Emotional Vocalizations Alter Behaviors and Neurochemical Release into the Amygdala

Zahra Ghasemahmad[1,2], Aaron Mrvelj[1], Rishitha Panditi[1], Bhavya Sharma[1], Karthic Drishna Perumal[1], and Jeffrey J. Wenstrup[1,2,3•]

[1]Department of Anatomy and Neurobiology and Hearing Research Group, Northeast Ohio Medical University, Rootstown, Ohio, USA

[2]School of Biomedical Sciences, Kent State University, Kent, Ohio, USA

[3]Brain Health Research Institute, Kent State University, Kent, Ohio, USA

* Correspondence:

Jeffrey J. Wenstrup

jjw@neomed.edu

Keywords: acetylcholine, basolateral amygdala, dopamine, estrus, mating, microdialysis, mouse, restraint, vocalization playback

ABSTRACT

The basolateral amygdala (BLA), a brain center of emotional expression, contributes to acoustic communication by first interpreting the meaning of social sounds in the context of the listener's internal state, then organizing the appropriate behavioral responses. We propose that modulatory neurochemicals such as acetylcholine (ACh) and dopamine (DA) provide internal-state signals to the BLA while an animal listens to social vocalizations. We tested this in a vocal playback experiment wherein we sampled fluids within the BLA and observed behavioral responses of male and female mice while presenting highly affective vocal sequences associated with either mating or restraint behaviors. In male mice, playback of restraint vocalizations increased ACh release and decreased DA release, while playback of mating sequences evoked the opposite neurochemical release patterns. In non-estrus female mice, the ACh and DA release patterns to mating playback were similar to males. Estrous females, however, showed increased ACh, associated with vigilance, as well as increased DA, associated with reward-seeking. Across these groups, increased ACh concentration was correlated with an increase in defensive behavior. These neurochemical release patterns and several behavioral responses depended on 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 modulates their output to downstream brain regions responsible for appropriate behavioral outcomes.

INTRODUCTION

In social interactions involving vocal communication signals, an animal receives and analyzes acoustic information, compares it with previous experiences, identifies the salience and valence of such information, and responds with appropriate behaviors. These integrated functions depend on brain circuits that include the amygdala, a region located within the temporal lobe that is recognized to play a role in orchestrating responses to salient sensory stimuli (LeDoux, 2000, 2003; McGaugh, 2002; Sah et al., 2003; Wenstrup et al., 2020). The amygdalar target of auditory inputs from the thalamus and cortex is the basolateral amygdala (BLA) (J. E. LeDoux et al., 1991; X. F. Li et al., 1996; Sacchetti et al., 1999; Keifer et al., 2015). Through reciprocal connections with areas such as the hippocampus, the BLA compares received sensory information with previous experiences (Pitkänen et al., 2000). By integrating this information with other sensory inputs and inputs from other limbic areas, BLA neurons shape appropriate behavioral responses (Namburi et al., 2015, 2016; Beyeler et al., 2016; Gründemann et al., 2019) 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 (Goosens et al., 2000; Sander et al., 2005; Wiethoff et al., 2009; Leitman et al., 2010; Parsana et al., 2012; Grimsley et al., 2013; Gadziola et al., 2016; Li et al., 2017; Gründemann et al., 2019). Contextual information may arise from inputs associated with other sensory modalities (e.g., somatic sensation or olfaction) (Mcdonald, 1998;

68 Lanuza et al., 2004; Grimsley et al., 2013b), but in other cases the contextual
69 information is associated with an animal's internal state. Sources of internal state cues
70 to BLA include brain circuits involving modulatory neurochemicals (i.e.,
71 neuromodulators), known to affect the processing of sensory signals, thus shaping
72 attention, emotion, and goal-directed behaviors (Smythies, 2005; Krichmar, 2008;
73 Bargmann, 2012; Picciotto et al., 2012; Schofield & Hurley, 2018; Likhtik & Johansen,
74 2019).

75 This study investigates contextual information provided by neuromodulatory inputs to
76 the BLA in response to vocal communication signals. Our hypothesis is that these salient
77 vocalizations elicit distinct patterns of neuromodulator release into the BLA, by which
78 they shape the processing of subsequent meaningful sensory information. We further
79 hypothesize that these neuromodulatory patterns may depend on longer term
80 processes that are critical to vocal communication: to experience with these behaviors
81 and the accompanying vocalizations, to sex, and to estrous stage in females. To test
82 these hypotheses, we conducted playback experiments in a mouse model to understand
83 the behavioral and neuromodulator responses to salient vocalizations associated with
84 very different behavioral states.

85 **RESULTS**

86 To study how vocalizations affect behaviors and release of neurochemicals within BLA,
87 we first developed highly salient vocal stimuli associated with appetitive (mating) and
88 aversive (restraint) behaviors of CBA/CaJ mice (Figs. 1, S1). During interactions between

89 adult male and female mice, we analyzed mating-related behaviors and vocalizations to
90 assess the intensity of the interactions (see Materials and Methods). Comparing lower
91 and higher intensity interactions, we found significant differences in acoustic features
92 representing emotional intensity of the vocalizations produced (Fig. S2) (Lahvis et al.,
93 2011; Altenmüller et al., 2013; Cowen et al., 2019; Huang et al., 2021). From higher
94 intensity mating interactions, we selected several sequences of vocalizations to form a
95 20-minute mating vocal stimulus. These sequences included ultrasonic vocalizations
96 (USVs) with harmonics, steps, and complex structure, mostly emitted by males, and low
97 frequency harmonic calls (LFHs) emitted by females (Figs. 1A, S1A,C, S2B)
98 (Ghasemahmad, 2020). During short periods of restraint, mice produce distinctive
99 vocalizations that are associated with anxiety-related behaviors and increased release of
100 the stress hormone corticosterone (Grimsley et al., 2016). From vocal sequences
101 obtained in restrained mice, we created a 20-minute vocal stimulus, primarily containing
102 mid-frequency vocalizations (MFVs) and fewer USV and LFH syllables (Figs. 1B, S1B,C).

103 We next asked whether these salient vocal stimuli, associated with very different
104 behavioral states, could elicit distinct behaviors and patterns of neuromodulator release
105 into the BLA, where neurons respond to vocalizations in context-dependent ways
106 (Grimsley et al., 2013; Gadziola et al., 2016; Matsumoto and Okanoya, 2016). Because
107 previous studies suggest that interaction of cholinergic and dopaminergic systems
108 shapes the emission of positive and negative vocalizations (Silkstone & Brudzynski,
109 2020; Inagaki et al., 2020; Rojas-Carvajal et al., 2022), we specifically hypothesized that
110 patterns of acetylcholine (ACh) and dopamine (DA) release show differences in response

111 to playback of mating and restraint vocalizations. We further hypothesized that sex,
112 female estrous stage, and prior behavioral experience shape the behavioral and
113 neurochemical responses to vocal playback. We tested these hypotheses through
114 experiments combining playback of the vocal stimuli, behavioral tracking and
115 observations, and microdialysis of BLA extracellular fluid in freely moving mice (Figs. 1C,
116 S1D). Fluids were analyzed using a liquid chromatography-mass spectrometry (LC-MS)
117 technique that allowed simultaneous measurement of several neurochemicals and their
118 metabolites in the same dialysate samples, including ACh, DA and the serotonin
119 metabolite 5-HIAA (see Materials and Methods).

120 Prior to the study, male and female mouse subjects had no experience with sexual or
121 restraint behaviors. On the first two days of the experiment, mice in the experienced
122 group (EXP, n=31) were each exposed to 90-min sessions with mating and restraint
123 behaviors in a counterbalanced design (Fig. 1C). Mice in the inexperienced group (INEXP,
124 n=21) received no mating or restraint experience when placed in the behavioral arena
125 on those days. All mice were then implanted with a guide cannula for microdialysis (Fig.
126 1C). On the playback/sample collection day (Day 6), a microdialysis probe was inserted
127 into the guide cannula. After a 4-hour period of mouse habitation and probe
128 equilibration, we recorded behavioral reactions and sampled extracellular fluid from the
129 BLA before (Pre-Stim) and during a 20 min playback period, divided into two 10-min
130 collection/observation periods (Stim 1 and Stim 2) (Fig. 1C, S1D). Each mouse received
131 playback of either the mating or restraint stimuli, but not both. Data are reported only

132 from mice with more than 75% of the microdialysis probe implanted within the BLA
133 (Figs. 1D, S3).

134 We first tested whether playback of mating and restraint vocalizations results in
135 different behavioral responses in male mice. In both playback groups (n_{mating}=7,
136 $n_{restraint}$=6), increased attending (Fig 2A) indicated a generalized response to the vocal
137 stimuli. Other behaviors differed between groups, however. For instance, still-and-alert
138 and flinching behaviors displayed pronounced increases with restraint playback that
139 were not observed in the mating playback group (Fig. 2B,C).

140 For these groups of male mice, analysis of microdialysis samples revealed distinct,
141 vocalization-dependent modulation of cholinergic and dopaminergic release in the BLA.
142 That is, in response to mating vocalizations, ACh release decreased below pre-stimulus
143 levels during both playback windows, while restraint vocalizations resulted in an
144 increase compared to pre-stimulus ACh concentration (Fig. 2D). DA release, however,
145 displayed opposite patterns to ACh, increasing during playback of mating vocalizations
146 but decreasing during playback of restraint vocal sequences (Fig. 2E). In contrast to
147 these distinct patterns, the serotonin metabolite 5-HIAA showed no significant change
148 over time as a result of playback of either vocal stimulus (Fig. S5A). These findings
149 suggest that both behavioral responses and ACh and DA release are modulated in
150 listening male mice by the affective content of social vocalizations. Further, some
151 behavioral and neurochemical responses were significantly correlated, especially the
152 percentage change of ACh concentration (re Pre-Stimulus) with the number of flinching
153 behaviors (Fig. 2F).

154 As male and female mice emit different vocalizations (Neunuebel et al., 2015; Sangiamo
155 et al., 2020; Warren et al., 2020) during mating, we sought to understand whether
156 playback of vocalizations associated with intense mating interactions results in different
157 behavioral and neurochemical responses in listening male and female mice. Since our
158 testing included females in both estrus and non-estrus stages, we further examined the
159 estrous effect on neurochemical release and behavioral reactions. We used a
160 generalized linear model with repeated measure comparisons to examine how estrous
161 stage of females on the day of microdialysis affected neurochemical levels and
162 behaviors during playback of mating vocalizations.

163 Playback of mating vocalizations resulted in some general and sex-based differences in
164 behavioral responses. For instance, all groups displayed increased attending behavior
165 (Fig. S4A). In females, regardless of estrous stage, exploratory behaviors such as rearing
166 were significantly reduced during playback (Fig. 3A), while still-and-alert behavior
167 increased (Fig. S4B), compared to male mice. Other behaviors differed by estrous stage
168 during mating playback: females in estrus displayed a strikingly higher number of
169 flinching behaviors and reduced self-grooming, compared to males, while females in
170 non-estrus stages did not (Fig. 3B,C).

171 We then examined neuromodulator responses to mating vocalization playback for sex-
172 or estrous-stage-dependent patterns of release. This analysis revealed an estrous-
173 dependent modulation of ACh levels during playback: ACh concentration in estrus
174 females increased significantly compared to males during the first and second playback
175 windows (Fig. 3D), but decreased in both non-estrus females and males. DA release,

however, showed a consistent increase with mating playback across all three experimental groups (Fig. 3E). Similar to male groups in restraint and mating playback, the 5-HIAA release patterns in females showed no modulation during mating vocal playback (Fig. S5B).

Like male mice exposed to restraint vocalizations, estrus females showed increases in flinching behavior in response to mating vocalizations. Both of these groups displayed increased ACh release. Among all mice exposed to mating playback, we observed a positive correlation between ACh concentration and the number of flinching behaviors (Fig. 3F). 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.

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. 1C). Does such experience shape the release patterns of these neuromodulators in response to vocal playback? We tested male and female mice under identical vocal playback conditions as previous groups, except that they did not receive the restraint and mating experiences (INEXP groups). Since only one INEXP female was in a non-estrus stage during the playback session, our analysis of the effect of experience included only estrus females and males.

Several behavioral responses to vocalization playback differed between EXP and INEXP mice in a sex- or context-dependent manner. For example, only estrus females showed experience-dependent increases in flinching behaviors (Fig. 4A) and experience-

197 dependent decreases in attending behaviors (not shown) in response to mating vocal
198 sequences. These experience effects were not observed in males in response to mating
199 or restraint vocal playback. Males, however, showed striking experience-dependent
200 increases in rearing behaviors (Fig. 4B) and locomotion in response to mating
201 vocalizations (not shown), but this pattern was not observed during restraint playback
202 for males or mating playback in estrus females. These findings indicate that behavioral
203 responses to salient vocalizations result from interactions between sex of the listener or
204 context of vocal stimuli with the previous behavioral experience associated with these
205 vocalizations.

206 A major finding is that prior experience with mating and restraint behaviors also shaped
207 patterns of ACh and DA release in response to vocal playback (Fig. 4C,D, S6). Our results
208 show that the significant effects of vocal stimulus type and of sex on ACh and DA
209 release, observed in our EXP groups, were absent in the INEXP groups (Figs. 4C,D,
210 S6A,B). For example, recall that in EXP males, restraint vocalizations increased ACh and
211 decreased DA, while mating vocalizations decreased ACh and increased DA. When male
212 mice lacked the previous mating and restraint experiences, the concentrations of ACh
213 and DA showed similar, non-significant changes in response to both vocal playback
214 types (Figs. 4C,D, S6A,B).

215 Also recall that in EXP animals exposed to mating vocalization playback, ACh release
216 increased in estrus females while decreasing in males and non-estrus females. In INEXP
217 estrus females, however, the ACh pattern was similar to INEXP male mice. Although we
218 did not include INEXP non-estrus females, the current data suggests that the effect of

219 estrus in modulating ACh release is experience dependent (Figs. 4C, S6A). DA release
220 during mating playback also appears to be experience-dependent: in both male and
221 female INEXP groups, DA release during mating playback remained similar to baseline
222 levels, unlike the comparable EXP groups (Fig. 4D). Further, 5-HIAA concentrations,
223 which were unaffected by sex, estrous stage, or playback type, were also unaffected by
224 experience (Fig. S6C). Finally, INEXP groups showed no significant correlations between
225 concentrations of ACh with behavior in response to vocalization playback.

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

229

230 **DISCUSSION**

231 In a mouse model of acoustic communication, we showed that the motivational state of
232 a "sender" is reflected in the acoustics of social vocalizations. We then showed that
233 these vocalizations related to intense experiences of restraint and mating affect
234 behavioral responses in listening conspecifics. Further, behavioral responses to mating
235 vocalizations are shaped by sex and estrous stage. The link between hearing and
236 responding to vocal communication signals is formed by brain circuits that integrate
237 acoustic information in vocal signals with other sensory inputs, with internal state
238 signals, and with previous experiences of the listener (Gadziola et al., 2016; Rouby et al.,
239 2016; Wenstrup et al., 2020; Frühholz and Schweinberger, 2021). With access to centers

shaping behavioral responses to these sensory cues, including those involved in social communication (Bickart) (Sah et al., 2003; Bickart et al., 2014; Hsu et al., 2014; Wenstrup et al., 2020), the amygdala participates substantially in this process. 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 in estrous- and context-dependent manners. These release patterns are expected to alter the functional properties of the vocal communication circuits that evoke behavioral responses.

Neuromodulatory Contributions to Behavioral Output

Functional imaging studies in humans and mechanistic studies in other species provide substantial evidence of amygdalar involvement in processing vocalizations (Sander et al., 2003; Andics et al., 2010; Frühholz et al., 2012, 2014, 2016; Viinikainen et al., 2012; Abrams et al., 2016; Liebenthal et al., 2016; Pannese et al., 2016), in valence coding of appetitive and aversive cues (Kyriazi et al., 2018; O'Neill et al., 2018; Pignatelli & Beyeler, 2019; W. C. Huang et al., 2020; Kong & Zweifel, 2021; Smith & Torregrossa, 2021), and in shaping appropriate behavioral responses to these cues (Lim et al., 2009; Senn et al., 2014; Zhang & Li, 2018; Gründemann et al., 2019). 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 (Carlsen et al., 1985; Asan, 1997, 1998; Bigos et al., 2008; Bocchio et al., 2016; Aitta-aho et al., 2018), and since the role of these neurochemicals in providing internal state and contextual information is well-proven

262 (Bocchio et al., 2016; L. Jiang et al., 2016; Likhtik & Johansen, 2019), we hypothesized
263 that release patterns of neuromodulators into the BLA provide contextual information
264 during processing of affective vocalizations. Our results show that these emotionally
265 charged vocalizations result in distinct release patterns of acetylcholine (ACh) and
266 dopamine (DA) into the BLA of male and female mice. Further, female hormonal state
267 appears to influence ACh but not DA release into the BLA when processing mating
268 vocalizations. Such context- or state-dependent changes were not observed in release
269 patterns of other neurochemicals (e.g., 5-HIAA). These data indicate that during analysis
270 of affective vocalizations in the BLA, ACh and DA provide state- and context-related
271 information that can, potentially, modulate sensory processing within the BLA and thus
272 shape an individual's response to these vocalizations.

273 The BLA receives strong cholinergic projections from the basal forebrain (Carlsen et al.,
274 1985; Zaborszky et al., 1999; Aitta-aho et al., 2018) that contribute to ACh-dependent
275 processing of aversive cues and fear learning in the amygdala (Mascagni et al., 2009;
276 Baysinger et al., 2012; Pidoplichko et al., 2013; Tingley et al., 2014; Gorka et al., 2015; L.
277 Jiang et al., 2016; Minces et al., 2017). Our findings support these studies by
278 demonstrating increased ACh release in BLA in response to playback of aversive
279 vocalizations. Although the exact mechanism by which ACh affects vocal information
280 processing in BLA is not clear yet, the result of ACh release onto BLA neurons seems to
281 enhance arousal during emotional processing (Likhtik & Johansen, 2019). Several studies
282 suggest mechanisms by which vocalizations affect ACh release and in turn drive
283 behavioral responses (Fig. 5A). Cholinergic modulation in the BLA is mediated via

284 muscarinic and nicotinic ACh receptors on BLA pyramidal neurons and inhibitory
285 interneurons (Mesulam et al., 1983; Pidoplichko et al., 2013; Unal et al., 2015; Aitta-aho
286 et al., 2018). During the processing of sensory information in the BLA, partially non-
287 overlapping populations of neurons respond to cues related to positive or negative
288 experiences (Paton et al., 2006; Shabel & Janak, 2009; Namburi et al., 2015; Beyeler et
289 al., 2018). These neurons then project to different target areas involved in appetitive or
290 aversive behaviors—the nucleus accumbens or central nucleus of the amygdala,
291 respectively (Namburi et al., 2015).

292 In response to an aversive cue or experience (Fig. 5A), released ACh affects neurons
293 according to their activity. If projection neurons are at rest, ACh may exert an inhibitory
294 effect by activating nicotinic ACh receptors on local GABAergic interneurons, which in
295 turn synapse onto the quiescent pyramidal neurons. This results in GABA-A mediated
296 inhibitory postsynaptic potentials (IPSPs) in the pyramidal neurons. Alternately, direct
297 activation of M1 ACh receptors on pyramidal neurons, activating inward rectifying K+
298 currents, may result in additional ACh-mediated inhibition (Fig. 5A.) (Pidoplichko et al.,
299 2013; Unal et al., 2015; Aitta-aho et al., 2018). When BLA pyramidal neurons are already
300 active due to strong excitatory input associated with aversive cues, M1 receptor
301 activation can result in long afterdepolarizations that produce persistent firing lasting as
302 long as ACh is present (Unal et al., 2015; Jiang et al., 2017). Such a process may explain
303 persistent firing observed in single neuron responses to aversive social vocalizations in
304 bats (Gadziola et al., 2012; Peterson & Wenstrup, 2012). Through this process of
305 inhibiting quiescent neurons and enhancing activation and persistent firing in active

306 neurons, ACh sharpens the population signal-to-noise ratio (SNR) during the processing
307 of salient, aversive signals in the BLA. These neurons, processing negative cues, likely
308 project to the central nucleus of the amygdala (CeA) to regulate defensive behaviors
309 such as escape and avoidance (Fig. 5A) (Namburi et al., 2015; Beyeler et al., 2016, 2018).
310 In agreement, our behavioral findings show increased behaviors such as flinching
311 correlated with increased release of ACh during processing of aversive vocalizations.
312 Such prolonged afterdepolarizations provide the appropriate condition for associative
313 synaptic plasticity (Likhtik & Johansen, 2019) that underlies an increase in AMPA/NMDA
314 currents in CeA-projecting neurons during processing of aversive cues (Namburi et al.,
315 2015; van Vugt et al., 2020).

316 Dopaminergic innervation from the ventral tegmental area (VTA) (Asan, 1997, 1998)
317 acts on BLA neurons via D1 and D2 receptors, both G-protein coupled receptors.
318 Dopamine is important in reward processing, fear extinction, decision making, and
319 motor control (Di Ciano & Everitt, 2004; Ambroggi et al., 2008; Lutas et al., 2019). We
320 observed increased DA release in the BLA in response to mating vocalizations both for
321 males and for females across estrous stages. Electrophysiological studies show that DA
322 enhances sensory processing in BLA neurons by increasing the population response SNR
323 in a process like ACh (See et al., 2001; Kröner et al., 2005; Vander Weele et al., 2018).
324 Thus, during processing of mating vocalizations or those related to other rewarding
325 experiences, DA release in the vicinity of BLA pyramidal neurons and interneurons is
326 enhanced (Fig. 5B). For neurons with elevated spiking activity during processing of
327 appetitive vocalizations or other sensory stimuli, DA acts on D2 receptors of pyramidal

328 cells to further enhance neuronal firing and result in persistent firing of these projection
329 neurons. Conversely, in BLA projection neurons that do not respond to such positive
330 cues, such as CeA-projecting neurons, DA exerts a suppressive effect directly via D1
331 receptors and indirectly by activating inhibitory interneuron feedforward inhibition
332 (Kröner et al., 2005). The net result of this process in response to appetitive
333 vocalizations is an enhancement of activity in the reward-responding neurons and
334 suppression of activity in aversive-responding neurons. This process likely depends on
335 the increase in synaptic plasticity via enhanced AMPA/NMDA current during processing
336 such cues in NAc-projecting neurons in the BLA (Otani et al., 2003; Namburi et al., 2015;
337 van Vugt et al., 2020). Our findings suggest that this may occur in the BLA in response to
338 appetitive vocalizations.

339 **Influence of sex, hormonal state, and experience**

340 As the results with males listening to restraint vocalizations demonstrate, increased ACh
341 release is associated with processing aversive cues. How, then, should the increased
342 ACh release in estrus females during mating vocal playback be interpreted? Many
343 studies have suggested changes in forebrain activity and neuromodulation due to
344 hormonal changes in males and females (Egozi et al., 1986; van Huizen et al., 1994;
345 Matsuda et al., 2002; Sosa et al., 2004; Kirry et al., 2019; Mizuno et al., 2022; Krentzel et
346 al., 2022). For instance, the cholinergic neurons that project to the BLA, originating in
347 the basal forebrain, exhibit high expression of estrogen receptors that is influenced by a
348 female's hormonal state (Shughrue et al., 2000). During estrus, the enhanced circulating
349 estrogen affects release of ACh and may influence neuronal networks and behavioral

350 phenotypes in a distinct manner (Gibbs, 1996; McEwen, 1998). Thus, increased ACh
351 release in estrus females may underlie increased attentional and risk assessment
352 behaviors in response to vocalization playback. Combined with DA increase, it may
353 trigger both NAc and CeA circuit activation, resulting in both reward-seeking and
354 cautionary behaviors in estrus females.

355 Our results demonstrate the strong impact of even limited experience in shaping
356 behavioral and neuromodulatory responses associated with salient social vocalizations.
357 In the adult mice, a single 90-min session of mating and of restraint, occurring 4-5 days
358 prior to the playback experiment, resulted in consistent behavioral responses and
359 consistent and enhanced ACh and DA release into BLA for both vocalization types. As
360 previous work shows (Pawlak et al., 2010; S. Y. Huang et al., 2012; Nadim & Bucher,
361 2014) neuromodulatory inputs play crucial roles in regulating experience-dependent
362 changes in the brain. However, it remains unclear whether the experience shapes
363 neuromodulator release, or whether neuromodulators deliver the experience-related
364 effect into the BLA.

365 The interaction between ACh and DA is thought to shape motor responses to external
366 stimuli (Lester et al., 2010). Our results support the view that a balance of DA and ACh
367 may regulate the proper behavioral response to appetitive and aversive auditory cues.
368 For instance, increased reward-seeking behavior (rearing and locomotion) in EXP males
369 after mating vocalizations playback may result from the differential release of the two
370 neuromodulators—decreased ACh and increased DA. Further, the lack of this
371 differential release may be the underlying cause for the lack of such responses in INEXP

372 male mice. This supports the role of experience in tuning interactions of these two
373 neuromodulators throughout the BLA, for shaping appropriate behaviors. Overall, the
374 behavioral changes orchestrated by the BLA in response to emotionally salient stimuli
375 are most likely the result of the interaction between previous emotional experiences,
376 hormonal state, content of sensory stimuli, and sex of the listening animals.

377

ACKNOWLEDGMENTS

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

Funding: This work was supported by research grant R01 DC00937 to J.J.W and Alexander Galazyuk from the National Institute on Deafness and Other Communication Disorders of the U.S. Public Health Service. Additional support was through awards to Z.G from the Kent State University Graduate Student Senate and Northeast Ohio Medical University Biomedical Sciences Graduate Program Committee.

Author Contributions (CreDit). https://credit.niso.org: Conceptualization: ZG, JJW; Data curation: ZG; Formal analysis: ZG, AM, RP, BS, KDP; Funding acquisition: ZG, JJW; Investigation: ZG; Methodology: ZG, JJW; Project Administration: ZG, JJW; Resources ZG, JJW; Supervision: JJW; Validation: ZG; Visualization: ZG, JJW, AM; Writing—original draft: ZG, JJW; Writing—review and editing: ZG, JJW.

Main Figures

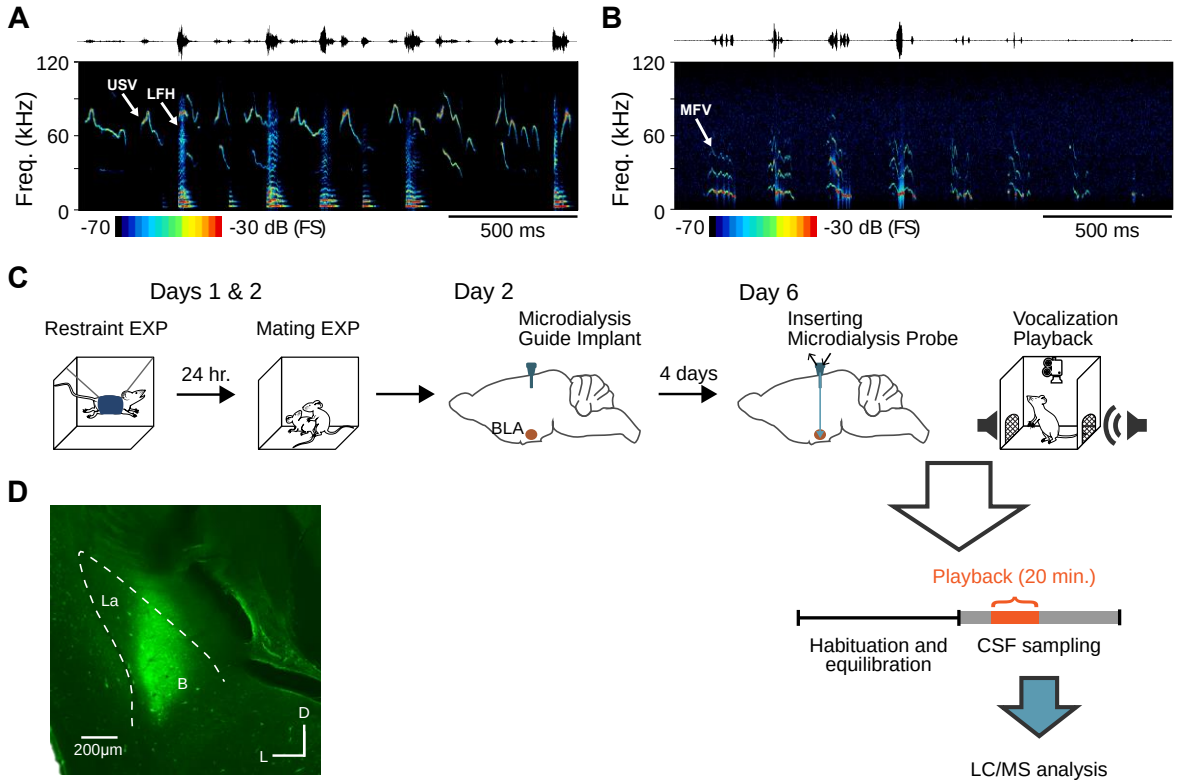


Figure 1. Behavioral / microdialysis experiments test how playback of affective vocal signals alters behaviors and neuromodulator release into the BLA. A. Short sample of mating vocal sequence used in playback experiments. Recording was obtained during high-intensity mating interactions and included USVs, likely emitted by males, as well as LFH calls likely emitted by females. **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 MFV syllables. **C**. 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. **D.** Location of microdialysis probe in BLA was confirmed by perfusion of dextran-fluorescein through probe at the end of experiment. Abbreviations: *B,* basal nucleus of the amygdala; *La*, lateral nucleus of the amygdala.

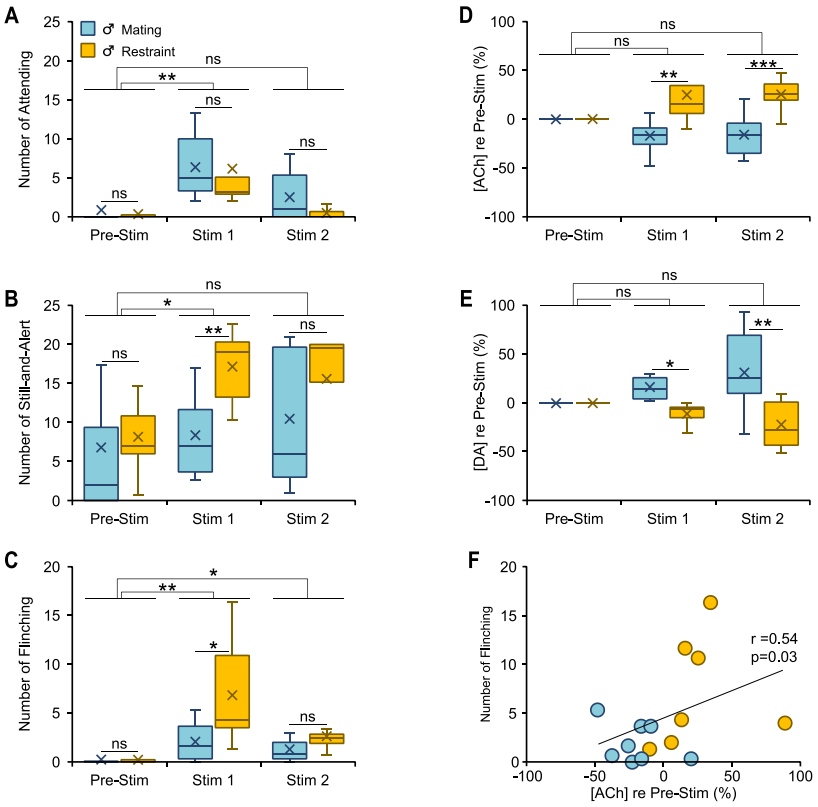


Figure 2. Behavioral and neuromodulator responses to vocal playback in male mice differ by behavioral context of vocalizations. A-C. Boxplots show number of occurrences of specified behavior in 10-min observation periods before (Pre-Stim) and during (Stim 1, Stim 2) playback of mating or restraint vocal sequences ($n_{Mating} = 7$, $n_{Restraint} = 6$). Note that playback sequences during Stim 1 and Stim 2 periods were identical within each group. **A.** Both mating and restraint vocal playback similarly increased attending behaviors (time: F (2,22) =19.3, p=0.0001, partial η^2=0.64; time*context: F (2,22) =0.53, p=0.6, η^2=0.05). **B, C.** During playback, the restraint group increased still-and-alert (time: F(2,22) =6.8, p=0.005, η^2=0.4; context: F (1,11) = 9.6, p=0.01, η^2=0.5) and flinching (time: F (1.1,12.2) =14.2, p=0.002, η^2=0.6; time*context: F (1.1,12.2) =6.3, p=0.02, η^2=0.4) behaviors compared to the mating group. **D, E.** Boxplots show differential release of acetylcholine (ACh) or dopamine (DA), relative to the Pre-Stim period, during mating and restraint vocal playback ($n_{Mating} = 9$, $n_{Restraint} = 7$). **D.** Significant differences in ACh for mating (decrease) vs restraint (increase) playback in stim 1 and stim2 (time: F(2,28)=0.3, p=0.7, η^2=0.02; time*context: F(2,28)=6.6,p=0.004, η^2=0.32) **E.** Significant differences for DA for mating (increase) vs restraint (decrease) playback (time: F(2,28)=0.25,p=0.8, η^2=0.02; time*context: F(2,28)=6.9,p=0.004, η^2=0.33). **F.** Across EXP male subjects in vocal playback, the number of flinching behaviors was positively correlated with change in ACh concentration relative to the Pre-Stim period. (n=15, Pearson r=0.54, p=0.03); **A-E.** GLM repeated measures with Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001.

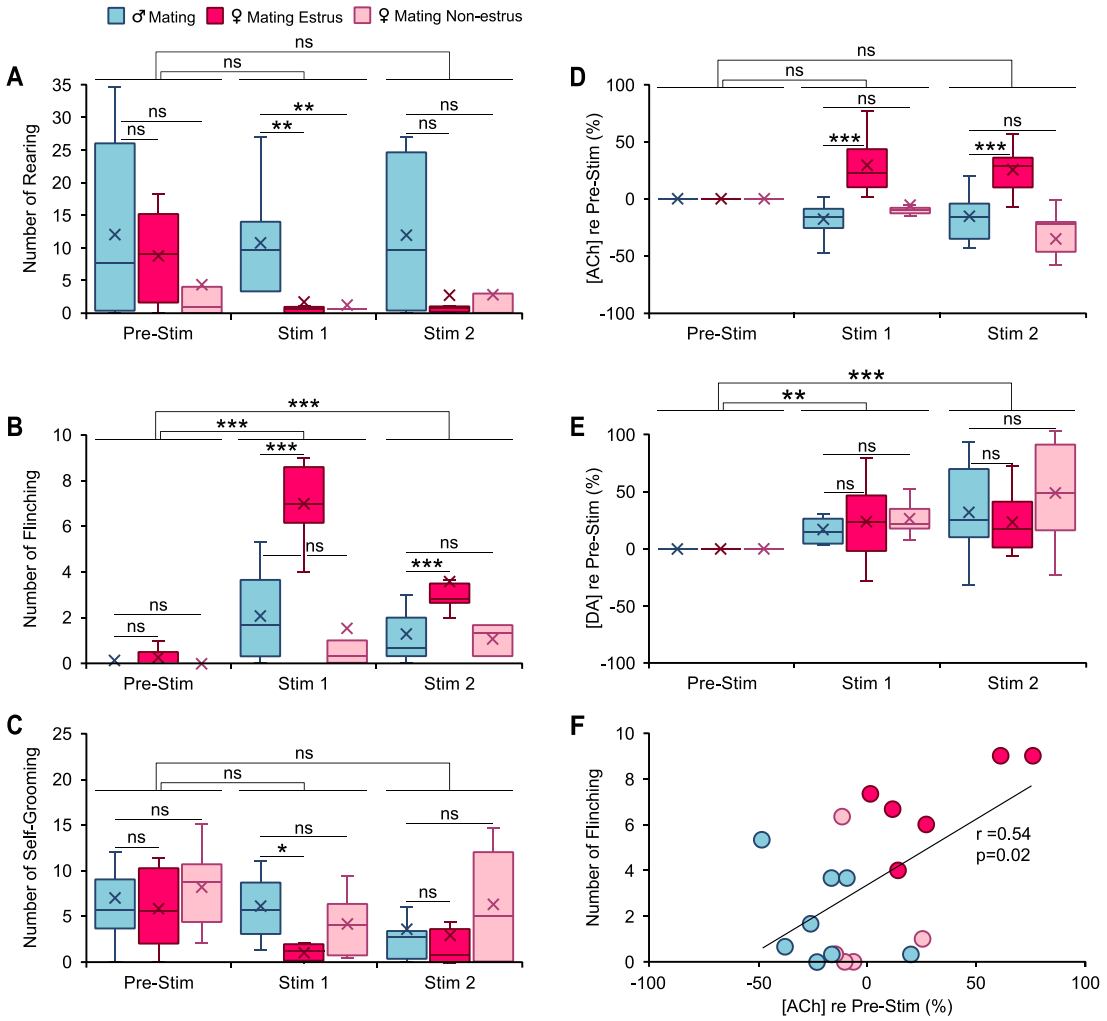


Figure 3. Behavioral and neuromodulator responses to playback of mating vocal sequences differ by sex and female estrous stage. A-C. Occurrences of specified behaviors in 10-min periods before (Pre-Stim) and during (Stim 1, Stim 2) mating vocal playback (n_{Male} = 7, $n_{Estrous\ Fem}$ = 6, $n_{Non\text{-}estrous\ Fem}$ = 5). **A.** Females reared less than males during playback (time: (1.4,20.8) =1.8, p=0.2, η^2=0.1; sex: F (1,15) =10.22, p=0.006; η^2=0.4; estrus: F (1,15) =0.2, p=0.7, η^2=0.01). **B, C**. In comparison to males, estrus females but not non-estrus females showed a significant increase in flinching behavior (time: F(2,30) =20.5, p=0.000, η^2= 0.6; time* sex: F(2,30) =3.1, p=0.06, η^2= 0.2; time*estrus: F (2,30) =9.0, p=0.001, η^2=0.4) and reduced self-grooming (time: F (2,30) =2.4, p=0.1, η^2 =0.14 ; sex: F(1,15) =0.34, p=0.6, η^2= 0.02; estrus for stim1: estrous female vs male: t =2.5, p=0.02, η^2=0.3; non-estrous female vs male: t =0.7, p=0.5, η^2=0.03). **D, E.** Changes in concentration of acetylcholine (ACh) or dopamine (DA) relative to the Pre-Stim period, evoked during Stim 1 and Stim 2 periods of vocal playback (n_{Male} = 9, $n_{Estrous\ Fem}$ = 8, $n_{Non\text{-}estrous\ Fem}$ = 7). **D.** Release of ACh during mating playback showed a significant estrous effect (F (2,42) =10.0, p=0.000, η^2=0.32), increasing above Pre-Stim period in estrous females (time: F (2,42) =1.9 p=0.2, η^2=0.08). **E.** DA release during mating playback increased in all groups relative to Pre-

Stim period (time: $F(2,42) = 12.4$, $p=0.000$, $\eta^2=0.4$), with no significant sex ($F(1,21) =0.2$, $p=0.6$, $\eta^2=0.01$) or estrous effect ($F(1,21) =0.8$, $p=0.4$, $\eta^2=0.04$). **F.** Across EXP subjects responding to mating playback, number of flinching behaviors was positively correlated with change in ACh concentration, relative to Pre-Stim period (n=18, Pearson $r=0.54$, $p=0.02$). A-E: GLM repeated measures with Bonferroni post hoc test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

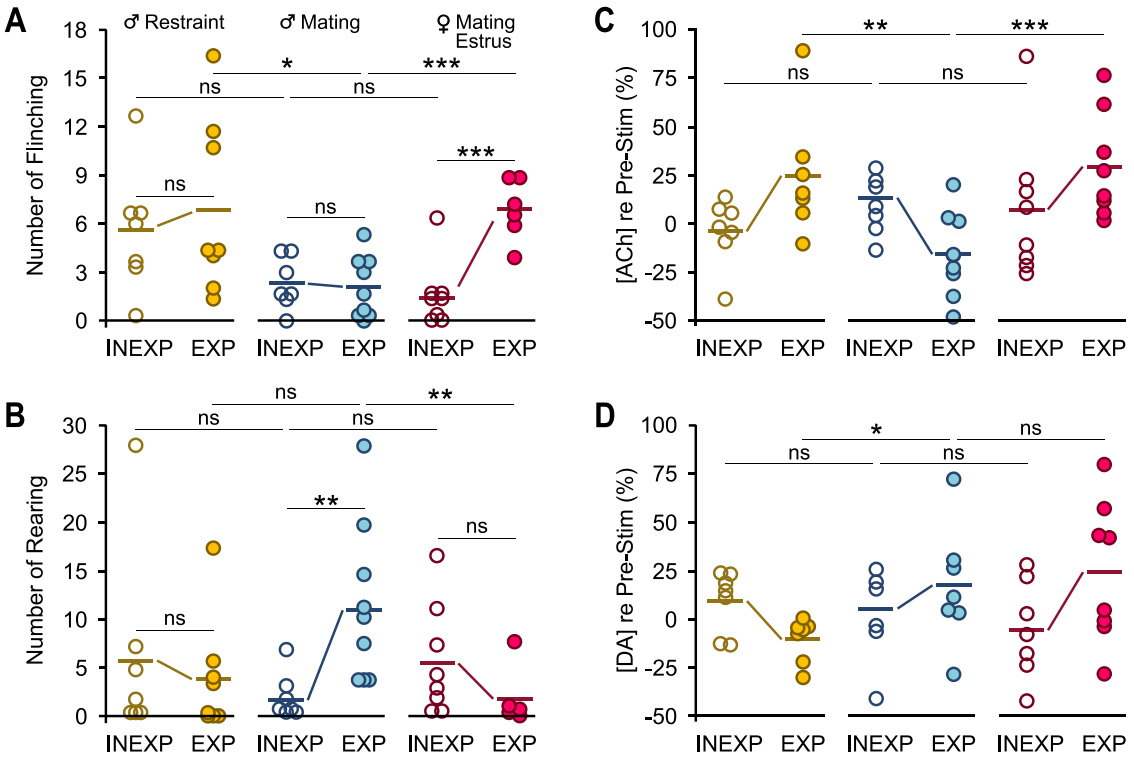


Figure 4. A single bout each of mating and restraint experience altered behaviors and enhanced neuromodulator release in response to vocal playback. In all graphs, dots represent measures from individual animals obtained during the first 10-min playback period; connected thick lines represent mean values across subjects. **A.** Experience increased flinching responses in estrus female mice, but not in males in mating or restraint vocal playback (Females: $n_{EXP} = 6$, $n_{INEXP} = 8$, t=5.0, p=0.000; Male-mating: $n_{EXP} = 8$, $n_{INEXP} = 7$, t=0.4, p=0.7; Male-restraint: $n_{EXP} = 7$, $n_{INEXP} = 7$, t=0.6, p=0.6). **B.** Experience increased rearing responses in males exposed to mating playback (Male-mating: $n_{EXP} = 8$, $n_{INEXP} = 7$, t=3.2, p=0.007), but not in females (t=1.4, p=0.2) or males in restraint playback (t=0.4, p=0.7). **C.** EXP mice displayed consistent estrus-(estrous female vs male: t=4.5, p=0.000) and context-dependent (mating vs restraint: t=3.2, p=0.006) changes in Ach. Such changes were not observed in INEXP groups during playback period (estrous female vs male: t= 0.1 , p=0.9; mating vs restraint: t=1.6, p=0.1). **D**. Context dependent increase of DA in EXP mice during mating playback (estrous female vs male: t=0.5, p=0.6, mating vs restraint: t=2.5, p=0.02) was absent in INEXP animals (estrous female vs male: t=0.7, p=0.5, mating vs restraint: t=0.5, p=0.6). A-D. GLM repeated measures with Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001.

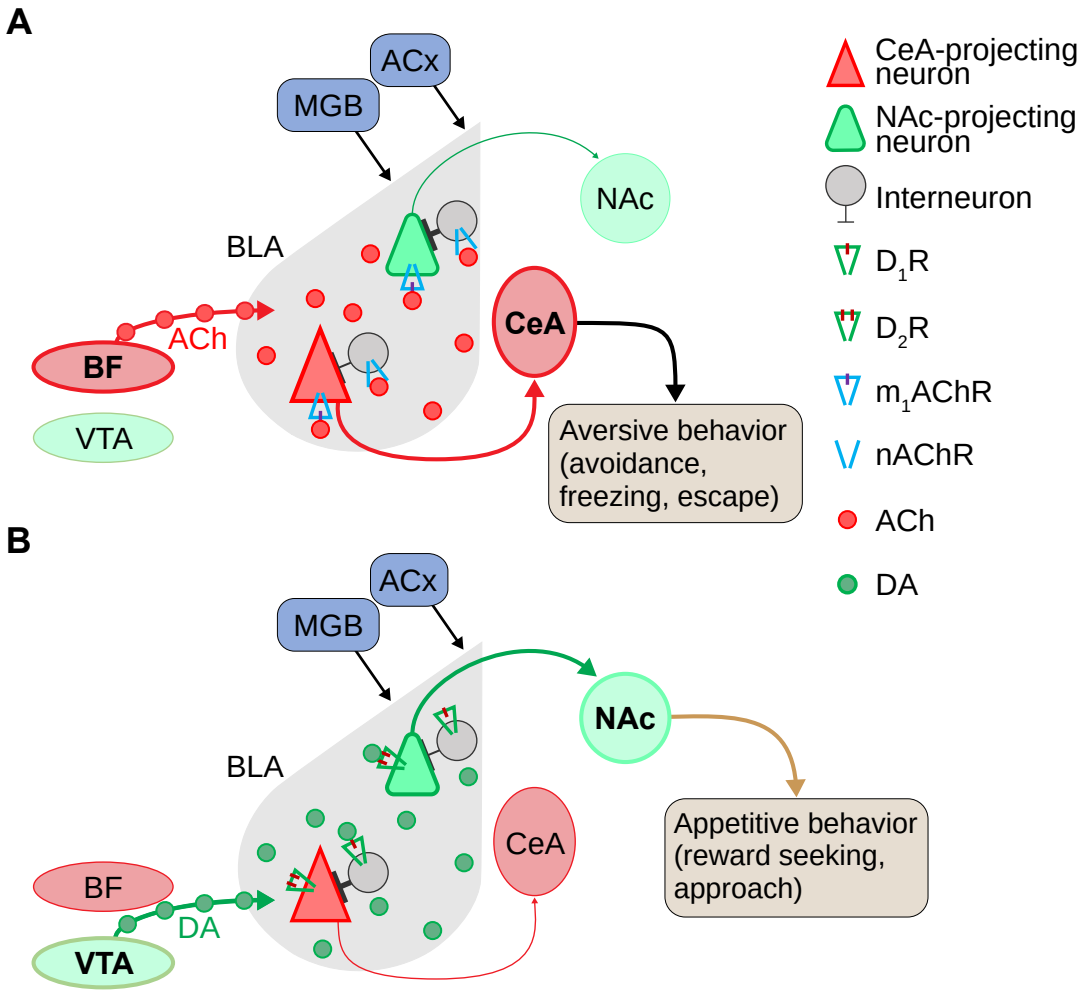
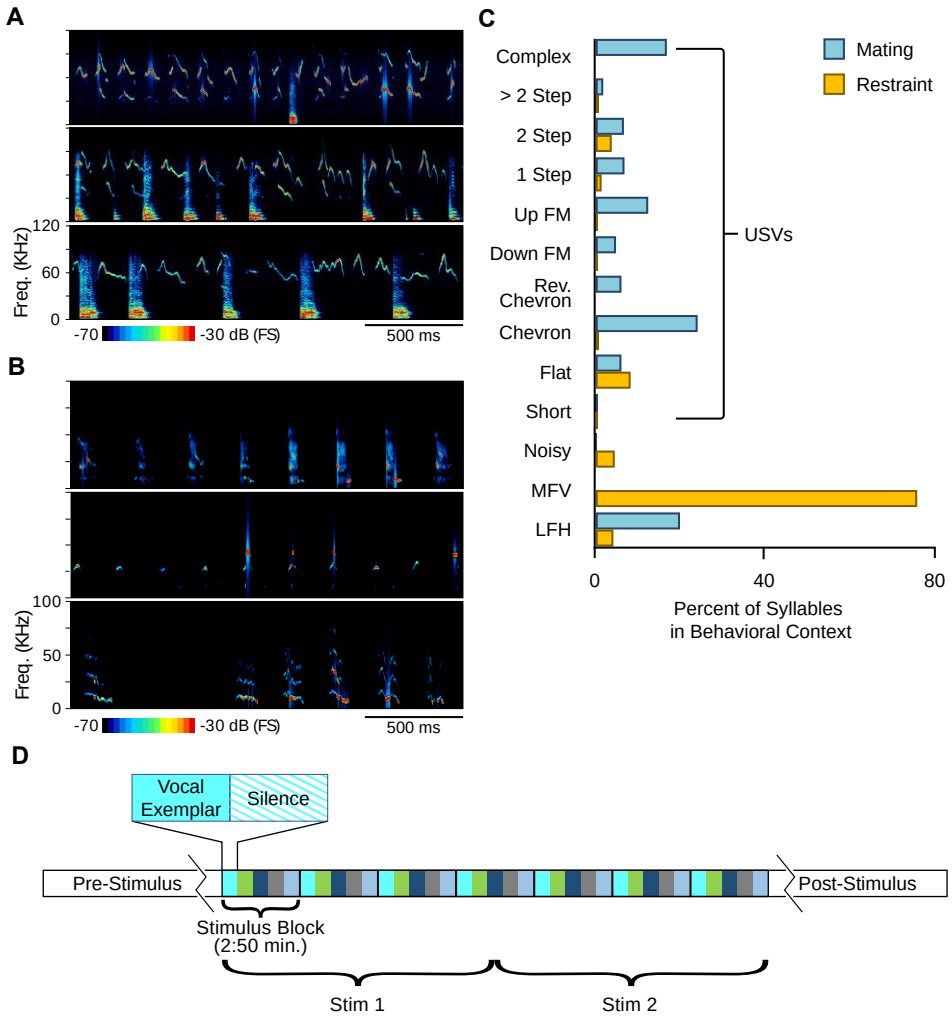
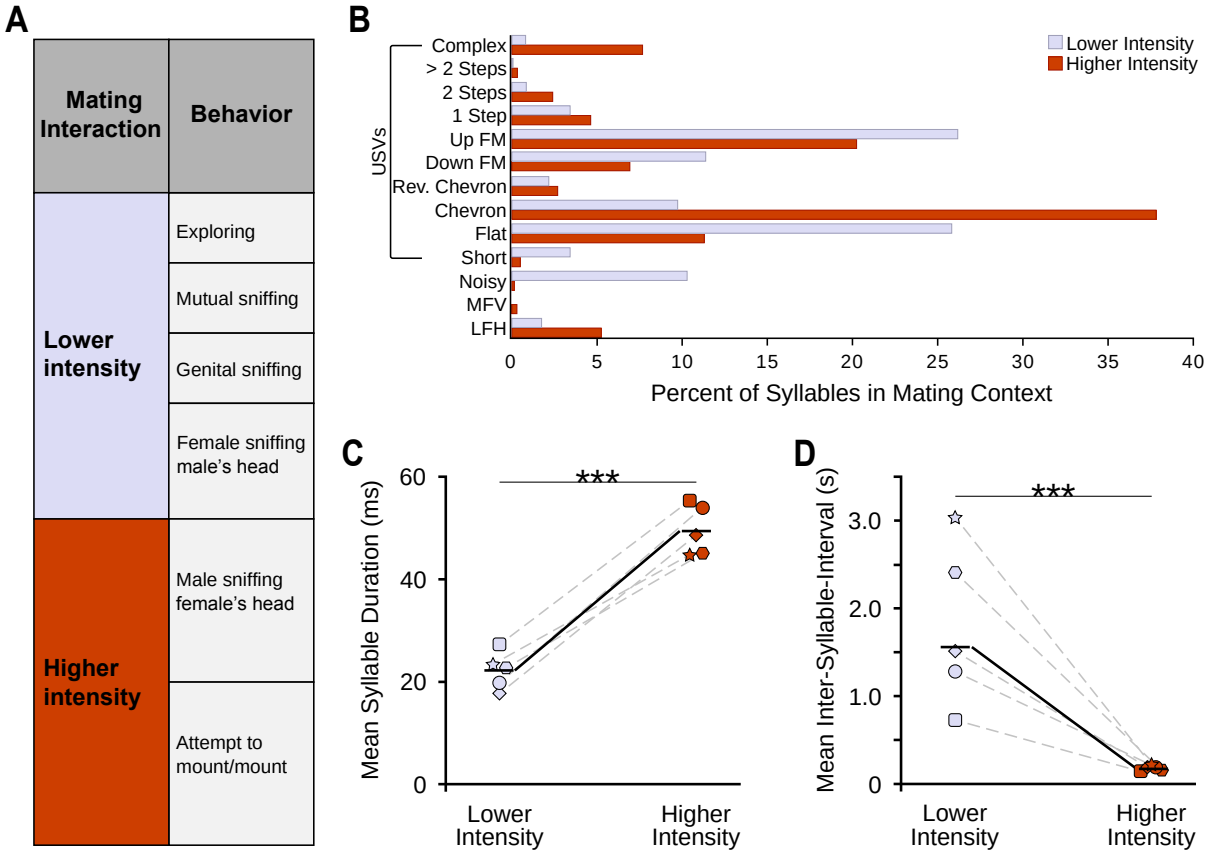


Figure 5. Proposed model for neuromodulation of salient vocalization processing via acetylcholine (ACh) and dopamine (DA) in the basolateral amygdala (BLA). A. Cholinergic modulation of CeA-projecting neurons during aversive vocalization cue processing in the BLA. In the presence of aversive cues, ACh released from the basal forebrain acts on m1 ACh receptors (m1AChRs) to enhance the cue-induced excitatory responses of CeA -projecting neurons. In contrast, NAc-projecting neurons are quiescent, because they do not respond to aversive cues and are inhibited by interneurons that are activated through nicotinic ACh receptors (nAChRs). **B.** Dopaminergic modulation and enhancement of signal-to-noise ratio in response to reward-associated cues (appetitive vocalizations). When vocalizations or other rewarding cues are present, release of DA from VTA enhances D2R-mediated excitation in NAc-projecting neurons that are responsive to positive cues. In contrast, CeA -projecting neurons are not responsive to rewarding vocalizations and are inhibited by local interneurons. DA is thought to act on D1 DA receptors (D1Rs) in these local interneurons to shape a direct inhibition onto CeA-projecting neurons. Abbreviations: ACx: auditory cortex; BF, basal forebrain; CeA, central nucleus of amygdala; MGB, medial geniculate body; NAc, nucleus accumbens; VTA, ventral tegmental area.

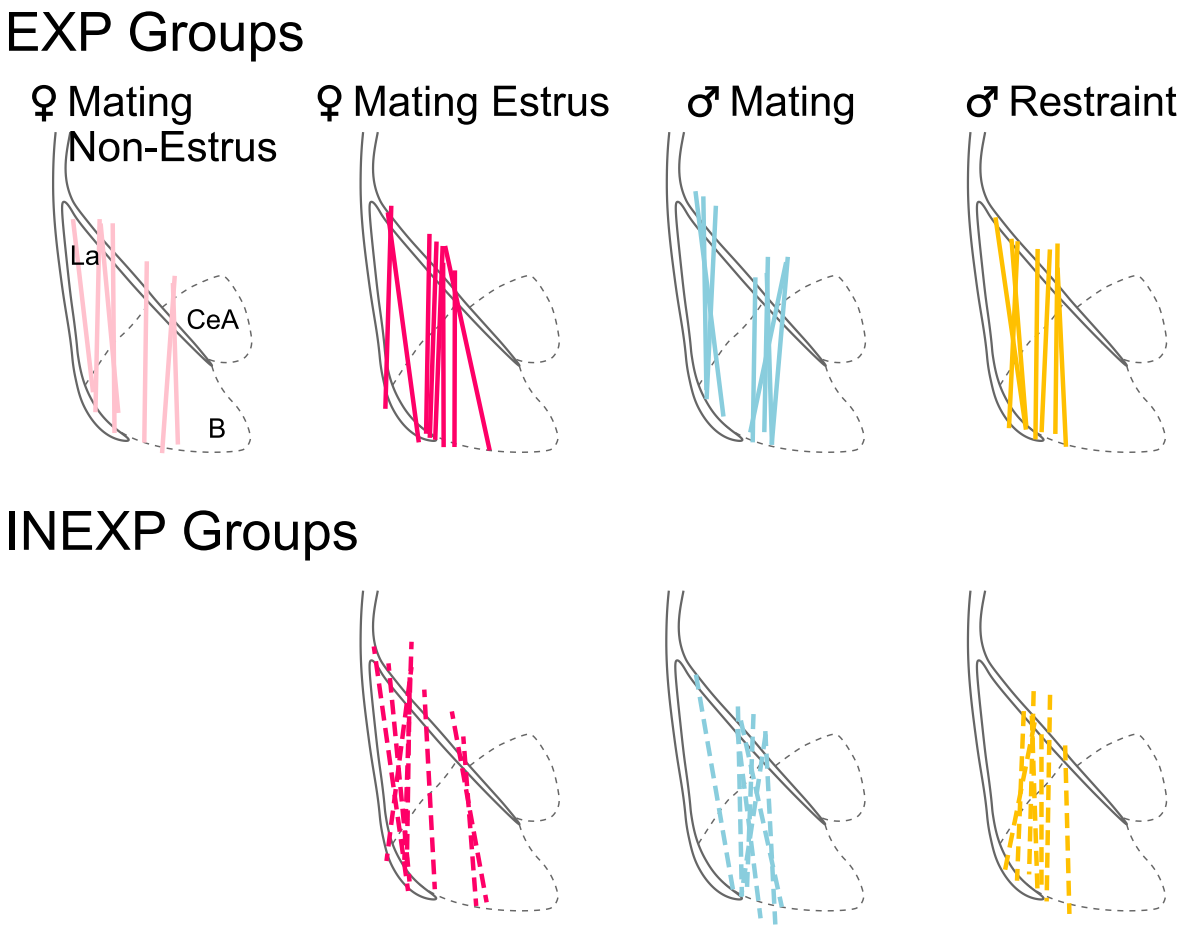
Supplemental Figure Captions



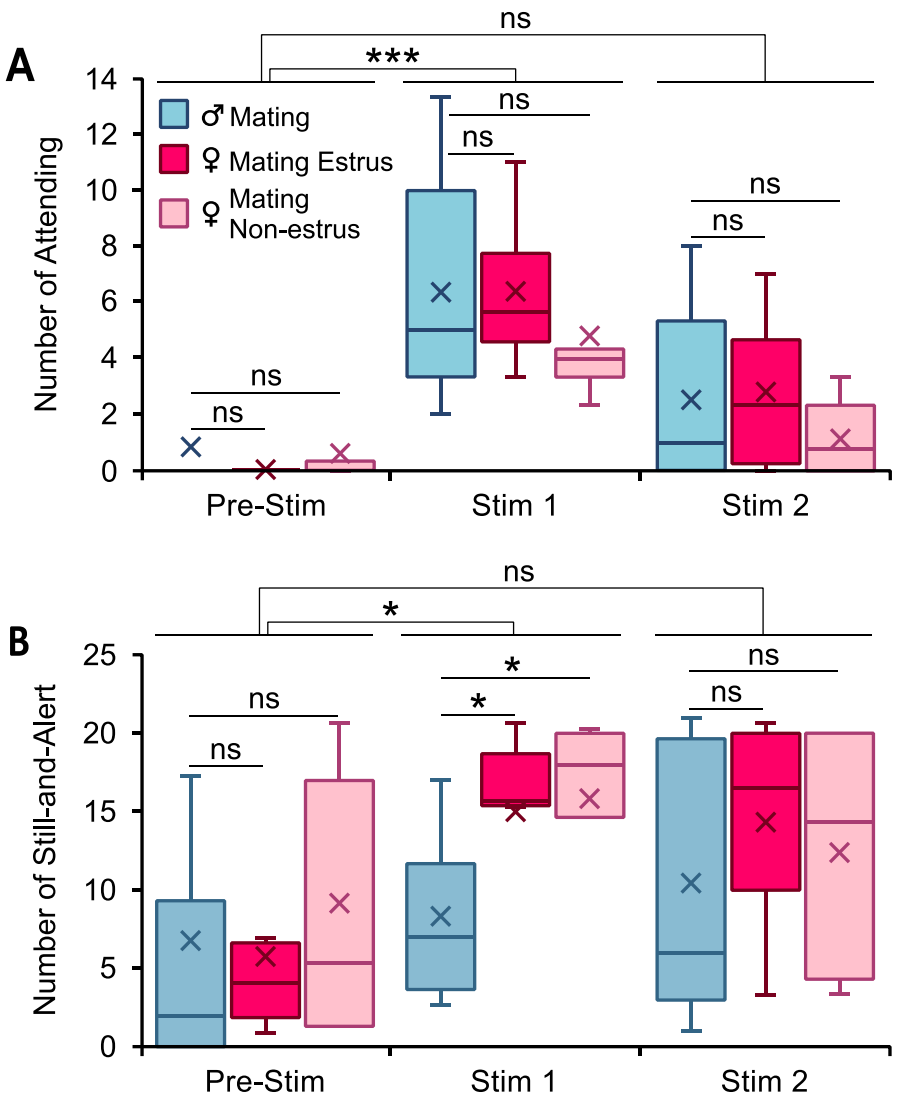
Supplemental Figure 1. Features of vocal stimuli in playback experiments. A. Two-second segments from three different vocal sequence exemplars in mating illustrate common syllable types and sequencing. **B.** Two-second segments from three different vocal sequence exemplars in restraint illustrate common syllable types and sequencing. **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.** Detailed sequencing of vocal stimuli, shown here for mating playback. A 20-min period of vocal playback was formed by seven repeated stimulus blocks of 170 s. The stimulus blocks were composed of five vocal exemplars of variable length, with each exemplar followed by an equal duration of silence. Stimuli during the Stim1 and Stim 2 playback windows thus included identical blocks but in slightly altered patterns.



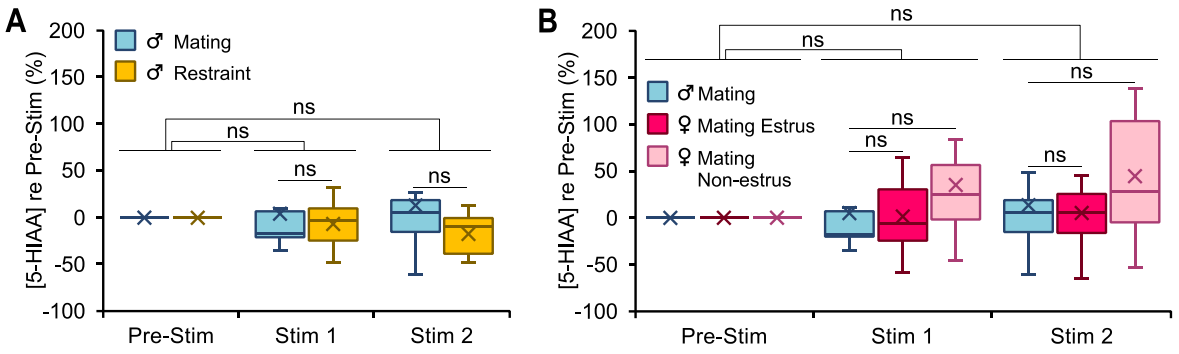
Supplemental Figure 2. Features of mating vocalizations differ based on interaction intensity. **A.** Behaviors associated with lower and higher intensity of mating, respectively. **B.** Percentage of different syllable types associated with lower and higher intensity mating interactions (n_{pairs} =5; recording duration, 30 min each). **C.** The mean duration of syllables is significantly higher when emitted during higher intensity mating interactions. Each line represents one mating pair (n_{LI} = 3107, n_{HI} = 3093 syllables, F (12, 6168.1) = 289.7, p=0.000, linear mixed model). **D.** The mean inter-syllable interval is significantly lower when emitted during higher intensity mating interactions. Each line represents one mating pair (n_{LI} = 2944, n_{HI} = 2920 syllables, F (12,5826.3) =3.31, p<0.001, linear mixed model). *p<0.05, **p<0.01, ***p<0.001.



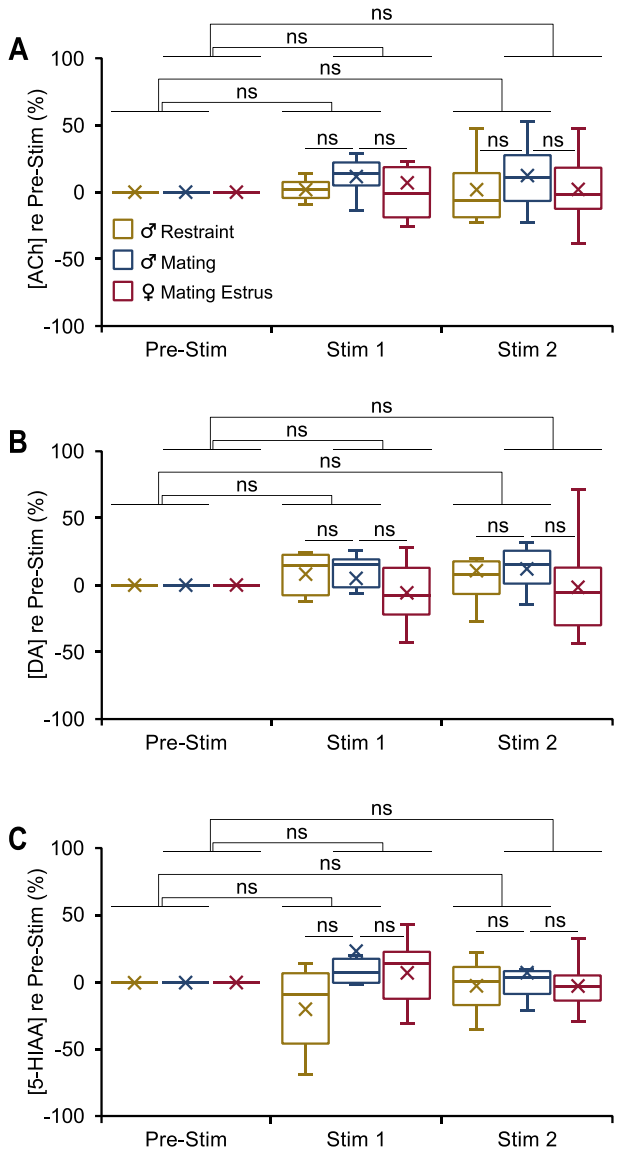
Supplemental Figure 3. Microdialysis probe locations for EXP and INEXP Groups. Colored lines indicate recovered probe tracks that resulted from infusion of fluorescent tracers. Black solid lines indicate external capsule; black dashed lines indicate major amygdalar subdivisions. Abbreviations: *B,* basal nucleus of amygdala; *CeA*, central nucleus of amygdala; *La*, lateral nucleus of amygdala.



Supplemental Figure 4. Behavioral changes during mating vocal playback in EXP male and female mice. Boxplots show number of occurrences of the specified behavior in 10-min periods before (Pre-Stim) and during (Stim 1, Stim 2) vocal sequence playback in EXP mice. **A.** Attending behavior increased in response to mating vocal playback, regardless of sex or estrus stage (time: $F(2,30) = 32.6$; $p<0.001$, $\eta 2=0.7$; time*sex: $F(2,30) = 0.12$; $p=0.9$, $\eta 2=0.008$ time*estrus: $F(2,30) = 1.1$; $p=0.4$, $\eta 2=0.07$). **B.** Still-and-Alert behavior increased in response to mating vocalizations for female but not male mice (time: $F(2,30) = 4.8$; $p=0.01$, $\eta 2=0.24$; Sex: $F(1,15) =5.17$, $p=0.04$, partial $\eta 2= 0.3$, estrus: $(1,15) =0.07$, $p=0.8$, partial $\eta 2= 0.005$). GLM repeated measures with Bonferroni post hoc test, *$p<0.05$, ***$p<0.001$.



Supplemental Figure 5. Concentration of serotonin metabolite 5-HIAA in EXP animals showed no significant context-, sex- or estrous-dependent changes with vocal stimulus playback. Boxplots show change in concentration of 5-HIAA in 10-min periods from the baseline level (Pre-Stim) during (Stim 1, Stim 2) vocal sequence playback. **A.** Comparison of mating and restraint groups of male mice (n_{Mating}=9, $n_{Restraint}$=7; time: F (2,28) =0.02, p=0.9, η^2 =0.001; time*context: F (2,28) = 0.97, p=0.4, η^2 =0.06). **B.** Comparison of male and female mice during mating sequence playback (n_{Male} = 9, $n_{Estrous\ Fem}$ = 8, $n_{Non\text{-}estrous\ Fem}$ = 7, time: F (2,42) =1.7, p=0.2, η^2 =0.07; time*sex: F (2,42) = 0.2, p=0.8, η^2 =0.01; time*estrus: F (2,42) =1.2, p=0.3, η^2 =0.05). GLM repeated measures with Bonferroni post hoc test.



Supplemental Figure 6. In INEXP mice, vocal playback failed to evoke significant differences in neuromodulator release between experimental groups. Boxplots show change in concentration of the specified neuromodulator in 10-min periods compared to the baseline level (Pre-Stim) during (Stim 1, Stim 2) vocal sequence playback**. A.** ACh release shown for both playback periods (mating vs restraint: time: F (2,24) =0.14; p=0.9; time*context: F (2,24) =0.56; p=0.6; male vs female: time: F (2,24) =0.9; p=0.4; time*sex**:** F (2,24) =0.01; p=0.9). **B.** DA release shown for both playback periods (mating vs restraint: time: F (2,24) =1.2; p=0.3; time*context**:** F (2,24) = 0.2; p=0.8; male vs female: time: F (2,24) =0.4; p=0.7; time*sex: F (2,24) = 0.4; p=0.7). **C.** 5-HIAA concentration shown for both playback periods (mating vs restraint: time: F (1.3,15.6) =0.005, p=0.9; time*context: F (1.3,15.6) = 2.1; p=0.17; male vs female: time: F (1.3,15.7) =0.9; p=0.4; time* sex: F (1.3,15.7) = 0.3; p=0.7). GLM repeated measure with Bonferroni post hoc test, n_{INEXP} = 21.