RESEARCH STRATEGY

Significance:

The auditory cortex (AC) is a key site for processing conspecific vocalizations, a complex and critical function of the auditory system (3). AC is also critical in auditory learning, as it exhibits long-term, experience-dependent plasticity for behaviorally relevant stimuli (4, 5). The processing and learning of conspecific auditory cues can be profoundly modulated by steroid hormones such as estrogen (E) in many species (6-9). Despite E's modulation of acoustic communication perception (7, 10-12) and social recognition (13, 14); the expression of E's receptors (ER) along the auditory pathway including the AC (15, 16); and E's memory enhancing mechanisms for non-social learning (17), there is a gap in knowledge concerning how E can mechanistically promote the formation and longevity of social auditory memories. *The proposed research is significant because it will for the first time investigate whether critical molecular signaling pathways for establishing (Aim 1) and maintaining (Aim 2) memories are modulated by E in the mammalian AC during experience-dependent, communication sound learning.*

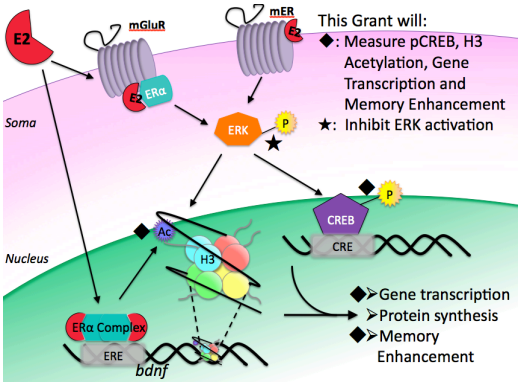

Figure 1: Estrogen's molecular mechanisms enhance memory. Simplified figure: black star represents a manipulation, while squares represent proteins examined in this grant proposal; estradiol is E2, metabotropic glutamate receptors is mGluR, membrane bound estrogen receptor is mER, estrogen receptor alpha is ERα, extracellular regulated kinase is ERK, cAMP response element binding protein is CREB, phosphorylation is P, histone 3 is H3, acetylation modification is (Ac), estrogen regulatory element is ERE, CREB response element is CRE, and brain derived neurotropic factor is the *bdnf* gene.

The ultrasonic vocalization (USV) system between mouse pups and adult female mice is an ideal ethological model for studying the auditory cortical mechanisms for communication sound processing and learning (18). Mouse pups emit USVs when isolated, eliciting a search-and-retrieval response from mothers (Dam). Dams preferentially approach pup call models over neutral sounds (19), demonstrating their recognition of USVs as behaviorally relevant. Importantly, this recognition memory can be learned, since virgin females with sufficient experience caring for pups (co-carers, Coc) also display this preference (19). Learning to respond to pup USVs requires left AC (20, 21), where electrophysiological mechanisms triggered by pup cues support acquiring the behavioral significance of calls (21, 22). This results in long-term changes that improve neural detection, discrimination and categorization of those calls by dams, lasting weeks after maternal experience (23-25). Recent studies suggest that maternal hormones can accelerate learning (21) and prolong recognition memory for pup USVs (1), highlighting the importance of hormone-sensory experience interactions. These enhanced abilities can also be directly attributed to E (11, 19), yet the molecular mechanism that E acts through to strengthen this social auditory learning and memory is unknown.

Memory-associated molecular candidates for E-enhanced social auditory learning include classic molecular cascade activation (26, 27) as well as newly identified long-lasting epigenetic modifications (28). Through the activation of kinase-associated molecular cascades within neurons, E causes the phosphorylation of cyclic adenosine monophosphate (cAMP) responsive element binding protein (pCREB), a universal modulator of memory formation (Fig 1) (27, 29, 30) linked to auditory learning (31). Activating this transient molecular cascade leads to long-lasting transcriptional activation of genes, which contain the cAMP response element (CRE) (29) (Fig 1). E can also induce the transcription of plasticity and memory-associated genes such as brain derived neurotropic factor (*bdnf*) through these kinase cascades (32), direct binding to an E response element (ERE) (33), or long-lasting epigenetic modifications (34) (Fig 1). E's promotion of epigenetic changes explains how E is capable of inducing stable changes in genetic expression (35). Although changes in neuronal gene expression via epigenetic modifications in the sensory cortex are known to promote long-lasting changes in neuronal activity (32, 36), in the persuit of understanding social auditory communication the proposed research will be the first to investigate hormonally linked epigenetic changes in the AC.

Aim 1 will use the maternal mouse model to determine if E's activation of critical memory-modulating molecules during social experience generates long-term plasticity in the AC. **Aim 2** will determine if 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.

Understanding how social neuromodulators affect social learning and behavior will allow future researchers to develop focused therapeutics, improving the lives of individuals with deficits in auditory communication. Disorders such as autism, schizophrenia, and deafness can impose deficits on conspecific vocalization recognition (37-40). Deficits in autism and schizophrenia can be specific for social auditory stimuli, suggesting that unique mechanisms underlie the recognition of social auditory information (38, 41). Because older deaf

individuals who receive cochlear implants require significantly more time to learn auditory speech than younger implant receivers (42) there is a need for therapeutic enhancement of adult language learning. In fact, cochlear implants induce CREB activation and *bdnf* expression in AC (43). Therefore, the therapeutic targeting of downstream effectors of E has the potential to improve social auditory attention and language learning in patient populations.

Approach:

General Experimental Design: Our *central hypothesis* is that processing and remembering behaviorally relevant sounds is modulated by an animals' hormonal state, which can induce molecular and behavioral changes detectable at the time of memory encoding (Aim 1) and memory recall (Aim 2). By examining molecular, epigenetic, and behavioral changes, this research will determine how E and pup experience affects AC neurons and behavior in the context of natural acoustic communication sound learning.

Animal Groups: Dams are responsive immediately after parturition and retain long-term responsiveness to pup-calls (Fig 5b) (1, 19) and thus will serve as a baseline for how hormones and experience naturally induce long-term pup-call responsiveness. Hormone exposure will manipulated by providing adult ovariectomized (OVXed) females with E2 implants (+E), or vehicle/blank implants (+B). E2 implants deliver high concentrations of estradiol, comparable to the levels found in pregnant dams (44), allowing manipulation of E exposure (11). This controls for estrous phase fluctuation of model pup-call responses rates (12). To produce pup-call experienced animals, OVXed implanted animals will experience pup socialization. This grant will use animals that are; naïve to pups (Nïv), obtain limited (hour-long) experience with pups, or are co-housed with mothers to receive pup cocaring experience (Coc).

Justification: Since recent literature has suggested an important role of E in pup-call recognition (45, 46), the laboratory has precisely construct animal groups as described above to better interpret electrophysiological, molecular and behavioral changes associated with social auditory learning.

Behavior: In both Aims, two behavioral assays will be used to assess pup-call responsiveness: pup search and retrieval and a W-maze. The search-and-retrieval behavior elicited in female mice by pups scattered outside the nest, a now established mammalian model of social communication (18), will determine maternal responsiveness. First, a subject is placed in a cage with a nest occupied by pups. Second, the experimenter will remove three pups from the nest and place each in three different corners of the cage, inducing USVs by the pups. If the subject returns the three pups to the nest, the experimenter will scatter the three pups into three corners once more. A subject is described as maternally responsive if they are able to return all three scattered pups to the nest two times within fifteen minutes.

The W-maze is a two-alternative choice memory recall task developed to assess approach behavior of Cocs, Dams and Nïvs, elicited by speaker playback of pup-calls, tones at the same frequency range (65 to 74kHz) of pup-calls or behaviorally irrelevant tones (20kHz) (1). Animal subjects with pups are placed in a nest with servo-controlled doors blocking the maze entrance. Speakers on opposing sides of the maze play sounds alternately. When the doors are opened, the phonotaxis of the animal is measured once it crosses a threshold in either of the two arms, indicating an approach decision toward one of the two speakers. The W-maze measures preferred approach as the ratio of pup-like to control sound approaches.

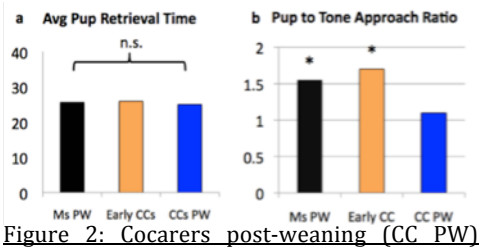

Figure 2: Cocarers post-weaning (CC PW) show degradation of pup-call memory that is not attributable to maternal responsiveness. (a) Mothers post-weaning (Ms PW), cocarers with 5 days of pup experience (Early CCs) and CCs PW show no significant difference in the average time in seconds to search and retrieve scattered pups. (b) Using a W-maze to assess pup-call memory, CCs PW show degradation of pup-call memory (1, 2).

Priming the maze with pups encourages maze exploration during testing but unfortunately exposes animals to vocalizing pups before pup vocalization memory testing (1, 47). To circumvent this confound, postnatal day (p)5-p7 pups who have undergone a survival surgery in which the inferior laryngeal nerve is unilaterally dissected, preventing the production of ultrasonic vocalizations (48) will be scattered throughout the maze before memory testing. Thus, subjects will be exposed to positive-reinforcing pup social contact (49), yet not be exposed to pup USVs until speaker playback.

Justification: After giving birth or sufficient pup caring experience, female mice will approach pup-calls because they are maternally motivated. However, their preferential approach to pup USVs over other sounds in a two-alternative choice test reflects a mnemonic recognition for calls that goes beyond simple maternal motivation. By testing animals' search-and-retrieval behavior with devocalized pups, and only using maternally responsive females, maternal responsiveness can be experimentally separated from vocalization recognition to examine social auditory long-term memory (1, 47). In fact, differences between groups cannot be attributed to maternal motivation since the

average latencies to retrieve pups was not significantly different (Fig 2a) (1).

Dams post-weaning and Cocs with five days of pup experience display significant pup-call preferred approach, however cocarers post-weaning do not preferentially approach pup-calls over tones (Fig 2b), suggesting that maternal levels of reproductive hormones such as E during pup care experience are important for the persistent recognition of calls. Perhaps surprisingly, endogenous E post social-experience is not necessary to assist maternal approach behavior in rodents (11, 50). This evidence points to the existence of memory mechanisms, induced by E during pup-call learning with long lasting affects, even in the absence of E.

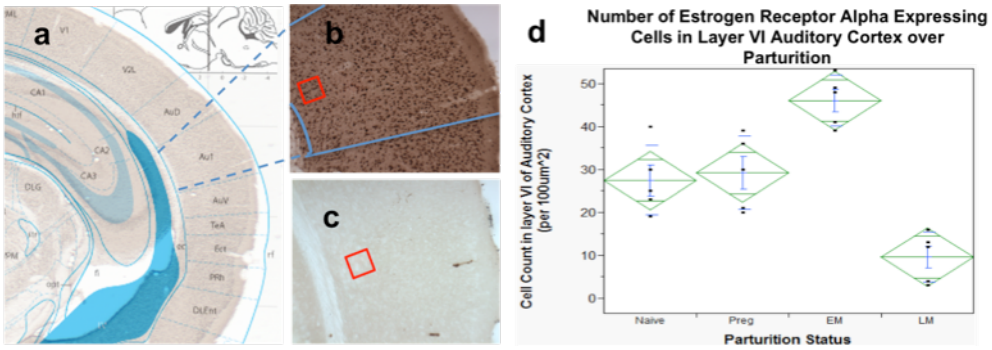

Figure 3: Neurons expressing ERα in the AC change significantly over parturition: (a) Primary auditory cortex (Au1) identified in 45mm sections cut from Bregma -2.18mm to -3.64mm of the C57BL/J6 strain mouse. Red box in 20x stain (b) and blank (c) sections are 100µm² size cell count areas in cortical layer VI. (d) Graph shows average number of ERα labeled neurons in cortical layer VI of the auditory cortex (AC) in: Naïve, n=4; Pregnant (Preg), prenatal day 12, n=4; Early Mother (EM), post natal day 4, n=4; Late Mother (LM), postnatal 21, post weaning 2 days, n=4. [F(3,20)= 21.46, p <.0001]. Mean diamonds in green. Mean and standard deviation in blue. Methods: Immunohistochemistry with ERα antibody raised against C-terminus of mouse ERα. These data are relevant because they show over the course of parturition, neurons in the AC change phenotype and possibly their sensitivity to estrogen. AC estrogen sensitivity could be essential to rapid pup call learning. These techniques will be used in Aim1.

Molecular: As previously mentioned, E has a direct effects on a memory-associated molecular cascade, which activates CREB by phosphorylation (Fig 1). Upstream of CREB is extracellular signal regulating kinase (ERK), preceded by mitogen-activated kinase-kinase (MEK). MEK inhibitors, such as U0126, cause decreases in pCREB and learning deficits (30, 51, 52) and thus are commonly used to determine if a type of memory formation is ERK-pathway dependent. For example, antagonist infusions in the auditory forebrain of songbirds determined that tutor song learning is ERK-dependent (53). Infusions of 0.04 to 1.0ul per hemisphere of the MEK inhibitors U0126 administered pre- or post- learning experience are frequently employed to identify ERK-dependent memory formation (51-53). To assess pCREB in the mammalian AC, a pilot immunohistochemistry study will determine the time of maximal pCREB expression post pup-call playback of limited pup experienced animals, without U0126 infusions. pCREB expression occurs rapidly after re-exposure to a recently learned stimuli, often taking only 30-60 minutes before maximum expression (54) therefore, pup experience sessions in U0126 experiments will be short for the rapid assessment of CREB activation. An additional pilot study will determine the optimal dose of U0126 to induce auditory memory deficits in the AC.

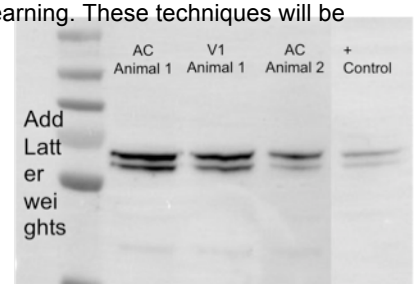

Figure 4 Western Blot verification of ERα in AC. Stained using same antibody used for staining (MC-20 SC, Fig X), confirms the presence of ERα in the Auditory, and Visual cortex, as well as the MPOA/BNST positive control. Assay optimization is necessary to yielded adequate protein concentrations. This technique will be used in Aim 1 and 2.

Justification: Immunohistochemistry, Western Blotting, and ChIP assays are ready for utilization to address these aims and alternative strategies if needed. Our preliminary immunohistochemistry data found more neurons in the AC express E receptor α (ERα) than previously thought (Fig 3) (16). Anti-body specificity was confirmed using AC tissue in Western Blots (Fig 4). In addition to this increased expression discovery, we found the highest density of ERα in AC occurs during late pregnancy, followed by a precipitous drop by p5. This suggests that as E concentrations reach their height in late pregnancy, the AC increases it's receptiveness by increasing receptors as well as the molecular signaling described above, creating a cortex primed for plasticity at the exact time that pup stimuli will be experienced. This finding adds credence to the hypothesis that E primes the AC for the rapid learn of infant vocalizations(45, 46). Therefore, the following studies are the next logical steps for discovering how estrogen changes the molecular activity and structure of AC neurons.

Specific Aim 1: ***Determine estrogen and auditory experience's role in the expression of behavior, memory-associated molecules, and the regulation of genes in mouse auditory cortex:*** The objective of Aim 1 is to determine how hormonal state and social auditory experience influence the expression of memory associated molecules and behavioral responsiveness at the time of memory encoding. To achieve this

objective, *we will test the hypothesis that estrogen modulates the transcription of RNA, and activation of proteins associated with memory formation in the AC of animals that have recently received pup experience, directly affecting infant cue processing.* To test our hypothesis four separate studies will be employed, each manipulating E, social auditory experience or both. Aim 1.1 will determine if a MEK phosphorylation antagonist can decrease pCREB and social auditory memory formation. E's specific targeting of histone 3 (H3) for acetylation (H3-acetyl) in the hippocampus is linked to increases in memory (34, 35) and might be responsible for E's ability to regulate the transcription of genes such as *bdnf* (34, 55-57). To understand how E can alter gene transcription, Aim 1.2 will determine if social auditory experience during E exposure modulates AC's *bdnf* gene transcription, global H3-acetyl and H3-acetyl at *bdnf* promoter regions in.

When the proposed studies for Aim 1 have been completed, it is our *expectation* that the role of ERK and pattern of H3-acetyl in AC social auditory learning will be determined. We will know if pCREB and behavioral recognition is MEK/ERK cascade mediated. We will also determine if at the time of pup-call experience H3-acetyl occurs globally, if it occurs in the promoter region *bdnf* and if there are changes in *bdnf* transcription. The outcome of this experiment will be the identification of the specific mechanisms responsible for social auditory learning. Future research can focus on the discovered mechanisms of E enhanced social auditory learning to develop targeted pharmaceuticals, aiding individuals with auditory communication deficits.

Research Design:

Aim 1.1: We will determine if treatment with a MEK phosphorylation antagonist effects CREB activation and social auditory memory formation.

In this experiment OVXed animals will receive E or blank implants and receive U0126 or vehicle injections in association with pup experience. At the time of OVX and implant surgery, animals will receive bilateral canalization of the AC and will be randomly assigned to U0126 or vehicle groups. No Nïv animals will be used .+E or +Bs will receive one hour of pup experience followed by pup scattering and while pup-call playback occurs. Animals will then be anesthetized, placed in a stereotaxic instrument and microinjected bilaterally with U0126 or vehicle, allowing five minutes for diffusion before syringe withdraw. Animals will be allowed 24 hours to recover before pCREB or behavioral assays.

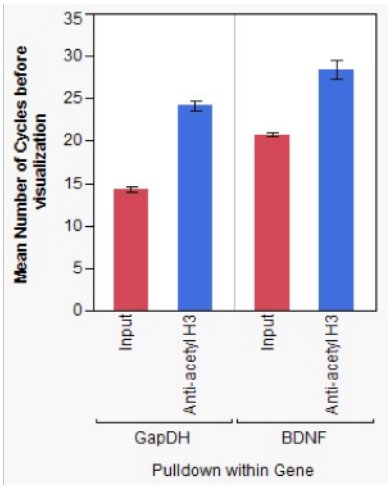

Figure 5: Acetylated H3 antibody pulls down genes in chromatin immunoprecipitase (ChIP) assay. A protocol for *bdnf* associated H3 has been successfully developed and run with AC tissue samples, n=12, no experimental groups. *GapDH* is run as a control. The number of gene amplification cycles run before illumination for H3 associated *GapDH* is only an average of 9 cycles more than the cycles necessary to visualize the non-pulled down input DNA. This method will be used in Aim 1.2.

pCREB Assay:+E and +B animals will be re-exposed to pup-call playback in an anechoic chamber 24 hours after initial pup experience. Animals will be sacrificed and AC tissue collected at the time of maximal pCREB expression after playback. Western blotting will incubate samples with CREB, and pCREB antibodies. Chemiluminescence measurements between bands will quantify protein density. These values will be normalized to the total amount of pCREB and CREB obtained from each sample. Based on previous studies (58), G*Power software determined a two-way ANOVA with f= 4.25 for ERK-dependent CREB activation would require a total of 32 animals, with 8 animals in each of the four groups for an α of 0.05.

Behavioral Assay: An age and condition-matched group of animals will be tested in a W-maze 24 hours post pup experience (Fig 3). The latency of pup-call approach and ratio of pup versus non-pup tone approach will be measured. Based on previous learning behavior research using ERK inhibitors (35), G*Power software requires the use 32 animals to an α of 0.05 in this experiment.

Aim 1.2: We will determine if social auditory experience during estrogen exposure modulates H3 acetylation and bdnf *transcription in the auditory cortex shortly after pup-experience.*

This experiment will use OVXed +E or +B and Coc or Niv animals. Coc will be allowed 5 days of pup experience with pup scattering and playback of pup calls on p3, p4 and p5. In parallel, Niv animals will also experience playback of pup calls but without social experience with pups. Pup-call playback will serve to control for variations in pup litter vocalizations and pup-call exposure (Fig 7).

RNA and Protein Expression: At 95 days of age for Nivs or 30 minutes after exposure to p5 pup scattering with pup-call playback, AC tissue of adult animals will be collected for *bdnf* RNA quantification or histone protein quantification using western blot. Samples will be homogenized and incubated with antibodies: H3, and H3-acetyl antibodies to measure global histone acetylation. *Bdnf* complementary strands tagged with

phosphorescent markers will be used for polymerase chain reaction assays to measure *bdnf* transcription. Based on previous research (35), G*Power software computes a two-way ANOVA with f= 8.92 would require six animals for each group; 24 animals for the RNA quantification and 24 for the H3 western for an α of 0.05.

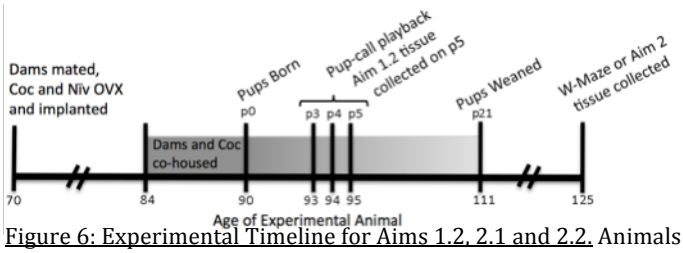

Figure 6: Experimental Timeline for Aims 1.2, 2.1 and 2.2. Animals assigned to the dams, cocarer (Coc) or naïve (Nïv) experimental group. At 70 days of age dams will be mated and Coc and Nïvs will be ovariectomized (OVX), and implanted. After recovery, dams and Cocs will be co-housed before pups are born. At age 93, 94 and 95 or on p3, p4, and p5, Nïs and Cocs will experience pup-call playback in an anechoic chamber. For Cocs, pups will be scattered during playback. In Aim 1.2, animals will be sacrificed on the last playback day. In Aim 2, 14 days post weaning or 125 days of age, animals will be behaviorally tested (2.1) or have tissue collected (2.2).

H3-acetyl at bdnf *promoter region:* AC tissue from Cocs at p5 or 95-day-old Nïvs will be collected for ChIP (Fig 6). This might require tissue pooling within groups. *GapDH* will serve as a control, because learning does not change its acetylation (59). Using the dam condition as a baseline, the H3-acetyl at promoter regions will be expressed as a fold change. This will determine association between promoter regions and H3-acetyl. G*Power software computes a two-way ANOVA with f= 2.31, based on previous research on cortical expression of H3-acetyl post-learning (59), would require 12 samples, each containing two to four animals for an α of 0.01, meaning a total of 24 to 48 animals.

Expected Outcomes for Aim 1: With the research of Aim 1 complete, we can identify multiple methods by which E modulates the neurons of the AC during pup-experience. In Aim 1.1, if E enhances social auditory memory in an ERK-dependent manner, then we would expect this enhancement to be blocked by U0126. The condition with the highest ratios of pCREB/CREB and most responsive the pup-calls is predicted to be +E/vehicle animals. If pCREB ratios do not positively correlate with higher approach ratios or decreased approach latencies, then we will know E enhancements are not MEK-dependent and will investigate the activation/expression of other molecules or proteins such as c-fos. In Aim 1.2, if E treatment and social auditory experience induce stable H3-acetyl lasting five days in the AC, then we predict Coc+E animals will have the highest ratios of H3-acetyl to H3+H3-acetyl and the Nï+B condition will have the lowest ratio of the conditions tested. In Aim 1.2, H3-acetyl at *bdnf* promoter regions in +E animals would suggest a link between changes in *bdnf* transcription and promoter region histone acetylation. We predict the Coc+E condition will have the highest fold change of H3-acetyl at *bdnf* promoter regions and Nïv+B condition will have the lowest. If the goals of Aim 1 are reached, we will determine if the MEK/ERK cascade and histone acetylation are potential targets for the development of social auditory learning enhancing therapies.

Alternative Strategies: We expect our experimental design to control for both hormones and experience to determine E's role in social auditory learning. In Aim 1.2 depending on ERα distribution, a lack of cell-type specificity in Aim 1.1's western blot, could wash out significant differences in pCREB expression. If this is the case, we will use histology cell counts to quantify cell-type specific expression of pCREB (as used in Fig 3 preliminary data). RNA quantification was suggested as a strong correlate between H3-acetyl and gene transcription; however, we may also decide to examine protein expression for BDNF isoforms. Similar to methylation, histone acetylation has typically been studied in the hippocampus, which experiences short lived changes in acetylation (60). Because others have found acetylation patterns in the sensory cortex which maintain cellular phenotype into adulthood, we hypothesize these changes will have more longevity in the AC (58). Earlier time points could be used to determine when histone acetylation is occurring.

Specific Aim 2: ***Determine estrogen and auditory experience's role in long-term social auditory memory formation by measuring the longevity of vocal recognition and molecular changes in the auditory cortex:*** Unlike Aim 1, Aim 2 will focus on the longevity of behavioral and epigenetic changes when E is present during social auditory cue encoding. While dams experience an influx of E during parturition and maintain pup-call preference post-weaning, Cocs do not, suggesting motherhood and perhaps E induce in long-term auditory memory (1, 19). However, one month after pup experience the presence of E is not necessary to assist pup-call approach behavior (50). The objective of Aim 2 is to determine if E's presence at the time of pup-call encoding is sufficient to induce long-term memory. To achieve this objective, *we will test the hypothesis that, during the encoding of pup-call memories, estrogen enacts long-term molecular changes increasing future behavioral responsiveness to pup-calls*. To test our hypothesis two separate studies will be employed, each manipulating E concurrently with a pup-care experience.

The *rationale* for Aim 2 is that while previous studies have investigated pup-call response in OVXed Cocs and in dams (19), there is a gap in knowledge concerning how E's presence specifically during pup exposure

affects the longevity of vocalization recognition. OVXed, non-implanted Coc's latency to retrieve pups one month after pup experience has been assessed and was not significantly different than their latency after five days of experience (61). However, this research did not compare Cocs+E versus Cocs+B and focused on maternal responsiveness, not auditory cues. Thus it did not address our question of sensory memory formation. Our research will include E depleted females, and animals with available E at the time of pup experience. By testing animals post-weaning, we will assess if E at the time of social auditory experience leads to stable increased global levels of H3-acetyl and/or H3-acetyl at the *bdnf* promoter region in the AC.

Research Design:

Aim 2.1: Determine estrogen and social experience's role in long-term behavioral and histone acetylation in response to social auditory stimuli:

In Aim 2.1, CoC and Niv animals will be OVXed and implanted with +E or +B. Cocs will be paired with a mother and remain co-housed with pups until weaning on p21 (Fig 7). All animals will be exposed to pup-call playback and for Cocs this will occur on p3, p4, and p5, with pup scattering. At the time of weaning, all experimental animals will have their implants removed and replaced with new, blank implants to homogenize the hormonal conditions during the W-maze memory recall task. This will allow us to assess pup-call memory without E motivating pup retrieval. Devocalized pups will be scattered in the maze and only retrieving animals will proceed with the memory recall task. Speakers will alternate playing pup-calls, 65 to 74kHz tones or 20kHz tones, and approach ratio will be measured (62). With a small effect size of f=0.50, G*Power analysis predicts the use of 55 animals for this experiment.

Aim 2.2: Determine estrogen's role in long-term epigenetic to social auditory memory.

Here we will determine if E exposure at the time of pup experience changes H3-acetyl long-term. The experimental timeline will be identical to Aim 2.1 (Fig. 7). Cocs and Nïvs will be sacrificed after pup-call playback, 14 days post-weaning and samples from each group pooled, similar to Aim 1.2. Samples for global histone analysis will be homogenized and incubated with H3 and H3-acetyl antibodies and protein concentrations quantified. These values will then be normalized to the total amount of H3 obtained from each sample. Based on previous research (35), G*Power software computes that a two-way ANOVA with f= 8.92 would require 24 animals, for an α of 0.05.

Determining H3-acetyl at the bdnf promoter region will be be similar to 1.2. AC tissue from Cocs 14 days post-weaning or 125-day-old Nïvs will be collected for ChIP after pup-call playback (Fig 6). Pooling tissue might be required within groups. *GapDH* will serve as a control (59). Using the dam condition as a baseline, the H3-acetyl at promoter regions will be expressed as a fold change. This will determine association between promoter regions and H3-acetyl. G*Power software computes a two-way ANOVA with f= 2.31, based on previous research on cortical expression of H3-acetyl post-learning (59), would require 12 samples, each containing two to four animals for an α of 0.01, meaning a total of 24 to 48 animals.

Expected Outcomes for Aim 2: With this research complete, we will understand the long-lasting changes in behavior and histone acetylation associated with estrogen exposure during social auditory experience. In Aim 2.1, if E enhances social auditory memory retention, then we would expect to see increases in approach ratios in the +E/CC condition. Aim 2.2 has the potential to identify a specific histone acetylation associated with social auditory long-term memory. High ratios of H3-acetyl to H3+H3-acetyl in +E animals at this time point would suggest that E is associated with long-term epigenetic modifications at the site of auditory processing and that H3-acetyl could be responsible for enhancing behavioral response. If this is the case then the Nï+B condition will have the lowest ratios of the groups. With the goals of Aim 2 reached, we will determine the long-term nature of E's memory enhancing mechanisms and identify a potential epigenetic modification responsible for social auditory long-term memory.

Alternative Strategies for Aim 2: We expect our experimental design will allow us to assess the role of E on long-term memory at the time of pup experience. Before behavioral or histone acetylation testing, implants will be removed in all experimental groups. Equalizing the endogenous E during recall testing allows us to focus on the role of E during pup experience. If insufficient motivation to retrieve is observed prior to memory recall task, all experimental groups will receive E implants, producing either basal or parturition levels of E. In either case, the levels of endogenous E in all experimental animals will be the same during recall testing. If changes in molecular structure are not significant, it would suggest that E act through neuromodulatory areas of the brain to enhance auditory responsiveness, rather than by direct action on the AC. Neuromodulatory brain areas will be collected as well to determine significant changes in molecular composition.