) affects the expression of the IEG *c-Fos* in response to the playback of pup isolation calls, while controlling for systemic estrogen (E2 vs. B). Animals were exposed to a pup-call sound stimulus at 13.4±0.9 weeks, 19±4 days after hormone (or vehicle) implant surgery. We measured c-Fos-IR in the LC, as well as in cortical areas including the typical physiological extent of AC core, a high frequency AC subregion and A2, to characterize the response and relationship between these brain regions to novel or familiar social auditory stimuli.

Figure 3

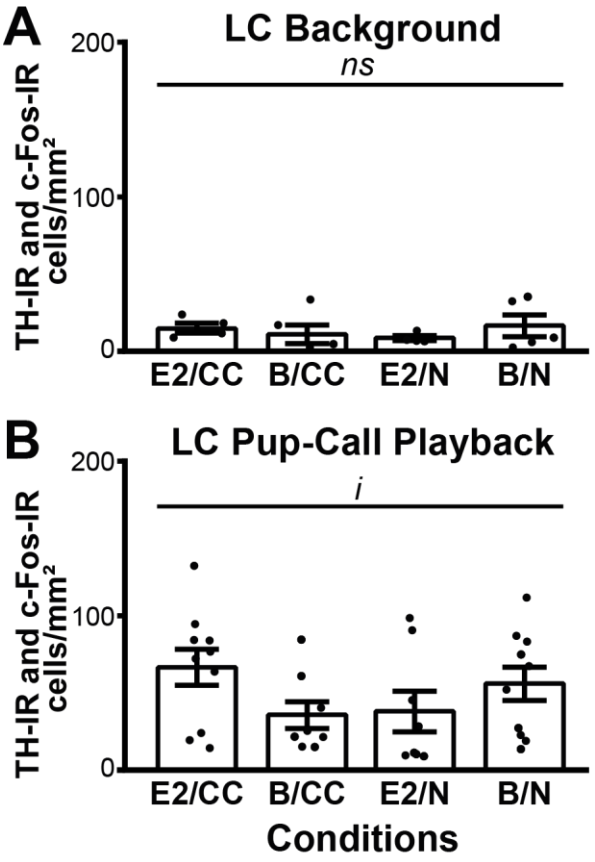

Figure 2-3 *LC c-Fos-IR expression varies between stimuli, with pup-call playback producing differences in expression dependent on pup experience and hormone treatment.*

C-Fos-IR per mm² in relation to pup experience and hormonal state conditions. ***(A)*** *LC response to playback of sound stimuli filtered to remove pup-calls (background). No significant difference between groups.* ***(B)*** *LC c-Fos-IR in response to unfiltered sound stimuli containing pup-calls, showed an interaction between pup experience and hormone state but no main effects. ns, not significant; i, interaction,* $p < 0.5$.

2.5.1 Differential impact of experience and hormone on pup-call-induced LC and AC c-Fos-IR

We quantified c-Fos-IR using multiple tissue sections from the three brain regions of each animal (see Section 2.7). We determined that a sound stimulus containing background noise without pup-calls did not modulate LC expression of c-Fos-IR across our animal groups ($F_{1,34} = 0.45$, $p = 0.715$), where expression levels were overall fairly low (Figure 3A). However, there was a significant difference between the LC c-Fos-IR of animals who experienced background sound playback and those who experienced pup-call playback (χ^2 (1) = 19.39, $p < .0001$, not shown). In the LC of animals hearing pup-calls, there was no main effect of experience ($F_{1,34} = 0.64$, $p = 0.21$) or hormonal treatment ($F_{1,34} = 0.44$, $p = 0.51$) of c-Fos-IR. However, there was a significant interaction between the two factors ($F_{1,34} = 5.20$, $p = 0.029$) (Figure 3B), indicating LC

activity following pup-call exposure depends on both hormonal state and prior pup experience. No post-hoc significant differences between groups were found.

In quantifying AC expression, we first examined sections taken over the entire rostral-to-caudal extent of the anatomically defined AC core region, averaging cell counts for each animal (1.46 mm span, see Methods). Here, we found a main effect of experience leading to a decrease in c-Fos-IR ($F_{1,12} = 12.54$, $p = 0.004$), but neither a main effect of hormone ($F_{1,12} = 2.05$, $p = 0.17$) nor an interaction between hormone and experience ($F_{1,12} = 0.10$, $p = 0.76$).

Figure 4

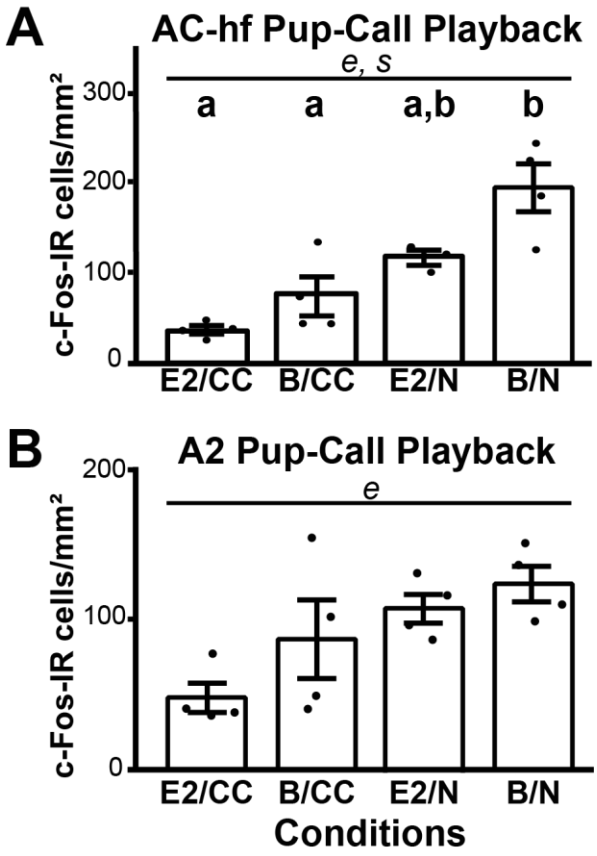

Figure 2-4 *C-Fos-IR expression in response to pup-call playback varies within layers V/VI of the Auditory Cortex, and depends on pup experience and hormone treatment.*

C-Fos-IR per mm² in relation to pup experience and hormone exposure conditions. ***(A)*** *AC-high frequency region and* ***(B)*** *A2.* ***(A)*** *In the AC-hf region there was an effect of hormonal state. Both in the AC-hf region and* ***(B)*** *in the A2 there is a main effect of*

experience. Points indicate average c-Fos-IR in 1mm² and bars indicate standard error. e, experience, $p < 0.5$; s, hormonal state, $p < 0.5$.

Previous suggested a region within the AC core, near the point of tonotopic map reversal between A1 and AAF where neurons are particularly sensitive to vocalizations (Issa et al., 2014). Thus, we also performed a restricted analysis on this physiologically validated (see Section 2.4) high frequency region of AC core (AC-hf). In the AC-hf we saw a significant decrease in c-Fos-IR, associated with a main effect of pup-care experience ($F_{3,12} = 28.43$, $p = 0.0002$). Additionally, a main effect of E2 treatment ($F_{3,12} = 9.33$, $p = 0.011$) emerged (Figure 4A). Unlike the LC, there was no interaction between hormone and experience ($F_{3,12} = 1.22$, $p = 0.29$), and instead, it appeared that both E2 and pup experience tended to reduce c-Fos-IR. Indeed, post-hoc analyses found a significant difference between E2/CC and B/N animals ($p = 0.0003$, Tukey-Kramer) in this AC-hf region, as well as between B/N and B/CC animals ($p = 0.002$, Tukey-Kramer). Thus, five days of social experience, and to some extent hormone state, led to decreased c-Fos-IR in response to social auditory stimuli in the AC-hf subregion.

We next quantified animal-averaged c-Fos-IR over a physiologically validated A2 region (Figure 2E). We found a main effect of experience ($F_{1,12} = 11.91$, $p = 0.004$), but not of hormone ($F_{1,12} = 2.98$, $p = 0.10$). There was also an absence of an interaction ($F_{1,12} = 0.36$, $p = 0.55$) (Figure 4B). Unequal variance between groups ($p = 0.03$, Levene) can be attributed to the significance high variance in B/CC group's c-Fos expression,

necessitated non-parametric post hoc tests that found no significant post-hoc differences (Steel-Dwass). Nevertheless, 5 days of social experience led to the decrease in c-Fos-IR in the A2, as in AC core and AC-hf. Notably, out of the 3 regions examined, the effect of experience was the greatest in the AC-hf.

Figure 5

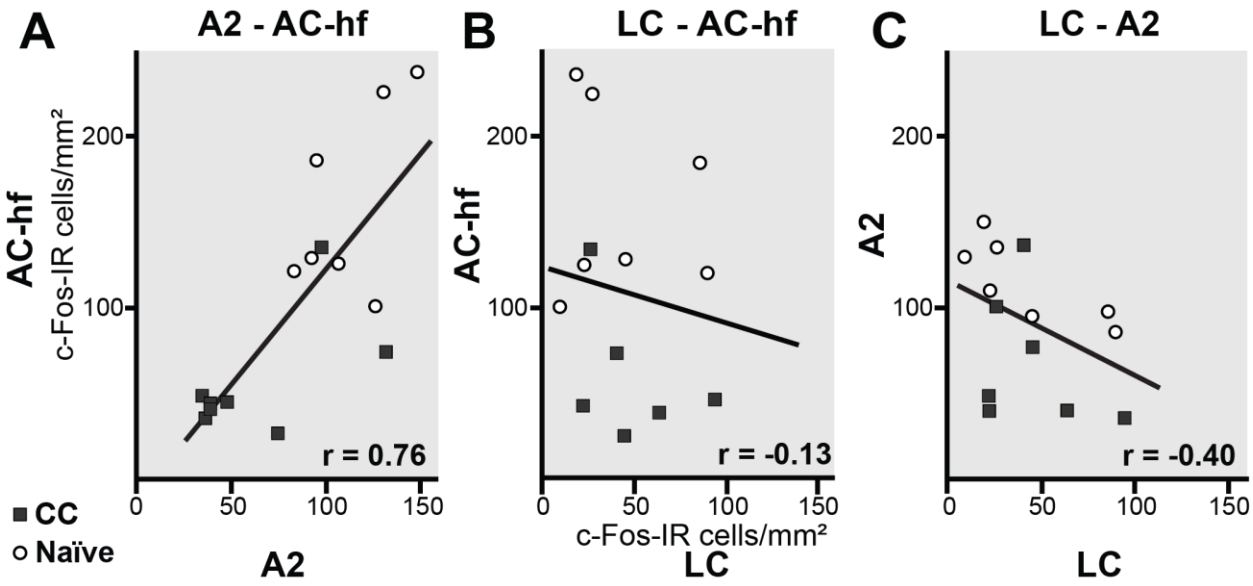

Figure 2-5 *Correlation between brain regions.*
C-Fos-IR expression averages per animal and brain region with solid line being best fit; ***(A)*** *AC-hf and A2 (r = 0.76, p = 0.0009)* ***(B)*** *AC-hf and LC (r = 0.13, p = 0.64) and* ***(C)*** *A2 and LC (r = -0.40, p = 0.14).*

Lastly, we collected and analyzed sections from all three brain regions in a subset of animals to consider the cross-region covariance (Figure 5). There was a high correlation

between the two auditory cortical areas, AC core and A2 (r = 0.83, $p < 0.0001$, not shown) and between the AC-hf and A2 (r = 0.76, $p = 0.0009$, Figure 5A), agreeing with the expectation that sound-driven AC core and A2 activation in the same animal should be related. There was little to no correlation between LC and AC core (r = - 0.21, $p = 0.46$, not shown) or AC-hf (r = - 0.13, $p = 0.65$, Figure 5B), and a non-significant negative correlation between LC and A2 (r = -0.40, $p = 0.14$, Figure 5C).

Table 2-1 ***GLMM confidence-interval, z-value and p-value results.*** *A GLMM was run using all available data in the LC, AC-hf and A2 to determine the fixed effects of estrogen, experience and possible interactions on c-Fos-IR.*

Fixed Effect	*z*-value	*p*-value	Confidence Interval	
			lower	upper
E2 on AC-hf:	-0.4981	0.115	-1.125	0.035
E2 on A2:	-0.1163	0.6207	-0.574	0.343
E2 on LC	-0.5921	0.0780	-1.275	0.060
CC experience on AC-hf	-1.0584	5.50e-06 ***	-1.498	-0.550
CC experience on A2	-0.5172	0.0180 *	-0.935	-0.055
CC experience on LC	-0.4041	0.2211	-1.046	0.224
Hormone/Experience Interaction in AC-hf	-0.298	0.6917	-0.997	0.570
Hormone/Experience Interaction in A2	-1.109	0.3042	-0.990	0.570
Hormone/Experience Interaction in LC	1.4002	0.0030 **	0.447	2.344

2.5.2 GLMM Analysis Confirms Differential Effects of Experience and Hormone on LC and AC

The analyses above relied on c-Fos-IR expression averaged within animal, reducing the ability to leverage the statistical power of multiple observations within an animal. To further confirm the strength of our results, we next applied a GLMM (see Section 2.9) to the data from each tissue section. The mixed model accounted for sampling differences

between brain regions, the fact that observation from the same animal are not independent and considered individual animal variation in overall c-Fos-IR expression. Our measurements were standardized as cells per mm² in the AC-hf, A2 and LC. B/N animals were set as the baseline, and the analysis was bootstrapped with 1000 resamples (Table 1). In situations with several experimental variables, this approach can yield higher statistical power than traditional methods that make it difficult to account for numerous sources of experimental variance (Li et al., 2013).

Figure 6

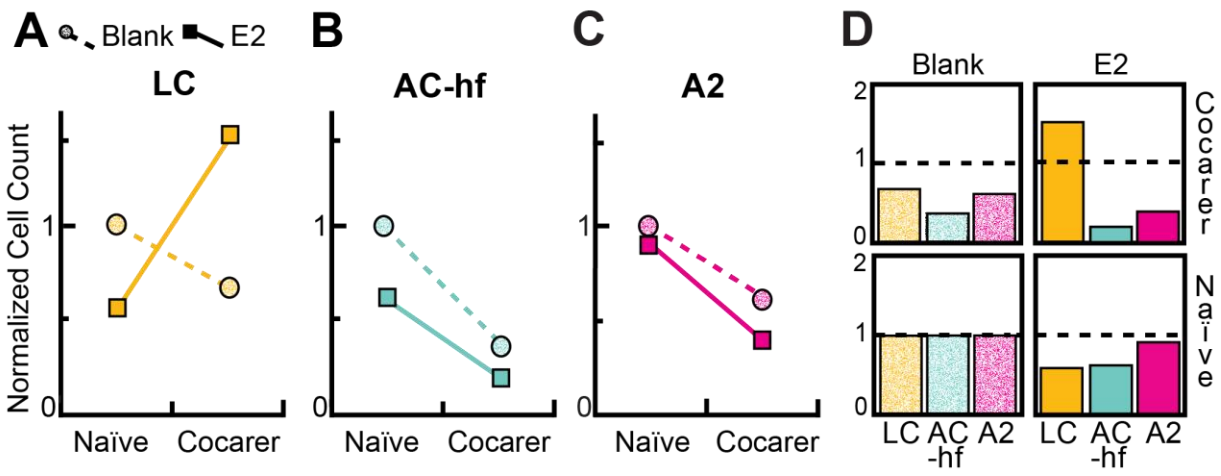

Figure 2-6 *GLMM analysis verifies majority of ANOVA findings.*

(A) *In the LC, there is an interaction between pup experience and hormone state* (p = *0.008) but no main effects.* ***(B)*** *The Au1-high frequency region displayed a robust main effect of experience* (p = *6.55e-06) significantly decreasing c-Fos-IR with experience.* ***(C)*** *The A2 there was a main effect of experience. A2 c-Fos-IR per mm²*

*also decreases with pup experience (*p = *0.020). **(D)** Bar graph representation of normalized results indicates c-Fos-IR in each of the three brain regions for the 2x2 experimental conditions. Normalized to blank/naïve condition values and set at 1 (represented by dashed line).*

As with the animal-averaged ANOVA analyses, the GLMM found a significant interaction between E2 availability and pup experience in the LC (p = 0.008), as demonstrated by the crossed-lines in Figure 6A showing the normalized cell counts. Further supporting the ANOVA findings, the GLMM also found decreases in c-Fos-IR expression as a main effect of pup experience in the AC-hf ($p = 5.50^{-6}$), and in A2 (p = 0.018) (Figure 6B-C). A main effect of hormone on AC-hf expression, reported in the ANOVA analysis, was trending (p = 0.093), as was a similar main effect on LC (p = 0.058), suggesting a weak direct hormone effect in these brain areas.

Figure 7

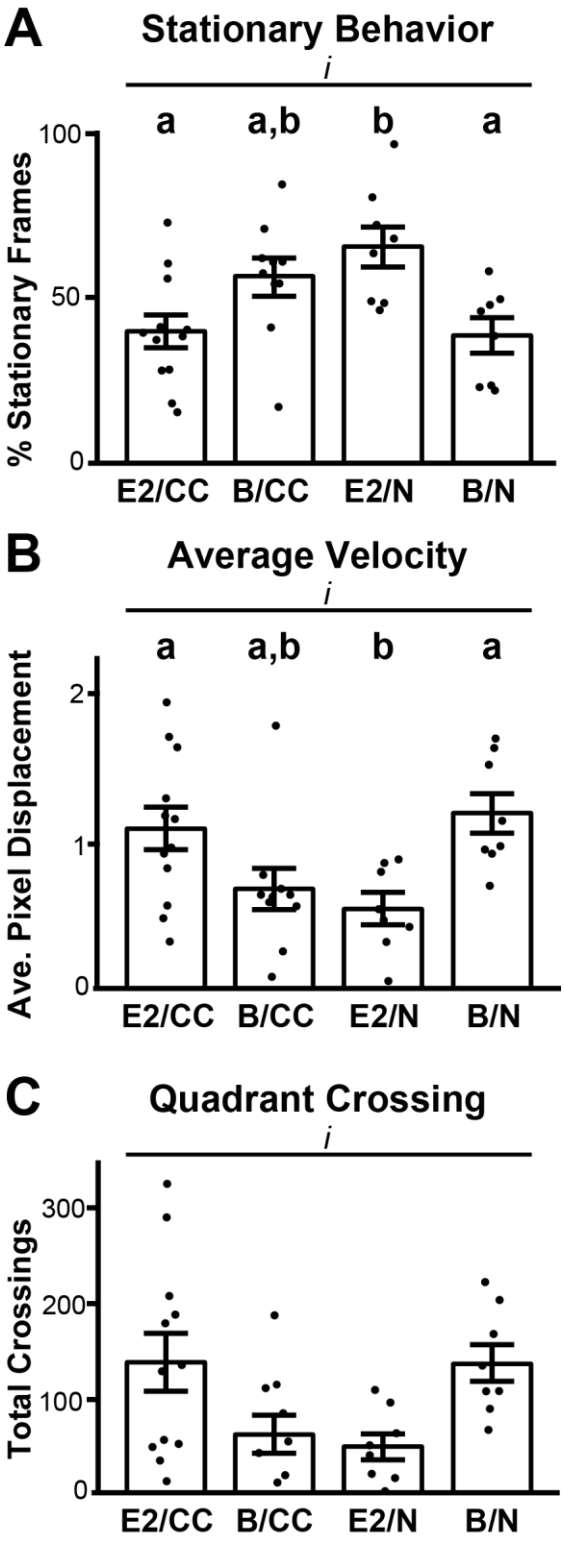
A
Stationary Behavior
i
a
a,b
b
a
% Stationary Frames
100
50
0
E2/CC
B/CC
E2/N
B/N
B
Average Velocity
i
a
a,b
b
a
Ave. Pixel Displacement
2
1
0
E2/CC
B/CC
E2/N
B/N
C
Quadrant Crossing
i
Total Crossings
300
200
100
0
E2/CC
B/CC
E2/N
B/N

Figure 2-7 *Behavior during 10 minute playback.*
(A) *Percent stationary frames per condition. Bars are standard error. No significant difference between social experience and hormone state, but a significant interaction between experience and hormone (*p = *.0004). Post-hoc, there is a significant difference within E2 treatment group (E2/N versus E2/CC, p = 0.009, Tukey-Kramer), as well as the naïve condition group (E2/N versus B/N,* p = *0.01, Tukey-Kramer).* ***(B)*** *Average velocity during movement showed no significant main effect of experience or hormone, yet a significant interaction between experience and hormones (*p = *0.0006) and post-hoc significant differences within the E2 treatment group (*p = *0.03, Tuckey-Kramer) and the naïve condition group (*p = *0.02).* ***(C)*** *Number of quadrant crossings within the time of playback revealed no significant effects for experience or hormone but the behavior was significantly affected by an interaction of E2 and pup experience (*p = *0.001).*

2.5.3 Differential effects of experience and hormone on behavioral response to pup-call playback

In a subset of animals (n = 38), videos of the 10-min playback session were analyzed to assess coarse behavioral differences across groups in response to pup-call stimuli

playback. The behaviors analyzed included the percentage of time spent stationary, average velocity, and number of quadrant crossings. All three behaviors showed significant interaction between hormone treatment and pup-experience.

Although data on the percentage of time spent stationary (Figure 7A) revealed no significant effect for experience ($F_{1, 35} = 0.53$, $p = 0.47$) or hormone ($F_{1, 35} = 0.83$, $p = 0.36$), we found a significant interaction between experience and hormone ($F_{1, 35} = 15.63$, $p = 0.0004$). E2/CC and B/N groups showed the lowest levels of stationary frames. Post-hoc analyses showed a significant difference within E2 treatment groups (E2/N versus E2/CC, $p = 0.009$, Tukey-Kramer), as well as a significant difference within the naïve condition groups (E2/N versus B/N, $p = 0.01$, Tukey-Kramer).

When animals were moving, an analysis of average velocity (Figure 7B) found no significant main effect of experience or hormone ($F_{1, 34} = 0.01$, $p = 0.90$; $F_{1, 34} = 0.68$, $p = 0.41$). Yet like stationary behavior, this behavior measure displayed a significant interaction between experience and hormones ($F_{1, 34} = 14.25$, $p = 0.0006$). Significant post hoc differences were present within the E2 treatment groups (E2/N versus E2/CC, $p = 0.03$, Tukey-Kramer), as well as within the naïve condition groups (E2/N versus B/N, $p = 0.02$, Tukey-Kramer). The quadrant crossings data (Figure 7C) also revealed no significant effects for experience or hormone ($F_{1, 34} = 0.53$, $p = 0.47$; $F_{1, 34} = 0.47$, $p = 0.40$) but the behavior was significantly affected by an interaction of E2 and pup experience $F_{1, 34} = 11.52$, $p = 0.001$). Number of quadrant crossing did not show

significant post hoc differences, though there was a trend towards significance within the E2 treatment group (E2/N versus E2/CC, $p = 0.055$, Tukey-Kramer).

The interaction between experience and E2 treatment for our three behaviors resembled the interaction we found in LC c-Fos-IR (Figure 3B), though correlations between behavioral measures and LC c-Fos-IR for the smaller group of animals where we obtained both data (n=12) did not reach significance (stationary behavior r = -0.45, $p = 0.13$; average velocity r = 0.46, $p = 0.12$; quadrant crossing r = 0.43, $p = 0.15$).

2.6 DISCUSSION

Our work used an ethological learning context (Bennur et al., 2013) to study how the sensory plasticity-related, noradrenergic neuromodulatory area LC is engaged as a function of prior experiences and internal hormonal state. We exploited a mouse model of maternal recognition of pup-calls, which arises through experience caring for pups and is facilitated by the hormone E2 (Stolzenberg et al., 2009; Koch et al., 1989a). Our manipulation of E2 availability and pup care experience in virgin females allowed us to model the physiological and experiential state of motherhood (Miranda et al., 2014) to test how these factors affect neural responsiveness in both LC and AC to infant vocalizations when they are either novel or familiar.

When we exposed female mice to the sound of pup vocalizations, but not a sound containing behaviorally irrelevant background noise, we found prior experience caring for pups and the presence of maternal levels of E2 interacted to alter the expression of the plasticity-related IEG, *c-Fos*, in noradrenergic LC neurons. Estrodiol-treated females with pup experience (E2/CC) and naïve females without estradiol (B/N) showed the highest LC c-Fos-IR, average velocity behavior and number of quadrant crossing behavior as well as the lowest amount of stationary time during the pup call playback. Familiarity with pup-calls significantly decreased the c-Fos-IR expression in both AC core and A2, especially in the central AC-hf region, previously linked to vocalization processing (Tsukano et al., 2015; Issa et al., 2014). Thus, far from being stereotyped and hard-wired, the brain's response to natural vocalizations is situationally dependent on hormonal state and prior familiarity with the vocal category. Below we interpret our results in light of the current literature on LC and AC, placing them in the context of a working model wherein a social stimulus' familiarity leads to the genomic activation of a reduced subset of auditory cortical neurons compared to when the sounds are initially novel.

2.6.1 LC processing of infant cues

We found playback of the pup-calls, but not background noise, led to elevated LC c-Fos-IR, indicating that species-specific infant vocalizations engage this key neuromodulatory area implicated in cortical plasticity (Edeline et al., 2011; Martins et al., 2015). This result is consistent with the importance of the neuromodulator NE in maternal learning and behavior. Dams lacking the enzyme dopamine beta-hydroxylase, which synthesizes NE from dopamine, fail to exhibit maternal responsiveness (Thomas et al., 1997). Curiously, restoring the ability to synthesize NE in these knock-out animals by administering before first parturition a synthetic precursor for NE production reestablishes maternal behavior for the first litter and subsequent litters, suggesting that NE not only affects initial maternal responsiveness but also maternal memory (Scanlan et al., 2006; Levy et al., 1990). That such a memory could involve NE-mediated experience-dependent plasticity for the sensory cues associated with infants has been suggested previously (Miranda et al., 2009; Banerjee et al., 2013).

C-Fos expression is facilitated by phasic firing (Labiner et al., 1993; Dragunow et al., 1989b), which is induced in LC by arousing stimuli (Devilbiss et al., 2011; Martins et al., 2015). Our significant interaction between experience and hormones in LC c-Fos-IR therefore suggests the identical stimulus is differentially arousing to these animal groups, a result reinforced by our behavioral analysis (Figure 7). On the one hand, although pup-calls can be arousing when novel, inducing higher LC c-Fos-IR in blank-

implanted naïve mice, elevated systemic E2 dampens this response, consistent with E2 reducing general stress and hyperactivity (Tang et al., 2005). On the other hand, while calls are familiar and perhaps less arousing to blank-implanted cocarers, E2 heightens their arousal and LC c-Fos-IR, potentially reflecting an ability of reproductive hormones to enhance maternal responsiveness to previously experienced pup cues (Fleming et al., 1990). Notably, the contrast in LC's versus AC's pattern of c-Fos-IR expression across groups further reinforces the conclusion that the auditorineural drive is not the only determinant of LC response in this sound exposure paradigm, which must also depend on both internal physiological factors and prior experience.

The prior experience we highlighted here was pup care, but it should be noted that our housing condition also introduced a separate experiential factor that could potentially impact our findings. Specifically, our naïve animals were individually rather than socially housed in the days between ovariectomy and sound exposure, unlike our cocarers who were housed with mothers and their pups. Even though long-term social isolation in mice can lead to abnormalities such as more anxiety-like behavior, greater hyperactivity, impaired recognition memory and changes in social interactions (Valzelli, 1973; Koike et al., 2009; Voikar et al., 2005) our naïves' social isolation alone likely cannot explain their pattern of behavioral and LC c-Fos-IR results. First, social isolation effects on behavior are usually studied after at least 4-weeks or more of isolation after

weaning – longer than the period that our naïve, adult animals were isolated. Second, even though *c-Fos* expression in socially isolated rodents is associated with increased levels of catecholamines such as norepinephrine (Heritch et al., 1990), we did not see a baseline elevation of average LC activation in naïve vs. cocarer animals hearing background noise (Figure 3A). Third, pup-call playback also did not change the average LC activation between these two groups, which might have been expected based on social isolation effects on *c-Fos* in other brain areas after brief social encounters (Ahern et al, 2016). Hence, our findings of significant experience and hormone interaction rather than main effects on LC c-For-IR likely do not arise from our naïve subjects' social isolation.

2.6.2 Experience-dependent changes in AC

As noted above, we found c-Fos-IR in AC (Figures 4 and 6) had a strikingly different dependence on hormones and experience compared to LC. Familiarity with a social auditory cue was associated with decreased c-Fos-IR in both auditory cortical sites examined. Previous literature has differed on whether familiarity with a cue increases or decreases c-Fos expression within the AC. Rats listening to complex, non-social sounds show no difference in the primary AC c-Fos-IR between novel and familiar sound playback while the secondary associated area displays a significant decrease in c-Fos-IR

when the sound is familiar (Wan et al., 2001). On the other hand, in the ultrasonic vocalization literature, stimulation with pure tone models of pup-calls induce lower levels of c-Fos-IR in the ultrasonic field of the core AC in dams compared to naïve females, while the former expresses higher secondary AC c-Fos-IR than the latter (Fichtel et al., 1999). The A2 response was associated with the "news-worthy" nature of the stimuli (Geissler et al., 2016). This discrepancy with our results for A2 may be due to our use of natural pup-calls, which, while technically novel, potentially sound more acoustically familiar to dams than tonal models of calls, and thus be less "news-worthy."

In considering our AC c-Fos-IR results, it is important to note that animals were free to move within the cage, and thus could have experienced different sound levels for calls during the playback. Despite this caveat, which undoubtedly creates some variability across animals, it is unlikely that variation in sound level from the positioning of the animal could explain the large, systematic group differences in auditory cortical responses (Figure 4). In fact, movement is not correlated with AC c-Fos expression, as is particularly obvious for E2/CC vs. B/N animals (compare Figure 7 to Figure 4, showing similar average movement, but vastly different AC c-Fos-IR).

Mouse pup communication sound processing is believed to be lateralized to the left hemisphere (Geissler et al., 2004; Ehret et al., 1987; Marlin et al., 2015). Here, we did not consider hemispheric cell expression in the collection of our data, but the persistence of a significant effect of pup experience despite this homogenization speaks

to the robustness of our observed effects. Within core AC, an AC-hf sub-region has been linked to species-specific vocalization processing in mice (Issa et al., 2014; Tsukano et al.). In fact, Tsukano et al. delineate a part of this transition area as a dorsomedial sub-field that is particularly sensitive to ultrasonic vocalizations. Our finding that pup familiarity effects on pup-call induced responses were highest in this region, which we delineated through alignment to electrophysiological maps (in separate animals), adds further support for this region's heightened importance within AC for communication sound processing. Further research is needed though to determine whether this area specifically processes vocalizations such as regions identified in the human AC (Belin et al., 2000), or one that is activated by ultrasonic vocalizations simply because of its high frequency responsiveness.

That experience with pups consistently reduces pup-call induced c-Fos-IR in core AC may seem counter-intuitive, especially if increased AC activation is presumed to underlie sound recognition. In fact though, expansion in the area of auditory cortex responding to a newly behaviorally relevant signal is not observed as pup-calls gain significance (Shepard et al., 2015b). Instead, one should consider that sensory cortices may balance stable representations of previously learned stimuli against plasticity for newly relevant stimuli. Since our pup experienced animals had five-days of experience with vocalizing pups by the time we exposed them to call playback, these subjects may have already experienced substantial cortical plasticity to optimize pup-call recognition.

Since *c-Fos* expression not only reflects neural activation but also active cellular mechanisms for learning (Mayer et al., 2010; de Hoz et al., 2018), c-Fos-IR would then be low in response to the already familiar auditory cues. Diminished production of a plasticity associated protein in response to a familiar stimuli, that previously gained meaning through social experience, might be reflected in a reduced subset of neurons constituting the most behaviorally useful circuit (Kilgard, 2012) and preserving established sensorineural representation of those sounds.

While the effects of experience on AC c-Fos-IR were quite robust, we only found trending significance associated with estradiol exposure for AC and AC-hf, once we took our GLMM results into account. There are two possibilities as to why we did not observe a robust E2 effect, even if it were present. First, since our c-Fos-IR measure (assayed 90 minutes after sound playback began) decreased with experience, any modulation of plasticity that strengthens the effects of experience in AC would likely further decrease c-Fos-IR, which then runs the danger of being subject to a floor effect. Second, E2 may have a more obvious effect on AC responses at earlier stages of experience learning about the social meaning of the sounds, and our 5-day time point misses this. Future studies will need to resolve this uncertainty.

2.6.3 Correlation among brain regions

A correlation between cortical and subcortical brain regions was not observed (Figure 5), regardless of whether the sounds were novel or familiar. This lack of correlation was not just due to errors in our ability to measure c-Fos-IR, since we did find a strong correlation between AC fields (Figure 5A), which can be explained as a consistent, within-individual, sound-driven neural response. The lack of a strong correlation between c-Fos-IR in auditory processing regions and LC (Figure 5B-C), despite both brain areas being sound-responsive, presumably reflects the fact that canonical sensory pathways do not directly feed into LC, which instead is integrating both external and internal factors, producing arousal (Samuels et al., 2008). Moreover, LC's widespread projections, including to the cochlear nucleus (Ebert, 1996; Klepper et al., 1991) and sensory neocortex (Jones et al., 1985; Berridge et al., 2003), are presumably only one potential modulator of genomic responses in auditory cortex, so that any c-Fos-IR correlation based on this direction of connectivity would be weak. Intriguingly though, a previous study did find that pairing exogenous electrical stimulation of the LC with novel auditory stimuli in mice leads to plasticity in LC electrophysiological activity, and long-lasting improvements of AC electrophysiological responses to the paired stimuli (Martins et al., 2015). This raises the possibility that a stronger LC-AC correlation might be observed between an animal's initial sensory-evoked LC c-Fos-IR when sounds are novel, and its later AC c-Fos-IR when the sound is

familiar. Nevertheless, our results showing different patterns of c-Fos-IR across our experimental groups suggests at a minimum that the genomic response of these two sound processing and plasticity regions is situation-dependent.

2.7 CONCLUSION

We sought to determine how estradiol, social experience and the activity of a key neuromodulatory area contributes to enhancing processing of social auditory stimuli. We found further evidence for neural plasticity at key sites of auditory processing (AC-hf and A2) as social stimuli transitioned from being novel to familiar. Moreover, social sound-evoked activation of an important neuromodulatory center for sensory plasticity, LC, was not directly coupled to activity levels in cortical regions processing sounds, but instead reflected how behavioral arousal elicited by the sound can be affected by estradiol and prior sound experience. Together, these results highlight how the specific combination of internal hormonal state and past experience differentially impacts how individuals process the same sound (Figure 6D).

3 BDNF TRANSCRIPTION IN RESPONSE TO INFANT AUDITORY EXPERIENCE IN THE FEMALE MOUSE AUDITORY CORTEX

Authors: Amielle Moreno, Swetha Ragagopalan, Matthew Tucker, Parker Lunsford and Robert C. Liu

The following experiments and text were designed, conducted, analyzed and written with the collaboration of the authors listed above. Amielle Moreno's contribution to this project included, but was not limited to, experimental design, surgeries, running habituation and experiment day conditions and video recordings, brain tissue collection, RNA isolation, cDNA preparation and qPCR protocol development and conducting, ANOVA and other data analyses, final figure creation, writing and editing. Swetha Ragagopalan contributed to running habituation and experiment day and video recording, video behavior scoring, tissue collection, RNA isolation, data synthesis and analyses as well as coding for figure creation. Matthew Tucker developed RNA isolation and qPCR protocol with Amielle Moreno, video behavior scoring, performed tissue collection, RNA isolation and cDNA preparation, and contributed text for the methods section. Parker Lunsford developed tissue collection protocol and behavior scoring protocol with Amielle Moreno, conducted tissue collection and behavior scoring, and contributed text for the methods section. Experiments were conducted in the laboratory and with the guidance and editing support of Robert C. Liu.

3.1 ABSTRACT

While infant cues are often assumed to innately motivate maternal response, recent research highlights how infant cue processing can be enhanced through sensory cortical plasticity. Infant vocalizations are important social signals for caregivers, and evidence from mice suggests experience caring for pups induces plasticity in the auditory cortex (AC), which improves pup-vocalization detection and discrimination at the neural level. However, little is known about the molecular mediators for such AC plasticity during the initial pup experience. Here, we used the maternal mouse model, ovariectomizing and implanting virgin female mice with estradiol (E2) or blank (Bk) capsules to explore the behavior and AC molecular changes induced by the very first pup caring and vocalization experience. As expected, E2 increases the overall time spent performing maternal behavior during 1-hour of pup experience, with a lower latency to crouch over a nest of pups and less time engaged in non-maternal behaviors. We used qRT-PCR to assay how the memory-associated gene brain derived neurotrophic factor's *(Bdnf)* transcription is altered by the playback of pup-calls depending on the availability of pups to retrieve. Subjects hearing the pup-calls with no pups present had significantly lower AC *Bdnf* mRNA compared to females with pups present, suggesting the social context of vocalizations can induce immediate molecular changes at the site of auditory cortical processing. While E2 influenced the rate of maternal behavior, it did not have a significant effect on *Bdnf* mRNA transcription in the AC, ventral hippocampus or visual

cortex. Using a maternal mouse model for communication, we observed differential regulation of *Bdnf* exon IV in the AC after pup-vocalizations were paired with maternal experience. To our knowledge, this is the first time *Bdnf* has been associated with processing vocalizations in the AC. The BDNF protein is a potential molecular component responsible for enhancing future recognition of infant cues by contributing to AC plasticity.

3.2 HIGHLIGHTS

- E2 affected crouching behavior and time engaged with pups but not retrieval behavior.
- E2 implant did not affect *Bdnf* mRNA in the AC in response to social auditory cue experience.
- The exon IV transcript of *Bdnf* mRNA increases in the AC only when auditory cues were presented in a social context.
- *Bdnf* mRNA did not increase in the ventral hippocampus to social auditory cue experience.

3.3 INTRODUCTION

Across social species, auditory cues such as vocalizations, play an important role in communicating vital information between conspecifics (Poremba et al., 2013). Through social vocalizations, the sender and receiver can potentially gain beneficial outcomes, ultimately enhancing the chance of survival of oneself, kin, and one's genes through inclusive fitness (Dawkins, 1989). To convey their needs to caregivers, mammalian infants can use vocal cues to garner attention and care, insuring their development and strengthening the bond between caregiver and infant (Okabe et al., 2012). The detection and recognition of infant vocalizations through neural processing has the power to release a maternal response.

The subcortical circuitry mediating maternal behavior is highly conserved and maintains the essential components that direct an innate response to offspring (Numan, 2006; Kohl et al., 2017b). As caretakers gain experience with infants, plasticity at the genetic, molecular, and neuroendocrine levels occur in these canonical regions mediating maternal behavior (Fleming et al., 1990; Bridges, 2015; Stolzenberg et al., 2012). However, in most of these studies, the sensory representations that come to trigger maternal behaviors are assumed to already be attuned to species-specific infant cues.

In rodent research, the auditory system has demonstrated plasticity in the sensory representations of infant (pup) sounds after caring experience (Liu et al., 2006; Liu et al., 2007), and similar to what is seen in subcortical regions, reproductive hormones can

contribute to these changes (Ehret et al., 2009; Marlin et al., 2015). Even if much of maternal behavior is hard-wired within a species (Wu et al., 2014), learning about infants is also important, driving an interest in what mechanisms underlie neural plasticity in this and other social contexts, especially in the sensory cortex.

When an adult mouse gains experience caring for pups, their auditory cortex (AC) undergoes plasticity leading to significant differences in processing infant sounds between those with and without pup-caring experience (Fichtel et al., 1999; Liu et al., 2006). It is thought this plasticity allows for better detection and discrimination of ultrasonic vocalizations (USVs) emitted by mouse pups (Liu et al., 2007). Mouse pups innately produce bouts of USVs when separated from the nest, which signal their isolation and triggers caretakers to approach the isolated pup (Branchi et al., 2001). The maternal mouse model of communication capitalizes on this robust approach response to determine the social and hormonal components that drive pup-call salience (Koch et al., 1989a; Miranda et al., 2009; Marlin et al., 2015; Ehret, 2005).

Much of the research into auditory plasticity in the maternal context has compared the sensory representations of pup-experienced animals with those naïve to infant vocalizations (Geissler et al., 2004; Geissler et al., 2016). This leaves open the question of how AC plasticity is induced molecularly during and after initial pup experience. We investigated the hypothesis that molecular changes occur at the site of auditory processing after initial pup caring and vocalization experience.

In the maternal settings, initial pup experience coincides with high concentrations of estrogens, which are theorized to work in tandem with neurotrophic factors to enhance cognition and learning (Zhou et al., 2005; Singh et al., 1999; Pluchino et al., 2013). One likely factor for mediating cortical plasticity in concert with estrogens is brain derived neurotrophic factor, BDNF (Luine et al., 2013; Scharfman et al., 2006; Fortress et al., 2014b). This memory-associated trophic factor is affiliated with neuronal activity, memory formation, and spine density in response to experience-dependent learning (Marty et al., 1997; Lubin et al., 2008; Alonso et al., 2005). The visual, somatosensory, and auditory cortices express BDNF during developmental critical periods and sensory stimulation to induce plasticity (Huang et al., 1999; Rocamora et al., 1996; Tan et al., 2008; Anomal et al., 2013).

Bdnf gene transcription is controlled by multiple promoters that drive transcription of exons with unique regulation properties (Timmusk et al., 1993; Lauterborn et al., 1996). For example, the mRNA isoform containing the ‘5 exon IV displays activity-dependent transcription much like an immediate early gene (Lauterborn et al., 1996). This isoform is associated with sensory cortex expression (Pattabiraman et al., 2005; Nanda et al., 1998) as well as GABAergic interneuron activity of the prefrontal cortex (Sakata et al., 2009). In addition, epigenetic modifications to *Bdnf*’s exon IV promoter region, which contains a likely estrogen receptor complex binding site, arise during learning and extinction processes in the cortex (Sohrabji et al., 1995; Bredy et al., 2007).

Here, we explored the molecular nature of AC plasticity by measuring *Bdnf* transcription evoked in the female mouse AC after the first social experience with infant vocalizations as well as estradiol exposure. We designed our experiment around 4 animal groups, which varied in their pup social experience and hormone exposure (Moreno et al., 2018). Ovariectomized mice with or without systemic estradiol implants were exposed to 1 hour (hr) of pup social experience and with up to 15 minutes (min) of pup retrieval experience or remained naïve to pup contact. Both groups heard the playback of pup-vocalizations that was either occurred during retrieval or was outside of a social context.

We found that while estradiol influenced a variety of maternal behaviors displayed by subjects, the expression of *Bdnf* in the AC was not affected by the hormone when measured 2 hours after initial pup exposure. When we collapsed hormonal condition within pup experience groups, the AC of subjects with pup-vocalizations and social experience demonstrated a significant increase in transcription of *Bdnf* exon IV isoform and not total *Bdnf* transcription (exon IX). This research suggests the social context of vocalization exposure affects the molecular response of the AC. During the first social experience caring for infants, AC neurons experience a transcriptional response, increasing the mRNA of a memory-associated gene, which highlights *Bdnf* as a player of socially induced cortical plasticity.

3.4 METHODS

3.4.1 Animal Use

The Emory University Institutional Animal Care and Use Committee approved all procedures involved in this study. Experiments were performed on adult virgin female *CBA/CaJ* mice with no prior adult experience caring for pups. Subject animals were weaned at 21 days, placed in single-sex ALPHA-dri bedded housing with 2 to 5 animals per cage under a reverse-light cycle (14 hours of light /10 hours of dark), and had access to food and water *ad libitium*. All experiments were performed during the animal's dark phase, under red light.

3.4.2 Hormonal Manipulations

Animals (n = 104) were randomly divided into 1 of 2 hormonal implant treatments; estradiol (E2; n = 55) or blank (Bk; n = 51). At the average age of 12 weeks (± 11 days), all mice were bilaterally ovariectomized and implanted with a subcutaneous capsule (2 mm Silastic tubing, sealed with silicone aquarium sealant) containing either estradiol benzoate dissolved in sesame oil (50 µl at 3 mg/ml, Selleck Chemicals LLC, Houston, TX, USA) or a blank control containing only sesame oil; (Figure 1 A) (Moreno et al.,

2018; Miranda et al., 2014). Prior to implantation, all capsules were sterilized using 0.9% saline solution and then hydrogen peroxide gas for 29 min. Subjects were singly housed for 3 days of post-surgery monitoring and recovery before experiment habituation. The experimenters were not blind to implant type on the Experiment Day but were for behavior scoring.

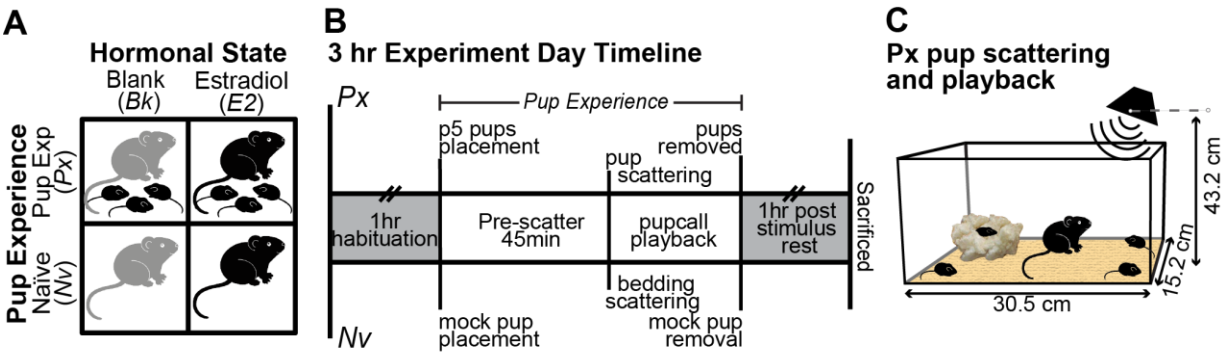

Figure 3-1 *Hormonal treatment and pup-vocalization exposure, with or without pup experience.*

(A) *Virgin female mice were ovariectomized and implanted with either a blank (Bk) or estradiol (E2) implant, creating 2 hormonal conditions. On the Experiment Day, half of the subjects received 1 hour (hr) of social experience with pups (Px), while the other half remained naïve (Nv) to pups, creating a 2x2 experimental design.* **(B)** *Experiments were run inside an anechoic chamber, beginning with 1 hr of solo habituation. Then, Px subjects had 4 pups placed in their cage while Nv subjects*

experienced mock pup placement. **(C)** *After 45 min, a pup-isolation call stimulus was played during a pup scattering session or a nest disturbance session to mimic pup scattering for Nv subjects. Px animals experienced a pup search and retrieval session during speaker playback providing social context, while Nv animals experienced the pup-isolation call playback with no behavioral association to actual pups. Pups were removed after 1 hr. Subjects were sacrificed 2 hr after pup or mock pup placement to assess gene transcription.*

3.4.3 Auditory Stimulus

Two sound stimuli were used in the course of this experiment. The creation and descriptions of these stimuli were described previously (Liu et al., 2003; Ivanova et al., 2017; Moreno et al., 2018). Briefly, pup-isolation call audio was used on the Experiment Day. This stimulus was a 10 min long recording of concatenated isolation pup-calls. It contained a dense series of 1055 pup-isolation calls grouped in bouts, ranging from 50 to 100 kHz, produced by 10 different *CBA/CaJ* mouse pups. A second, 10 min long stimulus was created by filtering out all calls from the pup-isolation call stimulus, generating a background noise stimulus.

In a subset of 4 pup naïve subjects, pups placed in their cage produced an average of 389 calls in 45 min, nearly 3 times fewer calls than within our pup-isolation call stimulus. Audio playback of the pup-isolation call stimulus ensured all subjects were exposed to a minimum baseline of social sound during the pup scattering or mock pup scattering session on the Experiment Day.

3.4.4 Habituation

Habituation ensured familiarity with all non-pup procedures associated with the Experiment Day. After surgical recovery, OVXed and implanted female subjects were assigned to 1 of 2 different experimental groups: those remaining naïve to pups as an adult (Nv) or those gaining 1 hr of pup experience (Px). All subjects then experienced habituation once a day for 3 days. Habituation included transportation to and from the procedure room, portion-cup water access, exposure to the silence of an anechoic chamber (44” x 27” x 24”, W × D × H inner dimensions, IAC Acoustics), and random playback of a background noise stimulus from a speaker at least 3 times per habituation. Mock pup scattering sessions were conducted during the playback of the background

noise stimulus, involving a gloved experimenter gently distributing the subject's bedding material around their cage.

3.4.5 Sound Stimuli Playback and Context

On the Experiment Day, female subjects were an average of 13 weeks of age (± 12 days; Figure 1 B). A portion of the subject's home cage bedding was removed and reserved for later use and their cage placed inside an anechoic chamber underneath a video camera.

All subjects habituated solo inside the anechoic chamber for 1 hr. For Px subjects, we removed post-natal day 5 or 6 (p5-6) pups from a donor dam's cage and rubbed them with, and placed them into a nest made of, the Px subject's reserved bedding. After the Px subject's solo habituation, video recording began and the nest with pups was transferred to a corner of the subject's cage. Px animals then experienced 45 min of uninterrupted social time with pups. For Nv subjects, procedures were identical except that the reserved bedding without any pups was returned to the subject's cage, and the animals were left for 45 min.

After 45 min, we began the playback of the pup-isolation calls, and accompanied this with either actual pup scattering (for Px subjects) or mock pup scattering (for Nv subjects), as described next. The pup search and retrieval session involved multiple

scattering and retrieval trials. Each trial comprised removing 3 pups from the nest and scattering/placing them throughout the cage, while 1 pup was left in the nest area (Figure 1 C). Pup retrieval was characterized by the adult mouse approaching, picking up, and returning all displaced pups to the nest, completing a trial (Pedersen et al., 1979; Coutellier et al., 2008). Px animals were given ~4 min to complete a trial before the experimenter would return the pups by hand to the nest. Thirty seconds after subject or experimenter retrieval, 3 pups were re-scattered to initiate a new retrieval trial. Px animals that successfully retrieved all 3 pups on 3 trials were classified as maternally responsive "retrievers." To insure adequate pairing between pup-calls and maternal behavior response, scatterings continued through the 10 min playback and into a subsequent 5 min period, as needed to meet retriever status. Nv subjects had small portions of their bedding material scattered at least 5 times during their mock-pup scattering session.

After a total of 1 hr of pup experience, we removed the foster pups from the cage of the Px subjects. We simulated the same disturbance for Nv subjects. The subjects then remained in the anechoic chamber for another 1 hr before being sacrificed to assess gene transcription. The combination of hormone exposure and pup experience created a 2x2 experimental design (Figure 1 A).

3.4.6 Behavior Assessment

All scoring of behavior from recorded video was performed blind to the hormonal condition of the subject. Over the 1 hr experience, common maternal behaviors were scored using the Observer XT application (Noldus Information Technology, Leesburg, VA, USA). Behaviors were judged as mutually exclusive from one another, so total time of mothering behavior could not exceed the 1 hr experiment time spent with pups. Common maternal behaviors were scored for duration and number of instances, including licking/grooming, nest building, crouching over a nest with pups and the latency to retrieve a displaced pup, all according to established ethogram procedures (Weber et al., 2008).

Repetitive head bobbing of the adult in close proximity of a pup in the nest, leading to the gentle rocking of the pup, was judged as sniffing/licking/grooming. Because of the similarity in the way licking and sniffing appear during video review, these 2 behaviors were scored as the same. After scattering commenced, this behavior was only scored for pups in the nest.

Crouching consists of an adult mouse's stationary position over the nest with her ventral side bowed out towards the pups (Pedersen et al., 1979; Pedersen et al., 2006). Hovering consists of the adult mouse laying or sitting on top of the nest (Coutellier, 2008). Because of difficulty distinguishing these 2 behaviors on video, they were scored as the same. We distinguished this behavior by an increase in the animal's apparent size due to sedentary position, and minimal head movement. Walking over the nest was not

scored as hovering/crouching. After scattering, this behavior was only scored for pups in the nest.

Nest building was judged as when the adult mouse moved or displaced the bedding material around the established nest or pups or otherwise manipulated it. The mouse could push her snout around in the bedding by the nest, manipulate pieces of bedding, or bring larger pieces of bedding over to the nest. This did not include walking over the nest, because any movement of the bedding was likely inadvertent (Pedersen et al., 1979).

These 3 behaviors —licking/grooming, crouching, and nest building— were only scored if they persisted for more than 2 seconds (Pedersen et al., 1979; Lucas et al., 1998; Coutellier et al., 2008; Pedersen et al., 2006). Total durations of these behaviors were calculated for the first 45 min (pre-scattering) and over the 1hr of pup experience, which included 10 min pup-isolation call playback, pup scattering by an experimenter and pup retrieval by the subject for up to 15 min (Figure 1 B).

Mice display rapid onset of retrieval behavior while other rodents, such as rats, may take days if inexperienced with infant care (Fleming et al., 1990). Mother mice perform retrieval behavior to ensure wandering pups are transferred to the safety of the nest and pup retrieval is an often used as an experimental measure of maternal response (Branchi et al., 2001; Thomas et al., 1997).

The latency for a subject to retrieve scattered pups was calculated by taking the time the pup was retrieved by the subject back to the nest, minus the time when the

experimenter removed the pups from the nest. A successful retrieval was scored at the moment the adult mouse let go of the pup in the nest. Attempts at retrieval in which the subject did not bring the pup all the way back to the nest did not count as a retrieval (Lucas et al., 1998). The latency to retrieve during a retrieval trial began at the time the experimenter removed 3 pups from the nest and ended when all pups were returned, with 3 retrievals possible per trial. We also measured the time to retrieve the 1st,2nd, and 3rd pups during each retrieval trial.

Both ovariectomized and intact nulliparous female mice are known to engage in maternal behaviors when exposed to pups (Thomas et al., 1997), although aggression towards pups does occur (Calamandrei et al., 1994). Video of the subject during pup experience was monitored online for aggressive behavior towards foster pups and recorded onto DVD for later maternal behavioral scoring. Attack behavior was grounds for the immediate removal of pups from the subject's cage. Of the 104 animals in this study, 72 were Px females, of which 21 attacked their foster pups (Bk = 11, E2 = 10) usually within 10 min of placement. There was no difference in rate of attack between the 2 hormonal conditions (Fisher's exact test, $p = 0.612$). Animals that attacked were not included in behavior scoring or brain processing.

Total animals that did not attack broke down into the following groups: PxE2 n = 27, PxBk n= 24, NvE2 n=18, and NvBk n=14. Of these, 69 animals contributed behavior videos for scoring; 14 others could not due to technical issues with the recordings. Two

PxE2 animals were removed from analyses because they covered both themselves and the pups with a square block of nesting material, making it impossible to score licking/grooming versus crouching behavior. One PxE2 animal was removed from our analyses of behavior over the total 1 hr, but not the first 45 min, because even though it was a retriever, a technical failure caused no pup-isolation call stimulus playback during the scattering session. Of the 69 subject videos' scored, 3 failed to learn to retrieve scattered pups (2 PxE2 and 1 PxBk) and an additional 2 animals did not display maternal behavior including retrieval (1 PxE2 and 1 PxBk).

3.4.7 Tissue Collection and Processing

After a final solo hour in the anechoic chamber, subjects were incapacitated with CO_2 and decapitated. Mouse brains were removed, preserved by rapid freezing using liquid nitrogen and Tissue-Tek OCT compound (Sakura Fine Technical, Tokyo, Japan) and stored at -80°C. Samples of desired brain regions —the medial pre-optic area (MPOA), the primary auditory cortex (AC), the primary visual cortex (V1), and the ventral hippocampus (VH)— were collected bilaterally on a microtome via a 1mm micro tissue puncher and stored in 70 micro-liters (µl) of RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at -20 C° until RNA isolation.

Total RNA was extracted from brain tissue using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. On-column DNAse (Qiagen) treatment was used to eliminate genomic DNA. Purity of RNA was assessed by 260/280 ratio and concentration analyzed using Gen5 Take3 software on a BioTek Snergy HT RNA quantification machine (BioTek, Winooski, VT, USA). RNA was reverse transcribed into cDNA using the Superscript III reverse transcriptase kit (Introvigen Tokyo, Japan), standardizing to 150ng/20µl using RNA concentration measurements.

Quantitative real-time polychain reaction (qRT-PCR) was conducted with validated TaqMan® probes and TaqMan® Universal PCR Master Mix (Applied Biosystems, Austin, TX, USA) on the ABI Step-One-Plus PCR system using Step-One software by Life Technologies (Applied Biosystems). Each sample well contained the following: 10 µl of PCR Master Mix, 1.7 µl of cDNA (150ng/20 µl), 7.3 µl of nuclease-free water (Sigma-Aldrich, St Louis, MO, USA) and 1 µl of the specific TaqMan® Gene Expression Assay. Each qRT-PCR run contained the following: 5 samples for each of the 4 experimental conditions, in duplicate for the gene of interest and a positive reference gene; a no-RT sample and a water sample for each primer as internal controls. The following schedule was used for the qRT-PCR program: 50°C for 2 min, 95°C for 10 min; cycling stage: 95°C for 15 sec then 60°C for 1 min for 40 cycles. Each qRT-PCR result was verified with an additional run of independent samples (See *Statistical Analysis Section 3.4.8*).

Experimental TaqMan® primers for mice (Thermo Fisher Scientific) included one for the 3' coding exon of *Bdnf* exon IX (Mm04230607_s1; coding to Chr.2: 109,674,700 – 109,727,043) which allows for the evaluation of total *Bdnf* transcription (i.e. the sum of all isoforms transcribed). Another primer identified the site-specific transcription of the *Bdnf* exon IV isoform (Mm00432069_m1; coding to Chr2: 109,692,436- 109,692,774). Housekeeping primers included β-actin (Mm02619580_g1 Chr.5: 142,903,116 – 142,906,724, used for MPOA, AC and V1) and Ribosomal protein eL19 (RPL19; Mm02601633_g1, Chr.11: 98,023,080 – 98,030,493, used for VH) purchased from Thermo Scientific (Thermo Fisher Scientific).

Of the 82 Nv and non-attacking Px animals that experienced our paradigm, 68 were used for qRT-PCR analyses. The samples from 12 animals were consumed during processing and protocol optimization and did not contribute to BDNF mRNA data.

3.4.8 Statistical Analysis

Data were analyzed with JMP data analysis software (SAS, Cary, NC, USA). Before analyses, behavioral data were screened for outliers and points that met the JMP software's Robust Fit (Huber M-estimation method) criteria were removed (Huber,

1973). Maternal behavioral data were assessed for the first 45 min and for the total 1 hr (including mock or real pup scattering).

Fisher's exact test was used to determine the independence of one nominal variable's relative proportions to a second variable's, such as the proportions of attacking subjects per hormone condition. Other results were subjected to statistical analyses using two-way Analysis of Variance (ANOVA) for experiments with 4 groups and Student's T test for comparing 2 groups. Two-group analyses were always checked for unequal variances using the Levene test and, if non-significant, a Student's T test result was reported. All Student T-test analyses were conducted under the hypothesis that E2 animals would be more likely to express maternal behavior (Stolzenberg et al., 2009; Koch et al., 1989a; Rosenblatt et al., 1988). We also held the hypothesis that Px subjects would have lower cycles to threshold (Ct's) for *Bdnf* transcripts in our qRT-PCR, indicating more transcription, possibly isoform specific, of *Bdnf* mRNA in response to the social experience gained in our paradigm, than Nv (Lubin et al., 2008; Bredy et al., 2007). Differences were considered to be statistically significant at $p < 0.05$ and expressed as mean ± SEM.

Data from qRT-PCR were analyzed using the 2-ΔΔCt method (Livak et al., 2001). Data were normalized to the naïve blank (NvBk) condition for two-way ANOVA and Nv for Student's T-test. Data entered into analyses were the average Ct for a sample's gene of interest (all replicates) minus the average Ct for that same sample's reference control gene, arriving at the average delta (Δ)Ct value. Results from qRT-PCR were verified by

running an additional set of independent samples from different animals. Data reported here represent the conservative ANOVA results from each brain region and gene transcript of interest. Reference control genes were verified using the $2^{-\Delta Ct}$ method, where ΔCt is the average of a sample's replicate Ct's minus the average Ct of the normalized group (Schmittgen et al., 2000): $2^{-[Ct(\text{average of each sample's replicates}) - Ct(\text{average of normalized group's replicates})]}$.

The MPOA was initially a region of interest because it had previously demonstrated significant increase in the immediate early gene *c-Fos* in both intact and ovariectomized nulliparous female mice with 30 min of pup experience (Calamandrei et al., 1994). However, we found a significant effect of hormones on the expression of our endogenous control gene, β-actin, in the MPOA ($F_{3,17} = 8.2627$, $p = 0.011$) during verification and therefore could not verify *Bdnf* expression for the MPOA. Further research is needed to determine whether β-actin transcription in the MPOA changes with hormone treatment.

3.5 RESULTS

Using a maternal mouse model, we examined the response of adult female mice to pup-calls, to determine how the initial social context when first hearing those calls

affects molecular changes in brain regions of interest. Adult females were ovariectomized and estradiol (E2) or blank (Bk) implanted, to control for the potential neuromodulatory role of E2. Half of the subjects had foster p5-6 pups placed in their cage (Px) or remained naïve to pups (Nv) for 1 hr, over which time their behavior was scored (Figure 1B). All subjects experienced a 10 min pup-isolation call sound stimulus paired with (Px) or without (Nv) a pup search and retrieval session lasting up to 15 min. All subjects were sacrificed after an additional hour, to measure changes in transcription of the memory-associated gene *Bdnf* in the auditory cortex (AC), ventral hippocampus (VH), and visual cortex (V1).

3.5.1 Behavior

3.5.1.1 Experiment Day Behavior During 1 hr Post Pup or Mock Pup Placement

Px animals with 1 hr of pups experience displayed significant differences in their behavior compared to Nv subjects. The 1 hr experience with pups included a 45 min pre-scattering period, followed by 10 min playback of pup-isolation call sound stimulus in conjunction with a pup search and retrieval session for up to 15 min. Nv animals were exposed to the same actions by the experimenter and pup-isolation call sound stimulus but without live pups. The amount of total time and instances of maternal behaviors such as pup licking/grooming, nest crouching, and nest building were measured.

Both Nv and Px subjects displayed nest building with Px subjects spending significantly more time engaged in nest building than Nvs (two-way ANOVA, $F_{(3,62)} = 23.8919$, $p < 0.0001$; Figure 2 A). There was neither a main effect of hormone exposure (two-way ANOVA, $F = 0.4466$, $p = 0.5064$), nor an interaction between hormone and experience variables (two-way ANOVA, $F = 0.455$, $p = 0.5025$) on nest building behavior.

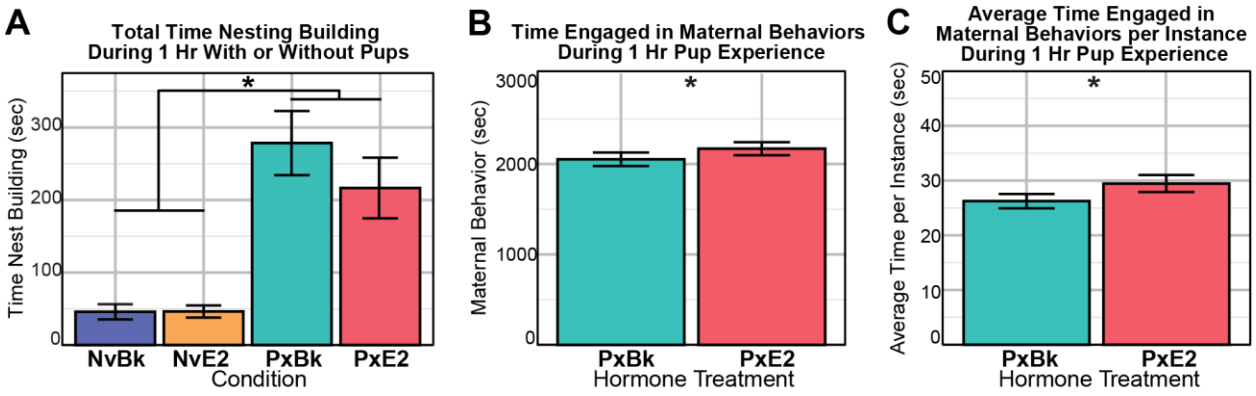

Figure 3-2 *The presence of pups and E2 affected the behavior displayed by subjects.*

(A) *There was a main effect of pup experience on the total time spent engaged in nest building behavior ($p < 0.0001$, $n = 65$) with the presence of pups increasing the total time over the course of an hr.* **(B)** *Within the Px group, PxE2s spent significantly more time engaged in the licking/grooming, crouching over nest, and nest building maternal behaviors than PxBk subjects ($p = 0.04$, $n = 42$).* **(C)** *When engaged in licking/grooming, crouching over nest, and nest building, the PxE2 animals on*

average spent significantly more time engaged than PxBk per instance ($p = 0.04$, $n = 42$). (*) indicates p-values < 0.05. Error bars represent SEM.

Given E2's intrinsic association with maternal behavior (Numan et al., 1977; Ogawa et al., 1998; Stolzenberg et al., 2009), we hypothesized E2 would increase the time subjects engaged with pups. During the full 1 hr of pup experience, PxE2 subjects spend significantly more time displaying maternal behaviors such as licking/grooming, crouching over the nest, and nest building than PxBk animals (one-tail Student's T-test: $p = 0.04$; Figure 2 B). PxE2 animals also spent more time engaged in maternal behavior per instance (one-tail Student's T-test: $p = 0.04$; Figure 2 C). Hence, our hormone manipulation had a significant effect on the time subjects engaged in maternal behavior, providing a positive control for the behavioral effects of our E2 implants.

3.5.1.2 Experiment Day Behavior During 45 min Pre-Scatter Experience

During the pre-scattering portion of the experimental day, Px subjects had 45 min of uninterrupted experience socializing with pups. E2 and Bk subjects demonstrated significant differences in specific maternal behaviors. The PxE2 subjects had significantly decreased latency to crouch over pups (one-tailed Student's T-test: $p < 0.01$) which resulted in more time crouching than PxBk subjects (one-tailed Student's T-

test: p = 0.04; Figure 3 A, 3 B). PxBk subjects spent significantly more time outside of licking/grooming, crouching, and nest building behaviors (one-tailed Student's T-test: p = 0.02; Figure 3 B, 3 C). There was no difference in the total time licking/grooming (one-tailed Student's T-test: p = 0.62) or nest building (one-tailed Student's T-test: p = 0.69; Figure 3 B, 3 C).

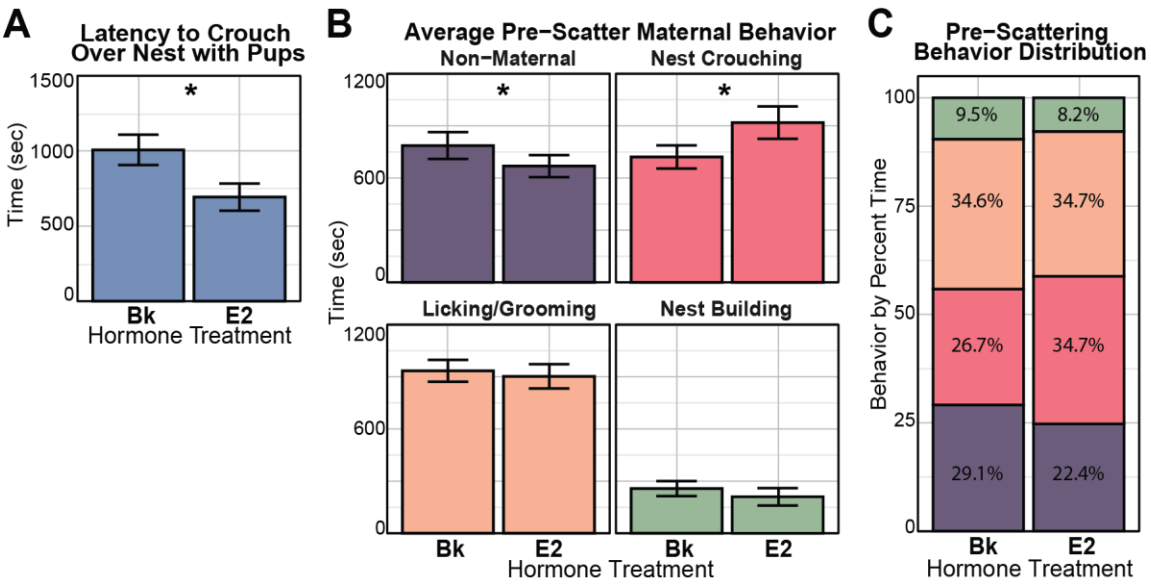

Figure 3-3 *There were specific maternal behavior differences between PxE2 and PxBk subjects (n =43) during the first 45 min of pup experience.*

(A) *During their first pup experience, PxE2 subjects had a shorter latency before crouching over a nest of foster pups compared to PxBks (p < 0.01).* **(B)** *Time spent engaged in maternal or non-maternal behavior by hormone implant type in seconds (sec) and* **(C)** *by percentage of time indicate that during the first 45 min of pup*

experience, there were significant differences between hormone groups and the behaviors they engaged in. The PxBk group showed more time engaged in non-maternal behaviors than the PxE2 group (p = 0.02). The PxE2 group also spent more time engaged in nest crouching behavior (p = 0.04). Time engaged in licking/grooming (p = 0.62) and nest building (p = 0.69) were not significantly different between hormone treatment. (*) indicates p-values < 0.05. Error bars represent SEM.

3.5.1.3 Retrieval Experience

Retrieval of rodent pups by a caretaker begins with active, goal-directed searching in response to isolated pup cues and ends with delivery of infants to the safety of the nest (Terkel et al., 1979a; Numan et al., 2010; Numan et al., 2009). This appetitive maternal response in rodents requires both the recognition of pup cues, such as vocalizations, and maternal responsiveness (Hansen et al., 1991; Stern, 1996; Sewell, 1970). Experimental induction of retrieving has been used to assess molecular and transcriptional changes in brain regions involved in the complex behavior (Ehret, 2005; Krishnan et al., 2017; Stolzenberg et al., 2012).

In our study, Px subjects experienced a pup search and retrieval session with repeated trials of pup retrieval, paired with natural pup-isolation calls, creating a social

context for those vocalization cues. For Nv subjects, with no pups present there was a lack of social context during the playback of pup-isolation calls.

During the pup-isolation call playback and the pup search and retrieval session, we saw no retrieval differences between E2 and Bk implanted Px animals. Px subjects displayed the longest latency to retrieve all 3 pups on their first retrieval trial (one-way ANOVA, $F_{(3,114)} = 21.126$, $p < 0.0001$). The likelihood of successfully retrieving all 3 pups during the first retrieval was no greater for E2 than Bk treated subjects (Fisher's exact test, $p = 0.47$). The latency to successfully retrieve their first pup during the first scattering was no lower for E2 than Bk subjects (two-tailed Student's T-test: $p = 0.22$). Also, the latency to retrieve all pups during the first scattering was not lower for E2 subjects (two-tailed Student's T-test: $p = 0.81$). There was no difference in the average number of pups either group retrieved (two-tailed Student's T-test: $p = 0.49$).

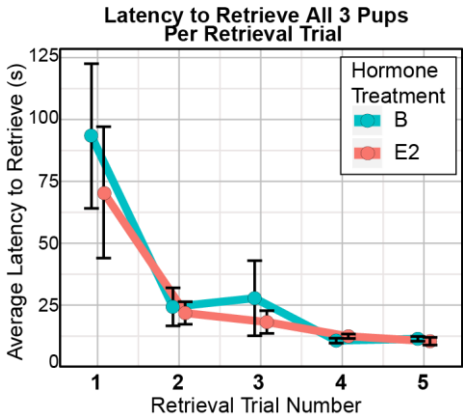

Figure 3-4 *Latency to Retrieve All 3 Pups Per Retrieval Trial*

During the pup-isolation call playback and pup search and retrieval session, the latency to retrieve pups did not differ depending on the subject's hormone treatment. E2 subject's latency to retrieve all 3 pups was not significantly lower than Bk subjects, including the first retrieval trial (p = 0.81).

The majority of subjects gained retrieval experience during natural pup vocalization playback. Non-retrievers were removed from the study. PxE2 and PxBk implanted subjects displayed equivalent search and retrieval rates of isolated pups and thus received equivalent experience. While increased time spent engaged in other maternal behaviors provided a positive control, showing the E2 implant had an effect, hormone treatment neither enhanced nor interfered with retrieval behavior during the search and retrieval session. Thus, all Px subjects included in in *Bdnf* analyses had a standard behavior experience of vocalization associated pup-retrieval.

3.5.2 Bdnf Expression

Our primary interest was identifying signs of sensory cortical plasticity as a result of experience hearing social sounds during a behaviorally relevant task. We hoped to gain a

better understanding of how the first such experience may affect learning-associated transcription in areas with demonstrated plasticity for sensory cues that drive behavior.

We measured *Bdnf* mRNA from 3 brain regions of virgin female mice shortly after their first pup experience. The transcription of total *Bdnf* transcripts, and the exon IV specific transcript, which is adjacent to a likely estrogen response element (Sohrabji et al., 1995) were assessed. Given the exon IV isoform's association with activity-dependent induction (Lauterborn et al., 1996), memory formation (Lubin et al., 2008), and the epigenetic regulation of adjacent histones mediated by E2 and learning tasks (Fortress et al., 2014b), we investigated transcription of exon IV in association with our E2 manipulation.

Brain Region	Statistics	*Bdnf* transcript	
		All	*Exon 4*
AC	*ANOVA*	$F_{3,14} = 1.274$, $p = 0.324$	$F_{3,17} = 1.368$, $p = 0.288$
	Hormone	$F = 2.944$, $p = 0.11$	$F = 1.040$, $p = 0.323$
	Experience	$F = 1.475$, $p = 0.246$	$F = 2.669$, $p = 0.121$
	Hormone x Experience	$F = 0.183$, $p = 0.675$	$F = 0.002$, $p = 0.961$
	Student's T-test for Experience	Nv mRNA < Px mRNA $p = 0.799$	Nv mRNA < Px mRNA $p = 0.044$ *
V1	*ANOVA*	$F_{3,15} = 0.820$ $p = 0.504$	$F_{3,14} = 0.726$, $p = 0.554$
	Hormone	$F = 1.221$, $p = 0.287$	$F = 1.811$, $p = 0.201$
	Experience	$F = 0.539$, $p = 0.474$	$F = 0.051$, $p = 0.824$
	Hormone x Experience	$F = 0.563$, $p = 0.465$	$F = 0.239$, $p = 0.632$
	Student's T-test for Experience	Nv mRNA < Px mRNA $p = 0.79$	Nv mRNA < Px mRNA $p = 0.506$
VH	*ANOVA*	$F_{3,15} = 1.249$, $p = 0.329$	$F_{3,16} = 0.06$, $p = 0.98$
	Hormone	$F = 1.832$, $p = 0.197$	$F = 0.012$, $p = 0.914$
	Experience	$F = 0.131$, $p = 0.722$	$F = 0.148$, $p = 0.705$
	Hormone x Experience	$F = 1.656$, $p = 0.219$	$F = 0.011$, $p = 0.916$
	Student's T-test for Experience	Nv mRNA < Px mRNA $p = 0.683$	Nv mRNA < Px mRNA $p = 0.338$

Table 3-1 Statistical values reported here represent the result of 1 of 2 RT-qPCR runs of independent samples, with the highest ANOVA p-values of the 2 runs.

Our hypothesis for Student T-test is Cts (cycle thresholds) for Nv animals would be greater than those of Px for our gene and transcript of interest and that the mRNA for Nv animals would be less than Px animals for the AC and the VH. The p-value the AC's Student's T-test for Experience met the criterial of significance on both runs. (*) indicates p-value < 0.05

3.5.2.1 ANOVA Analyses

We used a two-way ANOVA to determine how *Bdnf* transcription may vary depending on hormone treatment and pup experience. When measuring total *Bdnf* mRNA levels (exon IX), our analyses yielded no significant differences between our groups in the AC (two-way ANOVA, $F_{(3,14)}= 1.27$, $p = 0.32$), or VH (two-way ANOVA, $F_{(3, 15)} = 1.25$, $p = 0.33$; Table 1, Figure 5 A, 5 C). There was also no significant difference in the transcription of the *Bdnf* exon IV isoform in the AC (two-way ANOVA, $F_{(3, 17)} = 1.368$, $p = 0.288$), or VH (two-way ANOVA, $F_{(3, 16)} = 0.06$, $p = 0.98$; Figure 5 B, 5 D). In the AC, there was a trend towards a main effect of experience, with Px subjects displaying what appeared to be higher levels of mRNA than Nvs ($F = 2.669$, $p = 0.121$; Figure 5 B).

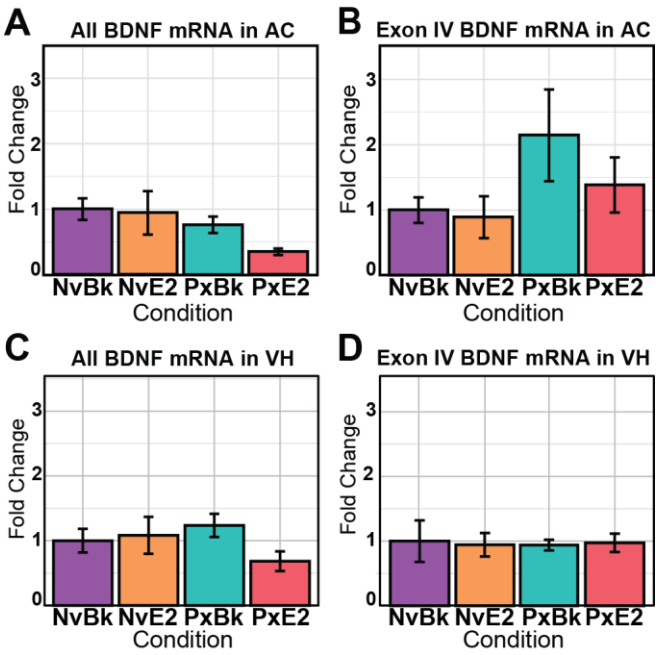

Figure 3-5 *No effect of 2x2 conditions on Bdnf mRNA levels in AC or VH*
In the AC, neither mRNA from total Bdnf transcripts **(A)** *nor exon IV transcripts* **(B)** *demonstrated significant main effect of hormone or an interaction between experience and hormone. AC exon IV transcripts showed a trending main effect of experience (p = 0.121). In the VH, neither the mRNA from total BDNF transcripts* **(C)** *nor exon IV transcripts* **(D)** *demonstrated a significant main effect of experience, hormone or an interaction between the two. No p-values < 0.05. Error bars represent SEM.*

3.5.2.2 Student T-test Analyses

Considering that during our search and retrieval session and auditory playback our hormonal manipulation did not alter retrieval behavior, we collapsed our data across Bk and E2 subjects to separately test whether pup experience, irrespective of E2 availability, affects *Bdnf* expression. When the 2 hormone conditions were collapsed, we found 1 hr of pup experience led to a significant increase in the expression of *Bdnf*'s IV mRNA transcript in the AC (one-tailed Student's T-test: $p = 0.044$; Table 1; Figure 6 A). The same was not true for VH (one-tailed Student's T-test: $p = 0.338$; Table 1; Figure 6 B).

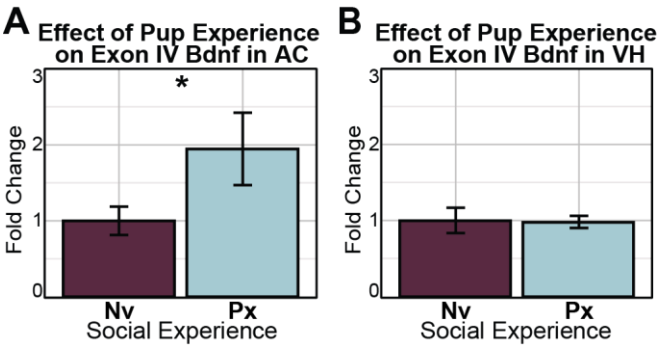

Figure 3-6 *When subject hormone condition was collapsed within experience group, a transcript specific effect was found in the AC.*

(A) *There was a significant effect of experience with pups on the transcription of Bdnf exon IV but not total Bdnf transcripts (Table 1).* **(B)** *An effect of experience on exon IV*

transcription was not found in the VH. (*) indicates p-values < 0.05. Error bars represent SEM.

Bdnf transcription in V1 was also included as a negative control, to ensure the experimental paradigm was not inducing *Bdnf* transcription across all sensory cortical regions. Given the experiment took place during the subject's dark phase, under red filtered light, plasticity induced within a visual region was unlikely. V1 did not display transcriptional differences in *Bdnf* mRNA as a result of associative-pairing vocalizations with the natural behavior they elicit (Table 1). There was no difference in total *Bdnf* gene mRNA levels (two-way ANOVA, $F_{(3,15)} = 0.820$, $p = 0.504$) or the exon IV transcript ($F_{(3,14)} = 0.726$, $p = 0.554$). V1 also did not show an effect of experience when collapsed within experience groups (one-tailed Student's T-test, $p = 0.506$). This negative results is consistent with other molecular studies where an auditory learning experience does not engage visual cortical plasticity (Banerjee et al., 2017).

Our results therefore suggest the first experience with pup-vocalizations in conjunction with pup retrieval caused molecular changes at the site of cortical auditory processing, increasing the site specific transcription of *Bdnf*'s exon IV transcript in the AC.

3.6 DISCUSSION

Our study provides the first evidence that *Bdnf* transcription increases in the AC after social auditory experience in an exon transcript specific manner. *Bdnf*'s exon IV mRNA concentrations increased in the AC when pup-vocalizations were paired with pup experience. The exposure to pup-call vocalization playback from recording did not influence *Bdnf* exon IV transcripts in subject's AC unless pups were present. E2 treatment did not influencing the transcription of total *Bdnf* mRNA within subject's AC, V1, or VH whether pups were present or not during pup-call playback. Memory-associated gene expression increases in the AC with vocalization experience in a social context, potentially triggering plasticity to enhance future presentations of these new and behaviorally relevant sounds.

3.6.1 Auditory Plasticity Induced by BDNF

With maternal experience comes familiarity with a new set of infant associated sounds. Prior research in the field of maternal auditory experience examined the outcome of plasticity. By comparing naïve animals to those days after their social auditory experience, significant differences have been identified between subjects familiar or naïve to social sound stimuli (Moreno et al., 2018; Ivanova et al., 2017;

Geissler et al., 2004). Here, instead we attempted to identify molecular events that may contribute to initiating those changes.

The AC responds to vocalization experience within social context with plasticity, thought to enhance the recognition of these vocalizations (Liu, 2011). Inhibitory mechanisms play an important role in this plasticity. In the AC of mother mice, inhibitory neurons that express parvalbumin shift their best frequency (BF) up an octave and closer to USV frequencies (Cohen et al., 2015). Furthermore, USVs evoke a stronger call-inhibited response in so-called lateral band regions of the core auditory cortex of mothers (Galindo-Leon et al., 2009). Neurons in these regions have BFs tuned just below the USV frequencies, and stronger evoked suppression of neural activity. Changes in the parvalbumin-expressing neural population's BF, would potentially improve neural contrast in the cortical representation of pup-vocalizations. By suppressing call-evoked activity in the lateral bands, inhibitory plasticity enhances the processing of behaviorally relevant vocalizations long-term for mothers (Lin et al., 2013). Moreover, computational models suggest cortical inhibition might be necessary for complex sound processing (Narayan et al., 2005). Models of bird song processing demonstrate improved discrimination of songs when an inhibitory multi-circuit, inspired by GABAergic interneurons, is included. As the identify of molecules and proteins upregulated in response to social auditory experience are discovered, it is constructive to consider how they might be associated with changes in inhibitory neuron activity.

The experience-dependent increase in *Bdnf* mRNA found in this study might be a mechanism driving the AC inhibitory plasticity associated with vocalization processing. BDNF is released by pyramidal cells, targeting GABAergic interneurons and in turn, regulating excitation in the neocortex and hippocampus (Marty et al., 1997). BDNF receptors are expressed abundantly by parvalbumin-positive interneurons (Gorba et al., 1999). The protein also influences GABAergic interneurons in part by increasing GABA and its synthetic enzyme (Mizuno et al., 1994; Woo et al., 2006). BDNF treatment of visual cortex neuron cultures can preserve GABA-positive neurons and GABA-mediated inhibition of pyramidal neurons during activity blockades (Rutherford et al., 1997). The *Bdnf* exon IV transcript is associated with GABAergic signaling of cortical neuron and its absence impairs auditory processing (Sakata et al., 2009; Hill et al., 2016).

The significant change in the activity-associated isoform of *Bdnf* (Lauterborn et al., 1996; Tao et al., 1998; Timmusk et al., 1993; Nanda et al., 1998) found in our AC study calls for further study of mammalian AC plasticity at a molecular level. Next, the specific types of neurons producing this mRNA within the AC should be confirmed to verify interneuron expression. *In situ* immunohistochemistry experiments or genetic tagging (Tasaka et al., 2018) could determine the AC layer and cell type responsible for pup experience-dependent increased *Bdnf* exon IV mRNA in the AC.

3.6.2 Effect of Estrogens on Auditory Processing

Estrogens have well established effects on the processing of vocalizations within higher order auditory sites (Caras, 2013). There are also connections between estrogens and the site specific transcription of the exon IV isoform of *Bdnf* (Fortress et al., 2014b; Sohrabji et al., 1995). However, the exact mechanism employed by estrogens to enhance social auditory processing remain unclear.

Though estradiol within our study was not found to regulate the transcription of the interneuron-associate *Bdnf* transcript IV, a recent publication indicated estrogens' effect on inhibitory cortical activity in the sensory cortex. The natural cycling of estrogens during estrus influenced somatosensory processing in rats. Clemens et al. (2019) recorded activity of the barrel cortex of female rats throughout their estrous cycle while the rats experienced social touch by a conspecific. The activity of fast-spiking interneurons was strongly activated by social facial touch with estrus cycle and estradiol increasing excitability. Findings such as these encourage future research into the ability of hormones to modulate social sensory representations.

It is possible our results were a true reflection of the experience-dependent, not estrogen influenced, transcriptional changes of the sensory cortex. *Bdnf* exon IV mRNA has been associated with experience-dependent memory formation (Lauterborn et al., 1996; Lubin et al., 2008) and sensory cortical processing (Nanda et al., 1998;

Pattabiraman et al., 2005; Hong et al., 2008) outside of estrogen modulated conditions. Pups can be pleasurable and thus the stimuli they produce can reinforce social interaction. Therefore, they can acquire enhanced behavioral significance to caretakers without estrogens. But given the extensive evidence of the role of estrogens in auditory plasticity (Miranda et al., 2009) and social experience (Choleris et al., 2012), it is likely that aspects of our experiment prevented the observation of plasticity associated molecular events.

Estrogens might act to preserve changes in auditory cortical circuits via mechanisms that are evident at other times post-experience. For example, the long-lasting changes in AC plasticity induced by maternity (Lin et al., 2013) suggest maternal hormones might utilize epigenetic modification abilities to stabilize adult plasticity. Estrogens' promotion of epigenetic changes (Zhao et al., 2010) might explains how the hormone class is capable of inducing stable changes in genetic expression. Perhaps estrogens act to increase DNA methylation or histone acetylation at *Bdnf* promoter regions in the AC and hippocampus during maternal experience similarly to during other learning experiences (Zhao et al., 2010; Frick et al., 2011; Lubin et al., 2008; Bredy et al., 2007). Indeed, changes in neuronal gene expression via epigenetic modifications in the sensory cortex are known to promote long-lasting changes in neuronal activity (Swank et al., 2001; Silingardi et al., 2010). Now that a relationship has been established between *Bdnf* and auditory experience, additional research could examine the epigenetic state of the *Bbdf* promoter regions post-social auditory experience in association with estrogens.

Future research should also examine the dorsal hippocampus, where estradiol increases histone H3 acetylation at *Bdnf* promoter regions with object and spatial memory consolidation (Fortress et al., 2014b).

Our manipulation of estrogens was systemic, based on ovariectomizing and replacing gonadal estrogens with a silastic implant. An important caveat of this approach, relevant for neurophysiology, is that there is increasing evidence brain-derived (neuroestrogens) rather than gonadal estrogens can play an important role in neuromodulation. The song-bird auditory forebrain circuit experiences rapid regulation by neuroestrogens during sound processing in social contexts (Remage-Healey et al., 2012; London et al., 2006; Remage-Healey et al., 2010). Furthermore, the presence of aromatase in mammalian axonal processes suggests rapid neuroestrogen modulation of neural activity via activation of kinase cascades in rodent models (Naftolin et al., 1996; Hojo et al., 2004). A recent publication by Lu et al. utilized a forebrain neuron-specific aromatase knockout (FBN-ARO-KO) mouse to block all synthesis of neuroestrogens in forebrain excitatory neurons (Lu et al., 2019). Ovariectomized FBN-ARO-KOs experienced deficits in forebrain spine and synaptic density as well as spatial, recognition and contextual fear memory. However, heterozygous, ovariectomized females demonstrated no change in BDNF, pERK or pCREB levels in the cortex, even though these memory-associated markers decreased in ovariectomized FBN-ARO-KOs. This finding suggests a compensatory mechanism whereby the loss of gonadal E2 might

be alleviated by neuroestrogens in our ovariectomized Bk subjects. To fully appreciate the potential critical role E2 plays in social auditory experiences, conditional KO or knock-down models should be utilized during future work.

3.6.3 Linking Behavior Findings to Behavior and Circuitry

Our E2 treated animals presented decreased latency to crouch as well as increased time spent crouching over foster pups. Crouching over a nest with pups to provide insulation and access to the mammary region is a highly stereotyped maternal response displayed by rodents (Numan et al., 2003). In rats, the exposure of the ventral trunk to pups is so reflexive, it is displayed even by adults unable to nurse such as males and virgin females (Stern, 1991, 1996). Crouching is a consummatory maternal behavior governed by the canonical maternal circuitry including the MPOA of the hypothalamus (Stern, 1996; Hansen et al., 1991). Expression of the transcription factor FosB in the MPOA is critical for nursing and crouching behavior (Brown et al., 1996). From our findings, we can speculate that while the majority of our subjects displayed crouching over pups without previous experience or influence from the hormone estradiol, the rate for displaying this behavior was enhanced by the presence of estradiol implants. Our subjects did not, however, display differences in the appetitive maternal behavior of pup approach and retrieval.

We found no difference between E2 or Bk implant subjects in their retrieval behavior within 45 minutes of their first exposure to pups. The MPOA is believed to mediate maternal behaviors such as pup retrieval, by inhibiting nucleus accumbens, which releases ventral pallidum from inhibition (Numan et al., 2005). MPOA galanin neurons that project to the ventral tegmental area were shown to mitigate the approach behavior of male and female adults towards out-of-nest pups (Kohl et al., 2018). An alternative pathway via the basolateral amygdala and prefrontal cortex is known to activate in response to pup stimuli and influence nucleus accumbens and ventral pallidum to initiate goal-directed maternal response (Numan et al., 2009). This mesolimbic dopamine activation of the nucleus accumbens drives maternal response including retrieval. While consummatory behavior was enhanced, appetitive retrieval behavior unaffected by our E2 implants, and our paradigm of pup exposure was sufficient to release retrieval behavior in virgin pup-naïve and ovariectomized females regardless of E2 treatment.

3.6.4 Ventral Hippocampus and Social Memory

Because of its connection to social recognition and memory, the ventral hippocampus was an additional brain region of interest for our social experience study.

The complex task of social information processing and memory is an open area of hippocampus research (Montagrin et al., 2018; Broad et al., 2002). Recently, a population of CA1 pyramidal neurons projecting to the nucleus accumbens were identified as responsible for social recognition (Okuyama et al., 2016). The hippocampus's dorsal, intermediate, and ventral subregions and cell types are now appreciated as distinct, and their functions are being teased apart experimentally (Fanselow et al., 2010).

It was reasonable to expect a social paradigm such as ours, which manipulated estrogens and measured hippocampal mRNA would yield significant effects on *Bdnf* transcription due to the social nature of estrogens and estrogens' ability to induce hippocampal *Bdnf* transcription. First, estrogens are often thought of as social neuromodulators, because of their ties to social recognition (Choleris et al., 2003), social motivation (Tang et al., 2005; Imwalle et al., 2002), as well as the fact that social experiences can initiate changes in their production and receptor expression (Remage-Healey et al., 2008; Ehret et al., 1993; Ehret et al., 1994). Second, estradiol increases the release of BDNF from the dentate gyrus in hippocampal slices (Sato et al., 2007). In vivo, ovariectomized female rats had their low levels of BDNF in hippocampal CA3, CA4, and dentate gyrus neurons rescued by estradiol replacement (Singh et al., 1995). In another study, 4 hours after high estradiol treatment CA3, but not CA1, neurons synthesize high amounts of BDNF (Scharfman et al., 2007). However, hippocampal subregions and cell groups appear to react differently to estrogens, with other

researchers finding estradiol-induced increases in BDNF mRNA in the CA1 and CA3 but not the CA2 or dentate gyrus (Zhou et al., 2005).

It is with these studies in mind that we expected to see an estradiol effect on *Bdnf* transcription in the ventral hippocampus. However, those works also highlight a limitation of our use of simply punching out the entire VH for analysis. The broad nature of punch collection could in part be the reason for our inability to detect increases in *Bdnf* transcription that may occur only within subregions of the VH. Our question could be better addressed by utilizing a more precise *in situ* hybridization approach, which allows for the identification of hippocampal sub regions and neurons (Singh et al., 1995).

3.7 CONCLUSION

We demonstrated that hearing natural pup vocalizations during pup experience enacted immediate transcriptional changes in the AC. Specifically, *Bdnf* exon IV transcript, which is associated with neuronal activity and experience-dependent synaptic plasticity, increases in the AC when female mice experience a social context with infant vocalizations. The transcript type suggests a link to inhibitory neuron plasticity, which is known to occur as a result of motherhood and infant experience in

mice. This increase in transcription during the initial pup experience is potentially one of the first transcriptional changes in a series, leading to synaptic and then circuit level modifications improving a caretaker's AC representation of vocalizations. Thus, our findings add to previous AC research indicating experience caring for infants enacts molecular changes and lasting enhanced-processing of infant vocalizations.

4 GENERAL DISCUSSION AND FUTURE DIRECTIONS:

4.1 Memory in the Sensory Cortex: How does the research here broaden the field?

Our experiences shape our perspective of the world, allowing the lessons of the past to guide responses in the present. The sensory cortices detect, discriminate, and categorize cues to insure the appropriate behavioral response to a cue is enacted. It had been assumed the function of the sensory cortex was to perform the final stage of sensory processing and then pass that information to association areas. Now, the larger neuroscience community is beginning to appreciate that sensory cortical areas are more than just sites for online sensory processing, but are essential for sensory memory storage and retrieval (Harris et al., 2001; Kosslyn et al., 2001).

Sensory cortical processing areas are sites where top-down information concerning the context of a stimulus and bottom-up information on the characteristics of the stimulus converge. For instance, the AC can process emotional and behavioral associations concomitantly with the features of a sound stimulus (Polley et al., 2006; Letzkus et al., 2011; Scheich et al., 2011). There is substantial evidence that sensory cortices, such as the AC, are responsible for sensory memory storage. During sensory memory retrieval, these regions display a reemergence of patterns associated with the sensory stimuli being recalled (Harris et al., 2002; Sakai et al., 1991; Buckley et al., 1998; Alain et al., 1998; Miranda et al., 2004; Hoffman et al., 2002). Thus, as a sound

transitions from being novel to meaningful, cortical plasticity incorporates sound elements with the context of that sound.

By using complex and ethologically relevant stimuli, the breath of AC's abilities can be studied. Researchers have utilized the transition that occurs during infant rearing, when a new appreciation for infant vocalizations emerges, to examine the range of AC's plasticity during adulthood. The AC's role in emotional sound processing (LeDoux, 2000), makes it a logical site for studying the learning of conspecific vocalization, which can possess negative or positive valences (Morton, 1977). Because of altricial offspring vulnerability, infant care is a natural example of when detection, discrimination, and categorization of conspecific vocalizations can be critical for survival.

An important question for the science of auditory processing, as well as maternal behavior studies, is how do vocalizations transition from being novel to familiar and meaningful sounds? Auditory neuroscience research attempts to better understand how experience with infants leads to changes in the processing of their vocalizations. Evidence from electrophysiological studies suggest there are measurable differences in auditory cortical responses between the states of familiarity and unfamiliarity (Galindo-Leon et al., 2009; Shepard et al., 2016; Liu et al., 2006; Rothschild et al., 2013; Liu et al., 2007). To understand how these differences are formed and maintained, cellular and molecular research is warranted.

Strengthening synaptic connections between neurons is an underlying mechanism of learning and memory. Transcriptional regulation is often considered a signature of memory formation (Alberini, 2009). In a mature neuron, the transition from one transcriptional state to another re-shapes its functional capabilities (Tischmeyer et al., 1999).

The transcription of *c-Fos* and *Bdnf* genes are both associated with experience-dependent plasticity and memory formation. *C-Fos* is an IEG and transcription factor expressed in recently active neurons likely to experience plasticity. Due to its transcription start site being rich in paused RNA Polymerase II (Adelman et al., 2012), regulatory-factor-accessible and with maintained high levels of histone acetylation, the *c-Fos* gene has been considered constitutively permissive, not requiring *de novo* protein synthesis for transcription (Fowler et al., 2011). The resulting Fos family of proteins dimerize with other transcription factors such as Jun (named for the Japanese word for 17, ju-nana) or activator protein 1 (AP-1) to transcribe genes containing AP-1 promoter binding sites (Chiu et al., 1988; Rauscher III et al., 1988). They are associated with hippocampal dependent learning, NMDA receptor-dependent LTP, long-term synaptic changes, and memory formation through its transcriptional activity (Fleischmann et al., 2003; Alberini, 2009). In fact, it has been demonstrated that populations of neurons expressing c-Fos immediately after a learning experience go on to form the memory

engram of that experience (Minatohara et al., 2015; Liu et al., 2012). Up to 24 hours post-experience, *c-Fos* transcription aids in memory associated changes (Katche et al., 2010).

Within minutes of a contextual learning event, the *bdnf* gene can be transcribed in the hippocampus and sensory cortex, increasing the concentrations of the BDNF protein (Hall et al., 2000; Tokuyama et al., 2000; Alonso et al., 2005). The BDNF protein rapidly modifies synaptic components involved at the site of active synapses, encouraging the connection between neurons (Poo, 2001). After transcriptional activation due to experience-dependent learning, the *bdnf* gene may exhibit epigenetic modifications (Lubin et al., 2008; Bredy et al., 2007), which make its transcription more likely. Both the c-Fos and BDNF proteins provide or physically interact with the building blocks of synapses. They are important aspects of neural circuitry assembly and impact systems level activity to enhance the processing of environmental stimuli.

The c-Fos protein is commonly used to understand AC's response to vocalizations in mother and non-mother mice (Geissler et al., 2016; Fichtel et al., 1999; Wan et al., 2001). New studies using c-Fos expression as a measure of the molecular state in response to auditory stimuli can easily be compared to previous research. In Chapter 2's experiments, virgin female mice that were pup-experienced or naïve to pup-caring, heard pup-calls and then had the number of c-Fos positive neurons in their ACs assessed. By ovariectomizing and exposing subjects to estradiol or blank treatments, we

were able to control for both the potential neuromodulatory effects of estradiol and social experience with pups on the molecular response to a pup-call stimulus. We found experience decreased the number of c-Fos-IR neurons of the primary and secondary AC compared to the pup-naïve group, similar to previous research on familiar sound processing (Fichtel et al., 1999; Wan et al., 2001). Experience but not hormonal state, lead to significant differences in the response of AC neurons to pup-calls. This is in spite of the estrogen response element located in the *c-fos* promoter region (Weisz et al., 1990). The possible contributions of estradiol on novel or familiar sound processing was not measured through *c-fos* positive neuron totals.

Nevertheless, these results helped us form a clearer picture of AC's role as a site for sensory memory storage. *C-Fos* transcription is an essential component in AC memory acquisition and behavioral response (de Hoz et al., 2018). In our study, the AC of naïve subjects were processing the novel pup-call sounds and in response the AC c-Fos expression was preparing for plasticity. Previous experience cocaring for pups had presumably already altered the AC of our experienced female group. A state of familiarity within the AC requires stability and thus it may not be advantageous to induce plasticity during re-presentation and retrieval. Therefore, the adaptive response to a familiar stimuli in the AC would be to limit changes to established connectivity via low levels of c-Fos expression.

Sensory cortical areas must balance stable representations of previously learned stimuli against the plasticity for newly relevant stimuli. It is our interpretation that the AC processes familiar and novel social sounds differently, to enact plasticity when the sounds are behaviorally relevant or decrease plasticity when an already learned, familiar sound category is presented. Decreased expression of a protein that causes plasticity is adaptive for memory retrieval to familiar infant cues and a product of infant cue experience/learning.

The AC expression of c-Fos-IR was strikingly different than that of the neuromodulatory region the locus coeruleus, an area associated with attention and the production of norepinephrine. In the LC, there was an interaction between experience and estradiol treatment, with estradiol animals familiar with pup-calls displaying increases in LC c-Fos-IR. Behavior during the pup-call playback, resembled the interaction we found in LC c-Fos-IR. There was increased movement displayed by estrogen treated, pup-experienced subjects. While the AC decreased c-Fos-IR neurons with familiar social sound exposure, the LC c-Fos-IR increased. The role of the LC is to continue to be reactive to relevant stimuli once they are familiar. Perhaps the high c-Fos-IR in the LC of estradiol treated experiences females was because of the presence of a behaviorally relevant stimuli under the unique circumstances of pup-calls in the

absence of pups. This was a new context for the familiar stimuli that potentially induced the expression of c-Fos within the LC during its first occurrence.

The time of first infant interaction could be when the first molecular changes are modulating sensory representations for infant vocalizations. There is a gap in knowledge concerning the molecular mechanisms enacted during initial sensory experience and how they might induce the neural circuitry refinement for familiar sound processing and response. A number of sensory areas express the neurotrophin BNDF during sensory dependent learning tasks (Alonso et al., 2005; Klintsova et al., 2004; Tokuyama et al., 2000). For example, during development the BDNF protein participates in tone induced plasticity in the AC (Anomal et al., 2013). The regulation of BNDF transcription and its mnemonic effects is also associated with estrogens (Pluchino et al., 2013; Gibbs, 1999; Scharfman et al., 2006). However, BDNF has never been examined in the adult AC during social auditory experience. I therefore hypothesized that initial social experience with pups and their vocalizations and estrogen availability would increase the transcription of the *Bdnf* gene.

In a new experimental paradigm presented in Chapter 3, I examined BDNF transcription during initial sound experience within and outside of a social context. Subjects had their estrogens manipulated using ovariectomy and estradiol or blank capsule implants, and had 1 hour of pup experience or remain naïve, creating a 2x2 experimental design. Contrary to my initial hypothesis, estradiol treatment neither

increased the expression of total or isoform-specific *Bdnf* transcription nor affected retrieval behavior, so experimental groups were collapsed across their hormonal condition. Subjects hearing an audio recording of pup-calls during pup experience had significantly higher and isoform specific *Bdnf* mRNA transcription in their AC compared to pup naïve females. The social context of vocalizations induced immediate transcriptional response at the site of auditory cortical processing. This was specific to a BDNF transcript associated with experience-dependent learning and inhibitory interneuron functioning (Sakata et al., 2009; Timmusk et al., 1993; Lauterborn et al., 1996).

For the first time, the transcription of *Bdnf* was found to increase in the AC in response to social sound processing. It is likely that BDNF contributes to new plasticity in the AC after behaviorally relevant vocalization experience. This finding paves the way for AC studies on the epigenetic modifications of the BDNF promoter region, associated with long-term memory formation and extinction (Lubin et al., 2008; Fortress et al., 2014b; Bredy et al., 2007). Much like the findings in Chapter 2, the interpretation of our results remains that the AC processes familiar and novel social sounds differently. At the time of initial experience with a sound that is behaviorally relevant, increased transcription of synaptic modifying mRNA occurs.

Even though we did not find an effect of estradiol manipulation in our *Bdnf* study, other work has shown that memory consolidation is dependent on molecular and

cellular mechanisms and influenced by the hormonal state (McGaugh, 2000). Previous research suggested that hormones such as estrogens have an effect on memory and that similar markers of memory formation are induced in the hippocampus and sensory cortex. For example, one of the first major findings of estrogens' effect on memory formation was that estradiol treatment induces an increase in hippocampal neurons spine density (Woolley et al., 1993). Twenty years later other researchers found estradiol increased spine density in the somatosensory cortex during a somatosensory experience (Khan et al., 2013). I had therefore hypothesized that the effects of estradiol treatment would lead to measurable molecular changes within the AC at the time of auditory processing, including initial exposure of a relevant social sound.

With my thesis projects, I was unable to identify a point during initial vocalization experience or familiarity at which mnemonic effects of estrogens were evident in the AC. I found systemic estradiol manipulation affected neither AC c-Fos-IR during experience hearing vocalizations, nor BDNF mRNA transcription during initial experience with pups and their vocalizations. These findings failed to support my initial hypothesis. Estrogens decrease the latency of maternal response across species (Maestripieri et al., 1998; Champagne et al., 2001), so it was hypothesized that the AC might also be subject to swift plasticity events driven by the hormone class during and immediately after a learning event. It remains possible that estrogens' role in the AC might be restricted only to a narrower time window to aid in the rapid transition of pup-call processing to a

familiar state. There is evidence estrogens derived by neurons in the AC of mice have an effect on molecular states (Lu et al., 2019).The possible influence of neuron-derived estrogens at the site of auditory processing was not controlled for in our experimental designs (See sections 3.6.2 and 4.3 for discussion). Additionally, differences between our hormone treatment and the natural composition and fluctuation of hormones across pregnancy and parturition might contribute to our inability discover a hormonal effect in the AC.

However, my results did not conclusively close the door on future research of estrogens effect on AC plasticity. It is my hope the knowledge gained here can help focus the field of AC plasticity to identify new time points, and molecular mechanisms of interest. The molecular findings described above suggest various fruitful paths for future research on the underpinnings of social associated AC plasticity in social communication contexts.

In this final chapter, I suggest possible directions for future research, building on the results presented here. First, the limited expression of c-Fos in the AC neurons of pup familiar subjects could represent a mechanism of sensory processing and memory storage called “sparse coding”. Second, I will discuss the possibilities that initial experience was too early for estradiol to exhibit an effect on *Bdnf* transcription and that the effects of estradiol could be uncovered within a secondary signaling pathway. Third,

a way to identify how social auditory experience alters the transcriptional response of AC neurons long-term will be covered. Estrogen did not increase *Bdnf* transcription, but estrogens are associated with epigenetic modifications of the *Bdnf* promoter. Since we identified an increase in *Bdnf* transcription as a result of vocalization experience, it is possible epigenetic modification is where we might see the evidence of hormones on AC plasticity. Finally, this and other research on the role of hormones in AC plasticity has the potential to help half of the human population transition into low estrogen states such as menopause without cognitive deficits (Jenkins et al., 2004; Bender et al., 2006).

4.2 Sparse Encoding of AC Stimuli and Memories

One of the major questions facing neuroscience is, how is the need for cortical plasticity balanced with the need for stable memories? In order to preserve important neural circuitry, sensory cortices must balance stable representations of previously learned stimuli against plasticity for newly relevant stimuli. One strategy of memory storage in the cortex, which minimizes the disruption of previous memories, is the theory of "sparse coding". The theory of sparse coding suggests that only a small number of neurons, firing simultaneously, are necessary for sensory information processing and retrieval (Olshausen et al., 2004). A sparse coding method of storing auditory cue

memories could explain the results presented here, in Chapter 2 (Moreno et al., 2018), and elsewhere.

One of the benefits offered to a neural network using the sparse coding model is energy efficiency. The brain consumes a massive amount of metabolic energy and a strategy of sparse coding substantially decreases the energy necessary to process environmental information (Laughlin, 2001). Another benefit is with more simplistic firing patterns there is a decreased risk of cross-talk between unrelated memory traces. Finally, sparse coding means easier storing of smaller memory traces, however this needs to be balanced with increased trace size increasing stability.

The recent activity of neurons has commonly been measured by the translation and transcription of IEGs such as c-Fos. Using immunohistochemistry, neurons labeled positive for c-Fos were thought to have recently participated in neural activity. It was speculated that these AC neurons were involved in sound processing. More recent research has shifted the view of c-Fos from a marker of activity, to an instigator of plasticity. When a stimulus causes c-Fos expression in a hippocampal neuron, that neuron may become part of a memory trace for that stimulus (Minatohara et al., 2015; Liu et al., 2012). Thus, instead of indicating which AC neurons are just responding to a stimulus, c-Fos expression more likely reveals neuron response as well as which neurons are likely to experience changes in their connectivity and response to the stimulus in the future.

Within the experiments of Chapter 2, we found that recognition of pup-calls produced lower primary, and secondary AC c-Fos-IR than when the same stimulus was perceived by a naïve subject (Moreno et al., 2018). This is consistent with the hypothesis that sound familiarity leads to less c-Fos-mediated plasticity, and less disrupted sensory representations of a meaningful call category. Decreased c-Fos labeled neurons in the auditory pathway in response to familiar auditory cues is not a new finding (Wan et al., 2001). Previous research had found maternal experience associated with decreases in c-Fos-IR of AC primary neurons in response to pup-associated sounds compared to naïve subjects (Fichtel et al., 1999). If c-Fos is considered a marker of plasticity, the AC c-Fos-IR in response to a familiar stimulus provides a glimpse at the neurons which have become part of the memory trace, and due to the new context of the familiar sound, might alter their connectivity.

Research that includes naïve and familiar states of experience, are opportunities to view nascent and transitioning memory traces. Fichtel et al. (1999) concluded that the behavioral significance of a pup sound is correlated with reduced and "focused neuronal activity in the primary auditory field". The authors went on to describe the qualitative difference in immunohistochemistry labeling of Fos-positive cells. Subjects displayed two distinct patterns: mothers' c-Fos-IR "appeared generally well ordered in cortical columns" and "focused" while the staining in virgin females appeared "scattered"

(Fichtel et al., 1999). While subjective, this observation suggests that when cortical neurons are allocated with encoding a memory trace, the physical distance between neurons might be a factor to increase efficiency. This, paired with the decreased c-Fos-IR intimates that established sound engrams are smaller than the initial molecular response and appear to possess order.

One rationale for decreased c-Fos-IR expression during the retrieval of auditory memories is to increase metabolic efficiency. An engram requiring a smaller amount of neuronal firing, perhaps by limiting the number of neurons activated, could indicate more economic memory storage. The sparse c-Fos-IR displayed by experienced care-giver subjects could represent the efficient storage of pup-call memory because of the decreased number of neurons necessary for recall than the naïve perception of the same sound.

Efficient storage of sensory memory engrams would be a potential interpretation of the reduced number of c-Fos-IR neurons in the AC in response to familiar sound playback. There is evidence in the somatosensory cortex of rats which suggests that associative learning of a whisker stimulation leads to sparse encoding of that stimuli (Gdalyahu et al., 2012). In one study, whisker stimulation was paired with a foot-shock in rats to determine how the associative fear learning affected the population response to that stimuli in the primary sensory cortex. When the association was formed, fewer neurons responded to the same stimulation but the neurons that did respond displayed

increased response magnitude as measured by calcium imaging fluorescence change over baseline. The authors here described this as increased optimization of sensory memory encoding or increased "response fidelity." There is also evidence of sparse coding in the AC's response to sensory stimuli. Previous auditory studies have presented evidence of sparse coding of auditory related stimuli in the amygdala (Rogerson et al., 2014) and the AC (Terashima et al., 2013; Hromádka et al., 2008).

Thus, the c-Fos-IR neurons in the AC in the Chapter 2 study represented a small population of neurons allocated with holding the auditory memory trace of infant vocalizations as well as other neurons encoding new aspects of the calls being presented during the experiment. By visualizing and quantifying recently active neurons, future research could test an AC theory of sparse memory utilizing the ecologically valid stimuli incorporated within the methods of this thesis. If sparse coding is a strategy employed by the AC for vocalizations, I would expect calcium imaging, similar to that used by Gdalyahu et al. (2012) would be able to determine the difference in number and response during the processing of novel or familiar pup cues. Advances in imaging techniques such as genetic tagging of active neurons could potentially allow researchers to examine these differences within the same subjects, before and after they gain pup experience (Tasaka et al., 2018).

4.3 THE IMMEDIATE MOLECULAR CHANGES WITH INITIAL PUP EXPERIENCE

By examining the changes enacted in the AC during initial pup vocalization experience, the underpinnings of AC plasticity for complex and behaviorally relevant sounds can be determined. Future research on this initial time point should begin by determining the type of cells transcribing *Bdnf*. The BDNF protein, including the exon IV gene transcript, is associated with inhibitory plasticity during development and adulthood (Huang et al., 1999; Hong et al., 2008). Given this association, I would hypothesize that it is likely inhibitory interneurons in the AC are responsible for the increase of *Bdnf* mRNA in response to behaviorally relevant pup-call processing.

Even though c-Fos at the time of recognition or novelty, and BDNF at initial experience did not show evidence of estrogen modulation, there are still multiple alternative routes estrogens could utilize to influence memory-associated molecules and affect AC plasticity during pup-call experience (Figure 4-1). Through the activation of second messenger systems, estrogens can influence modulators of memory formation (Abraham et al., 2004; Zhou et al., 1996; Silva et al., 1998) linked to auditory learning (Han et al., 2008; London et al., 2008). The most conceivable pathway for estrogens to impact auditory plasticity would be through the memory associated mitogen activated protein kinase (MAPK) pathway (Sweatt, 2001; Abraham et al., 2004).

When estrogens bind with estrogen receptor alpha, beta, or G-protein coupled receptors, they can rapidly activate MAPK cascades including that of extracellular signal

regulated kinase (ERK). This signaling leads to the phosphorylation of CREB (cyclic adenosine monophosphate response element binding protein) at its Serine 133 residue (pCREB; Figure 4-1) (Gonzalez et al., 1989; Boulware et al., 2005; Marino et al., 2006; Zhou et al., 1996).

CREB is a well-studied transcription factor in the nervous system, involved with memory formation (Frank et al., 1994; Silva et al., 1998; Lonze et al., 2002). Phosphorylation of CREB can occur via the molecule cyclic adenosine monophosphate (cAMP), the influx of calcium during membrane depolarization, or by the converging of these two systems (Sheng et al., 1990). In the sensory cortex, CREB activation can be experience-induced, occurring within the neural circuits that encode the salient features of the experience (Desmedt et al., 2003). In rats, estrogens are thought to increase somatosensory cortex spine density through pCREB (Khan et al., 2013). If estrogens play a similar role in learning auditory social cues via the non-genomic estrogen signaling pathway, then one would expect pCREB to increase in the AC as it does in the hippocampus and sensory cortex areas during learning (Figure 4.1).

Using similar experimental paradigms described earlier, one could test the hypothesis that initial pup-auditory exposure increases the ratio of pCREB to CREB in the AC of female mice depending on whether pups and estrogens are present. Estrogens might have an additive or multiplicative effect on pCREB. Regarding estrogens and manipulating hormones, the various sources of estrogens must be experimentally

controlled by researchers. A recent publication in mice suggested brain-derived estrogens are capable of preventing depletion of pCREB due to ovariectomy (Lu et al., 2019). This is one factor that might have contributed to the inability of my designs to identify a role of estrogens.

Female mice in any future study into estrogenic modulation of pCREB should also knock down aromatase in the brain regions of interest, instead of relying on ovariectomy to deplete available estrogens. Conducting immunohistochemistry to visualize pCREB in future research could determine CREB's response to social auditory learning. This is one potential method of determining if estrogens rapidly modulate the activation of a well-known molecular cascade in the AC during pup vocalization exposure.

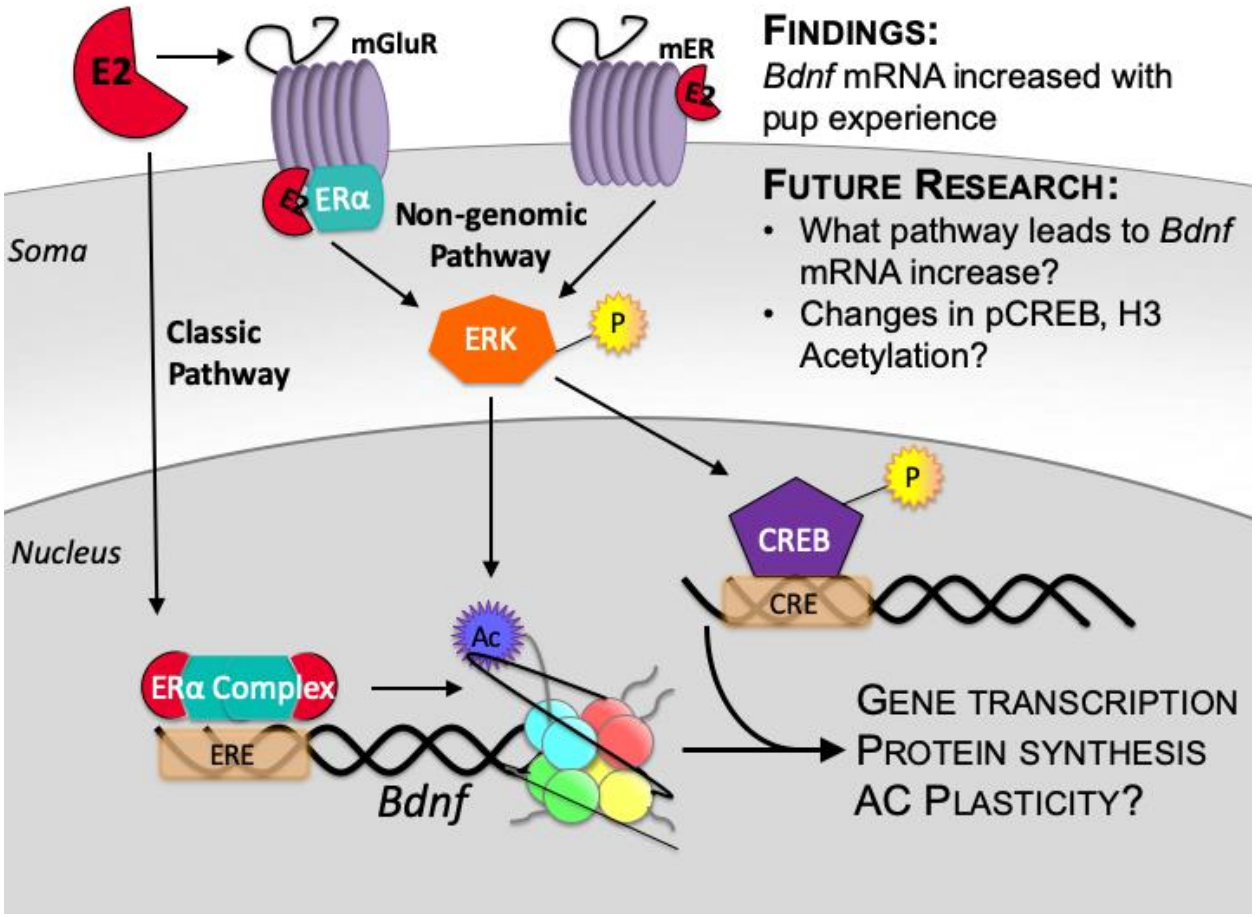

Figure 4-1 *Estrogen-Sensitive Molecular Pathways*

This simplified figure outlines estrogens' molecular mechanisms associated with enhanced memory formation. Abbreviations: Estradiol (E2), metabotropic glutamate receptors (mGluR), membrane bound estrogen receptor (mER), estrogen receptor α

(ERα), extracellular regulated kinase (ERK), cAMP response element binding protein (CREB), phosphorylation (P), histone 3 (H3), acetylation modification (Ac), estrogen regulatory element (ERE), cyclic AMP response element (CRE), brain derived neurotropic factor (Bdnf gene).

4.4 Long-Term Changes of Bdnf Transcription: Epigenetic Changes

Chapter 3 addressed potential mechanisms for hormonal modulation of sound encoding at the point of the first pup experience. However, there remains a gap in knowledge concerning how the maternal physiological state, specifically during pup exposure, affects the longevity of vocalization recognition. The cortical plasticity in a mother's AC for pup vocalizations appears to be more resilient to decay than experience-only sensitization without maternal hormones (Lin et al., 2013). Here, I will review studies that suggest a mother's hormonal state, when she first gains social experience with pups, may be critical for the long-lasting neural encoding of infant cries.

Mother mice maintain maternal responsiveness to pup-stimuli for longer than non-mothers with the same level of experience behavior (Rosenblatt et al., 1981; Stolzenberg et al., 2011). While mothers experience pups for the first time after an extreme influx of hormones, these hormones are not necessary for the reinstatement of maternal behavior. Maternal responses to 50 kHz pup-model calls could be elicited from mouse

mothers 1 month after weaning, even when these mothers were ovariectomized post-weaning (Ehret et al., 1989). The same was not true for pup-experienced virgin cocarers. Perhaps hormones play a role in the initial consolidation and not during later presentations of infant cues. The research of Chapter 3 stopped short of investigating the long-term nature of auditory plasticity for infant vocalizations that occurs in the presence of maternal hormones. With the discovery of increased *Bdnf* transcription upon initial pup experience, next it can be determined how and where the protein is translated, as well as possible epigenetic mechanisms for its continued transcription in nascent memory traces.

The physiological state of motherhood appears to contribute to AC plasticity. Call inhibited single-unit responses in the A1 and AAF regions lateral to pup-call sensitive areas change depending on motherhood, not simply pup-caring experience. Virgin female mice with 5 days of pup caring experience (early cocarers) and post-weaning (21 days) mothers had similar call inhibited single-unit responses in their A1 and AAF regions lateral to the high frequency pup-sensitive region. However, when cocarers were tested after their foster pups were weaned, their call inhibited response had decayed (Lin et al., 2013). In other words, cocarers' consolidation of the meaningfulness of pup vocalizations in the AC dissipated by the time their foster pups stopped producing calls, while mothers maintained enhanced processing post weaning. The neural results found by Lin et al. (2013) paralleled their behavioral results, wherein they found that while

mothers and early cocarers preferentially approached natural pup-calls over a neutral sound in a two-alternative choice task, post-weaning cocarers did not, suggesting a loss of the call's salience.

The longevity of maternal responsiveness by mothers suggests epigenetic modifications might maintain long-term consolidation (Stolzenberg et al., 2012). Perhaps, the intense concentrations and precipitous drop of hormones right at parturition cement modifications in AC plasticity, which leads to long-term auditory memory formation. As I explain next, future studies could consider how sensory memories in virgin mice might be prolonged through hormonal treatments that engage epigenetic mechanisms.

Epigenetic modifications in the subcortical MPOA support maternal behavior. These alterations were found at estrogen and oxytocin receptor promoter regions, influencing gene expression as well as that of CREB associated histone acetyltransferase (Stolzenberg et al., 2012; Champagne et al., 2006; Stolzenberg et al., 2016; Stolzenberg et al., 2014). It is possible that epigenetic modifications within the AC could also result in long-term plasticity in mothers. Future research should determine whether fluctuations of maternal hormones at the time of pup-call encoding induces long-term AC plasticity by enacting epigenetic modifications.

A likely target of epigenetic modification by estrogen or other hormonal induced mechanisms is the promoter region of the *Bdnf* gene. There is substantial overlap

between the actions of estrogens and the neurotrophic factor *Bdnf,* as well as evidence that they might act synergistically to increase spine formation and memory enhancement (Luine et al., 2013). Estrogens can affect the transcription of plasticity and memory-associated genes such as *Bdnf* by activating kinase cascades (Swank et al., 2001; Sohrabji et al., 1995). But most important for long-term memory, estrogens can change neuronal response by enacting long-lasting epigenetic modifications (Fortress et al., 2014b). For example, estrogens are associated with an increase in the acetylation of histone protein 3 (H3) specifically at *Bdnf*'s exon II and exon IV promoter regions, in a MAPK/ERK pathway dependent manner (Zhao et al., 2010). Acetylation of H3 in association with estrogen treatment leads to an increase in BDNF protein in the hippocampus (Fortress et al., 2014b) and improved novel object recognition and location memory.

Although changes in neuronal gene expression via epigenetic modifications in the sensory cortex are known to promote long-lasting changes in neuronal activity (Swank et al., 2001), epigenetic research to understand social auditory communication in the AC is still forthcoming. Now that we know that *Bdnf* transcription increases in response to pup-experience, we can examine whether epigenetic mechanism are responsible for long lasting changes in Bdnf response. A power analysis of the single-sided t-test on experience presented in Chapter 3 for the AC, with a larger common standard deviation, indicates that 10 subjects per condition is required for a power of 0.8 ($\mu1 = 1$, $\mu2 = 1.89$,

σ = 0.8, 1-sided test of experience, alpha = 0.05, power = 0.8; University of British Columbia Power/Sample Size Calculator). To confirm the results of chapter 3, another cohort of subjects of the same size should be used. Perhaps a role for estrogens could be to aid in epigenetic modifications (Zhao et al., 2010; Silingardi et al., 2010; Frick et al., 2014) in the AC, which then lead to long-term changes in *Bdnf* transcription. Future research could use the maternal mouse model to determine whether estrogen's presence at the time of pup call experience is necessary and sufficient to induce more enduring social auditory learning by enacting long-lasting molecular changes.

Questions such as this could be answered by studying natural mothers, and experimentally delivering hormones to virgin females to replicate maternal hormone fluctuations. Virgin female mice can receive ovariectomies and remain gonadal estrogen-free or receive estrogens, including the placenta-associated estriol, before and at the time of pup experience. Hormone treatment could be ceased post-sensitization or around 5 days of pup-caring experience. By testing subjects post-weaning, one could then assess the long-term effects of estrogens when present at the time of the first social auditory experience such as epigenetic modifications and long-lasting behavioral response.

There are a number of different epigenetic modifications that could be examined in these animal groups. For example, estrogen exposure during social auditory experience

could lead to an increase in global levels of H3-acetyl or an increase in the ratio of H3-acetyl to global H3 in the AC, while naïve and vehicle treated subjects would likely have lower ratios. Chromatin immunoprecipitation could determine if *Bdnf* promoter regions specifically experience histone acetylation. Alternatively, mother mice could be treated with histone deacetylase inhibitors in their AC once a day for the first 5 days of pup experience. These mothers could then be behaviorally assessed for their preferential approach to pup calls, or have their average call-inhibited SU spike rates measured (Galindo-Leon et al., 2009; Lin et al., 2013). This future research has the potential to identify specific epigenetic changes in the AC in relation to long-term memory of social auditory cues. Projects such as these could determine whether intrinsic maternal physiological state can act as a facilitator of auditory plasticity through epigenetic changes to genes associated with memory.

4.5 Applications: Female Post-Menopausal Memory Deficits

Post-menopausal women experience difficulty in verbal-information learning and auditory stimulus processing (Jenkins et al., 2004; Bender et al., 2006). Effective menopausal treatments can include exogenous estrogens, which can improve this population's verbal-memory deficits (Wroolie et al., 2011; Sherwin, 2005; Kampen et al., 1994). However, due to estrogen receptor expression throughout the body, hormone

therapies come with the risk of peripheral side effects, such as breast cancer and cardiovascular disease (Chlebowski et al., 2003; Rossouw, 2002). Additionally, not only can some hormone treatments negatively impact verbal memory (Resnick et al., 2006), but they may also cause the acceleration of age-related hearing loss (Price et al., 2009). To improve the verbal-specific deficits in post-menopausal memory, research at the cortical site of memory consolidation, is justified that spans estrogens, aging, and auditory communication. By elucidating the role of estrogens in adult cortical plasticity, auditory neuroscientists have an opportunity to uncover the verbal-processing mechanisms naturally strengthened by estrogens in adult women, which are lost to aging in the auditory cortex.

Research in the hippocampus provides a template for how one might examine estrogen's role in the AC, believed to be the site of neural traces of auditory memories (Banerjee et al., 2013). Estrogen receptor activation in the hippocampus is linked to enhanced learning and memory formation via multiple well established molecular pathways (Khan et al., 2013; Woolley et al., 1993; Boulware et al., 2013). Auditory neuroscientists should follow the lead of previous work in the hippocampus, identifying the biochemical mechanisms downstream from hormone receptors. Such an approach, to catalog nonsteroidal targets for pharmaceuticals, could avoid the negative side effects of current hormone (Frick, 2012).

As discussed earlier, a number of estrogens' rapid memory enhancing actions occur by activating second messenger systems have been identified in hippocampal neurons. Estrogens can increase the phosphorylation of CREB within hippocampal neurons through activation of cellular kinase cascades (Figure 4-1). CREB phosphorylation is associated with synaptic plasticity, dendritic spine formation, and learning (Alonso et al., 2005; Thomas et al., 2004; Murphy et al., 1997). Once phosphorylated, CREB increases the transcription of genes containing a cAMP response element within their promoter regions (Montminy, 1997; West et al., 2001; Zhou et al., 1996). In this way, estrogens can help initiate experience dependent expression and synthesis for memory-associated proteins such as BDNF (Luine et al., 2013). This is a critical avenue to explore given that aging women with low levels of plasma BDNF have increased risk of impaired verbal memory (Komulainen et al., 2008). The close relationship between steroid hormones and trophic factors could yield insights on the neuroprotective effects of both (Sohrabji et al., 1995; Pluchino et al., 2013; Wise et al., 2001; Brann et al., 2007).

Unlike the hippocampus' rapid learning, the cortex allows for new information to be gradually incorporated and organized, producing stable and long-lasting consolidation (McClelland et al., 1995). Thus, some memory mechanism are likely to be more prominent in the cortex than in the hippocampus. One long-term memory mechanism likely to play a prominent role in cortical memory is epigenetic modification. Identified

in the hippocampus and cortex, epigenetic modifications have yet to be fully examined in the AC. Epigenetics has advanced memory research by examining how molecular constructs such as DNA methylation and histone modifications alter neuronal gene expression in response to learned experiences (Day et al., 2013). It is theorized the epigenetic code is responsible for stable, long-term memory formation, by keeping new synaptic connections supplied with protein products (Sweatt et al., 2013; Miller et al., 2010), and that estrogens influence *de novo* DNA modifications (Zhao et al., 2010). Knowing how the sensory cortex employs epigenetic modifications in adulthood and estrogen's faciliatory role is a fruitful path for translational discovery.

With available techniques, crucial memory elements within the AC can be identified as new targets for drug development. The maternal mouse model is well suited for exploring how hormonal state facilitates learning of conspecific vocalizations through epigenetic memory formation. Using this model as well as other available molecular techniques, the differences between the molecular state of pre- and post-menopausal females can be compared. Expanding memory research by examining the role of hormones can lead to better treatment of individuals with neurological disorders affecting memory (Frick, 2012; Luine, 2014). The proposed research will impact the future study of long-term memory, epigenetics, cortical plasticity, and auditory processing by examining an inducible period of neural plasticity in adulthood.

5 APPENDIX

5.1 ADVICE FOR FUTURE RESEARCHERS

5.1.1 qPCR Housekeeping Genes

I did considerable research in order to find the best housekeeping gene for a qPCR on mice gaining a new behavioral experience with an estrogen manipulation. Previous work had used glyceraldhyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene in paradigms studying the effects of estradiol on memory formation in the hippocampus (Zhao et al., 2010). However, in the central nervous system (CNS), estrogen and the GAPDH protein have an established relationship. Whole rat brain CNS membrane preparations are used to explore the membrane binding sites of steroids. Preparations run though estrogen affinity columns found GAPDH binds to the steroids estradiol and to a lesser extent, progesterone (Ramirez et al., 2001). The same group of researchers also established estradiol increasing the catalytic activity of GAPDH in vitro (Joe et al., 2001). In addition to this *in vitro* relationship, in vivo effects of estrus cycle were seen with increased fractions of GAPDH coincided with the high estrogen proestrus phase in both the hippocampus and the cerebellum (Ramirez et al., 2001). Kuroda et al., failed to see a significant increase in *Bdnf* mRNA in the hippocampus of rodents after pup exposure using GAPDH as a reference gene (Kuroda et al., 2007), but it may be that better housekeeping controls should have been run

GAPDH has various roles in cellular activity for which estrogen could effect, including but not limited to metabolism, synaptic remodeling and apoptosis. Therefore clear conflicts exist with the use of GAPDH as a housekeeping gene in hippocampal, memory, and/or estrogen studies and perhaps steroid studies in general.

In the hippocampus, Ribosomal protein L19 (RPL19) has been established as a valid hippocampus housekeeping gene even during periods of vast structural changes such as development (Al-Bader et al., 2005). It has also been used as a reference gene during estrogen treatment experiments (Takeo et al., 2009). I decided this was the best for use in my hippocampus samples.

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