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

Short title: The Lateral Habenula Regulates Fear Conditioning.

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Abstract

Fear is an extreme form of aversion that, if inappropriately generalized, initiates pathological conditions such as panic or anxiety disorders. Fear conditioning (FC) is the best understood model of fear learning.

FC forms two associations that independently link the cue and the training context to fear responses. The lateral habenula (LHb) encodes aversive information. However, how the LHb participates in fear learning has not been intensively studied. Here we studied the role of the LHb in FC learning using optogenetics and pharmacological tools in rats. We found that disrupting neuronal activity of the LHb during training abolishes independent expression of contextual and cued memories, yet memory is normally expressed when the cue is played in the training context. Our results show that neuronal activity at the LHb regulates independent expression of sub-components of a fear memory, assigning to that structure a previously uncharacterized role as regulator of fear memories.

Introduction

Fear is an extreme form of aversion characterized by an uncontrollable reaction to threatening stimulus. In nature, stimuli to be fear of will never be identical, thus fear is adaptively generalized to some degree (Asok, Kandel and Rayman, 2019). However, exacerbated fear generalization is supposed to be the basis to the development of psychopathological conditions like panic or anxiety disorders, highlighting the relevance of the study of basic mechanisms of fear learning (Lissek *et al.*, 2010, 2014; Dunsmoor and Paz, 2015).

Fear conditioning (FC) is probably the most studied and best understood model of fear learning (Maren and Quirk, 2004; Maren, 2008). 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; LeDoux and Phillips, 1992). Consequently, subjects will display defensive responses, characterized by freezing behavior, when re-exposed to the training context or to the cue presented in a novel and neutral context.

The lateral habenula (LHb) is a central hub for the processing of aversive information in the brain. Aversion related information reaches the LHb from numerous structures of the limbic system and basal ganglia. In turn, the LHb projects to the brain stem, where it is one of the few structures that control (directly and indirectly) both serotoninergic and dopaminergic systems (Hikosaka, 2010; Proulx, Hikosaka and Malinow, 2014; Hu, Cui and Yang, 2020). That connectivity places the LHb in a central position to control aversive associative learning. However, how the LHb is related to fear learning has not been intensively studied yet.

52 In a previous work, using the Inhibitory Avoidance paradigm, we found that inactivation of the
53 LHb during training affects temporal stability of the memory but not its formation (Tomaiuolo *et al.*,
54 2014). To better understand how the LHb influences aversive learning, in this work we studied its role in
55 FC learning by means of pharmacological and optogenetics manipulations. We show that interfering
56 with neuronal activity of the LHb during FC training severely impairs recall of contextual and cued FC
57 memories. However, when the cue is played in the training context, a conserved FC memory is
58 evidenced. Thus, our results suggest that the formation of two independent memory traces during FC
59 requires an intact LHb activity, placing this structure in a central position in the neuronal circuits
60 mediating FC learning.

61

62 ## Results

63 ### Pharmacological inactivation of the LHb impairs contextual fear conditioning

64 To examine the role of the LHb in contextual FC, we analyzed the effect of its inactivation during
65 training. For that purpose, we performed surgeries, in rats, to implant bilateral intracerebral cannulae
66 aimed at the LHb. After a recovery time of 10 to 12 days, animals were bilaterally infused the GABA-A
67 agonist muscimol or vehicle in the LHb, and were trained in contextual FC thirty minutes later. During
68 training rats were placed in a FC chamber and presented with four unsignaled mild foot-shocks (Figure
69 1A). We observed that the infusion of muscimol in the LHb did not modify freezing behavior during FC
70 training (Figure 1B and Supplementary Figure 1). Also, in a separate cohort of animals, we observed that
71 such procedure did not modify locomotion or exploratory behavior in an open field (OF; Supplementary
72 Figure 2), indicating that inactivation of the LHb does not have a general effect over motility or context
73 exploration. To evaluate contextual FC, animals were placed back in the conditioning chamber 7 days
74 after training and freezing behavior was quantified. Muscimol group displayed significantly lower levels
75 of freezing than the control group, suggesting that inactivation of the LHb during contextual FC training
76 impairs long-term memory formation (Figure 1C).

77 Long-term memories tend to decay over time, a phenomenon that could already be evident 7
78 days after memory formation (Bekinschtein *et al.*, 2007; Rossato *et al.*, 2009). To test whether an
79 accelerated decay of contextual FC memory could account for the memory deficits observed in the
80 muscimol group, we repeated our previous experiment but tested contextual FC memory 24 hours after
81 training. Contextual FC memory deficits were also evident 24 hours after training (Figure 1 D), further
82 suggesting that inactivation of the LHb during contextual FC training has a detrimental effect over long-
83 term memory formation.

84 Importantly, muscimol infusion in the LHb before exposure to an OF did not impact on the
85 decrease in exploratory behavior observed upon a subsequent re-exposure performed 48 hours later
86 (Supplementary Figure 2), which is acts as a measure of contextual memory on that test (Vianna, 2000;
87 Etkin *et al.*, 2006). That observation suggests that the inactivation of the LHb by muscimol does not
88 induce a general deficit in context encoding.

89 Pharmacological inactivation of the LHb impairs cued fear conditioning

90 Having observed that inactivation of the LHb impairs the formation of contextual FC we
91 extended our experiments analyzing the effect of LHb inactivation on cued FC memory formation.
92 Animals were infused muscimol or vehicle in the LHb, and 30 minutes later were trained in cued FC, in
93 which each of the 4 foot-shocks was preceded by a 17 seconds long tone (Figure 2A). Cued FC memory
94 was tested 24 hours or 7 days later by exposing the animals to the tone in a novel context (Context B).
95 Behavior during training confirmed that inactivation of the LHb does not affect the surge of freezing
96 elicited by shock exposure (Figure 2B, Supplementary Figure 1B). During tests, presentation of the cue
97 elicited a robust freezing in the vehicle groups that was markedly reduced in the muscimol groups
98 (Figure 2C, D). These results indicate that cued FC memory is also impaired by the inactivation of the LHb
99 during training. Thus, inactivation of the LHb during FC training impairs formation of both contextual and
100 cued FC long-term memories.

101 To control for spatial specificity of muscimol infusion we implanted cannulae targeting areas
102 around the LHb and infused muscimol before cued FC training (Supplementary Figure 3A, B). During
103 tests, performed 7 days later, we found that neither infusion of muscimol 1 mm lateral, dorsal or ventral
104 to the LHb affected freezing behavior (Supplementary Figure 3 B, C). Moreover, we did not see a
105 reduction in freezing behavior during cued FC test in muscimol infused animals implanted with cannulae

106 aimed at the LHb in which histological control showed cannulations bilaterally missed the LHb
107 (Supplementary Figure 3A, C).

108 To control for non-transient effects of muscimol infusion, we trained in cued FC the animals that
109 had previously undergone muscimol inactivation of the LHb before exposure to an OF (Supplementary
110 Figure 4A). In that group of animals, we found no differences in freezing behavior during either cued FC
111 training or testing (Supplementary Figure 4B, C), demonstrating the transient nature of that
112 manipulation. Thus, deficits in contextual and cued FC in the muscimol groups should be attributed to a
113 transient inactivation of the LHb during training.

114 Context + tone test evidences a conserved fear conditioning memory

115 To further investigate to what extent inactivation of the LHb impairs FC learning, we performed
116 additional experiments in which freezing to the cue was evaluated in the training context (Context A), a
117 condition we called context + tone (Figure 3A). In that test the pre-tone period is a readout of the
118 contextual component of the memory after a cued FC training. Indeed, during the pre-tone period we
119 observed a clear reduction of freezing in the muscimol group, confirming that inactivation of the LHb
120 during training impairs contextual FC (Figure 3B). Presenting the tone further increased freezing in the
121 control group and, most notably, also induced a robust freezing in the muscimol group, which reached
122 freezing values equivalent to the control group (Figure 3B). Thus, under inactivation of the LHb, a FC
123 memory is formed that could be effectively retrieved when the cue is presented in the conditioning
124 context.

125 In a previous paper we demonstrated that the inactivation of the LHb impairs long-term stability
126 of the Inhibitory Avoidance memory (Tomaiuolo *et al.*, 2014). To investigate long-term stability of FC
127 memory formed under inactivation of the LHb, we evaluated freezing in context + tone conditions 3

128 weeks after training in a new set of animals (Figure 3 A, C). Freezing levels of the muscimol group in
129 context + tone condition were lower than in the vehicle group 3 weeks after training (Figure 3C),
130 showing that FC training under inactivation of the LHb generates a weak memory that is harder to
131 retrieve and is temporarily less stable.

132 Context + tone retrieval reconsolidates cued but not contextual memory

133 In animals that were trained in FC under inactivation of the LHb we observed conserved freezing
134 responses in context + tone conditions. However, it could not be known if that freezing represents the
135 retrieval of the contextual memory, the cued memory, both or neither of them. To get an insight on
136 that, we analyzed if memory retrieval in context + tone conditions induces the reconsolidation of cued
137 and contextual memories. Animals were infused muscimol in the LHb and trained in cued FC as
138 described above. Seven days later they went through a recall session in which the cue was presented in
139 the training context (*i.e.* as in context + tone test). Twenty-four hours later they went through a test
140 session in which we evaluated reconsolidation of cued and contextual memories. To evaluate
141 reconsolidation of the cued memory, animals were placed in Context B and exposed to the cue
142 (reconsolidation group, Figure 4A). To evaluate reconsolidation of thecontextual memory, animals were
143 re-exposed to the training context (Context A; Figure 4C). In addition, at the end of this latter
144 experiment the cue was presented, configuring a re-test in context + tone conditions (Figure 4C). A "no-
145 reconsolidation" group was also included as a control for cued memory tested "reconsolidation" group.
146 In the no-reconsolidation group, cued memory was tested 8 days after training in Context B without any
147 recall session (Figure 4A). Notably, during cued memory test, the "reconsolidation" group that went
148 through the recall session displayed higher levels of freezing to the cue than the no-reconsolidation
149 group (Figure 4B). In fact, in the reconsolidation group levels of freezing to the tone during test session
150 were equivalent to those shown during the recall session (Figure 4B). In contrast, in the group in which

151 we tested reconsolidation of contextual memory, we did not observe any increase in freezing to the
152 context between test and recall session (Figure 4D). Then, levels of freezing remained low even though
153 those animals effectively displayed freezing in context + tone condition in the recall session (Figure 4D).
154 Also, freezing in context + tone conditions was not different between the recall and the test session and
155 was high in both cases (Figure 4D). Those results suggest the recall session reconsolidated the cued
156 memory but not the contextual memory, arguing that freezing observed during context + tone condition
157 mainly reflects the reactivation of the cued memory trace. Furthermore, they suggest that FC training
158 under inactivation of the LHb creates a cued memory whose retrieval depends on contextual
159 information.

160 Optogenetic inactivation of the LHb impairs cued fear conditioning

161 To get a better spatial and temporal control of the interventions over the LHb we manipulated
162 its activity using optogenetic tools. To first confirm and support results obtained from muscimol
163 inactivation of the LHb, we injected an adeno-associated virus (AAV) encoding the inhibitory light-
164 dependent proton pump Archaerhodopsin (ArchT) fused to the fluorescent protein GFP (AAV-CamKIIα-
165 ArchT-GFP) bilaterally in the LHb of rats (Figure 5A). Control rats were injected with an AAV encoding the
166 fluorescent protein GFP (AAV-CamKIIα-GFP). In both groups, optic fiber cannulae were implanted with
167 the tip immediately above the LHb (Figure 5A, B). Whole cell patch-clamp recordings confirmed that LHb
168 neurons could be inhibited by ArchT (Supplementary Figure 5). Light was applied during the FC training
169 session starting at tone onset and stopping 5 s after shock termination (Figure 5A). We did not observe
170 differences in freezing between ArchT and GFP groups during FC training (Supplementary Figure 6A). In
171 accordance with pharmacological results, we found that freezing to the tone during cued memory test
172 was lower in the ArchT than in the GFP group. (Figure 5C, E). On the other hand, in contrast to
173 pharmacological manipulation, contextual memory was not affected by optogenetic inhibition of the

174 LHb during cue and US (Figure 5D, E). Additionally, as observed in pharmacological experiments, context
175 + tone condition evoked similar freezing in ArchT and GFP groups (Figure 5D, E).

176 Artificial activation of the LHb impairs cued and contextual fear conditioning

177 To rigorously test the involvement of the LHb in contextual FC using optogenetic tools, it would
178 be necessary to disturb neuronal activity at the LHb during context encoding as well (*i.e.* during whole
179 training period). Optogenetic inhibition mediated by light activated hyperpolarizing pumps such as
180 ArchT or NpHR, require sustained illumination which could create confounding effects, such as
181 temperature changes, when prolonged for long periods like the one involved in our protocols
182 (Supplementary Figure 7). Also, sustained on-off patterns of optogenetic inhibition induce burst activity
183 of LHb neurons that induce depression-like symptoms (Yang *et al.*, 2018; Supplementary Figure 5D). To
184 avoid those potential artifacts, we decided to take a different approach. We reasoned that neuronal
185 encoding would be disturbed by any major intervention over neuronal activity. Thus, we opted to
186 disrupt endogenous activity of the LHb by imposing an artificial excitation during the whole training.
187 Animals were injected with an AAV encoding the fast excitatory opsin oChIEF (Lin *et al.*, 2009), and
188 implanted with optic fibers bilaterally in the LHb (Figure 6A, B). Three weeks later they were trained in
189 cued FC under a sustained 20 Hz excitation drive with light pulses of 5 ms (Figure 6A), which has been
190 shown before not to induce depression-like symptoms (Yang *et al.*, 2018; Figure 6A, B). Modelling of
191 light induced temperature changes supports the idea that such protocol would result in minimal
192 temperature changes in the illuminated tissue (Stujenske, Spellman and Gordon, 2015; Supplementary
193 Figure 7). We found that artificial stimulation of the LHb during FC training mostly replicates results
194 obtained by pharmacological inhibition (Figure 6). oChIEF activation did not modify behavior during FC
195 training (Supplementary Figure 6B). During cued memory test, oChIEF stimulated animals showed lower
196 freezing to the tone than the control group (Figure 6C, E). Next day, during context + tone test session,

197 oChIEF stimulated animals showed almost no freezing to the context but displayed freezing to the cue,
198 reaching levels higher than in cued memory test performed the previous day (Figure 6D, E). Thus,
199 disrupting the encoding of the LHb during whole training has a deleterious effect over FC learning
200 equivalent to that of pharmacological inhibition. Remarkably, AAVs did not infect medial habenula
201 (Figure 5B, 6B). Thus, results from optogenetics experiments could not be attributed to an effect over
202 that structure, a criticism valid for muscimol mediated inactivation.

203

204 Discussion

205 Previous reports indirectly suggested that the LHb could play a role in FC learning (Wang *et al.*,
206 2017; Trusel *et al.*, 2019). However, the requirement of the LHb for FC learning has received little
207 attention (Wang *et al.*, 2013; Chan *et al.*, 2017; Durieux *et al.*, 2020). In this work, we directly addressed
208 that subject. Our findings reveal that the LHb is a relevant component in FC learning circuits, playing a
209 role in the formation of both cued and contextual memories. In the following subsections we discuss our
210 findings in detail.

211 During FC training, subjects infer which stimuli have predictive value over the arrival of the US to
212 organize proper defensive behavioral responses. In normal conditions, that processing leads to a double
213 association that assigns a predictive value to both the context and the cues. Prevailing models postulate
214 that context-US association involves context encoding circuits centered in the hippocampus which send
215 contextual representation to the amygdala where the association takes place (Maren, Phan and
216 Liberzon, 2013; Kim and Cho, 2020), and locate cue-US association in the amygdala (Bocchio, Nabavi and
217 Capogna, 2017). Particularly, solid evidence indicates that tone-US association takes place in cortical and
218 thalamic auditory inputs to the lateral amygdala (Rumpel *et al.*, 2005; Sah, Westbrook and Lüthi, 2008;
219 Nabavi *et al.*, 2014). Thus, as postulated in that model, cued memory is assumed to be intrinsically
220 context-independent. However, our experiments suggest that the formation of a cued memory that
221 could be expressed in a context-independent manner requires signaling by the LHb during memory
222 formation.

223 Two observations lead us to reach that conclusion. First, we found that disrupting neuronal
224 activity at the LHb during FC training, either by inhibition (pharmacological or optogenetic), or by
225 sustained optogenetic activation, results in a condition in which retrieval of FC memory requires that the
226 cue is presented in the training context. This observation implies an interaction between the context

227 and the cue in guiding memory retrieval. Second, cued memory is recovered following that retrieval.
228 Since contextual FC memory is not restored by the same manipulation, it could be inferred that the
229 freezing observed when the cue is played in the training context is mostly attributable to the
230 reactivation of the cued memory. In addition, the latter observation argues that freezing in context +
231 tone conditions does not reflect the retrieval of two weak fear memories (contextual and cued) that add
232 up to trigger freezing behavior. Altogether, those two observations suggest that, under inactivation of
233 the LHb, a cued FC memory is formed whose retrieval depends on the context in which the cue is
234 presented.

235 It has been shown that during FC training, activity of the LHb increases in response to cue
236 presentation, paralleling the appearance of conditioned responses (Wang *et al.*, 2017; Trusel *et al.*,
237 2019). Though, no mechanistic information about how the LHb influences cued memory formation could
238 be inferred from the present work, a tempting hypothesis is that the emergence of responses of the LHb
239 to the cue acts as a prediction error signal over the arriving of the US that, when confirmed,
240 emancipates the cue-US association from the context where it was formed.

241 Contextual FC is also blocked by pharmacological inactivation or artificial excitation of the LHb
242 during whole training. It has been proposed elsewhere that the LHb influences context encoding
243 through a functional interaction with the hippocampus (Goutagny *et al.*, 2013; Baker *et al.*, 2019). That
244 hypothesis has received support from physiological data generated by other authors and us showing
245 that firing of the neurons of the LHb is synchronized with hippocampal theta rhythm (Aizawa *et al.*,
246 2013; Goutagny *et al.*, 2013; Bertone-Cueto *et al.*, 2020). In addition, two recent papers from Nyiri group
247 showed that LHb-innervated Nucleus Incertus (Szőnyi, Sos, *et al.*, 2019) and Dorsal Raphe neurons
248 (Szőnyi, Zichó, *et al.*, 2019) modulate hippocampal theta. Thus, based on that evidence, deficits in
249 contextual FC could be attributed to a disrupted context encoding.

250 However, three results presented here suggest that deficits in contextual FC induced by
251 disrupting neuronal activity of the LHb during training reflect deficits in context-US association rather
252 than in context representation. First, freezing in context + tone conditions, evidences the retention and
253 effective recall of contextual information. Second, as discussed above, retrieval in context + tone
254 conditions is not sufficient to reconsolidate contextual FC memory, suggesting that the contextual
255 memory retrieved is not linked to fear response. Third, inactivation of the LHb does not affect the
256 decrease in the exploration of an OF upon re-exposure, showing, in a different paradigm, that
257 inactivation of the LHb does not impede the formation of a contextual memory. More experiments
258 would be required to determine if the LHb participates in associative or representational components of
259 contextual FC.

260 How easy a memory is retrieved could be read as a parameter of memory strength. Also, in
261 temporal dimension, the strength of a memory could be read from how long it lasts (Bekinschtein *et al.*,
262 2008). In our previous work we described that inactivation of the LHb before training in the Inhibitory
263 Avoidance generates a memory that is normally expressed 24 hours after training but could not be
264 evidenced 7 days later (Tomaiuolo *et al.*, 2014). In that paradigm, temporal stability of the memory is
265 defined by shock intensity (*i.e.* by the salience of the US), thus it could be evaluated as a parameter of
266 memory strength. There, we also found that a series of manipulations known to extend temporal
267 stability of weak Inhibitory Avoidance memories did also extend temporal stability of the memory
268 formed under inactivation of the LHb (Tomaiuolo *et al.*, 2014). Based on those findings, we postulated
269 that the main effect of the inactivation of the LHb was to reduce memory strength. Our present results
270 support and extend that hypothesis to the FC paradigm. In one side, we found FC memory is harder to
271 retrieve, since matching between training and testing conditions should be increased in order to
272 effective retrieval. In addition, we also found that the FC memory expressed under context + tone
273 conditions is temporarily less stable (Figure 3C). Thus, FC memory formed under inactivation of the LHb

274 could be considered weaker in those two aspects. Taken together those results indicate a general role of
275 the LHb in defining aversive memory strength.

276 Inappropriate fear generalization has been postulated as the trigger to psychopathological
277 conditions like panic or anxiety disorders (Lissek *et al.*, 2010, 2014). Notably, studies on fear
278 generalization have focused on single components of FC, analyzing either generalization of cued or
279 contextual components of fear memories, but the interaction between them has not received much
280 attention (Asok, Kandel and Rayman, 2019). The results presented here suggest that contextual
281 information influences retrieval of cued memory, then, cued memory generalization could actually
282 reflect the interaction between cue and contextual memories. Moreover, FC memory formed without
283 proper LHb signaling could be considered an example of below-normal levels of generalization.
284 Therefore, it could be speculated the LHb could also influence fear generalization. Particularly it would
285 be interesting to analyze fear generalization in described models of LHb hyperactivity induced mood
286 disturbances (Hu, Cui and Yang, 2020).

287

288 ## Materials and Methods

289 ### Animals

290 Experiments were performed in male Wistar rats obtained from the vivariums of the Faculty of
291 Pharmacy and Biochemistry of the Buenos Aires University, Argentina, and Janvier Labs, France. Animals
292 were 5-6 weeks old at the time of surgery. Animals were housed 4 to 6 per cage, with *ad libitum* access
293 to food and water, under 12-hour light/dark cycle (lights on at 7:00 am), at constant temperature of 22 ±
294 2 °C. All the procedures where performed during light hours. Experimental procedures were approved
295 by the Animal Care and Use Committee of the University of Buenos Aires (CICUAL), and the Autonomous
296 Community of Madrid (PROEX 167/18).

297 ### Surgeries

298 **Pharmacology:** rats under deep ketamine/xylazine anesthesia (100 and 5 mg/kg respectively)
299 were bilaterally implanted with 22-Gauge guide cannulae aimed 2.0 mm above the LHb (AP -3.0 mm, ML
300 ± 0.7 mm, DV -3.8 mm from Bregma), or 1.0 mm dorsal, ventral or lateral to LHb coordinates in specific
301 experiments. Cannulae were fixed to the skull with three surgical steel screws and dental acrylic. At the
302 end of surgery, animals received a dose of analgesic (meloxicam 0.6 mg/kg) and antibiotic (gentamicin 3
303 mg/kg). Behavioral procedures began 7-9 days after surgery.

304 **Optogenetics:** rats under deep isofluorane anesthesia (4 % induction, 1-2 % maintenance, in 0.8
305 L/minute oxygen) were bilaterally injected with 300 nl of viral vector (AAV8-CamKIIα-ArchT-GFP, 6.2 x
306 10^{12} viral particles/ml from UNC Vector Core, AAV8-CamKIIα-GFP, 6.3 x 10^{12} viral particles/ml from UNC
307 Vector Core, or AAV8-hSyn-oChIEF-tdTomato, homemade as described (*34*) per side at the LHb (AP -2.9
308 mm, ML ± 0.7 mm, DV -5.0 mm from Bregma). Subsequently, optical fiber implants (200 µm core, 0.45
309 NA) were bilaterally inserted with the tips aiming just above the LHb, with 4 degrees angle from the

310 sagittal plane (AP -3.0 mm, ML ± 1.05 mm, DV -4.5 mm from Bregma), and fixed to the skull with three
311 surgical steel screws and dental acrylic. During surgery, animals received a dose of analgesic (meloxicam
312 0.6 mg/kg) and antibiotic (gentamicin 3 mg/kg). Behavioral procedures began three weeks after surgery.

313 Contextual and Cued Fear Conditioning Training

314 For three consecutive days before the FC training, rats were habituated to handling once a day.
315 There, animals were grasped by hand and slightly restrained against the chest of the investigator for 1-3
316 minutes a manipulation similar to the one performed during intracerebral drug infusions. On training
317 day, animals were transferred inside their home cage to a room contiguous to the training room where
318 they acclimatized for 1 hour before the beginning of any other procedure. FC training took place in
319 Context A (electric foot-shock delivering grid floor, black plastic walls and ceiling with one transparent
320 plexiglass side, 50x25x30 cm wide, width and height, illuminated by white light). Cued FC protocol
321 consisted of a 180 s baseline period of free exploration followed by 4 tone-shock presentations (17 s, 3
322 kHz, 80 dB tone followed by 3 s, 0.60 mA, square monophasic 60 Hz electric shock) with an inter-
323 stimulus interval (ISI) of 70 s. Thirty seconds after the last shock, animals were returned to their home
324 cage in the acclimation room. When training session ended, home cages were left in the acclimation
325 room for at least one hour before returning them to the animal facility. Ethanol 50 % was used to clean
326 the conditioning cage between subjects. For contextual FC training procedures were equal but the tone
327 was omitted during conditioning. Training was always video recorded for posterior analysis.

328 **Pharmacology experiments:** after the acclimation period, rats were randomly assigned to
329 receive bilateral intra LHb infusions of GABAa agonist, muscimol (Sigma, 60 ng/µl in saline solution, 0.5
330 µl/side), or saline. Fluorescent green beads were added to both solutions to aid in checking the infusion
331 site (1:1000 dilution of concentrated 1 µm diameter fluorescent green beads, Bangs Laboratories, USA).
332 During infusions animals were grasped by hand and slightly restrained in the lap or the arm of the

333 investigator. Infusions were delivered at a rate of 0.5 µl/minute through a 30-Gauge needle extending
334 2.0 mm beyond the tip of implanted guide cannula and connected to a 10 µl Hamilton syringe by a
335 polyethylene tube. Infusion needle was left in place for an additional minute to minimize backflow. After
336 that was animals were returned to their home cage. FC training began 30 minutes later.

337 **Optogenetics experiments**: after the acclimation period, rats were taken to the FC room and
338 were bilaterally connected to 1 to 2 optic fiber branching patch-cords (200 µm core each, Doric Lenses,
339 Canada). The single end of the branching patch-cord (400 µm core) was connected to a rotary joint
340 (Doric Lenses, Canada) that was attached to the laser source by a simple patch-cord (400 µm core, 0.45
341 NA, ThorLabs, USA). FC chamber (Context A) had a modification in the ceiling (10 cm hole in the center)
342 to allow optic fiber patch-cord movement. For the ArchT experiment, continuous light of 532 nm at 10
343 mW at the tip of the fiber implant was delivered by a laser source (CNI, China), starting at tone onset
344 and stopping 5 s after shock termination. For the oChIEF experiment, 5 ms pulses of 447 nm at 20 Hz
345 with an intensity of 10 mW at the tip of the fiber implant, were delivered by laser source (Tolket,
346 Argentina) during the whole training. A mixed control group composed of oChIEF injected without light
347 stimulation and saline injected animals with light stimulation was used in that experiment since an AAV-
348 tdTomato was not available. At the end of FC training, patch-cord was disconnected from the fiber
349 implants and animals were returned to home cage.

350 <u>Contextual FC Test</u>

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

355 Cued FC Test

356 Several precautions were taken to avoid generalization during cued test. Home cages were
357 moved to a different acclimation room of the one used in the training day, the investigator used latex
358 gloves, animals were transported between the acclimatation room and the test chamber within a plastic
359 box, and acetic acid 1 % m/v was employed for cleaning between subjects. Test was performed in
360 Context B, a white acrylic box with a transparent plexiglass ceiling (25 x 25 x 40 cm wide, width and
361 height) with floor covered in wood shaving and lit up with red light. Cue test consisted of a pre-tone
362 period of 180 s during which animals could freely explore followed by 60 s tone (same as training tone)
363 and 30 s post-shock period. After that, animals were removed from the test cage and placed back in the
364 home cage. Test was video recorded for posterior analysis. 1 hour after finishing tests, home cages were
365 taken back to the animal facility.

366 Context + Tone Test

367 Context + tone test proceeded as contextual FC test but after 180 s in the test cage the tone was
368 presented for 60 s. 30 s after tone ending animals were returned to their home cage.

369 Open Field

370 Animals were placed for 15 minutes into an open field (OF; square grey plastic floor and walls 50
371 cm side x 35 cm height) and video recorded. Behavior during OF was analyzed offline with ANY-Maze
372 video tracking system (v4.82, Stoelting Co., Wood Dale, USA).

373 Histologic Control

374 Within a week after the end of behavioral procedures animals were euthanized with a lethal
375 dose of ketamine/xylazine, 50 ml of ice-cold phosphate-buffered saline (PBS) 0.1 M was transcardially
376 perfused, followed by 50 ml of paraformaldehyde 4 %. After that, the brain extracted and incubated at 4

377 °C in paraformaldehyde 4 % overnight. Then, brain was transferred to 30 % m/v sucrose in PBS 0.1 M,
378 incubated for three days at 4 °C and coronal sections (150 µm) were cut at a freezing microtome.
379 Sections were mounted with glycerol 50 % v/v in PBS 0.1 M, examined and photographed at 40X
380 magnification with GFP and DAPI filters. For the pharmacological experiments, injection sites were
381 determined by the presence of green beads. Animals in which both infusions were correct were included
382 in the analysis. Animals from the cohorts used in Figure 2B-C in which both infusions were outside the
383 LHb were included in the miss group presented in Figure S3. For the optogenetics experiments, animals
384 with transfection and/or optic fiber misses were not considered in the analysis.

385 Freezing analysis

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

392 Statistical Analysis

393 Each animal was taken as an independent measure. During test session of cued or context +
394 tone experiments, average freezing before and during tone presentation were treated as repeated
395 measures. Thus, in most experiments analysis was done using a two-way repeated measures design in
396 which the analyzed factors were “test stage” (pre-tone or tone) and “treatment” (muscimol,
397 optogenetic excitation or inhibition, or the corresponding control group). In experiments in which
398 subjects were tested more than one day, “testing day” was considered an additional factor. Freezing
399 during training session was analyzed separately. There the factor stage comprised freezing during the

400 tone or in the inter-tone period. Statistical analysis of FC experiments was done by generalized linear
401 mixed modelling (GzLMM). Typical distribution of individuals freezing values accumulated at lower and
402 higher values with few individuals freezing around the group mean, indicating a non-normal distribution.
403 We therefore compared models with beta and gaussian distribution (rescaling freezing to y"=[y'(n-
404 1)+0.5]/n as suggested in (Smithson and Verkuilen, 2006)). For the model fitting in R (R Foundation for
405 Statistical Computing, Vienna, Austria) we used the 'glmmTMB' package (Brooks *et al.*, 2017). Beta
406 distribution provided better adjustment and less tendencies between group residues than gaussian
407 distribution (data not shown). Logit link function was employed. For the GzLMM we considered every
408 interaction between the corresponding fixed factors (test stage, treatment, testing day) and subject was
409 treated as a random factor with random intercepts. ANOVA and contrasts were done applying 'car' and
410 'emmeans' packages (Weisberg, 2019; Lenth, 2020) where GzLMM was used. GzLMM ANOVAs used
411 Wald χ^2 statistics for comparisons since neither likelihood ratio tests nor *F* tests are supported.
412 Assumptions of homoscedasticity and normal residues were met in the open field experiment;
413 therefore, it was analyzed via conventional two-way repeated measures ANOVA. Each experiment was
414 repeated at least twice in separate cohorts of animals. Each cohort included all the treatments. Sample
415 size calculations were done using power analysis. From pilot context test experiments, we considered a
416 standard deviation of aprox. 20 % aiming to detect a minimal difference among groups of 50 %, with an
417 80 % power, and a type I error of 5 %. Based on these parameters and first assuming gaussian
418 distribution, initially we aimed at an n = 14. Later we readjusted to n = 6 on cued memory tests, where
419 the observed difference between vehicle and muscimol infused groups (aprox. 60 %), standard deviation
420 (aprox. 25 %) and beta distribution at tone tests allowed to maintain about 80 % power and 5 % type I
421 error. For experiment in Figure 4C (reconsolidation of contextual memory) statistical evaluation of the
422 effect of the recall session was done following a within subject design since recall session on 7th day and
423 test session on 8th day were replicates. In bar graphs, freezing during training is included as a parameter

424 of the freezing of a naïve animal but, as mentioned above, freezing during training was analyzed
425 separately.

426

427 Supplementary Material:

428 Fig. S1. Freezing during training for experimental groups included in Figures 1-3.

429 Fig. S2. Inactivation of the LHb does not affect locomotion, exploration, or habituation to an Open Field.

430 Fig. S3. Controls of specificity of muscimol inactivation of the LHb.

431 Fig. S4. Inactivation of the LHb does not permanently block FC learning.

432 Fig. S5. Optogenetic inhibition of LHb neurons by ArchT.

433 Fig. S6. Freezing during training for experimental groups included in Figures 5 and 6.

434 Fig. S7. Modelling of temperature increase induced by optogenetic stimulation.

435

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539

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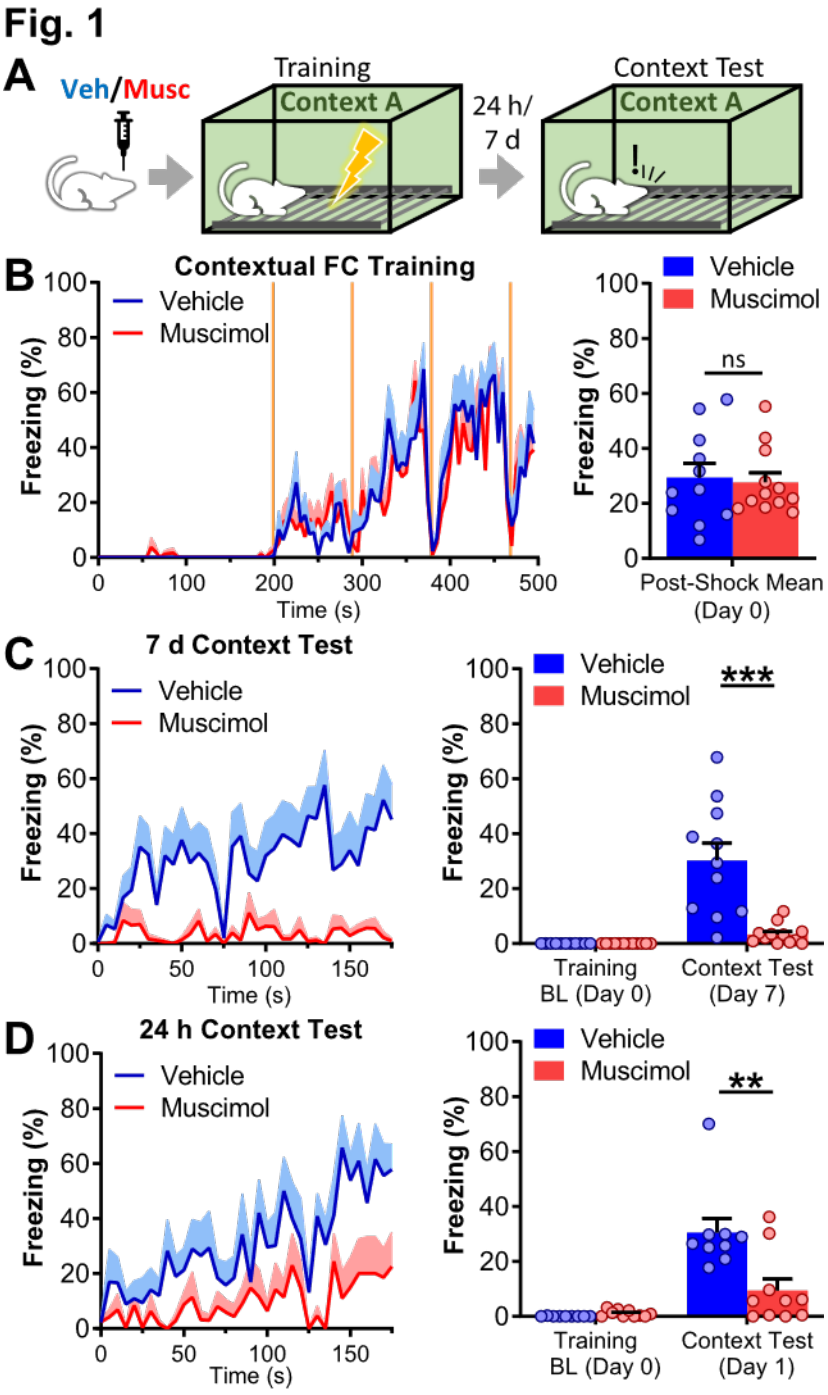
554 Figures



555

556

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

558 **A)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes
559 before training. Contextual FC was tested 24 hours or 7 days later. During training subjects were let to
560 freely explore the cage for a baseline period of 197 s. After that they were exposed to 4 foot-shocks (0.6
561 mA, 3 s) interspaced by 70 s. In the test session animals were re-exposed to the training context for 180
562 s and freezing was quantified.

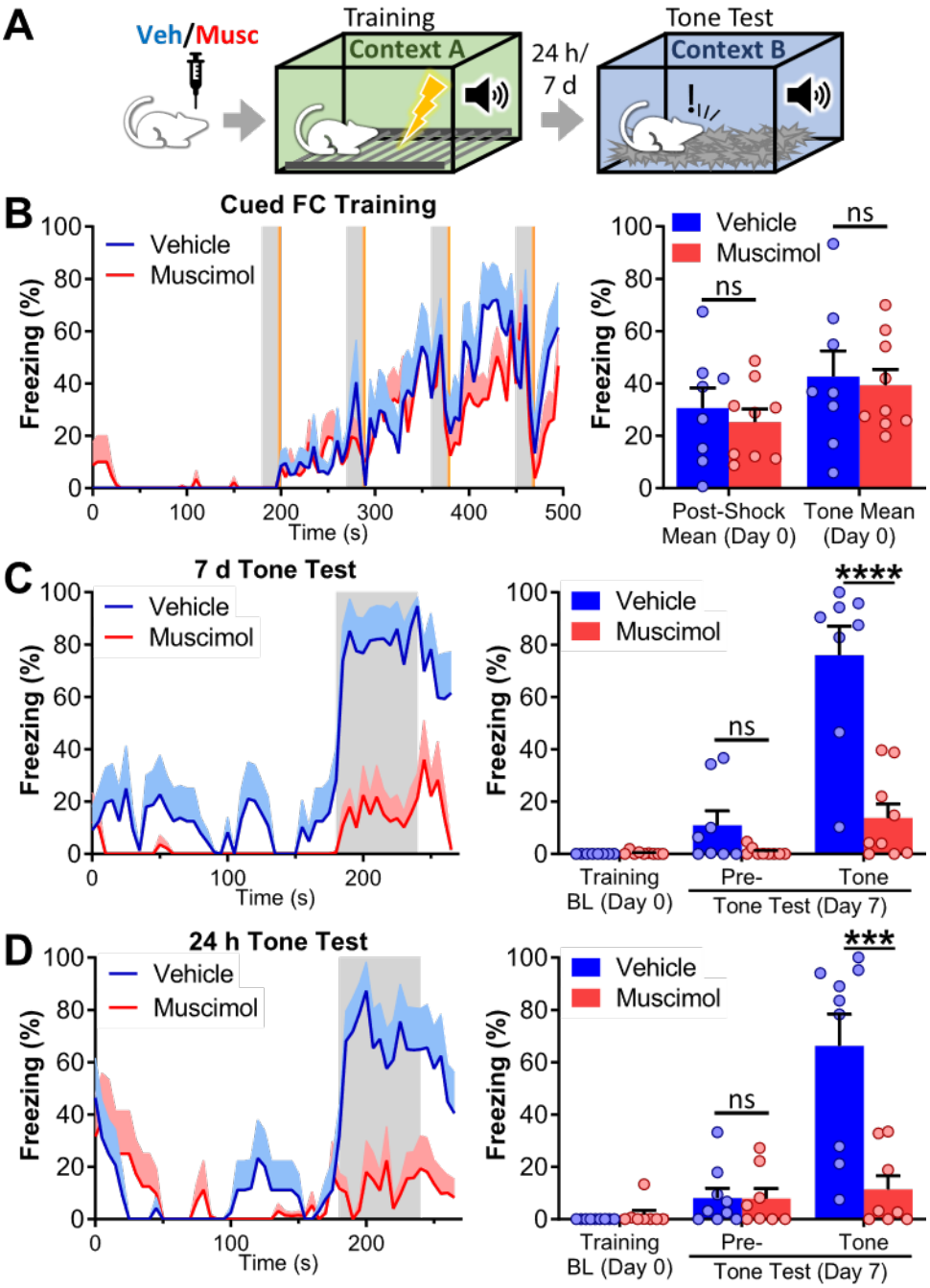
563 **B)** Freezing during cued FC training for the group tested 7 days after training. Left panel: freezing over
564 time, shocks were presented at times indicated by the orange shaded areas. Right panel: average
565 freezing at inter-shock periods. Freezing in both groups was equivalent (GzLMM t test: $t_{(20)} = 0.046$, $p =$
566 0.9635; $n_{vehicle} = 11$, $n_{muscimol} = 12$).

567 **C, D)** Seven days and 24 hours context tests. Left panels: freezing over time for groups tested at 7 days
568 (C) and 24 hours (D). Right panels: average freezing. In both experiments freezing in the muscimol group
569 was lower than in the control group (GzLMM t test for 7 days context test: $t_{(20)} = 4.294$, $p = 0.0004$, $n_{vehicle}$
570 $= 11$, $n_{muscimol} = 12$; GzLMM t test for 24 hours context test: $t_{(16)} = 3.964$, $p = 0.0011$; $n_{vehicle} = 9$, $n_{muscimol} =$
571 10.

572 In freezing over time plots, lines represent intersubjects' mean, and shaded area represents +SEM. In
573 bar plots, each dot represents a subject and bars represent mean and errors bars +SEM. ns $p > 0.05$, ** p
574 < 0.01, *** $p < 0.001$.

575

Fig. 2


A
Veh/Musc
Training
Context A
24 h/
7 d
Tone Test
Context B
B
Cued FC Training
Vehicle
Muscimol
Freezing (%)
Time (s)
ns
Post-Shock
Mean (Day 0)
Tone Mean
(Day 0)
C
7 d Tone Test
****
Training
BL (Day 0)
Pre-
Tone
Tone Test (Day 7)
D
24 h Tone Test
***

576

577

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

579 **A)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes
580 before training. Cued FC was tested 24 hour or 7 days later. During training, subjects were let to freely
581 explore the cage for a baseline period of 180 s that was followed by 4 tone-shock pairings (17 s of tone
582 followed by 3 s, 0.6 mA shock) interspaced by 70 s. During test, tone was presented for 60 s after a pre-
583 tone period of 180 s.

584 **B)** Freezing during cued FC training for the group tested 7 days after training. Left panel: freezing over
585 time. Tones were presented during the times indicated by the gray shaded areas. Shocks were
586 presented at times indicated by the orange shaded areas. Right panel: mean freezing during tone and
587 post-shock period. Freezing in both groups was equivalent. GzLMM Two-way repeated measures ANOVA
588 of treatment, training stage, and interaction: χ^2 treatment $_{(1)}$ = 0.185, p = 0.6668; χ^2 stage $_{(1)}$ = 6.308, p =
589 0.0120; χ^2 interaction $_{(1)}$ = 0.028, p = 0.8661. Sidak's multiple comparisons test: Post-shock freezing:
590 vehicle vs. muscimol: $t_{(28)}$ = 0.430, p = 0.8912; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.297, p =
591 0.9463; $n_{vehicle}$ = 8, $n_{muscimol}$ = 9.

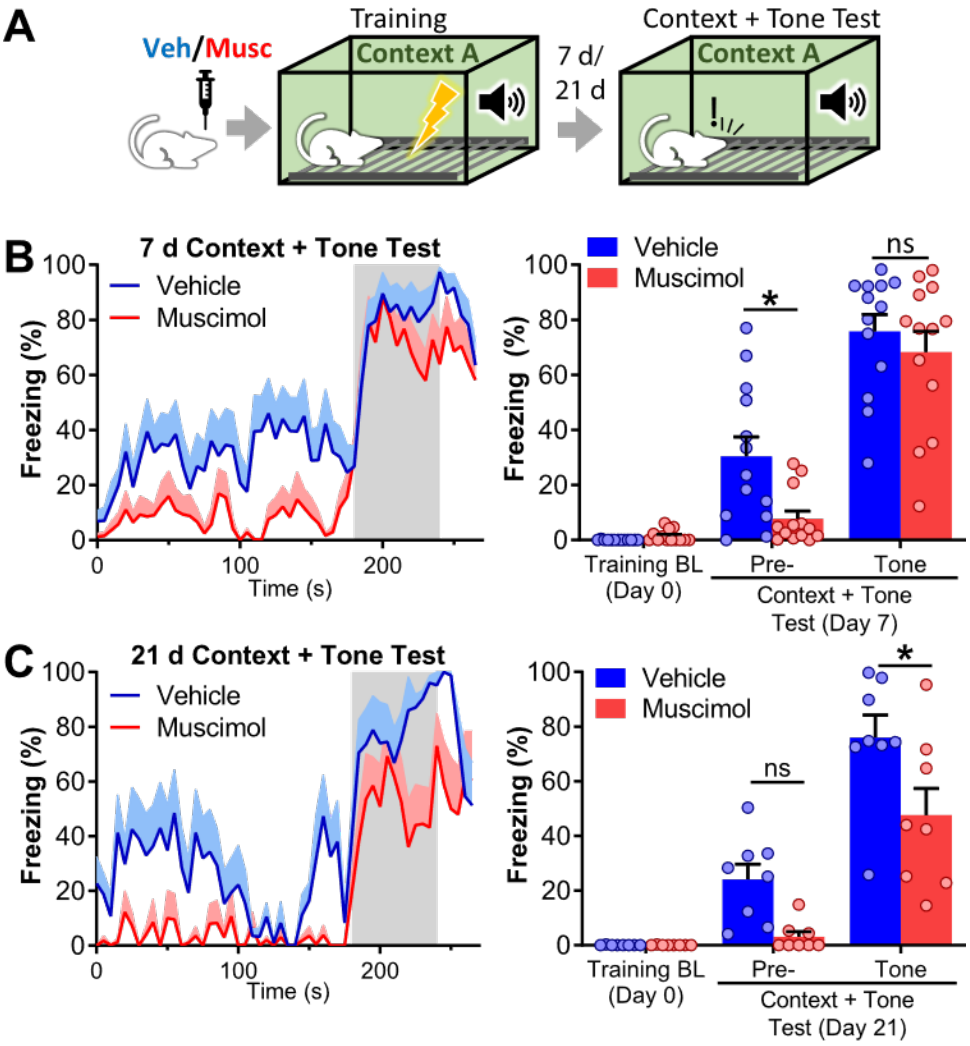
592 **C, D)** Seven days and 24 hours cued FC tests. Left panels: freezing over time for groups tested 7 days (C)
593 and 24 hours (D) after training. Gray areas indicate tone presentation. Right panels: average freezing for
594 pre- and tone period. Freezing during pre-tone period was not different between vehicle and muscimol
595 groups in either experiment. In contrast, during tone presentation, a highly significant reduction in
596 freezing was observed in the muscimol group in both experiments. GzLMM Two-way repeated measures
597 ANOVA of treatment, test stage, and interaction for 7 days experiment: χ^2 treatment $_{(1)}$ = 2.157, p =
598 0.1420; χ^2 stage $_{(1)}$ = 19.100, p < 0.0001; χ^2 interaction $_{(1)}$ = 32.939, p < 0.0001. Sidak's multiple
599 comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 1.469, p = 0.2828; Tone freezing: vehicle
600 vs. muscimol: $t_{(28)}$ = 5.396, p < 0.0001; $n_{vehicle}$ = 8, $n_{muscimol}$ = 9. GzLMM Two-way repeated measures

601 ANOVA of treatment, test stage, and interaction for 24 hours experiment: χ^2 treatment $_{(1)}$ = 0.024, p =
602 0.8781; χ^2 stage $_{(1)}$ = 0.108, p = 0.7428; χ^2 interaction $_{(1)}$ = 12.447, p = 0.0004. Sidak's multiple
603 comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.153, p = 0.9854; Tone freezing: vehicle
604 vs. muscimol: $t_{(28)}$ = 4.441, p = 0.0003; $n_{vehicle}$ = 9, $n_{muscimol}$ = 8.

605 In freezing over time plots, line represents intersubjects' mean, and shaded area represents SEM. In bar
606 plots each dot represents a subject and bars represent mean + SEM. ns p > 0.05, *** p < 0.001, **** p <
607 0.0001.

608

Fig. 3


A
Veh/Musc
Training
Context A
7 d/
21 d
Context + Tone Test
Context A
B
7 d Context + Tone Test
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
ns
*
Training BL
(Day 0)
Pre-
Tone
Context + Tone
Test (Day 7)
C
21 d Context + Tone Test
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
ns
*
Training BL
(Day 0)
Pre-
Tone
Context + Tone
Test (Day 21)

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

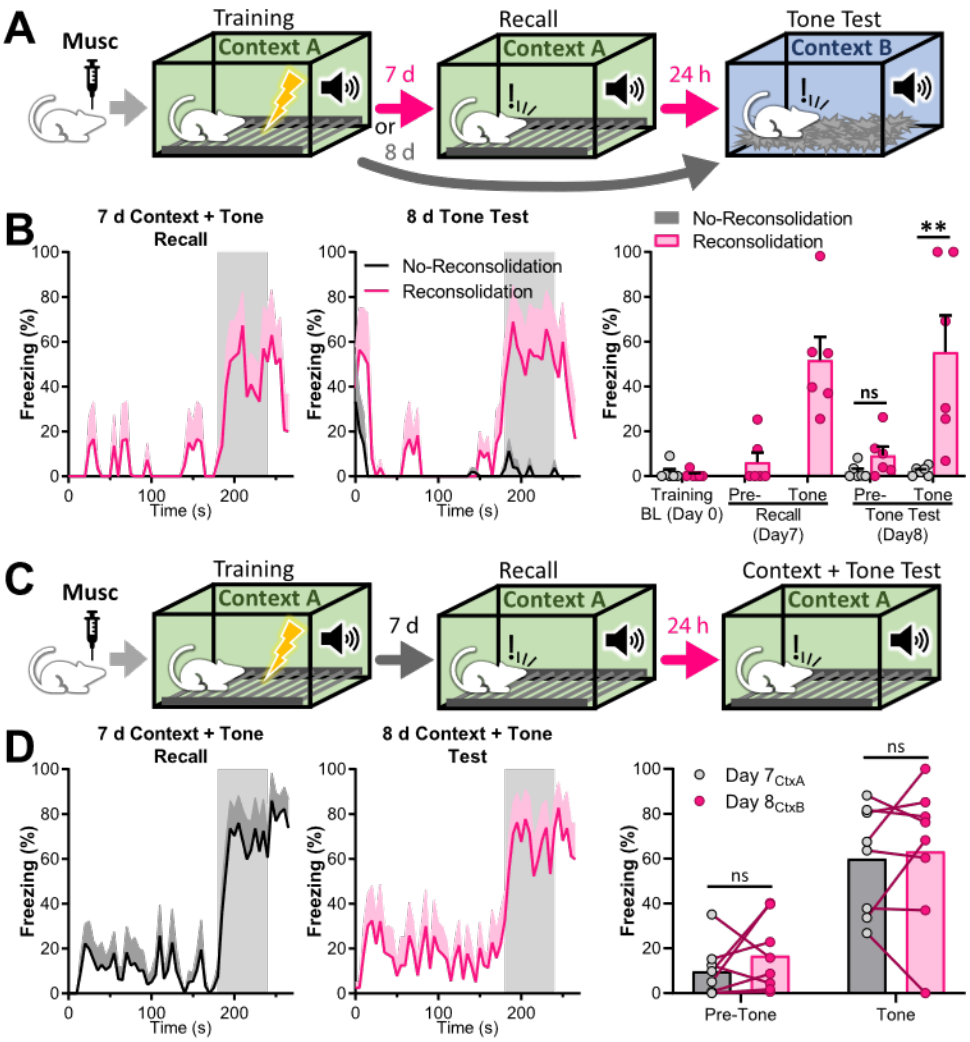
613 **A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes
614 before training. Animals were trained in cued FC. Test was performed 7 or 21 days later in the same
615 context used for training (Context A). The tone was presented for 60 s after a pre-tone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

616 **B)** Freezing during context + tone test 7 days after training. Left panel: freezing over time. Gray area
617 indicates tone presentation. Right panel: average freezing for pre- and tone period. Freezing to the
618 context was higher in vehicle group. However, tone elicited a robust freezing in the muscimol group that
619 reached values equivalent to the vehicle group. GzLMM Two-way repeated measures ANOVA of
620 treatment, test stage, and interaction: χ^2 treatment $_{(1)}$ = 6.830, p = 0.0090; χ^2 stage $_{(1)}$ = 44.992, p <
621 0.0001; χ^2 interaction $_{(1)}$ = 3.161, p = 0.0754. Sidak's multiple comparisons test: Pre-tone freezing: vehicle
622 vs. muscimol: $t_{(46)}$ = 2.613, p = 0.0474; Tone freezing: vehicle vs. muscimol: $t_{(46)}$ = 0.899, p = 0.8460;
623 Vehicle: pre-tone vs. tone freezing: $t_{(46)}$ = 6.014, p < 0.0001; muscimol: pre-tone vs. tone freezing: $t_{(46)}$ =
624 6.708, p < 0.0001; $n_{vehicle}$ = 13, $n_{muscimol}$ = 13.

625 **C)** Freezing during context + tone test 21 days after training. Left panel: freezing over time. Gray area
626 indicates tone presentation. Right panel: average freezing for pre- and tone period. 21 days after
627 training freezing in context + tone condition in the muscimol group was lower than in the vehicle group
628 indicating a diminished temporal stability of context + tone memory. GzLMM Two-way repeated
629 measures ANOVA of treatment, test stage, and interaction: χ^2 treatment $_{(1)}$ = 7.123, p = 0.0076; χ^2 stage
630 $_{(1)}$ = 41.364, p < 0.0001; χ^2 interaction $_{(1)}$ = 0.176, p = 0.6751. Sidak's multiple comparisons test: Pre-tone
631 freezing: vehicle vs. muscimol: $t_{(26)}$ = 2.669, p = 0.0508; Tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 2.683,
632 p = 0.0491; Vehicle: pre-tone vs. tone freezing: $t_{(26)}$ = 7.354, p < 0.0001; muscimol: pre-tone vs. tone
633 freezing: $t_{(26)}$ = 6.431, p < 0.0001; $n_{vehicle}$ = 8, $n_{muscimol}$ = 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, $p > 0.05$, * $p < 0.05$.

Fig. 4


A
Musc
Training
Context A
7 d
or
8 d
Recall
Context A
24 h
Tone Test
Context B
B
7 d Context + Tone Recall
8 d Tone Test
No-Reconsolidation
Reconsolidation
Freezing (%)
Time (s)
**
ns
Training BL (Day 0)
Pre-
Tone
Recall (Day7)
Tone Test (Day8)
C
Musc
Training
Context A
7 d
Recall
Context A
24 h
Context + Tone Test
Context A
D
7 d Context + Tone Recall
8 d Context + Tone Test
Day 7CtxA
Day 8CtxB
ns
Pre-Tone
Tone

639 Figure 4. Retrieval of FC memory in context + tone conditions reconsolidates cued but not contextual
640 memories.

641 **A)** Diagram of reconsolidation experiment of cued FC memory: bilateral intra LHb muscimol infusions
642 were performed 30 minutes before cued FC training as described previously. Seven days later, animals
643 of the reconsolidation group were subjected to a recall session in which the cue was presented in the
644 training context. The following day, reconsolidation of cued memory was tested in context "B". The no-
645 reconsolidation group was not exposed to the recall session and was tested for cued memory in context
646 "B" 8 days after training.

647 **B)** Retrieval of FC memory in context + tone conditions restores cued FC memory. Left and center:
648 freezing over time for the recall and the cued memory test sessions, respectively. Animals of the no-
649 reconsolidation group showed very low levels of freezing, confirming the impairment in cued memory
650 induced by inactivation of the LHb during training. In contrast animals of the reconsolidation group
651 presented a marked freezing to the tone during the test session, reaching levels of freezing equivalent to
652 context + tone condition during the recall session. Right: quantification of freezing. Freezing to the tone
653 in the test session was significantly higher in the reconsolidation group. GzLMM Two-way repeated
654 measures ANOVA of reconsolidation, test stage, and interaction during tone test session: χ^2
655 reconsolidation $_{(1)}$ = 0.207, p = 0.6489; χ^2 stage $_{(1)}$ = 0.009, p = 0.9232; χ^2 interaction $_{(1)}$ = 9.170, p =
656 0.0025. Sidak's multiple comparisons test: Pre-tone freezing: reconsolidation vs. no-reconsolidation: $t_{(18)}$
657 = 0.455, p = 0.8805; Tone freezing: reconsolidation vs. no-reconsolidation: $t_{(18)}$ = 4.219, p = 0.0010;
658 $n_{reconsolidation}$ = 6, $n_{NO\text{-}reconsolidation}$ = 6.

659 **C)** Diagram of the reconsolidation experiment of contextual FC memory: bilateral intra LHb muscimol
660 infusions were performed 30 minutes before cued FC training as described previously. Seven days later,

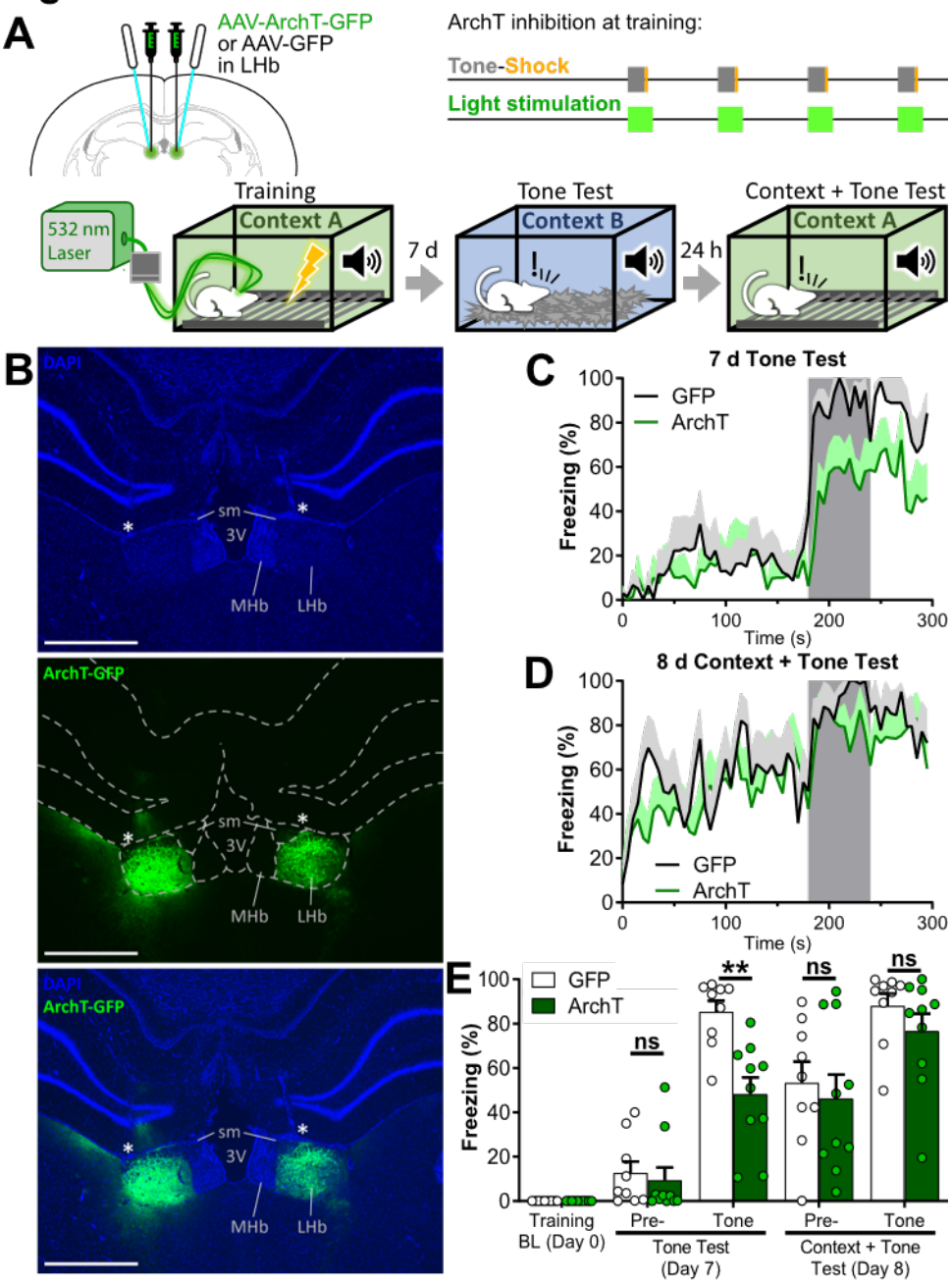
661 animals were subjected to a recall session in which the cue was presented in the training context. The
662 following day animals underwent the same procedure.

663 **D)** Retrieval of FC memory in context + tone conditions does not restore contextual FC memory. Left and
664 center: freezing over time for the recall and the test sessions, respectively. Right: paired comparison of
665 freezing for each subject on the recall and the test session. No differences in freezing were observed
666 between sessions indicating that retrieval of FC memory during the recall session did not restore
667 contextual FC memory, as it did with cued memory. GzLMM Two-way repeated measures ANOVA of
668 session day, test stage, and interaction: χ^2 day $_{(1)}$ = 0.514, p = 0.4732; χ^2 stage $_{(1)}$ = 20.398, p < 0.0001; χ^2
669 interaction $_{(1)}$ = 0.240, p = 0.6240. Sidak's multiple comparisons test: Pre-tone freezing: recall vs. test
670 day: $t_{(26)}$ = 0.717, p = 0.7292; Tone freezing: recall vs. test day: $t_{(26)}$ = 0.088, p = 0.9952; n = 6.

671 In freezing over time plots, line represents intersubjects' mean and shaded area represents +SEM, and
672 gray area indicates tone presentation. In bar plots each dot represents a subject and bars represent
673 mean + SEM. ns p > 0.05, ** p < 0.01.

674

Fig. 5


A
AAV-ArchT-GFP
or AAV-GFP
in LHb
ArchT inhibition at training:
Tone-Shock
Light stimulation
Training
Tone Test
Context + Tone Test
532 nm
Laser
Context A
Context B
Context A
7 d
24 h
B
DAPI
sm
3V
MHb
LHb
ArchT-GFP
DAPI
ArchT-GFP
C
7 d Tone Test
GFP
ArchT
Freezing (%)
Time (s)
D
8 d Context + Tone Test
GFP
ArchT
Freezing (%)
Time (s)
E
GFP
ArchT
Freezing (%)
ns
**
ns
ns
Training
BL (Day 0)
Pre-
Tone
Tone Test
(Day 7)
Pre-
Tone
Context + Tone
Test (Day 8)

675

676

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

679 **A)** Experiment diagram: Top-Left: animals were bilaterally transfected with AAV-ArchT-GFP or AAV-GFP
680 in the LHb and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during
681 training, optogenetic light stimulation was delivered starting with the tone and stopping 5 seconds after
682 the shock (tone and shock presentations were as previously described for cued FC). Bottom: diagram of
683 training and tests. Cued memory was tested 7 days after training in Context B. The same animals were
684 tested the following day in context + tone condition to evaluate contextual FC memory and freezing in
685 context + tone condition.

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

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

692 **E)** Average freezing on tone test, and context + tone test sessions. During tone test ArchT group
693 displayed lower levels of freezing to the tone than GFP group. The following day, during context + tone
694 test, freezing levels of the ArchT group to both to the context and to the tone were equivalent to those
695 of the GFP group.

696 GzLMM Three-way repeated measures ANOVA of test day, test stage, optogenetic inhibition and
697 interactions: χ^2 day $_{(1)}$ = 24.559, $p < 0.0001$; χ^2 stage $_{(1)}$ = 23.070, $p < 0.0001$; χ^2 inhibition $_{(1)}$ = 0.5568, p =
698 0.4555; χ^2 interaction(Day×Stage) $_{(1)}$ = 1.659, p = 0.1978; χ^2 interaction(Day×Inhibition) $_{(1)}$ = 0.294, p =

699 0.5879; χ^2 interaction(Stage×Inhibition) $_{(1)}$ = 5.006, p = 0.0253; χ^2 interaction(Day×Stage×Inhibition) $_{(1)}$ =

700 1.088, p = 0.2970. Sidak's multiple comparisons test: Day7: Pre-tone freezing: GFP vs. ArchT: $t_{(66)}$ = 0.746,

701 p = 0.9747; Day 7: Tone freezing: GFP vs. ArchT: $t_{(66)}$ = 3.410, p = 0.0067; Day 8: Pre-tone freezing: GFP

702 vs. ArchT: $t_{(66)}$ = 0.178, p = 1.0000; Day 8: Tone freezing: GFP vs. ArchT: $t_{(66)}$ = 1.145, p = 0.8308; ArchT:

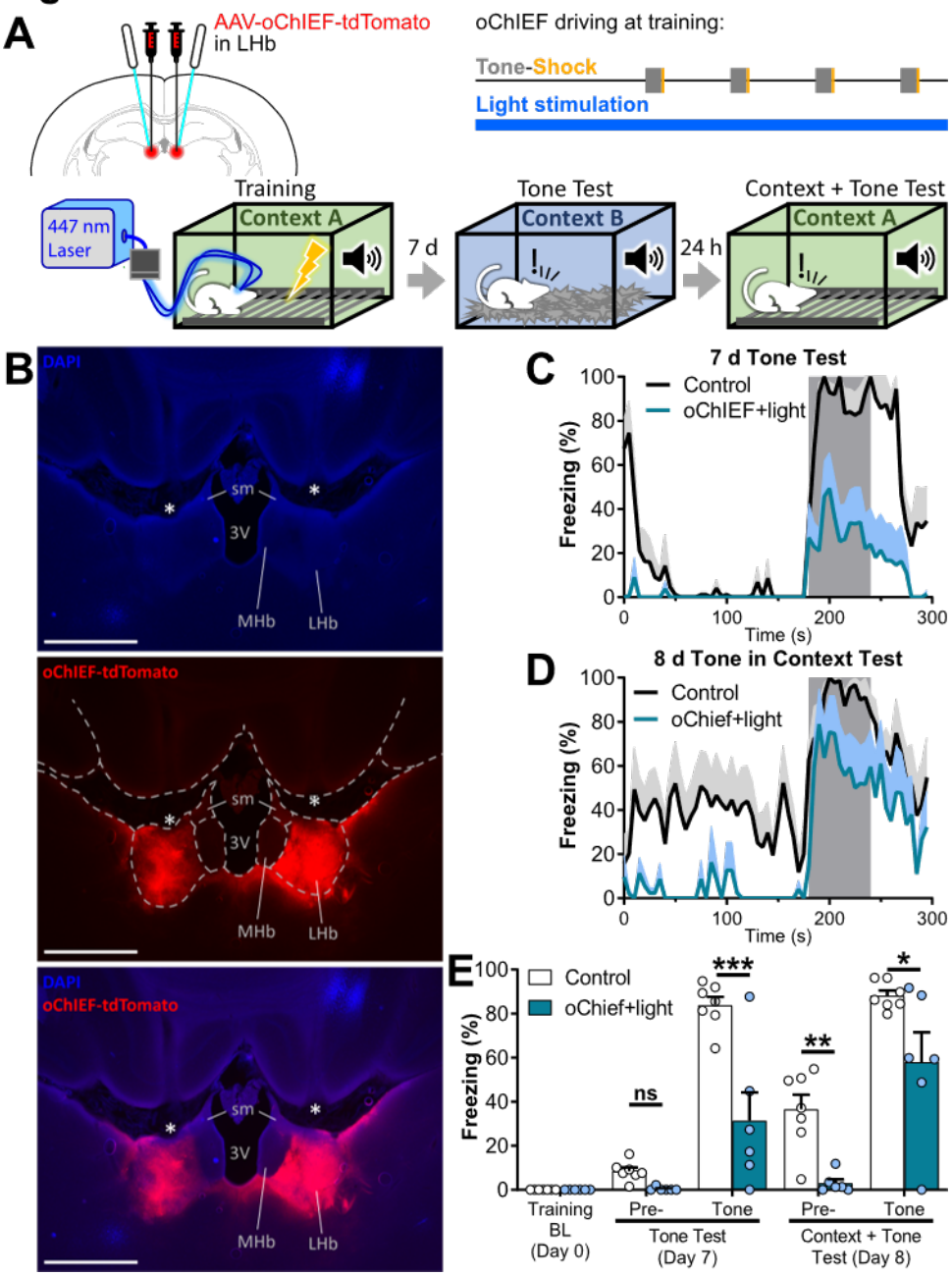
703 Tone freezing: Day 7 vs. Day 8: $t_{(66)}$ = 3.366, p = 0.0076; Day 8: ArchT: Pre-tone vs. Tone freezing: $t_{(66)}$ =

704 3.475, p = 0.0054; n_{GFP} = 9, n_{ArchT} = 10. In bar plots each dot represents a subject and bars represent

705 mean + SEM. ns p > 0.05, ** p < 0.01.

706

Fig. 6


A
AAV-oChIEF-tdTomato in LHb
oChIEF driving at training:
Tone-Shock
Light stimulation
Training
Tone Test
Context + Tone Test
447 nm Laser
Context A
Context B
Context A
7 d
24 h
B
DAPI
sm
3V
MHb
LHb
oChIEF-tdTomato
DAPI
oChIEF-tdTomato
C
7 d Tone Test
Control
oChIEF+light
Freezing (%)
Time (s)
D
8 d Tone in Context Test
Control
oChief+light
Freezing (%)
Time (s)
E
Control
oChief+light
Freezing (%)
ns
***
**
*
Training BL (Day 0)
Pre-
Tone
Tone Test (Day 7)
Pre-
Tone
Context + Tone Test (Day 8)

707

708

709 Figure 6. Disruption of LHb neuronal activity during complete FC training impairs contextual and cued FC
710 memories.

711 **A)** Experiment diagram: Top-Left: animals were bilaterally infected with AAV-oChIEF-tdTomato in the
712 LHb and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during whole
713 training session the LHb was stimulated with 5 ms light pulses at 20 Hz to disrupt endogenous neuronal
714 activity. Bottom: diagram of training and tests. Cued memory was tested 7 days after training in Context
715 B. The same animals were tested the following day in context + tone condition to evaluate contextual FC
716 memory and freezing in context + tone condition.

717 **B)** Microphotographs of the AAV-oChIEF-tdTomato infection (top, middle, bottom: DAPI, tdTomato
718 fluorescence, and merge respectively). Dashed white lines in the middle panel delimitates brain
719 structures. * indicates the optic fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third
720 ventricle. Scale bars: 1 mm.

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

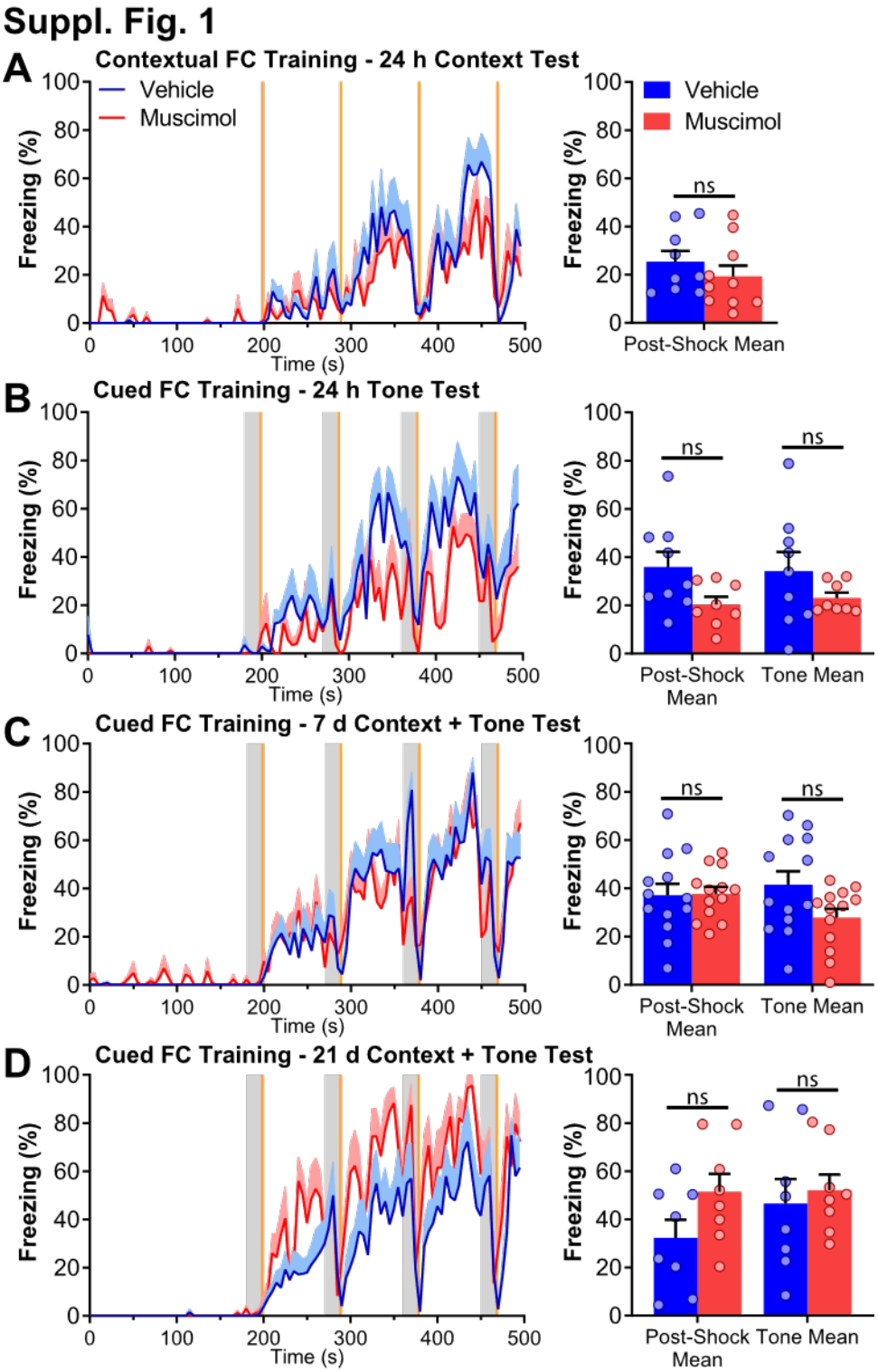
724 **E)** Average freezing of tone test and context + tone test sessions. Disruption of endogenous LHb activity
725 during training by optogenetic stimulation induced deficits in cued and contextual memories:

726 GzLMM Three-way repeated measures ANOVA of test day, test stage, optogenetic stimulation and
727 interactions: χ^2 day $_{(1)}$ = 20.439, $p < 0.0001$; χ^2 stage $_{(1)}$ = 100.217, $p < 0.0001$; χ^2 stimulation $_{(1)}$ = 5.135, p
728 = 0.0234; χ^2 interaction(Day×Stage) $_{(1)}$ = 5.805, p = 0.0160; χ^2 interaction(Day×stimulation) $_{(1)}$ = 2.048, p =
729 0.15237; χ^2 interaction(Stage×stimulation) $_{(1)}$ = 2.489, p = 0.1146; χ^2 interaction(Day×Stage×stimulation)
730 $_{(1)}$ = 4.905, p = 0.0268. Sidak's multiple comparisons test: Day 7: Pre-tone freezing: Control vs. oChIEF:

731 $t_{(42)}$ = 2.266, p = 0.1601; Day 7: Tone freezing: Control vs. oChIEF: $t_{(42)}$ = 4.454, p = 0.0004; Day 8: Pre-
732 tone freezing: Control vs. oChIEF: $t_{(42)}$ = 3.874, p = 0.0022; Day 8: Tone freezing: Control vs. oChIEF: $t_{(42)}$ =
733 2.995, p = 0.0272; oChIEF: Tone freezing: Day 7 vs. Day 8: $t_{(42)}$ = 3.810, p = 0.0027; Day 8: oChIEF: Pre-
734 tone vs. Tone freezing: $t_{(42)}$ = 7.283, p < 0.0001; $n_{Control}$ = 7, n_{oChIEF} = 6. In bar plots, each dot represents a
735 subject and bars represent mean + SEM. ns p > 0.05, * p < 0.05, ** p < 0.01 *** p < 0.001.

736

Supplementary Material



740 Supplementary Figure 1. Freezing during training for experimental groups included in Figures 1-3.

741 **A-D)** Left: temporal course of freezing during training in the experiments specified in the plot title. Right:
742 average freezing of those experiments. In either condition no significant differences were observed
743 between muscimol and vehicle group. In freezing over time plots orange shaded areas indicate shock
744 presentation, gray shaded areas indicate tone presentation, line plots represent intersubjects' mean and
745 shaded area over line plots represents +SEM. In bar plots each dot represents a subject and bars
746 represent mean +SEM. ns $p > 0.05$.

747 Statistics:

748 - Contextual FC training – 24 hours test (A): GzLMM t test: $t_{(16)} = 1.217$, $p = 0.2414$. $n_{vehicle} = 9$, $n_{muscimol}$
749 $= 10$.
750 - Cued FC training – 24 hours test (B): GzLMM two-way repeated measures ANOVA of treatment,
751 training stage, and interaction: χ^2 treatment $_{(1)} = 3.796$, $p = 0.0514$; χ^2 stage $_{(1)} = 0.550$, $p = 0.4584$; χ^2
752 interaction $_{(1)} = 0.852$, $p = 0.3560$. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs.
753 muscimol: $t_{(28)} = 1.948$, $p = 0.1192$; Tone freezing: vehicle vs. muscimol: $t_{(28)} = 1.245$, $p = 0.3972$.
754 $n_{vehicle} = 9$, $n_{muscimol} = 8$.
755 - Cued FC training - 7 days context + tone test (C): GzLMM two-way repeated measures ANOVA of
756 treatment, training stage, and interaction: χ^2 treatment $_{(1)} = 0.037$, $p = 0.8471$; χ^2 stage $_{(1)} = 8.741$, p
757 $= 0.0031$; χ^2 interaction $_{(1)} = 8.674$, $p = 0.0032$. Sidak's multiple comparisons test: Post-shock
758 freezing: vehicle vs. muscimol: $t_{(46)} = 0.193$, $p = 0.9769$; Tone freezing: vehicle vs. muscimol: $t_{(46)} =$
759 2.216, $p = 0.0624$. $n_{vehicle} = 13$, $n_{muscimol} = 13$.
760 - Cued FC training - 21 days context + tone test (D): GzLMMtwo-way repeated measures ANOVA of
761 treatment, training stage, and interaction: χ^2 treatment $_{(1)} = 3.723$, $p = 0.0537$; χ^2 stage $_{(1)} = 0.023$, $p =$
762 0.8802; χ^2 interaction $_{(1)} = 4.535$, $p = 0.0332$. Sidak's multiple comparisons test: Post-shock freezing:

763 vehicle vs. muscimol: $t_{(26)} = 1.930$, $p = 0.1251$; Tone freezing: vehicle vs. muscimol: $t_{(26)} = 0.521$, $p =$

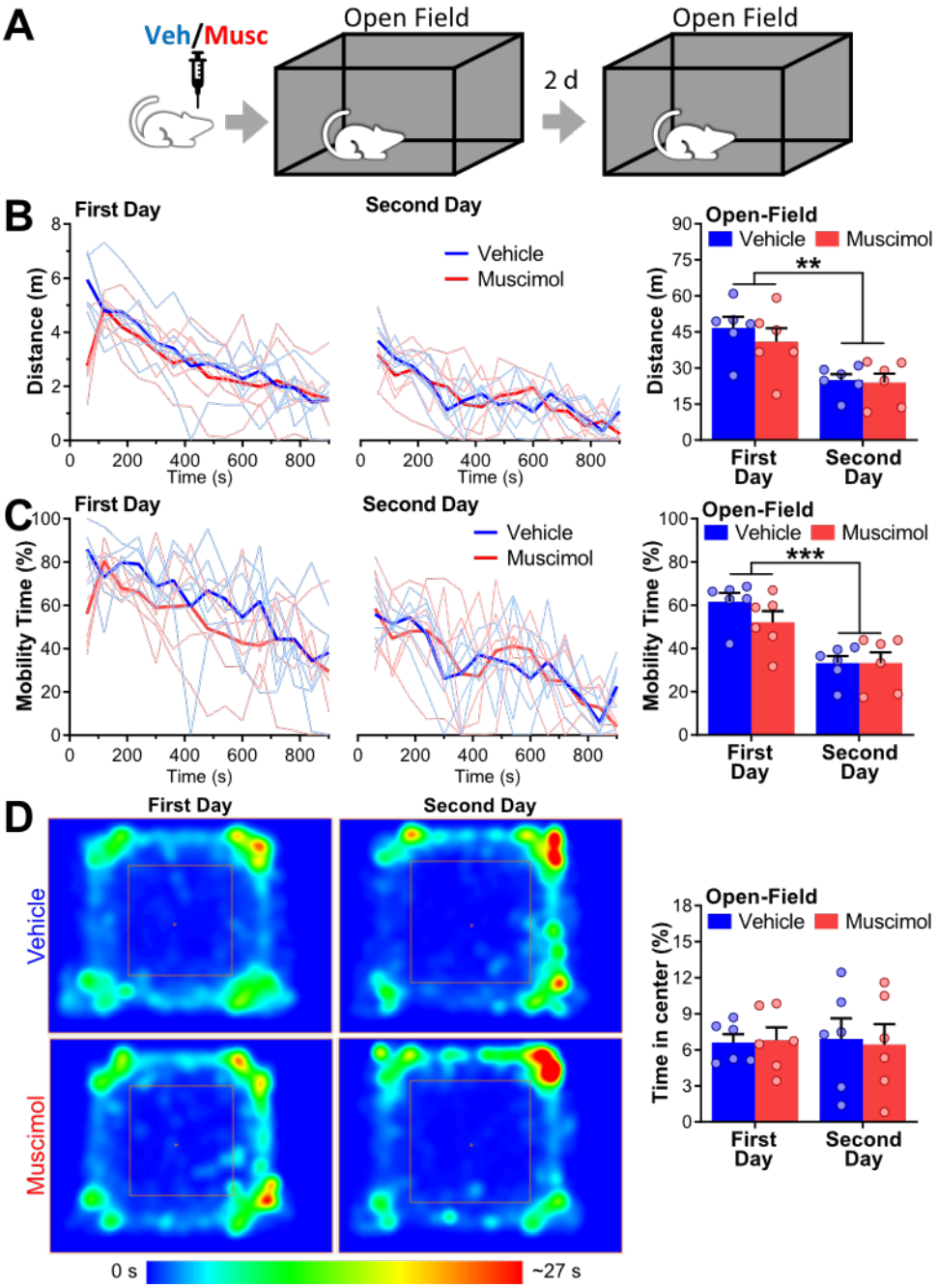
764 0.8454. $n_{vehicle} = 8$, $n_{muscimol} = 8$.

765 For cued FC trainings (B, C, D), stage factor refers to freezing between shock and tone, or during tone

766 (post-shock or tone respectively).

767

Suppl. Fig. 2


A
Veh/Musc
Open Field
2 d
Open Field
B
First Day
Second Day
Vehicle
Muscimol
Distance (m)
Time (s)
Open-Field
Vehicle
Muscimol
**
First Day
Second Day
C
First Day
Second Day
Vehicle
Muscimol
Mobility Time (%)
Time (s)
Open-Field
Vehicle
Muscimol
***
First Day
Second Day
D
First Day
Second Day
Vehicle
Muscimol
0 s
~27 s
Open-Field
Vehicle
Muscimol
Time in center (%)
First Day
Second Day

770 Supplementary Figure 2. Inactivation of the LHb does not affect locomotion, exploration, or habituation
771 to an Open Field.

772 **A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes
773 before exposing the animals to an OF for 15 minutes. 48 hours later animals were exposed to the same
774 OF for a second time.

775 **B)** Distance traveled over time (60 s bins) during the first day (left) and the second day (right) in the OF.
776 Thin lines represent individuals, thick lines represent the average. Right: total distance traveled.
777 Distance traveled by both groups diminished the second day. No differences between groups were
778 observed (two-way repeated measures ANOVA of treatment, day, and interaction: $F_{treatment\ (1, 10)}$ =
779 0.9320, p = 0.3571; $F_{day\ (1, 10)}$ = 15.92, p = 0.0026; $F_{interaction\ (1, 10)}$ = 0.2279, p = 0.6434. Sidak's
780 multiple comparisons test: Day 1: vehicle vs. muscimol: t = 0.9534, p = 0.5798; Day 2: vehicle vs.
781 muscimol: t = 0.1807, p = 0.9800).

782 **C)** Percentage of mobility time in the Open Field in each day. Left: percentage of mobility over time (60 s
783 bins) during the first day (left) and the second day (right). Thin lines represent individuals, thick lines
784 represent the average. Right: percentage of mobility time during whole OF assay. Mobility of both
785 groups diminished the second day. No differences between groups were observed (two-way repeated
786 measures ANOVA of treatment, day, and interaction: $F_{treatment\ (1, 10)}$ = 1.670, p = 0.2253; $F_{day\ (1, 10)}$ =
787 21.48, p = 0.0009; $F_{interaction\ (1, 10)}$ = 0.8727, p = 0.3722. Sidak's multiple comparisons test: First day:
788 vehicle vs. muscimol: t = 1.512, p = 0.2708; Second day: vehicle vs. muscimol: t = 0.005307, p > 0.9999).

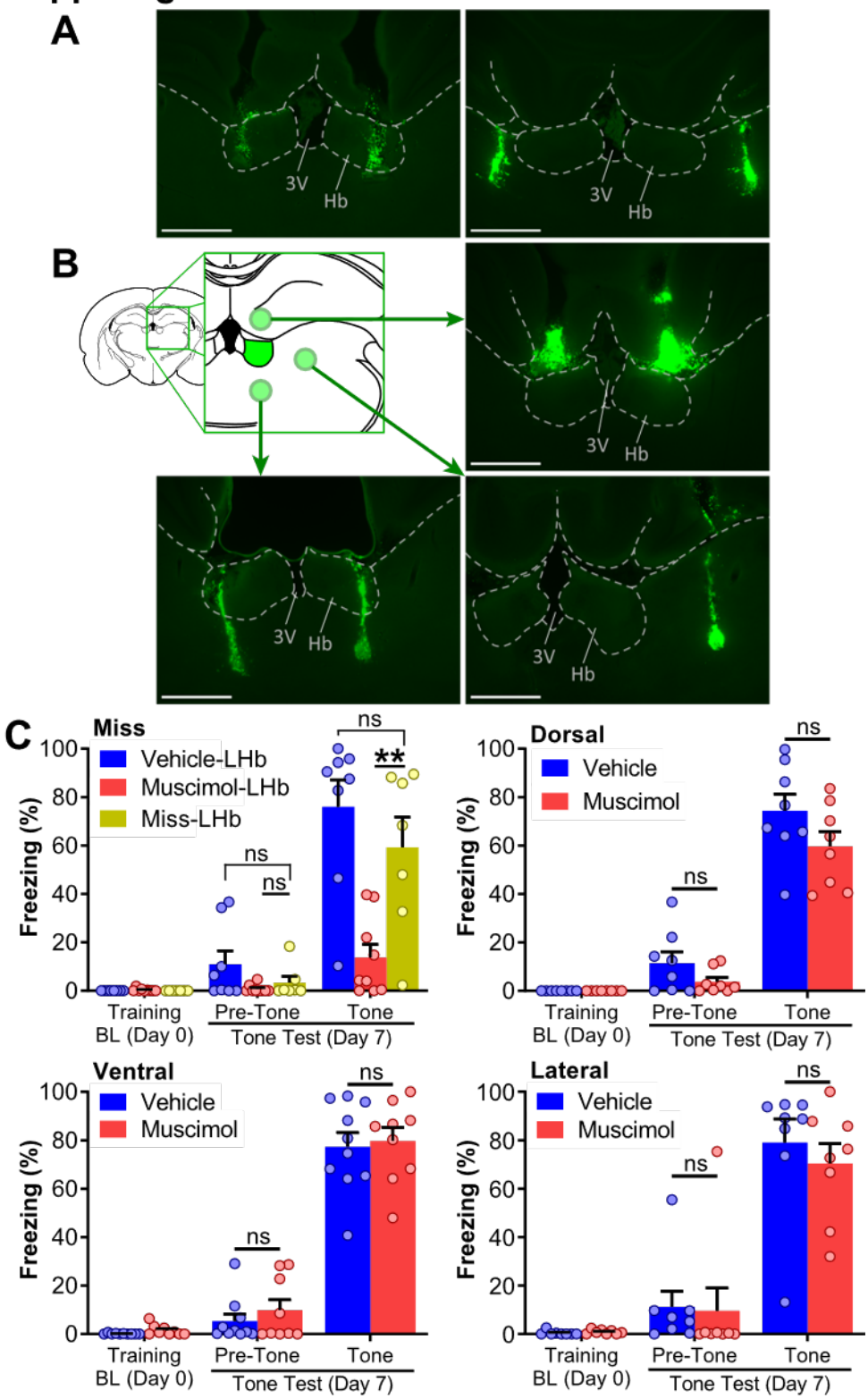
789 **D)** Heat map representation of spatial occupancy during OF. Left: average heat maps vehicle (top) and
790 muscimol (bottom) groups on the first day (left), and the second day (right). The center zone is indicated
791 by the orange square. Right: percentage of time in the center zone in both groups at each day. Time in

792 the center did not change between days and was equivalent among groups (two-way repeated
793 measures ANOVA of treatment, day, and interaction: $F_{treatment\ (1, 10)} = 0.01082$, $p = 0.9192$; $F_{day\ (1, 10)}$
794 $= 0.0003566$, $p = 0.9853$; $F_{interaction\ (1, 10)} = 0.05192$, $p = 0.8244$. Sidak's multiple comparisons test: Day
795 1: vehicle vs. muscimol: $t = 0.1082$, $p = 0.9928$; Day 2: vehicle vs. muscimol: $t = 0.2416$, $p = 0.9645$).

796 ** $p < 0.01$, *** $p < 0.001$.

797

Suppl. Fig. 3



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799

800 Supplementary Figure 3. Controls of specificity of muscimol inactivation of the LHb.

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

803 B) Diagram and representative photomicrographs of controls of specificity of muscimol infusion.
804 Infusions were aimed 1 mm dorsal (top right picture), ventral (bottom left picture) or lateral (bottom
805 right picture). References: Hb: habenula; 3V: third ventricle.

806 C) Freezing during cued memory testing in miss-LHb group, dorsal, ventral, and lateral controls. Miss-
807 LHb group was generated by selecting animals infused with muscimol in which both cannulae did not
808 target the LHb from the same cohorts included in Figure 2C. Dorsal, ventral, and lateral groups were
809 done as separate experiments. As in figure 2, infusions were done 30 minutes before training and test
810 was performed 7 days later. Miss-LHb group showed higher freezing to the tone than muscimol-LHb
811 group and equal freezing to the tone than the vehicle-LHb group. Muscimol infusion dorsal, ventral, or
812 lateral to the LHb did not affect tone evoked freezing. In bar plots each dot represents a subject and
813 bars represent mean + SEM. Muscimol-LHb and vehicle-LHb groups are taken from Figure 2C. ns $p >$
814 0.05, ** $p < 0.001$. Scale bars: 1 mm.

815 Statistics:

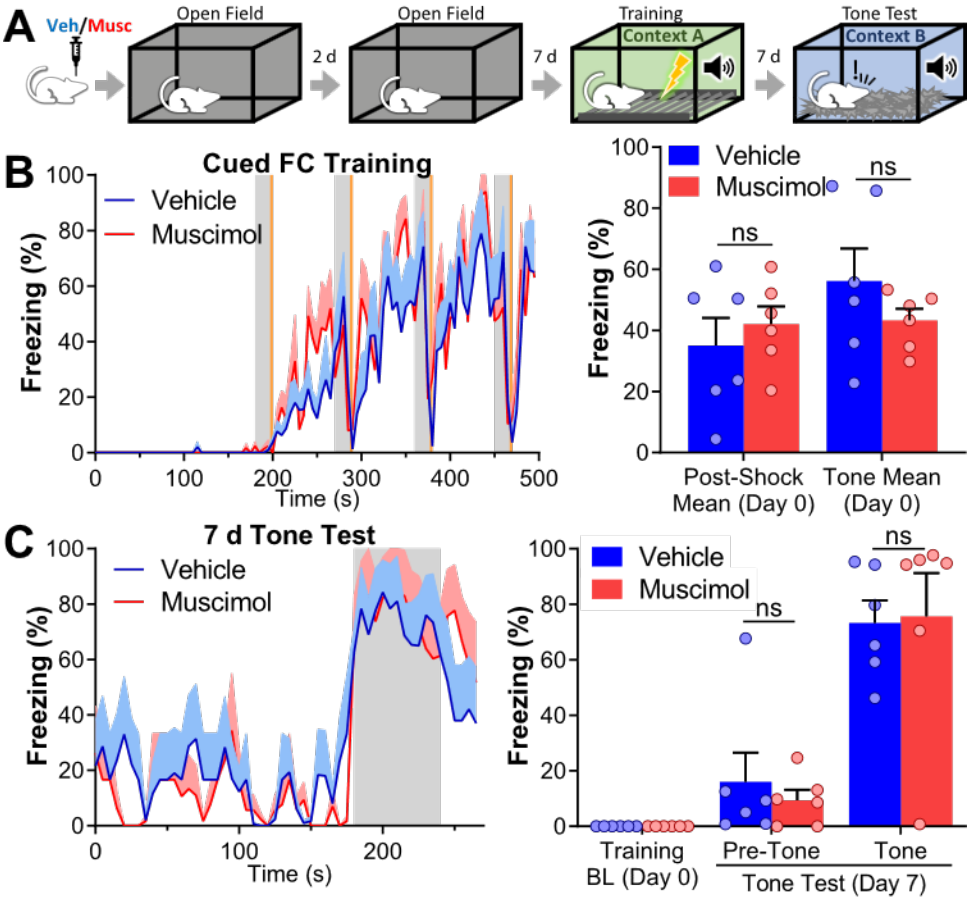
816 - GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for miss
817 graph: χ^2 treatment $_{(2)}$ = 2.5079, $p = 0.2854$; χ^2 stage $_{(1)}$ = 85.1604, $p < 0.0001$; χ^2 interaction $_{(2)}$ =
818 33.459, $p < 0.0001$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(40)}$ =
819 1.457, $p = 0.6306$; Pre-tone freezing: vehicle vs. miss: $t_{(40)}$ = 1.228, $p = 0.7863$; Pre-tone freezing:
820 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: $t_{(40)} = 3.559$, $p = 0.0058$; $n_{vehicle} = 8$, $n_{muscimol} = 9$, $n_{miss} = 7$.

- GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for dorsal graph: χ^2 treatment $_{(1)} = 1.7632$, $p = 0.1842$; χ^2 stage $_{(1)} = 102.565$, $p < 0.0001$; χ^2 interaction $_{(1)} = 0.133$, $p = 0.7153$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(26)} = 1.328$, $p = 0.3532$; Tone freezing: vehicle vs. muscimol: $t_{(26)} = 1.916$, $p = 0.1283$; $n_{vehicle} = 8$, $n_{muscimol} = 8$.
- GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for ventral graph: 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: $t_{(32)} = 0.360$, $p = 0.9224$; $n_{vehicle} = 10$, $n_{muscimol} = 9$.

- GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for lateral graph: 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: $t_{(26)} = 0.605$, $p = 0.7978$; $n_{vehicle} = 8$, $n_{muscimol} = 8$.

Suppl. Fig. 4



838

839

840 Supplementary Figure 4. Inactivation of the LHb does not permanently block FC learning.

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

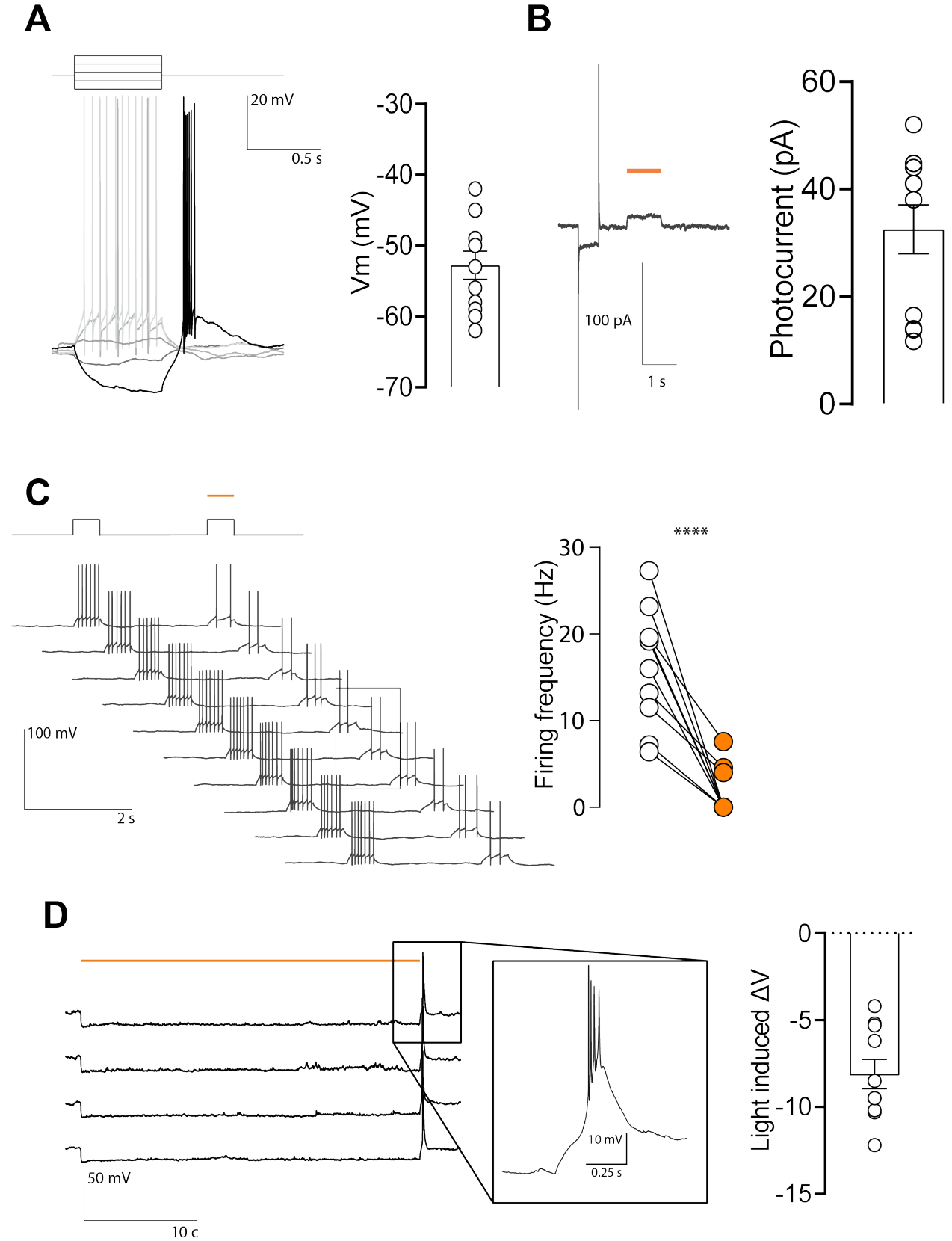
844 **B)** Cued FC training of the animals that were previously exposed to the OF. Left panel: freezing over
845 time. Tones were presented at times indicated by the gray shaded areas. Shocks were presented at
846 times indicated by the orange shaded areas. Right panel: mean freezing during tone and post-shock
847 period. Freezing in both groups was equivalent. GzLMM Two-way repeated measures ANOVA of
848 treatment, training stage, and interaction: χ^2 treatment $_{(1)}$ = 0.685, p = 0.4077; χ^2 stage $_{(1)}$ = 0.050, p =
849 0.8231; χ^2 interaction $_{(1)}$ = 7.700, p = 0.0055. Sidak's multiple comparisons test: Post-shock freezing:
850 vehicle vs. muscimol: $t_{(18)}$ = 0.828, p = 0.6620; Tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 1.265, p =
851 0.3945. $n_{vehicle}$ = 6, $n_{muscimol}$ = 6.

852 **C)** Test of cued FC memory on the same animals. Left panel: freezing over time. Gray area indicates tone
853 presentation. Right panels: average freezing for pre- and tone period. Freezing was not different
854 between vehicle and muscimol groups. GzLMM Two-way repeated measures ANOVA of treatment, test
855 stage, and interaction: χ^2 treatment $_{(1)}$ = 0.0697, p = 0.7918; χ^2 stage $_{(1)}$ = 15.254, p < 0.0001; χ^2
856 interaction $_{(1)}$ = 0.103, p = 0.7478. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs.
857 muscimol: $t_{(18)}$ = 0.264, p = 0.9579; Tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.190, p = 0.9780; $n_{vehicle}$ =
858 6, $n_{muscimol}$ = 6.

859 In freezing over time plots, line represents intersubjects' mean and shaded area represents SEM. In bar
860 plots each dot represents a subject and bars represent mean + SEM. ns p > 0.05.

861

Suppl. Fig. 5



862

863 Supplementary Figure 5. Optogenetic inhibition of LHb neurons by ArchT.

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

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

870 **C)** Light induced action potential suppression in ArchT expressing LHb neurons. Light pulse (590 nm,
871 orange line) was applied every second time neurons were depolarized with positive current injection.
872 Left, representative recordings. Right, data summary. Light activation of ArchT significantly decreases
873 the number of action potentials evoked by current steps in LHb neurons (paired t test, $t_{(9)} = 6.25$, $p <$
874 0.001); n = 9 neurons from 3 rats.

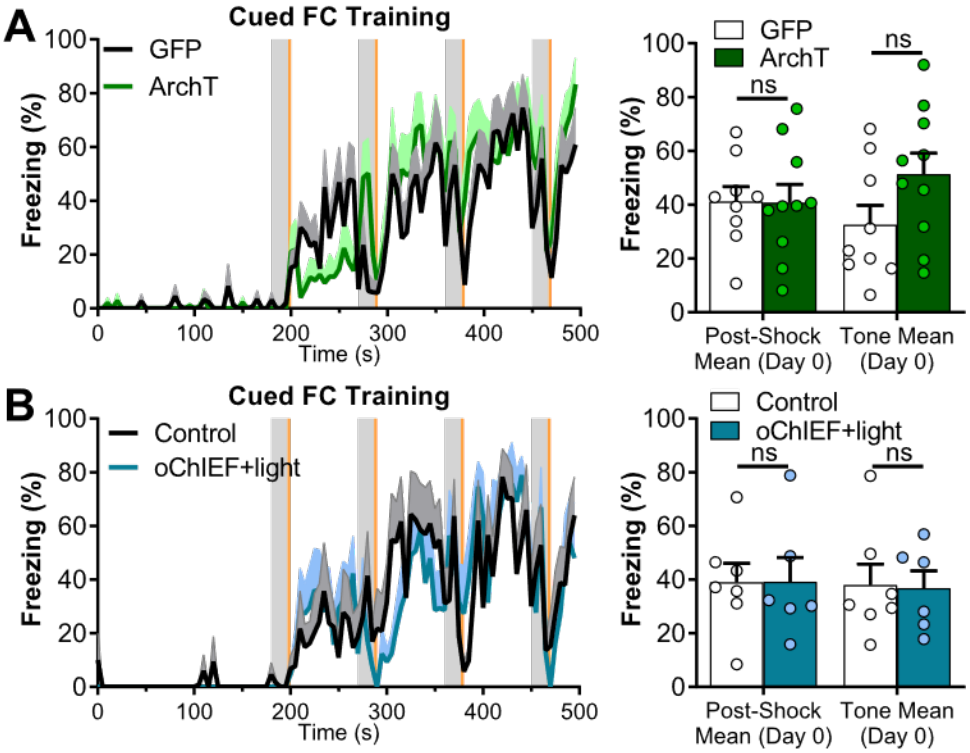
875 **D)** Membrane potential response to a 30 s light pulse (orange line) in LHb neurons. Rebound spiking was
876 observed after termination of light pulse, a representative trace is expanded in the middle traces. This
877 behavior confirms the reported rebound responses of the LHb neurons following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

880 Methods details for this experiment: Surgery was similar to the optogenetic manipulation experiments
881 with the following differences: 3 weeks old rats were employed, the viral vector injection sites were
882 adjusted according to Bregma-Lambda distance (Bλ) of each rat (AP = -2.9 × Bλ ÷ 8.5 mm, ML = ± 0.7 × Bλ
883 ÷ 8.5 mm from Bregma and DV -4.3 mm from dura mater), no fiber optic implants, nor screws or acrylic
884 was used, and the skin was stitched at the end of surgery. Recordings were obtained from ArchT

885 expressing LHb neurons in acute brain slices prepared 3 weeks after infection. General methods for slice
886 preparation and recordings were replicated from previous publications of the group (Stefanelli *et al.*,
887 2016).

888

Suppl. Fig. 6


A
Cued FC Training
GFP
ArchT
Freezing (%)
Time (s)
ns
Post-Shock Mean (Day 0)
Tone Mean (Day 0)
B
Cued FC Training
Control
oChIEF+light
Freezing (%)
Time (s)
ns
Post-Shock Mean (Day 0)
Tone Mean (Day 0)

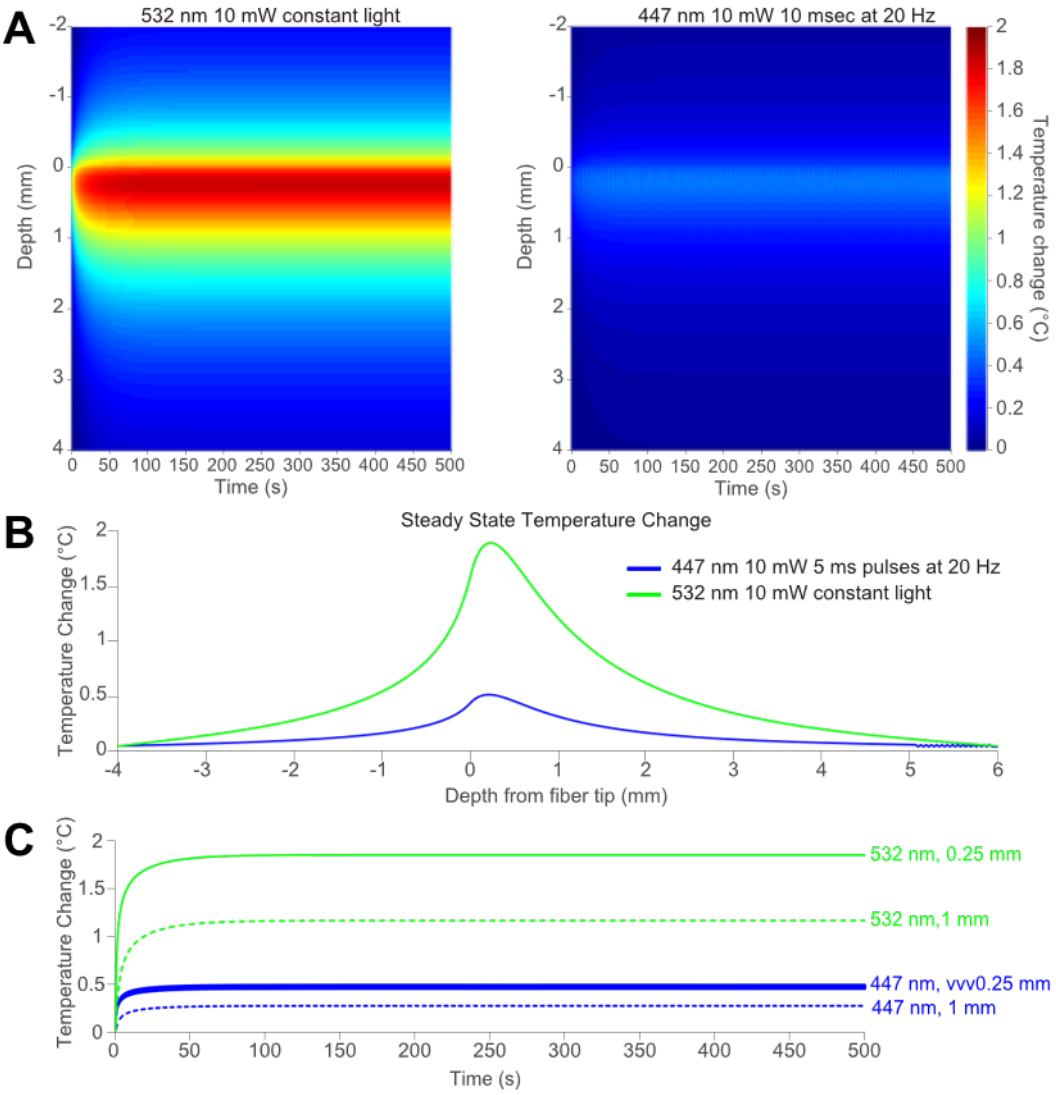
891 Supplementary Figure 6. Freezing during training for experimental groups included in Figures 5 and 6.

892 **A-B)** Left: temporal course of freezing during training in the ArchT (A) and oChIEF (B) stimulation
893 experiments. Right: average freezing on the same experiments. In freezing over time plots vertical
894 orange shaded areas indicate shock presentation, gray shaded areas indicate tone presentation, line
895 plots represent intersubjects' mean and shaded area over line plots represents +SEM. In either condition
896 no significant differences were observed between control and optogenetic stimulated groups.

897 Statistics:

898 - For ArchT experiment: GzLMM two-way repeated measures ANOVA of optogenetic inhibition,
899 training stage, and interaction: χ^2 inhibition $_{(1)}$ = 0.002, p = 0.9631; χ^2 stage $_{(1)}$ = 3.173, p = 0.0749; χ^2
900 interaction $_{(1)}$ = 5.000, p = 0.0254. Sidak's multiple comparisons test: Post-shock freezing: GFP vs.
901 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} =
902 10.
903 - For oChIEF experiment: GzLMM two-way repeated measures ANOVA of optogenetic disruption,
904 training stage, and interaction: χ^2 disruption $_{(1)}$ = 0.007, p = 0.9358; χ^2 stage $_{(1)}$ = 0.018, p = 0.8946; χ^2
905 interaction $_{(1)}$ = 0.0650, p = 0.7988. Sidak's multiple comparisons test: Post-shock freezing: Control
906 vs. oChIEF: $t_{(20)}$ = 0.081, p = 0.9960; Tone freezing: Control vs. oChIEF: $t_{(20)}$ = 0.113, p = 0.9921. $n_{Control}$
907 = 7, n_{oChIEF} = 6.
908 Stage factor refers to freezing between shock and tone, or during tone (post-shock or tone respectively).
909

Suppl. Fig. 7


A
532 nm 10 mW constant light
447 nm 10 mW 10 msec at 20 Hz
Depth (mm)
Time (s)
Temperature change (°C)
B
Steady State Temperature Change
447 nm 10 mW 5 ms pulses at 20 Hz
532 nm 10 mW constant light
Temperature Change (°C)
Depth from fiber tip (mm)
C
532 nm, 0.25 mm
532 nm,1 mm
447 nm, vvv0.25 mm
447 nm, 1 mm
Temperature Change (°C)
Time (s)

912 Supplementary Figure 7. Modelling of temperature increase induced by optogenetic stimulation.

913 Simulation was implemented using the toolbox developed by Stujenske, Spellman and Gordon (2015)
914 using parameters similar to the one used in our real experiments: optic fiber: radius = 0.2 mm, NA =
915 0.45, wavelength: 532 nm for ArchT and 447 nm for oChIEF, laser intensity 10 mW. Additional
916 parameters were taken from (Stujenske, Spellman and Gordon, 2015) (montecarlolight_function:
917 modelnumber = 1; HeatDiffusionLight: U_STEP = 0.03 mm, T_SAVE = 500, R_AVG = 0.25 mm).

918 **A**) Heat plot of predicted temperature changes at different distances from the optic fiber tip induced by
919 optogenetic stimulation during FC training. Left: temperature changes induced by constant light
920 stimulation required for inhibition with ArchT. Right: temperature changes induced by pulsed light
921 stimulation used for oChIEF activation.

922 **B**) Depth profile of temperature change in the steady state (calculated as the mean in the 250-300 s
923 interval). Constant light stimulation with 532 nm light would induce a significant temperature increase
924 that would extend over more than 1 mm and would induce non-controllable out of target changes in
925 neuronal activity. In contrast, pulsed activation of oChIEF would not induce physiologically relevant
926 temperature changes at any depth (Stujenske, Spellman and Gordon, 2015).

927 **C**) Temporal profile of temperature change at 0.25 and 1 mm from the fiber tip for both stimulation
928 protocols.