Independence of Cued and Contextual Components of Fear Conditioning is Gated by the Lateral Habenula

Tomas E. Sachella [1], Marina R. Ihidoype [1], Christophe D. Proulx [2], Diego E. Pafundo [1], Jorge H. Medina [3], Pablo Mendez [4], Joaquin Piriz [1] † *.

[1] Grupo de Neurociencia de Sistemas, Instituto de Fisiología y Biofísica "Bernardo Houssay" (IFIBIO "Houssay"), Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina.

[2] CERVO Brain Research Center, Department of Psychiatry and Neurosciences, Université Laval.

[3] Instituto de Biología Celular y Neurociencia "Dr. Eduardo De Robertis" (IBCN), Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina.

[4] Instituto Cajal, CSIC, Madrid, España.

† Present address: Instituto de Fisiología Biología Molecular y Neurociencias (IFIBYNE), Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina.

* Correspondence:

Joaquin Piriz

Email: pirizjoaquin@gmail.com

Abstract

Fear is an extreme form of aversion that, if inappropriately generalized, initiates pathological conditions such as panic or anxiety. Fear conditioning (FC) is the best understood model of fear learning. During FC two independent associations link the cue and the training context to fear expression. The lateral habenula (LHb) is a general encoder of aversion. However, its role in fear learning has not been intensively studied. Here we studied the role of the LHb in FC using optogenetics and pharmacological tools in rats. Disrupting the neuronal activity of the LHb during training abolishes the expression of fear to isolated presentation of the training context or the cue, yet the recall of both associations when the cue is played in the training context reveals a conserved memory. Our results demonstrate that the LHb is required for the formation of independently expressible contextual and cued memories, a previously uncharacterized role in FC.

Introduction

Fear is an extreme form of aversion characterized by an uncontrollable reaction to a threatening stimulus. Pavlovian fear conditioning (FC) is probably the most studied and best understood model of fear learning (Maren, 2008; Maren and Quirk, 2004). In FC an association is made between a neutral stimulus and a biologically-relevant aversive stimulus called unconditioned stimulus (US). Most frequently, FC training involves the pairing of a tone, named cue, and an electric foot-shock US. It has long been known that such protocol leads to two independent associations, one relating the cue to the US, and the other linking the context where the training took place to the US (Kim and Fanselow, 1992; Phillips and Ledoux, 1992). Prevailing models postulate that tone-US association takes place in cortical and thalamic auditory inputs to the lateral amygdala (Bocchio et al., 2017; Nabavi et al., 2014; Rumpel et al., 2005; Sah et al., 2008), while context-US association involves context encoding circuits centered in the hippocampus which send contextual representation to the amygdala (Kim and Cho, 2020; Maren et al., 2013).

41 The lateral habenula (LHb) is a hub for the processing of aversive information in the brain.
42 Aversion-related information reaches the LHb from the basal ganglia and numerous structures of the
43 limbic system including the Central Amygdala (Hu et al., 2020). In turn, the LHb projects to the brain stem,
44 where it is one of the few structures that controls both serotoninergic and dopaminergic systems
45 (Hikosaka, 2010; Hu et al., 2020; Proulx et al., 2014). Notably the LHb and its downstream targets are
46 activated by electric foot-shocks (Lecca et al., 2020; Szőnyi et al., 2019b; Trusel et al., 2019; Wang et al.,
47 2017) and develop responses to cues that predict these aversive stimuli along with the appearance of
48 conditioned responses (Lazaridis et al., 2019; Trusel et al., 2019; Wang et al., 2017). This suggests the LHb
49 may play a role in FC. However, only a few studies examined this possibility (Durieux et al., 2020; Song et
50 al., 2017; Wang et al., 2013). Thus, in this work we investigated the participation of the LHb in the
51 acquisition of contextual and cued FC by means of pharmacological and optogenetics manipulations. We
52 show that interfering with the neuronal activity of the LHb during FC training severely impairs the recall
53 of contextual and cued FC memories. However, when the cue is played in the training context, an intact
54 FC memory is expressed. Thus, our results suggest that the LHb plays a central position in FC learning,
55 affecting not only context and cued FC but also,, remarkably, theirs interaction.

56 Results

57 Pharmacological inactivation of the LHb impairs contextual fear conditioning.

58 To examine the role of the LHb in contextual FC, we analyzed the effect of its inactivation during
59 training. For that purpose, we performed surgeries in rats, to implant bilateral intracerebral cannulae
60 aimed at the LHb. After a recovery time of 10 to 12 days, animals were bilaterally infused with the GABA-
61 A agonist, muscimol, or vehicle in the LHb and were trained in contextual FC thirty minutes later. During
62 training rats were placed in a FC chamber and presented with four unsignaled mild foot-shocks (**Figure**
63 **1A**). We observed that the infusion of muscimol in the LHb did not modify freezing behavior during FC

64 training (**Supp. Fig. 1**). To evaluate contextual FC, animals were placed back in the conditioning chamber
65 7 days after training and freezing behavior was quantified. Muscimol group displayed significantly lower
66 levels of freezing than the control group, suggesting that inactivation of the LHb during contextual FC
67 training impairs long-term memory formation (**Figure 1B**).

68 In a separate cohort of animals, we observed that the infusion of muscimol in the LHb before the
69 exposure to an open field (OF) did not modify locomotion or exploratory behavior (**Supp. Fig. 2**), indicating
70 that inactivation of the LHb does not have a general effect over mobility or context exploration. Moreover,
71 upon a subsequent re-exposure to the OF 48 hours later, muscimol infused animals displayed a decrease
72 in exploratory behavior equivalent to controls (**Supp. Fig. 2**). This decrease in exploration is an index of a
73 non-associative habituation memory that depends on context encoding by the hippocampus (Vianna,
74 2000; Winograd and Viola, 2004). Thus, this observation suggests that the inactivation of the LHb by
75 muscimol does not induce a general deficit in context encoding.

76 <u>Pharmacological inactivation of the LHb impairs cued fear conditioning</u>

77 Having observed that the inactivation of the LHb impairs the formation of contextual FC we
78 extended our experiments analyzing the effect of LHb inactivation on cued FC memory formation. Animals
79 were infused with muscimol or vehicle in the LHb, and 30 minutes later they were trained with cued FC,
80 in which each of the 4 foot-shocks was preceded by a 17-second-long tone (**Figure 2A**). Cued FC memory
81 was tested 7 days later by exposing the animals to the tone in a novel context (Context B). Behavior during
82 training confirmed that the inactivation of the LHb does not affect the surge of freezing elicited by shock
83 exposure (**Supp. Fig. 3**). During test, the presentation of the cue elicited a robust freezing in the vehicle
84 group that was markedly reduced in the muscimol group (**Figure 2B**). These results indicate that cued FC
85 memory is also impaired by the inactivation of the LHb during training. Thus, inactivation of the LHb during
86 FC training impairs both contextual and cued FC long-term memories.

87 Long-term memories tend to decay over time, a phenomenon that could be already evident 7
88 days after memory formation (Bekinschtein et al., 2007; Rossato et al., 2009). To test whether an
89 accelerated decay could account for deficits of the contextual and cued fear memories, we repeated our
90 previous experiment but tested cued and contextual memories 24 hours after training. Deficits of both
91 memories were also evident at that time point (**Supp. Fig. 4**), further suggesting that inactivation of the
92 LHb during FC training has a detrimental effect over the formation of long-term contextual and cued FC
93 memories.

94 As a control for spatial specificity of muscimol infusion we implanted cannulae targeting areas
95 around the LHb and infused muscimol before cued FC training (**Supp. Fig. 5A**). During tests, performed 7
96 days later, we found that neither infusion of muscimol 1 mm dorsal, lateral, or ventral to the LHb affected
97 freezing behavior (**Supp. Fig. 5B**). Moreover, we did not see a reduction in freezing behavior during cued
98 or contextual FC test in muscimol infused animals implanted with cannulae aimed at the LHb in which
99 histological control showed cannulations bilaterally missed the LHb (**Supp. Fig. 6**).

100 To control for non-transient effects of muscimol infusion, we trained in cued FC the animals that
101 had previously undergone muscimol inactivation of the LHb before exposure to an OF (**Supp. Fig. 2**). In
102 these animals, we found no differences in freezing behavior during either cued FC training or testing
103 (**Supp. Fig. 7**), demonstrating the transient nature of that manipulation. Thus, deficits in contextual and
104 cued FC in the muscimol groups should be attributed to a transient inactivation of the LHb during training.

105 <u>Context + tone test reveals a conserved fear conditioning memory</u>

106 To further investigate the extent to which inactivation of the LHb impairs FC learning, we
107 performed additional experiments where freezing to the cue was evaluated in the training context
108 (Context A), a condition we called context + tone (Figure 3A, Supp. Fig. 8). In that test the pre-tone period
109 is a readout of the contextual component of the memory after a cued FC training. Indeed, during the pre-

110 tone period we observed a clear reduction of freezing in the muscimol group, confirming that the
111 inactivation of the LHb during training impairs contextual FC (**Figure 3B**). Presenting the tone further
112 increased freezing in the control group and, most notably, it also induced a robust freezing in the muscimol
113 group, which reached freezing values equivalent to the control group (**Figure 3B**). Thus, under the
114 inactivation of the LHb, a FC memory is formed that could be effectively retrieved when the cue is
115 presented in the conditioning context.

116 In a previous work we demonstrated that the inactivation of the LHb impairs long-term stability
117 of the Inhibitory Avoidance memory (Tomaiuolo et al., 2014). To investigate long-term stability of FC
118 memory formed under the inactivation of the LHb, we evaluated, in a new set of animals, freezing in
119 "context + tone" conditions 3 weeks after training (**Figure 3A, C**). Freezing levels of the muscimol group in
120 the "context + tone" condition was lower than in the vehicle group 3 weeks after training (**Figure 3C**),
121 showing that FC training under the inactivation of the LHb generates a weak memory that is harder to
122 retrieve and is also temporarily less stable.

123 <u>Artificial activation of the LHb impairs cued and contextual fear conditioning</u>

124 The increase in the neuronal activity of the LHb has been linked to aversion, which is at the core
125 of fear learning (Barker et al., 2017; Lazaridis et al., 2019; Li et al., 2011; Meye et al., 2015; Shabel et al.,
126 2012; Stamatakis and Stuber, 2012). On the other hand, it has been shown that neuronal activity of the
127 LHb is co-modulated with hippocampal theta rhythm (Aizawa et al., 2013; Bertone-Cueto et al., 2020;
128 Goutagny et al., 2013), and it has been postulated that such modulation reflects an influence of the LHb
129 in context encoding at the hippocampus (Baker et al., 2019; Goutagny et al., 2013). Those two seemingly
130 unrelated observations delineate two different scenarios about how the LHb could participate in FC. In
131 the first one, an increase or decrease of neuronal activity of the LHb would lead to changes in aversion
132 and consequently in FC, in the second one the LHb would participate in FC through a dynamically

133 modulated pattern of activity which, if disrupted, would affect FC. To get an insight onto these possibilities
134 we analyzed the impact of sustained excitation of the LHb on FC. For this purpose, we used optogenetics
135 to optically stimulate the LHb during training. We chose an optogenetic over chemogenetic approach
136 since it allows excitation to be limited to the training period, discarding potential effects of the LHb during
137 the consolidation phase. To achieve sustained stimulation of the LHb during training we infused adeno-
138 associated viruses (AAVs) encoding the fast opsin oChIEF (Lin et al., 2009) and implanted optic fibers
139 bilaterally immediately above the LHb (**Figure 4A, B**). Three weeks later subjects were trained in cued FC
140 under a sustained 20 Hz excitation drive with light pulses of 5 ms (**Figure 4A**), which has been shown not
141 to induce depression-like symptoms (Yang et al., 2018). Patch-clamp recordings from oChIEF expressing
142 neurons confirmed that responses of LHb neurons to optogenetic stimulation is stable for the duration of
143 the training period (**Supp. Fig. 9**). We also observed that light stimulation did not modify freezing during
144 FC training (**Supp. Fig. 10**). During cued memory test, oChIEF stimulated animals showed lower freezing
145 to the tone than the control group (**Figure 4C, E**). Next day, during "context + tone" test session, oChIEF
146 stimulated animals showed almost no freezing to the context but displayed freezing to the cue, reaching
147 levels higher than in the cued memory test performed the previous day (**Figure 4D, E**). Thus, the excitation
148 of the LHb during the whole training has a deleterious effect over FC learning mostly equivalent to that of
149 pharmacological inhibition, suggesting that proper encoding of the LHb during FC is required for the
150 formation of independent cued and contextual FC memories.

151 <u>Optogenetic inactivation of the LHb impairs cued fear conditioning</u>

152 Previous articles have described that neuronal activity at the LHb increases during presentation
153 of the cue and the US in both FC and active avoidance paradigms (Trusel et al., 2019; Wang et al., 2017).
154 We thus tested if inhibiting the LHb during cue and US presentation affects FC. For that purpose, we
155 injected an AAV encoding the inhibitory light-dependent proton pump Archaerhodopsin (ArchT) fused to

156 the fluorescent protein GFP (AAV-CamKIIα-ArchT-GFP) bilaterally in the LHb of rats (**Figure 5A**). Control
157 rats were injected with AAVs encoding the fluorescent protein GFP (AAV-CamKIIα-GFP) and fiber cannulae
158 were implanted with the tip above the LHb in both groups (**Figure 5A, B**). Whole cell patch-clamp
159 recordings confirmed that LHb neurons could be inhibited by ArchT (**Supp. Fig. 11**). Light was applied
160 during the FC training session starting at tone onset and stopping 5 s after shock termination (**Figure 5A**).
161 We did not observe differences in freezing between ArchT and GFP groups during FC training (**Supp. Fig.**
162 **12**). During cued memory test we found that freezing to the tone was lower in the ArchT than in the GFP
163 group. (**Figure 5C, E**). On the other hand, in contrast to pharmacological manipulation, contextual memory
164 was not affected by optogenetic inhibition of the LHb during cue and US (**Figure 5D, E**). Additionally, as
165 observed in pharmacological experiments, context + tone condition evoked similar freezing in ArchT and
166 GFP groups (**Figure 5D, E**). ArchT mediated inhibition of the LHb for the same amount of time but during
167 inter tone period did not affect cued memory (**Supp. Fig. 13**). Thus, those results show that the inhibition
168 of the LHb during cue and US presentation is sufficient for the disruption of cued FC but does not affect
169 its contextual component.

170 <u>Discussion</u>

171 Few previous articles had studied the LHb in the context of FC learning (Barrett and Gonzalez-
172 Lima, 2018; Durieux et al., 2020; Song et al., 2017; Wang et al., 2013). In this work, we directly investigated
173 this subject expanding previous results. We found that disrupting the neuronal activity of the LHb during
174 FC training, either by inhibition, or by sustained optogenetic activation, impairs cue and contextual FC
175 when tested independently. However, presenting the cue in the conditioning context is sufficient to
176 express FC memory, but with a reduced temporal stability. These results suggest that interfering with
177 neuronal activity of the LHb during FC leads to the formation of a weak memory that is harder to retrieve
178 and temporally unstable. In this regard, in a previous article we have shown that the inactivation of the

179 LHb before training in the Inhibitory avoidance generates a temporally less stable memory (Tomaiuolo et
180 al., 2014). Taken together these two observations support the idea that the LHb calibrates strength of
181 aversive memories during acquisition phase.

182 Circuits of the LHb related to FC

183 Considering its connectivity and its role as a general encoder of aversion it is not surprising that
184 the LHb participates in FC. Seminal works by Matsumoto and Hikosaka demonstrated that the LHb
185 encodes punishment and punishment predictive cues (Matsumoto and Hikosaka, 2007) as a phasic
186 increase in activity. More recent works in rodents showed that appearance of LHb responses to cue CSs
187 parallels conditioning in FC (Lazaridis et al., 2019; Wang et al., 2017) and active avoidance (Trusel et al.,
188 2019).

189 The LHb projects to both dopaminergic and serotoninergic systems both by direct projections as
190 well as indirectly through it projection to the Rostro Medial Tegmental Nucleus (RMTg) (Jhou et al., 2009)
191 and both neuromodulators regulate fear learning (Bissieére et al., 2003; Burghardt and Bauer, 2013; Kwon
192 et al., 2015; Sengupta and Holmes, 2019). In addition, the RMTg itself has been shown to regulate FC (Jhou
193 et al., 2009). Thus, the structures downstream of the LHb participate in FC.

194 Structures upstream of the LHb have also been implicated in FC learning, most notably the Central
195 Amygdala, which send a dense projection to the LHb (Zhou et al., 2019), and the Medial Prefrontal Cortex
196 (Herry and Johansen, 2014), but also sensory and motivational information relevant to FC reach the LHb
197 from upstream structures such as the Lateral Hypothalamus (Lazaridis et al., 2019; Trusel et al., 2019), the
198 Entopeduncular Nucleus (Li et al., 2019a), the Medial Septum (Pathway et al., 2018), the Median Raphe
199 (Szőnyi et al., 2019b), or the Lateral Preoptic Area (Barker et al., 2017). Thus, the LHb could control FC by
200 gating aversive signal transmission from different sources to serotonergic and dopaminergic centers
201 which ultimately control the formation of aversive memories in its target structures.

202 The LHb cued and contextual fear learning

203 Given the phasic response of the LHb to cue and US (Trusel et al., 2019; Wang et al., 2017) and its
204 role as source of aversive prediction error in monkeys (Matsumoto and Hikosaka, 2009) and rats (Li et al.,
205 2019b), it is conceivable that during cued conditioning the LHb encodes a predictive signal of cue-US
206 contingency as a transient increase in neuronal activity (as postulated also by Li et al., 2019b). Indeed, we
207 found that optogenetic inhibition of the LHb during tone and shock presentation is sufficient to impair
208 cued memory. The importance of the phasic activation of the LHb by the cue and US for cue conditioning
209 could be inferred from our work. It would not be required for the cue-US association to be made but it
210 would regulate the strength of the memory formed. Without proper LHb signaling during training the
211 memory could not be evoked by the cue alone and requires matching contextual information.

212 Electrophysiological (Congiu et al., 2019; Cui et al., 2018; Lecca et al., 2017; Matsumoto and
213 Hikosaka, 2009, 2007; Yang et al., 2018) and calcium imaging studies (Shabel et al., 2019) showed that 30-
214 50% of the LHb neurons are activated by aversive stimuli such as foot-shock. That subpopulation of LHb
215 neurons activated by foot-shocks would be candidate to be involved in cued conditioning. Recently,
216 transcriptional profiling of LHb neurons identified a specific cluster of neurons in which foot-shocks
217 modulate expression of immediate early genes (Hashikawa et al., 2020). That cluster of LHb cells could be
218 the population identified by electrophysiological recordings. However, this has not been proven yet.
219 Moreover, the lack of a systematic study analyzing the connectivity of foot-shock excited LHb neurons
220 make it impossible to define an LHb centered circuit participating in cued FC with the available
221 information.

222 In contrast to cued FC, inhibiting LHb during cue and US has no effect on contextual FC, which is
223 instead affected by whole training manipulations of neuronal activity of the LHb (either excitation or
224 inhibition). This discrepancy suggests that the participation of the LHb in contextual conditioning extends
225 beyond its activation by the cue and the US.

226 It has been proposed elsewhere that the LHb influences context encoding through a functional
227 interaction with the hippocampus (Baker et al., 2019; Goutagny et al., 2013). That hypothesis has received
228 support from physiological data generated by us and other authors showing that firing of the neurons of
229 the LHb is synchronized with hippocampal theta rhythm (Aizawa et al., 2013; Bertone-Cueto et al., 2020;
230 Goutagny et al., 2013). In addition, two recent papers from Nyiri group showed that LHb-innervated
231 Nucleus Incertus (Szőnyi et al., 2019a) and Median Raphe neurons (Szőnyi et al., 2019b) modulate
232 hippocampal theta. Thus, a considerable amount of data supports the hypothesis that the LHb modulates
233 context encoding by the hippocampus. This favors the idea that deficits in contextual FC induced by
234 manipulations of the LHb are consequence of a disturbed encoding of the context. However, we found
235 that, although context by itself does not elicit freezing, it is required for cue evoked freezing, evidencing
236 the retention of contextual information. In addition, inactivation of the LHb does not affect habituation
237 to an OF, which is a form of non-associative learning that is also dependent on contextual representation
238 on the hippocampus (Vianna, 2000; Winograd and Viola, 2004). These two observations contradict the
239 hypothesis that impairment of contextual FC induced by disruption of neuronal activity of the LHb simply
240 represent a deficit in contextual memory. In this regard, the most accepted model of contextual FC
241 postulates that learning take place in two stages (Fanselow, 2010; Rudy et al., 2004), a representative
242 stage in which a cognitive representation of the context is formed in the hippocampus, and a later
243 associative stage in which that contextual representation is associated with the US. The substrates for
244 that association have not been understood. It is tempting to speculate that manipulations of the LHb could
245 help to elucidate them.

246 Concluding remarks

247 Expression of cued and contextual memories have always been considered independent
248 processes. However, our results indicate that such independence depends on LHb signaling during

249 conditioning. Hence, if conditioning takes place without proper activity of the LHb, neither context nor
250 cued memory traces could be independently expressed during test. This observation is novel and assigns
251 the LHb a previously undescribed role within the brain circuits implicated in FC. The circuits implicated in
252 the synergistic action of the context and the cue in fear retrieval are still unknown, thus, their relevance
253 needs to be studied.

254 Based on our current and previous works we propose that the LHb is a regulator of the aversive
255 memory strength. In FC, the LHb would regulate the strength of both cued and contextual memories
256 probably by different mechanisms. A detailed assessment of that hypothesis could provide valuable
257 information about how the interaction of the hippocampus, the amygdala and the LHb regulates the
258 formation and strength of aversive memories.

259 Materials and Methods

260 Animals

261 Experiments were performed in male Wistar rats obtained from the vivariums of the Faculty of
262 Pharmacy and Biochemistry of the Buenos Aires University, Argentina, and Janvier Labs, France. Animals
263 were 5-6 weeks old at the time of surgery. Animals were housed 4 to 6 per cage, with *ad libitum* access to
264 food and water, under 12-hour light/dark cycle (lights on at 7:00 am), at constant temperature of 22 ± 2
265 °C. All the procedures were performed during light hours. Experimental procedures were approved by the
266 Animal Care and Use Committee of the University of Buenos Aires (CICUAL), and the Autonomous
267 Community of Madrid (PROEX 167/18).

268 Surgeries

269 **Pharmacology:** rats under deep ketamine/xylazine anesthesia (100 and 5 mg/kg respectively)
270 were bilaterally implanted with 22-Gauge guide cannulae aimed 2.0 mm above the LHb (AP -3.0 mm, ML
271 ±0.7 mm, DV -3.8 mm from Bregma), or 1.0 mm dorsal, ventral or lateral to LHb coordinates in specific
272 experiments. Cannulae were fixed to the skull with three surgical steel screws and dental acrylic. During
273 surgery, animals received a dose of analgesic (meloxicam 0.6 mg/kg) and antibiotic (gentamicin 3 mg/kg).
274 Behavioral procedures began 7-9 days after surgery.

275 **Optogenetics:** rats under deep isoflurane anesthesia (4 % induction, 1-2 % maintenance, in 0.8
276 L/minute oxygen) were bilaterally injected with 300 nl of viral vector (AAV8-CamKIIα-ArchT-GFP, 6.2 x 10^{12}
277 viral particles/ml from UNC Vector Core, AAV8-CamKIIα-GFP, 6.3 x 10^{12} viral particles/ml from UNC Vector
278 Core, or AAV8-hSyn-oChIEF-tdTomato homemade as described in (Proulx et al., 2018) per side at the LHb
279 (AP -2.9 mm, ML ± 0.7 mm, DV -5.0 mm from Bregma). For the ArchT experiment, injection of GFP or
280 ArchT-GFP vector was assigned randomly and was balanced across home cages. For the oChIEF
281 experiment, oChIEF-tdTomato vector or saline was assigned randomly and at a 3:1 proportion across
282 home cages. Subsequently, optical fiber implants (200 µm core, 0.45 NA) were bilaterally inserted with
283 the tips aiming just above the LHb, with 4 degrees angle from the sagittal plane (AP -3.0 mm, ML ±1.05
284 mm, DV -4.5 mm from Bregma), and fixed to the skull with three surgical steel screws and dental acrylic.
285 During surgery, animals received a dose of analgesic (meloxicam 0.6 mg/kg) and antibiotic (gentamicin 3
286 mg/kg). Behavioral procedures began three weeks after surgery.

287 **Electrophysiology:** surgery was similar to the optogenetic manipulation experiments with the
288 following differences: 3 weeks old rats were employed, the viral vector injection sites were adjusted
289 according to Bregma-Lambda distance (Bλ) of each rat (AP = -2.9 × Bλ ÷ 8.5 mm, ML = ± 0.7 × Bλ ÷ 8.5 mm
290 from Bregma and DV -4.3 mm from dura mater), no fiber optic implants, nor screws or acrylic was used,
291 and the skin was stitched at the end of surgery.

292 Contextual and Cued Fear Conditioning Training

293 For three consecutive days before the FC training, rats were habituated to handling once a day.
294 There, animals were grasped by hand and slightly restrained against the chest of the investigator for 1-3
295 minutes, a manipulation similar to the one performed during intracerebral drug infusions. On training day,
296 animals were transferred inside their home cage to a room contiguous to the training room where they
297 acclimatized for 1 hour before the beginning of any other procedure. FC training took place in Context A
298 (electric foot-shock delivering grid floor, black plastic walls and ceiling with one transparent plexiglass
299 side, 50 x 25 x 30 cm wide, width and height, illuminated by white light). Cued FC protocol consisted of a
300 180 s baseline period of free exploration followed by 4 tone-shock presentations (17 s, 3 kHz, 80 dB tone
301 followed by 3 s, 0.60 mA, square monophasic 60 Hz electric shock) with an inter-stimulus interval of 70 s.
302 Thirty seconds after the last shock, animals were returned to their home cage in the acclimation room.
303 The experimenter who transported the animals between home cages and FC chamber remained blind to
304 the animals' treatment. When training session ended, home cages were left in the acclimation room for
305 at least one hour before returning them to the animal facility. Ethanol 50 % was used to clean the
306 conditioning cage between subjects. For contextual FC training procedures were equal but the tone was
307 omitted during conditioning. Training was video recorded for posterior analysis.

308 **Pharmacology experiments:** after the acclimation period, rats were randomly assigned (balanced
309 across home cages) to receive bilateral intra LHb infusions of GABAa agonist, muscimol (Sigma, 60 ng/µl
310 in saline solution, 0.5 µl/side), or saline. Fluorescent green beads were added to both solutions to aid in
311 checking the infusion site (1:1000 dilution of concentrated 1 µm diameter fluorescent green beads, Bangs
312 Laboratories, Indiana, USA). During infusions animals were grasped by hand and slightly restrained in the
313 lap or the arm of the investigator. Infusions were delivered at a rate of 0.5 µl/minute through a 30-Gauge
314 needle extending 2.0 mm beyond the tip of implanted guide cannula and connected to a 10 µl Hamilton

315 syringe by a polyethylene tube. Infusion needle was left in place for an additional minute to minimize
316 backflow. After that animals were returned to their home cage. FC training began 30 minutes later.

317 **Optogenetics experiments**: after the acclimation period, rats were taken to the FC room and were
318 bilaterally connected to 1 to 2 optic fiber branching patch-cords (200 µm core each, Doric Lenses, Canada).
319 The single end of the branching patch-cord (400 µm core) was connected to a rotary joint (Doric Lenses,
320 Canada) that was attached to the laser source by a simple patch-cord (400 µm core, 0.45 NA, ThorLabs,
321 USA). FC chamber (Context A) had a modification in the ceiling (10 cm hole in the center) to allow optic
322 fiber patch-cord movement. For the ArchT experiment, continuous light of 532 nm at 10 mW at the tip of
323 the fiber implant was delivered by a laser source (CNI, China), starting at tone onset and stopping 5 s after
324 shock termination. For the oChIEF experiment, 5 ms pulses at 20 Hz of 447 nm light at 10 mW at the tip
325 of the fiber implant, were delivered by laser source (Tolket, Argentina) during the whole training. A mixed
326 control group composed of oChIEF injected without light stimulation and saline injected animals with light
327 stimulation was used in that experiment since an AAV-tdTomato was not available. Animals injected with
328 oChIEF on each cage were randomly assigned to light or no-light stimulation. At the end of FC training,
329 patch-cord was disconnected from the fiber implants and animals were returned to home cage.

330 Contextual FC Test

331 The contextual test was performed maintaining the same conditions of the FC training day (*e.g.*
332 acclimation room, FC chamber lights and investigator's nitrile gloves) and the experimenter remained
333 blind to the animals' treatments. During test, animals were placed in the training context (Context A) for
334 180 s and then returned to its home cage. Test was video recorded for posterior analysis.

335 Cued FC Test

336 Several precautions were taken to avoid generalization during cued test. Home cages were moved
337 to a different acclimation room of the one used in the training day, the investigator used latex gloves,

338 animals were transported between the acclimation room and the test chamber within a plastic box, and
339 acetic acid 1 % m/v was employed for cleaning between subjects. Experimenter remained blind to the
340 animals' treatments. Test was performed in Context B, a white acrylic box with a transparent plexiglass
341 ceiling (25x25x40 cm wide, width and height) with floor covered in wood shaving and lit up with red light.
342 Cue test consisted of a pre-tone period of 180 s, followed by 60 s tone (same as training tone) and 30 s
343 post-shock period. After that, animals were removed from the test cage and placed back in the home
344 cage. Test was video recorded for posterior analysis. 1 hour after finishing tests, home cages were taken
345 back to the animal facility.

346 Context + Tone Test

347 Context + tone test proceeded as contextual FC test but after 180 s in the test cage the tone was
348 presented for 60 s. 30 s after tone ending animals were returned to their home cage. Experimenter
349 remained blind to the animals' treatments.

350 Open Field

351 Animals were placed for 15 minutes into an open field (OF; square grey plastic floor and walls 50
352 cm side x 35 cm height) and video recorded. Experimenter remained blind to the animals' treatments.
353 Behavior during OF was analyzed offline with ANY-Maze video tracking system (v4.82, Stoelting Co., Wood
354 Dale, USA).

355 Histologic Control

356 Within a week after the end of behavioral procedures animals were euthanized with a lethal dose
357 of ketamine/xylazine, 50 ml of ice-cold phosphate-buffered saline (PBS) 0.1 M was transcardially perfused,
358 followed by 50 ml of paraformaldehyde 4 %. After that, the brain extracted and incubated at 4 °C in
359 paraformaldehyde 4 % overnight. Then, brain was transferred to 30 % m/v sucrose in PBS 0.1 M, incubated

360 for three days at 4 °C and 150 µm coronal sections were cut at a freezing microtome. Sections were
361 mounted with glycerol 50 % v/v in PBS 0.1 M, examined and photographed at 40X magnification with GFP
362 and DAPI filters. For the pharmacological experiments, injection sites were determined by the presence
363 of green beads. Animals in which both infusions were correct were included in the analysis. Animals from
364 the cohorts used in Figure 2B-C in which both infusions were outside the LHb were included in the miss
365 group presented in Figure S3. For the optogenetics experiments, animals with transfection and/or optic
366 fiber misses were not considered in the analysis.

367 Freezing analysis

368 Freezing was manually scored offline. Experimenter was blinded for animal´s treatment. An
369 Arduino custom system was used for the manual scoring. Briefly, a button was used to synchronize the
370 start of the video and another button was used to register the freezing state while pressed. The Arduino
371 system communicated time and freezing-button state at 10 Hz to the computer, and then saved as CSV
372 file for later analysis. For freezing over time graphs, data was binned in 5 s intervals, and inter-subjects´
373 mean and SEM was calculated.

374 Electrophysiological recordings

375 General methods for slice preparation and recordings were replicated from previous publications
376 of the group (Stefanelli et al., 2016).

377 Statistical Analysis

378 Each animal was taken as an independent measure. During test session of cued or context + tone
379 experiments, average freezing before and during tone presentation were treated as repeated measures.
380 Thus, in most experiments analysis was done using a two-way repeated measures design in which the
381 analyzed factors were “test stage” (pre-tone or tone) and “treatment” (muscimol, optogenetic excitation

382 or inhibition, or the corresponding control group). In experiments in which subjects were tested more
383 than one day, “testing day” was considered an additional factor. Freezing during training session was
384 analyzed separately. There the stage factor comprised freezing during tone or the inter-stimulus period.
385 Statistical analysis of FC experiments was done by generalized linear mixed modelling (GzLMM). Typical
386 distribution of individuals freezing values accumulated at lower and higher values with few individuals
387 freezing around the group mean, indicating a non-normal distribution. We therefore compared models
388 with beta and gaussian distribution (rescaling freezing to $y''=[y'(n-1)+0.5]/n$ as suggested in (Smithson and
389 Verkuilen, 2006). For the model fitting in R (R Foundation for Statistical Computing, Vienna, Austria) we
390 used the ‘glmmTMB’ package (Brooks et al., 2017). Beta distribution provided better adjustment and less
391 tendencies between group residues than gaussian distribution (data not shown). Logit link function was
392 employed. For the GzLMM we considered every interaction between the corresponding fixed factors (test
393 stage, treatment, testing day) and subject was treated as a random factor with random intercepts. ANOVA
394 and contrasts were done applying ‘car’ and ‘emmeans’ packages (Lenth, 2020; Weisberg and Sanford,
395 2019). Where GzLMM was used. GzLMM ANOVAs used Wald χ^2 statistics for comparisons since neither
396 likelihood ratio tests nor *F* tests are supported. Assumptions of homoscedasticity and normal residues
397 were met in the open field experiment; therefore, it was analyzed via conventional two-way repeated
398 measures ANOVA. Each experiment was repeated at least twice in separate cohorts of animals. Each
399 cohort included all the treatments. Sample size calculations were done using power analysis. From pilot
400 context test experiments, we considered a standard deviation of approximately 20 % aiming to detect a
401 minimal difference among groups of 50 %, with an 80 % power, and a type I error of 5 %. Based on these
402 parameters and first assuming gaussian distribution, initially we aimed at an n = 14. Later we readjusted
403 to n = 6 on cued memory tests, where the observed difference between vehicle and muscimol infused
404 groups (aprox. 60 %), standard deviation (aprox. 25 %) and beta distribution at tone tests allowed to
405 maintain about 80 % power and 5 % type I error.

The R scripts generated for the analysis are included as supplemental material.

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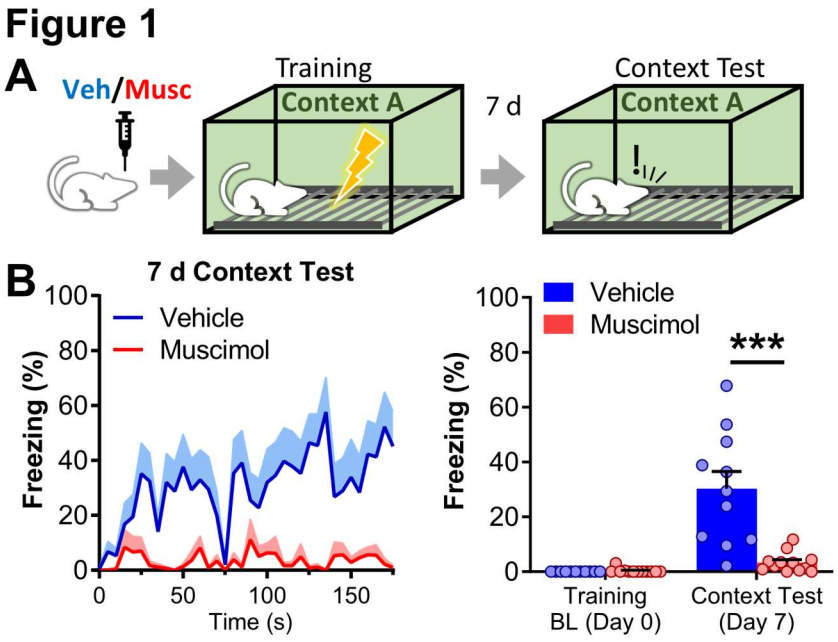
583 Figures



584

585 Figure 1. Inactivation of the LHb blocks the formation of contextual FC long-term memory.

586 **A)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes before
587 training. Contextual FC was tested 7 days later. During training subjects were let to freely explore the cage
588 for a baseline period of 197 s. After that they were exposed to 4 foot-shocks (0.6 mA, 3 s) interspaced by
589 87 s. In the test session animals were re-exposed to the training context for 180 s and freezing was
590 quantified.

591 **B)** Test of contextual FC memory. Left panel: freezing over time. Right panel: average freezing. Freezing in
592 the muscimol group was lower than in the control group. ($n_{vehicle} = 11$, $n_{muscimol} = 12$). In freezing over time
593 plot, line represents intersubjects' mean, and shaded area represents +SEM. In bar plot, each dot
594 represents a subject and bars represent mean and errors bars +SEM. *** $p < 0.001$. Additional statistics
595 information could be found in the Statistics Details supplementary file.

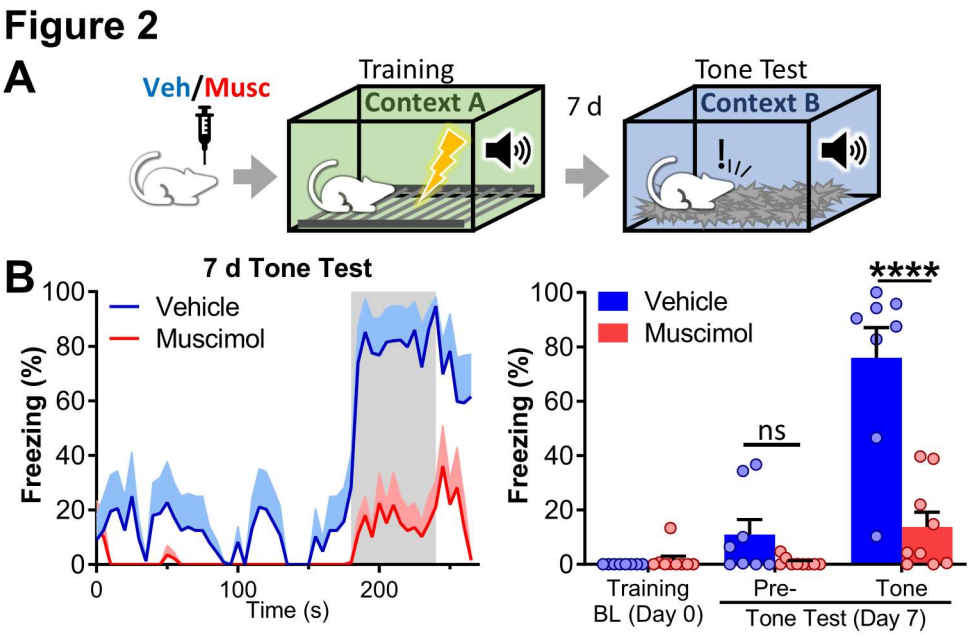


Figure 2. Inactivation of the LHb blocks the formation of cued FC long-term memory.

A) Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes before training. Cued FC was tested 7 days later. During training, subjects were let to freely explore the cage for a baseline period of 180 s that was followed by 4 tone-shock pairings (17 s of tone followed by 3 s, 0.6 mA shock) interspaced by 70 s. During test, tone was presented for 60 s after a pre-tone period of 180 s.

B) Left panel: freezing over time. Gray areas indicate tone presentation. Right panel: average freezing for pre- and tone periods. Freezing during pre-tone period was not different between vehicle and muscimol groups. In contrast, during tone presentation, a highly significant reduction in freezing was observed in the muscimol group. ($n_{vehicle}$ = 8, $n_{muscimol}$ = 9). In freezing over time plot, line represents intersubjects' mean, and shaded area represents +SEM. In bar plot each dot represents a subject and bars represent mean + SEM. ns $p > 0.05$, **** $p < 0.0001$. Additional statistics information could be found in the Statistics Details supplementary file.

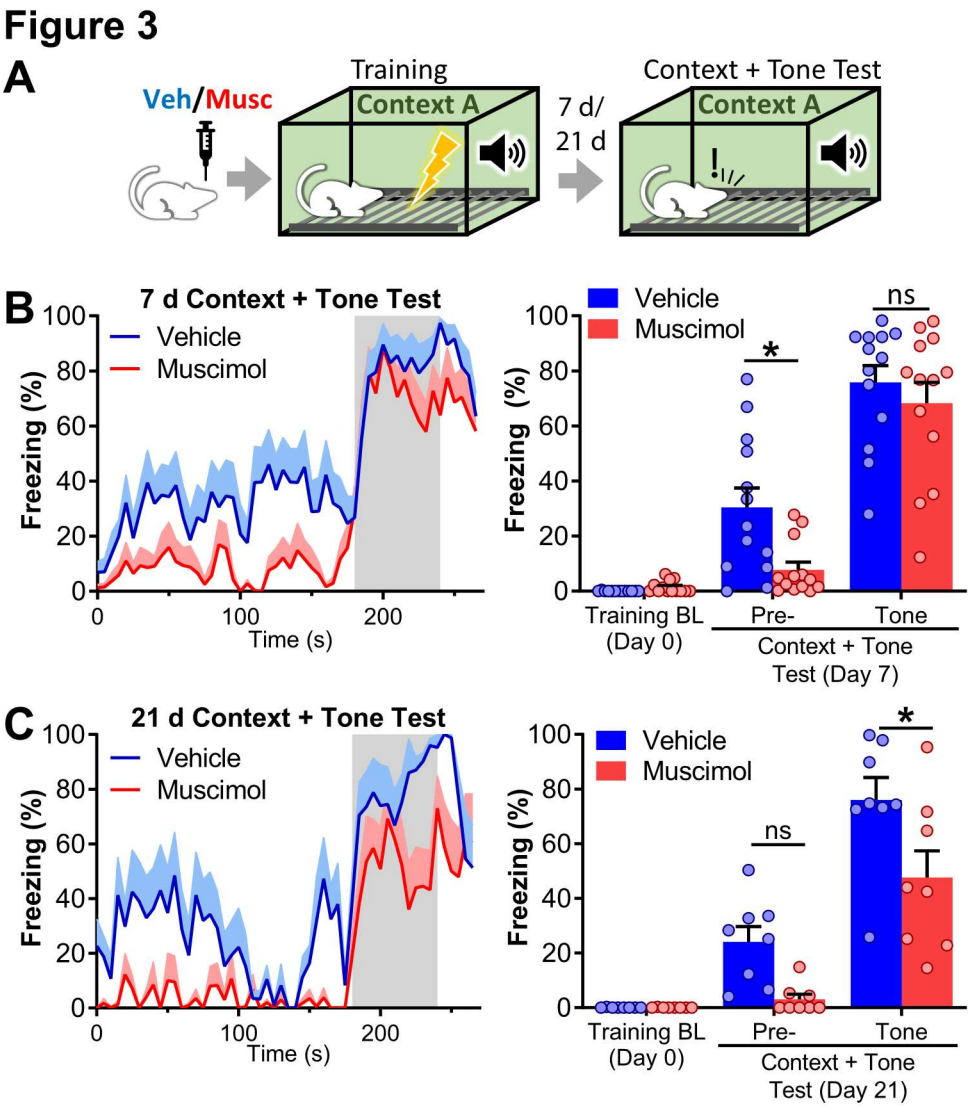


Figure 3. FC memory formed under inactivation of the LHb could be retrieved in context + tone conditions and showed decreased temporal stability.

A) Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes before training. Animals were trained in cued FC. Test was performed 7 or 21 days later in the same context used for training (Context A). The tone was presented for 60 s after a pre-tone period of 180 s.

B) Freezing during context + tone test 7 days after training. Left panel: freezing over time. Gray area indicates tone presentation. Right panel: average freezing for pre- and tone period. Freezing to the

617 context was higher in vehicle group. However, tone elicited a robust freezing in the muscimol group that

618 reached values equivalent to the vehicle group. $n_{vehicle} = 13$, $n_{muscimol} = 13$.

619 **C)** Freezing during context + tone test 21 days after training. Left panel: freezing over time. Gray area

620 indicates tone presentation. Right panel: average freezing for pre- and tone periods. 21 days after training

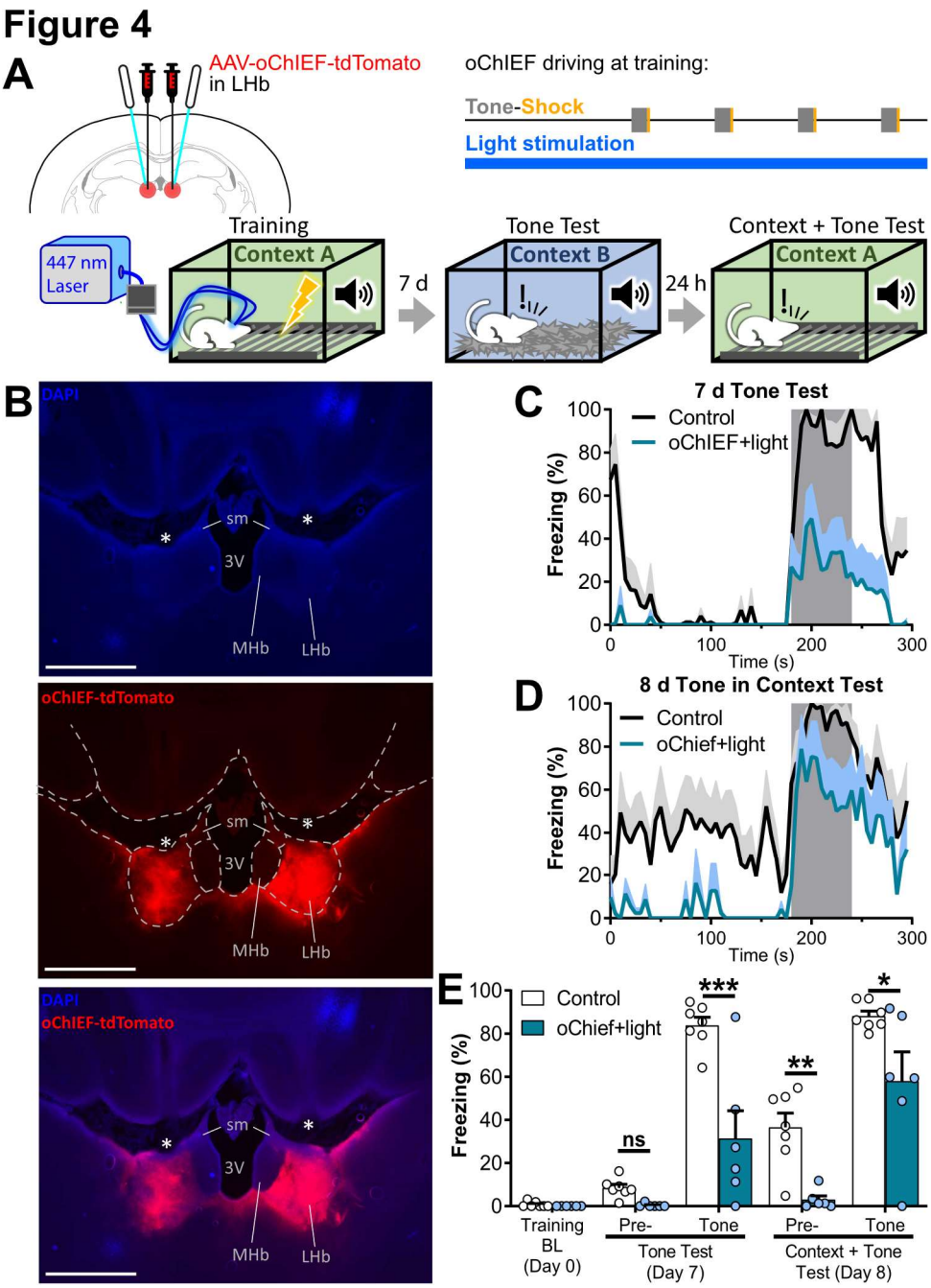
621 freezing in context + tone conditions in the muscimol group was lower than in the vehicle group indicating

622 a diminished temporal stability of context + tone memory. $n_{vehicle} = 8$, $n_{muscimol} = 8$.

623 In freezing over time plots, line represents intersubjects' mean, and shaded area represents +SEM. In bar

624 plots each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$, * $p < 0.05$. Additional

625 statistics information could be found in the Statistics Details supplementary file.



626

627 Figure 4. Optogenetic excitation of the LHb during complete FC training impairs contextual and cued FC

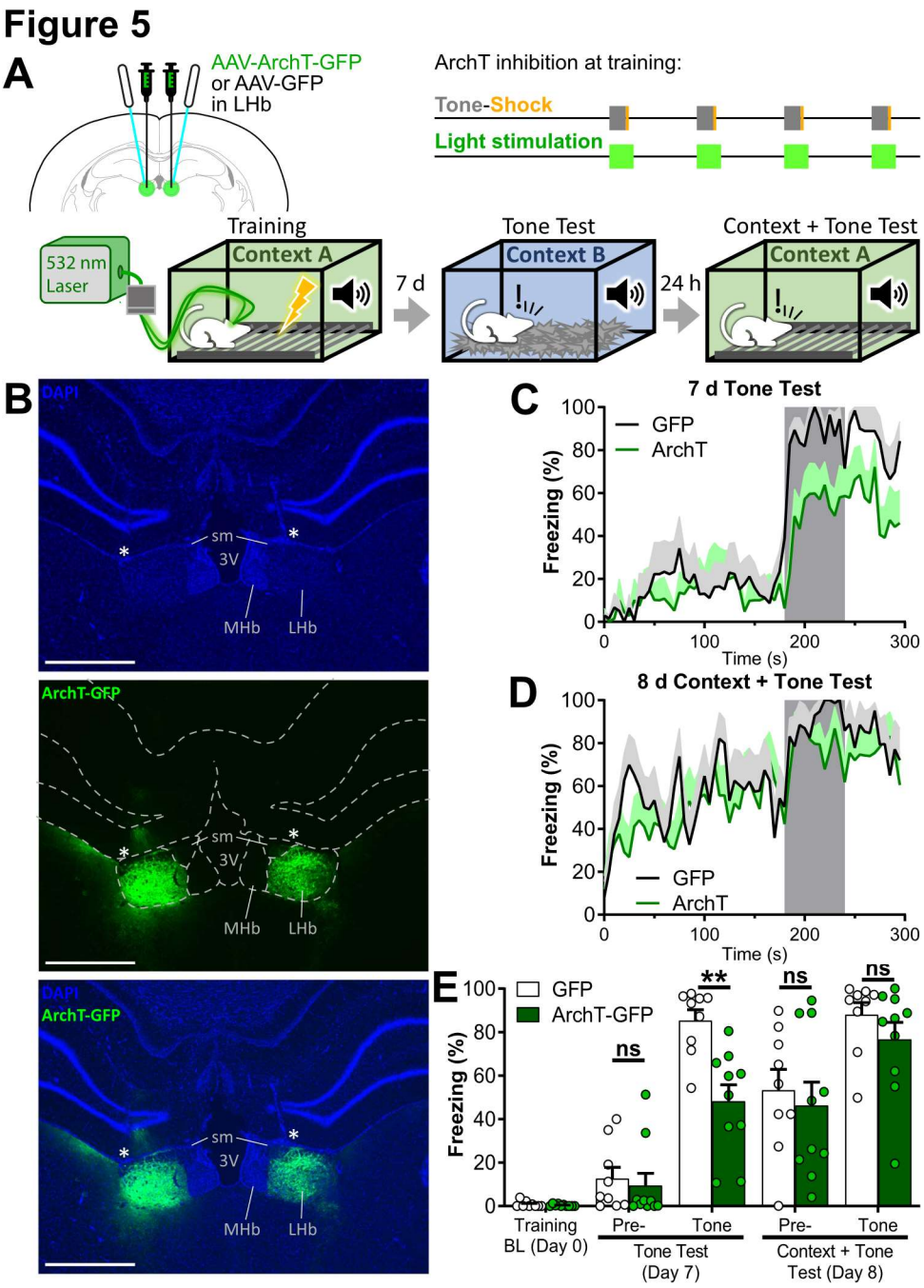
628 memories.

629 **A)** Experiment diagram: Top-Left: animals were bilaterally infected with AAV-oChIEF-tdTomato in the LHb

630 and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during whole training

631 session the LHb was stimulated with 5 ms light pulses at 20 Hz to disrupt endogenous neuronal activity.

632 Bottom: diagram of training and tests. Cued memory was tested 7 days after training in Context B. The
633 same animals were tested the following day in context + tone conditions to evaluate contextual FC
634 memory and freezing in context + tone condition.
635 **B)** Microphotographs of the AAV-oChIEF-tdTomato infection (top, middle, bottom: DAPI, tdTomato
636 fluorescence, and merge respectively). Dashed white lines in the middle panel delimitates brain
637 structures. * indicates the optic fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third ventricle.
638 Scale bars: 1 mm.
639 **C, D)** Freezing over time during cued test at day 7 (**C**) and during context + tone test at day 8 (**D**). Gray
640 area indicates tone presentation. Line represents intersubjects' mean, and shaded area represents +SEM.
641 **E)** Average freezing of tone test and context + tone test sessions. Disruption of endogenous LHb activity
642 during training by optogenetic stimulation induced deficits in cued and contextual memories. In bar plots,
643 each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ *** $p <$
644 0.001. $n_{Control} = 7$, $n_{oChIEF} = 6$. Additional statistics information could be found in the Statistics Details
645 supplementary file.



646

647 Figure 5. Optogenetic inactivation of the LHb during cue and US, reduced cued but not contextual FC

648 memories.

649 **A)** Experiment diagram: Top-Left: animals were bilaterally transfected with AAV-ArchT-GFP or AAV-GFP in

650 the LHb and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during training,

651 optogenetic light stimulation was delivered starting with the tone and stopping 5 seconds after the shock

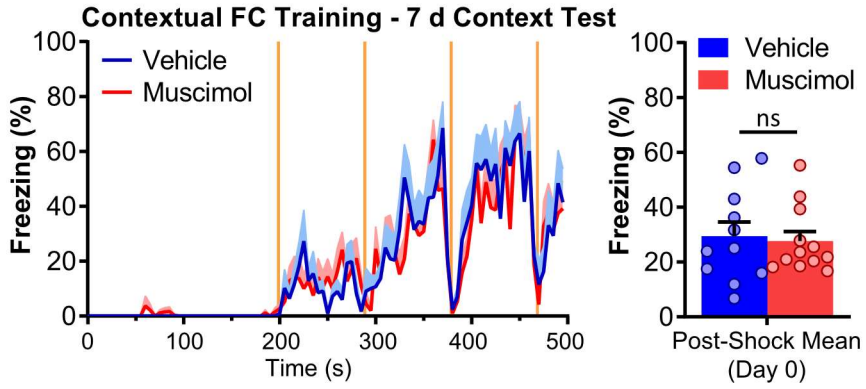
652 (tone and shock presentations were as previously described for cued FC). Bottom: diagram of training and
653 tests. Cued memory was tested 7 days after training in Context B. The same animals were tested the
654 following day in context + tone conditions to evaluate contextual FC memory and freezing in context +
655 tone condition.

656 **B)** Microphotographs of the AAV-ArchT-GFP infection (top, middle, bottom: DAPI, GFP, and merge
657 respectively). Dashed white lines in the middle panel delimitates brain structures. * indicates the optic
658 fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third ventricle. Scale bars: 1 mm.

659 **C, D)** Freezing over time during tone test at day 7 (**C**) and during context + tone test at day 8 (**D**). Gray area
660 indicates tone presentation. Line represents intersubjects' mean, and shaded area represents +SEM.

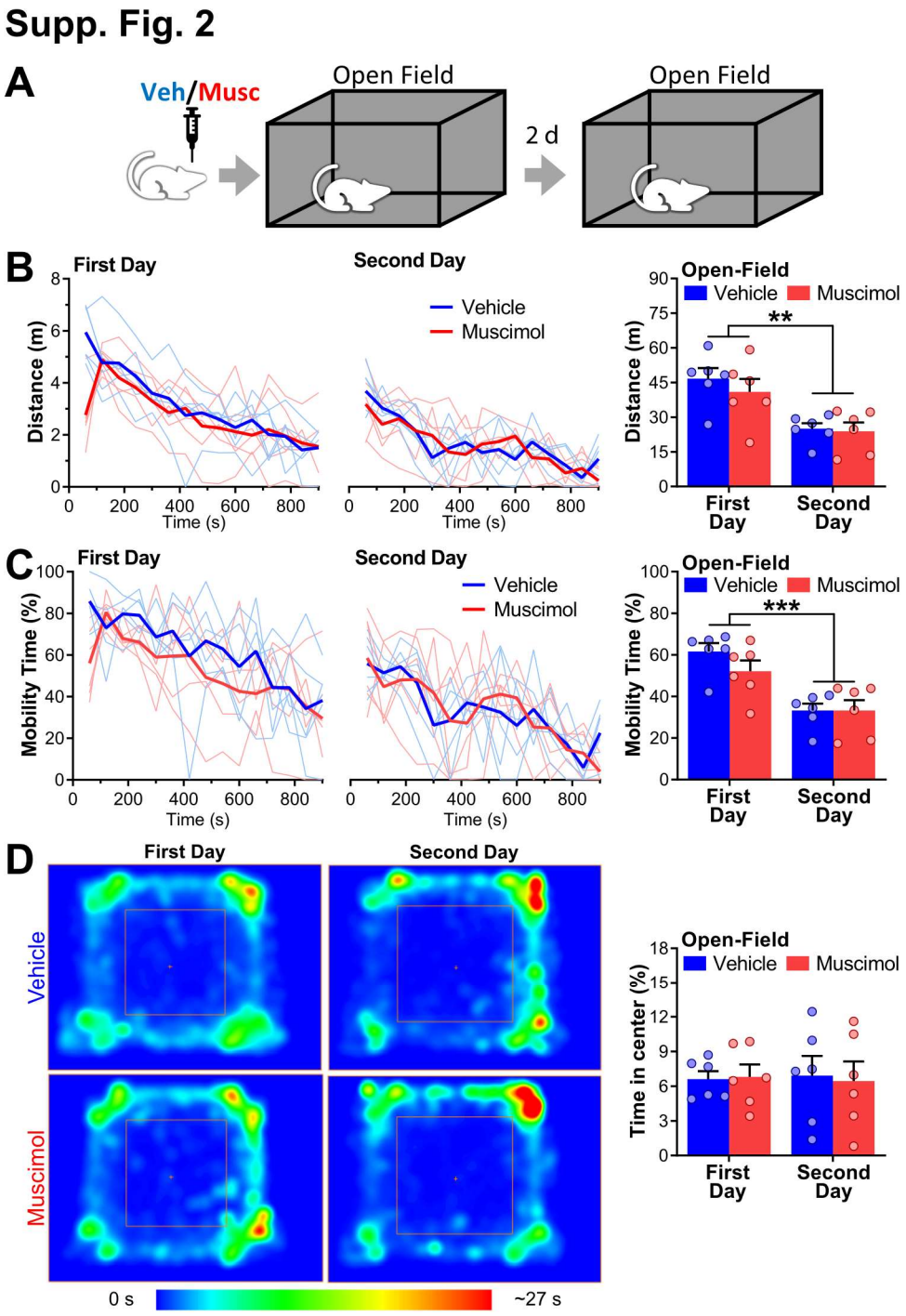
661 **E)** Average freezing on tone test, and context + tone test sessions. During tone test ArchT group displayed
662 lower levels of freezing to the tone than GFP group. The following day, during context + tone test, freezing
663 levels of the ArchT group to both the context and the tone were equivalent to those of the GFP group.
664 In bar plots each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$, ** $p < 0.01$. n_{GFP} =
665 9, n_{ArchT} = 10. Additional statistics information could be found in the Statistics Details supplementary file.

Supp. Fig. 1



Supplementary Figure 1. Freezing during training for groups in Figure 1.

Left: temporal course of freezing during training. Right: average freezing. No significant differences were observed between muscimol and vehicle group. In freezing over time plot orange shaded areas indicate shock presentation, line plot represent intersubjects' mean and shaded area over line plot represents +SEM. In bar plot each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$. $n_{vehicle} = 11$, $n_{muscimol} = 12$. Additional statistics information could be found in the Statistics Details supplementary file.



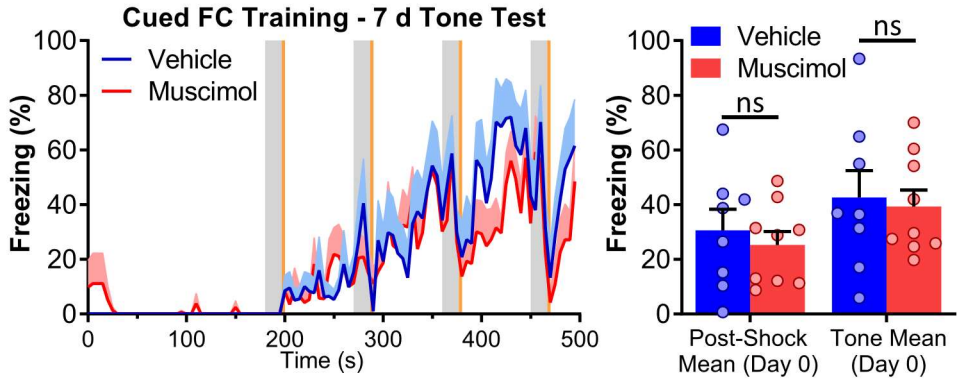
673

674 Supplementary Figure 2. Inactivation of the LHb does not affect locomotion, exploration, or habituation

675 to an Open Field.

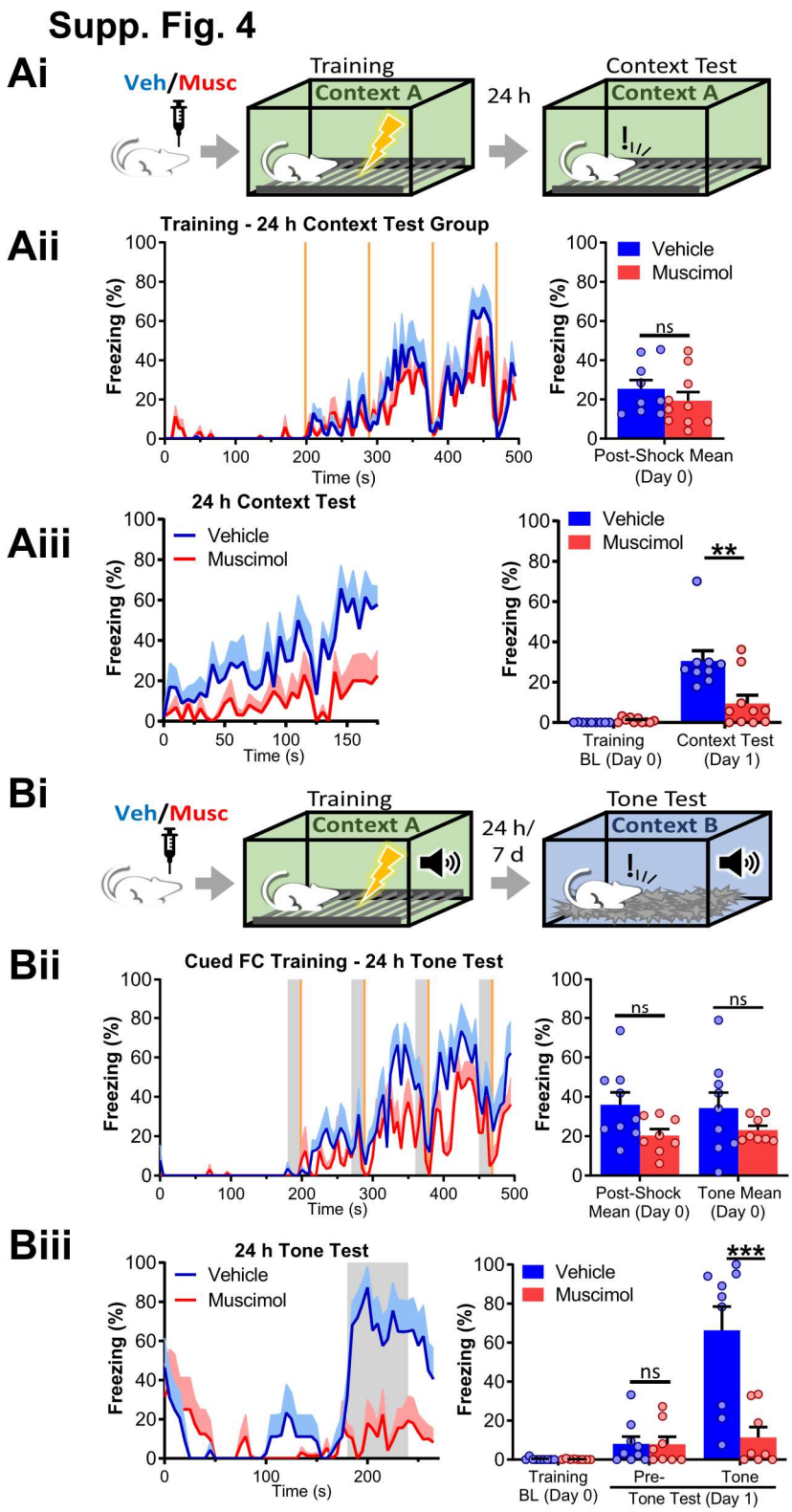
676 **A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes
677 before exposing the animals to an OF for 15 minutes. 48 hours later animals were exposed to the same
678 OF for a second time.
679 **B)** Distance traveled over time (60 s bins) during the first day (left) and the second day (right) in the OF.
680 Thin lines represent individuals, thick lines represent the average. Right: total distance traveled. Distance
681 traveled by both groups diminished the second day. No differences between groups were observed.
682 **C)** Percentage of mobility time in the Open Field in each day. Left: percentage of mobility over time (60 s
683 bins) during the first day (left) and the second day (right). Thin lines represent individuals, thick lines
684 represent the average. Right: percentage of mobility time during whole OF assay. Mobility of both groups
685 diminished the second day. No differences between groups were observed.
686 **D)** Heat map representation of spatial occupancy during OF. Left: average heat maps vehicle (top) and
687 muscimol (bottom) groups on the first day (left), and the second day (right). The center zone is indicated
688 by the orange square. Right: percentage of time in the center zone in both groups at each day. Time in
689 the center did not change between days and was equivalent among groups.
690 $n_{vehicle} = 6$, $n_{muscimol} = 6$. ** $p < 0.01$, *** $p < 0.001$. Additional statistics information could be found in the
691 Statistics Details supplementary file.

Supp. Fig. 3



Supplementary Figure 3. Freezing during training for groups in Figure 2.

Left: temporal course of freezing during cued FC training. Right: average freezing between stimuli and during tone presentation. In either condition no significant differences were observed between muscimol and vehicle group. In freezing over time plots gray and orange shaded areas indicate tone and shock presentation respectively, line plots represent intersubjects' mean and shaded area over line plots represents +SEM. In bar plots each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$, $n_{vehicle} = 8$, $n_{muscimol} = 9$. Additional statistics information could be found in the Statistics Details supplementary file.



701

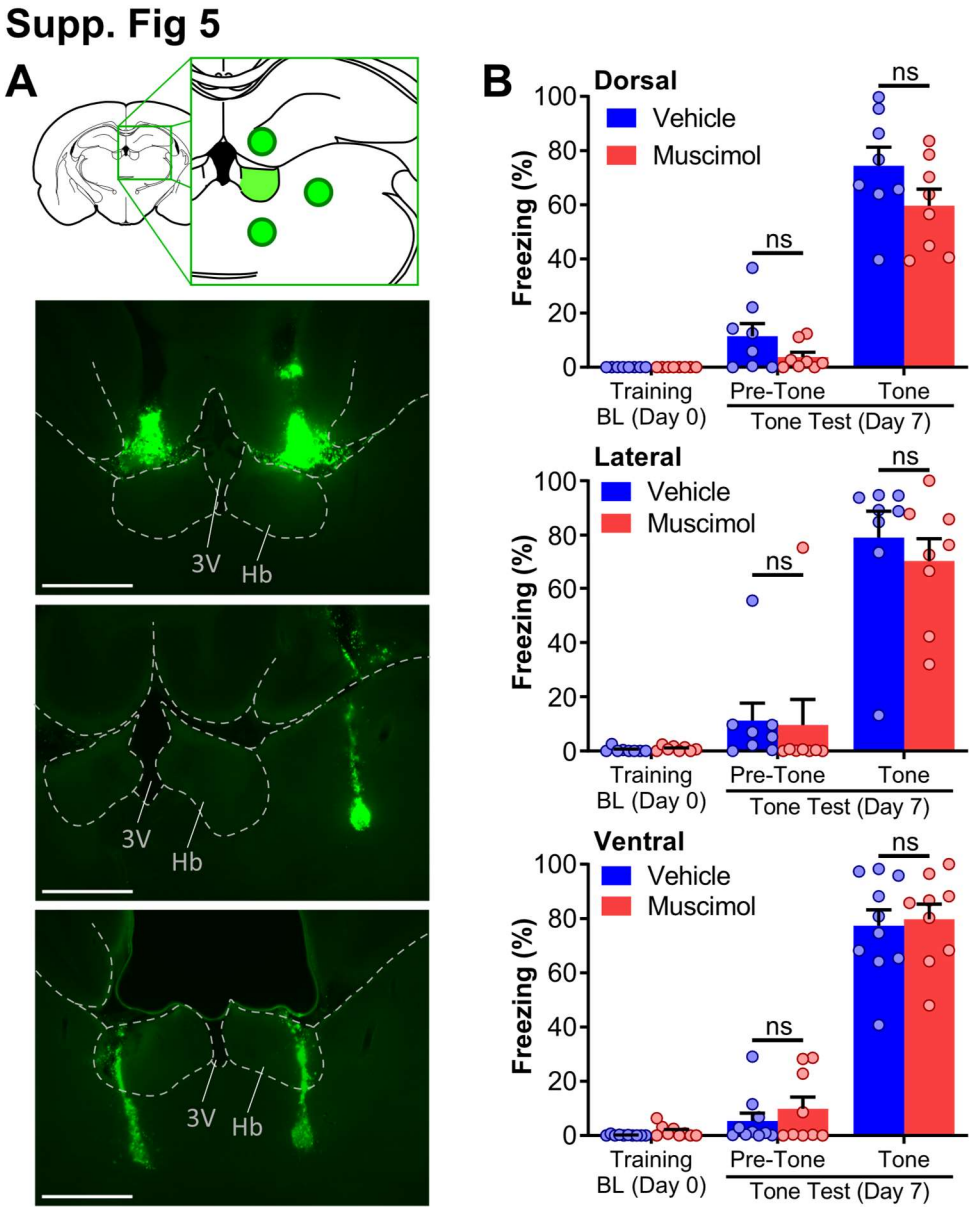
702 Supplementary Figure 4. Inactivation of the LHb during training impairs contextual and cued FC memories

703 evaluated 24 hours after training.

A) Inactivation of the LHb during training impairs 24 h contextual FC memory. **i)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes before training contextual fear memory was evaluated 24 hours after training. **ii)** Left: temporal course of freezing during training. Right: average freezing. No significant differences were observed between muscimol and vehicle groups. **iii)** 24 hours test of contextual fear memory. Left panel: freezing over time. Right panel: average freezing. Freezing in the muscimol group was lower than in the control group ($n_{vehicle} = 9$, $n_{muscimol} = 10$).

B) Inactivation of the LHb during training impairs 24 h cued FC memory. **i)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes before training. Cued FC was tested 24 hour later. **ii)** Left: temporal course of freezing during cued FC training. Right: average freezing between stimuli and during tone presentation. No significant differences were observed between muscimol and vehicle groups. **iii)** 24 hours cued FC test. Left panel: freezing over time. Gray areas indicate tone presentation. Right panel: average freezing for pre- and tone period. Freezing during pre-tone period was not different between vehicle and muscimol groups. In contrast, during tone presentation, a highly significant reduction in freezing was observed in the muscimol group ($n_{vehicle} = 9$, $n_{muscimol} = 8$).

In freezing over time plots, line represents intersubjects' mean, and shaded area represents +SEM. In bar plots each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Additional statistics information could be found in the Statistics Details supplementary file.



721

722 Supplementary Figure 5. Controls of specificity of muscimol inactivation of the LHb.

723 **A)** Diagram and representative photomicrographs of controls of specificity of muscimol infusion. Infusions

724 were aimed 1 mm dorsal (top picture), lateral (middle picture) or ventral (bottom picture). References:

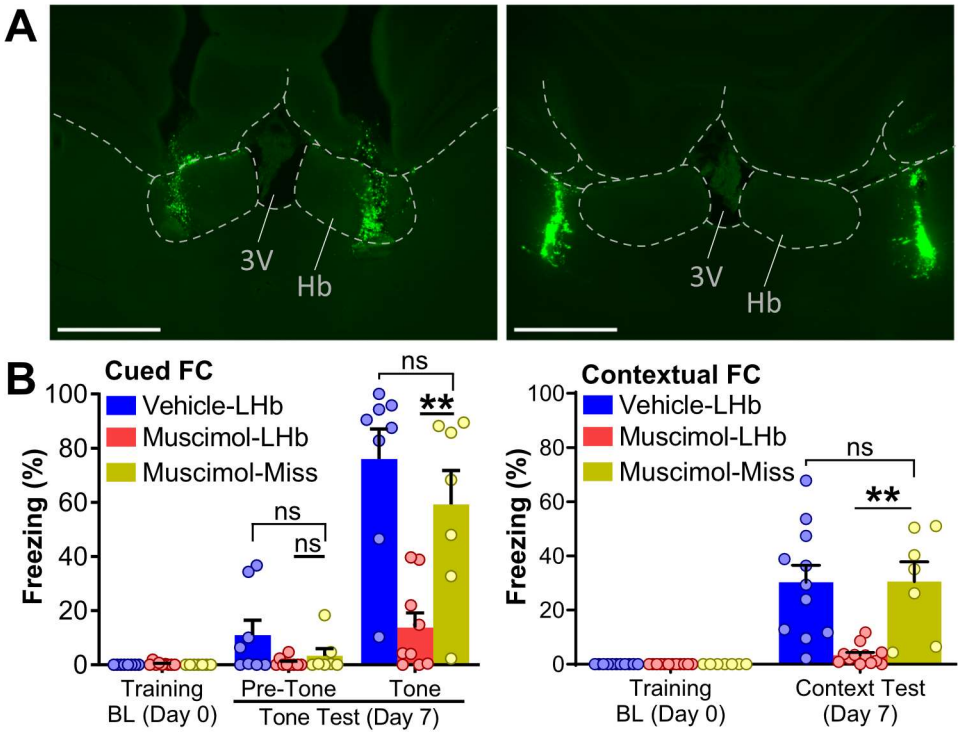
725 Hb: habenula; 3V: third ventricle.

726 **B)** Freezing during cued memory testing in dorsal, lateral, and ventral controls. Dorsal, ventral, and lateral

727 groups were done as separate experiments. As in Figure 2, infusions were done 30 minutes before training

728 and test was performed 7 days later. Muscimol infusion dorsal, ventral, or lateral to the LHb did not affect
729 tone evoked freezing. In bar plots each dot represents a subject and bars represent mean + SEM. ns $p >$
730 0.05. For Dorsal experiment: $n_{vehicle} = 8$, $n_{muscimol} = 8$. For Lateral experiment: $n_{vehicle} = 8$, $n_{muscimol} = 8$. For
731 Ventral experiment: $n_{vehicle} = 10$, $n_{muscimol} = 9$. Additional statistics information could be found in the
732 Statistics Details supplementary file.

Supp. Fig. 6



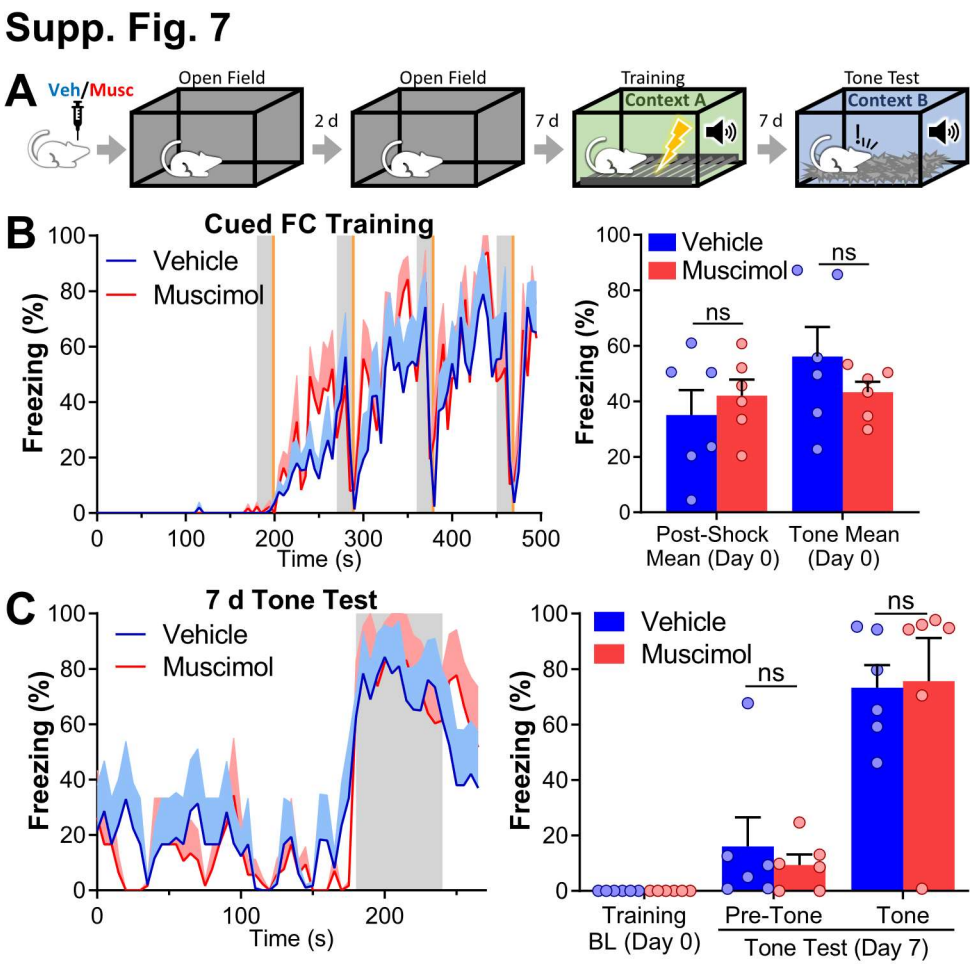
Supplementary Figure 6. Infusion of muscimol in non-voluntarily bilateral miss-cannulated animals does not block cued or contextual FC.

A) Photomicrographs of green beads fluorescence of a representative infusion on the LHb (left) or a double miss infusion (right). References: Hb: habenula; 3V: third ventricle. Scale bars: 1 mm.

B) Freezing during cued (left) or contextual (right) memory testing in miss-LHb groups. Miss-LHb groups consists of animals infused with muscimol in which the two cannulae did not hit the LHb from the same cohorts included in Figures 1B and 2B. As in Figures 1 and 2, infusions were done 30 minutes before training and test was performed 7 days later. At cued memory test (left) Miss-LHb group showed higher freezing to the tone than muscimol-LHb group and equal freezing to the tone than the vehicle-LHb group. The profile was equivalent at the contextual memory test (right), with Miss-LHb and vehicle-LHb groups freezing equally to the tone and each of them higher than the muscimol-LHb group. In bar plots each dot represents a subject and bars represent mean + SEM. Muscimol-LHb and vehicle-LHb groups are taken from Figures 1B and 2B. ns $p > 0.05$, ** $p < 0.01$. For cued FC experiment: $n_{vehicle} = 8$, $n_{muscimol} = 9$, $n_{miss} = 7$.

747 For contextual FC experiment: $n_{vehicle} = 11$, $n_{muscimol} = 12$, $n_{miss} = 7$. Additional statistics information could be

748 found in the Statistics Details supplementary file.



Supplementary Figure 7. Inactivation of the LHb does not permanently block FC learning.

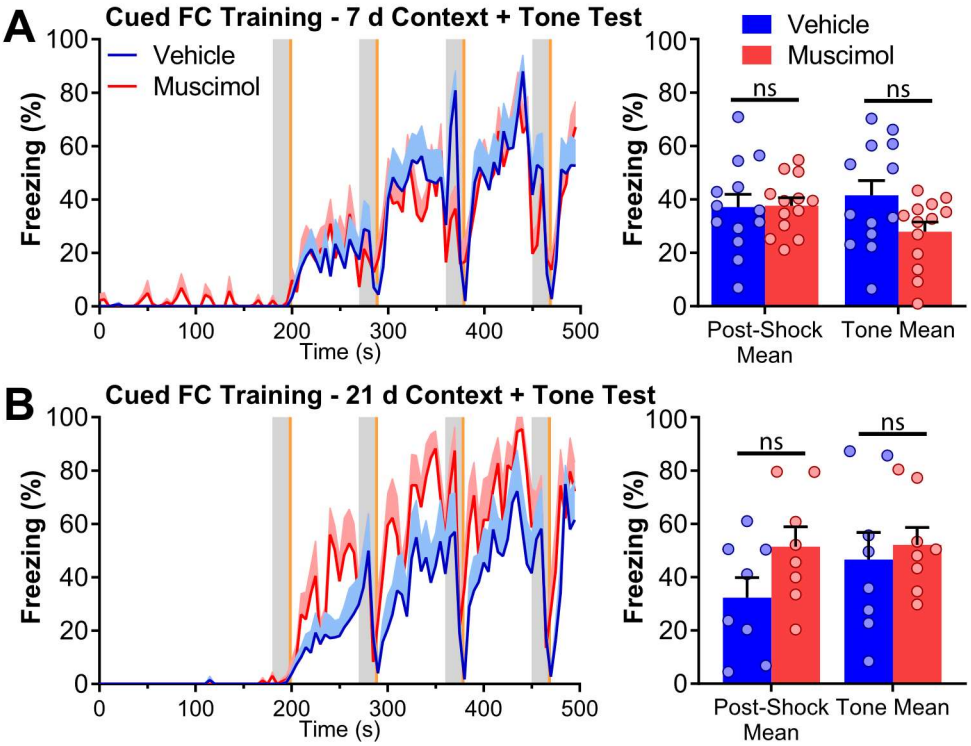
A) Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed in the animals that went through the OF (Supplementary Figure 2). Seven days after the second exposure to the OF animals were trained in cued FC. Memory was tested 7 days later.

B) Cued FC training of the animals that were previously exposed to the OF. Left panel: freezing over time. Tones were presented at times indicated by the gray shaded areas. Shocks were presented at times indicated by the orange shaded areas. Right panel: mean freezing during tone and post-shock period. Freezing in both groups was equivalent.

C) Test of cued FC memory on the same animals. Left panel: freezing over time. Gray area indicates tone presentation. Right panels: average freezing for pre- and tone period. Freezing was not different between vehicle and muscimol groups.

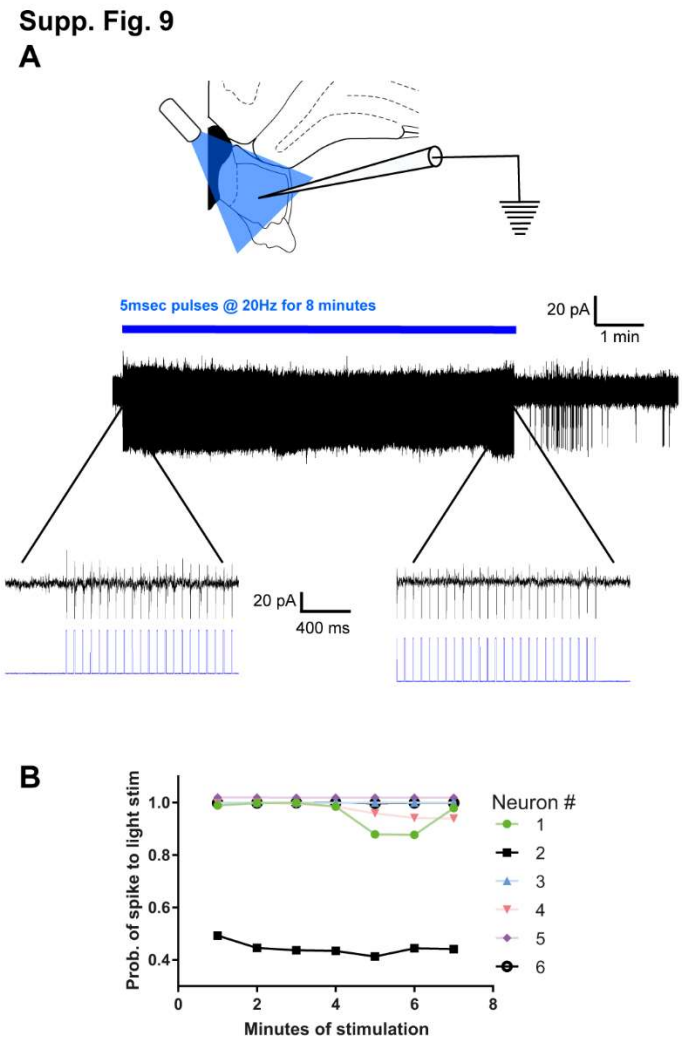
In freezing over time plots, line represents intersubjects' mean and shaded area represents +SEM. In bar plots each dot represents a subject and bars represent mean + SEM. ns $p > 0.05$. $n_{vehicle} = 6$, $n_{muscimol} = 6$. Additional statistics information could be found in the Statistics Details supplementary file.

Supp. Fig. 8



Supplementary Figure 8. Freezing during training for groups in Figure 3.

A, B) Left: temporal course of freezing during training for the experiments tested 7 (**A**) or 21 (**B**) days after training. Right: average freezing of those experiments. In either condition no significant differences were observed between muscimol and vehicle group. In freezing over time plots orange shaded areas indicate shock presentation, gray shaded areas indicate tone presentation, line plots represent intersubjects' mean and shaded area over line plots represents +SEM. In bar plots each dot represents a subject and bars represent mean + SEM. ns $p > 0.05$. For 7 days test experiment: $n_{vehicle} = 13$, $n_{muscimol} = 13$. For 21 days test experiment: $n_{vehicle} = 8$, $n_{muscimol} = 8$. Additional statistics information could be found in the Statistics Details supplementary file.



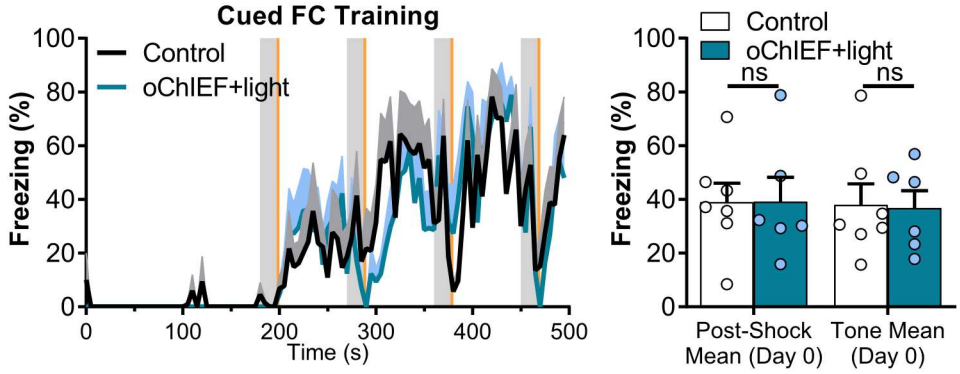
774

775 Supplementary Figure 9. Sustained optogenetic excitation of LHb neurons.

776 **A)** Top, experimental diagram. Neurons of the LHb were recorded in cell-attach configuration and
777 optogenetically stimulated mimicking in vivo stimulation during FC training. Bottom, example of neuron
778 recorded using that protocol. Left and right zoom-in traces show the beginning and the end of the
779 stimulation.

780 **B)** Quantification of all neurons recorded. The graph shows the mean probability of activation by light
781 pulses over a minute of stimulation. The probability of activation did not decrease over time indicating
782 that LHb neurons could sustain responses to optogenetic stimulation for the duration of our behavior
783 protocol.

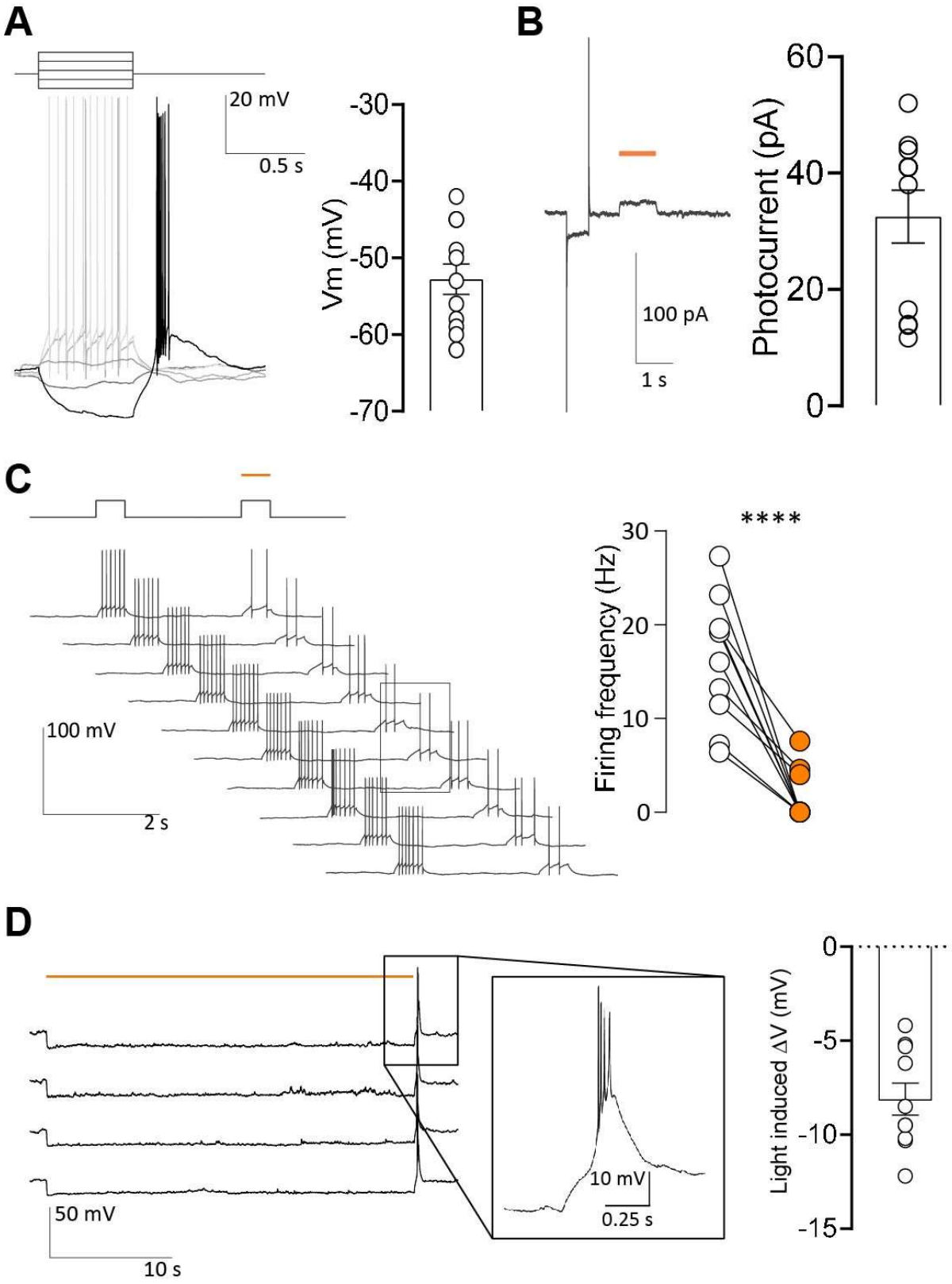
Supp. Fig. 10



Supplementary Figure 10. Freezing during training for groups in Figure 4.

Left: temporal course of freezing during training in the optogenetics stimulation experiment. Right: average freezing on the same experiment. In freezing over time plot vertical orange shaded areas indicate shock presentation, gray shaded areas indicate tone presentation, line plots represent intersubjects' mean and shaded area over line plots represents +SEM. No significant differences were observed between control and optogenetically stimulated groups. ns $p > 0.05$. $n_{Control} = 7$, $n_{oChIEF} = 6$. Additional statistics information could be found in the Statistics Details supplementary file.

Supp. Fig. 11



792

Supplementary Figure 11. Optogenetic inhibition of LHb neurons by ArchT.

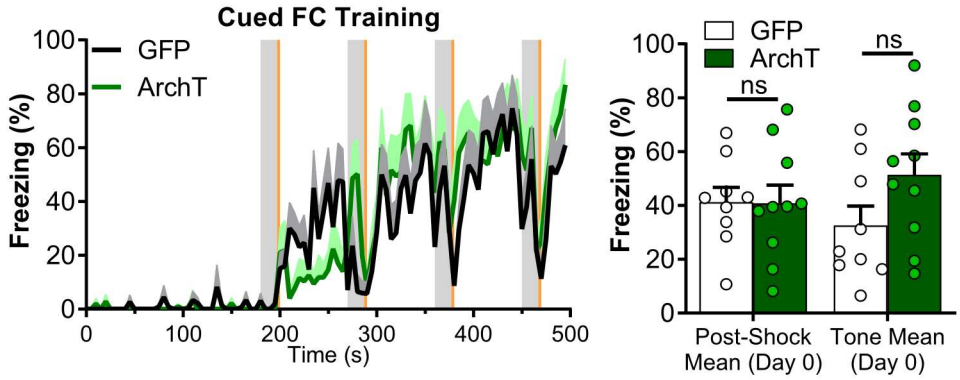
A) Example of membrane potential response to 600 ms current injection of an ArchT expressing LHb neuron (-40, -10, 15, and 40 pA steps). Rebound spiking was observed after cessation of large hyperpolarizing currents. Right graph: resting membrane potential of recorded neurons; n = 13 neurons from 3 rats.

B) Light induced photocurrent. ArchT was activated with 590 nm LED for 1 s (orange line) with a nominal power of 0.15 mW; n = 11 neurons from 3 rats.

C) Light induced action potential suppression in ArchT expressing LHb neurons. Light pulse (590 nm, orange line) was applied every second time neurons were depolarized with positive current injection. Left, representative recordings. Right, data summary. Light activation of ArchT significantly decreases the number of action potentials evoked by current steps in LHb neurons (paired t test, $t_{(9)} = 6.25$, $p < 0.001$); n = 9 neurons from 3 rats.

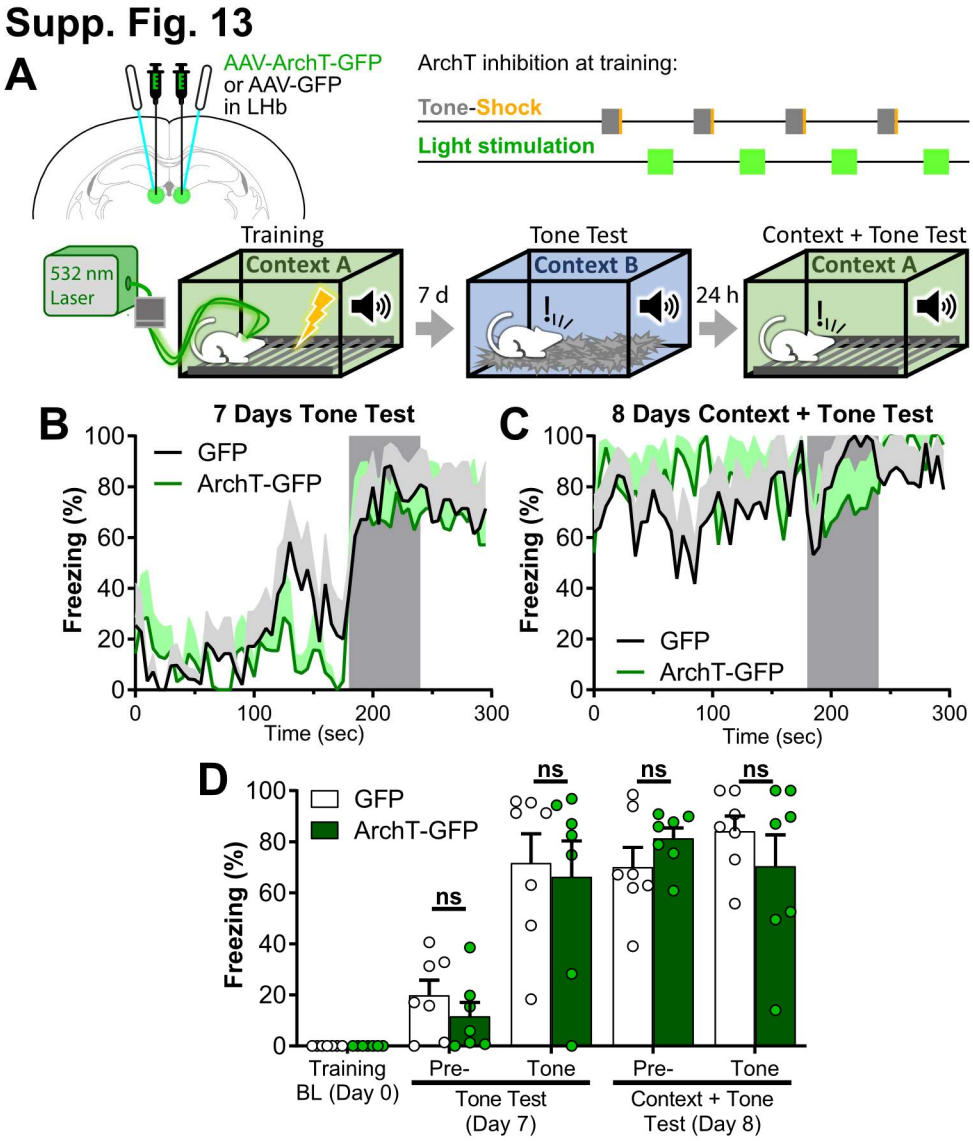
D) Membrane potential response to a 30 s light pulse (orange line) in LHb neurons. Rebound spiking was observed after termination of light pulse, a representative trace is expanded in the middle traces. This behavior confirms the reported rebound responses of the LHb neurons following long periods of optogenetic inhibition reported previously (Yang et al., 2018). Right: quantification of the stationary light induced ΔVm during the last 5 s of the 30 s pulse; n = 10 neurons from 3 rats.

Supp. Fig. 12



Supplementary Figure 12. Freezing during training for groups in Figure 5.

Left: temporal course of freezing during training in the experiments of optogenetic inhibition with ArchT. Right: average freezing on the same experiment. In freezing over time plot vertical orange shaded areas indicate shock presentation, gray shaded areas indicate tone presentation, line plots represent intersubjects' mean and shaded area over line plots represents +SEM. No significant differences were observed between control and optogenetically stimulated groups. ns $p > 0.05$. $n_{GFP} = 9$, $n_{ArchT} = 10$. Additional statistics information could be found in the Statistics Details supplementary file.



818

819 Supplementary Figure 13. Optogenetic inactivation of the LHb during inter stimulus period, did not affect

820 cued nor contextual FC memories.

821 **A)** Experiment diagram: Top-Left: animals were bilaterally transfected with AAV-ArchT-GFP or AAV-GFP in

822 the LHb and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during training,

823 optogenetic light stimulation was delivered for 25 seconds during inter stimulus period, starting 30

824 seconds after each shock termination (tone and shock presentations were as previously described for

825 cued FC). Bottom: diagram of training and tests. Cued memory was tested in Context B 7 days after

826 training. The same animals were tested the following day in context + tone conditions to evaluate
827 contextual FC memory and freezing in context + tone conditions.
828 **B, C)** Freezing over time during tone test at day 7 (**B**) and during context + tone test at day 8 (**C**). Gray area
829 indicates tone presentation. Line represents intersubjects' mean, and shaded area represents +SEM.
830 **D)** Average freezing on tone test, and context + tone test sessions. ArchT-GFP and GFP groups had
831 equivalent freezing over tone test and context + tone test days at each pre-tone and tone phases.
832 In bar plots each dot represents a subject and bars represent mean +SEM. ns $p > 0.05$. nGFP = 7, nArchT
833 = 7. Additional statistics information could be found in the Statistics Details supplementary file.
834

835 Statistics details

836 Figure 1. Statistics

837 **B)** GzLMM t test for 7 days context test: $t_{(20)} = 4.294$, $p = 0.0004$, $n_{vehicle} = 11$, $n_{muscimol} = 12$.

838 Figure 2. Statistics

839 **B)** GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for 7 days tone
840 test: χ^2 treatment $_{(1)} = 2.157$, $p = 0.1420$; χ^2 stage $_{(1)} = 19.100$, $p < 0.0001$; χ^2 interaction $_{(1)} = 32.939$, $p <$
841 0.0001. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(28)} = 1.469$, $p = 0.2828$;
842 Tone freezing: vehicle vs. muscimol: $t_{(28)} = 5.396$, $p < 0.0001$; $n_{vehicle} = 8$, $n_{muscimol} = 9$.

843 Figure 3. Statistics

844 **B)** GzLMM two-way repeated measures ANOVA of treatment, test stage, and interaction for 7 d context
845 + tone test: χ^2 treatment $_{(1)} = 6.830$, $p = 0.0090$; χ^2 stage $_{(1)} = 44.992$, $p < 0.0001$; χ^2 interaction $_{(1)} = 3.161$,
846 $p = 0.0754$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(46)} = 2.613$, $p =$
847 0.0474; Tone freezing: vehicle vs. muscimol: $t_{(46)} = 0.899$, $p = 0.8460$; Vehicle: pre-tone vs. tone freezing:
848 $t_{(46)} = 6.014$, $p < 0.0001$; muscimol: pre-tone vs. tone freezing: $t_{(46)} = 6.708$, $p < 0.0001$; $n_{vehicle} = 13$, $n_{muscimol}$
849 $= 13$.
850 **C)** GzLMM two-way repeated measures ANOVA of treatment, test stage, and interaction for 21 d context
851 + tone test: χ^2 treatment $_{(1)} = 7.123$, $p = 0.0076$; χ^2 stage $_{(1)} = 41.364$, $p < 0.0001$; χ^2 interaction $_{(1)} = 0.176$,
852 $p = 0.6751$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(26)} = 2.669$, $p =$
853 0.0508; Tone freezing: vehicle vs. muscimol: $t_{(26)} = 2.683$, $p = 0.0491$; Vehicle: pre-tone vs. tone freezing:
854 $t_{(26)} = 7.354$, $p < 0.0001$; muscimol: pre-tone vs. tone freezing: $t_{(26)} = 6.431$, $p < 0.0001$; $n_{vehicle} = 8$, $n_{muscimol}$
855 $= 8$.

856 Figure 4. Statistics

857 **E)** GzLMM three-way repeated measures ANOVA of test day, test stage, optogenetic stimulation and
858 interactions for oChIEF stimulated experiment: χ^2 day $_{(1)} = 20.439$, $p < 0.0001$; χ^2 stage $_{(1)} = 100.217$, $p <$
859 0.0001; χ^2 stimulation $_{(1)} = 5.135$, $p = 0.0234$; χ^2 interaction(Day×Stage) $_{(1)} = 5.805$, $p = 0.0160$; χ^2
860 interaction(Day×stimulation) $_{(1)} = 2.048$, $p = 0.15237$; χ^2 interaction(Stage×stimulation) $_{(1)} = 2.489$, $p =$
861 0.1146; χ^2 interaction(Day×Stage×stimulation) $_{(1)} = 4.905$, $p = 0.0268$. Sidak's multiple comparisons test:

862 Day 7: Pre-tone freezing: Control vs. oChIEF: $t_{(42)} = 2.266$, $p = 0.1601$; Day 7: Tone freezing: Control vs.
863 oChIEF: $t_{(42)} = 4.454$, $p = 0.0004$; Day 8: Pre-tone freezing: Control vs. oChIEF: $t_{(42)} = 3.874$, $p = 0.0022$; Day
864 8: Tone freezing: Control vs. oChIEF: $t_{(42)} = 2.995$, $p = 0.0272$; oChIEF: Tone freezing: Day 7 vs. Day 8: $t_{(42)} =$
865 3.810, $p = 0.0027$; Day 8: oChIEF: Pre-tone vs. Tone freezing: $t_{(42)} = 7.283$, $p < 0.0001$; $n_{Control} = 7$, $n_{oChIEF} =$
866 6.

867 Figure 5. Statistics

868 **E)** GzLMM three-way repeated measures ANOVA of test day, test stage, optogenetic inhibition and
869 interactions for CS-US ArchT inhibited experiment: χ^2 day $_{(1)} = 24.559$, $p < 0.0001$; χ^2 stage $_{(1)} = 23.070$, $p <$
870 0.0001; χ^2 inhibition $_{(1)} = 0.5568$, $p = 0.4555$; χ^2 interaction(Day×Stage) $_{(1)} = 1.659$, $p = 0.1978$; χ^2
871 interaction(Day×Inhibition) $_{(1)} = 0.294$, $p = 0.5879$; χ^2 interaction(Stage×Inhibition) $_{(1)} = 5.006$, $p = 0.0253$;
872 χ^2 interaction(Day×Stage×Inhibition) $_{(1)} = 1.088$, $p = 0.2970$. Sidak's multiple comparisons test: Day7: Pre-
873 tone freezing: GFP vs. ArchT: $t_{(66)} = 0.746$, $p = 0.9747$; Day 7: Tone freezing: GFP vs. ArchT: $t_{(66)} = 3.410$, p
874 $= 0.0067$; Day 8: Pre-tone freezing: GFP vs. ArchT: $t_{(66)} = 0.178$, $p = 1.0000$; Day 8: Tone freezing: GFP vs.
875 ArchT: $t_{(66)} = 1.145$, $p = 0.8308$; ArchT: Tone freezing: Day 7 vs. Day 8: $t_{(66)} = 3.366$, $p = 0.0076$; Day 8: ArchT:
876 Pre-tone vs. Tone freezing: $t_{(66)} = 3.475$, $p = 0.0054$; $n_{GFP} = 9$, $n_{ArchT} = 10$.

877 Supplementary Figure 1. Statistics

878 GzLMM t test for contextual FC training of 7 d tested group: $t_{(20)} = 0.046$, $p = 0.9635$; $n_{vehicle} = 11$, $n_{muscimol}$
879 $= 12$.

880 Supplementary Figure 2. Statistics

881 $n_{vehicle} = 6$, $n_{muscimol} = 6$.
882 **B)** Two-way repeated measures ANOVA of treatment, day, and interaction: F treatment $_{(1, 10)} = 0.9320$, p
883 $= 0.3571$; F day $_{(1, 10)} = 15.92$, $p = 0.0026$; F interaction $_{(1, 10)} = 0.2279$, $p = 0.6434$. Sidak's multiple
884 comparisons test: Day 1: vehicle vs. muscimol: $t = 0.9534$, $p = 0.5798$; Day 2: vehicle vs. muscimol: $t =$
885 0.1807, $p = 0.9800$.
886 **C)** Two-way repeated measures ANOVA of treatment, day, and interaction: F treatment $_{(1, 10)} = 1.670$, $p =$
887 0.2253; F day $_{(1, 10)} = 21.48$, $p = 0.0009$; F interaction $_{(1, 10)} = 0.8727$, $p = 0.3722$. Sidak's multiple comparisons
888 test: First day: vehicle vs. muscimol: $t = 1.512$, $p = 0.2708$; Second day: vehicle vs. muscimol: $t = 0.005307$,
889 $p > 0.9999$.

890 **D)** Two-way repeated measures ANOVA of treatment, day, and interaction: F treatment $_{(1, 10)}$ = 0.01082, p
891 = 0.9192; F day $_{(1, 10)}$ = 0.0003566, p = 0.9853; F interaction $_{(1, 10)}$ = 0.05192, p = 0.8244. Sidak's multiple
892 comparisons test: Day 1: vehicle vs. muscimol: t = 0.1082, p = 0.9928; Day 2: vehicle vs. muscimol: t =
893 0.2416, p = 0.9645.

894 Supplementary Figure 3. Statistics

895 GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction for cued FC
896 training of 7 d tested group: χ^2 treatment $_{(1)}$ = 0.185, p = 0.6668; χ^2 stage $_{(1)}$ = 6.308, p = 0.0120; χ^2
897 interaction $_{(1)}$ = 0.028, p = 0.8661. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs.
898 muscimol: $t_{(28)}$ = 0.430, p = 0.8912; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.297, p = 0.9463; $n_{vehicle}$ =
899 8, $n_{muscimol}$ = 9.

900 Supplementary Figure 4. Statistics

901 **Aii)** GzLMM t test for contextual FC training of 24 h tested group: $t_{(16)}$ = 1.217, p = 0.2414. $n_{vehicle}$ = 9,
902 $n_{muscimol}$ = 10.
903 **Aiii)** GzLMM t test for 24 hours context test: $t_{(16)}$ = 3.964, p = 0.0011; $n_{vehicle}$ = 9, $n_{muscimol}$ = 10.
904 **Bii)**: GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction for cued
905 FC training of 24 h tested group: χ^2 treatment $_{(1)}$ = 3.796, p = 0.0514; χ^2 stage $_{(1)}$ = 0.550, p = 0.4584; χ^2
906 interaction $_{(1)}$ = 0.852, p = 0.3560. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs.
907 muscimol: $t_{(28)}$ = 1.948, p = 0.1192; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 1.245, p = 0.3972. $n_{vehicle}$ =
908 9, $n_{muscimol}$ = 8.
909 **Biii)** GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for 24 hours
910 cued test: χ^2 treatment $_{(1)}$ = 0.024, p = 0.8781; χ^2 stage $_{(1)}$ = 0.108, p = 0.7428; χ^2 interaction $_{(1)}$ = 12.447, p
911 = 0.0004. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.153, p =
912 0.9854; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 4.441, p = 0.0003; $n_{vehicle}$ = 9, $n_{muscimol}$ = 8.

913 Supplementary Figure 5. Statistics

914 **B, Top (Dorsal)** GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for
915 dorsal experiment: χ^2 treatment $_{(1)}$ = 1.7632, p = 0.1842; χ^2 stage $_{(1)}$ = 102.565, p < 0.0001; χ^2 interaction $_{(1)}$
916 = 0.133, p = 0.7153. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(26)}$ =
917 1.328, p = 0.3532; Tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 1.916, p = 0.1283; $n_{vehicle}$ = 8, $n_{muscimol}$ = 8.

B, Middle (Lateral) GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for lateral experiment: χ^2 treatment $_{(1)}$ = 0.326, p = 0.5680; χ^2 stage $_{(1)}$ = 27.113, p < 0.0001; χ^2 interaction $_{(1)}$ < 0.0001, p = 0.9980. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 0.571, p = 0.8176; Tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 0.605, p = 0.7978; $n_{vehicle}$ = 8, $n_{muscimol}$ = 8.

B, Bottom (Ventral) GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for ventral experiment: χ^2 treatment $_{(1)}$ = 0.525, p = 0.4689; χ^2 stage $_{(1)}$ = 165.9408, p < 0.0001; χ^2 interaction $_{(1)}$ = 0.259, p = 0.6109. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(32)}$ = 0.724, p = 0.7235; Tone freezing: vehicle vs. muscimol: $t_{(32)}$ = 0.360, p = 0.9224; $n_{vehicle}$ = 10, $n_{muscimol}$ = 9.

<u>Supplementary Figure 6. Statistics</u>

B, Left (Cued FC) GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for miss cued test: χ^2 treatment $_{(2)}$ = 2.5079, p = 0.2854; χ^2 stage $_{(1)}$ = 85.1604, p < 0.0001; χ^2 interaction $_{(2)}$ = 33.459, p < 0.0001. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(40)}$ = 1.457, p = 0.6306; Pre-tone freezing: vehicle vs. miss: $t_{(40)}$ = 1.228, p = 0.7863; Pre-tone freezing: muscimol vs. miss: $t_{(40)}$ = 0.168, p = 1.0000; Tone freezing: vehicle vs. muscimol: $t_{(40)}$ = 5.191, p < 0.0001; Tone freezing: vehicle vs. miss: $t_{(40)}$ = 1.550, p = 0.5636; Tone freezing: muscimol vs. miss: $t_{(40)}$ = 3.559, p = 0.0058; $n_{vehicle}$ = 8, $n_{muscimol}$ = 9, n_{miss} = 7.

<u>Supplementary Figure 7. Statistics</u>

B) GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction for training of OF-inhibited animals: χ^2 treatment $_{(1)}$ = 0.685, p = 0.4077; χ^2 stage $_{(1)}$ = 0.050, p = 0.8231; χ^2 interaction $_{(1)}$ = 7.700, p = 0.0055. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.828, p = 0.6620; Tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 1.265, p = 0.3945. $n_{vehicle}$ = 6, $n_{muscimol}$ = 6.

C) GzLMM two-way repeated measures ANOVA of treatment, test stage, and interaction for cued test of OF-inhibited animals: χ^2 treatment $_{(1)}$ = 0.0697, p = 0.7918; χ^2 stage $_{(1)}$ = 15.254, p < 0.0001; χ^2 interaction $_{(1)}$ = 0.103, p = 0.7478. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.264, p = 0.9579; Tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.190, p = 0.9780; $n_{vehicle}$ = 6, $n_{muscimol}$ = 6.

<u>Supplementary Figure 8. Statistics</u>

945 **A)** GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction for cued
946 training of 7 d context + tone tested group: χ^2 treatment $_{(1)}$ = 0.037, p = 0.8471; χ^2 stage $_{(1)}$ = 8.741, p =
947 0.0031; χ^2 interaction $_{(1)}$ = 8.674, p = 0.0032. Sidak's multiple comparisons test: Post-shock freezing: vehicle
948 vs. muscimol: $t_{(46)}$ = 0.193, p = 0.9769; Tone freezing: vehicle vs. muscimol: $t_{(46)}$ = 2.216, p = 0.0624. $n_{vehicle}$
949 = 13, $n_{muscimol}$ = 13.
950 **B)** GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction for cued
951 training of 21 d context + tone tested group: χ^2 treatment $_{(1)}$ = 3.723, p = 0.0537; χ^2 stage $_{(1)}$ = 0.023, p =
952 0.8802; χ^2 interaction $_{(1)}$ = 4.535, p = 0.0332. Sidak's multiple comparisons test: Post-shock freezing: vehicle
953 vs. muscimol: $t_{(26)}$ = 1.930, p = 0.1251; Tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 0.521, p = 0.8454. $n_{vehicle}$
954 = 8, $n_{muscimol}$ = 8.
955 Stage factor refers to freezing between shock and tone, or during tone (post-shock or tone respectively).

956 <u>Supplementary Figure 10. Statistics</u>

957 GzLMM two-way repeated measures ANOVA of optogenetic disruption, training stage, and interaction for
958 oChIEF stimulated group: χ^2 disruption $_{(1)}$ = 0.007, p = 0.9358; χ^2 stage $_{(1)}$ = 0.018, p = 0.8946; χ^2 interaction
959 $_{(1)}$ = 0.0650, p = 0.7988. Sidak's multiple comparisons test: Post-shock freezing: Control vs. oChIEF: $t_{(20)}$ =
960 0.081, p = 0.9960; Tone freezing: Control vs. oChIEF: $t_{(20)}$ = 0.113, p = 0.9921. $n_{Control}$ = 7, n_{oChIEF} = 6. Stage
961 factor refers to freezing between shock and tone, or during tone (post-shock or tone respectively).

962 <u>Supplementary Figure 12. Statistics</u>

963 GzLMM two-way repeated measures ANOVA of optogenetic inhibition, training stage, and interaction for
964 cued training of CS-US ArchT inhibited group: χ^2 inhibition $_{(1)}$ = 0.002, p = 0.9631; χ^2 stage $_{(1)}$ = 3.173, p =
965 0.0749; χ^2 interaction $_{(1)}$ = 5.000, p = 0.0254. Sidak's multiple comparisons test: Post-shock freezing: GFP
966 vs. ArchT: $t_{(32)}$ = 0.046, p = 0.9987; Tone freezing: GFP vs. ArchT: $t_{(32)}$ = 2.098, p = 0.0859. n_{GFP} = 9, n_{ArchT} =
967 10. Stage factor refers to freezing between shock and tone, or during tone (post-shock or tone
968 respectively).

969 <u>Supplementary Figure 13. Statistics</u>

970 GzLMM three-way repeated measures ANOVA of test day, test stage, optogenetic inhibition and
971 interactions for inter stimulus ArchT inhibited experiment: χ^2 day $_{(1)}$ = 20.696, p < 0.0001; χ^2 stage $_{(1)}$ =
972 13.802, p = 0.0002; χ^2 inhibition $_{(1)}$ = 0.424, p = 0.5149; χ^2 interaction(Day×Stage) $_{(1)}$ = 7.247, p = 0.0071; χ^2

interaction(Day×Inhibition) $_{(1)}$ = 0.615, p = 0.4330; χ^2 interaction(Stage×Inhibition) $_{(1)}$ < 0.001, p = 0.9825; χ^2 interaction(Day×Stage×Inhibition) $_{(1)}$ = 0.294, p = 0.5876. Sidak's multiple comparisons test: Day7: Pre-tone freezing: GFP vs. ArchT: $t_{(46)}$ = 0.651, p = 0.9875; Day 7: Tone freezing: GFP vs. ArchT: $t_{(46)}$ = 0.704, p = 0.9813; Day 8: Pre-tone freezing: GFP vs. ArchT: $t_{(46)}$ = 0.456, p = 0.9982; Day 8: Tone freezing: GFP vs. ArchT: $t_{(46)}$ = 0.658, p = 0.9868; ArchT: Tone freezing: Day 7 vs. Day 8: $t_{(46)}$ = 0.937, p = 0.9271; Day 8: ArchT: Pre-tone vs. Tone freezing: $t_{(46)}$ = 0.046, p = 1.0000; n_{GFP} = 7, n_{ArchT} = 7.