Emotional Vocalizations Alter Behaviors and Neurochemical Release into the Amygdala

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Keywords:

acetylcholine, acoustic communication, basolateral amygdala, dopamine, estrus, mating, microdialysis, mouse, restraint, vocalization playback

ABSTRACT

The basolateral amygdala (BLA), a brain center of emotional expression, contributes to acoustic communication by first interpreting the meaning of social sounds in the context of the listener's internal state, then organizing the appropriate behavioral responses. We propose that modulatory neurochemicals such as acetylcholine (ACh) and dopamine (DA) provide internal-state signals to the BLA while an animal listens to social vocalizations. We tested this in a vocal playback experiment utilizing highly affective vocal sequences associated with either mating or restraint, then sampled and analyzed fluids within the BLA for a broad range of neurochemicals and observed behavioral responses of male and female mice. In male mice, playback of restraint vocalizations increased ACh release and usually decreased DA release, while playback of mating sequences evoked the opposite neurochemical release patterns. In non-estrus female mice, patterns of ACh and DA release with mating playback were similar to males. Estrus females, however, showed increased ACh, associated with vigilance, as well as increased DA, associated with reward-seeking. Across these groups, increased ACh concentration was correlated with an increase in an aversive behavior. These neurochemical release patterns and several behavioral responses depended on a single prior experience with the mating and restraint behaviors. Our results support a model in which ACh and DA provide contextual information to sound analyzing BLA neurons that modulate their output to downstream brain regions controlling behavioral responses to social vocalizations.

SIGNIFICANCE STATEMENT

In social communication by sound, an animal interprets the meaning of vocalizations based on its prior experience, other sensory stimuli, and its internal state. The basolateral amygdala (BLA), a brain center of emotional expression, contributes to this analysis. We found that the modulatory neurochemicals acetylcholine and dopamine were released differentially into the BLA depending on the emotional content of the vocalizations, the sex and hormonal state of the animal, as well as its prior experience. Our results suggest that acetylcholine and dopamine provide experience- and hormonal state-dependent contextual information to sound-analyzing BLA neurons that modulates their output to downstream brain centers controlling behavioral responses to social vocalizations.

INTRODUCTION

In social interactions utilizing vocal communication signals, an animal receives and analyzes acoustic information, compares it with previous experiences, identifies the salience and valence of such information, and responds with appropriate behaviors. These integrated functions depend on brain circuits that include the amygdala, a region located within the temporal lobe that is recognized to play a role in orchestrating responses to salient sensory stimuli (J. LeDoux, 2003; J. E. LeDoux, 2000; Sah et al., 2003).

The basolateral amygdala (BLA) receives auditory input from thalamus and cortex (J. LeDoux et al., 1984; Romanski & LeDoux, 1993; Shi & Cassell, 1997; Tsukano et al., 2019) and processes vocal and other acoustic information in a context-dependent manner (Gadziola et al., 2016; J. M. S. Grimsley et al., 2013; Matsumoto et al., 2016; Wenstrup et al., 2020; Wiethoff et al., 2009). Contextual information may arise from inputs associated with other sensory modalities (e.g., somatic sensation or olfaction) (J. M. S. Grimsley et al., 2013; Lanuza et al., 2004; McDonald, 1998), but in other cases the contextual information is associated with an animal's internal state. Sources of internal state cues to BLA include brain circuits involving modulatory neurochemicals (i.e., neuromodulators), known to affect the processing of sensory signals, thus shaping attention, emotion, and goal-directed behaviors (Bargmann, 2012; Likhtik & Johansen, 2019; Schofield & Hurley, 2018).

This study investigates contextual information provided by neuromodulatory inputs to the BLA in response to vocal communication signals. Our hypothesis is that these salient vocalizations elicit distinct patterns of neuromodulator release into the BLA, by which they shape the processing of subsequent meaningful sensory information. We further hypothesize that these neuromodulatory patterns may depend on longer term processes that are critical to vocal

communication: to experience with these behaviors and the accompanying vocalizations, to sex, and to estrous stage in females. To test these hypotheses, we conducted playback experiments in a mouse model to understand the behavioral and neuromodulator responses to salient vocalizations associated with very different behavioral states.

RESULTS

To study how vocalizations affect behaviors and release of neurochemicals within BLA, we first developed highly salient vocal stimuli associated with appetitive (mating) and aversive (restraint) behaviors of CBA/CaJ mice. From interactions between adult male and female mice that included female head-sniffing and attempted or actual mounting, we selected several sequences of vocalizations to form a 20-minute mating vocal stimulus (Gaub et al., 2016; Ghasemahmad, 2020). These sequences included ultrasonic vocalizations (USVs) with harmonics, steps, and complex structure, mostly emitted by males, and low frequency harmonic calls (LFHs) emitted by females (Fig. 1A,C) (Finton et al., 2017; Ghasemahmad, 2020; Hanson & Hurley, 2012). During short periods of restraint, mice produce distinctive mid-frequency vocalizations (MFVs) that are associated with anxiety-related behaviors and increased release of the stress hormone corticosterone (Dornellas et al., 2021; J. M. S. Grimsley et al., 2016a; Niemczura et al., 2020a). From vocal sequences produced by restrained mice, we created a 20-minute vocal stimulus, primarily containing MFVs and fewer USV and LFH syllables (Fig. 1B,C).

We next asked whether these salient vocal stimuli, associated with very different behavioral states, could elicit distinct behaviors and patterns of neuromodulator release into the BLA. We focused on the neuromodulators acetylcholine (ACh) and dopamine (DA), since previous work suggests their interaction in emission of positive and negative vocalizations (Rojas-Carvajal et

al., 2022; Silkstone & Brudzynski, 2020). Our experiments combined playback of the vocal stimuli, behavioral tracking and observations, and microdialysis of BLA extracellular fluid in freely moving mice (Fig. 1D). Fluids were analyzed using a liquid chromatography/mass spectrometry (LC/MS) technique that allowed simultaneous measurement of several neurochemicals and their metabolites in the same dialysate samples, including ACh, DA and the serotonin metabolite 5-HIAA (see Materials and Methods).

Prior to the study, male and female mouse subjects had no experience with sexual or restraint behaviors. On the first two days of the experiment, mice in an experienced group (EXP, n=31) were each exposed to 90-min sessions with mating and restraint behaviors in a counterbalanced design (Fig. 1D). Mice were then implanted with a guide cannula for microdialysis. On the playback/sample collection day (Day 6), a microdialysis probe was inserted into the guide cannula. After a 4-hour period of mouse habitation and probe equilibration, we recorded behavioral reactions and sampled extracellular fluid from the BLA before (Pre-Stim) and during a 20 min playback period, divided into two 10-min collection/observation periods (Stim 1 and Stim 2) (Fig. 1D,E). Each mouse received playback of either the mating or restraint stimuli, but not both. Data are reported only from mice with more than 75% of the microdialysis probe implanted within the BLA (Fig. S1).

We first describe tests to examine whether playback of mating and restraint vocalizations results in different behavioral and neurochemical responses in male mice. We observed that two behaviors, still-and-alert and flinching, displayed pronounced increases with restraint playback that were not observed in the mating playback group (Fig. 2A,B). There were also distinct, vocalization-dependent patterns of ACh and DA release into the BLA (Fig 2C,D). Thus, in response to restraint vocalizations, we observed a significant increase in ACh concentration

during both Stim 1 and Stim 2 playback windows. Mating vocalizations, however, resulted in decreased ACh release that was significant during the Stim 2 playback period. (Fig. 2C). DA release displayed opposite patterns to ACh, increasing significantly during playback of mating vocalizations but showing a decreasing pattern (although nonsignificant) during playback of restraint vocal sequences (Fig. 2D). For both ACh and DA, levels differed significantly between the mating and restraint groups during both Stim 1 and Stim 2 (Fig. 2C,D). In contrast, the serotonin metabolite 5-HIAA showed no significant change over time following playback of either vocal stimulus, nor significant differences between groups (Fig. S2A). These findings suggest that both behavioral responses and ACh and DA release are modulated in listening male mice by the affective content of social vocalizations. Further, some behavioral and neurochemical responses were significantly correlated, especially the percentage change of ACh concentration (Stim 1 re Pre-Stim) with the number of flinching behaviors (Fig. 2E).

As male and female mice emit different vocalizations during mating (Finton et al., 2017; J. M. S. Grimsley et al., 2013; Neunuebel et al., 2015; Sales (née Sewell), 1972), we tested whether playback of vocal interactions associated with mating (Fig. 1A) results in different behavioral and neurochemical responses in listening male and female mice. Since our testing included both estrus and non-estrus females, we further examined the estrous effect on neurochemical release and behavioral reactions.

Playback of mating vocalizations resulted in some general and sex-based differences in behavioral responses. For instance, all groups displayed increased attending behavior (Fig. 3A). In females, regardless of estrous stage, both rearing and still-and-alert behaviors increased relative to male mice (Fig. 3B,C). Other behaviors differed by estrous stage during mating playback: females in estrus displayed a strikingly higher number of flinching behaviors

compared to males and non-estrus females (Fig. 3D). Our analysis of neuromodulator responses to mating vocalization playback revealed an estrous-dependent modulation of ACh levels during playback. ACh concentration in estrus females increased significantly during both Stim 1 and Stim 2 periods, whereas ACh showed decreases in both males and non-estrus females that were significant in the Stim 2 period (Fig. 3E). Moreover, during playback, the ACh in estrus females was significantly higher than both males and non-estrus females. DA release showed a consistent increase during mating playback across all three experimental groups (Fig. 3F). Similar to male groups in restraint and mating playback, the 5-HIAA release patterns in females showed no modulation during mating vocal playback (Fig. S2B).

Like male mice exposed to restraint vocalizations, estrus females showed robust and significant increases in flinching behavior in response to mating vocalizations. Both groups displayed increased ACh release. Among all mice exposed to mating playback, we observed a positive correlation between ACh concentration and the number of flinching behaviors during Stim 1 (Fig. 3G). This supports the possible involvement of ACh in shaping such behavior in both males listening to restraint calls and in estrus females listening to mating vocalizations. Note that neuromodulator release, including ACh, has been previously linked to motor behaviors (Wall & Woolley, 2020), but the changes in ACh that we observed showed no correlations with behaviors involving motor activity such as rearing (Stim 1: n = , r=-0.02, p=.9) or locomotion (Stim 1: n= , r =-0.03, p=0.9). This suggests that the observed changes in ACh reflect the valence of these vocalizations.

All EXP mice used in the above experiments had undergone a single session each to experience mating and restraint conditions prior to the playback session on Day 6 (Fig. 1D). Does such experience shape the release patterns of these neuromodulators in response to vocal playback?

We tested male and female mice under identical vocal playback conditions as previous groups, except that they did not receive the restraint and mating experiences (INEXP groups). Since only one INEXP female was in a non-estrus stage during the playback session, our analysis of the effect of experience included only estrus females and males.

Several behavioral responses to vocalization playback differed between EXP and INEXP mice in a sex- or context-dependent manner. For example, only estrus females showed experience-dependent increases in flinching behaviors (Fig. 4A) in response to mating vocal sequences. These experience effects were not observed in males in response to mating or restraint vocal playback. Males, however, showed a striking experience-dependent increase in rearing behaviors (Fig. 4B) in response to mating vocalizations, but this pattern was not observed during restraint playback for males or mating playback in estrus females. These findings indicate that behavioral responses to salient vocalizations result from interactions between sex of the listener or context of vocal stimuli with the previous behavioral experience associated with these vocalizations.

A major finding is that prior experience with mating and restraint behaviors shaped patterns of ACh and DA release in response to vocal playback (Fig. 5). Thus, male and female mice lacking in previous mating and restraint experiences showed no significant change in ACh or DA concentrations in response to either vocal playback type (Fig. 5A,B). Further, there were no significant differences across groups during Stim 1 or Stim 2 periods. These results contrast sharply with those from all EXP groups, in which both ACh and DA release changed significantly during playback (Figs. 2C, 2D, 3E, 3F). Moreover, 5-HIAA concentrations, which were unaffected by sex, estrous stage, or playback type (Fig. S2A,B), were also unaffected by experience (Fig. S2C). Finally, INEXP groups showed no significant correlations between concentrations of ACh with behavior in response to vocalization playback ($n=22$, $r=0.1$, $p=0.6$).

Collectively, these data suggest that the playback vocalization type and estrous effects observed in neuromodulator release patterns and behavioral reactions are mediated by previous experience with the corresponding behaviors.

DISCUSSION

Functional imaging studies in humans and mechanistic studies in other species provide substantial evidence that the amygdala participates in circuits that process vocalizations (Frühholz et al., 2016; Liebenthal et al., 2016; Sander et al., 2003; Wenstrup et al., 2020), assess the valence of appetitive and aversive cues (Kyriazi et al., 2018; O'Neill et al., 2018; Pignatelli & Beyeler, 2019; Smith & Torregrossa, 2021b), and shape appropriate behavioral responses to these cues (Gründemann et al., 2019; Lim et al., 2009; Schönfeld et al., 2020; Zhang & Li, 2018). Here, in a mouse model of acoustic communication, we showed that the motivational state of a "sender" is reflected in the acoustics of social vocalizations. We then showed that these vocalizations, related to intense experiences of restraint and mating, affect behavioral responses in listening conspecifics. Our results show that these emotionally charged vocalizations result in distinct release patterns of acetylcholine (ACh) and dopamine (DA) into the BLA of male and female mice. Further, female hormonal state appears to influence ACh but not DA release into the BLA when processing mating vocalizations. Such context- or state-dependent changes were not observed in patterns of other neurochemicals (e.g., 5-HIAA). These data indicate that during analysis of affective vocalizations in the BLA, ACh and DA provide state- and context-related information that can, potentially, modulate sensory processing within the BLA and thus shape an individual's response to these vocalizations.

The BLA receives strong cholinergic projections from the basal forebrain (Aitta-aho et al., 2018; Carlsen et al., 1985) that contribute to ACh-dependent processing of aversive cues and fear learning in the amygdala (Baysinger et al., 2012; Gorka et al., 2015; Jiang et al., 2016; Tingley et al., 2014). Our findings support these studies by demonstrating increased ACh release in BLA in response to playback of aversive vocalizations. Although the exact mechanism by which ACh

affects vocal information processing in BLA is not clear yet, the result of ACh release onto BLA neurons seems to enhance arousal during emotional processing (Likhtik & Johansen, 2019a). Our results, in conjunction with previous work, suggest mechanisms by which vocalizations affect ACh release and in turn drive behavioral responses (Fig. 6A). Cholinergic modulation in the BLA is mediated via muscarinic and nicotinic ACh receptors on BLA pyramidal neurons and inhibitory interneurons (Aitta-aho et al., 2018; Mesulam et al., 1983; Pidoplichko et al., 2013; Unal et al., 2015). During the processing of sensory information in the BLA, partially non-overlapping populations of neurons respond to cues related to positive or negative experiences (Namburi et al., 2015; Paton et al., 2006; Smith & Torregrossa, 2021a). These neurons then project to different target areas involved in appetitive or aversive behaviors—the nucleus accumbens or central nucleus of the amygdala, respectively (Namburi et al., 2015).

In response to an aversive cue or experience (Fig. 6A), released ACh affects neurons according to their activity. If projection neurons are at rest, ACh may exert an inhibitory effect by activating nicotinic ACh receptors on local GABAergic interneurons, which in turn synapse onto the quiescent pyramidal neurons. This results in GABA-A mediated inhibitory postsynaptic potentials (IPSPs) in the pyramidal neurons. Alternately, direct activation of M1 ACh receptors on pyramidal neurons, activating inward rectifying K+ currents, may result in additional ACh-mediated inhibition (Fig. 6A) (Aitta-aho et al., 2018; Pidoplichko et al., 2013; Unal et al., 2015). When BLA pyramidal neurons are already active due to strong excitatory input associated with aversive cues, M1 receptor activation can result in long afterdepolarizations that produce persistent firing lasting as long as ACh is present (Jiang et al., 2016; Unal et al., 2015). Such a process may explain persistent firing observed in single neuron responses to aversive social vocalizations in bats (Gadziola et al., 2012; Peterson & Wenstrup, 2012). Through this process of

inhibiting quiescent neurons and enhancing activation and persistent firing in active neurons, ACh sharpens the population signal-to-noise ratio (SNR) during the processing of salient, aversive signals in the BLA. These neurons, processing negative cues, likely project to the central nucleus of the amygdala (CeA) to regulate defensive behaviors such as escape and avoidance (Fig. 6A) (Beyeler et al., 2016; Davis et al., 2010; Namburi et al., 2015). In agreement, our behavioral findings show increased behaviors such as flinching correlated with increased release of ACh during processing of aversive vocalizations. Such prolonged afterdepolarizations provide the appropriate condition for associative synaptic plasticity (Likhtik & Johansen, 2019) that underlies an increase in AMPA/NMDA currents in CeA-projecting neurons during processing of aversive cues.

Dopaminergic innervation from the ventral tegmental area (VTA) (Asan, 1998) acts on BLA neurons via D1 and D2 receptors, both G-protein coupled receptors. Dopamine is important in reward processing, fear extinction, decision making, and motor control (Ambroggi et al., 2008; Di Ciano & Everitt, 2004; Lutas et al., 2019). We observed increased DA release in the BLA in response to mating vocalizations both for males and for females across estrous stages. Electrophysiological studies show that DA enhances sensory processing in BLA neurons by increasing the population response SNR in a process like ACh (Kröner et al., 2005; Vander Weele et al., 2018). Thus, during processing of mating vocalizations or those related to other rewarding experiences, DA presence in the vicinity of BLA pyramidal neurons and interneurons is enhanced (Fig. 6B). For neurons with elevated spiking activity during processing of appetitive vocalizations or other sensory stimuli, DA acts on D2 receptors of pyramidal cells to further enhance neuronal firing and result in persistent firing of these projection neurons. Conversely, in BLA projection neurons that do not respond to such positive cues, such as CeA-projecting

neurons, DA exerts a suppressive effect directly via D1 receptors and indirectly by activating inhibitory interneuron feedforward inhibition (Kröner et al., 2005). The net result of this process in response to appetitive vocalizations is an enhancement of activity in the reward-responding neurons and suppression of activity in aversive-responding neurons. This process likely depends on the increase in synaptic plasticity via enhanced AMPA/NMDA current during processing such cues in NAc-projecting neurons in the BLA (Namburi et al., 2015; Otani et al., 2003; van Vugt et al., 2020). Our findings suggest that this may occur in the BLA in response to appetitive vocalizations.

As the results with males listening to restraint vocalizations demonstrate, increased ACh release is associated with processing aversive cues. How, then, should the increased ACh release in estrus females during mating vocal playback be interpreted? Previous work shows that neuromodulation of amygdalar and other forebrain activity is altered by sex hormone/receptor changes in males and females (Egozi et al., 1986; Kalinowski et al., 2023; Kirry et al., 2019; Matsuda et al., 2002; Mizuno et al., 2022; van Huizen et al., 1994). For instance, the cholinergic neurons that project to the BLA, originating in the basal forebrain, exhibit high expression of estrogen receptors that is influenced by a female's hormonal state (Shughrue et al., 2000). During estrus, the enhanced circulating estrogen affects release of ACh and may influence neuronal networks and behavioral phenotypes in a distinct manner (Gibbs, 1996; McEwen, 1998). Thus, increased ACh release in estrus females may underlie increased attentional and risk assessment behaviors in response to vocalization playback. Combined with DA increase, it may trigger both Nac and CeA circuit activation, resulting in both reward-seeking and cautionary behaviors in estrus females.

Our results demonstrate the strong impact of even limited experience in shaping behavioral and neuromodulatory responses associated with salient social vocalizations. In the adult mice, a single 90-min session of mating and of restraint, occurring 4-5 days prior to the playback experiment, resulted in consistent behavioral responses and consistent and enhanced ACh and DA release into BLA for both vocalization types. As previous work shows (Huang et al., 2012; Nadim & Bucher, 2014; Pawlak et al., 2010), neuromodulatory inputs play crucial roles in regulating experience-dependent changes in the brain. However, it remains unclear whether the experience shapes neuromodulator release, or whether neuromodulators deliver the experience-related effect into the BLA.

The interaction between ACh and DA is thought to shape motor responses to external stimuli (Lester et al., 2010). Our results support the view that a balance of DA and ACh may regulate the proper behavioral response to appetitive and aversive auditory cues. For instance, increased reward-seeking behavior (rearing and locomotion) in EXP males during mating vocalizations playback may result from the differential release of the two neuromodulators—decreased ACh and increased DA. Further, the lack of this differential release may be the underlying cause for the lack of such responses in INEXP male mice. This supports the role of experience in tuning interactions of these two neuromodulators throughout the BLA, for shaping appropriate behaviors. Overall, the behavioral changes orchestrated by the BLA in response to emotionally salient stimuli are most likely the result of the interaction between previous emotional experiences, hormonal state, content of sensory stimuli, and sex of the listening animals.

MATERIALS AND METHODS

Animals

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Northeast Ohio Medical University. A total of 83 adult CBA/CaJ mice (Jackson Labs, p90-p180), male and female, were used for this study. Animals were maintained on a reversed dark/light cycle and experiments were performed during the dark cycle. The mice were housed in same-sex groups until the week of the experiments, during which they were singly housed. Food and water were provided *ad libitum* except during the experiment.

The estrous stage of female mice was evaluated based on vaginal smear samples obtained by sterile vaginal lavage. Samples were collected using glass pipettes filled with double distilled water, placed on a slide, stained using crystal violet, and coverslipped for microscopic examination. Estrous stage was determined by the predominant cell type: squamous epithelial cells (estrus), nucleated cornified cells (proestrus), or leukocytes (diestrus) (McLean et al., 2012). To confirm that the stage of estrous did not change during the experiment day, samples obtained prior to and after data collection on the experimental day were compared.

Acoustic methods

Vocalization recording and analysis

To record vocalizations for use in playback experiments, mice were placed in an open-topped plexiglass chamber (width, 28 cm; length, 28 cm; height, 20 cm), housed within a darkened, single-walled acoustic chamber (Industrial Acoustics, New York, NY) lined with anechoic foam (J. M. S. Grimsley et al., 2016a; Niemczura et al., 2020b). Acoustic signals were recorded using ultrasonic condenser microphones (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany)

connected to a multichannel amplifier and A/D converter (UltraSoundGate 416H, Avisoft Bioacoustics). The gain of each microphone was independently adjusted once per recording session to optimize the signal-to-noise ratio (SNR) while avoiding signal clipping. Acoustic signals were digitized at 500 kHz and 16-bit depth, monitored in real time with RECORDER software (Version 5.1, Avisoft Bioacoustics), and Fast Fourier Transformed (FFT) at a resolution of 512 Hz. A night vision camera (VideoSecu Infrared CCTV), centered 50 cm above the floor of the test box, recorded the behaviors synchronized with the vocal recordings (VideoBench software, DataWave Technologies, version 7).

To record mating vocalizations, ten animals (5 male-female pairs) were used in sessions that lasted for 30 minutes. A male mouse was introduced first into the test box, followed by a female mouse 5 min later. Vocalizations were recorded using two ultrasonic microphones placed 30 cm above the floor of the recording box and 13 cm apart. See below for analysis of behaviors during vocal recordings.

To record vocalizations during restraint, six mice (4 male, 2 female) were briefly anesthetized with isoflurane and then placed in a restraint jacket as described previously (J. M. S. Grimsley et al., 2016b). Vocalizations were recorded for 30 minutes while the animal was suspended in the recording box. Since these vocalizations are usually emitted at lower intensity compared to mating vocalizations, the recording microphone was positioned 2-3 cm from the snout to obtain the best SNR.

Vocal recordings were analyzed offline using Avisoft-SASLab Pro (version 5.2.12, Avisoft Bioacoustics) with a hamming window, 1024 Hz FFT size, and an overlap percentage of 98.43. For every syllable the channel with the higher amplitude signal was extracted using a custom-written Python code and analyzed. Since automatic syllable tagging did not allow distinguishing

some syllable types such as noisy calls and mid-frequency vocalizations (MFVs) from background noise, we manually tagged the start and end of each syllable, then examined spectrograms to measure several acoustic features and classify syllable types based on Grimsley et al. (J. M. S. Grimsley et al., 2011, 2016a).

Vocalization playback

Vocalization playback lasting 20 min was constructed from a sequence of seven repeating stimulus blocks lasting 2:50 min each (Fig. 1E). Each block was composed of a set of vocal sequence exemplars that alternated with an equal duration of background sound ("Silence") associated with the preceding exemplar. The exemplars were recorded during mating interactions and restraint, and selected based on high SNR, correspondence with behavioral category by video analysis, and representation of the spectrotemporal features of vocalizations emitted during mating and restraint (Ghasemahmad, 2020; J. M. S. Grimsley et al., 2016a). Mating stimulus blocks contained five exemplars of vocal sequences emitted during mating interactions. These exemplars ranged in duration from 15.0 – 43.6 s. Restraint stimulus blocks included seven vocal sequences, emitted by restrained male or female mice, with durations ranging from 5.7 – 42.3 s. Across exemplars, each stimulus block associated with both mating and restraint included different sets of vocal categories (Fig. 1A-C).

Playback sequences, i.e., exemplars, were conditioned in Adobe Audition CC (2018), adjusted to a 65 dB SNR level, then normalized to 1 V peak-to-peak for the highest amplitude syllable in the sequence. This maintained relative syllable emission amplitude in the sequence. For each sequence, an equal duration of background noise (i.e., no vocal or other detected sounds) from the same recording was added at the end of that sequence (Fig. 1E). A 5-ms ramp was added at the beginning and the end of the entire sequence to avoid acoustic artifacts. A MATLAB app

(EqualizIR, Sharad Shanbhag; https://github.com/TytoLogy/EqualizIR) compensated and calibrated each vocal sequence for the frequency response of the speaker system. Vocal sequences were converted to analog signals at 500 KHz and 16-bit resolution using DataWave (DataWave SciWorks, Loveland, CO), anti-alias filtered (TDT FT6-2, fc=125KHz), amplified (HCA-800II, Parasound, San Francisco, CA), and sent to the speaker (LCY, K100, Ying Tai Audio Company, Hong Kong). Each sequence was presented at peak level equivalent to 85 dB SPL.

Behavioral methods

Behaviors during both vocalization recording and playback sessions were recorded using a night vision camera (480TVL 3.6mm, VideoSecu), centered 50 cm above the floor of the test box, and SciWorks (DataWave, VideoBench version 7) for video acquisition and analysis.

Analysis of mating behaviors during vocal recordings

Mating behaviors in video recordings were analyzed second-by-second and classified as described previously (Gaub et al., 2016; Heckman et al., 2016). All vocal sequences selected as exemplars for playback of mating vocalizations were recorded in association with the following male mating behaviors: head-sniffing, vocalizing, attempted mounting, or mounting. Vocalizations during these behaviors included chevron, stepped, and complex USVs emitted with longer durations and higher repetition rates, and more LFH calls (Ghasemahmad, 2020; Hanson & Hurley, 2012).

Experience and playback sessions

Prior to playback experiments, each animal underwent 90-min sessions on two consecutive days (Days 1 and 2) that provided both mating and restraint experiences to the EXP group (n = 31

animals) or no experiences of these to the INEXP group (n = 24 animals). EXP sessions were presented in a counterbalanced pattern across subjects (Fig. 1D).

After the Day 2 session, mice underwent surgery for implantation of a microdialysis guide canula (see below), then recovered for four days. On Day 6, the day of the playback experiment, male mice were randomly assigned to either restraint or mating vocal playback groups. Females were only tested with mating playback.

Video recording was performed simultaneously with microdialysis, beginning 10 min before vocal playback (pre-stimulus), continuing for 20 min during playback, and including another 10 min after playback ended (post-stimulus) (Fig. 1D). From the video recording, several behaviors were analyzed in 10-second intervals.

Analysis of behaviors during vocal playback

Behavioral analysis was based on previous descriptions (Bakshi & Kelley, 1993, 1994; Blanchard et al., 2003; Füzesi et al., 2016; C. A. Grimsley et al., 2015; Lezak et al., 2017; Saibaba et al., 1996) (Mouse Ethogram: https://mousebehavior.org). To examine the effects of vocal playback on mouse behavior, we first identified behaviors exhibited by a separate group of naïve male and female mice in the experimental arena over a 15 min period, without playback. These included rearing, self-grooming, locomotion, and still-and-alert. During vocal playback we observed three additional behaviors: flinching, attending, and stretch and attend posture (see Table 1 for description).

For analysis of behaviors during vocal playback, all videos were examined blind to the sex of the animal and the context of vocalizations. Video recordings (40 min) were analyzed manually (n=48 mice) second-by-second in 10-second intervals for the seven behaviors identified above. The number of occurrences of every behavior per 10-second block was marked. The numbers

were added for every block of 2:50 min of vocal playback (see Fig. 1E), then averaged for the Pre-Stim, Stim 1, and Stim 2 periods separately. Videos were further analyzed automatically using the video tracker within VideoBench (DataWave Technologies, version 7) for speed of locomotion, distance traveled, and time spent in the periphery and center of the test arena.

Procedures related to microdialysis

Surgery

Mice were anaesthetized with Isoflurane (2-4%, Abbott Laboratories, North Chicago, IL) and hair overlying the skull was removed using depilatory lotion. A midline incision was made, then the skin was moved laterally to expose the skull. A craniotomy ($\simeq$1 mm^2) was made above the basolateral amygdala (BLA) on the left side (stereotaxic coordinates from bregma: -1.65 mm rostrocaudal, +3.43 mm mediolateral). A guide cannula (CMA-7, CMA Microdialysis, Sweden) was implanted to a depth of 2.6 mm below the cortical surface and above the left BLA (Fig. 1D, Day 2), then secured using dental cement. After surgery, the animal received a subcutaneous injection of carprofen (4 mg/kg, s.c.) and topical anesthetic (lidocaine) and antibiotic cream (Neosporin). It was returned to its cage and placed on a heating pad until full recovery from anesthesia. The animal recovered in its home cage for four days before the playback experiment.

Microdialysis

On the day before microdialysis, the probe was conditioned in 70% methanol and artificial cerebrospinal fluid (aCSF) (CMA Microdialysis, Sweden). On playback day (Day 6), the animal was briefly anesthetized and the probe with 1 mm membrane length and 0.24 mm outer diameter (MWCO 6 kDa) was inserted into the guide cannula (Fig. 1D).

Using a spiral tubing connector (0.1 mm ID x 50 cm length) (CT-20, AMUZA Microdialysis, Japan), the inlet and outlet tubing of the probe was connected to the inlet /outlet Teflon tubing of the microdialysis lines. A swivel device for fluids (TCS-2-23, AMUZA Microdialysis, Japan), secured to a balance arm, held the tubing, and facilitated the animal's free movement during the experiment.

After probe insertion, a four-hour period allowed animals to habituate and the neurochemicals to equilibrate between aCSF fluid in the probe and brain extracellular fluid (ECF). Sample collection then began, in 10-min intervals, beginning with four background samples, then two samples during playback of restraint or mating vocal sequences (total 20 minutes), then one or more samples after playback ended. To account for the dead volume of the outlet tubing, a flow rate of 1.069 µl/min was established at the syringe pump to obtain a 1 µl sample per minute. Samples collected during this time were measured for volume to assure a consistent flow rate. To prevent degradation of collected neurochemicals, the outlet tubing was passed through ice to a site outside the sound-proof booth where samples were collected on ice, then stored in a −80°C freezer.

Neurochemical analysis

Samples were analyzed using a liquid chromatography (LC) / mass spectrometry (MS) technique (Fig. 1D) at the Vanderbilt University Neurochemistry Core. This method allows simultaneous measurement of several neurochemicals in the same dialysate sample: acetylcholine (ACh), dopamine (DA) and its metabolites (3,4-Dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)), serotonin (5-HT) and its metabolite (5-hydroxyindoleacetic acid (5-HIAA)), norepinephrine (NE), gamma aminobutyric acid (GABA), and glutamate. Due to low

recovery of NE and 5-HT from the mouse brain, we were unable to track these two neuromodulators in this experiment.

Before each LC/MS analysis, 5 µl of the sample was derivatized using sodium carbonate, benzoyl chloride in acetonitrile, and internal standard (Wong et al., 2016). LC was performed on a 2.0 × 50 mm, 1.7 µM particle Acquity BEH C18 column (Waters Corporation, Milford, MA, USA) with 1.5% aqueous formic acid as mobile phase A and acetonitrile as mobile phase B. Using a Waters Acquity Classic UPLC, samples were separated by a gradient of 98–5 % of mobile phase A over 11 min at a flow rate of 0.6 ml/min with delivery to a SCIEX 6500+ Qtrap mass spectrometer (AB Sciex LLC, Framingham, MA, USA). The mass spectrometer was operated using electrospray ionization in the positive ion mode. The capillary voltage and temperature were 4KV and 350°C, respectively (Wong et al., 2016; Yohn et al., 2020). Chromatograms were analyzed using MultiQuant 3.0.2 Software (AB SCIEX, Concord, Ontario, Canada).

Verification of probe location

To verify placement of the microdialysis probe within the BLA, each probe was perfused with 2% dextran-fluorescein (MW 4Kda) (Sigma Aldrich Inc, Atlanta, GA) at the end of the experiment. The location of the probe was then visualized in adjacent cleared and Nissl-stained sections (e.g., Fig. S1, inset). Sections were photographed using a SPOT RT3 camera and SPOT Advanced Plus imaging software (version 4.7) mounted on a Zeiss Axio Imager M2 fluorescence microscope. Adobe Photoshop CS3 was used to adjust brightness and contrast globally. Animals with less than 75% of the probe membrane located within the BLA were excluded from statistical analyses (Fig. S1).

Data analysis

For neurochemical analyses, the total numbers of animals used were N_{EXP}=31 and N_{INEXP}=21. Only mice with useable neurochemical data were further evaluated for behaviors and for correlation between behaviors and neuromodulators. This included 48 mice: (N_{EXP}=27 and N_{INEXP}=21). Note that some animals used in neurochemical analyses were not included in the behavioral analyses because their behavioral data were unavailable. Since only one INEXP female was in a non-estrus stage during the playback session, our analysis of the effect of experience included only estrus females and males.

Only the following sample collection windows were used for statistical analysis: Pre-Stim, Stim 1 (10 mins), and Stim2 (10 mins). All neurochemical data were normalized to the background level, obtained from a single pre-stimulus sample immediately preceding playback. This provided clarity in representations and did not result in different outcomes of statistical tests compared to use of three pre-stimulus samples. The fluctuation in all background samples was ≤20%. The percentage change from background level was calculated based on the formula: *% change from background = (100 x stimulus sample concentration in pg)/ background sample concentration in pg.* Values are represented as mean ± one standard error unless stated otherwise. Box plots indicate minimum, first quartile, median, third quartile, and maximum values.

All statistical analyses were performed using SPSS (IBM, V. 26 and 27). To examine vocalizations in different stages of mating, we used a linear mixed model to analyze the changes of interval and duration (dependent variables) of vocalizations based on mating interaction intensity (fixed effect). For behavioral and neurochemical analyses in playback experiments, we initially compared the output using a linear mixed model and a generalized linear model (GLM) with a repeated measure for neurochemical data. Since both statistical methods resulted in

similar findings, we chose to use the GLM for statistical comparisons of the neurochemicals and behaviors. Where Mauchly's test indicated that the assumption of sphericity had been violated, degrees of freedom were corrected using Greenhouse Geisser estimates of sphericity.

To further clarify the differences observed between groups for every comparison in the GLM repeated measure, multivariate contrast was performed. All multiple comparisons were corrected using Bonferroni post hoc testing. 95% confidence intervals were used to compare values between timepoints (Julious, 2004).

For both microdialysis and behavioral data, we tested the hypotheses that the release of neurochemicals into the BLA and the number of behaviors is differently modulated by the vocal playback type (mating and restraint) in male mice, by estrous stage of females or sex of the animals during mating vocal playback, and by previous experience in any of these groups. The GLM model for testing these hypotheses used time as a within-subject factor, with vocalization context, sex, estrous, or the interaction of these with EXP and time as the between-subject factors. Dependent (response) variables included the normalized concentration of ACh, DA, 5-HIAA or the numbers of behaviors as previously described in the behavioral analysis section.

Throughout the figures, box plots show distributions of data into first (lower) quartile, median, and third (upper) quartile. Whiskers show minimum and maximum values; the mean is marked with an "x".

ACKNOWLEDGMENTS:

We are indebted to Sharad Shanbhag and Daniel Gavazzi for software used to condition vocal sequences and analyze audio and behavioral data, respectively, to Kristin Yeager for statistical consulting, and to Sheila Fleming for advice on behavioral assessments. We thank Anthony Zampino, Austin Poth, Debin Lei, and Krish Nair for technical assistance. We further thank Vanderbilt University Neurochemistry Core, supported by Vanderbilt Brain Institute and the Vanderbilt Kennedy Center, that performed the neurotransmitter sample analyses. We are grateful to Drs. Alexander Galazyuk, Brett Schofield, Merri Rosen, Mahtab Tehrani, and Sharad Shanbhag for their comments on previous versions of this manuscript.

FUNDING:

National Institutes of Health grant R01DC00937 (JJW, Alexander Galazyuk)

Kent State University Graduate Student Senate (ZG)

Northeast Ohio Medical University Biomedical Sciences Program Committee (ZG)

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FIGURES AND TABLES

Figure 1

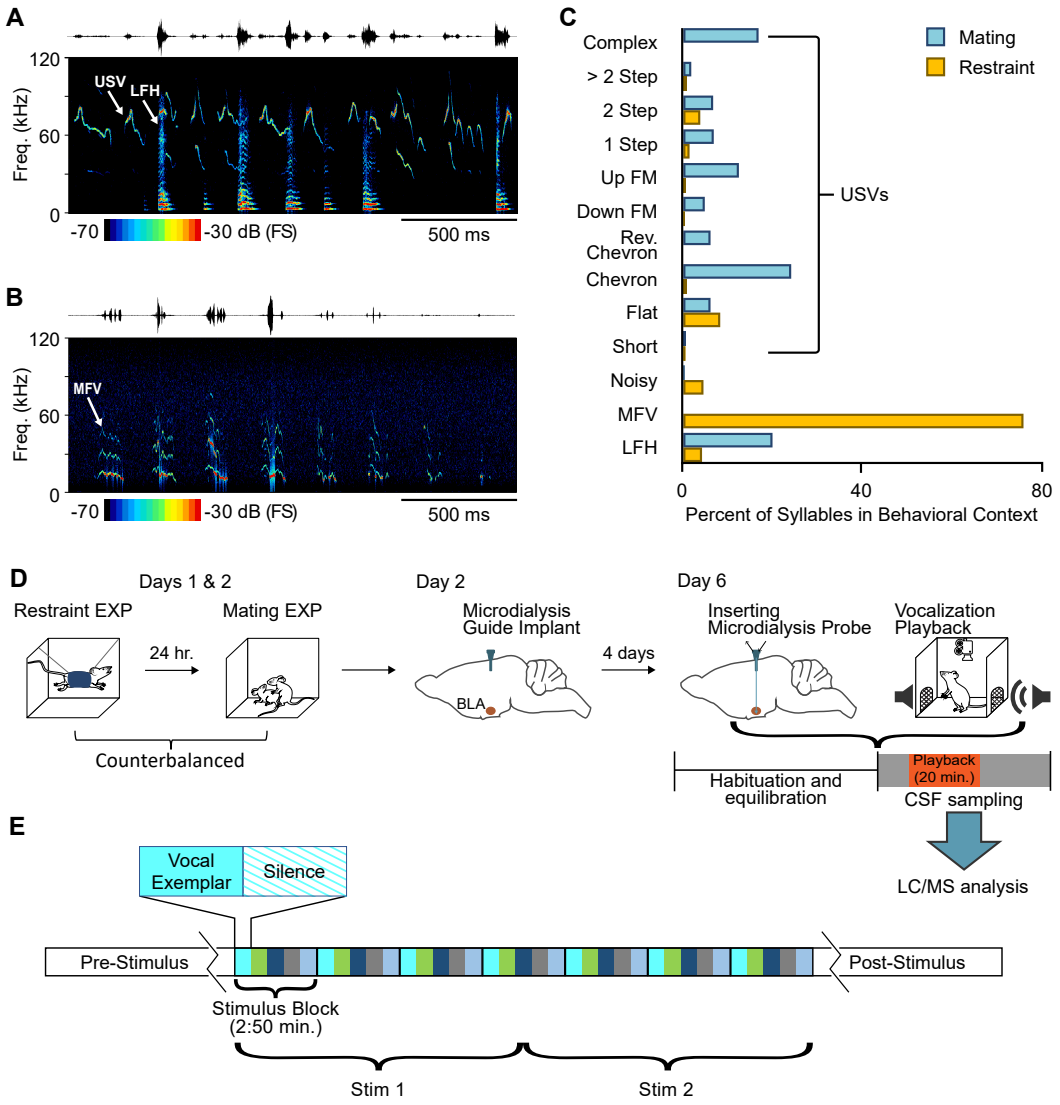

Figure 1. Behavioral / microdialysis experiments test how playback of affective vocal signals alters behaviors and neuromodulator release into the BLA. A. Short sample of mating vocal sequence used in playback experiments. Recording was obtained during high-intensity mating interactions and included ultrasonic vocalizations (USVs), likely emitted by the male, as well as low frequency harmonic (LFH) calls likely emitted by the female. **B.** Short sample of restraint vocal sequence used in playback experiments. Recording was obtained from an isolated, restrained mouse (see Material and Methods) and consisted mostly of mid-frequency vocalization (MFV) syllables. **C.** Syllable types in mating playback sequences differ substantially from those in restraint playback sequences. Percentages indicate frequency-of-occurrence of syllable types across all examplars used in mating or restraint vocal stimuli (n_{Mating} = 545, $n_{Restraint}$ = 622 syllables). **D**. Experimental design in playback experiment. Days 1 and 2:

each animal experienced restraint and mating behaviors (counterbalanced order across subjects). Day 2: a microdialysis guide tube was implanted in the brain above the BLA. Day 6: the microdialysis probe was inserted through guide tube into the BLA. Playback experiments began after several hours of habituation/equilibration. Behavioral observations and microdialysis sampling were obtained before, during, and after playback of one vocalization type. Microdialysis samples were analyzed using LC/MS method described in Materials and Methods. **E.** Detailed sequencing of vocal stimuli, shown here for mating playback. A 20-min period of vocal playback was formed by seven repeated stimulus blocks of 170 s. The stimulus blocks were composed of five vocal exemplars of variable length, with each exemplar followed by an equal duration of silence. Stimuli during the Stim1 and Stim 2 playback windows thus included identical blocks but in slightly altered patterns.

Figure 2

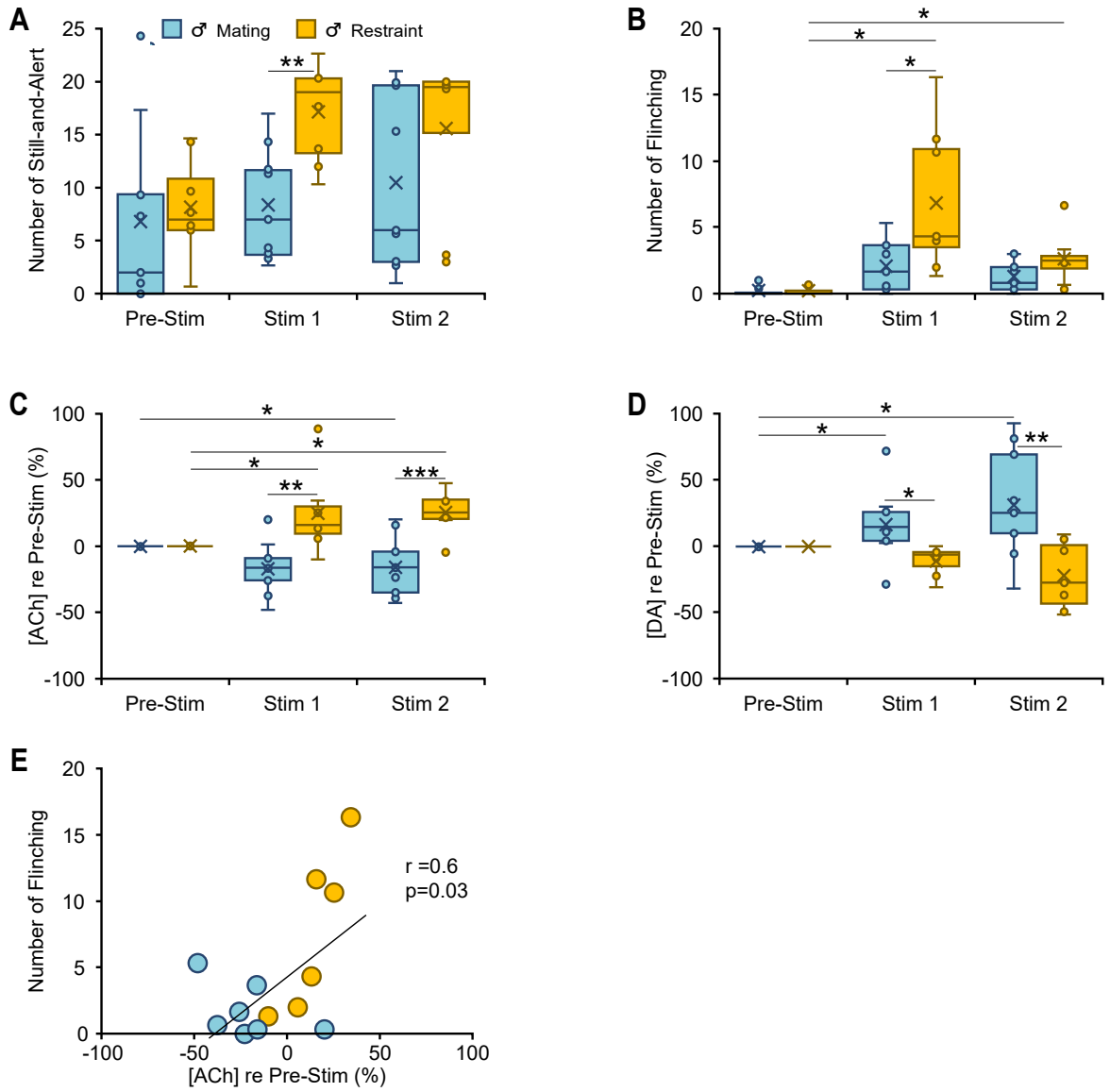

Figure 2. Behavioral and neuromodulator responses to vocal playback in male mice differ by behavioral context of vocalizations. A, B. Boxplots show number of occurrences of specified behavior in 10-min observation periods before (Pre-Stim) and during (Stim 1, Stim 2) playback of mating or restraint vocal sequences ($n_{Mating} = 7$, $n_{Restraint} = 6$). Note that playback sequences during Stim 1 and Stim 2 periods were identical within each group. During playback, the restraint group increased still-and-alert behavior compared to the mating group **(A)** (context: $F(1,11) = 9.6$, $p=0.01$, $\eta^2=0.5$), and increased flinching behavior **(B)** (time*context: $F(1.1,12.2) =6.3$, $p=0.02$, $\eta^2=0.4$) compared to the Pre-Stim baseline and to the mating group. **C, D.** Boxplots show differential release of acetylcholine (ACh) or dopamine (DA), relative to the Pre-Stim period, during mating and restraint vocal playback ($n_{Mating} = 9$, $n_{Restraint} = 7$). **C.** Significant differences in ACh for restraint (increase) playback in Stim 1 and Stim 2 vs Pre-Stim baseline and vs males in mating playback (time*context: $F(2,28)=6.6$, $p=0.004$, $\eta^2=0.32$). **D.** Significant

differences for DA for mating (increase) over time and vs restraint playback (time*context: $F(2,28)=6.9, p=0.004, \eta^2=0.33$). **E.** Across EXP male subjects in vocal playback, the number of flinching behaviors during Stim 1 was positively correlated with changes in ACh concentration relative to the Pre-Stim period. (n=13, Pearson r=0.6, p=0.03). **A-D.** Statistical testing examined all time windows within groups and all intergroup comparisons within time windows. Only significant tests are shown. Repeated measures GLM: *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc test). Time windows comparison: 95% confidence intervals. See Data Analysis section in *Materials and Methods* for description of box plots.

Figure 3

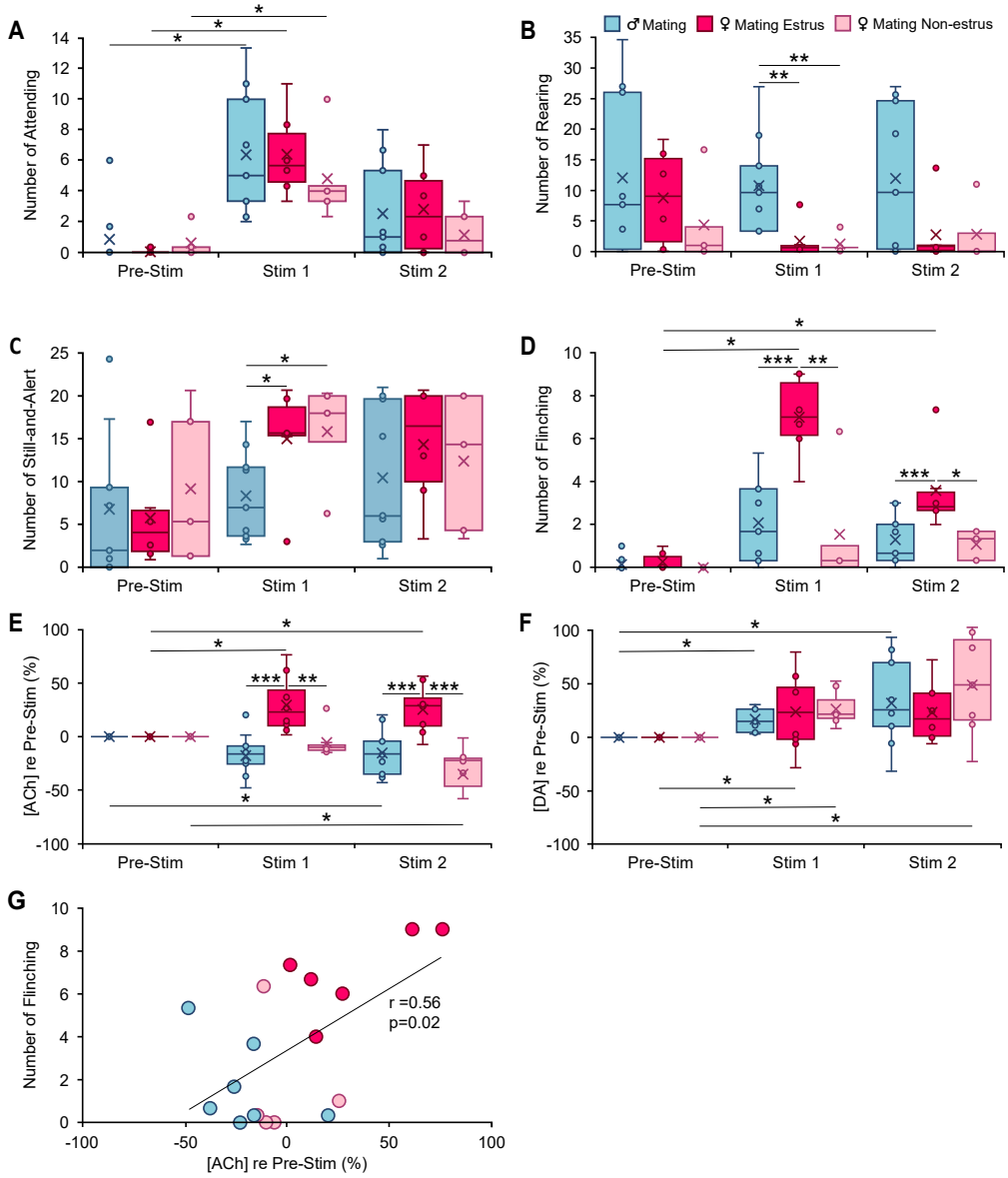

Figure 3. Behavioral and neuromodulator responses to playback of mating vocal sequences differ by sex and female estrous stage. A-D. Occurrences of specified behaviors in 10-min periods before (Pre-Stim) and during (Stim 1, Stim 2) mating vocal playback (n_{Male} = 7, $n_{Estrous\ Fem}$ = 6, $n_{Non\text{-}estrous\ Fem}$ = 5). **A.** Attending behavior increased during Stim 1 in response to mating vocal playback, regardless of sex or estrous stage (time*sex: F (2,30) = 0.12; p=0.9, ŋ2=0.008; time*estrous: F (2,30) = 1.1; p=0.4, ŋ2=0.07). **B-C.** Females regardless of estrous stage reared less (sex: F (1,15) =10.22, p=0.006; $ŋ^2$=0.4; estrous: F (1,15) =0.2, p=0.7, $ŋ^2$=0.01) and-displayed more Still-and-Alert behaviors (Sex: F (1,15) =5.17, p=0.04, partial ŋ2= 0.3, estrous: (1,15)

=0.07, p=0.8, partial ŋ2= 0.005) than males during mating vocal playback **D**. Estrus females, but not non-estrus females or males, showed a significant increase in flinching behavior during Stim 1 and Stim 2 periods (time*estrous: F (2,30) =9.0, p=0.001, $ŋ^2$=0.4). **E, F.** Changes in concentration of acetylcholine (ACh) or dopamine (DA) relative to the Pre-Stim period, evoked during Stim 1 and Stim 2 periods of vocal playback (n_{Male} = 9, $n_{Estrus\ Fem}$ = 8, $n_{Non\text{-}estrus\ Fem}$ = 7). **E.** Release of ACh during mating playback increased in estrus females (Stim1, Stim 2) but decreased in males and non-estrus females (Stim 2). Among groups, there was a significant estrous effect (time*estrous: F (2,42) =10.0, p<0.001, $ŋ^2$=0.32), increasing above Pre-Stim period in estrus females. **F.** DA release during mating playback increased in all groups relative to Pre-Stim period (time: F (2,42) = 12.4, p<0.001, $ŋ^2$=0.4), with no significant sex (F (1,21) =0.2, p=0.6, $ŋ^2$=0.01) or estrous effect (F (1,21) =0.8, p=0.4, $ŋ^2$=0.04). **G.** Across EXP subjects responding to mating playback, number of flinching behaviors during Stim 1 was positively correlated with change in ACh concentration, relative to Pre-Stim period (n=18, Pearson r=0.56, p=0.02). **A-F**: Repeated measures GLM: *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc test). Time windows comparison: 95% confidence intervals.

Figure 4

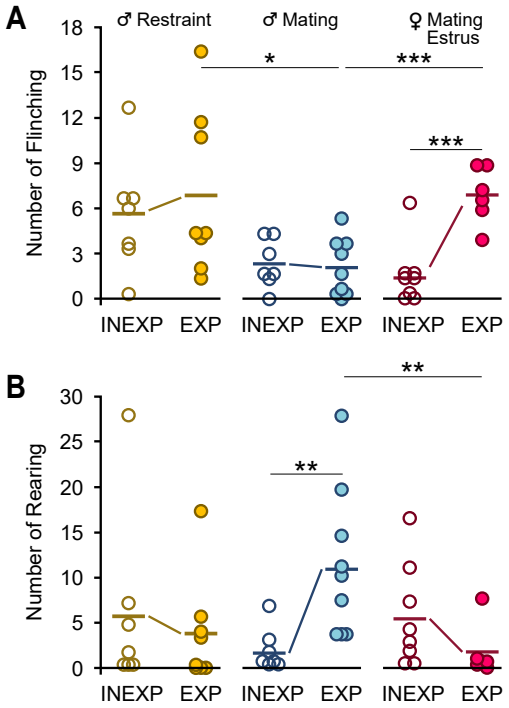

Figure 4. A single bout each of mating and restraint experience altered behaviors in response to vocal playback. In all graphs, dots represent measures from individual animals obtained during the Stim 1 playback period; connected thick lines represent mean values across subjects. **A.** Experience increased flinching responses in estrus female mice but not in males in mating or restraint vocal playback (Females: $n_{EXP} = 6$, $n_{INEXP} = 8$, t=5.0, p<0.001; Male-mating: $n_{EXP} = 8$, $n_{INEXP} = 7$, t=0.4, p=0.7; Male-restraint: $n_{EXP} = 7$, $n_{INEXP} = 7$, t=0.6, p=0.6); INEXP male mating vs restraint: t=2.08, p=0.06; mating female vs male: t=0.8, p=0.4; EXP male mating vs restraint: t= 2.5, p=0.03; mating female vs male: t=4.3, p<0.001. **B.** Experience increased rearing responses in males exposed to mating playback (Male-mating: $n_{EXP} = 8$, $n_{INEXP} = 7$, t=3.2, p=0.007), but not in females (t=1.4, p=0.2) or males in restraint playback (t=0.4, p=0.7); INEXP male mating vs restraint: t=1.07, p=0.3, mating female vs male: t=1.7, p=0.1; EXP male mating vs restraint: t=2.0, p=0.08; mating female vs male: t=3.4, p=0.004. GLM repeated measures with Bonferroni post hoc test: *p<0.05, **p<0.01, ***p<0.001.

Figure 5

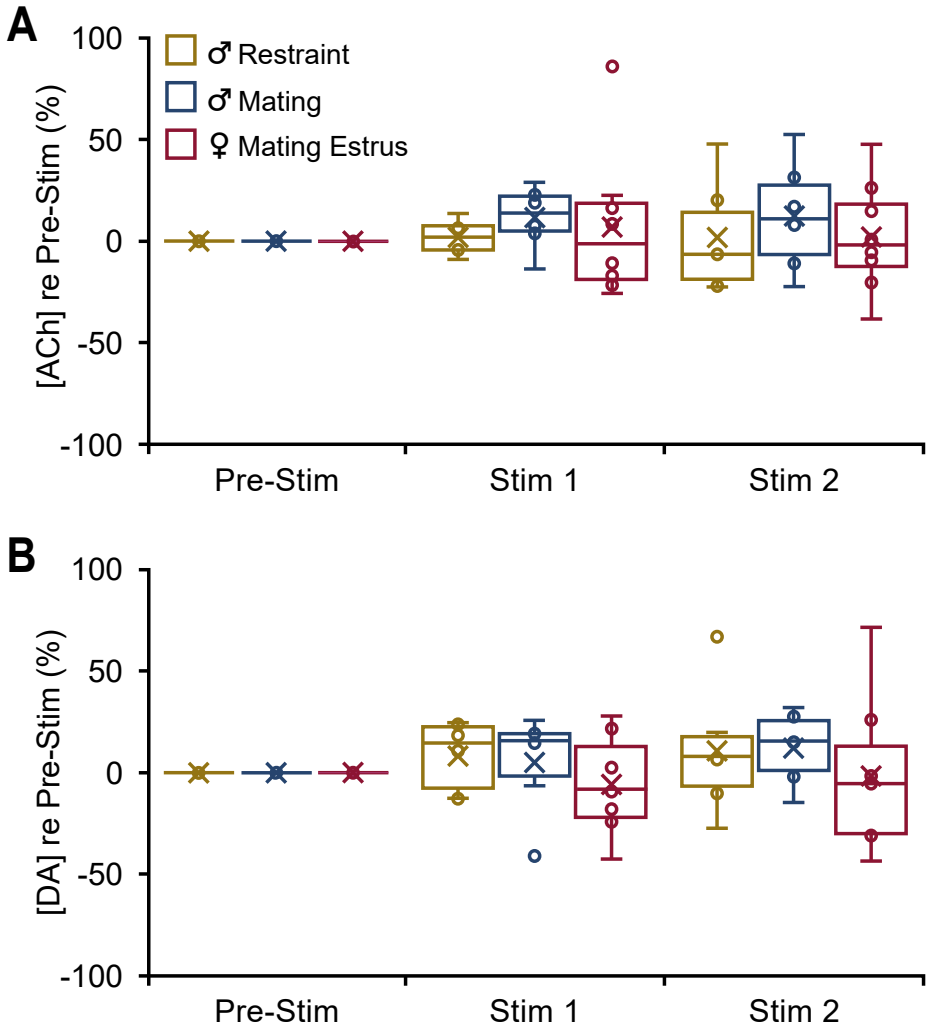

Figure 5. In INEXP mice, vocal playback failed to evoke significant changes in neuromodulator release. Boxplots show change in concentration of the specified neuromodulator in 10-min playback periods (Stim 1, Stim 2) compared to the baseline level (Pre-Stim). **A.** ACh release shown for both playback periods (mating vs restraint: time*context: F (2,24) =0.56; p=0.6; male vs female: time*sex: F (2,24) =0.01; p=0.9). **B.** DA release shown for both playback periods (mating vs restraint: time*context: F (2,24) = 0.2; p=0.8; male vs female: time*sex: F (2,24) = 0.4; p=0.7). **A, B**. Statistical testing examined all time windows within groups and all intergroup comparisons within time windows. No tests were significant. Repeated measures GLM (Bonferroni post hoc test). Time windows comparison: 95% confidence intervals.

Figure 6

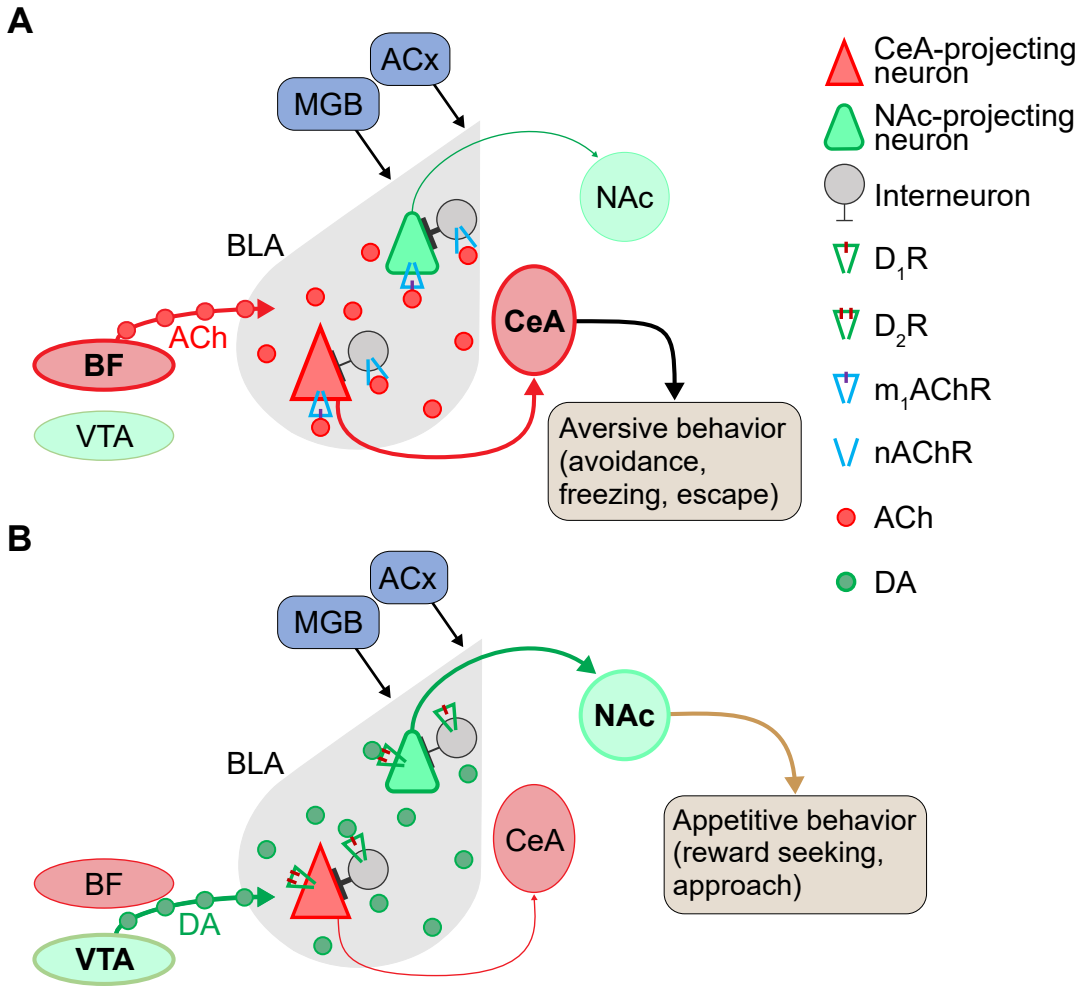

Figure 6. Proposed model for neuromodulation of salient vocalization processing via acetylcholine (ACh) and dopamine (DA) in the basolateral amygdala (BLA). A. Cholinergic modulation of CeA-projecting neurons during aversive vocalization cue processing in the BLA. In the presence of aversive cues, ACh released from the basal forebrain acts on m1 ACh receptors (m1AChRs) to enhance the cue-induced excitatory responses of CeA -projecting neurons. In contrast, NAc-projecting neurons are quiescent, because they do not respond to aversive cues and are inhibited by interneurons that are activated through nicotinic ACh receptors (nAChRs). **B.** Dopaminergic modulation and enhancement of signal-to-noise ratio in response to reward-associated cues (appetitive vocalizations). When vocalizations or other rewarding cues are present, release of DA from VTA enhances D2R-mediated excitation in NAc-projecting neurons that are responsive to positive cues. In contrast, CeA -projecting neurons are not responsive to rewarding vocalizations and are inhibited by local interneurons. DA is thought to act on D1 DA receptors (D1Rs) in these local interneurons to shape a direct inhibition onto CeA-projecting neurons. Abbreviations: ACx: auditory cortex; BF, basal forebrain; CeA, central nucleus of amygdala; MGB, medial geniculate body; NAc, nucleus accumbens; VTA, ventral tegmental area.

Table 1: Classification of behaviors during playback of vocalizations

Behavior	Definition
Abrupt attending	Sudden and quick pause in locomotion followed by abrupt change in head and body position lasting 2+ seconds. This behavior is accompanied by fixation of the eyes and ears during attending. Corresponds to freezing in some contexts.
Flinch	A short-duration twitch-like movement in response to vocal sequences, occurring at any location within arena. Distinguished by behavioral context (e.g., free-moving, 4-hr habituation period) and stimulus characteristics (e.g., non-repetitive over short term, variable level) from acoustic startle (Grimsley et al., 2015)
Locomotion	Moving all four limbs from one quadrant of the arena to another.
Rearing	A search behavior during which the body is upright and the head is elevated to investigate more distant locations.
Self-grooming	Licking fur and using forepaws to scratch and clean fur on head. The behavior can extend to other parts of the body.
Still and alert	Gradual reduction or lack of movement for 2+ seconds, during which animal appears to be listening or responding to external stimulus. Distinguished from abrupt freezing.
Stretch-attend posture	A risk-assessment behavior during which hind limbs are fixed while the head, forelimbs, and the body are stretched sequentially in different directions.

SUPPLEMENTARY MATERIALS

Supplemental Figure 1

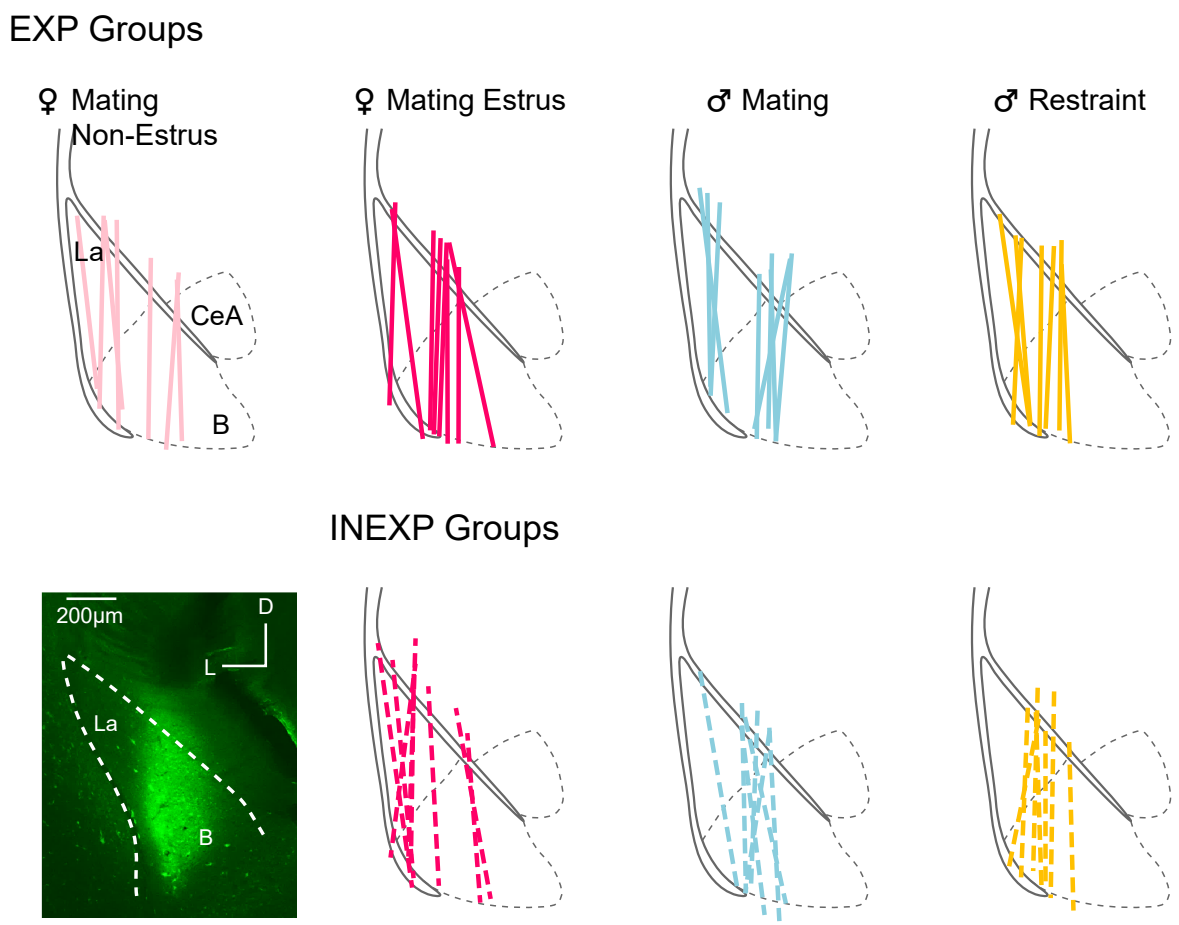

Figure S1. Microdialysis probe locations for EXP and INEXP Groups. Colored lines indicate recovered probe tracks that resulted from infusion of fluorescent tracers. Black solid lines indicate external capsule; black dashed lines indicate major amygdalar subdivisions. Inset: photomicrograph shows dextran-fluorescein labeling that marks the placement of the microdialysis probe. Abbreviations: *B,* basal nucleus of amygdala; *CeA*, central nucleus of amygdala; *La*, lateral nucleus of amygdala.

Supplemental Figure 2

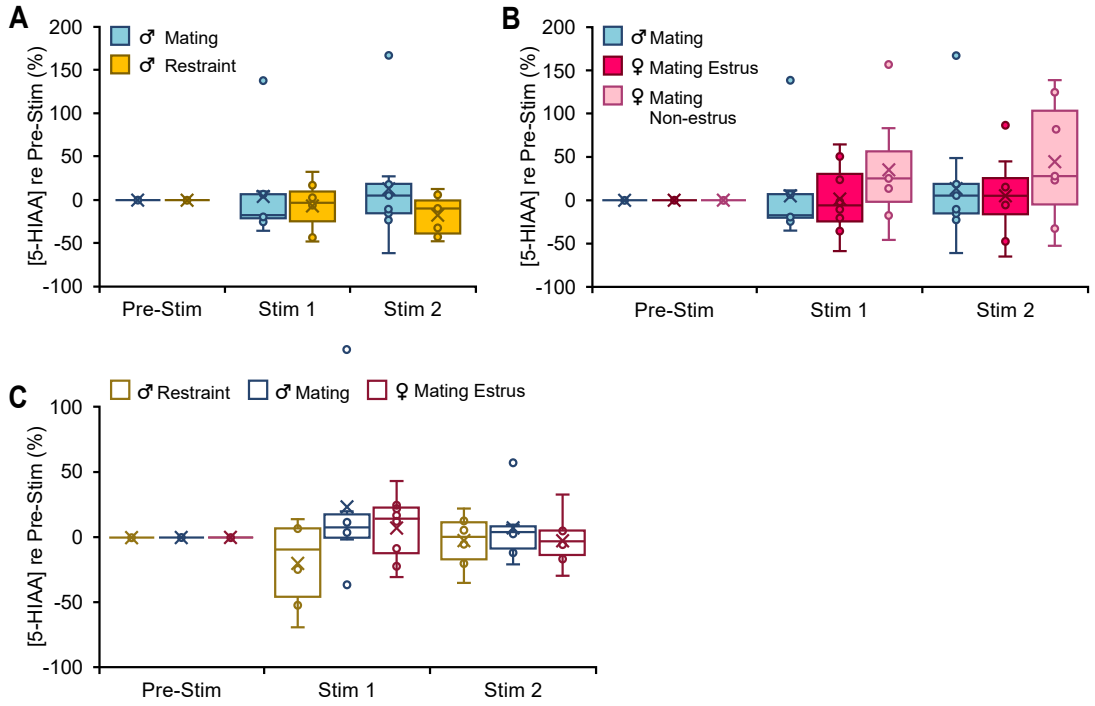

Figure S2. Concentration of serotonin metabolite 5-HIAA in EXP animals showed no significant context-, sex-, estrous-, or experience-dependent changes with vocal stimulus playback. Boxplots show change in concentration of 5-HIAA in 10-min periods from the baseline level (Pre-Stim) during (Stim 1, Stim 2) vocal sequence playback. **A.** Comparison of mating and restraint groups of male mice (n Mating=9, n Restraint=7; time*context: F (2,28) = 0.97, p=0.4, η^2 =0.06). **B.** Comparison of male and female mice during mating sequence playback (n_{Male} = 9, $n_{Estrous\ Fem}$ = 8, $n_{Non\text{-}estrous\ Fem}$ = 7, time*sex: F (2,42) = 0.2, p=0.8, η^2 =0.01; time*estrus: F (2,42) =1.2, p=0.3, η^2 =0.05). **C.** 5-HIAA concentration shown for both playback periods (mating vs restraint: time: F (1.3,15.6) =0.005, p=0.9; time*context: F (1.3,15.6) = 2.1; p=0.17; male vs female: time: F (1.3,15.7) =0.9; p=0.4; time* sex: F (1.3,15.7) = 0.3; p=0.7). nINEXP = 21. **A-C**. Statistical testing examined all time windows within groups and all intergroup comparisons within time windows. No tests were significant. Repeated measures GLM: ns: non-significant (Bonferroni post hoc test). Time windows comparison: 95% confidence intervals.