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Emotional Vocalizations Alter Behaviors and Neurochemical Release into the Amygdala

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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. Experimental groups that showed increased ACh release also showed the largest increases 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.

eLife assessment

This **important** study advances our understanding of how different types of communication signals differentially affect mouse behaviors and amygdala cholinergic/dopaminergic neuromodulation. Researchers interested in the complex interaction between prior experience, sex, behavior, hormonal status, and neuromodulation should benefit from this study. Nevertheless, some of the statistical comparisons using baseline normalized data might result in **inadequate** statistical power at this stage, requiring additional analysis to support the conclusions fully. With a few analytical parts strengthened, this paper will be of interest to neuroscientists and ethologists.

Introduction

In social interactions utilizing vocal communication signals, the acoustic features of the signals carry emotional information (Altenmüller et al., 2013; Darwin, 1872; Seyfarth & Cheney, 2003). Listeners receive and analyze the acoustic information, compare it with previous experiences, identify the salience and valence of such information, and respond 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 emotional responses to salient sensory stimuli (J. E. LeDoux, 2000; McGaugh, 2002; Sah et al., 2003). The amygdalar target of auditory inputs from the thalamus and cortex is the basolateral amygdala (BLA) (J. LeDoux et al., 1984; Romanski & LeDoux, 1993; Shi & Cassell, 1997; Tsukano et al., 2019) By integrating this auditory input with other sensory inputs and inputs from other limbic areas, BLA neurons shape appropriate behavioral responses (Beyeler et al., 2016; Gründemann et al., 2019; Namburi et al., 2015, 2016) via projections to downstream targets such as the nucleus accumbens (Ambroggi et al., 2008; Stuber et al., 2011) and central nucleus of the amygdala (Ciocchi et al., 2010).

The BLA processes vocal and other acoustic information in a context-dependent manner (Gadziola et al., 2016; 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) (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). While previous work has demonstrated a role for some neuromodulators—dopamine (DA) and acetylcholine (ACh)—in the production of social vocalizations (Rojas-Carvajal et al., 2022; Silkstone & Brudzynski, 2020), it remains unclear how these and other neuromodulators contribute to vocal processing in social interactions.

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 more intense, mating-related interactions between adult male and female mice that included female head-sniffing and attempted or actual mounting (Gaub et al., 2016; Ghasemahmad, 2020, see Materials and Methods), we selected several sequences of vocalizations to form a 20-minute mating vocal stimulus. 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; Gaub et al., 2016; 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; Grimsley et al., 2016; Niemczura et al., 2020). 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 (see **Table 1** for descriptions), 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). All neurochemical results during Stim 1 and Stim 2 periods are expressed as percent change from the Pre-Stim control period. However, raw values of ACh, DA, and 5-HIAA are reported in supplementary tables 1, 2, and 3, respectively.

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 habituation 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 stimulation/collection/observation periods that are designated Stim 1 and Stim 2 (**Fig. 1D,E**. See also Materials and Methods). Each mouse received playback of either the mating or restraint stimuli, but not both: same-day presentation of both stimuli would require excessively long playback sessions, the condition of the same probe would likely change on subsequent days, and quality of a second implanted probe on a subsequent day was uncertain. Data are reported only from mice with more than 75% of the microdialysis probe implanted within the BLA (**Fig. 2**).

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

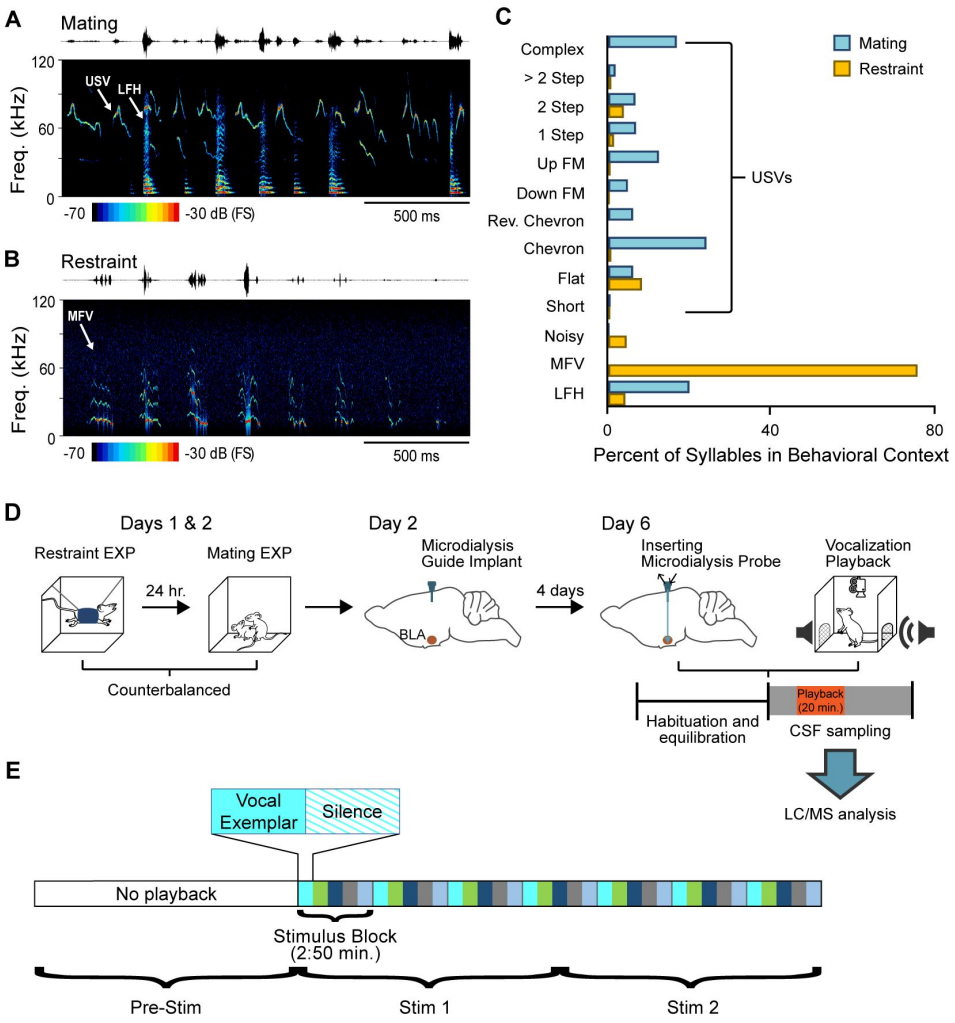

Figure 1.

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.** Schematic illustration of 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 (each represented by a different color) 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. See Material and Methods for in-depth description of vocalization playback.

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. Appears similar to "freezing" behavior described in fear conditioning, but occurs in the context of natural response to vocalizations.
Flinch	A short-duration twitch-like movement in response to vocal sequences, occurring at any location within arena. Unlike acoustic startle (Grimsley et al., 2015), this behavior occurs in free-moving animals in response to non-repetitive, variable-level vocal stimuli. Flinching movements are of smaller magnitude than those observed in acoustic startle.
Locomotion	Movement of 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 attending by gradual onset.
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.

Table 1

Classification of manually evaluated behaviors during playback of vocalizations

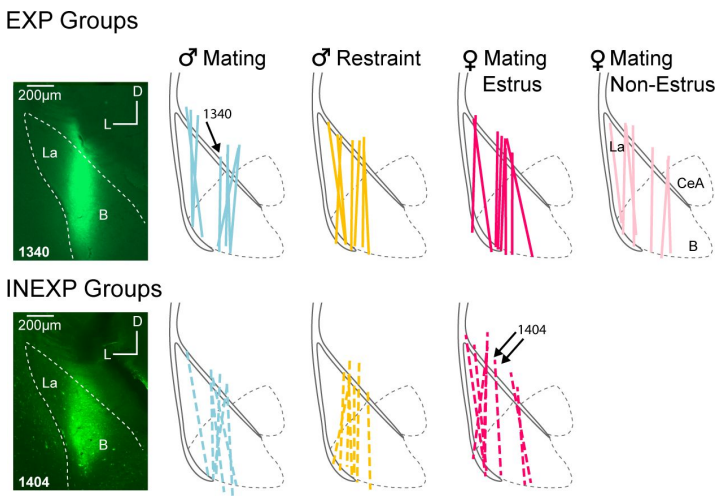

Figure 2.

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. Arrows indicate tracks related to insets. Insets: photomicrographs shows dextran-fluorescein labeling that marks the placement of the microdialysis probes for two cases, 1340 and 1404. Abbreviations: *B,* basal nucleus of amygdala; *CeA*, central nucleus of amygdala; *La*, lateral nucleus of amygdala.

that were not observed in the mating playback group (**Fig. 3A,B**). There were also distinct, vocalization-dependent patterns of ACh and DA release into the BLA (**Fig 3C,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 a significant decrease in ACh release during both playback periods. (**Fig. 3C**). DA release displayed opposite patterns to ACh, increasing significantly during Stim 2 playback of mating vocalizations but showing a decreasing pattern (although nonsignificant) during playback of restraint vocal sequences (**Fig. 3D**). For ACh, levels differed significantly between the mating and restraint groups during both Stim 1 and Stim 2, while DA levels differed significantly only during Stim 2 (**Fig. 3C,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. 3E**). 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.

As male and female mice emit different vocalizations during courtship and mating (Finton et al., 2017; 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. 4A**). In females, regardless of estrous stage, still-and-alert behaviors increased and rearing decreased relative to male mice (**Fig. 4B,C**). This change in motor activity was further supported by video tracking results showing a significant decrease in distance traveled by female mice in response to mating vocal playback (significant sex effect ($F(1,15) = 9.3$, $p=0.008$, $\eta 2=0.4$). Another behavior 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. 4D**). 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. 4E**). Moreover, during playback, the ACh in estrus females was significantly higher than both males and non-estrus females. DA release showed an increase during mating playback across all three experimental groups, although the time course differed (**Fig. 4F**). Similar to male groups in restraint and mating playback, the 5-HIAA release patterns in females showed no modulation during mating vocal playback (**Fig. 4G**).

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. 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 relationship with behaviors involving motor activity such as rearing (restraint males: Stim 1: $n = 6$, $r = 0.1$, $p = 0.8$; Est females: $n = 6$, $r = 0.7$, $p=0.08$), locomotion (Stim 1: restraint males, $n = 6$, $r = -0.2$, $p = 0.7$; Est females: $n = 6$, $r = 0.3$, $p = 0.6$), or distance traveled (Stim 1: restraint males, $n = 6$, $r = 0.16$, $p = 0.8$; Est females: $n = 6$, $r = -0.6$, $p = 0.2$). 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?

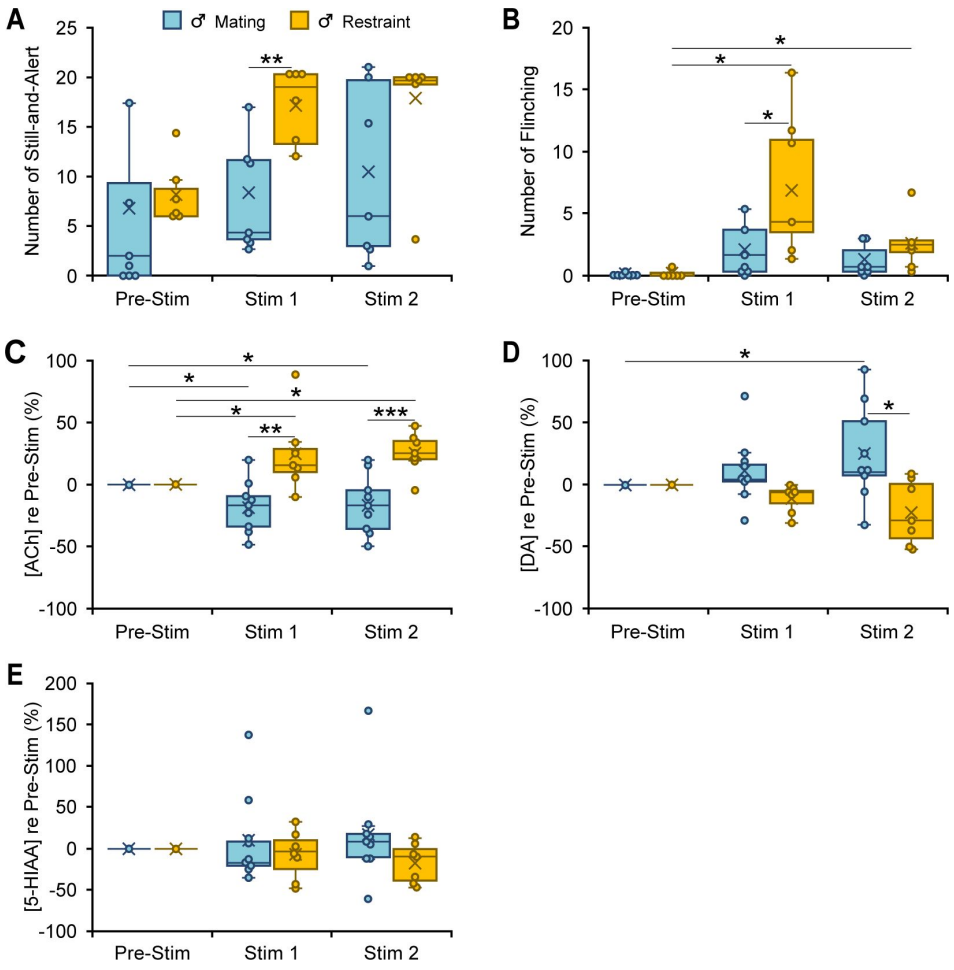

Figure 3.

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, η^2=0.5), and increased flinching behavior **(B)** (time*context: F (1.1,12.2) =6.3, p=0.02, η^2=0.4) compared to the Pre-Stim baseline and to the mating group. **C-E.** Boxplots show differential release of acetylcholine (ACh), dopamine (DA), and 5-HIAA 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, η^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, η^2=0.33). **E**. No significant changes in 5-HIAA during vocal playback in male mice (time*context: F (2,28) = 0.97, p=0.4, η^2 = 0.06). **A-E.** 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.

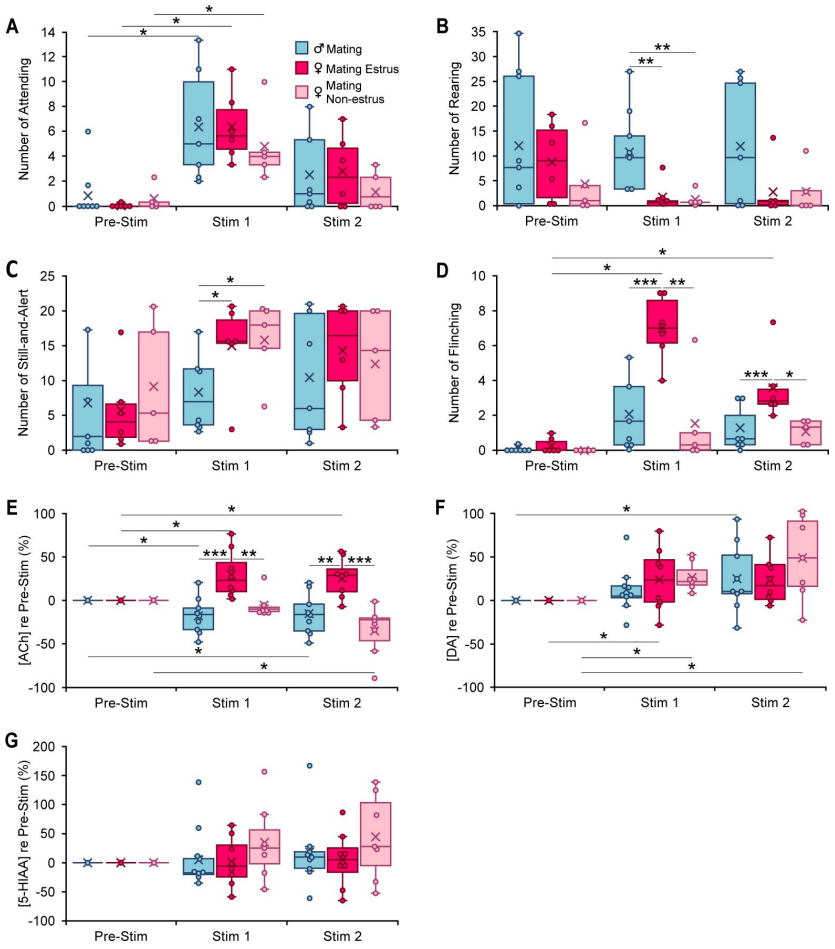

Figure 4.

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-G.** Changes in concentration of acetylcholine (ACh), dopamine (DA), and 5-HIAA 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) =9.9, 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) = 10.9, p<0.001, $ŋ^2$=0. 34), with no significant sex (F (1,21) =0.9, p=0.4, $ŋ^2$=0.04) or estrous effect (F (1,21) =0.9, p=0.4, $ŋ^2$=0.04). **G.** No significant changes in 5-HIAA during mating sequence playback (time*sex: F (2,42) = 0.08, p=0.9, $ŋ^2$=0.004; time*estrus: F (2,42) =1.2, p=0.3, $ŋ^2$ =0.05). **A-G**: Repeated measures GLM: *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc test). Time windows comparison: 95% confidence intervals.

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, EXP estrus females showed increased sound-evoked flinching behaviors compared to INEXP estrus females (**Fig. 5A**) in response to mating vocal sequences. These experience effects were not observed in males in response to mating or restraint vocal playback. Further, the differences in flinching behavior among EXP groups (male-mating vs male-restraint or estrus female-mating) were not evident among INEXP groups. For rearing behavior, EXP males responding to mating vocalizations showed an increase compared to INEXP males (**Fig. 5B**). 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. 6**). 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. 6A,B**). Further, there were no significant differences across groups during either Stim 1 or Stim 2 periods. These results contrast sharply with those from all EXP groups, in which release of ACh, DA, or both changed significantly during playback (**Figs. 3C**, **3D**, **4E**, **4F**). Moreover, 5-HIAA concentrations, which were unaffected by sex, estrous stage, or playback type (**Figs. 3E**, **4G**), were also unaffected by experience (**Fig. 6C**). Collectively, these data suggest that the playback vocalization type and estrous effects observed in neuromodulator release patterns and behavioral reactions depend on 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; Voytenko et al., 2023), assess the valence of appetitive and aversive cues (Kyriazi et al., 2018; O'Neill et al., 2018; Pignatelli & Beyeler, 2019; Smith & Torregrossa, 2021), and shape appropriate behavioral responses to these cues (Gründemann et al., 2019; Lim et al., 2009; Schönfeld et al., 2020; Zhang & Li, 2018). Nonetheless, how contextual information is delivered to the amygdala and contributes to vocal processing is not well understood. Since the amygdala receives strong projections from neuromodulatory brain centers (Aitta-aho et al., 2018; Asan, 1997, 1998; Bigos et al., 2008; Bocchio et al., 2016; Carlsen et al., 1985), and since the role of these neurochemicals in providing internal state and contextual information is well-proven (Bocchio et al., 2016; Jiang et al., 2016; Likhtik & Johansen, 2019), we hypothesized that release patterns of neuromodulators into the BLA provide contextual information during processing of affective vocalizations. 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). Finally, we showed that a single 90-min experience with intense behaviors is sufficient to establish strong, consistent patterns of neuromodulatory release into the amygdala. These data indicate that during analysis of affective

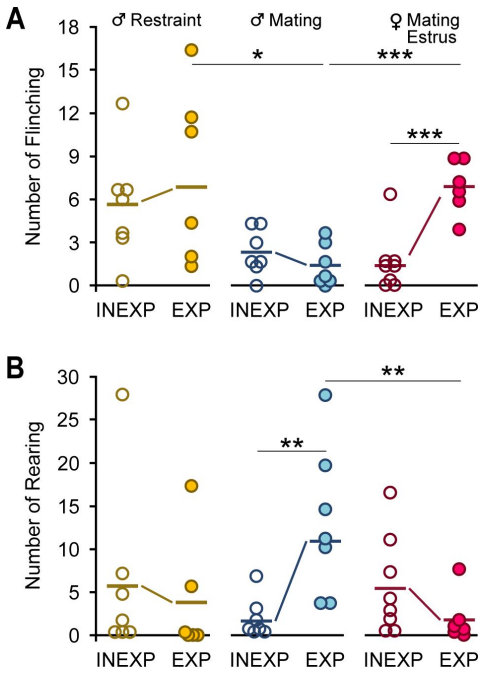

Figure 5.

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. There were no differences in Pre-Stim values between INEXP and EXP mice for any group (e.g., males-restraint, males-mating, estrus females-mating. **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} = 7, n_{INEXP} = 7, t= 0.4, p=0.7; Male-restraint: n_{EXP} = 6, 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: 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.

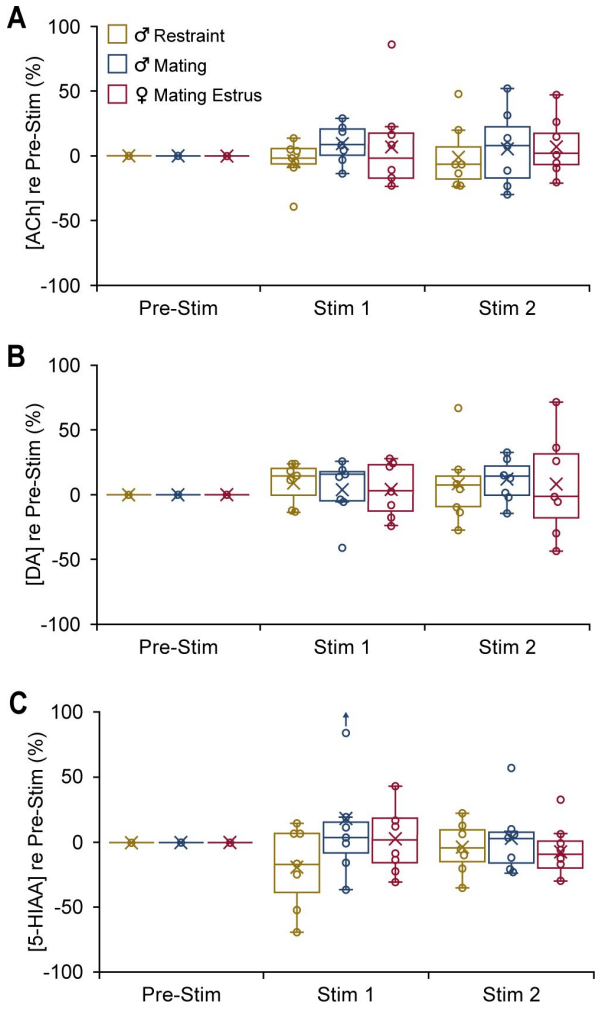

Figure 6.

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). Male-restraint, n_{INEXP} = 7; Male-mating, n_{INEXP} = 7; Estrus Female-mating, n_{INEXP} = 7). **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) =0.06; 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) = 0.03; p=0.97). **C.** 5-HIAA concentration shown for both playback periods (mating vs restraint: time: F (1.3,15.6) =0.005, p=0.97; time*context: F (1.3,15.6) = 2.1; p=0.17; male vs female: time: F (1.3,16.2) =0.8; p=0.4; time* sex: F (1.3,16.2) = 0.5; p=0.55). **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.

vocalizations in the BLA, ACh and DA provide experience-dependent, 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 linkage between neuromodulator release and behavioral responses to vocalizations varies. The strongest case is for a link between increased ACh release and flinching behavior. Males in the restraint playback group and estrus females in the mating playback group both displayed significantly increased ACh release and flinching behavior, occurring in both playback periods. This suggests a mechanistic relationship. Other significant behavioral responses to vocal stimuli did not closely match the timing of neurochemical changes, suggesting a weak mechanistic link. Overall, it is not surprising that most behaviors and neurochemicals do not match well, given the poor temporal resolution of the neurochemical sampling. We believe that our results show the need for a finer analysis of DA and ACh release based on techniques for high temporal resolution of neurochemical measurements (e.g., genetically encoded ACh indicator (Jing et al., 2018)), coupled with short interval behavioral observations. Only then can the role of the BLA and neuromodulator inputs in behavioral vocal responses be established more strongly. Further, we note that no conclusion can be made regarding relationships between noradrenalin and serotonin concentrations and vocal playback, since these were not detected by the neurochemical analysis.

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, 2019). Our results, in conjunction with previous work, suggest mechanisms by which vocalizations affect ACh release and in turn drive behavioral responses (**Fig. 7A**). 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, 2021). 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. 7A**), released ACh affects BLA 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. 7A**) (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. 7A**) (Beyeler et al., 2016; Davis et al., 2010; Namburi et al., 2015). In agreement, our behavioral findings show increased behaviors such as flinching along with increased release of

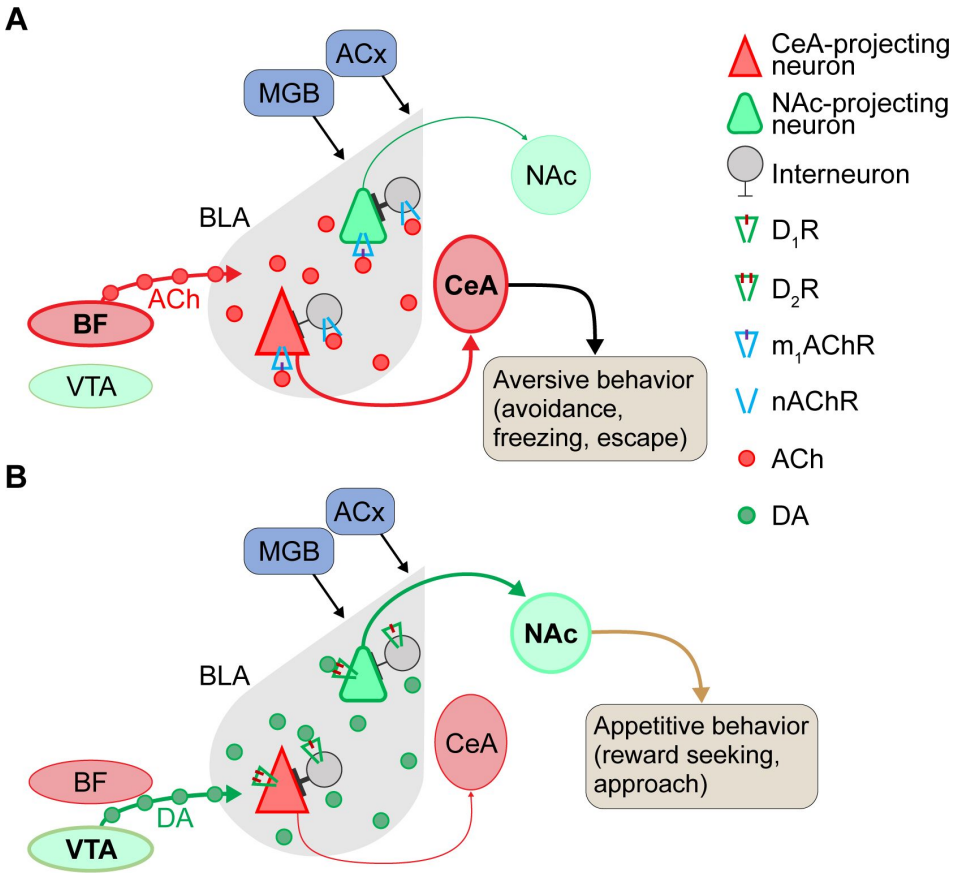

Figure 7.

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 (M1 mAChRs) 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.

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 the AMPA/NMDA current ratio 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. 7B**). 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 an enhanced AMPA/NMDA current ratio 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 in BLA 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, as we observed with flinching behavior in this experimental group. 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.

Experimental overview

The basic experimental structure that occurred over six days is illustrated in **Figure 1D**. Briefly, each mouse was placed in an arena on Days 1 and 2 to provide one-time experiences of mating and sustained restraint. After the experience on Day 2, the subject was anesthetized for implantation of a guide tube for the microdialysis probe. On Day 6, the vocalization playback session occurred. The mouse was briefly anesthetized for insertion of the microdialysis probe into the amygdala, followed by several hours recovery. Before, during, and after vocal playback, we sampled extracellular fluid from the amygdala through the microdialysis probe and recorded video to analyze the subject's behavior. A liquid chromatographic/mass spectrometric method was used to measure concentrations of several neurochemicals.

The microdialysis sampling interval of 10 mins was used to establish the temporal framework for vocal playback and analysis of neurochemicals and behaviors (**Fig. 1E**). The analysis compared neurochemicals and behaviors in a period immediately preceding the vocal stimuli (Pre-Stim) and in two 10-min periods featuring playback of the same sequence of vocal stimuli (Stim 1 and Stim 2). Each playback session featured only one category of vocalizations (associated with mating or restraint) and each animal participated in a single playback session. Additional details of this experimental structure are described in the sections below.

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 (Grimsley et al., 2016; Niemczura et al., 2020). 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 (Grimsley et al., 2016). 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. (Grimsley et al., 2011, 2016).

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; Grimsley et al., 2016). 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 1V 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

Interactions between male and female mice were video-recorded, then analyzed second-by-second. Among more general courtship interactions, we identified a set of behaviors that are mating-related, 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 these male mating behaviors: head-sniffing, 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 (Gaub et al., 2016; 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 = 22 animals). EXP sessions were presented in a counterbalanced pattern across subjects (**Fig. 1D**). For the mating experience, mounting or attempted mounting was required for the animal to be included in the remainder of the experiment. However, we did not record detailed behaviors or track estrous stage during the mating experience session. 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, since a preliminary behavioral study suggested no difference in male and female responses to restraint. Video recording was performed simultaneously with microdialysis, beginning 10 min before vocal playback (pre-stimulus) and continuing for 20 min during playback (**Fig. 1D**).

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; Grimsley et al., 2015; Lezak et al., 2017; Saibaba et al., 1996) (Mouse Ethogram: *https://mousebehavior.org*). The list of behaviors assessed was determined through pilot analysis and previous studies characterizing rodent defense and fear behaviors. They are defined as the following: abrupt attending, flinching, locomotion, rearing,

grooming, still-and-alert, and stretch-attend (description in **Table 1**; (Blanchard et al., 2003)). A behavior was only counted when its occurrence lasted two or more seconds, except for flinching, which could take place more quickly.

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 were analyzed manually (n=48 mice) 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 total distance traveled and time spent in the periphery and center of the test arena. For video-tracking analysis of time spent, the floor was divided into 16 equal squares with 4 central squares identified as “center” and the 4 corner squares identified as periphery. The two squares in the middle of each wall were excluded from the analysis to avoid attraction to the holes affect the animal’s preference for the periphery vs center.

Procedures related to microdialysis

Surgery

Since both left and right amygdala are responsive to vocal stimuli in human and experimental animal studies (Wenstrup et al., 2020), we implanted microdialysis probes into the left amygdala to maintain consistency with other studies in our laboratory. 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 (≃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. Recovery from the surgical procedures occurred in the animal’s home cage and was assessed by resumption of normal eating, drinking, exploratory, and grooming behaviors and postures. Playback experiments occurred in all recovered animals four days after surgery.

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. The choice of 10-min sampling intervals was based on the minimum collection time needed to provide 5µl samples both for immediate testing and for a backup sample in the event of catastrophic failure of the processes associated with the neurochemical analysis.

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

We verified placement of microdialysis probes to minimize variability that could arise due to placement in regions surrounding BLA that receive different sources of neurochemical inputs (e.g., cholinergic inputs to putamen and central amygdala). To verify probe placement 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. 2**, insets). 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 were included in statistical analyses only if ≥75% of the probe membrane was located within the BLA (**Fig. 2**).

Data analysis

For neurochemical analyses, the total numbers of animals used were N_{EXP}=31 and N_{INEXP}=22. Only mice with useable neurochemical data were further evaluated for behaviors. This included 46 mice: (N_{EXP}=25 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. Further, one INEXP female without detectable DA levels was removed from the INEXP group neurochemical analysis.

Only the following 10-min sample collection windows were used for statistical analysis: Pre-Stim, Stim 1, and Stim 2 (**Fig. 1E**). The purpose of the two collection windows during vocal playback was to allow detection of different epochs of neurochemical release, as may occur in ACh release into the amygdala (Aitta-aho et al., 2018). 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. Raw neurochemical values are reported in supplementary tables 1 (ACh), 2 (DA), and 3 (5-HIAA).

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, the distributions of data points are visualized by box plots, computed using the "inclusive median" formula in Excel. The box indicates first (Q1) and third (Q3) quartiles with the median line between the two. The whiskers normally stretch between minimum and maximum values, but this computation sometimes shows data points outside of the whiskers. Excel identifies these values as outliers in the boxplot function. However, our GLM statistical analysis includes these values because there was no compelling reason to exclude them. The mean value is marked with an "x".

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Reviewer #1 (Public Review):

The manuscript addresses a fundamental question about how different types of communication signals differentially affect brain state and neurochemistry. In addition, their manuscript highlights the various processes that modulate brain responses to communication signals, including prior experience, sex, and hormonal status. Overall, the manuscript is well-written and the research is appropriately contextualized.

That being said, it remains important for the authors to think more about their analytical approaches. In particular, the effect of normalization and the explicit outlining and interpretations of statistical models. As mentioned in the original review, the normalization of neurochemical data seems unnecessary given the repeated-measures design of their analysis and by normalizing all data to the baseline data and including this baseline data in the repeated measures analysis, one artificially creates a baseline period with minimal variation that dramatically differs in variance from other periods (akin to heteroscedasticity). If the authors want to analyze how a stimulus changes neurochemical concentrations, they could analyze the raw data but depict normalized data in their figures (similar to other papers). Or they could analyze group differences in the normalized data of the two stimulus periods (i.e., excluding the baseline period used for normalization).

It would also be useful for the authors to provide further discussion of the potential contributions of different types of experiences (mating vs. restraint) to the change in behavior and neurochemical responses to the vocalization playbacks and to try to disentangle sensory and motor contributions to neurochemical changes.

https://doi.org/10.7554/eLife.88838.2.sa1

Reviewer #3 (Public Review):

The work by Ghasemahmad et al. has the potential to significantly advance our understanding of how neuromodulators provide internal-state signals to the basolateral amygdala (BLA) while an animal listens to social vocalizations.

Ghasemahmad et al. made changes to the manuscript that have significantly improved the work. In particular, the transparency in showing the underlying levels of Ach, DA, and 5HIAA is excellent. My previous concerns have been adequately addressed.

https://doi.org/10.7554/eLife.88838.2.sa0

Author Response

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This important study advances our understanding of the ways in which different types of communication signals differentially affect mouse behaviors and amygdala cholinergic/dopaminergic neuromodulation. Researchers interested in the complex interaction between prior experience, sex, behavior, hormonal status, and neuromodulation should benefit from this study. Nevertheless, the data analysis is incomplete at this stage, requiring additional analysis and description, justification, and - potentially - power to support the conclusions fully. With the analytical part strengthened, this paper will be of interest to neuroscientists and ethologists.*

GENERAL COMMENTS ON REVIEWS AND REVISIONS

Experimental design

Here we address questions from several reviewers regarding our periods of neuromodulator and behavioral analysis. First, we recognize that the text would benefit from an overview of the experimental structure different from the narrative we provide in the first paragraphs of the Results. We now include this near the beginning for the Materials and Methods (page 17). We further articulate that the 10-minute time periods were dictated by the sampling duration required to perform accurate neurochemical analyses (and to reserve half of the sample in the event of a catastrophic failure of batch-processing samples). Since neurochemical release may display multiple temporal components (e.g., ACh: Aitta-aho et al., 2018) during playback stimulation, and since these could differ across neurochemicals of interest, we decided to collect, analyze, and report in two stimulus periods as well as one Pre-Stim control. We now clarify this in additional text in the Material and Methods (p. 24, lines 20-22; p. 26, lines 17-19). We decided not to include analyses of the post-stimulus period because this is subject to wider individual and neuromodulator-specific effects and because it weakens statistical power in addressing the core question—the change in neuromodulator release DURING vocal playback.

We also sought to clarify the meaning of the periods “Stim 1” and “Stim 2”; they are two data collection periods, using the same examplar sequences in the same order. We have added statements in the Material and Methods (p. 18, lines 4-7; Fig. caption, p. 39, lines 11-13) to clarify these periods.

For behavioral analyses, observation periods were much shorter than 10 mins, but the main purpose of behavioral analyses in this report is to relate to the neurochemical data. As a result, we matched the temporal features of the behavioral and neurochemical analyses (p. 22, lines 17-22). We plan a separate report, focused exclusively on a broader set of behavioral responses to playback, that may examine behaviors at a more granular level.

Data and statistical analyses

Reviewers 1 and 3 expressed concerns about our normalization of neurochemical data, suggesting that it diminishes statistical power or is not transparent. We note that normalization is a very common form of data transformation that does not diminish statistical power. It is particularly useful for data forms in which the absolute value of the measurement across experiments may be uninformative. Normalization is routine in microdialysis studies, because data can be affected by probe placement and factors affecting neurochemical recovery and processing. Recent examples include:

Li, Chaoqun, Tianping Sun, Yimu Zhang, Yan Gao, Zhou Sun, Wei Li, Heping Cheng, Yu Gu, and Nashat Abumaria. "A neural circuit for regulating a behavioral switch in response to prolonged uncontrollability in mice." Neuron (2023).

Gálvez-Márquez, Donovan K., Mildred Salgado-Ménez, Perla Moreno-Castilla, Luis Rodríguez-Durán, Martha L. Escobar, Fatuel Tecuapetla, and Federico Bermudez-Rattoni. "Spatial contextual recognition memory updating is modulated by dopamine release in the dorsal hippocampus from the locus coeruleus." Proceedings of the National Academy of Sciences 119, no. 49 (2022): e2208254119.

Holly, Elizabeth N., Christopher O. Boyson, Sandra Montagud-Romero, Dirson J. Stein, Kyle L. Gobrogge, Joseph F. DeBold, and Klaus A. Miczek. "Episodic social stress-escalated cocaine self-administration: role of phasic and tonic corticotropin releasing factor in the anterior and posterior ventral tegmental area." Journal of Neuroscience 36, no. 14 (2016): 4093-4105.

Bagley, Elena E., Jennifer Hacker, Vladimir I. Chefer, Christophe Mallet, Gavan P. McNally, Billy CH Chieng, Julie Perroud, Toni S. Shippenberg, and MacDonald J. Christie. "Drug-induced GABA transporter currents enhance GABA release to induce opioid withdrawal behaviors." Nature neuroscience 14, no. 12 (2011): 1548-1554.

However, since all reviewers requested raw values of neurochemicals, we provide these in supplementary tables 1-3. The manuscript references these table early in the Results (p. 6, lines 18-19) and in the Material and Methods (p. 27, lines 3-4)

All reviewers commented on correlation analyses that we presented, with different perspectives. Reviewer 2 questioned the validity of such analyses, performed across experimental groups, while Reviewer 1 pointed out that the analyses were redundant with the GLM. We agree with these criticisms, and note the challenges associated with correlations involving behaviors for which there is a “floor” in the number of observations. As a result, we have removed most correlation analyses from the manuscript. The text and figures have been modified accordingly. Due these changes, we have to decline requests of Reviewer 3 to include many more such analyses. While correlation analyses could still be performed between neurochemicals and behaviors for each group, the relatively small size of each experimental group, the large number of groups, and the even larger numbers of pairings between neurochemicals and behavior, the statistical power is very low. The only correlations we utilize in the manuscript concern the interpretation of our increased acetylcholine levels.

As part of this revision, we re-ran our statistical analyses on neuromodulators because of a calculation error in 3 animals (regarding baseline values). In a few instances, a significance level changed, but none of these changed a conclusion regarding neuromodulator changes under our experimental conditions.

Other revisions

INTRODUCTION: We modified the Introduction to provide both a more general framework and specific gaps in our understanding relating neuromodulators with vocal communication.

DISCUSSION: We have added material in the first two pages of the Discussion to provide more framework to our conclusions, to address the issues of the temporal aspects of neurochemical release and behavioral observations, and to identify limitations that should be addressed in future studies.

FIGURES: All figures are now in the main part of the manuscript. We modified most figures in response to reviewer comments. We removed neuromodulator – behavior correlations from several figures. We modified all box plots to ensure that all data points are visible. The visible data points match the numbers reported in figure captions. We brought 5-HIAA data into the main figures reporting on neuromodulator results.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *The manuscript addresses a fundamental question about how different types of communication signals differentially affect brain states and neurochemistry. In addition, the manuscript highlights the various processes that modulate brain responses to communication signals, including prior experience, sex, and hormonal status. Overall, the manuscript is well-written and the research is appropriately contextualized. The authors are thoughtful about their quantitative approaches and interpretations of the data.*

> *That being said, the authors need to work on justifying some of their analytical approaches (e.g., normalization of neurochemical data, dividing the experimental period into two periods (as opposed to just analyzing the entire experimental period as a whole)) and should provide a greater discussion of how their data also demonstrate dissociations between neurochemical release in the basolateral amygdala and behavior (e.g., neurochemical differences during both of the experimental periods but behavioral differences only during the first half of the experimental period). The normalization of neurochemical data seems unnecessary given the repeated-measures design of their analysis and could be problematic; by normalizing all data to the baseline data (p. 24), one artificially creates a baseline period with minimal variation (all are "0"; Figures 2, 3 & 5) that could inflate statistical power.*

Please see our general responses to structure of observation periods and normalization of neuromodulator data. Normalization is a common and appropriate procedure in microdialysis studies that does not alter statistical power.

We have included a section in the Discussion concerning the temporal relationship between behavioral responses and neurochemical changes in response to vocal playback (p. 12, lines 3-17). We note where the linkage is particularly strong (e.g., ACh release and flinching). This points to a need to examine these phenomena with finer temporal resolution, but also with the recognition that the brain circuits driving a behavioral response may extend beyond the BLA.

> *The Introduction could benefit from a priori predictions about the differential release of specific neuromodulators based on previous literature.*

We added some material to the Introduction to provide additional rationale for the study. However, we did not attempt to develop predictions for the range of neuromodulators that we sought to test. The literature can lead to opposite predictions for a given neuromodulator. For example, acetylcholine could be associated with both positive and negative valence. Instead, we note in the Introduction the association of both DA and ACh with vocalizations.

> *The manuscript would also benefit from a description of space use and locomotion in response to different valence vocalizations.*

We have provided additional descriptions of space use and video tracking data in Material and Methods (p. 23, lines 1-6). We now report a few correlations based on these data in the Results to demonstrate that increased ACh in Restraint males and Mating estrus females was not related to the amount of locomotion (p. 9, lines 8-14).

> *Nevertheless, the current manuscript seems to provide some compelling support for how positive and negative valence vocalizations differentially affect behavior and the release of acetylcholine and dopamine in the basolateral amygdala. The research is relevant to broad fields of neuroscience and has implications for the neural circuits underlying social behavior.*
>
> ***Reviewer #2 (Public Review):***
>
> *Ghasemahmad et al. report findings on the influence of salient vocalization playback, sex, and previous experience, on mice behaviors, and on cholinergic and dopaminergic neuromodulation within the basolateral amygdala (BLA). Specifically, the authors played back mice vocalizations recorded during two behaviors of opposite valence (mating and restraint) and measured the behaviors and release of acetylcholine (ACh), dopamine (DA), and serotonin in the BLA triggered in response to those sounds.*

> *Strength: The authors identified that mating and restraint sounds have a differential impact on cholinergic and dopaminergic release. In male mice, these two distinct vocalizations exert an opposite effect on the release of ACh and DA. Mating sounds elicited a decrease of Ach release and an increase of DA release. Conversely, restraint sounds induced an increase in ACh release and a trend to decrease in DA. These neurotransmission changes were different in estrus females for whom the mating vocalization resulted in an increase of both DA and ACh release.*
>
> *Weaknesses: The behavioral analysis and results remain elusive, and although addressing interesting questions, the study contains major flaws, and the interpretations are overstating the findings.*

Although Reviewer 2 raises several valid issues that we have addressed in our response and revision, we believe that none represent "major flaws" in the study that challenge the validity of our central conclusions. In brief, we will:

--provide enhanced description of behaviors (pp. 22-23 and Table 1)

--clarify / modify box-plot representations of data (p 28. Lines 3-9)

--point to our methods that describe corrections for multiple comparisons (p. 27; lines 15-16)

--revise figures to clarify sample size (Figs. 3-6)

> ***Reviewer #3 (Public Review):***
>
> *Ghasemahmad et al. examined behavioral and neurochemical responses of male and female mice to vocalizations associated with mating and restraint. The authors made two significant and exciting discoveries. They revealed that the affective content of vocalizations modulated both behavioral responses and the release of acetylcholine (ACh) and dopamine (DA) but not serotonin (5-HIAA) in the basolateral amygdala (BLA) of male and female mice. Moreover, the results show sex-based differences in behavioral responses to vocalizations associated with mating. The authors conclude that behavior and neurochemical responses in male and female mice are experience-dependent and are altered by vocalizations associated with restraint and mating. The findings suggest that ACh and DA release may shape behavioral responses to context-dependent vocalizations. The study has the potential to significantly advance our understanding of how neuromodulators provide internal-state signals to the BLA while an animal listens to social vocalizations; however, multiple concerns must be addressed to substantiate their conclusions.*
>
> *Major concerns:*
>
> 1. *The authors normalized all neurochemical data to the background level obtained from a single pre-stimulus sample immediately preceding playback. The percentage change from the background level was calculated based on a formula, and the underlying concentrations were not reported. The authors should report the sample and background concentrations to make the results and analyses more transparent. The authors stated that NE and 5-HT had low recovery from the mouse brain and hence could not be tracked in the experiment. The authors could be more specific here by relating the concentrations to ACh, DA, and 5-HIAA included in the analyses.*

Please see our general statement regarding normalization of neurochemical data. We have added supplemental tables that shows concentrations of dopamine, acetylcholine, 5-HIAA. We do not report serotonin or noradrenalin since these were below the detection threshold.

> 1. *For the EXP group, the authors stated that each animal underwent 90-min sessions on two consecutive days that provided mating and restraint experiences. Did the authors record mating or copulation during these experiments? If yes, what was the frequency of copulation? What other behaviors were recorded during these experiences? Did the experiment encompass other courtship behaviors along with mating experiences? Was the female mouse in estrus during the experience sessions?*

In the mating experience, mounting or attempted mounting was required for the animal to be included in subsequent testing. Since the session lasted 90 minutes, more general courtship behavior was likely. However, we did not record detailed behaviors or track estrous stage for the mating experience. See p. 21, line 20-22.

> 1. *For the mating playback, the authors stated that the mating stimulus blocks contained five exemplars of vocal sequences emitted during mating interactions. The authors should clarify whether the vocal sequences were emitted while animals were mating/copulating or when the male and female mice were inside the test box. If the latter was the case, it might be better to call the playback "courtship playback" instead of "mating playback".*

We have modified the Results (p. 5, lines 18-20) and Materials and Methods (p. 21, lines 8-15) to clarify our meaning. We continue to use the term “mating” because this refers to a specific set of behaviors associated with mounting and copulation, rather than the more general term “courtship”. We also indicate that we based these behaviors on previous work (e.g., Gaub et al., 2016).

> 1. *Since most differences that the authors reported in Figure 3 were observed in Stim 1 and not in Stim 2, it might be better to perform a temporal analysis - looking at behaviors and neurochemicals over time instead of dividing them into two 10-minute bins. The temporal analysis will provide a more accurate representation of changes in behavior and neurochemicals over time.*

Please see our general response to the structuring of experimental periods. The 10-min periods are the minimum for the neurochemical analyses, and we adopted the same periods for behavioral analyses to match the two types of observations. Our repeated measures analysis is a form of temporal analysis, since it compares values in three observation periods.

> 1. *In Figures 2 and 3, the authors show the correlation between Flinching behavior and ACh concentration. The authors should report correlations between concentrations of all neurochemicals (not just ACh) and all behaviors recorded (not just Flinching), even if they are insignificant. The analyses performed for the stim 1 data should also be performed on the stim 2 data. Reporting these findings would benefit the field.*

Please see general comments regarding correlation analyses. We removed almost all such analyses and references to them from the manuscript based on concerns of the other reviewers.

> 1. *The mice used in the study were between p90 - p180. The mice were old, and the range of ages was considerable. Are the findings correlated with age? The authors should also discuss how age might affect the experiment's results.*

Our p90-p180 mice are not “old”. CBA/CaJ mice display normal hearing for at least 1 year (Ohlemiller, Dahl, and Gagnon, JARO 11: 605-623, 2010) and adult sexual and social behavior throughout our observation period. They are sexually mature adults, appropriate for this study. We decline to perform correlation analyses with age, both because this was not a question for this study and because the very large number of correlations, for each experimental group (as requested by reviewer #2), render this approach statistically problematic.

> 1. *The authors reported neurochemical levels estimated as the animals listened to the sounds played back. What about the sustained effects of changes in neurochemicals? Are there any potential long-term effects of social vocalizations on behavior and neurochemical levels? The authors might consider discussing long-term effects.*

We have not included discussion of long term effects of neuromodulatory release, both because our data analysis doesn’t address it (see response to Comment #10) and because we desired to keep the Discussion focused on topics more closely related to the results.

> 1. *Histology from a single recording was shown in supplementary figure 1. It would benefit the readers if additional histology was shown for all the animals, not just the colored schematics summarizing the recording probe locations. Further explanation of the track location is also needed to help the readers. Make it clear for the readers which dextran-fluorescein labeling image is associated with which track in the schematic.*

Based on the recent publications cited in our overall response to reviewer comments about statistical methods, our reporting of histological location of microdialysis exceeds the standard. We believe that the inclusion of all histology is unnecessary and not particularly helpful. Raw photomicrographs do not always illustrate boundaries, so interpretation is required. However, we added a second photomicrograph example and we identified which tracks correspond to these photomicrographs (see Figure 2; now in main body of manuscript).

> 1. *The authors did not control for the sounds being played back with a speaker. This control may be necessary since the effects are more pronounced in Stim 1 than in Stim 2. Playing white noise rather than restraint or courtship vocalizations would be an excellent control. However, the authors could perform a permutation analysis and computationally break the relationship between what sound is playing and the neurochemical data. This control would allow the authors to show that the actual neurochemical levels are above or below chance.*

We considered a potential “control” stimulus in our experimental design. We concluded, based on our previous work (e.g., Grimsley et al., 2013; Gadziola et al., 2016), that white noise is not or not necessarily a neutral stimulus and therefore the results would not clarify the responses to the two vocal stimuli. Instead, we opted to use experience as a type of control. This control shows very clearly that temporal patterns and across-group differences in neurochemical response to playback disappear in the absence of experience with the associated behavior.

> *1. The authors indicated that each animal's post-vocalization session was also recorded. No data in the manuscript related to the post-vocalization playback period was included. This omission was a missed opportunity to show that the neurochemical levels returned to baseline, and the results were not dependent on the normalization process described in major concern #1. The data should be included in the manuscript and analyzed. It would add further support for the model described in Figure 6.*

We decided not to include analyses of the post-stimulus period because this period is subject to wider individual and neuromodulator-specific effects and because it weakens statistical power in addressing the core question—the change in neuromodulator release DURING vocal playback. We agree that the general question is of interest to the field, but we don't think our study is best designed to answer that question.

> *1. The authors could use a predictive model, such as a binary classifier trained on the CSF sampling data, to predict the type of vocalizations played back. The predictive model could support the conclusions and provide additional support for the model in Figure 6.*

We recognize that a binary classifier could provide an interesting approach to support conclusions. However, we do not believe that the sample size per group is sufficient to both create and test the classifier.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Major comments:*
>
> *• Introduction: It would be useful to set up an experimental framework before delving into the results. What are the predictions about specific neuromodulators based on previous literature?*

Because this narrative is laid out in the first two paragraphs of the Results, which immediately follow the Introduction, we believe that additional text in the Introduction on the experimental framework is redundant. As stated above, detailing predictions for a range of neuromodulators would make for a long and not particularly illuminating Introduction. We instead have related our findings to more general understanding of DA and ACh in the Discussion.

> *• There really isn't a major difference in stimuli during the "Stim 1" and "Stim 2" phases, and it's not clear why the authors divided the experimental period into two phases. Therefore, the authors need to justify their experimental approach. For example, the authors could first anecdotally mention that behavioral responses to playbacks seem to be larger in the first half of the playbacks than during the second half, therefore they individually analyzed each half of the experimental period. Or adopt a different approach to justify their design. Overall, the analytical approach is reasonable but it is currently not justified.*

See general comment for analysis periods. As noted, we clarified these issues in several locations with Materials and Methods (pp. 24, lines 20-22; p. 26, lines 17-19). We also sought to clarify the meaning of the periods "Stim 1" and "Stim 2"; they are two data collection periods, using the same examplar sequences in the same order. We have added statements in the Material and Methods (p. 18, lines 4-7; Fig. caption, p. 39, lines 11-13).

> *• The normalization of neurochemical data seems problematic and unnecessary. By normalizing all data to the baseline data (p. 24), one artificially creates a baseline period*

> *with minimal variation (all are "0"; Figures 2, 3 & 5) and this has implications for statistical power. Because the analysis is a within-subjects analysis, this normalization is not necessary for the analysis itself. It can be useful to normalize data for visualization purposes, but raw data should be analyzed. Indeed, behavioral data are qualitatively similar to the neurochemical data, and those data are not normalized to baseline values.*

Please see our general comment on this issue. We believe normalization does not affect statistical power and is both the standard way and an appropriate way to analyze microdialysis results. We include concentrations of ACh, DA, and 5-HIAA in supplementary tables?

> *• The authors should include a discussion (in the Discussion section) of how behavior and neurochemical release are associated during the first half of the experimental session but not in the second half (e.g., differences in Ach and DA release between mating and restraint groups during stim 1 and 2, but behavioral differences only during stim 1).*

We have included a section in the Discussion concerning the temporal relationship between behavioral responses and neurochemical changes in response to vocal playback. We note that the linkage is particularly strong in some cases (e.g., ACh release and flinching). This points to a need to examine these phenomena with finer temporal resolution, but also with the recognition that the brain circuits driving a behavioral response may extend beyond the BLA.

> *Minor comments:*
>
> *• Keywords: add "serotonin" (even though there are no significant differences on 5-HIAA, people interested in serotonin would find this interesting).*

Added to keywords list.

> *• Do the authors collect data on the vocalizations of mice in response to these playbacks?*

We monitored vocalizations during playback, noting that vocalizations–especially “Noisy” vocalization–were common. However, we did not record vocalizations and are therefore unable quantify our observations.

> *• First line of page 7: readers do not know about "stim 1" and "stim 2". Therefore, the authors need to describe their approach to analyzing behavior and neurochemical release.*

We first introduce these terms earlier, citing Figure 1D,E. We have added some additional wording for further clarification. page 7, lines 4-5.

> *• Make sure citations are uniformly formatted (e.g., Inconsistencies in: "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 have reviewed and corrected citations throughout the manuscript.

> *• Last paragraph of page 7: "attending behavior" has not been defined yet.*

Table 1 contains our description of the behaviors analyzed in this study. We have now inserted a reference to Table 1 earlier in the Results (p. 6, line 12).

> • *Figure 2E and 3G: I find these correlations to be redundant with the GLMs. This is because the significant relationship is likely to be driven by group differences in behavior and in neurochemical release.*

Please see general comments regarding correlation analyses. We removed such analyses and references to them from the manuscript.

> • *Page 2, 2nd paragraph, 2nd sentence: this paragraph seems to be rooted in comparing and contrasting experienced and inexperienced mice, so there should be explicit comparisons in each sentence. For example, the 2nd sentence should read: "Whereas EXP estrus females demonstrated increased flinching behaviors in response to mating vocalizations, INEXP ...." . This paragraph overall could use some refining.*

We believe this refers to page 9. We have revised the paragraph to clarify our findings (Beginning p. 9, line 23).

> • *Page 9: "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)." While I understand their perspective, this is misleading because changes were only observed during the Stim 1 period.*

We have slightly revised the wording in this paragraph, because the restraint males did not show significant ACh decreases. However, we do not believe our statements mislead readers just because some changes are observed in only one of the stimulation periods (p 10, lines 13-16).

> • *Last paragraph of page 14: it would be useful to mention the increase in flinching in experienced females in response to mating vocalizations.*

We have added a sentence in this paragraph relating flinching in estrus females to increased ACh (p. 15, lines 18-20).

> • *Was there a full analysis of locomotion in response to playbacks? I see that locomotion was correlated with neurochemical release but was it different in response to different stimuli? Were there changes to the part of the arena that mice occupied in response to restraint vs. mating vocalizations? Given their methods section, it would be useful for the authors to mention the results of the analyses of these aspects of movement.*

We have provided additional descriptions of space use and video tracking data in Material and Methods (p. 23, lines 1-6). We now report additional results associated with these analyses (p. 8, lines 13-15; p. 9, lines 8-14).

> • *I believe that each experimental mouse only heard one of the stimuli (given the analytical approach). Because it is plausible to measure neurochemical release in response to both types of stimuli, I encourage the authors to be more explicit about this aspect of the experimental design (e.g., mention in Results section).*

Sentence modified to read: “Each mouse received playback of either the mating or restraint stimuli, but not both: same-day presentation of both stimuli would require excessively long playback sessions, the condition of the same probe would likely change on subsequent days, and quality of a second implanted probe on a subsequent day was uncertain.” (p. 7, lines 5-9).

> *• Figure 1A and 1B: add labels to the panels so readers don't have to read the legend to know what spectrogram is associated with what context.*

We added these labels to Figure 1.

> *• Table 1: in the definition of "still and alert", should this mention "abrupt attending" instead of "abrupt freezing"? The latter isn't described.*

Yes, we intended “abrupt attending”, and now indicated that in Table 1

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Major comments:*
>
> - *The authors report they performed manual behavioral analysis, and provide a table defining the different behaviors. However, it remains unclear how some of these behaviors were detected (such as still-and-alert events). A thorough description of the criteria used to define these events needs to be provided.*

We have modified some descriptions of manually analyzed behaviors in Table 1, and have added additional description of how we developed this set of behaviors for analysis in the study (pp. 22-23).

> - *The box plots do not appear to represent the "minimum, first quartile, median, third quartile, and maximum values." as specified on page 24 (Methods). Indeed, the individual data points sometimes do not reach the max or min of the bar plot, and sometimes are way beyond them.*

We used the “inclusive median” function in Excel to generate final boxplots. These boxplots will sometimes result in a data point being placed outside of the whiskers. SPSS considers these to be “outliers”, but our GLM analysis includes these values. We describe this in Data Analysis section of Materials and Methods (p. 28, lines 3-9)

> - *Some of the data are replicated in different Figures: Figure 2A and Figure 3C. While this is acceptable, the authors did not correct for multiple comparisons (dividing the p value by the number of comparisons).*

Our analysis included corrections for multiple comparisons, as we have indicated on p. 27, lines 15-16.

> - *Overall, the sample sizes are too small (for example in Figure 3, non-estrus females are at n=3), and are different in experiments where they should be equal (Figure 2B: mating stim 1 is at n=5 and mating stim 2 is at n=3).*

We apologize that sample sizes were not properly displayed in figures. Please note that sample sizes are identified in the figure captions. For neuromodulator data, all sample sizes are at least 7. For behavioral data, the minimum sample size is 5. We have revised Figures 3-6 to ensure that all data points are visible.

> - *It remains unclear why the impact of mating vocalizations has been tested only in males.*

We assume the reviewer meant that only males were tested in restraint. We now indicate that our preliminary evidence indicated no difference in behavioral responses to restraint vocalization between males and females, so we opted to perform the neurochemical analysis for restraint only in males (page 22 lines 4-5). If there were no limitations to time and cost, we would have preferred to test responses to restraint in females as well. We note that such inclusion would have added up to 4 experimental groups (estrus and non-estrus groups in both EXP and INEXP groups).

> - *The correlation between the number of flinching and ACh release changes (Figure 2E) visually appears to be opposite between mating and restraint playbacks. The authors should perform independent correlations for these 2 playbacks.*

Please see general comments regarding correlation analyses. We removed such analyses and references to them from the manuscript.

> - *The authors state that their 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.". However, in male mice, they do not report any difference in previous experience on flinching for both restraint and mating sounds, as well as no difference in rearing for the restrain sounds (Figure 4A-B). Thus, the discussion of these results should be completely revisited.*

We revised the paragraph in question (p. 9, line 22 through p. 10, line 9). For instance, we note that significant differences between EXP male-mating and male-restraint flinching do not exist between the INEXP groups. We believe that the last sentence correctly summarizes findings described in this paragraph.

> - *For serotonin experiments in Figure S2 there are strong outliers (150% increase in 5HIAA release). Did the authors correlate these levels with the behavior of the animals?*

Outliers are identified by the Excel function that generated the boxplots, but we have no reason to consider these as outliers and exclude them. As noted above, we have clarified that these “outliers” are the result of the Excel function in the Materials and Methods (p. 28, lines 3-9) and we have revised the plotting of data points

> *Minor comments:*
>
> - *Mating vocalization playback is mainly emitted by males, thus, instead of a positive valence signal, this could also be interpreted as a competitive signal to other males.*

There is support in the literature for viewing our mating stimulus as having positive valence. Gaub et al., 2016 describe the emission of stepped calls, lower frequency harmonics, and increased sound level as indicators of “positive emotion”. We have shown (Grimsley et al, 2013) that the female LFH vocalization can be highly attractive to male mice, under the right conditions, indicating something like “sex is happening”. The inclusion of both the male and female vocalizations in our stimuli was a key piece of our experimental design, based on our understanding of the contributions of both vocalizations to the meaning of the overall acoustic experience.

- *Figure 1 should include panel titles.*

No change. This information is available in the Figure caption.

- *n=31 should be indicated in the EXP group.*

We're not sure where the reviewer is referring to this value.

- *The color legend of Figure 1E is absent, making the Figure not understandable.*

We added text in the Figure 1 caption to indicate that each color represents a different exemplar. We don't think a legend provides additional useful information.

- *The point of making two blocks (stim 1 and stim2) should be stated more clearly.*

Please see general statement regarding experimental blocks. We have modified our description of these in an Experimental overview section in the Material and Methods.

- *Including raw data of micro-dialysis in the supplementary figures would allow assessment of the variability and quality of the measurements.*

We have added concentrations of neurochemicals in supplemental tables 1-3.

- *Baseline (prestimulus) number of flinch and rearing should systematically be indicated (missing in Figure 4).*

The focus in this figure is on the differences that occur in Stim 1 values. There are no differences between EXP and INEXP animals of any group during the Pre-Stim period. We now state that in the Figure 4 caption.

- *Discussion: "increase in AMPA/NMDA currents". We believe the authors are referring to the ratio of AMPA to NMDA currents. This sentence should be reformulated.*

These are modified to refer to "… the AMPA/NMDA current ratio…" in two locations in the Discussion (p. 14, lines 8-9; p. 15, line 4)

- *Overall the discussion is very speculative and should rely more on the data.*

We believe that the Discussion provides appropriate speculation that is based on our experimental data and previous literature. We have added a paragraph to identify limitations of our findings and recommendations of future experiments to resolve some issues (p. 12, lines 3-17)

Reviewer #3 (Recommendations For The Authors):

Minor concerns:

> 1. *The authors stated that USVs are most likely to be emitted by males, and LFH are likely to be emitted by females. However, Oliveira-Stahl et al. 2023, Matsumoto et al. 2022, Warren et al. 2018, Heckman et al. 2017, Neunuebel et al., 2015 showed that females also emit USVs. The authors should mention that USVs are emitted by both males and females and discuss how the sex of the vocalizing animal (both males and females) can influence neuromodulator release.*

The reviewer slightly mis-stated the wording of our text, changing the meaning significantly. Our wording is "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)…" This phrasing is correct and carefully chosen. The Discussion in Oliveira-Stahl et al 2023 (p. 10-11) supports our statement: "The exact fraction of USVs emitted by females as concluded in all previous studies on dyadic courtship has varied, ranging from 18%, 17.5%, and 16% to 10.5% in the present study…".

> 1. *The authors should explain why ECF from BLA was collected unilaterally from the left hemisphere.*

p. 23, lines 9-11: We inserted a sentence to explain why we targeted the BLA unilaterally. "Since both left and right amygdala are responsive to vocal stimuli in human and experimental animal studies (Wenstrup et al., 2020), we implanted microdialysis probes into the left amygdala to maintain consistency with other studies in our laboratory.." Beyond that, the choice was arbitrary.

> 1. *The authors said each animal recovered in its home cage for four days before the playback experiment. A 4-day period may not be sufficient for every animal to recover from surgery, so the authors should describe how a mouse's recovery was assessed.*

p. 23, lines 20-23: We provide more description about the recovery and how it was assessed. Except for a few animals that were not included in the experiments, all animals recovered within 4 days.

> 1. *The authors stated that each animal was exposed to 90-min sessions with mating and restraint behaviors in a counterbalanced design. This description for Figure 1D should also include the duration of the mating and restraint experience.*

The Results that immediately precede citation to this figure include this information.

> 1. *The authors stated, "Data are reported only from mice with more than 75% of the microdialysis probe implanted within the BLA". What are the implications of having 25% of the probe outside the BLA? The authors should shed more light on this by discussing this issue as it relates to the findings and commenting on where the other 25% of the probe was located.*

We inserted a sentence to explain the rationale for this inclusion criterion. "We verified placement of microdialysis probes to minimize variability that could arise because regions surrounding BLA receive neurochemical inputs from different sources (e.g., cholinergic inputs to putamen and central amygdala)." (p. 25, lines 21-23).

All brain regions that surround BLA, dorsal, medial, ventral, or lateral, could have been sampled by the "other" 25%. Some of these, e.g., the central amygdala or caudate-putamen,

have different sources of cholinergic input that may not have the same release pattern. We do not think it is worthy of further speculation in the Discussion. Due to the high cost of the neurochemical analysis, we often did not process the neurochemistry data if histology indicated that a probe missed the BLA target.

> 1. *The authors confirmed that the estrus stage did not change during the experiment day by evaluating and comparing estrus prior to and after data collection. This strategy was a fantastic experimental approach, but the authors should have discussed the results. How did the results the authors included change when the females were in estrus before but not after data collection? What percentage of females started in estrus but ended in metestrus? Assuming that some females changed estrus state, were these animals excluded from the analyses?*

All animals were in the same estrus state at the beginning and end of the playback session.

> *7). Authors cite Neunuebel et al., 2015 for the sentence "As male and female mice emit different vocalizations during mating". However, Neunuebel et al., 2015 showed vocalizations emitted during chasing--not mating. If mating is a general term for courtship, then this reference is appropriate, but see major concern #3.*

In the Results (p. 8, line 5), we changed the phrasing to “courtship and mating” to include the Neunubel et al study.

As we indicate in our response to Public Comment #3, we have modified the Results (p. 5, lines 18-20) and Materials and Methods (p. 21, lines 8-15) to clarify our meaning. We continue to use the term “mating” because this refers to a specific set of behaviors associated with mounting and copulation, rather than the more general term “courtship”. We also indicate that we based these behaviors on previous work (e.g., Gaub et al., 2016).

> 1. *Authors interpret Figure 3F as DA release showed a "consistent" increase during mating playback across all three experimental groups. However, the increase in the estrus female group is inconsistent, as seen in the graph. This verbiage should be reworded to describe the data more accurately.*

p. 8, line 23 “consistent” was deleted.

> 1. *In all the box plots, multiple data points overlay each other. A more transparent way of showing the data would be adding some jitter to the x value to make each data point visible. The mean (X's) in Figure 3D (pre-stim mating and mating estrus) are difficult to see, as are all the data points in mating non-estrus. Adding all the symbols to the figure legend or a key in the figure instead of the method section would aid the reader and make the plots easier to interpret*

We have revised the boxplots to ensure that all data points are visible.

> 1. *Some verbiage used in the discussion should be toned down. For example, "intense" experiences and "emotionally charged" vocalizations should be removed.*

We have not changed these terms, which we believe are appropriate to describe these experiences and vocalizations.

> 1. *The authors include "Emotional Vocalizations" in the title. It would be beneficial if the authors included more detail and references in the introduction to help set up the emotional content of vocalizations. It may benefit a broader readership as typically targeted by eLife.*

We now cite Darwin and some more recent publications that articulate the general understanding that social vocalizations carry emotional content.