1 **Acetylcholine is released in the basolateral amygdala in response to predictors of**

2 **reward and enhances learning of cue-reward contingency**

3 Richard B. Crouse[1,2], Kristen Kim[1,2], Hannah M. Batchelor[1,2], Eric M. Girardi[1], Rufina

4 Kamaletdinova[1,3], Justin Chan[1], Prithviraj Rajebhosale[4,5], Steven T. Pittenger[1], Lorna

5 W. Role[5], David A. Talmage[6], Miao Jing[7], Yulong Li[8-10], Xiao-Bing Gao[11], Yann S.

6 Mineur[1], Marina R. Picciotto[1,2,*]

7 [1]Department of Psychiatry,

8 Yale University, 34 Park Street, 3rd Floor Research, New Haven, CT 06508, USA

9 [2]Yale Interdepartmental Neuroscience Program, New Haven, CT

10 [3]City University of New York, Hunter College, New York, NY

11 [4]Program in Neuroscience, Stony Brook University, NY

12 [5]National Institute of Neurological Disorders & Stroke (NINDS), Bethesda MD

13 [6]National Institute of Mental Health (NIMH), Bethesda MD

14 [7]Chinese Institute for Brain Research (CIBR), Beijing, China

15 [8]State Key Laboratory of Membrane Biology, Peking University School of Life Sciences,

16 Beijing, China.

17 [9]PKU-IDG/McGovern Institute for Brain Research, Beijing, China.

18 [10]Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary

19 Studies,

20 Peking University, Beijing, China.

21 [11]Section of Comparative Medicine, Yale University School of Medicine New Haven, CT

22

23 * To whom correspondence should be addressed

24   Marina R. Picciotto

25   Dept. of Psychiatry, Yale University School of Medicine

26   34 Park Street – 3rd floor research

27   New Haven, CT 06508

28   Phone: 203-737-2041; Fax: 203-737-2043; email: marina.picciotto@yale.edu

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30   **Running title**: BLA ACh enhances reward learning

Abstract

The basolateral amygdala (BLA) is critical for associating initially neutral cues with appetitive and aversive stimuli and receives dense neuromodulatory acetylcholine (ACh) projections. We measured BLA ACh signaling and activity of neurons expressing CaMKIIα (a marker for glutamatergic principal cells) in mice during cue-reward learning using a fluorescent ACh sensor and calcium indicators. We found that ACh levels and nucleus basalis of Meynert (NBM) cholinergic terminal activity in the BLA (NBM-BLA) increased sharply in response to reward-related events and shifted as mice learned the cue-reward contingency. BLA CaMKIIα neuron activity followed reward retrieval and moved to the reward-predictive cue after task acquisition. Optical stimulation of cholinergic NBM-BLA terminal fibers led to quicker acquisition of the cue-reward contingency. These results indicate BLA ACh signaling carries important information about salient events in cue-reward learning and provides a framework for understanding how ACh signaling contributes to shaping BLA responses to emotional stimuli.

Introduction

Learning how environmental stimuli predict the availability of food and other natural rewards is critical for survival. The basolateral amygdala (BLA) is a brain area necessary for associating cues with both positive and negative valence outcomes (Baxter & Murray, 2002; Janak & Tye, 2015; LeDoux et al., 1990). Recent work has shown that genetically distinct subsets of BLA principal neurons encode the appetitive and aversive value of stimuli (J. Kim et al., 2016). This encoding involves the interplay between principal neurons, interneurons, and incoming terminal fibers, all of which need to be tightly regulated to function efficiently.

The neuromodulator acetylcholine (ACh) is released throughout the brain and can control neuronal activity via a wide range of mechanisms. ACh signals through two families of receptors (nicotinic, nAChRs and muscarinic, mAChRs) that are differentially expressed on BLA neurons as well as their afferents (Picciotto et al., 2012). ACh signals through these receptors to increase signal-to-noise ratios and modify synaptic transmission and plasticity in circuits involved in learning new contingencies (Picciotto et al., 2012), especially in areas that receive dense cholinergic input, like the BLA (Woolf, 1991; Zaborszky et al., 2012). The effect of ACh signaling can differ depending on the receptor, as metabotropic mAChRs work on a slower timescale than the rapid, ionotropic nAChRs (Gu & Yakel, 2011; Picciotto et al., 2012). The overall impact of ACh signaling on the BLA is likely quite heterogeneous since mAChRs are coupled to both inhibitory and excitatory signaling cascades and nAChRs are found on both glutamatergic and GABAergic BLA neurons (Picciotto et al., 2012).

70   The basal forebrain complex is a primary source of ACh input to the BLA. In

71   particular, the nucleus basalis of Meynert (NBM) sends dense cholinergic projections to

72   the BLA (Woolf, 1991; Zaborszky et al., 2012). Optical stimulation of BLA-projecting

73   cholinergic terminal fibers (NBM-BLA) during fear conditioning is sufficient to strengthen

74   fear memories (Jiang et al., 2016) and may support appetitive behavior (Aitta-aho et al.,

75   2018). Cholinergic NBM neurons increase their firing in response to both rewarding and

76   aversive unconditioned stimuli (Hangya et al., 2015). Cholinergic signaling in the medial

77   prefrontal cortex and visual cortex has been linked to cue detection (Parikh et al., 2007)

78   and reward timing (Chubykin et al., 2013; Liu et al., 2015), respectively. A recent study

79   has also demonstrated that NBM cells fire in response to a conditioned stimulus during

80   trace fear conditioning, indicating that ACh signaling may be involved in learning about

81   cues that predict salient outcomes (Guo et al., 2019).

82   We hypothesized that ACh signaling in the BLA is a critical neuromodulatory

83   signal that responds to both unconditioned stimuli and cues that gain salience, thereby

84   coordinating activity in circuits necessary for learning cue-reward contingencies. To test

85   this hypothesis, we measured relative levels of BLA ACh (ACh signaling), cholinergic

86   NBM-BLA terminal fiber activity (BLA ACh signal origin), and the activity of BLA

87   principal neurons (BLA output) across all phases of learning in an appetitive operant

88   learning task to evaluate how BLA output and ACh signaling are related to behavioral

89   performance in this paradigm. We then optically stimulated cholinergic NBM fibers

90   locally in the BLA while mice learned to nose poke in response to an auditory cue to

91   receive a food reward to determine if accelerating the increase in ACh signaling that

92   occurs as mice learn the task would enhance performance. We also pharmacologically

93   blocked different ACh receptors during the learning task to determine the subtypes

94   involved, and varied the timing of optical stimulation of cholinergic NBM-BLA terminal

95   fibers to determine whether time-locked ACh release with the reward-predictive cue is

96   necessary for the improvement of the task performance. These studies provide a novel

97   framework for understanding how NBM ACh signaling in the BLA is recruited during

98   perception of novel stimuli and how it contributes to linking previously neutral cues to

99   predictions about future salient outcomes.

100

101   **Results**

102   **<u>Acetylcholine release in the BLA occurs at salient points in the cue-reward</u>**

103   **<u>learning task and shifts as mice learn the cue-reward contingency</u>**

104   The BLA is critical for learning that previously neutral cues can predict future

105   punishments or rewards and for assigning valence to those cues (Baxter & Murray,

106   2002; Janak & Tye, 2015). The BLA receives dense cholinergic input (Woolf, 1991;

107   Zaborszky et al., 2012) and we speculated that, since ACh signaling is involved in both

108   attention and several types of learning (Picciotto et al., 2012), it could be essential for

109   learning about cues that predict salient events, such as reward delivery. Based on data

110   showing that ACh neurons fire in response to unexpected or salient events (Hangya et

111   al., 2015), we also hypothesized that ACh release might vary as mice learn a cue-

112   reward contingency. Therefore, we designed a cue-reward learning task in which food-

113   restricted mice were trained to perform a nose poke when signaled by a cue (tone) to

114   receive a palatable reward (Ensure) on a 30 sec variable intertrial interval (ITI) (**Figure**

115   **1A-D**). We injected adeno-associated virus (AAV) carrying an improved version of the

116 fluorescent ACh sensor GRAB$_{ACh3.0}$ (ACh3.0; (Jing et al., 2018, 2019) construct into the

117 BLA of mice and implanted an optical fiber above the BLA to record ACh signaling

118 during the cue-reward learning task (**Figure 2A + Figure 2-figure supplement 1A**).

119   During the Pre-Training phase of the task, mice received reward and cue light

120 presentation for performing a nose poke in the active port during tone presentation

121 (**Figure 1C**, purple active nose poke coincident with tone) but there was no

122 consequence for an incorrect nose poke (**Figure 1C**, red active nose poke not

123 coincident with tone). Animals quickly learned to make a high number of responses over

124 the course of each Pre-Training session. In this paradigm, mice obtained most available

125 rewards by day 5 of Pre-Training (**Figure 2B,** blue shaded region). However, this phase

126 of training did not promote learning of the cue-reward contingency, (i.e. that they should

127 only nose poke during tone presentation) seen by the high number of incorrect nose

128 pokes (**Figure 2-figure supplement 2A**, blue shaded region). Mice performed roughly

129 8-fold more incorrect nose pokes than correct nose pokes, suggesting that mice were

130 not attending to the task contingency. The Training phase of the task was identical to

131 Pre-Training except incorrect nose pokes resulted in a 5 sec timeout, during which the

132 house light was illuminated, that concluded with a restarting of the ITI timer (**Figure 1D**,

133 red active nose poke not coincident with tone). On day 1 of the Training phase, all

134 animals earned fewer rewards (**Figure 2B**, pink shading) and, while still high, incorrect

135 nose pokes dropped (**Figure 2-figure supplement 2A**, pink shading). Animals that did

136 not meet acquisition criterion by day 9 (defined as consistently earning 20 or more

137 rewards per session, **Figure 2B**, white horizontal line) were moved to a 20 sec variable

138 ITI to promote responding (**Figure 2B**, pink shading day 10). Following the change in

ITI, mice acquired the cue-reward behavior at different rates. After acquisition, animals

were switched to Extinction training in which correct nose pokes did not result in reward

delivery, and all mice decreased nose poke responding (**Figure 2B** + **Figure 2-figure**

supplement 2A, orange shading).

During Pre-Training, when there were high numbers of both correct and incorrect

nose pokes, there was a large increase in ACh release following correct nose pokes,

which were followed by reward delivery and cue light, but not incorrect nose pokes

(**Figure 2C + Figure 2-figure supplement 1 B-C**). We used bootstrapped confidence

intervals (bCI) to determine when transients were statistically significant (bCI did not

contain the null of 0 (Jean-Richard-dit-Bressel et al., 2020)). Correct, but not incorrect,

nose poke trials consistently showed a sustained, significant increase in fluorescence

close to the time of nose poke onset (**Figure 2C)**. We also observed a significant

decrease in fluorescence for most mice around 2-4 sec after correct nose poke, which

corresponds to the time of reward retrieval.

ACh release occurred in response to different events as mice learned the task

(data for individual mice are shown in **Figure 2D** + **Figure 2-figure supplement 1D-G**

and averaged data across all mice at key time points in the task is shown in **Figure 2E**

+ Figure 2-figure supplement 1H). During Pre-Training rewarded trials, the highest

levels of ACh release occurred close to the time of correct nose pokes (NP), with a

smaller peak at the time of reward retrieval (entry into the reward receptacle, Rec). As

Training began, the ACh release during reward trials shifted dramatically toward the

time of reward retrieval, likely because the animals were learning that many nose pokes

did not result in reward delivery. Incorrect nose pokes that triggered a timeout were also

162 followed by a modest but non-significant increase in BLA ACh levels (**Figure 2-figure**

163 **supplement 2B-H**). As mice began to learn the contingency (**Figure 2E + Figure 2-**

164 **figure supplement 1H**, 10 rewards), the peak ACh release during rewarded trials

165 shifted back to the time of the correct nose poke response. As animals approached the

166 acquisition criterion (**Figure 2E + Figure 2-figure supplement 1H**, Acq.), ACh level

167 significantly increased at the time of the tone, suggesting that as animals learned the

168 cue-reward contingency, the tone became a more salient event. At this time point, there

169 was still a peak at the time of reward, but its magnitude was diminished. After task

170 acquisition, the increase in ACh following correct nose pokes remained but was

171 diminished, and incorrect nose pokes did not elicit apparent ACh release (**Figure 2-**

172 **figure supplement 2C-H**, Acq.). During Extinction, ACh release to tone onset

173 diminished. We replicated this experiment in an independent cohort of mice and found

174 similar results (**Figure 2-figure supplement 3-4**). Mice in this replicate cohort learned

175 in a similar fashion (**Figure 2-figure supplement 3B + 4A**) but met the acquisition

176 criteria faster than initial mice because aspects of the behavioral setup were optimized

177 (3D printed wall extensions) to allow the imaging apparatus to be used inside sound

178 attenuating chambers (see Methods section). One difference observed in this group that

179 learned the task more rapidly, was small magnitude, but significant, increases in BLA

180 ACh release following tone onset late in Pre-Training (**Figure 2-figure supplement 3C-**

181 **I**). As behavioral performance during the Training phase increased, ACh release to tone

182 onset became more pronounced, as in the initial cohort.

183   In order to determine the source of the ACh released in the BLA during cue-

184 reward learning, we recorded calcium dynamics as a measure of cell activity of ChAT[+]

185 NBM terminal fibers in the BLA (NBM-BLA), since the NBM is a major source of

186 cholinergic input to the BLA (Jiang et al., 2016; Woolf, 1991; Zaborszky et al., 2012).

187 We injected AAV carrying a Cre-recombinase-dependent, genetically-encoded calcium

188 indicator (DIO-GCaMP7s) into the NBM of ChAT-IRES-Cre mice and implanted an

189 optical fiber above the ipsilateral BLA (**Figure 2F + Figure 2-figure supplement 5A-C)**.

190 As with the ACh3.0 sensor, there was a significant increase in NBM-BLA cholinergic

191 terminal activity following correct, but not incorrect, nose pokes (**Figure 2H + Figure 2-**

192 **figure supplement 5D-E**). NBM-BLA cholinergic terminal activity evolved across

193 phases of the reward learning task as was seen for ACh levels in the BLA (data for each

194 mouse shown in **Figure 2I + Figure 2-figure supplement 5F-G**, averaged across all

195 mice at key time points in the task shown in **Figure 2J + Figure 2-figure supplement**

196 **7G**). Strikingly, NBM-BLA cholinergic terminal activity most closely followed correct

197 nose pokes in Pre-Training and shifted primarily to tone onset as mice learned the

198 contingency during Training. As in the replication cohort for the ACh sensor, small

199 magnitude, but significant, increases in terminal activity were observed following tone

200 onset late in Pre-Training (**Figure 2J + Figure 2-figure supplement 7G**). Incorrect

201 nose pokes that resulted in a timeout in Training sessions were followed by a modest

202 increase in NBM-BLA cholinergic terminal activity before task acquisition (**Figure 2-**

203 **figure supplement 6B-E**). During Extinction, activity of NBM-BLA terminals following

204 tone onset diminished. These findings were replicated in an independent cohort of mice,

205 which we combined for across-mouse statistical analyses (**Figure 2-figure supplement**

206 **7-8**).

207   In order to record NBM-BLA cholinergic terminal activity and BLA ACh levels

208   simultaneously in the same mouse, we injected AAV carrying a construct for Cre-

209   recombinase dependent red-shifted genetically-encoded calcium indicator (DIO-

210   jRCaMP1b) into the NBM of ChAT-IRES-Cre mice, ACh3.0 sensor into the ipsilateral

211   BLA, and implanted a fiber above the BLA (**Figure 2-figure supplement 9A-E**, mouse

212   1). DIO-jRCaMP1b was also injected into the NBM of a wild type littermate so Cre-

213   mediated recombination would not occur to control for any crosstalk between the

214   ACh3.0 and jRCaMP1b channels. While this was only a single animal and proof of

215   principle for future studies, we found that NBM-BLA cholinergic terminal activity

216   coincided with ACh levels (**Figure 2-figure supplement 9F-G**). Importantly, this

217   relationship between ACh release and NBM-BLA terminal fiber activity was not

218   explained by signal crosstalk (**Figure 2-figure supplement 9H-I**), further indicating that

219   the BLA ACh measured comes at least in part from the NBM.

220

221   **BLA principal neurons respond to reward availability and follows cue-reward**

222   **learning**

223   Glutamatergic principal cells are the primary output neurons of the BLA (Janak &

224   Tye, 2015), and their firing is modulated by NBM-BLA cholinergic signaling (Jiang et al.,

225   2016; Unal et al., 2015). BLA principal neurons can increase their firing in response to

226   cues as animals learn cue-reward contingencies (Sanghera et al., 1979; Schoenbaum

227   et al., 1998; Tye & Janak, 2007). Calcium/calmodulin-dependent protein kinase

228   (CaMKII) has been shown to be a marker for glutamatergic BLA principal cells (Butler et

229   al., 2011; Felix-Ortiz & Tye, 2014; McDonald, 1992; Tye et al., 2011). To determine

11

230 whether ACh modulates principal neuron activity during cue-reward learning, we

231 injected AAV carrying a Cre-recombinase dependent genetically encoded calcium

232 indicator (DIO-GCaMP6s) into the BLA of CaMKIIα-Cre mice to record BLA principal cell

233 activity during the learning task (**Figure 3A + Figure 3-figure supplement 1A**). As was

234 seen for BLA ACh levels, there was a significant increase in BLA CaMKIIα cell activity

235 following correct and a modest, but not significant, decrease in activity following

236 incorrect nose pokes on the last day of Pre-Training (**Figure 3B**). However, the activity

237 peaked later after the correct nose poke response (~2.5 sec) compared to the ACh3.0

238 signal (~0.5 sec) and appeared to align more tightly with reward retrieval (**Figure 3-**

239 **figure supplement 1B**). As mice learned the task (**Figure 3C + Figure 3-figure**

240 **supplement 2A**), BLA CaMKIIα cell activity increased first in response to reward and,

241 after acquisition of the task, to the reward-predictive cue (individual data for each mouse

242 shown in **Figure 3D + Figure 3-figure supplement 1E-F,** and averaged data across all

243 mice at key time points in task is shown in **Figure 3E** + **Figure 3-figure supplement**

244 **1G-H**).

245 During Pre-Training, the highest levels of BLA CaMKIIα cell activity followed

246 reward retrieval. In addition, during the first few days of Training, BLA CaMKIIα cell

247 activity after reward retrieval was higher than it was during Pre-Training, and the

248 magnitude of response decreased as mice learned the contingency and earned more

249 rewards, ultimately reaching similar intensity to that observed during Pre-Training.

250 Concurrently, as mice approached acquisition of the task (**Figure 3C**, white horizontal

251 line), BLA CaMKIIα cell activity significantly increased in response to tone onset (**Figure**

252 **3D-E + Figure 3-figure supplement 1E-H**, Acq.), suggesting that the recruitment of

253   BLA CaMKIIα cell activity likely reflects the association of the cue with a salient outcome

254   (Lutas et al., 2019; Sengupta et al., 2018). Incorrect nose pokes that triggered a timeout

255   did not elicit a different response in CaMKIIα cell activity compared to before timeouts

256   were incorporated (**Figure 3-figure supplement 2B-G**). In an independent cohort of

257   mice, those with more posterior fiber tip placements (mice 4 + 7) replicated the primary

258   findings (**Figure 3-figure supplement 3-4**).

259

260   **<u>Stimulation of cholinergic terminals in BLA improves cue-reward learning</u>**

261   Since ACh released by NBM-BLA terminals during Training shifted to tone onset

262   during acquisition of cue-reward learning **(Figure 2E, J)**, we hypothesized that ACh may

263   potentiate learning the cue-reward contingency. We therefore tested whether increasing

264   ACh release in BLA during learning could alter cue-reward learning by injecting AAV

265   carrying a Cre-recombinase-dependent channelrhodopsin-EYFP (AAV-DIO-ChR2-

266   EYFP) construct bilaterally into the NBM of ChAT-IRES-Cre transgenic mice and

267   placing fibers over the BLAs to optically stimulate cholinergic terminals originating from

268   the NBM selectively (**Figure 4A + Figure 4-figure supplement 1**). Optical control over

269   ChAT^{+} NBM cells was verified by *ex vivo* slice recordings, depolarizations followed light

270   flashes and clear action potentials were observed *ex vivo* (**Figure 4B + Figure 4-figure**

271   **supplement 2**). After operant familiarization, ChAT+ NBM-BLA terminals were

272   stimulated via bilateral optical fibers (2 sec, 20 Hz, 25 ms pulses) triggered by a correct

273   nose poke throughout both Pre-Training (**Figure 4C**) and Training (**Figure 4D**).

274   Stimulation usually occurred during at least a portion of all three components of a

275   rewarded trial: tone, correct nose poke, and reward retrieval, since these events were

276   often separated by short latencies.

277   As seen in previous experiments, during the Pre-Training phase animals made a

278   high number of nose poke responses over the course of each session, obtained most

279   available rewards by the last day (**Figure 4E + Figure 4-figure supplement 3A**, blue

280   shading), and committed a very high number of incorrect nose pokes (**Figure 4F +**

281   **Figure 4-figure supplement 3B**, blue shading). There were no differences in rewards

282   earned (main effect of group (EYFP vs. ChR2) in a two-way repeated-measures

283   ANOVA, $F_{(1, 9)} = 1.733$, $p = 0.2205$) or incorrect nose pokes (main effect of group

284   (EYFP vs. ChR2) in a two-way repeated-measures ANOVA, $F_{(1, 9)} = 0.002433$, $p =$

285   0.9617) between the EYFP control (n = 5) and ChR2 (n = 6) groups during the Pre-

286   Training phase (**Figure 4E-F + Figure 4-figure supplement 3A-B**, blue shading),

287   suggesting that increasing BLA ACh signaling was not sufficient to modify behavior

288   during the Pre-Training phase of the task.

289   On Day 1 of the Training phase, all animals earned fewer rewards (**Figure 4E +**

290   **Figure 4-figure supplement 3A**, pink shading) and incorrect nose pokes remained

291   high (**Figure 4F + Figure 4-figure supplement 3B**, pink shading). As the animals

292   learned that a nose poke occurring outside of the cued period resulted in a timeout, both

293   control EYFP and ChR2 groups learned the contingency and improved their

294   performance, resulting in acquisition of the cue-reward task (20 rewards earned).

295   However, significant group differences emerged, such that ChR2 mice earned

296   significantly more rewards than EYFP controls (**Figure 4E + Figure 4-figure**

297   **supplement 3A**, pink shaded; main effect of group (EYFP vs. ChR2) in a two-way

298   repeated-measures ANOVA, F (1, 9) = 9.434, p = 0.0133), and there was a significant

299   Day x Group (EYFP vs. ChR2) interaction (two-way repeated-measures ANOVA, F (11,

300   99) = 3.210, p = 0.0009). ChR2 mice also made significantly fewer incorrect nose pokes

301   than control mice (**Figure 4F + Figure 4-figure supplement 3B**, pink shaded; two-way

302   repeated-measures ANOVA, F (1, 9) = 12.67, p = 0.0061), suggesting that the ChR2

303   group learned the tone-reward contingency more quickly than the EYFP group. EYFP

304   mice were able to reach the same peak cue-reward performance as the ChR2 group

305   only after 4-6 additional days of training. Once peak performance was achieved, there

306   was no difference in extinction learning between the groups (main effect of group (EYFP

307   vs. ChR2) in a two-way repeated-measures ANOVA, F (1, 9) = 2.293, p = 0.1643).

308   While sex differences in the behavior were not formally tested side by side, an

309   independent cohort of male mice (EYFP n = 7, ChR2 n = 7, **Figure 4-figure**

310   **supplement 4**) was tested to determine whether both male and female mice would

311   respond to ACh stimulation, revealing similar trends during Training for rewards earned

312   (**Figure 4-figure supplement 3C,E**, pink shaded; two-way repeated-measures ANOVA,

313   Group main effect (EYFP vs. ChR2): F (1, 12) = 3.636, p = 0.0808, Day x Group

314   interaction: F (11, 132) = 3.033, p = 0.0012) and incorrect nose pokes (**Figure 4-figure**

315   **supplement 3D,F**, red shaded; two-way repeated-measures ANOVA, Group main

316   effect (EYFP vs. ChR2): F (1, 12) = 4.925, p = 0.0465).

317   In order to determine if optical stimulation of NBM-BLA cholinergic terminals

318   improved performance in the task by increasing the rewarding value of the outcome,

319   rather than enhancing cue-reward learning by some other means, we allowed mice to

320   nose poke for optical stimulation rather than for Ensure (**Figure 4-figure supplement**

321   **5A**). There were no differences between the EYFP control and ChR2 groups (two-way

322   repeated-measures ANOVA, F (1, 9) = 0.6653, p = 0.4357). We also tested whether

323   NBM-BLA cholinergic terminal activation was reinforcing on its own by stimulating these

324   terminals in a real-time place preference test. Mice were allowed to explore two similar

325   compartments to determine baseline preference, and NBM-BLA cholinergic terminals

326   were then stimulated in one of the two chambers to determine whether it increased time

327   spent in the simulation-paired chamber. There was no difference between groups

328   (**Figure 4-figure supplement 5B**, main effect of group (EYFP vs. ChR2) in a two-way

329   repeated-measures ANOVA, F (1, 9) = 0.1311, p = 0.7257) in place preference,

330   confirming that optical activation of NBM-BLA cholinergic terminals is not innately

331   rewarding. Stimulation of NBM-BLA cholinergic terminals also did not lead to changes in

332   nose poke behavior in an uncued progressive ratio task (**Figure 4-figure supplement**

333   **5C**, main effect of group (EYFP vs. ChR2) in a two-way repeated-measures ANOVA, F

334   (1, 12) = 0.0009814, p = 0.975). Locomotor behavior was also not significantly affected

335   by NBM-BLA cholinergic terminal activation (**Figure 4-figure supplement 5D**, two-way

336   repeated-measures ANOVA, F (1, 9) = 0.05804, p = 0.8150.) Finally, to determine

337   whether there was any effect of NBM-BLA cholinergic terminal stimulation on

338   preference for, or avoidance of, a stressful environment, mice were tested for changes

339   in time spent in the dark or light side due to laser stimulation in the Light/Dark Box test,

340   and there were no differences between the groups (**Figure 4-figure supplement 5E-F**,

341   unpaired t-tests, number of crosses: p = 0.3223; time in light side: p = 0.1565).

342

Muscarinic, but not nicotinic, receptors are required for acquisition of the cue-reward contingency

ACh signals through multiple receptor subtypes, with rapid, ionotropic signaling mediated through stimulation of nAChRs, and metabotropic signaling mediated through stimulation of mAChRs (Picciotto et al., 2012). To determine which ACh receptors were involved in this cue-reward learning task, mice were injected intraperitoneally with saline (n = 8), mecamylamine (non-competitive nicotinic antagonist, Mec, n = 9), scopolamine (competitive muscarinic antagonist, Scop, n = 8), or a combination of both antagonists (Mec+Scop, n = 9) 30 min prior to Pre-Training and Training, during the same epochs of the task in which optical stimulation was administered (**Figure 5A**). Like optical stimulation, blockade of ACh receptors during the Pre-Training phase of the task had no effect on rewards earned (**Figure 5B + Figure 5-figure supplement 1A**, blue shading, main effect of Group (antagonist) in a two-way repeated-measures ANOVA, $F_{(3, 30)}$ = 1.285, P=0.2973) or on the large number of incorrect nose pokes (**Figure 5C + Figure 5-figure supplement 1B**, blue shading, main effect of Group (antagonist) in a two-way repeated-measures ANOVA, $F_{(3, 30)}$ = 1.496, p = 0.2356). In contrast, blockade of muscarinic signaling abolished the ability of mice to learn the correct cue-reward contingency during the Training period (**Figure 5B + Figure 5-figure supplement 1A**, pink shading, two-way repeated-measures ANOVA, Antagonist main effect: $F_{(3, 30)}$ = 23.13, p < 0.0001, Day x Antagonist interaction: $F_{(33, 330)}$ = 10.79, p < 0.0001), with these mice maintaining high levels of incorrect nose pokes for the duration of Training compared to Saline and Mec treated mice (**Figure 5C + Figure 5-figure supplement 1B**, pink shading, main effect of Group (antagonist) in a two-way repeated-measures

366   ANOVA, F (3, 30) = 25.64, p < 0.0001). Saline and Mec groups were not significantly

367   different in any phase of the task, including across Extinction (**Figure 5B-C + Figure 5-**

368   **figure supplement 1A-B**, orange shading, main effect of Group (antagonist) in a two-

369   way repeated-measures ANOVA, F (1, 15) = 1.201, p = 0.2903). We have shown that

370   this dose of mecamylamine delivered i.p. has significant effects in tests of anxiety-like

371   behavior and responses to inescapable stress. In addition, chronic treatment with

372   mecamylamine at this dose is sufficient to decrease BLA c-fos immunoreactivity (Mineur

373   et al., 2007). Consistent with the inability to acquire the cue-reward contingency, mice

374   treated with Scop or Mec+Scop also obtained very few rewards during Extinction

375   (**Figure 5B + Figure 5-figure supplement 1A**, orange shading). The antagonists had

376   no effect on locomotion as measured by beam breaks (**Figure 5-figure supplement**

377   **1C**) one-way ANOVA, F (3, 30) = 0.5074, p = 0.6802).

378

379   **ACh-mediated accelerated cue-reward learning does not require contingent**

380   **stimulation of ChAT$^+$ NBM terminals in the BLA**

381   Acetylcholine is often thought of as a neuromodulator (Picciotto et al., 2012), and

382   the window for cholinergic effects on synaptic plasticity varies across ACh receptor

383   subtypes (Gu & Yakel, 2011). It is therefore possible that ACh signaling may result in

384   intracellular signaling changes that outlast the cue presentation window. In order to

385   determine if the effect of NBM-BLA stimulation is dependent upon the timing of correct

386   nose poke and laser stimulation contingency, we repeated the experiment in an

387   independent cohort of mice with an additional non-contingent ChR2 group that received

388   the same number of stimulation trains as the contingent ChR2 group, but in which light

389   stimulation was explicitly unpaired with task events (**Figure 6A + Figure 6-figure**

390   **supplement 1**). As in the previous experiment, there were no differences between the

391   EYFP control (n = 6) and stimulation groups (contingent-ChR2 n = 5 and non-contingent

392   ChR2 n = 5) during Pre-Training (**Figure 6B-C + Figure 6-figure supplement 2A-B**,

393   blue shading; main effect of group (EYFP vs. contingent-ChR2 vs. non-contingent

394   ChR2) two-way repeated-measures ANOVAs; rewards earned: $F_{(2, 13)} = 0.7008$, p =

395   0.5140; incorrect nose pokes: $F_{(2, 13)} = 0.3906$, p = 0.6843). However, the non-

396   contingent ChR2 group was not significantly different from the contingent ChR2 group

397   during the Training period with respect to number of rewards earned (two-way repeated-

398   measures ANOVA, $F_{(1, 8)} = 0.09147$, p = 0.7700) or incorrect nose pokes (two-way

399   repeated-measures ANOVA, $F_{(1, 8)} = 0.3681$, p = 0.5609), but both ChR2 groups were

400   significantly better than the EYFP control group (**Figure 6B-C + Figure 6-figure**

401   **supplement 2A-B**, pink shading; two-way repeated-measures ANOVAs; rewards

402   earned: Group (EYFP vs. contingent-ChR2 vs. non-contingent-ChR2) main effect: $F_{(2,}$

403    $_{13)} = 7.254$, p = 0.0077; Day x Group interaction: $F_{(22, 143)} = 1.861$, p = 0.0164.

404   Incorrect nose pokes: Group main effect: $F_{(2, 13)} = 4.884$, p = 0.0262.). These results

405   demonstrate that ChR2-mediated ACh release does not have to be time-locked to the

406   cue, nose poke, or reward retrieval to improve performance of the task, suggesting that

407   ACh may alter the threshold for neuronal plasticity for cue-reward pairing over a much

408   longer timescale than might be expected based on results from the ACh3.0 recording

409   and NBM-BLA recordings, which could be consistent with the involvement of mAChR

410   signaling in this effect. As in the previous experiment, once all groups reached criterion

411   for acquisition of the cue-reward contingency, there were no differences between any of

412   the groups during Extinction (**Figure 6B-C + Figure 6-figure supplement 2A-B**, orange

413   shaded; two-way repeated-measures ANOVA, $F_{(2, 13)} = 0.04229$, $p = 0.9587$).

414

415   **Discussion**

416   It is increasingly recognized that the BLA is involved in learning to predict both

417   positive and negative outcomes from previously neutral cues (Cador et al., 1989; Janak

418   & Tye, 2015; LeDoux et al., 1990). Cholinergic cells in the basal forebrain complex fire

419   in response to both positive and negative reinforcement (Hangya et al., 2015). The

420   results shown here indicate that ACh signaling in the BLA is intimately involved in cue-

421   reward learning. Endogenous ACh is released in the BLA in response to salient events

422   in the task, and ACh dynamics evolved as the subject formed associations between

423   stimuli and reward. While the pattern of ACh signaling in the BLA may seem reminiscent

424   of how dopamine neurons encode reward prediction errors as measured in other brain

425   areas (Schultz et al., 1997), the current results suggest that ACh release in the BLA

426   may instead be involved in signaling a combination of salience and novelty. ACh

427   release and NBM-BLA activity increased following correct nose poke and, around the

428   time that animals acquired the cue-reward task, following tone onset. However, earlier

429   in Training, incorrect nose pokes that resulted in a timeout were also followed by ACh

430   release, although this was smaller in magnitude. Further, stimulating NBM-BLA

431   cholinergic terminals during learning enhanced behavioral performance, but was not

432   intrinsically rewarding on its own and did not support responding for the tone alone.

433   Although ACh was released in the BLA at discrete points during the task, the effects of

434   heightened BLA ACh signaling were relatively long lasting, since it was not necessary

435   for stimulation to be time-locked to cue presentation or reward retrieval to enhance

436   behavioral performance. Thus, cholinergic inputs from the basal forebrain complex to

437   the BLA are a key component of the circuitry that links salient events to previously

438   neutral stimuli in the environment and uses those neutral cues to predict future

439   rewarded outcomes.

440

441   **BLA ACh signaling and CaMKIIα cell activity are related to cue-reward learning**

442   We have shown that ACh release in the BLA is coincident with the stimulus that

443   was most salient to the animal at each phase of the task. Use of the fluorescent ACh

444   sensor was essential in determining these dynamics (Jing et al., 2018, 2019). Previous

445   microdialysis studies have shown that ACh is released in response to positive, negative,

446   or surprising stimuli, but this technique is limited by relatively long timescales (minutes)

447   and cannot be used to determine when cholinergic transients align to given events in an

448   appetitive learning task and how they evolve over time (Sarter & Lustig, 2020). In this

449   cue-reward learning paradigm, when there was no consequence for incorrect nose-

450   poking (Pre-Training phase), animals learned to perform a very high number of nose

451   pokes and received a large number of rewards, and BLA ACh signaling peaked

452   following correct nose pokes. Both the behavioral response (nose poking that was not

453   contingent with the tone) and the ACh response (linked to the correct nose poke)

454   suggest that the animals were not attending to the tone during most of the Pre-Training

455   phase of the task, but rather were attending to the cues associated with reward delivery,

456   such as the reward light or the sound of the pump that delivered the reward. Consistent

457   with this possibility, in the next phase of the task when mice received a timeout for

458   responding if the tone was not presented, performance of all groups dropped

459   dramatically. Interestingly, in animals that had difficulty learning the cue-reward

460   contingency, during early Training sessions ACh release shifted to reward retrieval,

461   likely because this was the most salient aspect of the task when the majority of nose

462   pokes performed did not result in reward. Finally, as mice acquired the contingency

463   between tone and reward availability, the tone also began to elicit ACh release in the

464   BLA, suggesting that mice learned that the tone is a salient event predicting reward

465   availability. Since there are multiple sources of ACh input to the BLA, it was important to

466   determine whether NBM cholinergic neurons were active during the periods when ACh

467   levels were high (Woolf, 1991). Recordings from cholinergic NBM-BLA terminal fibers

468   showed similar dynamics to ACh measurements, suggesting that the NBM is a primary

469   source of ACh across the phases of cue-reward learning.

470   Perhaps the most well-known example of dynamic responding related to learning

471   cue-reward contingencies and encoding of reward prediction errors is the firing of

472   dopaminergic neurons of the ventral tegmental area (VTA; Schultz, 1998). After

473   sufficient pairings, dopaminergic neurons will fire in response to the cue that predicts

474   the reward, and no longer to the rewarding outcome, which corresponds with behavioral

475   changes that indicate an association has been formed between conditioned stimuli (CS)

476   and unconditioned stimuli (US). It should be noted that dopamine signaling is not unique

477   in this learning-related dynamic response profile. Serotonergic neuronal responses also

478   evolve during reward learning in a manner distinct from dopaminergic neurons (Zhong

479   et al., 2017). Plasticity related to learning has also been observed in cholinergic neurons

480   in the basal forebrain complex during aversive trace conditioning, such that after several

481   training days, neuronal activity spans the delay between CS and US (Guo et al., 2019).

482   Additionally, a recent study suggested that ACh may signal a valence-free

483   reinforcement prediction error (Sturgill et al., 2020). Future studies on the selective

484   inputs to NBM to BLA cholinergic neurons would be of interest to identify the links

485   between brain areas involved in prediction error coding.

486   We found that BLA CaMKIIα cells were most reliably activated following reward

487   retrieval before contingency acquisition (both when they were receiving several rewards

488   but no timeouts in Pre-Training and few rewards early in Training). Similar to the

489   recording of ACh levels, after acquisition, the tone began to elicit an increase in BLA

490   CaMKIIα cell population activity. However, activity of CaMKIIα neurons differed from

491   ACh signaling in the BLA in important ways. ACh was released in response to the

492   salient events in the task that were best able to predict reward delivery or availability. In

493   contrast, the activity of BLA CaMKIIα neurons was not tightly time-locked to correct

494   nose poking, and instead followed reward retrieval until acquisition, when activity

495   increased in response to tone onset. The divergent dynamics of ACh release and

496   CaMKIIα neuron activity underscores that ACh's role in the BLA is to modulate, rather

497   than drive, the activity of CaMKIIα neurons, and therefore may alter dynamics of the

498   network through selective engagement of different populations of GABA interneurons

499   (Unal et al., 2015).

500

501   **Increasing BLA acetylcholine levels enhances cue-reward learning**

502   Neuronal activity and plasticity in the BLA is required for both acquisition of

503   appetitive learning (conditioned reinforcement) and fear conditioning, however the

504   inputs that increase activity in the structure during salient events likely come from many

505   brain areas (McKernan & Shinnick-Gallagher, 1997; Rogan et al., 1997; Tye et al.,

506   2008). In particular, dopaminergic inputs to the BLA are important for acquisition of

507   conditioned reinforcement and for linking the rewarding properties of addictive drugs to

508   cues that predict their availability (Cador et al., 1989). Our results indicate that ACh is a

509   critical neuromodulator upstream of the BLA that is responsive to salient events, such

510   as reward availability, motor actions that elicit reward, and cues that predict reward. We

511   show here that increasing endogenous ACh signaling in the BLA caused mice to

512   perform significantly better than controls in an appetitive cued-learning task. Heightened

513   ACh release during learning of a cue-action-reward contingency led to fewer incorrect

514   responses and increased acquisition rate in both female and male mice. The optical

515   stimulation was triggered by correct nose poke, thus the cholinergic NBM-BLA terminal

516   fiber stimulation overlapped with all three salient events: tone, nose poke, and reward

517   retrieval, since the tone terminated 2 sec after correct nose poke. We chose this

518   stimulation pattern, as opposed to linking optical stimulation to tone onset, to ensure

519   stimulation was dependent on behavioral responses. Therefore, stimulation did not

520   precisely recapitulate the ACh release profile observed in mice that had already

521   acquired the task (when ACh increases following tone onset). This suggests that

522   behaviorally-contingent increases in BLA ACh are sufficient to enhance task acquisition

523   (but see below). It is also possible that optogenetic-mediated ACh release may last

524   longer than endogenous, tone-evoked release. A simultaneous stimulation and

525   recording approach would be required to compare ACh release under both conditions

526   (Pisansky et al., 2019). It is important to note that basal forebrain neurons have been

527   demonstrated to co-release ACh and GABA (Ma et al., 2018; Saunders et al., 2015),

528   and cholinergic basal forebrain neurons that project to the BLA have been shown to co-

529   express a glutamate transporter (Ma et al., 2018; Poulin et al., 2006). Thus, it is

530   possible that both fiber photometry and optogenetic results could be influenced, in part,

531   by co-release of other neurotransmitters from ChAT-positive neurons. Future studies

532   employing additional fluorescent neurotransmitter sensors (Marvin et al., 2013, 2018,

533   2019) could help understand the interaction between the different signals employed by

534   basal forebrain neurons.

535   It is possible that ACh improved learning by increasing the intensity of the

536   reward, potentiating the learned association, improving discrimination, or a combination

537   of these phenomena. However, increasing ACh release in the BLA was not inherently

538   rewarding, because it did not support self-stimulation or real-time place preference. This

539   is at odds with a recent study that found stimulation of NBM-BLA cholinergic terminals

540   could induce a type of place-preference and modest self-stimulation (Aitta-aho et al.,

541   2018). Perhaps slight differences in targeting of ChR2 infusion or differences in the

542   behavioral paradigm could be responsible for the lack of direct rewarding effects of

543   optical ChAT terminal stimulation in the current study. Other recent work (Jiang et al.,

544   2016) has demonstrated that stimulating this NBM-BLA cholinergic pathway is sufficient

545   to strengthen cued aversive memory, suggesting that the effect of ACh in the BLA may

546   not be inherently rewarding or punishing, but instead potentiates plasticity in the BLA,

547   allowing learning of cue-outcome contingencies. Similarly, it is possible that ACh alters

548   motor activity. However, there were no effects of optical stimulation on locomotion or

549   responding in the inactive nose poke port. In addition, during the Pre-Training phase

550   when there was no consequence for incorrect nose pokes, all groups earned the same

551   number of rewards, regardless of optical stimulation or pharmacological blockade of

552   ACh receptors, suggesting that ACh is not involved in the motor aspects of the task or

553   the value of the reward. Indeed, differences emerged only during the Training phase,

554   when attention to the tone was critical to earn rewards. Further, incorrect nose poking

555   remained high for mice administered scopolamine. This suggests that scopolamine-

556   treated animals were seeking the reward, as in the operant familiarization and Pre-

557   Training phases of training, but were unable to learn that they should only nose poke in

558   response to the tone.

559   Cell-type-specific expression of AChRs and activity-dependent effects place

560   cholinergic signaling at a prime position to shape BLA activity during learning. For

561   instance, late-firing interneurons in the BLA exhibit nAChR-dependent EPSP's when no

562   effect is seen on fast-spiking interneurons, while principal neurons can be either excited

563   or inhibited through mAChRs, depending on activity level of the neuron at the time of

564   cholinergic stimulation (Unal et al., 2015). BLA mAChRs can support persistent firing in

565   principal neurons and can be important for the expression of conditioned place

566   preference behavior, as well as trace fear conditioning (Baysinger et al., 2012; Egorov

567   et al., 2006; McIntyre et al., 1998). Similar to studies of trace fear conditioning, in which

568   activity of the network over a delay period must be maintained, we found that

569   metabotropic (mAChRs) but not ionotropic (nAChRs) ACh receptors were required for

570   learning the contingency of this cue-reward task. The timing of cholinergic signaling can

571   be a critical factor in the induction of synaptic plasticity in other brain regions, so we

572   hypothesized that the enhancement of cue-reward learning observed might be

573   dependent upon when NBM-BLA terminal fibers were stimulated with respect to tone

574   presentation and/or behavioral responses (Gu & Yakel, 2011). However, we found that

575   heightened ACh signaling in the BLA improved behavioral performance even when

576   stimulations were explicitly unpaired with the cue or correct nose poking. This suggests

577   that the effect of increased cholinergic signaling in the BLA is long lasting, and that

578   stimulation during a learning session is sufficient to potentiate synaptic events linking

579   the cue to a salient outcome—even if CS and/or reward delivery are presented tens of

580   seconds later. Given the findings from fiber photometry recordings, which showed

581   endogenous ACh release was time-locked to salient stimuli during the task and evolved

582   with learning, it is surprising that time-locking of exogenous ACh release was not

583   necessary for enhancement of cue-reward learning. Coupled with pharmacological

584   evidence demonstrating that muscarinic signaling is necessary for reward learning in

585   this task, these results suggest the involvement of metabotropic signaling downstream

586   of muscarinic receptors that outlasts the initial cholinergic stimulation.

587   To conclude, the abundant ACh input to the BLA results in ACh release in

588   response to stimuli that predict reward in a learned cue-reward task. Increasing

589   cholinergic signaling results in accelerated learning of the cue-reward contingency.

590   These findings are consistent with the hypothesis that ACh is a neuromodulator that is

591   released in response to salient stimuli and suggests that ACh signaling may enhance

592   neuronal plasticity in the BLA network, leading to accelerated cue-reward learning.

Materials and Methods

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
genetic reagent *(M. musculus)*	B6;129S6-*Chat$^{tm2(cre)Lowl/J}$*	Jackson Laboratory	Stock #: 006410 RRID:IMSR_JAX:006410	
genetic reagent *(M. musculus)*	C57BL/6J	Jackson Laboratory	Stock #: 000664 RRID:IMSR_JAX:000664	
genetic reagent *(M. musculus)*	Tg(*Camk2a-cre*)2Gsc	Günter Schütz, German Cancer Research Center	RRID:MGI:4457404	(Casanova et al., 2001; Wohleb et al., 2016)
antibody	anti-ChAT (goat polyclonal)	Millipore Sigma	Cat #: AB144P RRID:AB_2079751	(1:1000)
antibody	anti-GFP (chicken, polyclonal)	Thermo Fisher Scientific	Cat #: A10262, RRID:AB_2534023	(1:1000)
antibody	anti-DsRed (rabbit, monoclonal)	Takara Bio	Cat #: 632392, RRID:AB_2801258	(1:1000)
antibody	donkey anti-chicken 488 (secondary)	Jackson ImmunoResearch	Cat #: 703-545-155, RRID:AB_2340375	(1:1000)
antibody	donkey anti-rabbit 555 (secondary)	Thermo Fisher Scientific	Cat #: A-31572, RRID:AB_162543	(1:1000)
antibody	donkey anti-goat 555 (secondary)	Thermo Fisher Scientific	Cat #: A-21432, RRID:AB_141788	(1:1000)

antibody	donkey anti-goat 647 (secondary)	Thermo Fisher Scientific	Cat #: A-21447, RRID:AB_141844	(1:1000)
recombinant DNA reagent	AAV9 hSyn-ACh3.0	Yulong Li (Jing et al., 2018, 2019)	Cat #: YL10002-AV9	
recombinant DNA reagent	AAV1 Syn-FLEX-GCaMP6s-WPRE-SV40	Addgene	Cat #: 100845-AAV1 RRID:Addgene_100845	
recombinant DNA reagent	AAV1-Syn-FLEX-jGCaMP7s-WPRE	Addgene	Cat #: 104491-AAV1 RRID:Addgene_104491	
recombinant DNA reagent	AAV1 Syn-FLEX-NES-jRCaMP1b-WPRE-SV40	Addgene	Cat #: 100850-AAV1 RRID:Addgene_100850	
recombinant DNA reagent	AAV2 EF1a-DIO-EYFP	UNC Viral Vector Core	RRID: SCR_002448	
recombinant DNA reagent	AAV2 EF1a-DIO-hChR2(H134R)-EYFP	UNC Viral Vector Core	RRID: SCR_002448	
chemical compound, drug	mecamylamine hydrochloride	Millipore Sigma	Cat #: M9020	
chemical compound, drug	(-) scopolamine hydrochloride	Millipore Sigma	Cat #: S1013	
software, algorithm	MATLAB	MathWorks	RRID: SCR_001622	Version 2020a
software, algorithm	GraphPad Prism 8	GraphPad Software	RRID: SCR_002798	

software, algorithm	EthoVision XT 10	Noldus	RRID: SCR_000441	
software, algorithm	FV10-ASW	Olympus	RRID:SCR_014215	Version 04.02.03.06
software, algorithm	Doric Neuroscience Studio	Doric Lenses		Version 5.3.3.14
software, algorithm	MED-PC IV	Med Associates Inc.	RRID:SCR_012156	
other	Allen Reference Atlas	(Lein et al., 2007)	RRID:SCR_013286	
other	DAPI stain	Thermo Fisher Scientific	Cat #: 62248	1:1000

594

Animals

596   All procedures were approved by the Yale University Institutional Animal Care &

597 Use Committee (protocol: 2019-07895) in compliance with the National Institute of

598 Health's Guide for the Care and Use of Laboratory Animals. Experiments were

599 performed in mice of both sexes, in keeping with the NIH policy of including sex as a

600 biological variable. Sex of mice in behavioral graphs is indicated by circles for females

601 and squares for males.

602   Female and male heterozygous mice with Cre recombinase knocked into the

603 choline acetyltransferase (ChAT) gene (ChAT-IRES-Cre, B6;129S6-*Chat*$^{tm2(cre)Lowl/J}$,

604 Jackson Laboratory, Bar Harbor, ME) were bred in house by mating ChAT-IRES-Cre

605 with C57BL6/J mice. CaMKIIα-Cre (Tg(*Camk2a-cre*)2Gsc) mice obtained from Ronald

606 Duman (Casanova et al., 2001; Wohleb et al., 2016) were bred in house as above.

607 C57BL6/J mice were obtained from The Jackson Laboratory at 6-10 weeks of age, and

608 tested at 5-7 months of age, following at least one week of acclimation. All mice were

609 maintained in a temperature-controlled animal facility on a 12-hour light/dark cycle

610 (lights on at 7:00 AM). Mice were group housed 3-5 per cage and provided with *ad*

611 *libitum* food and water until undergoing behavioral testing. Mice were single housed 1-3

612 weeks before surgery to facilitate food restriction and body weight maintenance.

613

614 **Surgical procedures**

615   Surgical procedures for behavior were performed in fully adult mice at 4-6

616 months of age, age-matched across conditions. For viral infusion and fiber implantation,

617 mice were anesthetized using isoflurane (induced at 4%, maintained at 1.5-2%) and

618 secured in a stereotactic apparatus (David Kopf Instruments, Tujunga, CA). The skull

619 was exposed using a scalpel and Bregma was determined using the syringe needle tip

620 (2 μL Hamilton Neuros syringe, 30 gauge needle, flat tip; Reno, NV).

621   For fiber photometry surgeries, 0.4 μL of AAV9 hSyn-ACh3.0 (Vigene

622 Biosciences Inc.) to measure BLA ACh levels (**Figure 2A-E + Figure 2-figure**

623 **supplement 1-2**) was delivered unilaterally to the BLA (A/P; -1.34 mm, M/L + or - 2.65

624 mm, D/V -4.6 mm, relative to Bregma) of ChAT-IRES-Cre or wild-type C57BL6/J mice at

625 a rate of 0.1 μL/min. The needle was allowed to remain at the infusion site for 5 min

626 before and 5 min after injection. A mono fiber-optic cannula (1.25 mm outer diameter

627 metal ferrule; 6 mm long, 400 μm core diameter/430 μm outer diameter, 0.48 numerical

628 aperture (NA), hard polymer cladding outer layer cannula; Doric Lenses, Quebec City,

629 Quebec, Canada) was implanted above the BLA (A/P; -1.34 mm, M/L + 2.65 mm, D/V -

630　4.25 mm) and affixed to the skull using opaque dental cement (Parkell Inc., Edgewood,

631　NY). For BLA CaMKIIα cell calcium dynamic recordings (**Figure 3 + Figure 3-figure**

632　**supplement 1-2**), 0.5 µL of AAV1 Syn-FLEX-GCaMP6s-WPRE-SV40 (Addgene,

633　Watertown, MA) was injected into the left BLA using the same procedure and

634　coordinates but was injected into CaMKIIα-Cre mice. Cholinergic NBM-BLA terminal

635　fiber calcium dynamic recording (**Figure 2F-J + Figure 2-figure supplement 5-8**)

636　surgeries were performed as above except AAV1-Syn-FLEX-jGCaMP7s-WPRE

637　(Addgene) was infused unilaterally into the NBM (A/P: - 0.7 mm, M/L + or - 1.75 mm,

638　D/V – 4.5 mm) of ChAT-IRES-Cre mice, with the optical fiber being placed above the

639　ipsilateral BLA. The jRCaMP1b + ACh3.0 surgeries to simultaneously measure

640　cholinergic NBM-BLA terminal fiber calcium dynamics and BLA ACh levels (**Figure 2-**

641　**figure supplement 9**) consisted of both the NBM and BLA infusions above, except

642　AAV1 Syn-FLEX-NES-jRCaMP1b-WPRE-SV40 (Addgene) was infused the NBM of

643　ChAT-IRES-Cre mice. The RCaMP sham mouse (**Figure 2-figure supplement 9E,H**)

644　was a wild-type littermate and thus had no jRCaMP1b expression.

645　pAAV.Syn.Flex.GCaMP6s.WPRE.SV40 (Addgene viral prep # 100845-AAV1;

646　http://n2t.net/addgene:100845 ; RRID:Addgene_100845), pGP-AAV-syn-FLEX-

647　jGCaMP7s-WPRE was a gift from Douglas Kim & GENIE Project (Addgene viral prep #

648　104491-AAV1; http://n2t.net/addgene:104491 ; RRID:Addgene_104491), and

649　pAAV.Syn.Flex.NES-jRCaMP1b.WPRE.SV40 (Addgene viral prep # 100850-AAV1;

650　http://n2t.net/addgene:100850 ; RRID:Addgene_100850) were gifts from Douglas Kim &

651　GENIE Project (Chen et al., 2013; Dana et al., 2016, 2019).

652   Mice were allowed to recover in a cage without bedding with a microwavable

653   heating pad underneath it until recovery before being returned to home cage. For two

654   days following surgery, mice received 5 mg/Kg Rimadyl i.p (Zoetis Inc., Kalamazoo, MI)

655   as postoperative care.

656   For optical stimulation experiments (**Figure 4,6 + Figure 4-figure supplement 1-**

657   **5 + Figure 6-figure supplement 1-2**), surgeries were performed as above except as

658   follows: 0.5 µL of control vector (AAV2 EF1a-DIO-EYFP) or channelrhodopsin (AAV2

659   EF1a-DIO-hChR2(H134R)-EYFP; University of North Carolina Gene Therapy Center

660   Vector Core, Chapel Hill, NC) was delivered bilaterally into the NBM (A/P: - 0.7 mm, M/L

661   ± 1.75 mm, D/V – 4.5 mm) of ChAT-IRES-Cre mice. Mono fiber-optic cannulas (1.25

662   mm outer diameter zirconia ferrule; 5 mm long, 200 µm core diameter/245 µm outer

663   diameter, 0.37 NA, polyimide buffer outer layer cannula; Doric Lenses) were inserted

664   bilaterally above the basolateral amygdala (BLA, A/P; -1.22 mm, M/L ± 2.75 mm, D/V -

665   4.25 mm). Mice were randomly assigned to EYFP or ChR2 groups, controlling for

666   average group age.

667   For *ex vivo* local field potential electrophysiology experiments (**Figure 4B**), the

668   NBM was injected with DIO-ChR2-EYFP as described above, except mice were 8

669   weeks of age (see Supplemental Methods for current clamp recording methods). The

670   coronal brain slices containing the NBM were prepared after 2-4 weeks of expression.

671   Briefly, mice were anesthetized with 1X Fatal-Plus (Vortech Pharmaceuticals, Dearborn,

672   MI) and were perfused through their circulatory systems to cool down the brain with an

673   ice-cold (4°C) and oxygenated cutting solution containing (mM): sucrose 220, KCl 2.5,

674   NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$1, $MgCl_2$ 6 and glucose 10 (pH 7.3 with NaOH).

675   Mice were then decapitated with a guillotine immediately; the brain was removed and

676   immersed in the ice-cold (4°C) and oxygenated cutting solution to trim to a small tissue

677   block containing the NBM. Coronal slices (300 µm thick) were prepared with a Leica

678   vibratome (Leica Biosystems Inc., Buffalo Grove, IL) after the tissue block was glued on

679   the vibratome stage with Loctite 404 instant adhesive (Henkel Adhesive Technologies,

680   Düsseldorf, Germany). After preparation, slices were maintained at room temperature

681   (23-25 C°) in the storage chamber in the artificial cerebrospinal fluid (ACSF) (bubbled

682   with 5% CO_2 and 95% O_2) containing (in mM): NaCl 124, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2,

683   NaH_2PO_4 1.23, $NaHCO_3$ 26, glucose 10 (pH 7.4 with NaOH) for recovery and storage.

684   Slices were transferred to the recording chamber and constantly perfused with ACSF

685   with a perfusion rate of 2 ml/min at a temperature of 33 oC for electrophysiological

686   experiments. Cell-attached extracellular recording of action potentials was performed by

687   attaching a glass micropipette filled with ACSF on EYFP-expressing cholinergic neurons

688   with an input resistance of 10-20 MΩ under current clamp. Blue light (488 nm) pulse (60

689   ms) was applied to the recorded cells through an Olympus BX51WI microscope

690   (Olympus, Waltham, MA) under the control of the Sutter filter wheel shutter controller

691   (Lambda 10-2, Sutter Instrument, Novato, CA). All data were sampled at 3-10 kHz,

692   filtered at 3 kHz and analyzed with an Apple Macintosh computer using Axograph X

693   (AxoGraph). Events of field action potentials were detected and analyzed with an

694   algorithm in Axograph X as reported previously (Rao et al., 2008).

695

696   **Behavioral Testing**

697   ***Habituation***

698   One week after surgery, mice were weighed daily and given sufficient food

699   (2018S standard chow, Envigo, Madison, WI) to maintain 85% free-feeding body

700   weight. All behavioral tests were performed during the light cycle. Mice were allowed to

701   acclimate to the behavioral room for 30 min before testing and were returned to the

702   animal colony after behavioral sessions ended.

703   Two weeks after surgery, mice were handled 3 min per day for 7 days in the

704   behavioral room. Mice were given free access to the reward (Ensure[®]Plus Vanilla

705   Nutrition Shake solution mixed with equal parts water (Ensure); Abbott Laboratories,

706   Abbott Park, IL) in a 50 mL conical tube cap in their home cages on the last 3 days of

707   handling to familiarize them to the novel solution. Mice were also habituated to patch

708   cord attachment during the last 3 days of handling for optical stimulation and fiber

709   photometry experiments. Immediately before training each day, a patch cord was

710   connected to their optical fiber(s) via zirconia sleeve (s) (1.25 mm, Doric Lenses) before

711   being placed in the behavioral chamber.

712   ***Operant Training***

713   All operant training was carried out using Med Associates modular test chambers

714   and accessories (ENV-307A; Med Associates Inc., Georgia, VT). For optical stimulation

715   experiments, test chambers were housed in sound attenuating chambers (ENV-022M).

716   Two nose poke ports (ENV-313-M) were placed on the left wall of the chamber and the

717   reward receptacle (ENV-303LPHD-RL3) was placed on the right wall. The receptacle

718   cup spout was connected to a 5 mL syringe filled with Ensure loaded in a single speed

719   syringe pump (PHM-100). Nose pokes and receptacle entries were detected by infrared

720   beam breaks. The tone generator (ENV-230) and speaker (ENV-224BM) were placed

721   outside the test chamber, but within the sound attenuating chamber, to the left. The

722   house light (used for timeout, ENV-315M) was placed on top of the tone generator to

723   avoid snagging patch cords. Each chamber had a fan (ENV-025F28) running

724   throughout the session for ventilation and white noise. Behavior chambers were

725   connected to a computer running MEDPC IV to collect event frequency and timestamps.

726   For optical stimulation experiments, a hole drilled in the top of the sound attenuating

727   chambers allowed the patch cord to pass through. Initial BLA ACh3.0 (**Figure 2A-E**)

728   and BLA CaMKIIα GCaMP6s (**Figure 3**) fiber photometry recordings occurred in a

729   darkened behavioral room outside of sound attenuating chambers due to steric

730   constraints with rigid fiber photometry patch cords. Later behavioral chamber

731   optimization (wall height was extended with 3D printed and laser cut acrylic panels to

732   allow removing the test chamber lid while preventing escape) allowed all other fiber

733   photometry cohorts to be tested inside sound attenuating chambers. For fiber

734   photometry experiments, a custom receptacle was 3D printed that extended the cup

735   beyond the chamber wall to allow mice to retrieve the reward with more rigid patch

736   cords. Each mouse was pseudo-randomly assigned to behavioral chamber when

737   multiple chambers were used, counterbalancing for groups across boxes.

738   Three weeks after surgery, initial operant familiarization consisted of one 35 min

739   session of Free Reward to demonstrate the location of reward delivery; all other

740   sessions were 30 mins. During Free Reward, only the reward receptacle was

741   accessible. After 5 min of habituation, Ensure (24 µL over 2 seconds) was delivered in

742   the receptacle cup and a light was turned on above the receptacle. The receptacle light

743   was turned off upon receptacle entry. The next phase of operant familiarization, mice

744   learned to nose poke to receive reward on a fixed-ratio one (FR1) schedule of

745   reinforcement. Mice in experiments involving manipulations (optical stimulation and

746   antagonist studies) were pseudo-randomly assigned to left or right active (reinforced)

747   nose poke port. Mice in fiber photometry experiments were all assigned to right active

748   port to minimize potential across subject variability. The inactive (unreinforced) port

749   served as a locomotor control. During FR1 operant familiarization, each nose poke

750   response into the active port resulted in receptacle light and reward delivery. After the

751   mice reached criterion on FR1 operant familiarization (group average of 30 rewards for

752   2 consecutive days, usually 4-5 days), mice were advanced to the Pre-Training phase.

753   This phase incorporated an auditory tone (2.5-5 kHz, ~60 dB) that lasted for at most 10

754   seconds and signaled when active nose pokes would be rewarded. Only active nose

755   pokes made during the 10 sec auditory tone (correct nose pokes) resulted in reward

756   and receptacle light delivery. The tone co-terminated with Ensure delivery. During Pre-

757   Training, there was no consequence for improper nose pokes, neither in the active port

758   outside the tone (incorrect nose pokes) nor in the inactive port (inactive nose pokes).

759   The number of inactive nose pokes were typically very low after operant familiarization

760   and were not included in analysis. After reward retrieval (receptacle entry following

761   reward delivery) the receptacle light was turned off and the tone was presented again

762   on a variable intertrial interval schedule with an average interval of 30 sec (VI 30),

763   ranging from 10 to 50 sec (Ambroggi et al., 2008). After 4-5 days of Pre-Training, mice

764   progressed to the Training phase, which had the same contingency as Pre-Training

765   except incorrect nose pokes resulted in a 5 sec timeout signaled by house light

766   illumination, followed by a restarting of the previous intertrial interval. Mice were

767   considered to have acquired the task after earning 20 rewards during the Training

768   phase of the task. In order to promote task acquisition, mice that were not increasing

769   number of rewards earned reliably were moved to a VI 20 schedule after 9 days of VI 30

770   Training for BLA ACh3.0 or 6-7 days for BLA CaMKIIα cell mice. The VI 20 schedule

771   was only needed for the two groups that were trained outside of the sound attenuating

772   chambers. Mice progressed to Extinction after 12 days of Training or, in the case of

773   fiber photometry cohorts, once all mice met the acquisition criteria. Extinction was

774   identical to Training except no Ensure was delivered in response to correct nose pokes.

775   The replicate cohorts of the BLA CaMKIIα GCaMP6s and NBM-BLA terminal fiber

776   recording experiments were advanced to one day of Extinction after only 7 days of

777   Training due to the COVID-19 shutdown.

778   Between mice, excrement was removed from the chambers with a paper towel.

779   At the end of the day chambers were cleaned with Rescue Disinfectant (Virox Animal

780   Health, Oakville, Ontario, Canada) and Ensure syringe lines were flushed with water

781   then air. Mice were excluded from analyses if a behavioral chamber malfunctioned (e.g.

782   syringe pump failed) or they received the improper compound. Fiber photometry mice

783   were excluded from analyses if they did not meet the acquisition criterion by the last day

784   of Training. See **Supplementary File 1–Supplementary Table 1** for number of mice

785   that acquired, were excluded, and further explanations for behavioral paradigm

786   deviations.

787   ***Optical Stimulation***

788   Optical stimulation was generated by a 473 nm diode-pumped solid-state

789   continuous wave laser (Opto Engine LLC, Midvale, UT) controlled by a TTL adapter

790 (SG-231, Med Associates Inc.). The laser was connected to a fiber optic rotary joint

791 (Doric Lenses) via a mono fiber optic patch cord (200 μm core, 220 μm cladding, 0.53

792 NA, FC connectors; Doric Lenses). The rotary joint was suspended above the sound

793 attenuating chamber with a connected branching fiber optic patch cord (200 μm core,

794 220 μm cladding, 0.53 NA, FC connector with metal ferrule; Doric Lenses) fed into the

795 behavioral box. Laser power was adjusted to yield 10-12 mW of power at each fiber tip.

796 The stimulation pattern was 25 ms pulses at 20 Hz for 2 sec modified from parameters

797 in (Jiang et al., 2016). Jiang et al. used a 20Hz pulse frequency, 5 ms pulses, and 10-12

798 mW power at the fiber tips. In the current study we used a 2 sec stimulation duration

799 because it matched the time of syringe pump activation for reward delivery and co-

800 terminated with the pump and auditory tone. A 25 ms pulse width was used because

801 our lasers were not able to generate sufficient power with 5 ms pulses. Optical

802 stimulation was only delivered during the Pre-Training and Training phases of the

803 operant task. Both control (EYFP) and experimental (ChR2) groups received identical

804 light delivery, and stimulation was triggered by a correct nose poke and co-terminated

805 with the auditory tone and Ensure delivery. For the non-contingent experiment, the

806 number of light stimulations was yoked to the concurrently running ChR2 mouse. The

807 timing of the non-contingent stimulation was explicitly unpaired with correct nose pokes

808 or tones, and was held in queue until the mouse had not made a response in the last 2

809 sec, a tone was not going to be delivered within the next 2 sec, or at least 5 sec had

810 passed since the mouse entered the receptacle after earning reward.

811

812 **Fiber Photometry**

813 ***Acquisition***

814 Fluorescent measurements of ACh and calcium levels were recorded using two

815 Doric Lenses 1-site Fiber Photometry Systems: a standard 405/465 nm system and a

816 405/470/560 nm system. The standard 405/465 system was configured as follows: the

817 fiber photometry console controlled the two connectorized LEDs (CLEDs, 405 nm

818 modulated at 208.616 Hz and 465 nm modulated at 572.205 Hz) through the LED

819 module driver (Cassidy et al., 2019). Each CLED was connected via attenuating patch

820 cord to the five-port Fluorescence MiniCube (FMC5_AE(405)_AF(420-450)_E1(460-

821 490)_F1(500-550)_S). A pigtailed fiber optic rotary joint was connected to the MiniCube

822 and suspended above the behavioral chamber with a rotary joint holder in order to

823 deliver and receive light through the implanted optical fiber. The other end of the rotary

824 joint was connected to the mono fiber optic patch cord via M3 connector and attached

825 with a zirconia sleeve to the implanted fiber optic as above. The F1 (500-550 nm) port of

826 the MiniCube was connected to the photoreceiver (AC low mode, New Focus 2151

827 Visible Femtowatt Photoreceiver, New Focus, San Jose, CA) via a fiber optic adapter

828 (Doric Lenses) that was finally connected back to the fiber photometry console through

829 an analog port. The 405/470/560 nm system was set up similarly, except a 560 nm LED

830 was incorporated (modulated at 333.786 Hz), a six-port MiniCube with two integrated

831 photodetector heads was used (iFMC6_IE(400-410)_E1(460-490)_F1(500-

832 540)_E2(555-570)_F2(580-680)_S), and Doric Fluorescence Detector Amplifiers were

833 used (AC 1X or 10X mode, DFD_FOA_FC). A TTL adapter (SG-231, Med Associates

834 Inc.) was connected to the digital input/output port to allow for timestamping when

835 events occurred in the behavioral chamber. Signal was recorded using Doric

836   Neuroscience Studio (V 5.3.3.14) via the Lock-In demodulation mode with a sampling

837   rate of 12.0 kS/s. Data were decimated by a factor of 100 and saved as a comma-

838   separated file.

839   **Pre-Processing**

840   Preprocessing of raw data was performed using a modified version of a MATLAB

841   (MathWorks, Natick, MA) script provided by Doric. The baseline fluorescence (F0) was

842   calculated using a first order least mean squares regression over the ~30 min recording

843   session. Second order least mean squares regressions were used when

844   photobleaching of the sensor was more pronounced, as in the case of NBM-BLA

845   terminal fiber recordings. The change in fluorescence for a given timepoint (ΔF) was

846   calculated as the difference between it and F0, divided by F0, which was multiplied by

847   100 to yield %ΔF/F0. The %ΔF/F0 was calculated independently for both the signal

848   (465 nm) and reference (405 nm) channels to assess the degree of movement artifact.

849   Since little movement artifact was observed in the recordings (**Figure 2-figure**

850   **supplement 1B-C, Figure 2-figure supplement 5D-E, Figure 3-figure supplement**

851   **1C-D**, tan lines), the signal %ΔF/F0 was analyzed alone (the code provided allows for

852   running the entire analysis pipeline with the reference channel %ΔF/F0 if desired). The

853   %ΔF/F0 was z-scored to give the final Z%ΔF/F0 reported here. For the BLA CaMKIIα

854   cell recordings (**Figure 3-figure supplement 1C-D)**, the reference channel displayed

855   some mirroring (moving in the opposite direction) compared to the signal. This is likely

856   because 405 nm is not the "true" isosbestic point for GCaMP and we were instead

857   measuring some changes in calcium-unbound GCaMP rather than calcium-insensitive

858   GCaMP signal alone (Barnett et al., 2017; C. K. Kim et al., 2016; Sych et al., 2019).

859   Graphs and heatmaps for averaged traces aligned to actions were based on licking bout

860   epoch filtering code from TDT (Alachua, FL; link in code comments).

861   ***Heatmaps***

862   Combined action heatmaps were generated in MATLAB (2020a) by analyzing

863   data 5 sec preceding tone onset (rewarded trials only) to 5 sec after receptacle entry.

864   Actions were aligned despite variable latencies by evenly splitting a maximum of 4 sec

865   post-tone onset/pre-correct nose poke and 1 sec post-correct nose poke/pre-receptacle

866   entry for each trial within a day. The resulting aligned trials were averaged to generate

867   daily averages that made up the rows of the individual animal heatmaps. Blanks in the

868   rows of heatmaps (black time bins) indicate time bins added for alignment, meaning that

869   no trials for that day had a latency that stretched the entire window. Only rewarded trials

870   where the mouse entered the receptacle within 5 sec after nose poke were analyzed.

871   Full or partial training days were excluded from analysis if there were acquisition issues

872   such as the patch cord losing contact with the fiber or behavioral apparatus malfunction.

873   Lack of trials (fewer than three) for analysis or recording issues led to missing rows of

874   fiber photometry data in the heatmap despite having behavioral data, in which case

875   these rows were skipped rather than adding entire blank rows. Due to individual

876   differences in behavior, across-mouse average data was calculated by using a selection

877   of days in which behavior was roughly similar or milestones such as first and last day of

878   Pre-Training, first day earning 10 rewards in Training, first day crossing acquisition

879   threshold (and maintaining afterward), last day of Training, last day of Extinction (with 3

880   or more rewarded trials that met analysis criteria). Additional days were included in

881   across-mouse average heatmaps when possible. Incorrect nose poke heatmaps were

882 generated by averaging signals for 5 sec before and 5 sec after incorrect nose pokes

883 that were not preceded by an incorrect nose poke in the last 5 sec. The incorrect nose

884 poke heatmaps averaged across mice were generated using the same selection of days

885 as the combined action heatmaps for a given experiment.

886 **Bootstrapped Confidence Interval Analyses**

887 Bootstrapped Confidence Intervals (bCI's) of the Z-scored % ΔF/F0 fiber

888 photometry data within and across mice were generated using the methods described in

889 (Jean-Richard-dit-Bressel et al., 2020) for the following events: tone onset, correct nose

890 poke, receptacle entry, and incorrect nose poke. For the within-mouse analysis by day,

891 trials were aligned to event onset, and bCI's were generated for events that had at least

892 3 trials from 5 seconds prior to 10 seconds after each event. Each series of data were

893 bootstrapped 10,000 times and a two-sided 99% confidence interval was constructed.

894 Data were considered significantly different from baseline (Z% ΔF/F0 = 0) when their

895 99% bCIs did not contain zero for an interval of time designated by a consecutive

896 threshold of 0.5 sec.

897 To avoid comparing vastly different numbers of trials, in graphs where correct

898 and incorrect nose pokes were plotted together, incorrect nose pokes were

899 downsampled to match the number of correct nose poke trials. For Incorrect Nose

900 Pokes graphs where last Pre-Training Day and Training Day 1 were plotted together,

901 both days were downsampled to the number of correct nose pokes on the last Pre-

902 Training Day.

903 For the combined action bCI plots (tone onset, correct nose poke, and

904 receptacle entry), the selection of days for each mouse matched that of the cohort-

905   averaged combined action heatmaps. The three event plots were combined by cropping

906   to match the maximum latencies used in the combined action heatmaps. For the

907   across-mouse averaged bCI plots, analyses were carried out as above except the

908   bootstrapping used mouse trial averages. The mean lines for across-mouse averaged

909   bCI plots were calculated by taking the mean of all individual trials together. The NBM-

910   BLA cholinergic terminal fiber experiment required combining the two independent

911   cohorts to obtain n ≥ 3. For the incorrect nose poke bCI plots, the number of trials used

912   for each day were downsampled to 20 if a mouse performed more than 20.

913

914   **Pharmacology**

915   Male wildtype C57BL/6J mice were injected i.p. 30 min prior to each Pre-Training

916   and Training session with a volume of 10 mL/kg with the following compounds: 1X

917   DPBS (Thermo Fisher Scientific, Waltham, MA), 1 mg/kg mecamylamine hydrochloride

918   (Millipore Sigma, St. Louis, MO), 0.5 mg/kg (-) scopolamine hydrochloride (Millipore

919   Sigma), or 1 mg/kg mecamylamine + 0.5 mg/kg scopolamine (**Figure 5** + **Figure 5-**

920   **figure supplement 1**)

921

922   **Histology**

923   After completion of behavioral experiments, animals were anesthetized with 1X

924   Fatal-Plus (Vortech Pharmaceuticals). Once there was no response to toe-pinch, mice

925   were transcardially perfused with 20 mL ice cold 1X DPBS followed by 20 mL 4%

926   paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA). Brains were

927   extracted and post-fixed for at least 1 day in 4% PFA at 4°C and transferred to 30%

928   sucrose (Millipore Sigma) for at least 1 day at 4°C. Brains were sliced 40 μm thick on a

929   self-cooling microtome and stored in a 0.02% sodium azide (Millipore Sigma) PBS

930   solution. Brain slices were washed in PBS, blocked for 2-3 hours (0.3% Triton X-100,

931   American Bioanalytical, Canton, MA; 3% normal donkey serum, Jackson

932   ImmunoResearch, West Grove, PA), then incubated overnight with primary antibodies

933   (1:1000 + 1% normal donkey serum). Slices were then washed in PBS and incubated

934   with secondary antibodies (1:1000) for 2 hours, washed, stained with DAPI for 5 min,

935   washed, mounted, and coverslipped with Fluoromount-G (Electron Microscopy

936   Sciences). All incubations were at room temperature. Microscope slides were imaged

937   using a FLUOVIEW FV10i confocal microscope (Olympus). Injection sites and fiber

938   placements were designated on modified Allen Mouse Brain Atlas figures (Lein et al.,

939   2007). Mice were excluded from analyses if fluorescence was not observed at injection

940   sites or if fiber tips were not identified at the intended site.

941

942   **Statistical Analyses of Behavior**

943   Operant behavioral data saved by MEDPC IV was transferred to Excel using

944   MPC2XL. Data were organized in MATLAB and analyzed in Prism (V8.3.0, GraphPad

945   Software, San Diego, CA). Differences between groups and interactions across days for

946   Training were evaluated using Two-Way Repeated Measures ANOVAs. We computed

947   the required sample size for a 90% power level with an alpha of 0.05 by estimating the

948   control (EYFP) group mean would be 10 rewards and the mean experimental (ChR2)

949   group would be 20 rewards with a standard deviation of 5. We utilized a power

950   calculator for continuous outcomes of two independent samples, assuming a normal

951 distribution. The result was 6 samples per group. Each manipulation experiment started

952 with at least 6 mice included in each group (*Sealed Envelope | Power Calculator for*

953 *Continuous Outcome Superiority Trial*, n.d.). In each experiment, each animal within a

954 group served as a biological replicate. These studies did not include technical

955 replicates. Masking was not applied during data acquisition but data analyses were

956 semi-automated in MATLAB and performed blind to condition

957
958 **Supplemental Methods**

959 **Ex Vivo Electrophysiology**

960 ***Slice preparation***: Coronal brain slices were prepared from virus injected mice after 3

961 weeks from surgery. Animals were anesthetized with a mixture of ketamine and

962 xylazine (100 mg ketamine and 6 mg xylazine/kg body weight injected ip). Then the

963 mice were transcardially perfused with a sucrose-based solution (see below). After

964 decapitation, the brain was rapidly transferred into a sucrose-based cutting solution

965 bubbled with 95%O_2 and 5% CO_2 and maintained at ~3°C. This solution contained (in

966 mM): sucrose 230; KCl 2.5; $MgSO_4$ 10; $CaCl_2$ 0.5; NaH_2PO_4 1.25; $NaHCO_3$ 26; glucose

967 10 and pyruvate1.5. Coronal brain slices (300µm) were prepared using a Leica

968 VT1000S vibratome (Leica Biosystems Inc). Slices were equilibrated with a mixture of

969 oxygenated artificial cerebrospinal fluid (aCSF) and sucrose-based cutting solution at

970 room temperature (24-26°C) for at least 1 hour prior to transfer to the recording

971 chamber. Pyruvate (0.15–0.75 mM) was added to reduce oxidative damage and

972 enhance survival. With this protocol, slices are initially incubated in a mixture of 50%

973 cutting solution with pyruvate and 50% aCSF (in mM): sucrose 115; NaCl 63; KCl 2.5;

974 NaH_2PO_4 1.25; $MgSO_4$ 5; $CaCl_2$1.25; $MgCl_2$ 1; $NaHCO_3$ 26; glucose 10; and sodium

975   pyruvate 0.75 at 35°C for 30 min and then transferred to a mixture of 10% cutting

976   solution and 90% aCSF (in mM): sucrose 23; NaCl 113.4; KCl 2.5; NaH_2PO_4 1.25;

977   $MgSO_4$ 1; $CaCl_2$ 1.85; $MgCl_2$ 1.8; $NaHCO_3$ 26; glucose 10; and sodium pyruvate 0.15 at

978   35°C for 1–4 h prior to recording. The slices were continuously superfused with aCSF at

979   a rate of 2ml/min containing (in mM); NaCl 126, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26,

980   $CaCl_2$ 2, $MgCl_2$ 2 and glucose 10 bubbled with 95% O_2 and 5% CO_2 at room

981   temperature.

982   ***Electrophysiological recordings:*** Brain slices were placed on the stage of an upright,

983   infrared-differential interference contrast microscope (Olympus BX51WI, Olympus).

984   NBM neurons were visualized with a 40 X water-immersion objective by infrared

985   microscopy (COHU 4915 camera, COHU, Inc., Poway, CA). Patch electrodes with a

986   resistance of 4–6 MΩ were pulled with a laser-based micropipette puller (P-2000, Sutter

987   Instrument Company). Signals were recorded with a Multi Clamp 700A amplifier and

988   pClamp10 software (Molecular Devices, Inc., San Jose, CA). The pipette solution

989   contained (in mM) 130 K-gluconate, 2 KCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 1 ATP and

990   0.2 GTP (pH=7.3).

991   To examine action potential firing frequency, NBM neurons were recorded in a current

992   clamp configuration after forming a giga-ohm seal. Membrane potentials were clamped

993   at -60 mV by injecting 0-~50 pA current through the recording electrode as needed.

994   Cells that maintained steady membrane potentials for at least 5 mins were included in

995   the analysis.

996 ***Optogenetic stimulation ex vivo:*** Channelrhodopsin was activated with a train of light

997 flashes delivered through the 40x microscope objective. The light source was an

998 Olympus x-cite 120Q lamp (Olympus) gated with a TTL controlled shutter (LAMBDA

999 SC, Sutter Instrument). The filter cube contained an HQ480/40x excitation filter, a

1000 Q505lp bypass filter and an HQ535/50m emission filter (Chroma Technology Corp.,

1001 Bellows Falls, VT). The fluorescence illumination intensity delivered at the brain slices

1002 was adjusted to 1-3 mW/mm2, measured with a PM100D optical power and energy

1003 meter (Thorlabs Inc., Newton, NJ). In the NBM, cholinergic neurons were identified by

1004 EGFP fluorescence and light flashes were delivered at 1 Hz, 5 Hz, 10 Hz, 15Hz, 20 Hz,

1005 25 Hz, and 30 Hz.

1006 **Cued Self-Stimulation**

1007 After Extinction, responding was reinstated in Training for 2 days. Then mice

1008 underwent a modified Training paradigm where correct nose pokes yielded only laser

1009 stimulation, without Ensure delivery.

1010 **Real Time Place Preference**

1011 An empty, clear mouse cage (29.5 cm x 19 cm x 12.5 cm) had half of its floor

1012 covered in printer paper to provide a distinct floor texture. A video camera was placed

1013 above the cage and was connected to a computer running EthoVision XT (version

1014 10.1.856, Noldus, Wageningen, Netherlands) to track the position of the mouse and

1015 deliver optical stimulation when the mouse was on the laser-paired side (via TTL pulse

1016 to OTPG_4 laser controller (Doric Lenses) connected to the laser; 20 Hz, 25 ms

1017 pulses). Mice were randomly assigned and counterbalanced to receive laser stimulation

1018 only on one side of the cage. Mice were allowed free access to either side for 15 min

1019   during a session. Baseline was established in the absence of optical stimulation on Day

1020   1. Mice then received optical stimulation on Day 2 only when on the laser-paired side.

1021   Data are presented as percent time spent on the laser-paired side.

1022   **Progressive Ratio testing**

1023   In the progressive ratio test, mice were given 60 min to nose poke for Ensure and

1024   2 sec of optical stimulation on a progressive ratio schedule (escalations given below).

1025   Training Day escalation: 1, 2, 2, 2, 2, 3, 3, 3, 3, 3, 5, 5, 5, 5, 5, 8, 8, 8, 8, 8, 8, 11, 11,

1026   11, 11, 11, 11, 15, 15, 15, 15, 15, 15, 22, 22, 22, 22, 22, 33, 33, 33, 33, 33, 44, 44, 44,

1027   44, 44, 55, 66, 77, 88, 99, 133, 166, 199, 255, 313, 399, 499, 599, 777, 900,1222. Test

1028   Day escalation: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219,

1029   268, 328, 402, 492, 603, 777, 900, 1222.

1030   **Locomotor Activity**

1031   *Optical Stimulation*: Mice were placed in a square box (47 cm x 47 cm x 21 cm) for 20

1032   min with a floor of filter paper that was changed between mice. During the 3rd 5 min bin

1033   of the session, mice received optical stimulation (20 sec on/off, 20 Hz, 25 ms pulses).

1034   Locomotor activity was recorded via overhead camera and analyzed in 5 min bins with

1035   EthoVision.

1036   *Antagonists*: Locomotor data was collected using an Accuscan Instruments

1037   (Columbus, Ohio) behavior monitoring system and software. Mice were individually

1038   tested in empty cages, with bedding and nesting material removed to prevent

1039   obstruction of infrared beams. Mice were injected (i.p.) with saline, mecamylamine (1

1040   mg/kg, Sigma), scopolamine (0.5 mg/kg, Sigma), or mecamylamine+scopolamine (1

1041   mg/kg and 0.5 mg/kg, respectively) 30 min before locomotor testing. Locomotion was

1042   monitored for 20 min using 13 photocells placed 4 cm apart to obtain an ambulatory

1043   activity count, consisting of the number of beam breaks recorded during a period of

1044   ambulatory activity (linear motion rather than quick, repetitive beam breaks associated

1045   with behaviors such as scratching and grooming).

1046   **Light/Dark Box Exploration**

1047   A rectangular box was divided evenly into a light (clear top, illuminated by an 8W

1048   tube light) and dark (black walls, black top) side with a black walled divider in the middle

1049   with a small door. The lid and divider were modified to allow the optical fiber and patch

1050   cord to pass through freely. Mice were placed facing the corner on the light side furthest

1051   from the divider and the latency to crossing to the dark side was measured. The number

1052   of crosses and time spent on each side were measured for 6 min following the initial

1053   cross.

1054

References

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1269 **Figure 1**

1270 **Experimental timeline and cue-reward learning paradigm.**

1271 A) <u>Experimental timeline.</u> Mice began food restriction 7 days after surgery and were

1272 maintained at 85% free-feeding body weight for the duration of the experiment. After 7

1273 days of handling, 5-6 days of operant familiarization prepared the mice for the cue-

1274 reward learning task (Pre-Training through Extinction).

1275 B) <u>Behavioral chamber setup.</u> Mice were placed in modular test chambers that included

1276 two nose poke ports on the left wall (Active and Inactive) and the Reward Receptacle on

1277 the right wall. A tone generator and timeout light were placed outside the modular test

1278 chamber. For fiber photometry (FP) and optical stimulation (Laser) experiments, mice

1279 were tethered to a patch cord(s).

1280 <u>C-D) Details of the Cue-Reward Learning Paradigm</u> C) In Pre-Training, an auditory tone

1281 was presented on a variable interval 30 schedule (VI30), during which an active nose

1282 poke yielded Ensure reward delivery but there was no consequence for incorrect nose

1283 pokes (active nose pokes not during tone). D) Training was identical to Pre-Training,

1284 except incorrect nose pokes resulted in a 5 sec timeout, signaled by timeout light

1285 illumination, followed by a restarting of the intertrial interval (ITI).

1286

Figure 2

Basolateral amygdala (BLA) ACh signaling aligns with salient events during reward learning.

A) <u>Diagram and example of injection and fiber placement sites in the BLA for recording from mice expressing a fluorescent acetylcholine sensor (ACh3.0)</u>. Left: Diagram of BLA ACh3.0 injection and fiber tip placement. Right: Representative coronal brain slice with fiber tip and ACh3.0 expression. Blue: DAPI, Green: ACh3.0. White dashed line: BLA outline. Grey dashed rectangle: fiber track. Scale = 500 μm. Individual fiber placements are shown in **Figure 2-figure supplement 1A**.

B) <u>Behavioral responding of mice expressing ACh3.0 in BLA</u>. Individual mice acquired the task at different rates as measured by rewards earned. Horizontal white line: acquisition threshold, when a mouse began to earn 20 rewards consistently in Training. Incorrect nose pokes shown in **Figure 2-figure supplement 2A**. Pre-Training (PT): blue shaded area, Training: pink shaded area, Extinction (Ext): orange shaded area.

C) <u>Fluorescence traces from BLA of ACh3.0-expressing mouse</u>. A significant increase in fluorescence representing BLA ACh release consistently coincided with correct (green line) but not incorrect (grey line) nose pokes on last day of PT (data are shown from Mouse 1). Mean Z-scored (Z%ΔF/F0) overlaid on bootstrapped 99% confidence intervals (99% bCI's). Shaded significance bars under traces represent time points where 99% bCI's do not contain 0 for at least 0.5 sec. Correct: n = 24; downsampled incorrect: n = 24 of 58. Traces of signal and reference channels (%ΔF/F0) during nose pokes are shown in **Figure 2-figure supplement 1B-C**. Incorrect nose pokes on last day of PT vs Training Day 1 shown in **Figure 2-figure supplement 2B.**

1310   D) <u>Heatmap of BLA ACh signaling in Mouse 1 across all training phases, aligned to tone</u>

1311   <u>onset (Tone), correct nose poke (NP), and receptacle entry (Rec)</u>. Each row is the

1312   average of rewarded trials across a training session. White dashed horizontal line: first

1313   Training day earning 10 rewards. Horizontal white line: acquisition threshold, when a

1314   mouse began to earn 20 rewards consistently in Training. Black horizontal lines:

1315   divisions between training phases. Black vertical lines: divisions between breaks in time

1316   to allow for variable latencies in tone onset, correct nose poke, and receptacle entry

1317   (reward retrieval). bCI plot for Mouse 1 in **Figure 2-figure supplement 1G**. Individual

1318   heatmaps for mice 2-4 in **Figure 2-figure supplement 1D-F**. Incorrect nose pokes

1319   heatmaps for individual mice shown in **Figure 2-figure supplement 2C-F.**

1320   E) <u>Heatmap of BLA ACh signaling averaged across mice</u>. Signal aligned as in D) with a

1321   selection of data from key days in the behavioral paradigm shown. From bottom to top:

1322   PT Day 1, PT Day 5, Early Training Day, First Training day earning 10 rewards (white

1323   dashed horizontal line), Mid Training Day, Acquisition Day (white horizontal line), Last

1324   Training Day, Last Extinction Day. Black horizontal lines: divisions between training

1325   phases. Black vertical lines: divisions between breaks in time to allow for variable

1326   latencies in tone onset, correct nose poke, and receptacle entry. bCI plot for cohort

1327   averaged data in **Figure 2-figure supplement 1H.** Incorrect nose poke heatmap and

1328   bCI plot averaged across mice shown in **Figure 2-figure supplement 2G-H**

1329   F) <u>Diagram and example of Nucleus Basalis of Mynert (NBM)-BLA terminal fiber</u>

1330   <u>recordings</u>. Left: DIO-GCaMP7s was injected in the NBM of ChAT-IRES-Cre mice,

1331   individual injection sites are shown in **Figure 2-figure supplement 5A**. Representative

1332   coronal brain slice showing GCaMP7s expression. White dashed lines: internal capsule

1333 and globus pallidus outlines. Blue: DAPI, Green: GCaMP7s, Red: ChAT. Scale = 500

1334 µm; separate channels shown in **Figure 2-figure supplement 5B**. Right: An optical

1335 fiber was implanted above the ipsilateral BLA, individual fiber placements are shown in

1336 **Figure 2-figure supplement 5A**. Representative coronal brain slice showing GCaMP7

1337 expression and fiber tip placement. White dashed line: BLA outline. Grey dashed

1338 rectangle: fiber tract. Blue: DAPI, Green: GCaMP7s, Red: ChAT. Scale = 500 µm;

1339 separate channels shown in **Figure 2-figure supplement 5C**.

1340 G) <u>Behavioral responding of mice during NBM-BLA terminal fiber recordings.</u> Individual

1341 mice acquired the task at different rates as measured by rewards earned. White

1342 horizontal line: acquisition threshold, when a mouse began to earn 20 rewards

1343 consistently in Training. Incorrect nose pokes shown in **Figure 2-figure supplement**

1344 **6A.**

1345 H) <u>NBM-BLA terminal fiber activity is similar to ACh3.0 recordings</u>. NBM-BLA terminal

1346 fiber activity significantly increased with correct (green line) but not incorrect (grey line)

1347 nose pokes on last day of PT (data shown for Mouse 1). Mean Z%ΔF/F0 overlaid on

1348 bootstrapped 99% confidence intervals (99% bCI's). Shaded significance bars under

1349 traces represent time points where 99% bCI's do not contain 0 for at least 0.5 sec.

1350 Correct: n = 42; downsampled incorrect: n = 42 of 101. Signal and reference channels

1351 (%ΔF/F0) during nose pokes are shown in **Figure 2-figure supplement 5D-E**. Incorrect

1352 nose pokes on last day of PT vs Training Day 1 shown in **Figure 2-figure supplement**

1353 **6B.** See **Figure 2-figure supplement 9A-H** for simultaneous ACh3.0 and NBM-BLA

1354 terminal fiber recordings.

1355 I) <u>Heatmap of NBM-BLA terminal fiber activity in Mouse 1 across all training phases,</u>

1356 <u>aligned to tone onset (Tone), correct nose poke (NP), and receptacle entry (Rec)</u>. Each

1357 row is the average of rewarded trials across a training session. Horizontal white line:

1358 acquisition threshold, when a mouse began to earn 20 rewards consistently in Training.

1359 Black horizontal lines: divisions between training phases. Black vertical lines: divisions

1360 between breaks in time to allow for variable latencies in tone onset, correct nose poke,

1361 and receptacle entry (reward retrieval). Blanks in the heatmaps indicate time bins added

1362 for alignment. bCI plot for Mouse 1 in **Figure 2-figure supplement 7F**. Mouse 2

1363 individual heatmap shown in **Figure 2-figure supplement 5F**. Incorrect nose pokes

1364 heatmaps for individual mice shown in **Figure 2-figure supplement 6C-D.**

1365 J) <u>Heatmap of NBM-BLA terminal fiber activity averaged across mice</u>. Signal aligned as

1366 in D-E) with a selection of key days shown, from bottom to top: PT Day 1, PT Day 4,

1367 Early Training, Acquisition Day (white horizontal line), Last Training Day, Last Extinction

1368 Day. Black horizontal lines: divisions between training phases. Black vertical lines:

1369 divisions between breaks in time to allow for variable latencies in tone onset, correct

1370 nose poke, and receptacle entry (reward retrieval). bCI plot for cohort averaged data in

1371 **Figure 2-figure supplement 7G.** Incorrect nose poke heatmap and bCI plot averaged

1372 across mice shown in **Figure 2-figure supplement 6E + 8E.**

1373

Figure 3

BLA CaMKIIα neuron activity aligns to reward retrieval and cue-reward learning.

A) Diagram and example of injection and fiber placement sites in the BLA for recording

from CaMKIIα-Cre mice expressing a fluorescent calcium indicator (DIO-GCaMP6s).

Left: Diagram of injection and fiber placement. Right: Representative coronal brain slice

with fiber tip and GCaMP6s expression. White dashed line: BLA outline. Grey dashed

rectangle: fiber tract. Blue: DAPI, Green: GCaMP6s. Scale 500 μm. Individual fiber

placements are shown in **Figure 3-figure supplement 1A**.

B) Fluorescence traces from BLA of GCaMP6s-expressing CaMKIIα-Cre mice. During

the last day PT, (data shown for Mouse 1) correct nose pokes (green line) were

followed by a modest, but significant rise in BLA CaMKIIα cell activity that increased

steeply following receptacle entry (**Figure 3-figure supplement 1B**) while incorrect

nose pokes (grey line) were followed by a modest decrease in activity. Mean Z%ΔF/F0

overlaid on bootstrapped 99% confidence intervals (99% bCI's). Shaded significance

bars under traces represent time points where 99% bCI's do not contain 0 for at least

0.5 sec. Correct: n = 44; downsampled incorrect: n = 44 of141. Signal and reference

channels (%ΔF/F0) during nose pokes are shown in **Figure 3-figure supplement 1C-**

D. Incorrect nose pokes on last day of PT vs Training Day 1 shown in **Figure 3-figure**

supplement 2B.

C) Behavioral responding of CaMKIIα-Cre mice expressing GCaMP6s in BLA. Individual

mice acquired the task at different rates as measured by rewards earned. Horizontal

white line: acquisition threshold, when a mouse began to earn 20 rewards consistently

in Training. Incorrect nose pokes shown in **Figure 3-figure supplement 2A**.

1397   D) <u>Heatmap of BLA CaMKIIα cell activity (Mouse 1) across all training phases, aligned</u>

1398   <u>to tone onset (Tone), correct nose poke (NP), and receptacle entry (Rec)</u>. Each row is

1399   the average of rewarded trials across a training session. White horizontal line: Day

1400   acquisition threshold met, as determined by rewards earned. Black horizontal lines:

1401   divisions between training phases. Black vertical lines: divisions between breaks in time

1402   to allow for variable latencies in tone onset, correct nose poke, and receptacle entry.

1403   Blanks in the heatmaps indicate time bins added for alignment. bCI plot for Mouse 1 in

1404   **Figure 3-figure supplement 1G**. Individual heatmaps for mice 2-3 in **Figure 3-figure**

1405   **supplement 1E-F**. Incorrect nose pokes heatmaps for individual mice shown in **Figure**

1406   **3-figure supplement 2C-E.**

1407   E) <u>Heatmap of BLA CaMKIIα cell activity averaged across mice</u>. Signal aligned as in D)

1408   with a selection of key days shown, from bottom to top: PT Day 1, PT Day 4, Early

1409   Training Day, Acquisition Day (white horizontal line), Last Extinction Day. Black

1410   horizontal lines: divisions between training phases. Black vertical lines: divisions

1411   between breaks in time to allow for variable latencies in tone onset, correct nose poke,

1412   and receptacle entry. bCI plot for cohort averaged data in **Figure 3-figure supplement**

1413   **1H.** Incorrect nose poke heatmaps averaged across mice shown in **Figure 3-figure**

1414   **supplement 2F.**

1415

1416

1417 **Figure 4**

1418 **Stimulation of cholinergic terminal fibers in the BLA enhances cue-reward**

1419 **learning.**

1420 A) <u>Schematic of optical stimulation of ChAT$^+$ terminal fibers projecting to the BLA</u>. Left:

1421 Bilateral AAV injection into the NBM of ChAT-IRES-Cre mice to gain optical control over

1422 ChAT$^+$ NBM cells and representative coronal brain slice showing ChR2-EYFP

1423 expression. White dashed lines: internal capsule and globus pallidus outlines. Blue:

1424 DAPI, red: ChAT, green: ChR2-EYFP. Scale: 500 µm, individual injection sites shown in

1425 **Figure 4-figure supplement 1A** and separate channels shown in **Figure 4-figure**

1426 **supplement 1B**. Right: Bilateral optical fiber implantation above BLA to stimulate BLA-

1427 projecting ChAT$^+$ NBM cells. Representative coronal brain slice showing ChR2-EFYP

1428 expression and fiber tip placement. Grey dashed rectangle: fiber tract. White dashed:

1429 BLA outline. Blue: DAPI, red: ChAT, green: ChR2-EYFP. Scale: 500 µm, individual fiber

1430 tip placements shown in **Figure 4-figure supplement 1C** and separate channels shown

1431 in **Figure 4-figure supplement 1D**. Injection sites and fiber tip placements for males

1432 from **Figure 4-figure supplement 3C-F** shown in **Figure 4-figure supplement 4A-B.**

1433 B) <u>Optical stimulation validation *via* local field potential recordings</u>. Extracellular

1434 recording of action potentials induced by optical stimulation of ChAT$^+$ NBM cells

1435 expressing ChR2. Arrows indicate 60 ms laser pulse.

1436 <u>C-D) Details of the Cue-Reward Learning Paradigm</u> C) During Pre-Training, auditory

1437 tones were presented on a variable interval 30 schedule (VI30), during which an active

1438 nose poke (correct) yielded Ensure reward delivery and 2 sec of optical stimulation but

1439 there was no consequence for incorrect nose pokes (active nose pokes not during

1440 tone). D) Training was identical to Pre-Training, except incorrect nose pokes resulted in

1441 a 5 sec timeout, signaled by house light illumination, followed by a restarting of the ITI.

1442 E) Behavioral performance in a cue-reward learning task improves with optical

1443 stimulation of ChAT$^+$ fibers in BLA. EYFP- and ChR2-expressing mice earn similar

1444 numbers of rewards during PT (blue shaded region). ChR2-expressing mice more

1445 rapidly earn significantly more rewards than EYFP-expressing mice during Training

1446 (pink shaded region). No significant differences were observed during extinction training

1447 (orange shaded region). Horizontal white line: acquisition threshold, when a mouse

1448 began to earn ~20 rewards consistently in Training. Mean ± SEM, EYFP: n = 5, ChR2: n

1449 = 6. Individual data are shown in **Figure 4-figure supplement 3A**. Data for males

1450 shown in **Figure 4-figure supplement 3C,E.**

1451 F) EYFP- and ChR2-expressing mice made similar numbers of incorrect nose pokes

1452 during Pre-Training. ChR2-epxressing mice made significantly fewer incorrect nose

1453 pokes than EYFP-expressing mice in Training. No significant differences were observed

1454 during extinction training. Mean ± SEM, EYFP: n = 5, ChR2: n = 6. Individual data are

1455 shown in **Figure 4-figure supplement 3B**. Data for males shown in **Figure 4-figure**

1456 **supplement 3D,F.** Additional behavioral assays shown in **Figure 4-figure supplement**

1457 **5A-F**.

1458

1459 **Figure 5**

1460 **Muscarinic, but not nicotinic, ACh receptor antagonism prevents learning of a**

1461 **cue-reward contingency.**

1462 A) <u>Timeline of drug administration</u>. Saline or ACh receptor (AChR) antagonists were

1463 delivered i.p., 30 min before PT and Training sessions, the same phases of the task as

1464 optical stimulation in **Figure 4**.

1465 B) <u>Behavioral performance of mice administered AChR antagonists.</u> AChR antagonists

1466 had no significant effect on rewards earned during Pre-Training. Muscarinic AChR

1467 antagonism (Scop and Mec+Scop) resulted in significantly fewer rewards earned during

1468 Training. There was no significant difference between saline controls and those

1469 receiving the nicotinic AChR antagonist (Mec) during Training and mice extinguished

1470 responding at similar rates. Mean ± SEM Saline (n = 8), Mec (n = 9), Scop (n = 8),

1471 Mec+Scop (n = 9). Horizontal white line: acquisition threshold, when a mouse began to

1472 earn ~20 rewards consistently in Training. Individual data are shown in **Figure 5-figure**

1473 **supplement 1A**.

1474 C) <u>Incorrect nose pokes</u>. Incorrect nose poking was not affected by AChR antagonism

1475 during PT but Scop- and Scop+Mec-treated mice maintained high levels of incorrect

1476 nose pokes compared to Saline- and Mec-treated mice throughout Training. Mean ±

1477 SEM, Saline (n = 8), Mec (n = 9), Scop (n = 8), or Mec+Scop (n = 9). Individual data are

1478 shown in **Figure 5-figure supplement 1B**. AChR antagonist locomotor test shown in

1479 **Figure 5-figure supplement 1C.**

1480

1481 **Figure 6**

1482 **Non-contingent stimulation of cholinergic NBM-BLA terminals is sufficient to**

1483 **enhance cue-reward learning.**

1484 A) <u>Experimental details of laser stimulation in non-contingent ChR2mice.</u> Non-

1485 contingent-ChR2-expressing mice received the same number of light stimulations as

1486 contingent-ChR2-expressing mice, but stimulation was only given during the ITI, when

1487 non-contingent mice had not made a response within 2 sec. Injection sites and fiber

1488 placements are shown in **Figure 6-figure supplement 1A-B**.

1489 B) <u>Non-contingent NBM-BLA optical stimulation also improves behavioral performance</u>

1490 <u>in cue-reward learning task.</u> There was no significant difference in the number of

1491 rewards earned between EYFP (n = 6), contingent-ChR2 (n = 5), or non-contingent-

1492 ChR2 (n = 5) mice during Pre-Training. Contingent- and non-contingent-ChR2-

1493 expressing mice more rapidly earned significantly more rewards during Training than

1494 EYFP-expressing mice. No differences were observed between groups during extinction

1495 training. Mean ± SEM EYFP: n = 6, contingent-ChR2: n = 5, non-contingent-ChR2: n =

1496 5. Horizontal white line: acquisition threshold, when a mouse began to earn 20 rewards

1497 consistently in Training. Individual data are shown in **Figure 6-figure supplement 2A**.

1498 C) <u>Incorrect nose pokes</u>. There was no significant difference in the number of incorrect

1499 nose pokes between groups during Pre-Training. Contingent- and non-contingent-

1500 ChR2-expressing mice made significantly fewer incorrect nose pokes during Training

1501 than EYFP-expressing mice. No differences between groups were observed during

1502 extinction training. Mean ± SEM EYFP: n = 6, contingent-ChR2: n = 5, non-contingent: n

1503 = 5. Individual data are shown in **Figure 6-figure supplement 2B**.

1504 **Figure 2-figure supplement 1**

1505 **BLA ACh3.0 recording.**

1506 A) Squares indicate optical fiber tips for individual mice. 1 (red), 2 (blue), 3 (teal), 4

1507 (purple).

1508 B) Increase in fluorescence (%ΔF/F0) following correct nose pokes is specific to the

1509 signal (465 nm, green) channel and is not observed in the reference channel (405 nm,

1510 tan). Data from Mouse 1 PT Day 5 as in **Figure 2C**. Mean ± SEM, n = 24.

1511 C) Minimal increase in fluorescence (%ΔF/F0) following incorrect nose pokes. Signal

1512 (465 nm, grey) channel, reference channel (405 nm, tan). Data from Mouse 1 PT Day 5

1513 as in **Figure 2C**. Downsampled mean ± SEM, n = 24 of 58.

1514 D-F) Individual mouse data for mice 2-4 as shown in **Figure 2D**. Dashed white

1515 horizontal line: first Training day earning 10 rewards (10 Rew.). White horizontal line:

1516 acquisition threshold (Acq.).

1517 G) Mouse 1 combined action bCI plot for subset of days used in cohort averaged

1518 heatmap **Figure 2E** (From bottom to top: PT Day 1, PT Day 5, Early Training Day, First

1519 Training day earning 10 rewards, Mid Training Day, Acquisition Day, Last Training Day,

1520 Last Extinction Day). Mean overlaid on 99% bCI's for tone onset, correct nose poke,

1521 and receptacle entry. Pink and blue significance bars under traces denote time points

1522 where 99% bCI's are above or below 0 for at least 0.5 sec, respectively. Horizontal

1523 scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1524 H) Cohort averaged combined action bCI plot for subset of days used in G) and cohort

1525 averaged heatmap **Figure 2E**. Trial level mean overlaid on 99% bCI's for tone onset,

1526 correct nose poke, and receptacle entry. Pink and blue significance bars under traces

1527   denote time points where 99% bCI's are above or below 0 for at least 0.5 sec,

1528   respectively. Horizontal scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1529

Figure 2-figure supplement 2

BLA ACh3.0 recording: incorrect nose pokes.

A) Incorrect nose poking of individual mice throughout training.

B) Incorrect nose pokes that yield timeouts (downsampled Training Day 1, pink line, n = 24 of 66) result in a modest increase in BLA ACh signaling but incorrect nose pokes before timeouts are introduced (downsampled PT Day 5, blue line, n = 24 of 58) do not. Data from Mouse 1 as in **Figure 2C**, Mean Z%ΔF/F0 overlaid on bootstrapped 99% confidence intervals (99% bCI's). Shaded significance bars under traces represent time points where 99% bCI's do not contain 0 for at least 0.5 sec.

C-F) Individual mouse heatmaps of BLA ACh signaling across all training phases, aligned to incorrect nose poke. Each row is the average of incorrect nose pokes that led to (or would have led to for PT) a timeout across a session. White dashed horizontal line: first Training day earning 10 rewards. Horizontal white line: acquisition threshold, when a mouse began to earn ~20 rewards consistently in Training. Black horizontal lines: divisions between training phases.

G) Heatmap of BLA ACh signaling during incorrect nose poke averaged across mice. Signal aligned as in C-F) with a selection of data from key days in the behavioral paradigm shown. From bottom to top: PT Day 1, PT Day 5, Early Training Day, First Training day earning 10 rewards (white dashed horizontal line), Mid Training Day, Acquisition Day (white horizontal line), Last Training Day, Last Extinction Day. Black horizontal lines: divisions between training phases.

H) Cohort averaged bCI plot for subset of days used in G). Trial level mean (downsampled to 20) overlaid on 99% bCI's for incorrect nose poke. Pink and blue

1553 significance bars under traces denote time points where 99% bCI's are above or below

1554 0 for at least 0.5 sec, respectively. It is unclear how to interpret the timepoints

1555 significantly below 0 before and after incorrect nose pokes pulled out by bootstrapping.

1556 This may be an artefact of the small signal across incorrect trials, although this remains

1557 to be investigated. Horizontal scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1558

1559 **Figure 2-figure supplement 3**

1560 **BLA ACh3.0 recording replicate.**

1561 A) Squares indicate optical fiber tips for individual mice. 5 (orange), 6 (cyan), 7 (brown),

1562 8 (navy).

1563 B) Behavioral responding of mice during BLA ACh3.0 recordings. Individual mice

1564 acquired the task at different rates as measured by rewards earned. White horizontal

1565 line: acquisition threshold, when a mouse began to earn 20 rewards consistently in

1566 Training. Incorrect nose pokes shown in **Figure 2-figure supplement 4A.**

1567 C-F) Heatmaps of BLA ACh signaling in mice 5-8 across all training phases, aligned to

1568 tone onset (Tone), correct nose poke (NP), and receptacle entry (Rec). Each row is the

1569 average of rewarded trials across a training session. Horizontal white line: acquisition

1570 threshold, when a mouse began to earn 20 rewards consistently in Training. Black

1571 horizontal lines: divisions between training phases. Black vertical lines: divisions

1572 between breaks in time to allow for variable latencies in tone onset, correct nose poke,

1573 and receptacle entry (reward retrieval). Blanks in the heatmaps indicate time bins added

1574 for alignment.

1575 G) Heatmap of BLA ACh signaling averaged across mice 5-8. Signal aligned as in C-F)

1576 with a selection of key days shown, from bottom to top: PT Day 1, PT Day 4, Early

1577 Training, Acquisition Day (white horizontal line), Last Training Day, Last Extinction Day.

1578 Black horizontal lines: divisions between training phases. Black vertical lines: divisions

1579 between breaks in time to allow for variable latencies in tone onset, correct nose poke,

1580 and receptacle entry (reward retrieval).

1581   H) Mouse 5 combined action bCI plot for subset of days used in G). Mean overlaid on

1582   99% bCI's for tone onset, correct nose poke, and receptacle entry. Pink and blue

1583   significance bars under traces denote time points where 99% bCI's are above or below

1584   0 for at least 0.5 sec, respectively. Horizontal scale = 1 sec. Vertical scale = 5

1585   Z%ΔF/F0.

1586   I) Cohort (mice 5-8) averaged combined action bCI plot for subset of days used in G-H).

1587   Trial level mean overlaid on 99% bCI's for tone onset, correct nose poke, and

1588   receptacle entry. Pink and blue significance bars under traces denote time points where

1589   99% bCI's are above or below 0 for at least 0.5 sec, respectively. Horizontal scale = 1

1590   sec. Vertical scale = 5 Z%ΔF/F0.

1591

1592

Figure 2-figure supplement 4

BLA ACh3.0 recording replicate: incorrect nose pokes.

A) Incorrect nose poking of individual mice throughout training.

B-E) Individual mouse heatmaps of BLA ACh signaling across all training phases,

aligned to incorrect nose poke. Each row is the average of incorrect nose pokes that led

to (or would have led to for PT) a timeout across a session. Horizontal white line:

acquisition threshold, when a mouse began to earn 20 rewards consistently in Training.

Black horizontal lines: divisions between training phases.

F) Heatmap of BLA ACh signaling during incorrect nose poke averaged across mice.

Signal aligned as in B-E) with a selection of data from key days in the behavioral

paradigm shown. From bottom to top: PT Day 1, PT Day 4, Early Training Day,

Acquisition Day (white horizontal line), Last Training Day, Last Extinction Day. Black

horizontal lines: divisions between training phases.

G) Cohort averaged bCI plot for subset of days used in F). Downsampled trial level

mean overlaid on 99% bCI's for incorrect nose poke. Pink and blue significance bars

under traces denote time points where 99% bCI's are above or below 0 for at least 0.5

sec, respectively. Horizontal scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1611 **Figure 2-figure supplement 5**

1612 **NBM-BLA GCAMP7s recording in cholinergic terminal fibers.**

1613 A) Circles indicate NBM DIO-GCaMP7s injection sites for individual mice, 1 (red), 2

1614 (blue). Triangles indicate estimated optical fiber tips based on adjacent slices for

1615 individual mice. 1 (red), 2 (blue).

1616 B) Representative injection site coronal slice from **Figure 2F** with channels separated.

1617 Scale = 500 μm.

1618 C) Representative fiber tip site coronal slice from **Figure 2F** with channels separated.

1619 Scale = 500 μm.

1620 D) Increase in fluorescence (%ΔF/F0) following correct nose pokes is specific to the

1621 signal (465 nm, green) channel and is not observed in the reference channel (405 nm,

1622 tan). Data from Mouse 1 PT Day 4 as in **Figure 2H**. Mean ± SEM, n = 42.

1623 E) Minimal increase in fluorescence (%ΔF/F0) following incorrect nose pokes. Signal

1624 (465 nm, grey) channel, reference channel (405 nm, tan). Data from Mouse 1 PT Day 4

1625 as in **Figure 2H**. Downsampled mean ± SEM, n = 42 of 101.

1626 F) Individual data for mouse 2 as shown in **Figure 2I**. White horizontal line: acquisition

1627 threshold.

1628 G) Mouse 1 combined action bCI plot for subset of days used in cohort averaged

1629 heatmap **Figure 2J** (From bottom to top: PT Day 1, PT Day 4, Early Training Day,

1630 Acquisition Day, Last Training Day, Last Extinction Day). Mean overlaid on 99% bCI's

1631 for tone onset, correct nose poke, and receptacle entry. Pink and blue significance bars

1632 under traces denote time points where 99% bCI's are above or below 0 for at least 0.5

1633 sec, respectively. Horizontal scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1634 **Figure 2-figure supplement 6**

1635 **NBM-BLA GCAMP7s recording in cholinergic terminal fibers: incorrect nose**

1636 **pokes.**

1637 A) Incorrect nose poking of individual mice throughout training.

1638 B) Incorrect nose pokes that yield timeouts (downsampled Training Day 1, pink line, n =

1639 42 of 105) result in a significant increase in NBM-BLA terminal fiber activity, but

1640 incorrect nose pokes before timeouts are introduced (downsampled PT Day 4, blue line,

1641 n = 42 of 101) do not. Data from Mouse 1 as in **Figure 2H**, mean Z%ΔF/F0 overlaid on

1642 bootstrapped 99% confidence intervals (99% bCI's). Shaded significance bars under

1643 traces represent time points where 99% bCI's do not contain 0 for at least 0.5 sec.

1644 C-D) Individual mouse heatmaps of NBM-BLA terminal fiber activity across all training

1645 phases, aligned to incorrect nose poke. Each row is the average of incorrect nose

1646 pokes that led to (or would have led to for PT) a timeout across a session. Horizontal

1647 white line: acquisition threshold, when a mouse began to earn 20 rewards consistently

1648 in Training. Black horizontal lines: divisions between training phases.

1649 E) Heatmap of NBM-BLA terminal fiber activity during incorrect nose poke averaged

1650 across mice. Signal aligned as in C-D) with a selection of data from key days in the

1651 behavioral paradigm shown. From bottom to top: PT Day 1, PT Day 4, Early Training

1652 Day, Acquisition Day (white horizontal line), Last Training Day, Last Extinction Day.

1653 Black horizontal lines: divisions between training phases.

1654 **Figure 2-figure supplement 7**

1655 **NBM-BLA GCAMP7s recording in cholinergic terminal fibers replicate.**

1656 A) Circles indicate NBM DIO-GCaMP7s injection sites for individual mice, 3 (teal), 4

1657 (purple). Squares indicate optical fiber tips for individual mice, 3 (teal), 4 (purple).

1658 B) Behavioral responding of mice during NBM-BLA recordings. Individual mice acquired

1659 the task at different rates as measured by rewards earned. White horizontal line:

1660 acquisition threshold, when a mouse began to earn 20 rewards consistently in Training.

1661 Incorrect nose pokes shown in **Figure 2-figure supplement 8A.**

1662 C-D) Heatmaps of NBM-BLA terminal fiber activity in mice 3-4 across all training

1663 phases, aligned to tone onset (Tone), correct nose poke (NP), and receptacle entry

1664 (Rec). Each row is the average of rewarded trials across a training session. Horizontal

1665 white line: acquisition threshold, when a mouse began to earn 20 rewards consistently

1666 in Training. Black horizontal lines: divisions between training phases. Black vertical

1667 lines: divisions between breaks in time to allow for variable latencies in tone onset,

1668 correct nose poke, and receptacle entry (reward retrieval). Blanks in the heatmaps

1669 indicate time bins added for alignment.

1670 E) Heatmap of NBM-BLA terminal fiber activity averaged across mice 3-4. Signal

1671 aligned as in C-D) with a selection of key days shown, from bottom to top: PT Day 1, PT

1672 Day 4, Early Training, Acquisition Day (white horizontal line), Last Training Day, Last

1673 Extinction Day. Black horizontal lines: divisions between training phases. Black vertical

1674 lines: divisions between breaks in time to allow for variable latencies in tone onset,

1675 correct nose poke, and receptacle entry (reward retrieval).

1676 F) Mouse 3 combined action bCI plot for subset of days used in cohort averaged

1677 heatmap E). Mean overlaid on 99% bCI's for tone onset, correct nose poke, and

1678 receptacle entry. Pink and blue significance bars under traces denote time points where

1679 99% bCI's are above or below 0 for at least 0.5 sec, respectively. Horizontal scale = 1

1680 sec. Vertical scale = 5 Z%ΔF/F0.

1681 G) Cohort (mice 1-4) averaged combined action bCI plot for subset of days used in E-

1682 F). Trial level mean overlaid on 99% bCI's for tone onset, correct nose poke, and

1683 receptacle entry. Pink and blue significance bars under traces denote time points where

1684 99% bCI's are above or below 0 for at least 0.5 sec, respectively. Horizontal scale = 1

1685 sec. Vertical scale = 5 Z%ΔF/F0.

Figure 2-figure supplement 8

NBM-BLA GCAMP7s recording in cholinergic terminal fibers replicate: incorrect

nose pokes.

1689   A) Incorrect nose poking of individual mice throughout training.

1690   B-C) Individual mouse heatmaps of NBM-BLA terminal fiber activity across all training

1691   phases, aligned to incorrect nose poke. Each row is the average of incorrect nose

1692   pokes that led to (or would have led to for PT) a timeout across a session. Horizontal

1693   white line: acquisition threshold, when a mouse began to earn 20 rewards consistently

1694   in Training. Black horizontal lines: divisions between training phases.

1695   D) Heatmap of NBM-BLA terminal fiber activity during incorrect nose poke averaged

1696   across mice 3-4. Signal aligned as in B-C) with a selection of data from key days in the

1697   behavioral paradigm shown. From bottom to top: PT Day 1, PT Day 4, Early Training

1698   Day, Acquisition Day (white horizontal line), Last Training Day, Last Extinction Day.

1699   Black horizontal lines: divisions between training phases.

1700   E) Cohort (mice 1-4) averaged bCI plot for subset of days used in D). Trial level mean

1701   (downsampled to 20) overlaid on 99% bCI's for incorrect nose poke. Pink and blue

1702   significance bars under traces denote time points where 99% bCI's are above or below

1703   0 for at least 0.5 sec, respectively. As for other experiments measuring signal before

1704   and after incorrect nosepokes, it is unclear how to interpret the timepoints significantly

1705   below 0 pulled out by bootstrapping. As mentioned previously, this may be an artefact of

1706   the small signal across incorrect trials, although this remains to be investigated.

1707   Horizontal scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1708

Figure 2-figure supplement 9

Simultaneous BLA ACh3.0 + GCAMP7s recording in NBM-BLA cholinergic

terminal fibers.

A) Left: DIO-jRCaMP1b was injected in the NBM of ChAT-IRES-Cre mice.

Representative coronal brain slice showing jRCaMP1b expression. Yellow dashed lines:

internal capsule and globus pallidus outlines. Scale = 500 µm. White box: higher

magnification area shown in B).

B) Higher magnification of injection site. Scale = 100 µm.

C) Circle indicates NBM DIO-jRCaMP1b injection site for mouse 1.

D) ACh3.0 was injected into the ipsilateral BLA and an optical fiber was implanted

above the BLA. White dashed line: BLA outline. Scale = 500 µm.

E) Squares indicate optical fiber tips for individual mice. ACh3.0 + RCaMP (red),

ACh3.0 + RCaMP sham (grey),

F) A substantial increase in both fluorescence representing BLA ACh release (green

line) and NBM-BLA cholinergic terminal activity (magenta line) coincided with correct

nose pokes on last day of PT. Mean Z%ΔF/F0 overlaid on bootstrapped 99%

confidence intervals (99% bCI's). Shaded significance bars under traces represent time

points where 99% bCI's do not contain 0 for at least 0.5 sec. n = 42.

G) Modest increase in fluorescence in both channels following incorrect nose pokes on

last day of PT. Mean Z%ΔF/F0 overlaid on bootstrapped 99% confidence intervals (99%

bCI's). Shaded significance bars under traces represent time points where 99% bCI's

do not contain 0 for at least 0.5 sec. Downsampled n = 42 of 94.

1731   H) jRCaMP1b signal is not simply crosstalk from ACh3.0 channel. A substantial

1732   increase in fluorescence representing BLA ACh release (green line) following correct

1733   nose pokes did not necessitate signal in RCaMP sham red channel (grey line). Last day

1734   of PT. Mean Z%ΔF/F0 overlaid on bootstrapped 99% confidence intervals (99% bCI's).

1735   Shaded significance bars under traces represent time points where 99% bCI's do not

1736   contain 0 for at least 0.5 sec. n = 44.

1737   I) Incorrect nose pokes on Last day of PT, as in G), for ACh3.0 + RCaMP sham mouse.

1738   Downsampled n = 44 of 135.

1739

1740

1741 **Figure 3-figure supplement 1**

1742 **GCAMP6s recording in BLA CaMKIIα cells.**

1743 A) Squares indicate optical fiber tips for individual mice. 1 (red), 2 (blue), 3 (teal).

1744 B) Increase in fluorescence (Z%ΔF/F0) during last day of PT (data shown for Mouse 1)

1745 aligns more closely to receptacle entry (reward retrieval) on rewarded trials. Mean ±

1746 SEM, n = 44.

1747 C) Increase in fluorescence (%ΔF/F0) following correct nose pokes is specific to the

1748 signal (465 nm, green) channel and is not observed in the reference channel (405 nm,

1749 tan). Mirroring in reference channel following correct nose poke is likely due to a change

1750 in the pool of unbound tracer, because data are not acquired at the "true" isosbestic

1751 point of GCaMP (Barnett et al., 2017; C. K. Kim et al., 2016; Sych et al., 2019)). Data

1752 from Mouse 1, PT Day 4 as in **Figure 3B**. Mean ± SEM, n = 44.

1753 D) Decrease in fluorescence (%ΔF/F0) following incorrect nose pokes is seen in signal

1754 channel (465 nm, grey), but not reference channel (405 nm, tan). Data from Mouse 1,

1755 PT Day 4 as in **Figure 3B**. Downsampled mean ± SEM, n = 44 of 141.

1756 E-F) Individual data for mice not shown in **Figure 3D**. White horizontal line: acquisition

1757 threshold.

1758 G) Mouse 1 combined action bCI plot for subset of days used in cohort averaged

1759 heatmap **Figure 3E** (from bottom to top: PT Day 1, PT Day 4, Early Training Day,

1760 Acquisition Day, Last Extinction Day). Mean overlaid on 99% bCI's for tone onset,

1761 correct nose poke, and receptacle entry. Pink and blue significance bars under traces

1762 denote time points where 99% bCI's are above or below 0 for at least 0.5 sec,

1763 respectively. Horizontal scale = 1 sec. Vertical scale = 1 Z%ΔF/F0.

1764   H) Cohort averaged combined action bCI plot for subset of days used in cohort

1765   averaged heatmap **Figure 3E**. Trial level mean overlaid on 99% bCI's for tone onset,

1766   correct nose poke, and receptacle entry. Pink and blue significance bars under traces

1767   denote time points where 99% bCI's are above or below 0 for at least 0.5 sec,

1768   respectively. Horizontal scale = 1 sec. Vertical scale = 1 Z%ΔF/F0.

1769

Figure 3-figure supplement 2

GCAMP6s recording in BLA CaMKIIα cells: incorrect nose pokes.

A) Incorrect nose pokes of individual mice throughout training.

B) Both incorrect nose pokes that yield timeouts (downsampled Training Day 1, pink line, n = 44 of 124) and incorrect nose pokes before timeouts are introduced (downsampled PT Day 4, blue line, n = 44 of 141) result in a modest decrease in BLA CaMKIIα neuron activity. Data from Mouse 1 as in **Figure 3B**, mean Z%ΔF/F0 overlaid on bootstrapped 99% confidence intervals (99% bCI's). Shaded significance bars under traces represent time points where 99% bCI's do not contain 0 for at least 0.5 sec.

C-E) Individual mouse heatmaps of BLA CaMKIIα neuron activity across all training phases, aligned to incorrect nose poke. Each row is the average of incorrect nose pokes that led to (or would have led to for PT) a timeout across a session. Horizontal white line: acquisition threshold, when a mouse began to earn ~20 rewards consistently in Training. Black horizontal lines: divisions between training phases.

F) Heatmap of BLA CaMKIIα neuron activity during incorrect nose poke averaged across mice. Signal aligned as in C-E) with a selection of data from key days in the behavioral paradigm shown. From bottom to top: PT Day 1, PT Day 4, Early Training Day, Acquisition Day (white horizontal line), Last Extinction Day. Black horizontal lines: divisions between training phases.

G) Cohort averaged bCI plot for subset of days used in F). Trial level mean (downsampled to 20) overlaid on 99% bCI's for incorrect nose poke. Pink and blue significance bars under traces denote time points where 99% bCI's are above or below 0 for at least 0.5 sec, respectively. It is unclear how to interpret the substantial number

1793   of timepoints significantly below 0 before and after incorrect nose pokes and may be an

1794   artifact. Horizontal scale = 1 sec. Vertical scale = 1 Z%ΔF/F0.

1795

Figure 3-figure supplement 3

GCAMP6s recording in BLA CaMKIIα replicate.

A) Squares indicate optical fiber tips for individual mice. 4(purple), 5 (orange), 6 (cyan), 7 (brown).

B) Behavioral responding of mice during BLA CaMKIIα GCaMP6s recordings. Individual mice acquired the task at different rates as measured by rewards earned. White horizontal line: acquisition threshold, when a mouse began to earn 20 rewards consistently in Training. Incorrect nose pokes shown in **Figure 3-figure supplement 4A.**

C-F) Heatmaps of BLA CaMKIIα cell activity in mice 4-7 across all training phases, aligned to tone onset (Tone), correct nose poke (NP), and receptacle entry (Rec). Each row is the average of rewarded trials across a training session. Horizontal white line: acquisition threshold, when a mouse began to earn 20 rewards consistently in Training. Black horizontal lines: divisions between training phases. Black vertical lines: divisions between breaks in time to allow for variable latencies in tone onset, correct nose poke, and receptacle entry (reward retrieval). Blanks in the heatmaps indicate time bins added for alignment.

G) Mouse 4 combined action bCI plot for subset of days, from bottom to top: PT Day 1, PT Day 4, Early Training Day, Acquisition Day, Last Training Day, Last Extinction Day. Mean overlaid on 99% bCI's for tone onset, correct nose poke, and receptacle entry. Pink and blue significance bars under traces denote time points where 99% bCI's are above or below 0 for at least 0.5 sec, respectively. Horizontal scale = 1 sec. Vertical scale = 5 Z%ΔF/F0.

1819 **Figure 3-figure supplement 4**

1820 **GCAMP6s recording in BLA CaMKIIα replicate: incorrect nose pokes.**

1821 A) Incorrect nose poking of individual mice throughout training.

1822 B-E) Individual mouse heatmaps of BLA CaMKIIα cell activity across all training phases,

1823 aligned to incorrect nose poke. Each row is the average of incorrect nose pokes that led

1824 to (or would have led to for PT) a timeout across a session. Horizontal white line:

1825 acquisition threshold, when a mouse began to earn 20 rewards consistently in Training.

1826 Black horizontal lines: divisions between training phases.

1827

1828 **Figure 4-figure supplement 1**

1829 **Injection sites and optical fiber placements.**

1830 A) Circles indicate NBM injection sites for individual mice, EYFP (green) and ChR2

1831 (blue). Anterior/Posterior position relative to Bregma indicated.

1832 B) Representative injection site coronal slice from **Figure 4A** with channels separated.

1833 Scale = 500 µm.

1834 C) Squares indicate observable optical fiber tips for individual mice, EYFP- (green) and

1835 ChR2-expressing mice (blue). Triangles indicate estimated optical fiber tips based on

1836 adjacent slices. Anterior/Posterior position relative to Bregma indicated.

1837 D) Representative fiber tip site coronal slice from **Figure 4A** with channels separated.

1838 Scale = 500

1839

1840 **Figure 4-figure supplement 2**

1841 *Ex vivo* **electrophysiology.**

1842 A-G) Current clamp recordings of ChAT^{+} NBM cells expressing ChR2. Optical

1843 stimulation was delivered at the indicated frequency.

1844

1845 **Figure 4-figure supplement 3**

1846 **Individual behavioral data and male cohort.**

1847 A) Rewards earned for individual mice from **Figure 4E**. Horizontal white line: acquisition

1848 threshold, when a mouse began to earn ~20 rewards consistently in Training.

1849 B) Incorrect nose pokes for individual mice from **Figure 4F**.

1850 C) Optical stimulation of ChAT$^+$ NBM-BLA terminal fibers (ChR2-expressing mice, blue

1851 squares) had a similar effect on rewards earned during Training in male mice compared

1852 to female mice. Mean ± SEM, EYFP: n = 7, ChR2: n = 7. Horizontal white line:

1853 acquisition threshold, when a mouse began to earn ~20 rewards consistently in

1854 Training.

1855 D) Optical stimulation of ChAT$^+$ NBM-BLA terminal fibers (ChR2-expressing mice, blue

1856 squares) had a similar effect on incorrect nose pokes during Training in male mice

1857 compared to female mice. Mean ± SEM, EYFP: n = 7, ChR2: n = 7.

1858 E) Individual data for graph shown in C).

1859 F) Individual data for graph shown in D).

1860

1861   **Figure 4-figure supplement 4**

1862   **Injection sites and optical fiber placements.**

1863   A) Circles indicate NBM injection sites for individual mice, EYFP- (green) and ChR2-

1864   expressing mice (blue). Anterior/Posterior position relative to Bregma indicated.

1865   B) Squares indicate observable optical fiber tips for individual mice, EYFP- (green) and

1866   ChR2-expressing mice (blue). Triangles indicate estimated site of optical fiber tips

1867   based on adjacent slices. Anterior/Posterior position relative to Bregma indicated.

1868

Figure 4-figure supplement 5

Additional behavioral assays with NBM-BLA optical stimulation.

A) Stimulation of ChAT$^+$ NBM-BLA terminal fibers did not support self-stimulation. Mice were allowed to nose poke for 2 sec of stimulation in the Training paradigm. Data for female mice from **Figure 4**, **Figure 4-figure supplement 1, Figure 4-figure supplement 3A-B.**

B) Stimulation of ChAT$^+$ NBM-BLA terminal fibers did not support real time place preference. Mice were allowed to move freely between two sides of an empty cage with distinct floor contexts for 15 min. Data are reported as percent time spent on the laser-paired side. Closed circles: Mean ± SEM, open circles: data for individual mice. Data for female mice from **Figure 4**, **Figure 4-figure supplement 1, Figure 4-figure supplement 3A-B.**

C) Stimulation of ChAT$^+$ NBM-BLA terminal fibers during a progressive ratio test did not affect active nose poking. Closed squares: Mean ± SEM, open squares: individual mice. Data for male mice from **Figure 4-figure supplement 3C-F + 4**.

D) There were no differences between EYFP- and ChR2-expressing mice in locomotor activity. X-axis ticks = 5 min bins, Laser = 5 min of 20 sec on/off optical stimulation. Closed circles: Mean ± SEM, open circles: data for individual mice. Data for female mice from **Figure 4**, **Figure 4-figure supplement 1, Figure 4-figure supplement 3A-B.**

E-F) No difference in behavior was seen between EYFP- and ChR2-expressing mice on any measures in the Light/Dark Box Test. Data for female mice from **Figure 4**, **Figure 4-figure supplement 1, Figure 4-figure supplement 3A-B.**

1892 **Figure 5-figure supplement 1**

1893 **Individual behavioral data and locomotion.**

1894 A) Rewards earned for individual mice from **Figure 5B**. Horizontal white line: acquisition

1895 threshold, when a mouse began to earn ~20 rewards consistently in Training.

1896 B) Incorrect nose pokes for individual mice from **Figure 5C**.

1897 C) There were no differences in locomotion for antagonists.

1898

1899 **Figure 6-figure supplement 1**

1900 **Injection sites and optical fiber placements.**

1901 A) Circles indicate NBM injection sites for individual mice, EYFP-expressing (green),

1902 contingent-ChR2-expressing (blue), and non-contingent-ChR2-expressing mice (cyan).

1903 Anterior/Posterior position relative to Bregma indicated.

1904 B) Squares indicate observable optical fiber tips for individual mice, EYFP-expressing

1905 (green), contingent-ChR2-expressing (blue), and non-contingent-ChR2-expressing mice

1906 (cyan). Triangles indicate estimated site of optical fiber tips based on adjacent slices.

1907 Anterior/Posterior position relative to Bregma indicated.

1908

1909 **Figure 6-figure supplement 2**

1910 **Individual behavioral data.**

1911 A) Rewards earned for individual mice from **Figure 6B**. Horizontal white line: acquisition

1912 threshold, when a mouse began to earn ~20 rewards consistently in Training.

1913 B) Incorrect nose pokes for individual mice from **Figure 6C**.

1914

Supplementary File 1

Supplementary Table 1: Number of mice that acquired the reward learning behavior, number that were excluded and any training deviations.

A) Mice in the initial BLA ACh3.0 group were trained outside of the sound attenuating chambers. These mice had 5 days of Pre-Training because they were trained concurrently with another cohort of mice (not shown) that required an extra day to reach two consecutive days of 20 rewards earned and were advanced to a VI 20 schedule of reinforcement during Training after 9 days to promote responding. Training was extended to allow all mice to acquire. Due to time constraints during acquisition, mouse 3 in this cohort was moved to Extinction after 20 days of Training because it had acquired earlier, was earning the most rewards, and we wanted to record more extinction days.

B) Mice in the BLA ACh3.0 and NBM-BLA terminal fiber replicate experiments were advanced to one day of Extinction after only 7 days of Training due to the COVID-19 shutdown.

C) BLA ACh3.0 and NBM-BLA terminal fiber jRCaMP1b mice were analyzed as dual channel mice just through Pre-Training and were instead used as replicates of the BLA ACh3.0 experiment. One of the mice had apparatus errors during Training and had to be excluded.

D) Mice in the initial BLA CaMKIIα GCaMP6 were trained outside of the sound attenuating chambers. Mouse 1 progressed from Pre-Training to Training a day earlier than the rest of the group and was able to have an extra day of Training before the 2 days of Extinction. Mice in this group were advanced to a VI 20 schedule of

1938   reinforcement during Training after 6-7 days to promote responding. Training was

1939   extended to allow more mice to acquire.

1940

A)

Surgery — Start Food Restriction — Handling — Operant Familiarization — Pre-Training — Training — Extinction

| 7 days | 7 days | 7 days | 5 - 6 days | 4 - 5 days | 12 - 21 days | 2 - 4 days |

B)

FP Rig or Laser

Timeout Light

Tone

Nose Pokes: Inactive, Active

Reward Receptacle

C) Pre-Training

Tone — 10s — ITI ~ 30s

Active Nose Poke — Correct — Incorrect

Reward Delivery — 2s — 2s

Receptacle Light

Receptacle Entry

D) Training

Tone — 10s — Restart ITI — ITI ~ 30s

Active Nose Poke

Reward Delivery — 2s — 2s

Receptacle Light

Receptacle Entry

Timeout Light — 5s

A) ACh3.0

DAPI
ACh3.0

B) Rewards
PT   Training   Ext

C) PT Nose Pokes
Correct
Incorrect
Z % ΔF/F0
Time (s)

D) Mouse 1 Individual Data
Ext
Acq.
10 Rew.
Training
PT
Sessions
Z % ΔF/F0
Tone NP Rec
5s 2s 2s 5s
0.5s

E) ACh3.0 Averaged Data
Ext
Acq.
10 Rew.
Training
PT
Sessions
Z % ΔF/F0
Tone NP Rec
5s 2s 2s 5s
0.5s

F) DIO-GCaMP7s
DAPI
ChAT
GCaMP7s

G) Rewards
PT   Training   Ext

H) PT Nose Pokes
Correct
Incorrect
Z % ΔF/F0
Time (s)

I) Mouse 1 Individual Data
Ext
Acq.
Training
PT
Sessions
Z % ΔF/F0
Tone NP Rec
5s 2s 2s 5s
0.5s

J) NBM-BLA Averaged Data
Ext
Acq.
Training
PT
Sessions
Z % ΔF/F0
Tone NP Rec
5s 2s 2s 5s
0.5s

A) DIO-GCaMP6s

DAPI
GCaMP6s

B) PT Nose Pokes

Correct
Incorrect

Z % ΔF/F0

Time (s)

C) Rewards

1   3
2

Rewards

PT   Training   Ext

D) Mouse 1 Individual Data

Ext

Training

Acq.

PT

Z % ΔF/F0

Tone   NP   Rec

5s   2s   2s   5s

0.5s

E) Averaged Data

Ext

Training

Acq.

PT

Z % ΔF/F0

Tone   NP   Rec

5s   2s   2s   5s

0.5s

A)

DAPI
ChAT
ChR2-EYFP

DIO-ChR2-EYFP
or
DIO-EYFP

DAPI
ChAT
ChR2-EYFP

B)

5 mV

2 s

C) Pre-Training

Tone	10s		ITI ~ 30s	
Active Nose Poke	Correct	Incorrect		
Reward Delivery	2s		2s	
Laser	2s		2s	
Receptacle Light				
Receptacle Entry				

D) Training

Restart ITI

Tone	10s		ITI ~ 30s	
Active Nose Poke				
Reward Delivery	2s		2s	
Laser	2s		2s	
Receptacle Light				
Receptacle Entry				
Timeout Light			5s	

E) Rewards

Interaction: p = 0.0009
Main effect: p = 0.0133

EYFP
ChR2

PT   Training   Ext

F) Incorrect Nose Pokes

Main effect: p = 0.0061

EYFP
ChR2

PT   Training   Ext

A) Start Food Restriction — 7 days — Handling — 7 days — Operant Familiarization — 5 days — Pre-Training (4 days) — Training (12 days) — Extinction (3 days)

Daily i.p. injections
30 min before training

B) Rewards

Saline, Scop, Mec, Mec+Scop

Interaction: p < 0.0001
Main effect: p < 0.0001

PT — Training — Ext

C) Incorrect Nose Pokes

Main effect: p < 0.0001

Saline, Scop, Mec, Mec+Scop

PT — Training — Ext

A)

Non-Contingent-ChR2

Tone — 10s — ITI ~ 30s

Active Nose Poke

Reward Delivery — 2s

Laser — 2s

Receptacle Light

Receptacle Entry

B)

Rewards

- EYFP
- Contingent-ChR2
- Non-contingent-ChR2

Interaction: p = 0.0164
Main effect: p = 0.0077

Rewards

PT   Training   Ext

C)

Incorrect Nose Pokes

Main effect: p = 0.0262

- EYFP
- Contingent-ChR2
- Non-contingent-ChR2

Nose Pokes

PT   Training   Ext

A) Fiber Tip Placements

Anterior ← → Posterior

B) Correct Nose Pokes

C) Incorrect Nose Pokes

D) Mouse 2 Individual Data

E) Mouse 3 Individual Data

F) Mouse 4 Individual Data

G) Mouse 1 Individual Data

H) Averaged Data

A) Incorrect Nose Pokes

B) Incorrect Nose Pokes

C) Mouse 1 Individual Data

D) Mouse 2 Individual Data

E) Mouse 3 Individual Data

F) Mouse 4 Individual Data

G) ACh3.0 Averaged Data

H) Averaged Data

A) Fiber Tip Placements

Anterior ← → Posterior

B) Rewards

C) Mouse 5 Individual Data

D) Mouse 6 Individual Data

E) Mouse 7 Individual Data

F) Mouse 8 Individual Data

G) Averaged Data

H) Mouse 5 Individual Data

I) Averaged Data

A) Incorrect Nose Pokes

B) Mouse 5 Individual Data

C) Mouse 6 Individual Data

D) Mouse 7 Individual Data

E) Mouse 8 Individual Data

F) Averaged Data

G) Averaged Data

A) Injection Sites | Fiber Tip Placements

Anterior ←————→ Posterior

B) Merge | DAPI | GCaMP7 | ChAT

C) Merge | DAPI | GCaMP7 | ChAT

D) Correct Nose Pokes

Reference
Signal

% ΔF/F0

Time (s)

E) Incorrect Nose Pokes

Reference
Signal

% ΔF/F0

Time (s)

F) Mouse 2 Individual Data

Ext
Acq.
Training
PT

Sessions

Tone | NP | Rec

Z % ΔF/F0

5s | 2s | 2s | 5s

0.5s

G) Mouse 1 Individual Data

Ext
Acq.
Training
PT

Sessions

Tone | NP | Rec

5s | 2s | 2s | 5s

0.5s

A) Incorrect Nose Pokes

B) Incorrect Nose Pokes

C) Mouse 1 Individual Data

D) Mouse 2 Individual Data

E) NBM-BLA Averaged Data

A) Injection Sites / Fiber Tip Placements

Anterior ⟷ Posterior

B) Rewards

C) Mouse 3 Individual Data

D) Mouse 4 Individual Data

E) Averaged Data

F) Mouse 3 Individual Data

G) Averaged Data

A) Incorrect Nose Pokes

B) Mouse 3 Individual Data

C) Mouse 4 Individual Data

D) Averaged Data

E) Averaged Data

A) DIO-jRCaMP1b

Merge | DAPI
jRCaMP1b | ChAT

B)

Merge | DAPI
jRCaMP1b | ChAT

C) Injection Site

D) ACh3.0

Merge | DAPI | ACh3.0 | DIO-jRCaMP1b | ChAT

E) Fiber Tip Placements

ACh3.0 + RCaMP

ACh3.0 + RCaMP sham

F) Correct Nose Pokes

ACh3.0
RCaMP

Z % ΔF/F0

Time (s)

G) Incorrect Nose Pokes

ACh3.0
RCaMP

Z % ΔF/F0

Time (s)

H) Correct Nose Pokes

ACh3.0
RCaMP Sham

Z % ΔF/F0

Time (s)

I) Incorrect Nose Pokes

ACh3.0
RCaMP Sham

Z % ΔF/F0

Time (s)

A) Fiber Tip Placements

Anterior ⟷ Posterior

B) PT Receptacle Entry

C) Correct Nose Pokes

D) Incorrect Nose Pokes

E) Mouse 2 Individual Data

F) Mouse 3 Individual Data

G) Mouse 1 Individual Data

H) Averaged Data

A) Incorrect Nose Pokes

B) Incorrect Nose Pokes

C) Mouse 1 Individual Data

D) Mouse 2 Individual Data

E) Mouse 3 Individual Data

F) Averaged Data

G) Averaged Data

A) Fiber Tip Placements

Anterior ← → Posterior

B) Rewards

C) Mouse 4 Individual Data

D) Mouse 5 Individual Data

E) Mouse 6 Individual Data

F) Mouse 7 Individual Data

G) Mouse 4 Individual Data

A) Incorrect Nose Pokes

B) Mouse 4 Individual Data

C) Mouse 5 Individual Data

D) Mouse 6 Individual Data

E) Mouse 7 Individual Data

A) Injection Sites

Anterior

-0.22 mm

-0.70 mm

Posterior

-0.58 mm

-1.06 mm

B)

Merge

DAPI

ChR2

ChAT

C) Fiber Tip Placements

Anterior

-0.82 mm

-1.46 mm

Posterior

-1.34 mm

-1.82 mm

D)

Merge

DAPI

ChR2

ChAT

A) **1 Hz**

5 mV
1 s

B) **5 Hz**

5 mV
500 ms

C) **10 Hz**

5 mV
250 ms

D) **15 Hz**

5 mV
100 ms

E) **20 Hz**

5 mV
100 ms

F) **25 Hz**

5 mV
100 ms

G) **30 Hz**

5 mV
500 ms

A) Rewards Individual Data

B) Incorrect Nose Pokes Individual Data

C) Male Rewards
Interaction: p = 0.0012
Main effect: p = 0.0808

D) Male Incorrect Nose Pokes
Main effect: p = 0.0465

E) Male Rewards Individual

F) Male Incorrect Nose Pokes Individual

A)

Injection Sites

Anterior

-0.22 mm

-0.46 mm

-0.70 mm

Posterior

-0.34 mm

-0.58 mm

-0.82 mm

B)

Fiber Tip Placements

Anterior

-0.70 mm

-1.06 mm

-1.46 mm

Posterior

-0.94 mm

-1.34 mm

-1.70 mm

A) Cued Self Stimulation

B) Real Time Place Preference

C) Progressive Ratio

D) Locomotion

E) Light Dark Box
# of crosses

F) Light Dark Box
% Time in Light

A) Rewards Individual Data

B) Incorrect Nose Pokes Individual Data

C) Locomotion

A) Injection Sites

Anterior

Posterior

-0.34 mm

-0.58 mm

-0.70 mm

-0.94 mm

-1.06 mm

-1.22 mm

B) Fiber Tip Placements

Anterior

Posterior

-0.94 mm

-1.22 mm

-1.34 mm

-1.58 mm

-1.70 mm

-1.82 mm

A) Rewards Individual Data

EYFP
Contingent-ChR2
Non-contingent-ChR2

Rewards

PT   Training   Ext

B) Incorrect Nose Pokes Individual Data

EYFP
Contingent-ChR2
Non-contingent-ChR2

Nose Pokes

PT   Training   Ext