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

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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 et al., 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 (Dunsmoor & Paz, 2015; Lissek et al., 2010, 2014).

Fear conditioning (FC) is probably the most studied and best understood model of fear learning (Maren, 2008; Maren & 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 (J. Kim & Fanselow, 1992; LeDoux & 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; Hu et al., 2020; Proulx et al., 2014). 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.

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

61 ## Results

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

63 To examine the role of the LHb in contextual FC, we analyzed the effect of its inactivation during
64 training. For that purpose, we performed surgeries, in rats, to implant bilateral intracerebral cannulae
65 aimed at the LHb. After a recovery time of 10 to 12 days, animals were bilaterally infused the GABA-A
66 agonist muscimol or vehicle in the LHb, and were trained in contextual FC thirty minutes later. During
67 training rats were placed in a FC chamber and presented with four unsignaled mild foot-shocks (**Figure**
68 **1A**). We observed that the infusion of muscimol in the LHb did not modify freezing behavior during FC
69 training (**Figure 1B and Supplementary Figure 1**). Also, in a separate cohort of animals, we observed that
70 such procedure did not modify locomotion or exploratory behavior in an open field (**OF; Supplementary**
71 **Figure 2**), indicating that inactivation of the LHb does not have a general effect over motility or context
72 exploration. To evaluate contextual FC, animals were placed back in the conditioning chamber 7 days after
73 training and freezing behavior was quantified. Muscimol group displayed significantly lower levels of
74 freezing than the control group, suggesting that inactivation of the LHb during contextual FC training
75 impairs long-term memory formation (**Figure 1C**).
76 Long-term memories tend to decay over time, a phenomenon that could already be evident 7
77 days after memory formation (Bekinschtein et al., 2007; Rossato et al., 2009). To test whether an
78 accelerated decay of contextual FC memory could account for the memory deficits observed in the
79 muscimol group, we repeated our previous experiment but tested contextual FC memory 24 hours after
80 training. Contextual FC memory deficits were also evident 24 hours after training (**Figure 1 D**), further
81 suggesting that inactivation of the LHb during contextual FC training has a detrimental effect over long-
82 term memory formation.

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

88 Pharmacological inactivation of the LHb impairs cued fear conditioning

89 Having observed that inactivation of the LHb impairs the formation of contextual FC we extended
90 our experiments analyzing the effect of LHb inactivation on cued FC memory formation. Animals were
91 infused muscimol or vehicle in the LHb, and 30 minutes later were trained in cued FC, in which each of
92 the 4 foot-shocks was preceded by a 17 seconds long tone (**Figure 2A**). Cued FC memory was tested 24
93 hours or 7 days later by exposing the animals to the tone in a novel context (Context B). Behavior during
94 training confirmed that inactivation of the LHb does not affect the surge of freezing elicited by shock
95 exposure (**Figure 2B, Supplementary Figure 1B**). During tests, presentation of the cue elicited a robust
96 freezing in the vehicle groups that was markedly reduced in the muscimol groups (**Figure 2C, D**). These
97 results indicate that cued FC memory is also impaired by the inactivation of the LHb during training. Thus,
98 inactivation of the LHb during FC training impairs formation of both contextual and cued FC long-term
99 memories.
100 To control for spatial specificity of muscimol infusion we implanted cannulae targeting areas
101 around the LHb and infused muscimol before cued FC training (**Supplementary Figure 3A, B**). During tests,
102 performed 7 days later, we found that neither infusion of muscimol 1 mm lateral, dorsal or ventral to the
103 LHb affected freezing behavior (**Supplementary Figure 3 B, C**). Moreover, we did not see a reduction in
104 freezing behavior during cued FC test in muscimol infused animals implanted with cannulae aimed at the
105 LHb in which histological control showed cannulations bilaterally missed the LHb (**Supplementary Figure**
106 **3A, C**).
107 To control for non-transient effects of muscimol infusion, we trained in cued FC the animals that
108 had previously undergone muscimol inactivation of the LHb before exposure to an OF (**Supplementary**
109 **Figure 4A**). In that group of animals, we found no differences in freezing behavior during either cued FC
110 training or testing (**Supplementary Figure 4B, C**), demonstrating the transient nature of that manipulation.
111 Thus, deficits in contextual and cued FC in the muscimol groups should be attributed to a transient
112 inactivation of the LHb during training.

113 Context + tone test evidences a conserved fear conditioning memory

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

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

<u>Context + tone retrieval reconsolidates cued but not contextual memory</u>

In animals that were trained in FC under inactivation of the LHb we observed conserved freezing responses in context + tone conditions. However, it could not be known if that freezing represents the retrieval of the contextual memory, the cued memory, both or neither of them. To get an insight on that, we analyzed if memory retrieval in context + tone conditions induces the reconsolidation of cued and contextual memories. Animals were infused muscimol in the LHb and trained in cued FC as described above. Seven days later they went through a recall session in which the cue was presented in the training context (*i.e.* as in context + tone test). Twenty-four hours later they went through a test session in which we evaluated reconsolidation of cued and contextual memories. To evaluate reconsolidation of the cued memory, animals were placed in Context B and exposed to the cue (reconsolidation group, **Figure 4A**). To evaluate reconsolidation of the contextual memory, animals were re-exposed to the training context (**Context A; Figure 4C**). In addition, at the end of this latter experiment the cue was presented, configuring a re-test in context + tone conditions (**Figure 4C**). A “no-reconsolidation” group was also included as a control for cued memory tested “reconsolidation” group. In the no-reconsolidation group, cued memory

145 was tested 8 days after training in Context B without any recall session (**Figure 4A**). Notably, during cued
146 memory test, the "reconsolidation" group that went through the recall session displayed higher levels of
147 freezing to the cue than the no-reconsolidation group (**Figure 4B**). In fact, in the reconsolidation group
148 levels of freezing to the tone during test session were equivalent to those shown during the recall session
149 (**Figure 4B**). In contrast, in the group in which we tested reconsolidation of contextual memory, we did
150 not observe any increase in freezing to the context between test and recall session (**Figure 4D**). Then,
151 levels of freezing remained low even though those animals effectively displayed freezing in context + tone
152 condition in the recall session (**Figure 4D**). Also, freezing in context + tone conditions was not different
153 between the recall and the test session and was high in both cases (**Figure 4D**). Those results suggest the
154 recall session reconsolidated the cued memory but not the contextual memory, arguing that freezing
155 observed during context + tone condition mainly reflects the reactivation of the cued memory trace.
156 Furthermore, they suggest that FC training under inactivation of the LHb creates a cued memory whose
157 retrieval depends on contextual information.

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

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

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

175 To rigorously test the involvement of the LHb in contextual FC using optogenetic tools, it would
176 be necessary to disturb neuronal activity at the LHb during context encoding as well (*i.e.* during whole
177 training period). Optogenetic inhibition mediated by light activated hyperpolarizing pumps such as ArchT
178 or NpHR, require sustained illumination which could create confounding effects, such as temperature
179 changes, when prolonged for long periods like the one involved in our protocols (**Supplementary Figure**
180 **7**). Also, sustained on-off patterns of optogenetic inhibition induce burst activity of LHb neurons that
181 induce depression-like symptoms (Yang *et al.*, 2018; **Supplementary Figure 5D**). To avoid those potential
182 artifacts, we decided to take a different approach. We reasoned that neuronal encoding would be
183 disturbed by any major intervention over neuronal activity. Thus, we opted to disrupt endogenous activity
184 of the LHb by imposing an artificial excitation during the whole training. Animals were injected with an
185 AAV encoding the fast excitatory opsin oChIEF (Lin et al., 2009), and implanted with optic fibers bilaterally
186 in the LHb (**Figure 6A, B**). Three weeks later they were trained in cued FC under a sustained 20 Hz
187 excitation drive with light pulses of 5 ms (**Figure 6A**), which has been shown before not to induce
188 depression-like symptoms (Yang *et al.*, 2018; **Figure 6A, B**). Modelling of light induced temperature
189 changes supports the idea that such protocol would result in minimal temperature changes in the
190 illuminated tissue (Stujenske, Spellman and Gordon, 2015; **Supplementary Figure 7**). We found that
191 artificial stimulation of the LHb during FC training mostly replicates results obtained by pharmacological
192 inhibition (**Figure 6**). oChIEF activation did not modify behavior during FC training (**Supplementary Figure**
193 **6B**). During cued memory test, oChIEF stimulated animals showed lower freezing to the tone than the
194 control group (**Figure 6C, E**). Next day, during context + tone test session, oChIEF stimulated animals
195 showed almost no freezing to the context but displayed freezing to the cue, reaching levels higher than in
196 cued memory test performed the previous day (**Figure 6D, E**). Thus, disrupting the encoding of the LHb
197 during whole training has a deleterious effect over FC learning equivalent to that of pharmacological
198 inhibition. Remarkably, AAVs did not infect medial habenula (**Figure 5B, 6B**). Thus, results from
199 optogenetics experiments could not be attributed to an effect over that structure, a criticism valid for
200 muscimol mediated inactivation.

## 201 Discussion

202 Few articles had studied the LHb in the context of FC (Barrett & Gonzalez-Lima, 2018; Durieux et
203 al., 2020; Song et al., 2017; Wang et al., 2013). In this work we directly investigated that subject expanding
204 our understanding of the role of the LHb in FC learning. Our findings reveal that the LHb is a relevant
205 component of the FC circuit that plays a role in the formation of both cued and contextual memories, and,

206 most notably, in the interaction between them. In the following subsections we discuss our findings in
207 detail.

208 Cued memory and the LHb

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

220 Two observations lead us to reach that conclusion. First, we found that disrupting neuronal
221 activity at the LHb during FC training, either by inhibition (pharmacological or optogenetic), or by
222 sustained optogenetic activation, results in a condition in which retrieval of FC memory requires that the
223 cue is presented in the training context. This observation implies an interaction between the context and
224 the cue in guiding memory retrieval. Second, cued memory is recovered following that retrieval. Since
225 contextual FC memory is not restored by the same manipulation, it could be inferred that the freezing
226 observed when the cue is played in the training context is mostly attributable to the reactivation of the
227 cued memory. In addition, the latter observation argues that freezing in context + tone conditions does
228 not reflect the retrieval of two weak fear memories (contextual and cued) that add up to trigger freezing
229 behavior. Altogether, those two observations suggest that, under inactivation of the LHb, a cued FC
230 memory is formed whose retrieval depends on the context in which the cue is presented.

231 It has been shown that during FC training, activity of the LHb increases in response to cue
232 presentation, paralleling the appearance of conditioned responses (Trusel et al., 2019; D. Wang et al.,
233 2017). Though, no mechanistic information about how the LHb influences cued memory formation could
234 be inferred from the present work, a tempting hypothesis is that the emergence of responses of the LHb
235 to the cue acts as a prediction error signal over the arriving of the US that, when confirmed, emancipates
236 the cue-US association from the context where it was formed.

237 Contextual memory and the LHb

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

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

256 Retrieval and temporal stability, two faces of memory strength

257 How easy a memory is retrieved could be read as a parameter of memory strength. Also, in
258 temporal dimension, the strength of a memory could be read from how long it lasts (Bekinschtein et al.,
259 2008). In our previous work we described that inactivation of the LHb before training in the Inhibitory
260 Avoidance generates a memory that is normally expressed 24 hours after training but could not be
261 evidenced 7 days later (Tomaiuolo et al., 2014). In that paradigm, temporal stability of the memory is
262 defined by shock intensity (*i.e.* by the salience of the US), thus it could be evaluated as a parameter of
263 memory strength. There, we also found that a series of manipulations known to extend temporal stability
264 of weak Inhibitory Avoidance memories did also extend temporal stability of the memory formed under
265 inactivation of the LHb (Tomaiuolo et al., 2014). Based on those findings, we postulated that the main
266 effect of the inactivation of the LHb was to reduce memory strength. Our present results support and
267 extend that hypothesis to the FC paradigm. In one side, we found FC memory is harder to retrieve, since

268 matching between training and testing conditions should be increased in order to effective retrieval. In
269 addition, we also found that the FC memory expressed under context + tone conditions is temporarily less
270 stable (Figure 3C). Thus, FC memory formed under inactivation of the LHb could be considered weaker in
271 those two aspects. Taken together those results indicate a general role of the LHb in defining aversive
272 memory strength.

273 Concluding remarks

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

## 284 Materials and Methods

285 Animals

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

293 Surgeries

294 **Pharmacology:** rats under deep ketamine/xylazine anesthesia (100 and 5 mg/kg respectively)
295 were bilaterally implanted with 22-Gauge guide cannulae aimed 2.0 mm above the LHb (AP -3.0 mm, ML
296 ±0.7 mm, DV -3.8 mm from Bregma), or 1.0 mm dorsal, ventral or lateral to LHb coordinates in specific

297 experiments. Cannulae were fixed to the skull with three surgical steel screws and dental acrylic. During
298 surgery, animals received a dose of analgesic (meloxicam 0.6 mg/kg) and antibiotic (gentamicin 3 mg/kg).
299 Behavioral procedures began 7-9 days after surgery.

300 **Optogenetics:** rats under deep isofluorane anesthesia (4 % induction, 1-2 % maintenance, in 0.8
301 L/minute oxygen) were bilaterally injected with 300 nl of viral vector (AAV8-CamKIIα-ArchT-GFP, 6.2 x $10^{12}$
302 viral particles/ml from UNC Vector Core, AAV8-CamKIIα-GFP, 6.3 x $10^{12}$ viral particles/ml from UNC Vector
303 Core, or AAV8-hSyn-oChIEF-tdTomato homemade as described in Proulx et al. (2018)) per side at the LHb
304 (AP -2.9 mm, ML ± 0.7 mm, DV -5.0 mm from Bregma). Subsequently, optical fiber implants (200 µm core,
305 0.45 NA) were bilaterally inserted with the tips aiming just above the LHb, with 4 degrees angle from the
306 sagittal plane (AP -3.0 mm, ML ±1.05 mm, DV -4.5 mm from Bregma), and fixed to the skull with three
307 surgical steel screws and dental acrylic. During surgery, animals received a dose of analgesic (meloxicam
308 0.6 mg/kg) and antibiotic (gentamicin 3 mg/kg). Behavioral procedures began three weeks after surgery.

309 Contextual and Cued Fear Conditioning Training

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

324 **Pharmacology experiments:** after the acclimation period, rats were randomly assigned to receive
325 bilateral intra LHb infusions of GABAa agonist, muscimol (Sigma, 60 ng/µl in saline solution, 0.5 µl/side),
326 or saline. Fluorescent green beads were added to both solutions to aid in checking the infusion site (1:1000
327 dilution of concentrated 1 µm diameter fluorescent green beads, Bangs Laboratories, USA). During

328 infusions animals were grasped by hand and slightly restrained in the lap or the arm of the investigator.
329 Infusions were delivered at a rate of 0.5 µl/minute through a 30-Gauge needle extending 2.0 mm beyond
330 the tip of implanted guide cannula and connected to a 10 µl Hamilton syringe by a polyethylene tube.
331 Infusion needle was left in place for an additional minute to minimize backflow. After that animals were
332 returned to their home cage. FC training began 30 minutes later.

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

345 Contextual FC Test

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

350 Cued FC Test

351 Several precautions were taken to avoid generalization during cued test. Home cages were moved
352 to a different acclimation room of the one used in the training day, the investigator used latex gloves,
353 animals were transported between the acclimation room and the test chamber within a plastic box, and
354 acetic acid 1 % m/v was employed for cleaning between subjects. Test was performed in Context B, a
355 white acrylic box with a transparent plexiglass ceiling (25x25x40 cm wide, width and height) with floor
356 covered in wood shaving and lit up with red light. Cue test consisted of a pre-tone period of 180 s, followed
357 by 60 s tone (same as training tone) and 30 s post-shock period. After that, animals were removed from

358 the test cage and placed back in the home cage. Test was video recorded for posterior analysis. 1 hour
359 after finishing tests, home cages were taken back to the animal facility.

360 Context + Tone Test

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

363 Open Field

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

367 Histologic Control

368 Within a week after the end of behavioral procedures animals were euthanized with a lethal dose
369 of ketamine/xylazine, 50 ml of ice-cold phosphate-buffered saline (PBS) 0.1 M was transcardially perfused,
370 followed by 50 ml of paraformaldehyde 4 %. After that, the brain extracted and incubated at 4 °C in
371 paraformaldehyde 4 % overnight. Then, brain was transferred to 30 % m/v sucrose in PBS 0.1 M, incubated
372 for three days at 4 °C and 150 µm coronal sections were cut at a freezing microtome. Sections were
373 mounted with glycerol 50 % v/v in PBS 0.1 M, examined and photographed at 40X magnification with GFP
374 and DAPI filters. For the pharmacological experiments, injection sites were determined by the presence
375 of green beads. Animals in which both infusions were correct were included in the analysis. Animals from
376 the cohorts used in Figure 2B-C in which both infusions were outside the LHb were included in the miss
377 group presented in Figure S3. For the optogenetics experiments, animals with transfection and/or optic
378 fiber misses were not considered in the analysis.

379 Freezing analysis

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

386 Statistical Analysis

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

417 a parameter of the freezing of a naïve animal but, as mentioned above, freezing during training was
418 analyzed separately.

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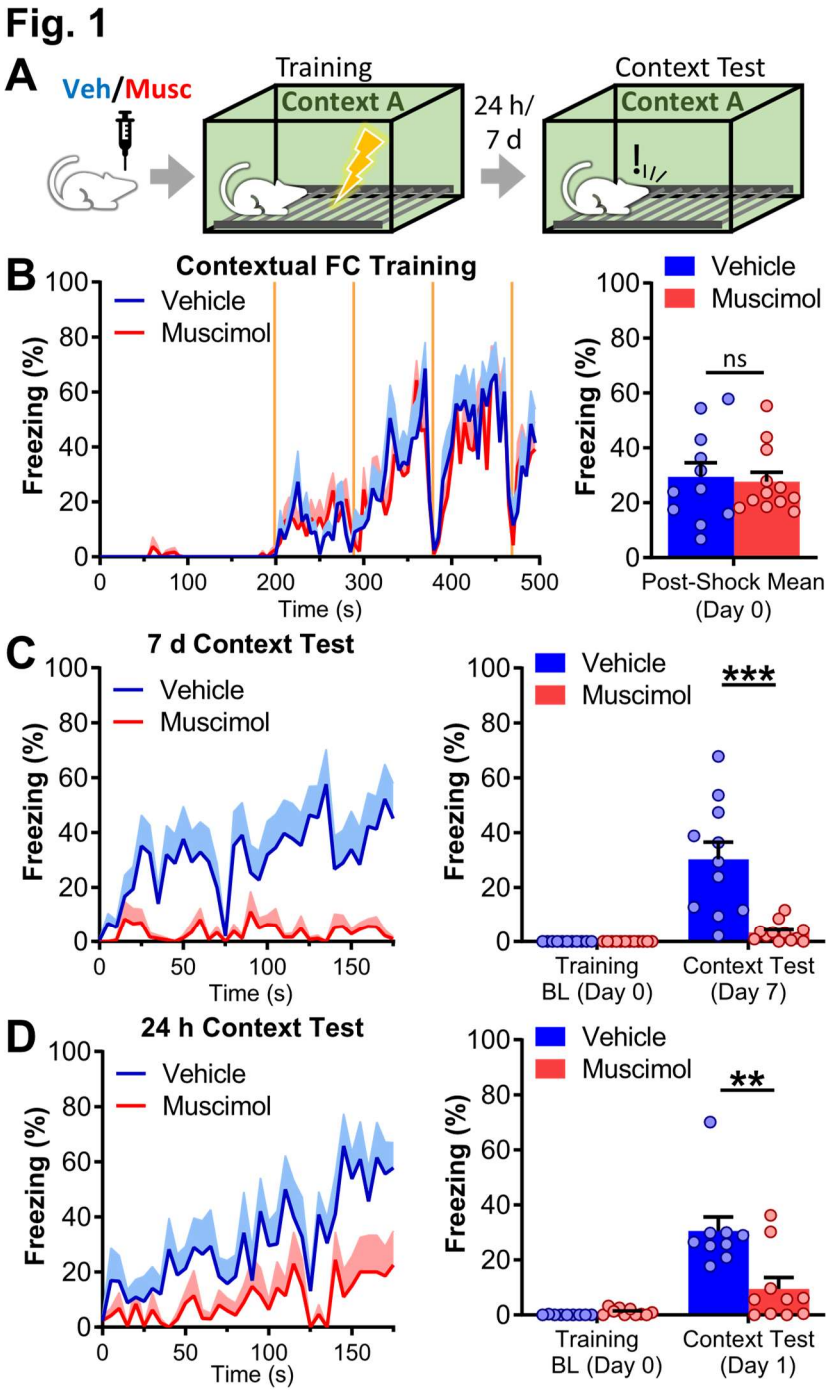
559 Figures




560

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

562 **A)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes before
563 training. Contextual FC was tested 24 hours or 7 days later. During training subjects were let to freely
564 explore the cage for a baseline period of 197 s. After that they were exposed to 4 foot-shocks (0.6 mA, 3

s) interspaced by 70 s. In the test session animals were re-exposed to the training context for 180 s and freezing was quantified.

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

**C, D)** Seven days and 24 hours context tests. Left panels: freezing over time for groups tested at 7 days (C) and 24 hours (D). Right panels: average freezing. In both experiments freezing in the muscimol group was lower than in the control group (GzLMM t test for 7 days context test: $t_{(20)} = 4.294$, $p = 0.0004$, $n_{vehicle} = 11$, $n_{muscimol} = 12$; GzLMM t test for 24 hours context test: $t_{(16)} = 3.964$, $p = 0.0011$; $n_{vehicle} = 9$, $n_{muscimol} = 10$. In freezing over time plots, lines represent intersubjects' mean, and shaded area represents +SEM. In bar plots, each dot represents a subject and bars represent mean and errors bars +SEM. ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$.

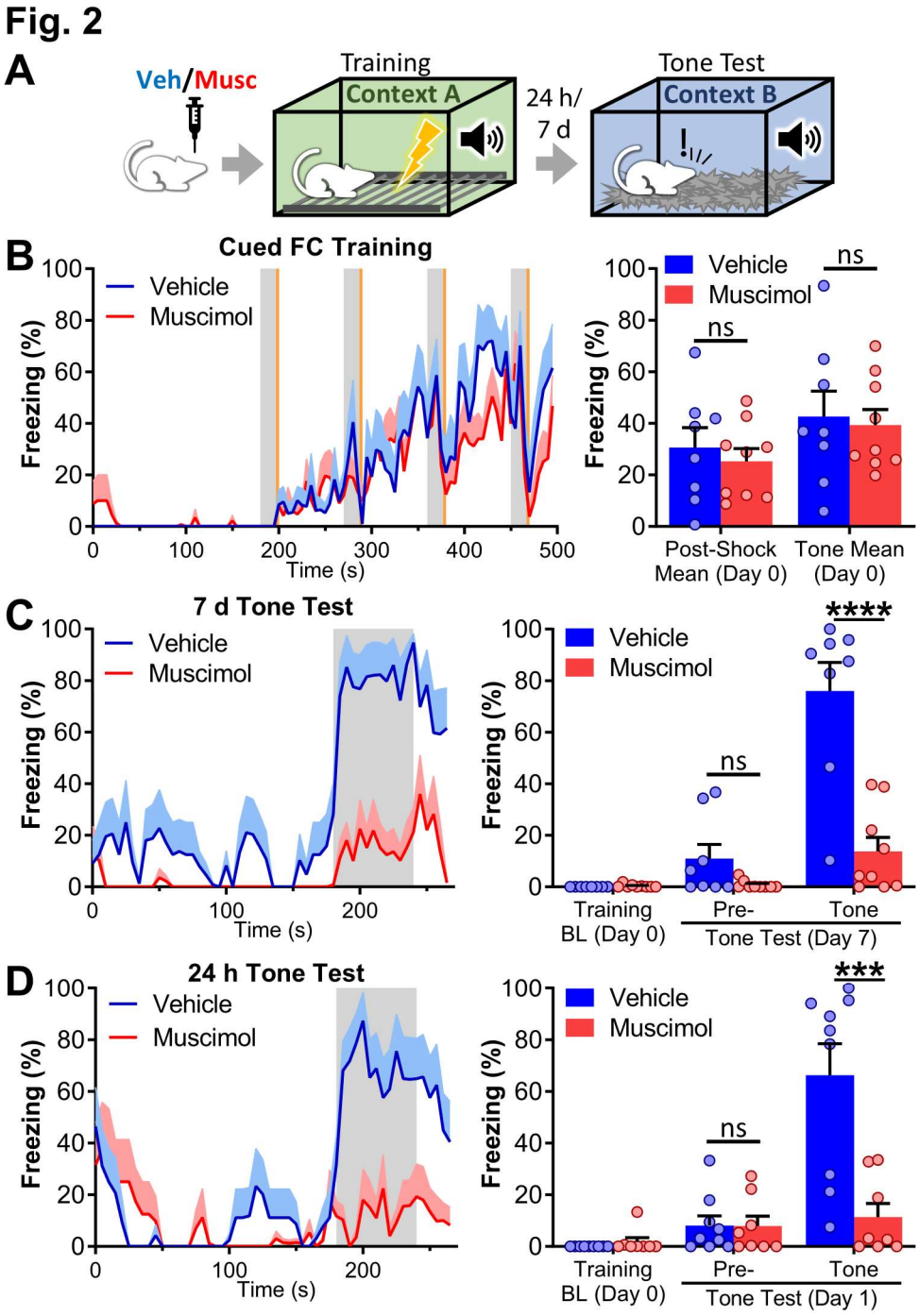


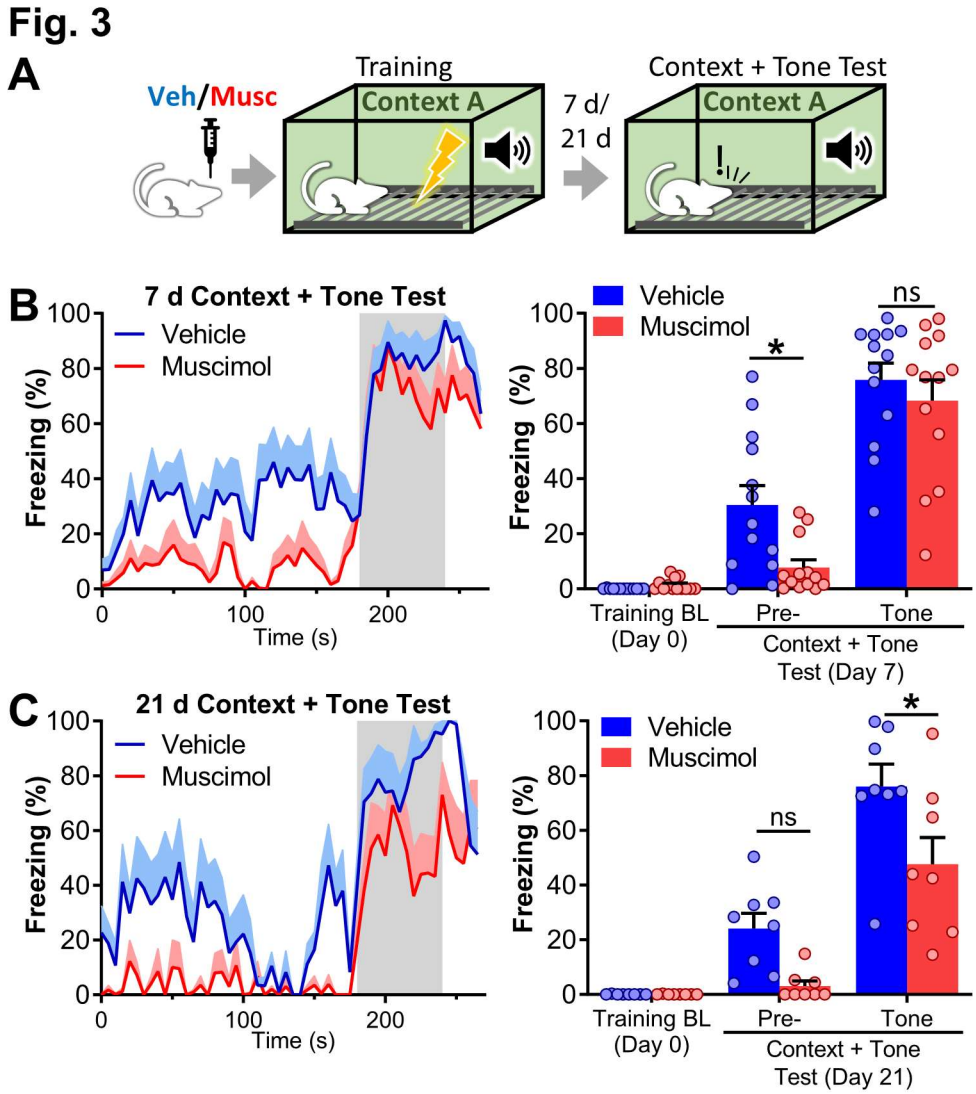

Figure 2. Inactivation of the LHb blocks the formation of cued FC long-term memory.

**A)** Experiment diagram: bilateral vehicle/muscimol intra LHb infusions were performed 30 minutes before training. Cued FC was tested 24 hour or 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)** Freezing during cued FC training for the group tested 7 days after training. Left panel: freezing over time. Tones were presented during the 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. GzLMM Two-way repeated measures ANOVA of treatment, training stage, and interaction: $\chi^2$ treatment $_{(1)}$ = 0.185, p = 0.6668; $\chi^2$ stage $_{(1)}$ = 6.308, p = 0.0120; $\chi^2$ interaction $_{(1)}$ = 0.028, p = 0.8661. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.430, p = 0.8912; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.297, p = 0.9463; $n_{vehicle}$ = 8, $n_{muscimol}$ = 9.

**C, D)** Seven days and 24 hours cued FC tests. Left panels: freezing over time for groups tested 7 days (C) and 24 hours (D) after training. Gray areas indicate tone presentation. Right panels: average freezing for pre- and tone period. Freezing during pre-tone period was not different between vehicle and muscimol groups in either experiment. In contrast, during tone presentation, a highly significant reduction in freezing was observed in the muscimol group in both experiments. GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for 7 days experiment: $\chi^2$ treatment $_{(1)}$ = 2.157, p = 0.1420; $\chi^2$ stage $_{(1)}$ = 19.100, p < 0.0001; $\chi^2$ interaction $_{(1)}$ = 32.939, p < 0.0001. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 1.469, p = 0.2828; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 5.396, p < 0.0001; $n_{vehicle}$ = 8, $n_{muscimol}$ = 9. GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for 24 hours experiment: $\chi^2$ treatment $_{(1)}$ = 0.024, p = 0.8781; $\chi^2$ stage $_{(1)}$ = 0.108, p = 0.7428; $\chi^2$ interaction $_{(1)}$ = 12.447, p = 0.0004. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.153, p = 0.9854; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 4.441, p = 0.0003; $n_{vehicle}$ = 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.




611

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

614 **A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes
615 before training. Animals were trained in cued FC. Test was performed 7 or 21 days later in the same
616 context used for training (Context A). The tone was presented for 60 s after a pre-tone period of 180 s.
617 **B)** Freezing during context + tone test 7 days after training. Left panel: freezing over time. Gray area
618 indicates tone presentation. Right panel: average freezing for pre- and tone period. Freezing to the
619 context was higher in vehicle group. However, tone elicited a robust freezing in the muscimol group that
620 reached values equivalent to the vehicle group. GzLMM Two-way repeated measures ANOVA of
621 treatment, test stage, and interaction: $\chi^2$ treatment $_{(1)}$ = 6.830, p = 0.0090; $\chi^2$ stage $_{(1)}$ = 44.992, p < 0.0001;
622 $\chi^2$ interaction $_{(1)}$ = 3.161, p = 0.0754. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs.

muscimol: $t_{(46)} = 2.613$, $p = 0.0474$; Tone freezing: vehicle vs. muscimol: $t_{(46)} = 0.899$, $p = 0.8460$; Vehicle: pre-tone vs. tone freezing: $t_{(46)} = 6.014$, $p < 0.0001$; muscimol: pre-tone vs. tone freezing: $t_{(46)} = 6.708$, $p < 0.0001$; $n_{vehicle} = 13$, $n_{muscimol} = 13$.

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

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, $p > 0.05$, * $p < 0.05$.

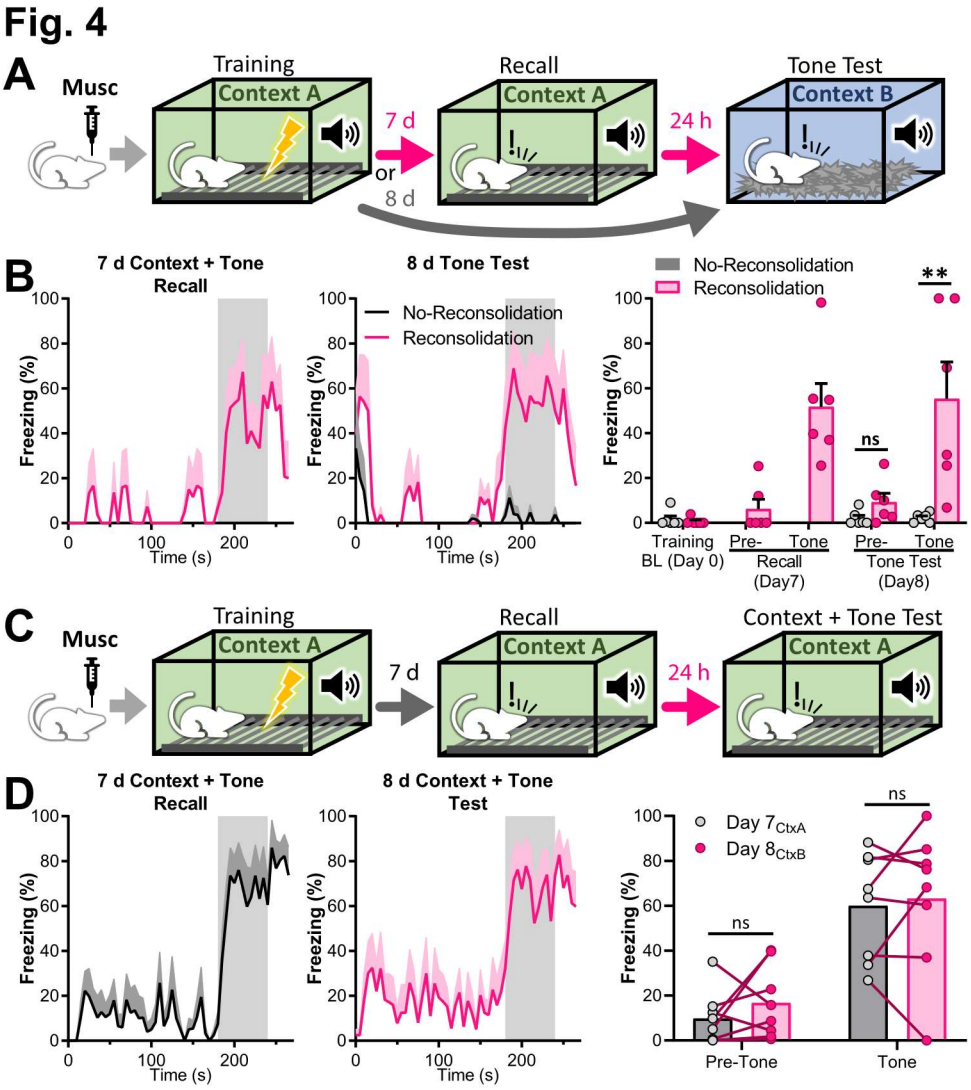



Figure 4. Retrieval of FC memory in context + tone conditions reconsolidates cued but not contextual memories.

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

**B)** Retrieval of FC memory in context + tone conditions restores cued FC memory. Left and center: freezing over time for the recall and the cued memory test sessions, respectively. Animals of the no-reconsolidation group showed very low levels of freezing, confirming the impairment in cued memory

induced by inactivation of the LHb during training. In contrast animals of the reconsolidation group presented a marked freezing to the tone during the test session, reaching levels of freezing equivalent to context + tone condition during the recall session. Right: quantification of freezing. Freezing to the tone in the test session was significantly higher in the reconsolidation group. GzLMM Two-way repeated measures ANOVA of reconsolidation, test stage, and interaction during tone test session: $\chi^2$ reconsolidation $_{(1)}$ = 0.207, p = 0.6489; $\chi^2$ stage $_{(1)}$ = 0.009, p = 0.9232; $\chi^2$ interaction $_{(1)}$ = 9.170, p = 0.0025. Sidak's multiple comparisons test: Pre-tone freezing: reconsolidation vs. no-reconsolidation: $t_{(18)}$ = 0.455, p = 0.8805; Tone freezing: reconsolidation vs. no-reconsolidation: $t_{(18)}$ = 4.219, p = 0.0010; $n_{reconsolidation}$ = 6, $n_{NO\text{-}reconsolidation}$ = 6.

**C)** Diagram of the reconsolidation experiment of contextual FC memory: bilateral intra LHb muscimol infusions were performed 30 minutes before cued FC training as described previously. Seven days later, animals were subjected to a recall session in which the cue was presented in the training context. The following day animals underwent the same procedure.

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

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

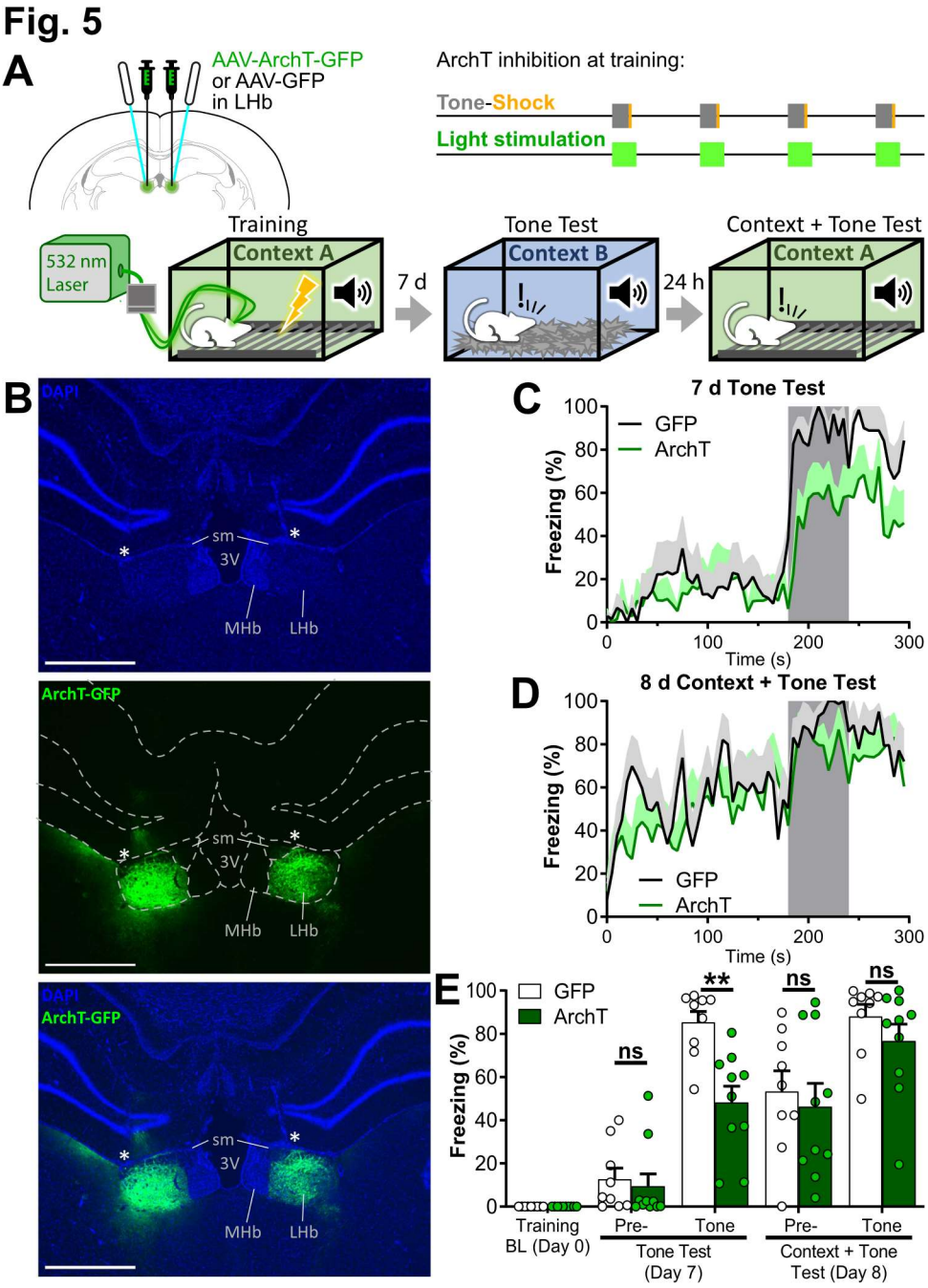



Figure 5. Optogenetic inactivation of the LHb during cue and US, reduced cued but not contextual FC memories.

**A)** Experiment diagram: Top-Left: animals were bilaterally transfected with AAV-ArchT-GFP or AAV-GFP in the LHb and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during training, optogenetic light stimulation was delivered starting with the tone and stopping 5 seconds after the shock (tone and shock presentations were as previously described for cued FC). Bottom: diagram of training and

682 tests. Cued memory was tested 7 days after training in Context B. The same animals were tested the
683 following day in context + tone condition to evaluate contextual FC memory and freezing in context + tone
684 condition.
685 **B)** Microphotographs of the AAV-ArchT-GFP infection (top, middle, bottom: DAPI, GFP, and merge
686 respectively). Dashed white lines in the middle panel delimitates brain structures. * indicates the optic
687 fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third ventricle. Scale bars: 1 mm.
688 **C, D)** Freezing over time during tone test at day 7 (C) and during context + tone test at day 8 (D). Gray area
689 indicates tone presentation. Line represents intersubjects' mean, and shaded area represents +SEM.
690 **E)** Average freezing on tone test, and context + tone test sessions. During tone test ArchT group displayed
691 lower levels of freezing to the tone than GFP group. The following day, during context + tone test, freezing
692 levels of the ArchT group to both to the context and to the tone were equivalent to those of the GFP
693 group.
694 GzLMM Three-way repeated measures ANOVA of test day, test stage, optogenetic inhibition and
695 interactions: $\chi^2$ day $_{(1)}$ = 24.559, $p < 0.0001$; $\chi^2$ stage $_{(1)}$ = 23.070, $p < 0.0001$; $\chi^2$ inhibition $_{(1)}$ = 0.5568, p =
696 0.4555; $\chi^2$ interaction(Day×Stage) $_{(1)}$ = 1.659, p = 0.1978; $\chi^2$ interaction(Day×Inhibition) $_{(1)}$ = 0.294, p =
697 0.5879; $\chi^2$ interaction(Stage×Inhibition) $_{(1)}$ = 5.006, p = 0.0253; $\chi^2$ interaction(Day×Stage×Inhibition) $_{(1)}$ =
698 1.088, p = 0.2970. Sidak's multiple comparisons test: Day7: Pre-tone freezing: GFP vs. ArchT: $t_{(66)}$ = 0.746,
699 p = 0.9747; Day 7: Tone freezing: GFP vs. ArchT: $t_{(66)}$ = 3.410, p = 0.0067; Day 8: Pre-tone freezing: GFP vs.
700 ArchT: $t_{(66)}$ = 0.178, p = 1.0000; Day 8: Tone freezing: GFP vs. ArchT: $t_{(66)}$ = 1.145, p = 0.8308; ArchT: Tone
701 freezing: Day 7 vs. Day 8: $t_{(66)}$ = 3.366, p = 0.0076; Day 8: ArchT: Pre-tone vs. Tone freezing: $t_{(66)}$ = 3.475, p
702 = 0.0054; $n_{GFP}$ = 9, $n_{ArchT}$ = 10. In bar plots each dot represents a subject and bars represent mean + SEM.
703 ns $p > 0.05$, ** $p < 0.01$.
704

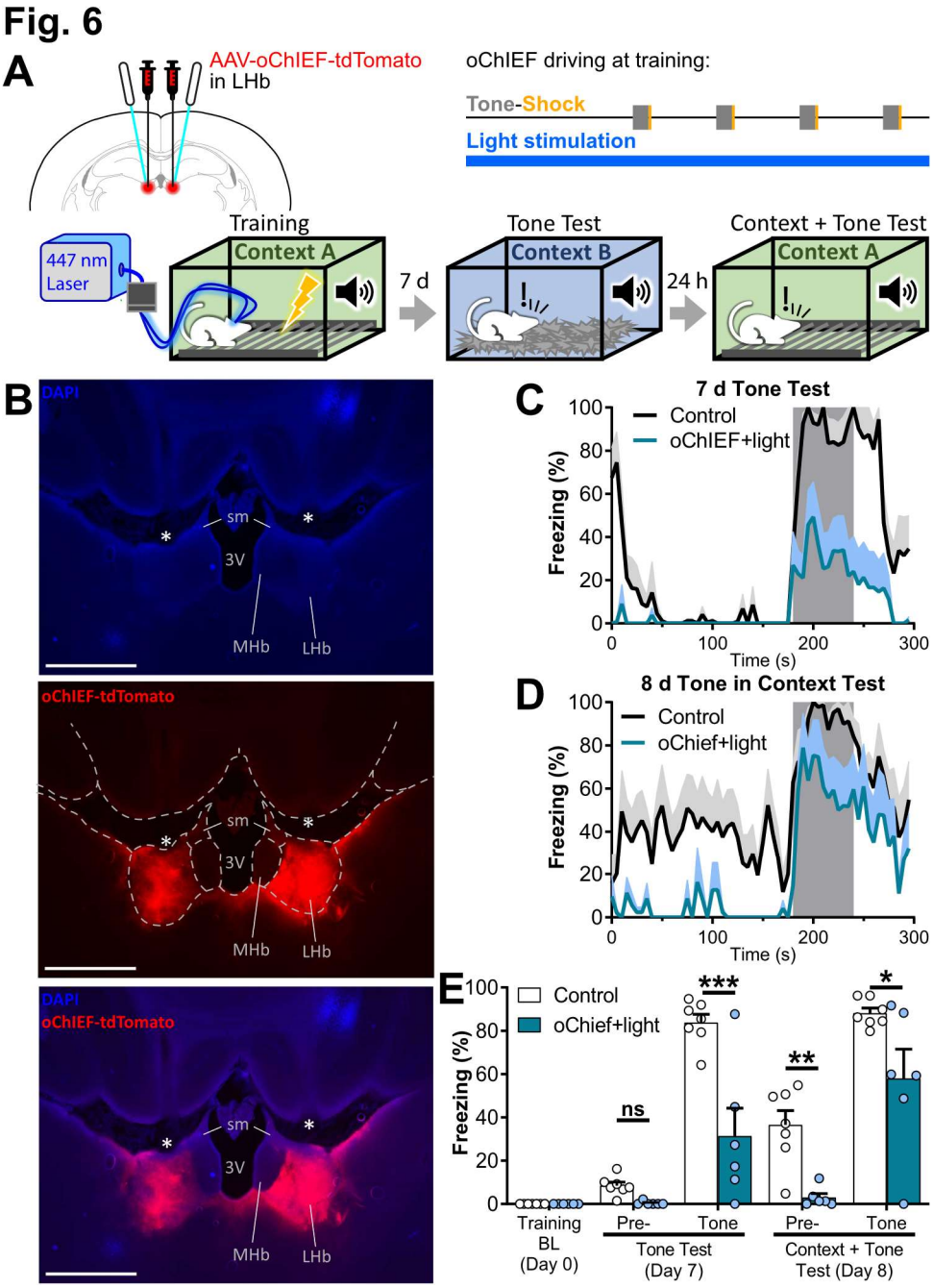
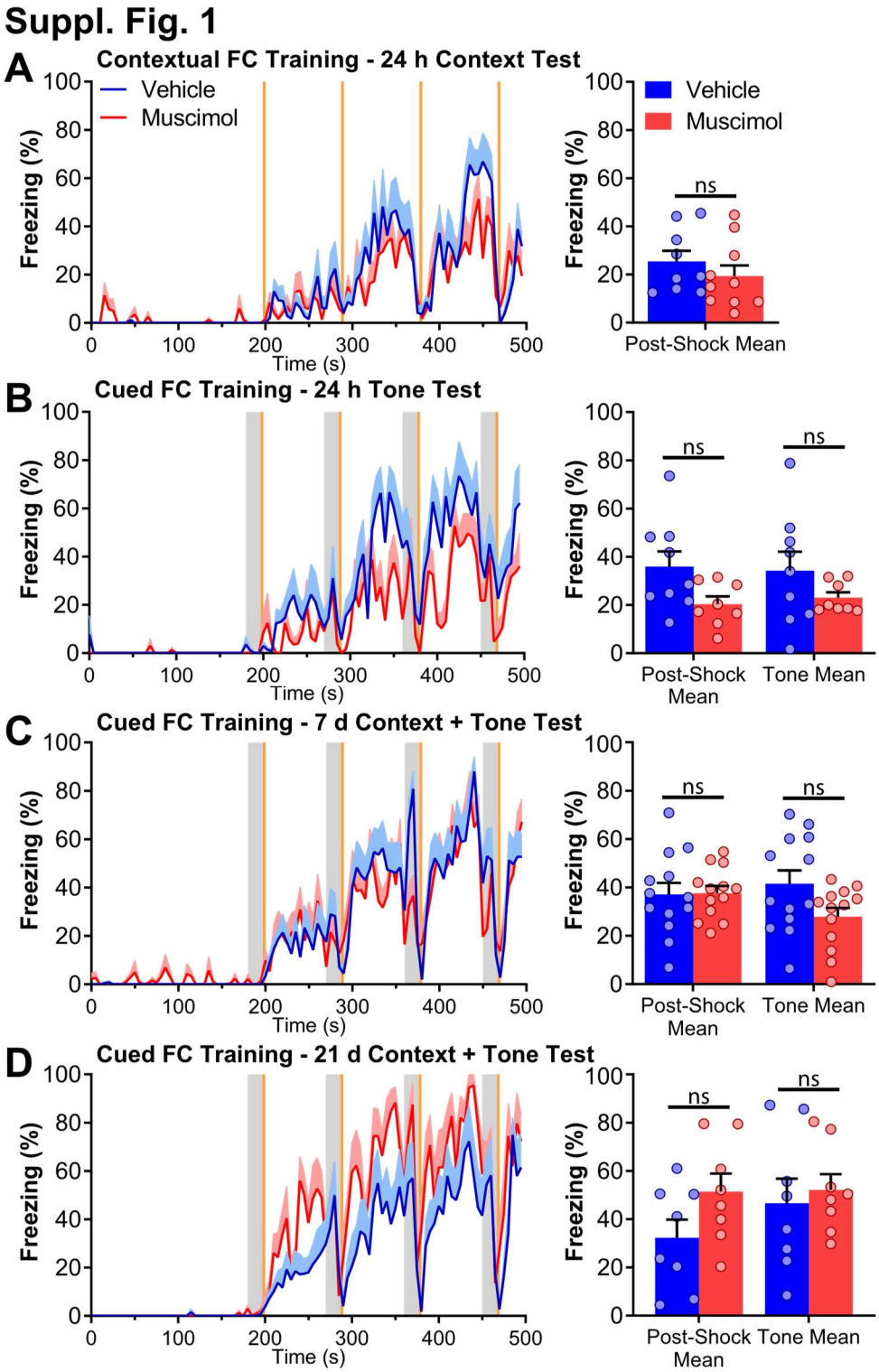



Figure 6. Disruption of LHb neuronal activity during complete FC training impairs contextual and cued FC memories.

**A)** Experiment diagram: Top-Left: animals were bilaterally infected with AAV-oChIEF-tdTomato in the LHb and implanted with optic fibers above the LHb 4 weeks before training. Top-Right: during whole training session the LHb was stimulated with 5 ms light pulses at 20 Hz to disrupt endogenous neuronal activity. Bottom: diagram of training and tests. Cued memory was tested 7 days after training in Context B. The

712 same animals were tested the following day in context + tone condition to evaluate contextual FC memory
713 and freezing in context + tone condition.
714 **B)** Microphotographs of the AAV-oChIEF-tdTomato infection (top, middle, bottom: DAPI, tdTomato
715 fluorescence, and merge respectively). Dashed white lines in the middle panel delimitates brain
716 structures. * indicates the optic fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third ventricle.
717 Scale bars: 1 mm.
718 **C, D)** Freezing over time during cued test at day 7 (C) and during context + tone test at day 8 (D). Gray
719 area indicates tone presentation. Line represents intersubjects' mean, and shaded area represents + SEM.
720 **E)** Average freezing of tone test and context + tone test sessions. Disruption of endogenous LHb activity
721 during training by optogenetic stimulation induced deficits in cued and contextual memories:
722 GzLMM Three-way repeated measures ANOVA of test day, test stage, optogenetic stimulation and
723 interactions: $\chi^2$ day $_{(1)}$ = 20.439, p < 0.0001; $\chi^2$ stage $_{(1)}$ = 100.217, p < 0.0001; $\chi^2$ stimulation $_{(1)}$ = 5.135, p =
724 0.0234; $\chi^2$ interaction(Day×Stage) $_{(1)}$ = 5.805, p = 0.0160; $\chi^2$ interaction(Day×stimulation) $_{(1)}$ = 2.048, p =
725 0.15237; $\chi^2$ interaction(Stage×stimulation) $_{(1)}$ = 2.489, p = 0.1146; $\chi^2$ interaction(Day×Stage×stimulation)
726 $_{(1)}$ = 4.905, p = 0.0268. Sidak's multiple comparisons test: Day 7: Pre-tone freezing: Control vs. oChIEF: $t_{(42)}$
727 = 2.266, p = 0.1601; Day 7: Tone freezing: Control vs. oChIEF: $t_{(42)}$ = 4.454, p = 0.0004; Day 8: Pre-tone
728 freezing: Control vs. oChIEF: $t_{(42)}$ = 3.874, p = 0.0022; Day 8: Tone freezing: Control vs. oChIEF: $t_{(42)}$ = 2.995,
729 p = 0.0272; oChIEF: Tone freezing: Day 7 vs. Day 8: $t_{(42)}$ = 3.810, p = 0.0027; Day 8: oChIEF: Pre-tone vs.
730 Tone freezing: $t_{(42)}$ = 7.283, p < 0.0001; $n_{Control}$ = 7, $n_{oChIEF}$ = 6. In bar plots, each dot represents a subject
731 and bars represent mean + SEM. ns p > 0.05, * p < 0.05, ** p < 0.01 *** p < 0.001.
732

733 Supplementary Information




734

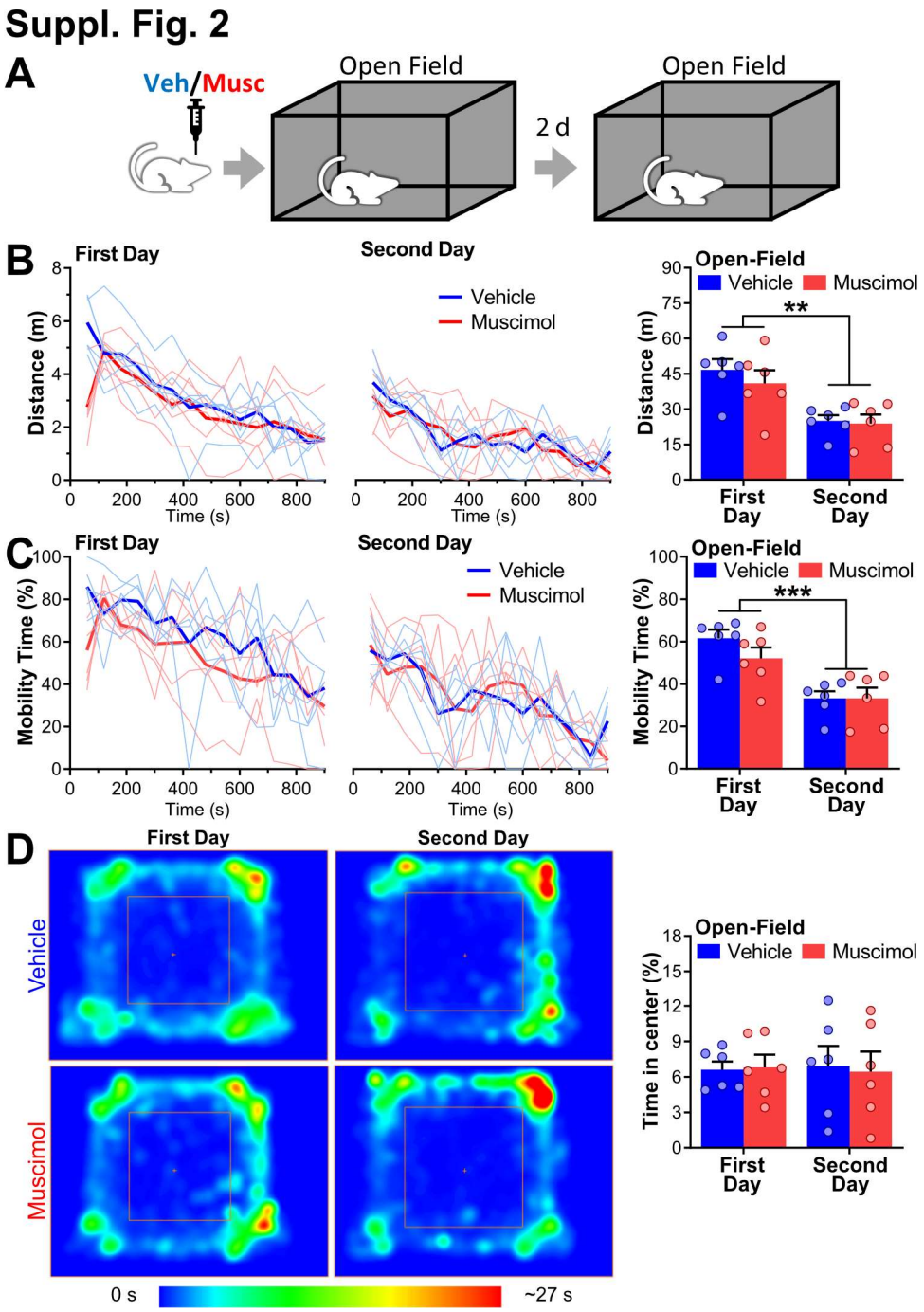
735 Supplementary Figure 1. Freezing during training for experimental groups included in Figures 1-3.

**A-D)** Left: temporal course of freezing during training in the experiments specified in the plot title. 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 $p > 0.05$.

Statistics:

- Contextual FC training – 24 hours test (A): GzLMM t test: $t_{(16)} = 1.217$, $p = 0.2414$. $n_{vehicle} = 9$, $n_{muscimol} = 10$.
- Cued FC training – 24 hours test (B): GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction: $\chi^2$ treatment $_{(1)} = 3.796$, $p = 0.0514$; $\chi^2$ stage $_{(1)} = 0.550$, $p = 0.4584$; $\chi^2$ interaction $_{(1)} = 0.852$, $p = 0.3560$. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs. muscimol: $t_{(28)} = 1.948$, $p = 0.1192$; Tone freezing: vehicle vs. muscimol: $t_{(28)} = 1.245$, $p = 0.3972$. $n_{vehicle} = 9$, $n_{muscimol} = 8$.
- Cued FC training - 7 days context + tone test (C): GzLMM two-way repeated measures ANOVA of treatment, training stage, and interaction: $\chi^2$ treatment $_{(1)} = 0.037$, $p = 0.8471$; $\chi^2$ stage $_{(1)} = 8.741$, $p = 0.0031$; $\chi^2$ interaction $_{(1)} = 8.674$, $p = 0.0032$. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs. muscimol: $t_{(46)} = 0.193$, $p = 0.9769$; Tone freezing: vehicle vs. muscimol: $t_{(46)} = 2.216$, $p = 0.0624$. $n_{vehicle} = 13$, $n_{muscimol} = 13$.
- Cued FC training - 21 days context + tone test (D): GzLMMtwo-way repeated measures ANOVA of treatment, training stage, and interaction: $\chi^2$ treatment $_{(1)} = 3.723$, $p = 0.0537$; $\chi^2$ stage $_{(1)} = 0.023$, $p = 0.8802$; $\chi^2$ interaction $_{(1)} = 4.535$, $p = 0.0332$. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs. muscimol: $t_{(26)} = 1.930$, $p = 0.1251$; Tone freezing: vehicle vs. muscimol: $t_{(26)} = 0.521$, $p = 0.8454$. $n_{vehicle} = 8$, $n_{muscimol} = 8$.

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




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

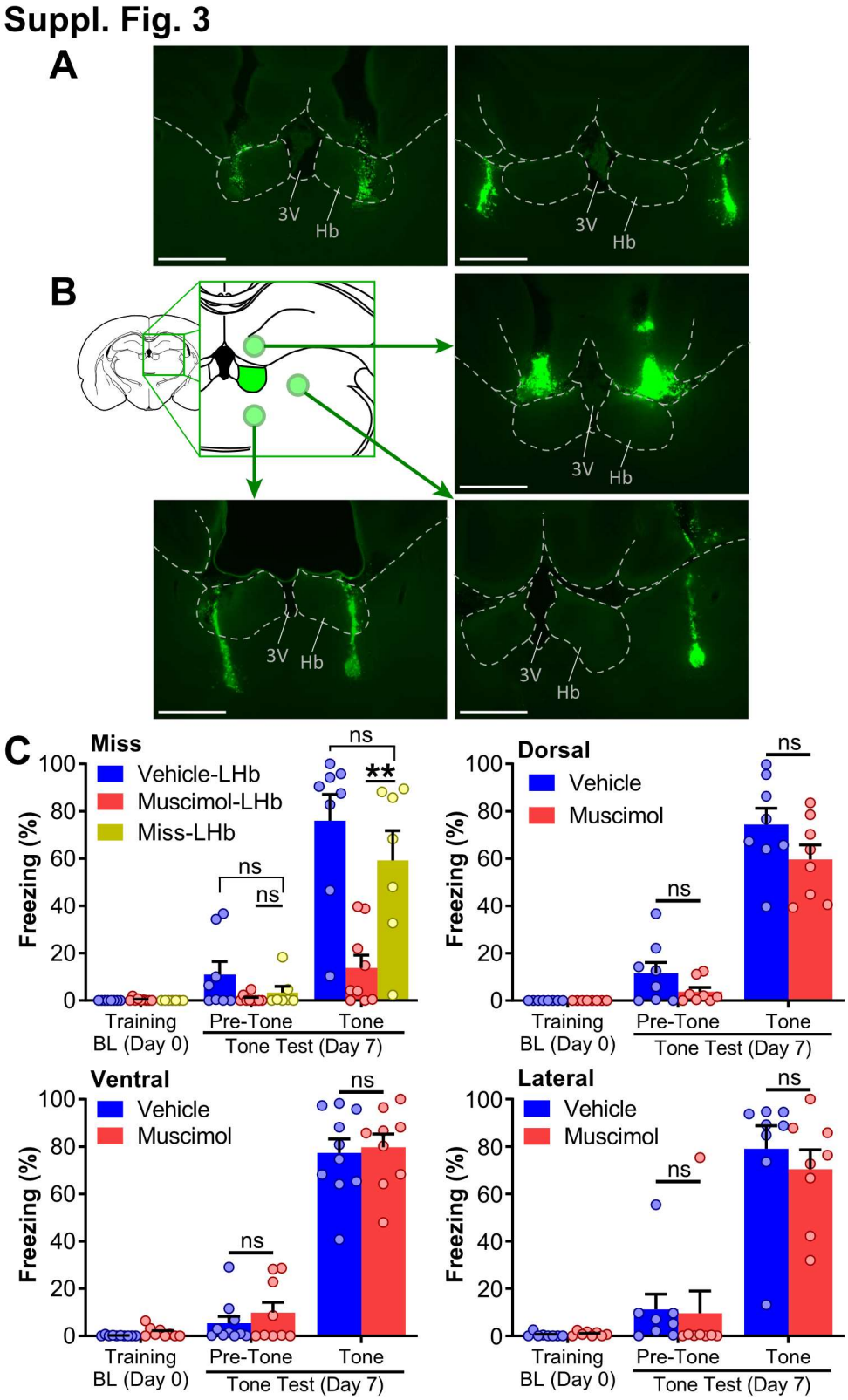
**A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes before exposing the animals to an OF for 15 minutes. 48 hours later animals were exposed to the same OF for a second time.

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

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

**D)** Heat map representation of spatial occupancy during OF. Left: average heat maps vehicle (top) and muscimol (bottom) groups on the first day (left), and the second day (right). The center zone is indicated by the orange square. Right: percentage of time in the center zone in both groups at each day. Time in the center did not change between days and was equivalent among groups (two-way repeated measures ANOVA of treatment, day, and interaction: $F_{treatment\ (1, 10)} = 0.01082$, $p = 0.9192$; $F_{day\ (1, 10)} = 0.0003566$, $p = 0.9853$; $F_{interaction\ (1, 10)} = 0.05192$, $p = 0.8244$. Sidak's multiple comparisons test: Day 1: vehicle vs. muscimol: $t = 0.1082$, $p = 0.9928$; Day 2: vehicle vs. muscimol: $t = 0.2416$, $p = 0.9645$).

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




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

**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.

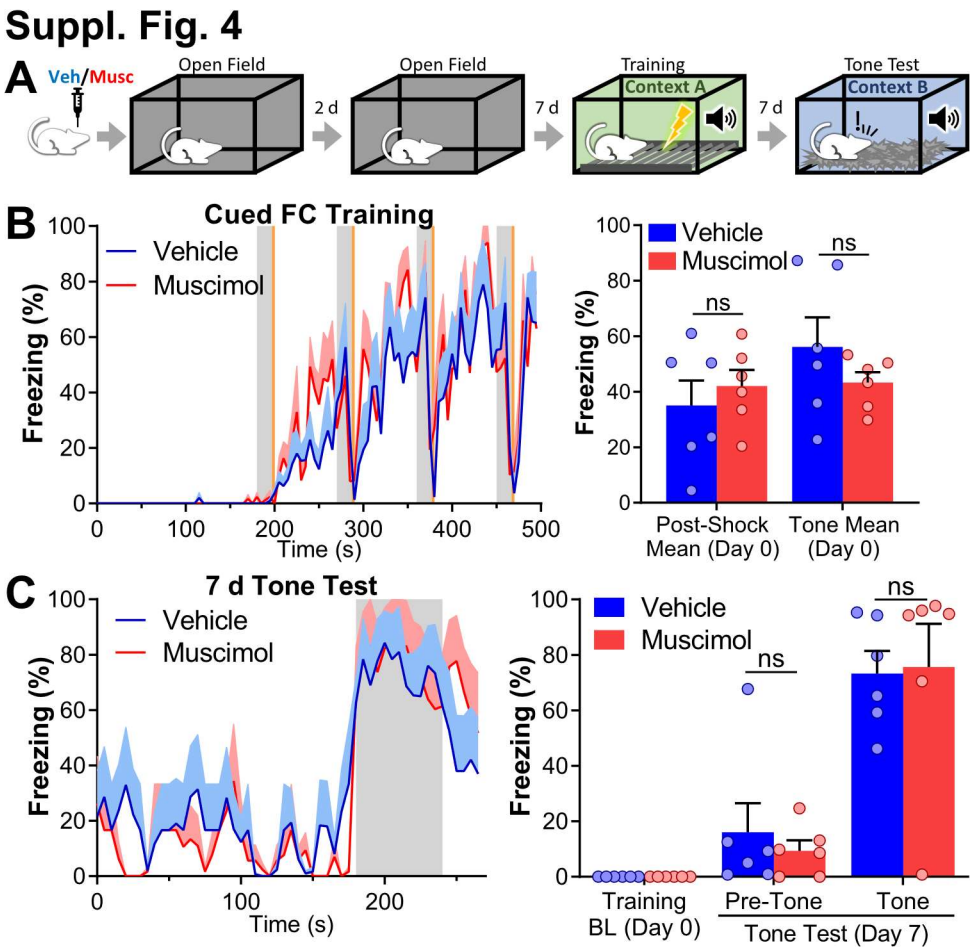
**B)** Diagram and representative photomicrographs of controls of specificity of muscimol infusion. Infusions were aimed 1 mm dorsal (top right picture), ventral (bottom left picture) or lateral (bottom right picture). References: Hb: habenula; 3V: third ventricle.

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

Statistics:

- GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for miss graph: $\chi^2$ treatment $_{(2)}$ = 2.5079, p = 0.2854; $\chi^2$ stage $_{(1)}$ = 85.1604, p < 0.0001; $\chi^2$ 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.
- GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for dorsal graph: $\chi^2$ treatment $_{(1)}$ = 1.7632, p = 0.1842; $\chi^2$ stage $_{(1)}$ = 102.565, p < 0.0001; $\chi^2$ 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: $\chi^2$ treatment $_{(1)}$ = 0.525, p = 0.4689; $\chi^2$ stage $_{(1)}$ = 165.9408, p < 0.0001; $\chi^2$ 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.

825 - GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for lateral
826 graph: $\chi^2$ treatment $_{(1)}$ = 0.326, p = 0.5680; $\chi^2$ stage $_{(1)}$ = 27.113, p < 0.0001; $\chi^2$ interaction $_{(1)}$ < 0.0001,
827 p = 0.9980. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 0.571,
828 p = 0.8176; Tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 0.605, p = 0.7978; $n_{vehicle}$ = 8, $n_{muscimol}$ = 8.
829



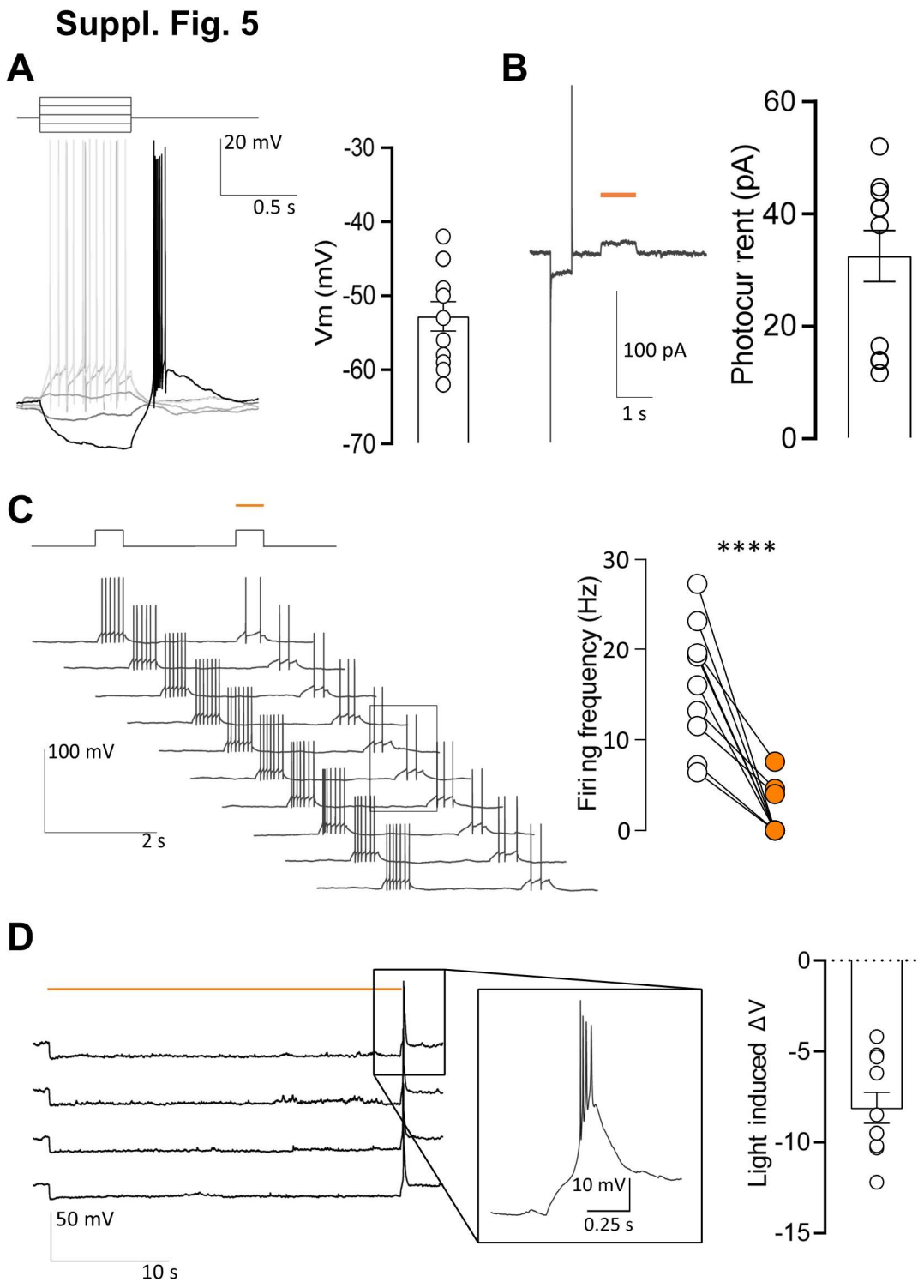

Supplementary Figure 4. Inactivation of the LHb does not permanently block FC learning.

**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. GzLMM Two-way repeated measures ANOVA of treatment, training stage, and interaction: $\chi^2$ treatment $_{(1)}$ = 0.685, p = 0.4077; $\chi^2$ stage $_{(1)}$ = 0.050, p = 0.8231; $\chi^2$ 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)** 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

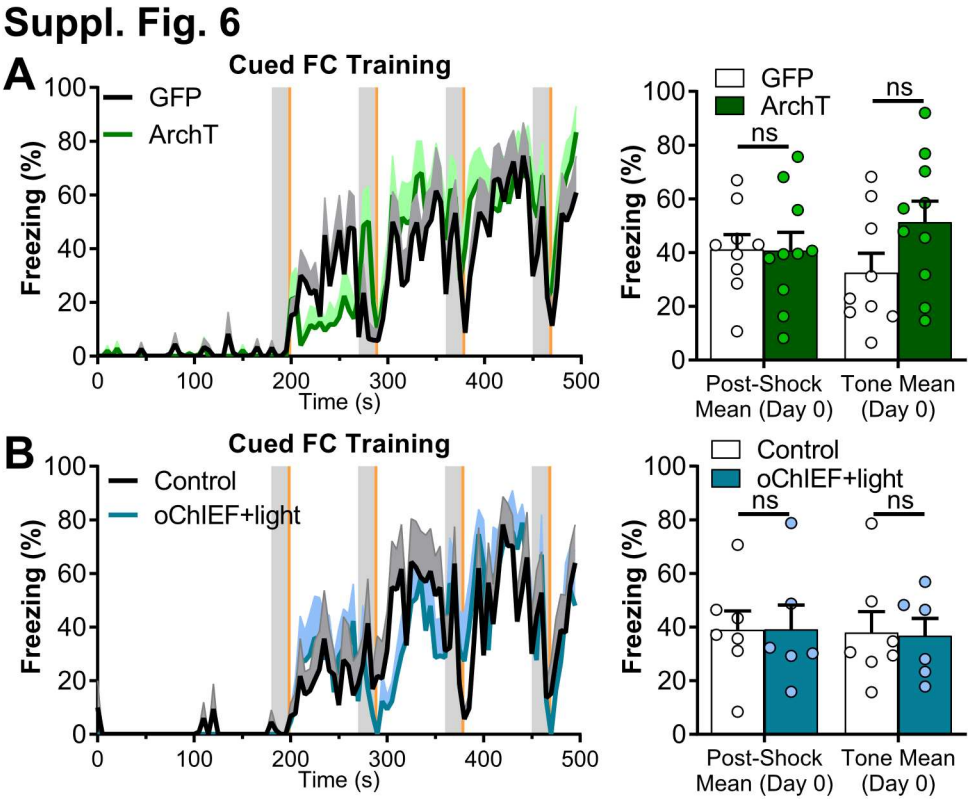
845 vehicle and muscimol groups. GzLMM Two-way repeated measures ANOVA of treatment, test stage, and
846 interaction: $\chi^2$ treatment $_{(1)}$ = 0.0697, p = 0.7918; $\chi^2$ stage $_{(1)}$ = 15.254, p < 0.0001; $\chi^2$ interaction $_{(1)}$ = 0.103,
847 p = 0.7478. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.264, p =
848 0.9579; Tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.190, p = 0.9780; $n_{vehicle}$ = 6, $n_{muscimol}$ = 6.
849 In freezing over time plots, line represents intersubjects' mean and shaded area represents SEM. In bar
850 plots each dot represents a subject and bars represent mean + SEM. ns p > 0.05.
851




Supplementary Figure 5. 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

856 hyperpolarizing currents. Right graph: resting membrane potential of recorded neurons; n = 13 neurons
857 from 3 rats.
858 **B)** Light induced photocurrent. ArchT was activated with 590 nm LED for 1 s (orange line) with a nominal
859 power of 0.15 mW; n = 11 neurons from 3 rats.
860 **C)** Light induced action potential suppression in ArchT expressing LHb neurons. Light pulse (590 nm,
861 orange line) was applied every second time neurons were depolarized with positive current injection. Left,
862 representative recordings. Right, data summary. Light activation of ArchT significantly decreases the
863 number of action potentials evoked by current steps in LHb neurons (paired t test, $t_{(9)} = 6.25$, $p < 0.001$);
864 n = 9 neurons from 3 rats.
865 **D)** Membrane potential response to a 30 s light pulse (orange line) in LHb neurons. Rebound spiking was
866 observed after termination of light pulse, a representative trace is expanded in the middle traces. This
867 behavior confirms the reported rebound responses of the LHb neurons following long periods of
868 optogenetic inhibition reported previously (Yang et al., 2018). Right: quantification of the stationary light
869 induced ΔVm during the last 5 s of the 30 s pulse; n = 10 neurons from 3 rats.
870 Methods details for this experiment: Surgery was similar to the optogenetic manipulation experiments
871 with the following differences: 3 weeks old rats were employed, the viral vector injection sites were
872 adjusted according to Bregma-Lambda distance (Bλ) of each rat (AP = -2.9 × Bλ ÷ 8.5 mm, ML = ± 0.7 × Bλ
873 ÷ 8.5 mm from Bregma and DV -4.3 mm from dura mater), no fiber optic implants, nor screws or acrylic
874 was used, and the skin was stitched at the end of surgery. Recordings were obtained from ArchT
875 expressing LHb neurons in acute brain slices prepared 3 weeks after infection. General methods for slice
876 preparation and recordings were replicated from previous publications of the group (Stefanelli et al.,
877 2016).
878



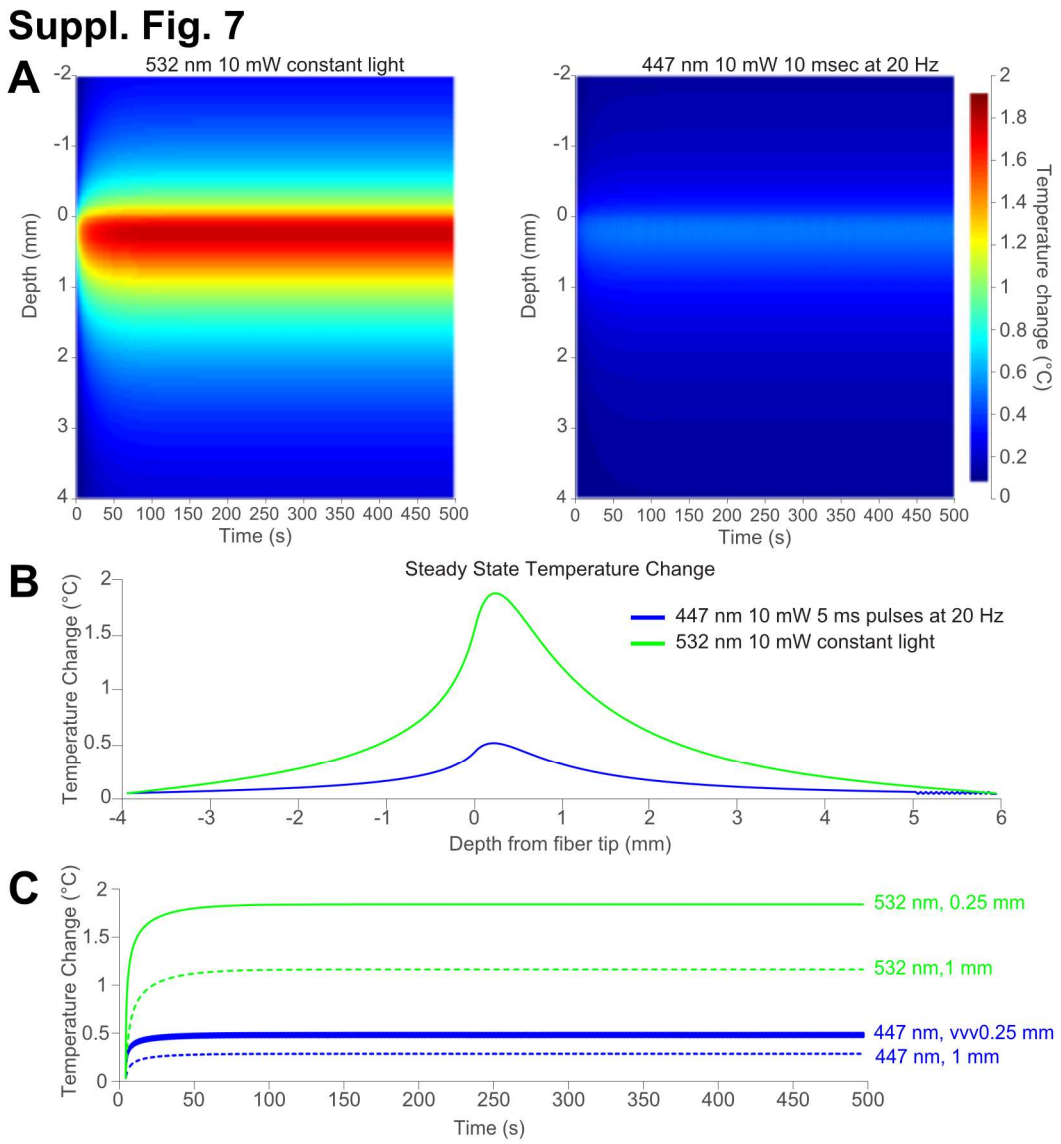

Supplementary Figure 6. Freezing during training for experimental groups included in Figures 5 and 6.

**A-B)** Left: temporal course of freezing during training in the ArchT (A) and oChIEF (B) stimulation experiments. Right: average freezing on the same experiments. In freezing over time plots 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. In either condition no significant differences were observed between control and optogenetic stimulated groups.

Statistics:

- For ArchT experiment: GzLMM two-way repeated measures ANOVA of optogenetic inhibition, training stage, and interaction: $\chi^2$ inhibition $_{(1)}$ = 0.002, p = 0.9631; $\chi^2$ stage $_{(1)}$ = 3.173, p = 0.0749; $\chi^2$ interaction $_{(1)}$ = 5.000, p = 0.0254. Sidak's multiple comparisons test: Post-shock freezing: GFP vs. ArchT: $t_{(32)}$ = 0.046, p = 0.9987; Tone freezing: GFP vs. ArchT: $t_{(32)}$ = 2.098, p = 0.0859. $n_{GFP}$ = 9, $n_{ArchT}$ = 10.
- For oChIEF experiment: GzLMM two-way repeated measures ANOVA of optogenetic disruption, training stage, and interaction: $\chi^2$ disruption $_{(1)}$ = 0.007, p = 0.9358; $\chi^2$ stage $_{(1)}$ = 0.018, p = 0.8946; $\chi^2$ interaction $_{(1)}$ = 0.0650, p = 0.7988. Sidak's multiple comparisons test: Post-shock freezing: Control vs. oChIEF: $t_{(20)}$ = 0.081, p = 0.9960; Tone freezing: Control vs. oChIEF: $t_{(20)}$ = 0.113, p = 0.9921. $n_{Control}$ = 7, $n_{oChIEF}$ = 6.

Stage factor refers to freezing between shock and tone, or during tone (post-shock or tone respectively).




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

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

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

910 **B**) Depth profile of temperature change in the steady state (calculated as the mean in the 250-300 s
911 interval). Constant light stimulation with 532 nm light would induce a significant temperature increase
912 that would extend over more than 1 mm and would induce non-controllable out of target changes in
913 neuronal activity. In contrast, pulsed activation of oChIEF would not induce physiologically relevant
914 temperature changes at any depth (Stujenske et al., 2015).
915 **C**) Temporal profile of temperature change at 0.25 and 1 mm from the fiber tip for both stimulation
916 protocols.