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

The lateral habenula (LHb) encodes aversive information. However, there is little understanding of how the information provided by the LHb influences learning and memory processes. Fear conditioning (FC) is probably the best understood model of aversive associative learning. Thus, FC constitutes a good behavioral paradigm to analyze LHb function in associative learning. In the present work we studied in rats the effect of interfering with neuronal activity of the LHb on FC using optogenetics and pharmacological tools. We found that exciting or inhibiting 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 reveal that the formation of independent cued and contextual FC memories requires intact signaling by the LHb assigning to this structure a previously uncharacterized integrative role in brain circuits required for FC learning.

Introduction

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 (*1–3*). That connectivity places the LHb in a central position to control aversive associative learning. However, how the LHb is involved in that process is not clear.

Fear conditioning (FC) is probably the most studied model of aversive associative learning (4, 5). 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 (*6*, *7*). 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.

In a previous work, using the Inhibitory Avoidance paradigm, we found that inactivation of the LHb during training affects temporal stability of the memory but not its formation (*8*). To better understand how the LHb influences aversive learning, in this work we studied its role in FC learning by means of pharmacological and optogenetics manipulations. We show that interfering with neuronal activity of the LHb during FC training severely impairs recall of contextual and cued FC memories. However, when the cue is played in the training context, a conserved FC memory is evidenced. Thus, our

results suggest that the formation of two independent memory traces during FC requires an intact LHb activity, placing this structure in a central position in the neuronal circuits mediating FC learning and revealing a new aspect of that behavioral paradigm.

54 ## Results

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

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

69 Long-term memories tend to decay over time, a phenomenon that could already be evident 7
70 days after memory formation (*9*, *10*). To test whether an accelerated decay of contextual FC memory
71 could account for the memory deficits that were observed, we repeated our previous experiment but
72 tested contextual FC memory 24 hours after training. Contextual FC memory deficits were also evident
73 24 hours after training (Figure 1 D), further suggesting that inactivation of the LHb during contextual FC
74 training has a detrimental effect over long-term memory formation.

75 Importantly, muscimol infusion in the LHb before exposure to an OF did not impact on the
76 decrease in exploratory behavior observed upon a subsequent re-exposure performed 48 hours later
77 (Supplementary Figure 2), which is acts as a measure of contextual memory on that test (*11*, *12*). That
78 observation suggests that the inactivation of the LHb by muscimol does not induce a general deficit in
79 context encoding.

80 Pharmacological inactivation of the LHb impairs cued fear conditioning

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

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

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

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

105 Context + tone test evidences a conserved fear conditioning memory

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

115 In a previous paper we demonstrated that the inactivation of the LHb impairs long-term stability
116 of the Inhibitory Avoidance memory (*8*). To investigate long-term stability of FC memory formed under
117 inactivation of the LHb, we evaluated freezing in context + tone conditions three weeks after training in

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

122 Context + tone retrieval reconsolidates cued but not contextual memory

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

140 increase in freezing to the Context A during the test session (Figure 4D). Then, levels of freezing
141 remained low even though those animals effectively displayed freezing in context + tone condition in
142 the recall session (Figure 4D). Also, freezing in context + tone conditions was not different between the
143 recall and the test session and was high in both cases (Figure 4D). Those results suggest the recall
144 session reconsolidated the cued memory but not the contextual memory, arguing that freezing
145 observed during context + tone condition mainly reflects the reactivation of the cued memory trace.
146 Furthermore, they suggest that FC training under inactivation of the LHb creates a cued memory whose
147 retrieval depends on contextual information.

148 Optogenetic inactivation of the LHb impairs cued fear conditioning

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

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

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

165 To rigorously test the involvement of the LHb in contextual FC using optogenetic tools, it would
166 be necessary to disturb neuronal activity at the LHb during context encoding as well (*i.e.* during whole
167 training period). Optogenetic inhibition mediated by light activated hyperpolarizing pumps such as
168 ArchT or NpHR, require sustained illumination which could create confounding effects, such as
169 temperature changes, when prolonged for long periods like the one involved in our protocols
170 (Supplementary Figure 7). Also, sustained on-off patterns of optogenetic inhibition induce burst activity
171 of LHb neurons that induce depression-like symptoms (*13,* Supplementary Figure 5D). To avoid those
172 potential artifacts, we decided to take a different approach. We reasoned that neuronal encoding would
173 be disturbed by any major intervention over neuronal activity. Thus, we opted to disrupt endogenous
174 activity of the LHb by imposing an artificial excitation during the whole training. Animals were injected
175 with an AAV encoding the fast excitatory opsin oChIEF (14), and implanted with optic fibers bilaterally in
176 the LHb (Figure 6A, B). Three weeks later they were trained in cued FC under a sustained 20 Hz
177 excitation drive with light pulses of 5 ms (Figure 6A), which has been shown before not to induce
178 depression-like symptoms (13; Figure 6A, B). Modelling of light induced temperature changes supports
179 the idea that such protocol would result in minimal temperature changes in the illuminated tissue (15;
180 Supplementary Figure 7). We found that artificial stimulation of the LHb during FC training mostly
181 replicates results obtained by pharmacological inhibition (Figure 6). oChIEF activation did not modify
182 behavior during FC training (Supplementary Figure 6B). During cued memory test, oChIEF stimulated
183 animals showed lower freezing to the tone than the control group (Figure 6C, E). Next day, during

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

190

Discussion

Previous reports indirectly suggested that the LHb could play a role in FC learning (*16*, *17*). However, the requirement of the LHb for FC learning was largely untested (*18*, *19*). In this work, we directly addressed that subject. Our findings reveal that the LHb is a relevant component in FC learning circuits, playing a role in the formation of both cued and contextual memories. In the following subsections we discuss our findings in detail.

Cued memory and the LHb

During FC training, subjects infer which stimuli have predictive value over the arrival of the US to organize proper defensive behavioral responses. In normal conditions, that processing leads to a double association that assigns a predictive value to both the context and the cues. Prevailing models postulate that context-US association involves context encoding circuits centered in the hippocampus which send contextual representation to the amygdala where the association takes place (*20*, *21*), and locate cue-US association in the amygdala (*22*). Particularly, solid evidence indicates that tone-US association takes place in cortical and thalamic auditory inputs to the lateral amygdala (*23*–*25*). Thus, as postulated in that model, cued memory is assumed to be intrinsically context-independent. However, our experiments suggest that the formation of a cued memory that could be expressed in a context-independent manner requires signaling by the LHb during memory formation.

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

212 and the cue in guiding memory retrieval. Second, in reconsolidation experiments we found that,
213 following retrieval in context + tone conditions, cued memory is reinstated. That result suggests that the
214 cued memory is reactivated when the cue is played in the training context. Moreover, since contextual
215 FC memory is not restored by the same manipulation, it could be inferred that freezing behavior during
216 context + tone is mostly attributable to the reactivation of the cued memory. In addition, the latter
217 observation argues that freezing in context + tone conditions does not reflect the retrieval of two weak
218 fear memories (contextual and cued) that have to add up to trigger freezing behavior. Altogether, those
219 two observations suggest that, under inactivation of the LHb, a cued FC memory is formed whose
220 retrieval depends on the context in which the cue is presented.

221 It has been shown that during FC training, activity of the LHb increases in response to cue
222 presentation, paralleling the appearance of conditioned responses (*16*, *17*). Though, no mechanistic
223 information about how the LHb influences cued memory formation could be inferred from the present
224 work, a tempting hypothesis is that the emergence of responses of the LHb to the cue acts as a
225 prediction error signal over the arriving of the US that, when confirmed, emancipates the cue-US
226 association from the context where it was formed.

227 <u>Contextual memory and the LHb</u>

228 Contextual FC is also blocked by pharmacological inactivation or artificial excitation of the LHb
229 during whole training. In contrast, temporally limited optogenetic inactivation of the LHb during cue and
230 US presentation did not affect contextual FC. The simplest explanation for this latter result is that
231 encoding of the US by the LHb is not involved in context-US association, which could be based on
232 representations of the US encoded in other structures, like the amygdala (*26*). Thus, deficits in

233 contextual FC induced by manipulations of the LHb might reflect disturbances in the encoding of the
234 context, or in the formation of the association between the context and the US.

235 In this regard, it has been proposed elsewhere that the LHb influences context encoding through
236 a functional interaction with the hippocampus (*27*, *28*). That hypothesis has received support from
237 physiological data generated by other authors and us showing that firing of the neurons of the LHb is
238 synchronized with hippocampal theta rhythm (*27*, *29*, *30*). In addition, two recent papers from Nyiri
239 group showed that LHb-innervated Nucleus Incertus (*31*) and Dorsal Raphe neurons (*32*) modulate
240 hippocampal theta. Thus, a considerable amount of data supports the hypothesis that the LHb
241 modulates context encoding, favoring the idea that disturbing neuronal activity of the LHb affects
242 context encoding and consequently the formation of contextual FC memory.

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

253 <u>Retrieval and temporal stability, two faces of memory strength</u>

How easy a memory is retrieved could be read as a parameter of memory strength. Also, in temporal dimension, the strength of a memory could be read from how long it lasts (*33*). In our previous work we described that inactivation of the LHb before training in the Inhibitory Avoidance generates a memory that is normally expressed 24 hours after training but could not be evidenced 7 days later (*8*). In that paradigm, temporal stability of the memory is defined by shock intensity (*i.e.* by the salience of the US), thus it could be evaluated as a parameter of memory strength. There, we also found that a series of manipulations known to extend temporal stability of weak Inhibitory Avoidance memories did also extend temporal stability of the memory formed under inactivation of the LHb. Based on those findings, we postulated that the main effect of the inactivation of the LHb was to reduce memory strength. Our present results support and extend that hypothesis to the FC paradigm. In one side, we found FC memory is harder to retrieve, since matching between training and testing conditions should be increased in order to effective retrieval. In addition, we also found that the FC memory expressed under context + tone conditions is temporarily less stable (Figure 3C). Thus, FC memory formed under inactivation of the LHb could be considered weaker in those two aspects. Taken together those results indicate a general role of the LHb in defining aversive memory strength.

Concluding Remarks

In summary, our results demonstrate that the LHb plays a role as a modulator of the associative learning of FC. Notably, the expression of the memory formed under the inhibition of the LHb could be described as an example of anti-generalization. Extrapolating that, it would be interesting to study whether increased activity of the LHb could lead to a behavior prone to the generalization of fear, a central characteristic of phobic and anxiety disorders.

276

277 ## Materials and Methods

278 ### Animals

279 Experiments were performed in male Wistar rats obtained from the vivariums of the Faculty of
280 Pharmacy and Biochemistry of the Buenos Aires University, Argentina, and Janvier Labs, France. Animals
281 were 5-6 weeks old at the time of surgery. Animals were housed 4 to 6 per cage, with *ad libitum* access
282 to food and water, under 12-hour light/dark cycle (lights on at 7:00 am), at constant temperature of 22 ±
283 2 °C. All the procedures where performed during light hours. Experimental procedures were approved
284 by the Animal Care and Use Committee of the University of Buenos Aires (CICUAL), and the Autonomous
285 Community of Madrid (PROEX 167/18).

286 ### Surgeries

287 **Pharmacology:** rats under deep ketamine/xylazine anesthesia (100 and 5 mg/kg respectively)
288 were bilaterally implanted with 22-Gauge guide cannulae aimed 2.0 mm above the LHb (AP -3.0 mm, ML
289 ± 0.7 mm, DV -3.8 mm from Bregma), or 1.0 mm dorsal, ventral or lateral to LHb coordinates in specific
290 experiments. Cannulae were fixed to the skull with three surgical steel screws and dental acrylic. At the
291 end of surgery, animals received a dose of analgesic (meloxicam 0.6 mg/kg) and antibiotic (gentamicin 3
292 mg/kg). Behavioral procedures began 7-9 days after surgery.

293 **Optogenetics:** rats under deep isofluorane anesthesia (4 % induction, 1-2 % maintenance, in 0.8
294 L/minute oxygen) were bilaterally injected with 300 nl of viral vector (AAV8-CamKIIα-ArchT-GFP, 6.2 x
295 10^{12} viral particles/ml from UNC Vector Core, AAV8-CamKIIα-GFP, 6.3 x 10^{12} viral particles/ml from UNC

296 Vector Core, or AAV8-hSyn-oChIEF-tdTomato, homemade as described (*34*) per side at the LHb (AP -2.9
297 mm, ML ± 0.7 mm, DV -5.0 mm from Bregma). Subsequently, optical fiber implants (200 µm core, 0.45
298 NA) were bilaterally inserted with the tips aiming just above the LHb, with 4 degrees angle from the
299 sagittal plane (AP -3.0 mm, ML ± 1.05 mm, DV -4.5 mm from Bregma), and fixed to the skull with three
300 surgical steel screws and dental acrylic. During surgery, animals received a dose of analgesic (meloxicam
301 0.6 mg/kg) and antibiotic (gentamicin 3 mg/kg). Behavioral procedures began three weeks after surgery.

302 Contextual and Cued Fear Conditioning Training

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

317 **Pharmacology experiments:** after the acclimation period, rats were randomly assigned to
318 receive bilateral intra LHb infusions of GABAa agonist, muscimol (Sigma, 60 ng/µl in saline solution, 0.5
319 µl/side), or saline. Fluorescent green beads were added to both solutions to aid in checking the infusion
320 site (1:1000 dilution of concentrated 1 µm diameter fluorescent green beads, Bangs Laboratories, USA).
321 During infusions animals were grasped by hand and slightly restrained in the lap or the arm of the
322 investigator. Infusions were delivered at a rate of 0.5 µl/minute through a 30-Gauge needle extending
323 2.0 mm beyond the tip of implanted guide cannula and connected to a 10 µl Hamilton syringe by a
324 polyethylene tube. Infusion needle was left in place for an additional minute to minimize backflow. After
325 that was animals were returned to their home cage. FC training began 30 minutes later.

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

339 Contextual FC Test

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

344 Cued FC Test

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

355 Context + Tone Test

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

358 Open Field

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

362 Histologic Control

363 Within a week after the end of behavioral procedures animals were euthanized with a lethal
364 dose of ketamine/xylazine, 50 ml of ice-cold phosphate-buffered saline (PBS) 0.1 M was transcardially
365 perfused, followed by 50 ml of paraformaldehyde 4 %. After that, the brain extracted and incubated at 4
366 °C in paraformaldehyde 4 % overnight. Then, brain was transferred to 30 % m/v sucrose in PBS 0.1 M,
367 incubated for three days at 4 °C and coronal sections (150 µm) were cut at a freezing microtome.
368 Sections were mounted with glycerol 50 % v/v in PBS 0.1 M, examined and photographed at 40X
369 magnification with GFP and DAPI filters. For the pharmacological experiments, injection sites were
370 determined by the presence of green beads. Animals in which both infusions were correct were included
371 in the analysis. Animals from the cohorts used in Figure 2B-C in which both infusions were outside the
372 LHb were included in the miss group presented in Figure S3. For the optogenetics experiments, animals
373 with transfection and/or optic fiber misses were not considered in the analysis.

374 Freezing analysis

375 Freezing was manually scored offline. Experimenter was blinded for animal´s treatment. An
376 Arduino custom system was used for the manual scoring. Briefly, a button was used to synchronize the
377 start of the video and another button was used to register the freezing state while pressed. The Arduino
378 system communicated time and freezing-button state at 10 Hz to the computer, and then saved as CSV

379 file for later analysis. For freezing over time graphs, data was binned in 5 s intervals, and inter-subjects´
380 mean and SEM was calculated.

381 Statistical Analysis

382 Each animal was taken as an independent measure. During test session of cued or context +
383 tone experiments, average freezing before and during tone presentation were treated as repeated
384 measures. Thus, in most experiments analysis was done using a two-way repeated measures design in
385 which the analyzed factors were "test stage" (pre-tone or tone) and "treatment" (muscimol,
386 optogenetic excitation or inhibition, or the corresponding control group). In experiments in which
387 subjects were tested more than one day, "testing day" was considered an additional factor. Freezing
388 during training session was analyzed separately. There the factor stage comprised freezing during the
389 tone or in the inter-tone period. Statistical analysis of FC experiments was done by generalized linear
390 mixed modelling (GzLMM). Typical distribution of individuals freezing values accumulated at lower and
391 higher values with few individuals freezing around the group mean, indicating a non-normal distribution.
392 We therefore compared models with beta and gaussian distribution (rescaling freezing to y"=[y'(n-
393 1)+0.5]/n as suggested in *(35)*. For the model fitting in R (R Foundation for Statistical Computing, Vienna,
394 Austria) we used the 'glmmTMB' package *(36)*. Beta distribution provided better adjustment and less
395 tendencies between group residues than gaussian distribution (data not shown). Logit link function was
396 employed. For the GzLMM we considered every interaction between the corresponding fixed factors
397 (test stage, treatment, testing day) and subject was treated as a random factor with random intercepts.
398 ANOVA and contrasts were done applying 'car' and 'emmeans' packages *(37, 38)* where GzLMM was
399 used. GzLMM ANOVAs used Wald χ^2 statistics for comparisons since neither likelihood ratio tests nor *F*
400 tests are supported. Assumptions of homoscedasticity and normal residues were met in the open field

experiment; therefore, it was analyzed via conventional two-way repeated measures ANOVA. Each experiment was repeated at least twice in separate cohorts of animals. Each cohort included all the treatments. Sample size calculations were done using power analysis. From pilot context test experiments, we considered a standard deviation of aprox. 20 % aiming to detect a minimal difference among groups of 50 %, with an 80 % power, and a type I error of 5 %. Based on these parameters and first assuming gaussian distribution, initially we aimed at an n = 14. Later we readjusted to n = 6 on cued memory tests, where the observed difference between vehicle and muscimol infused groups (aprox. 60 %), standard deviation (aprox. 25 %) and beta distribution at tone tests allowed to maintain about 80 % power and 5 % type I error. For experiment in Figure 4C (reconsolidation of contextual memory) statistical evaluation of the effect of the recall session was done following a within subject design since recall session on 7th day and test session on 8th day were replicates. In bar graphs, freezing during training is included as a parameter of the freezing of a naïve animal but, as mentioned above, freezing during training was analyzed separately.

415

416 Supplementary Material:

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

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

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

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

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

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

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

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516

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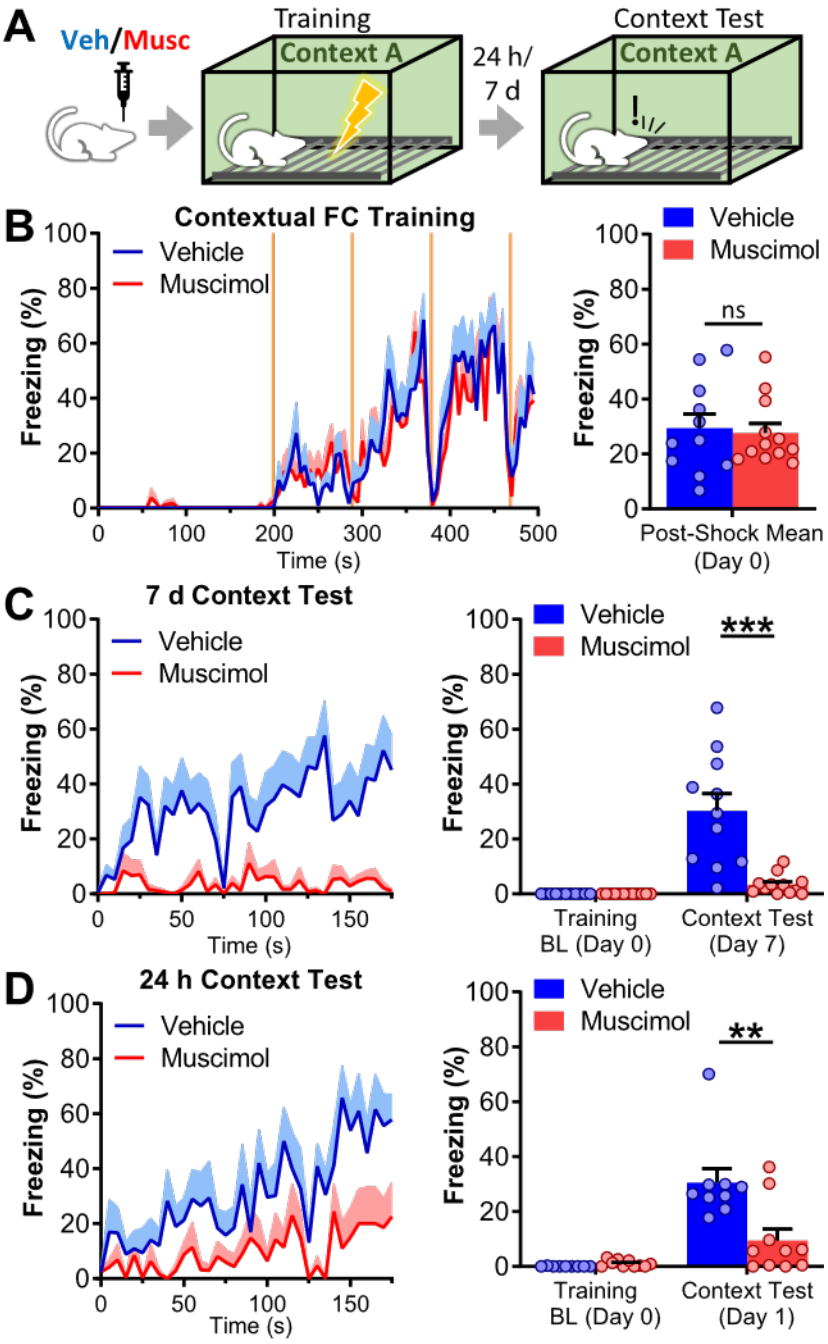
528 conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data
529 are available from authors upon request.

530 Figures

Fig. 1


A
Veh/Musc
Training
Context A
24 h/
7 d
Context Test
Context A
B
Contextual FC Training
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
ns
Post-Shock Mean
(Day 0)
C
7 d Context Test
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
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Training
BL (Day 0)
Context Test
(Day 7)
D
24 h Context Test
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
**
Training
BL (Day 0)
Context Test
(Day 1)

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

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

540 **B)** Freezing during cued FC training for the group tested 7 days after training. Left panel: freezing over
541 time, shocks were presented at times indicated by the orange shaded areas. Right panel: average
542 freezing at inter-shock periods. Freezing in both groups was equivalent (GzLMM t test: $t_{(20)} = 0.046$, $p =$
543 0.9635; $n_{vehicle} = 11$, $n_{muscimol} = 12$).

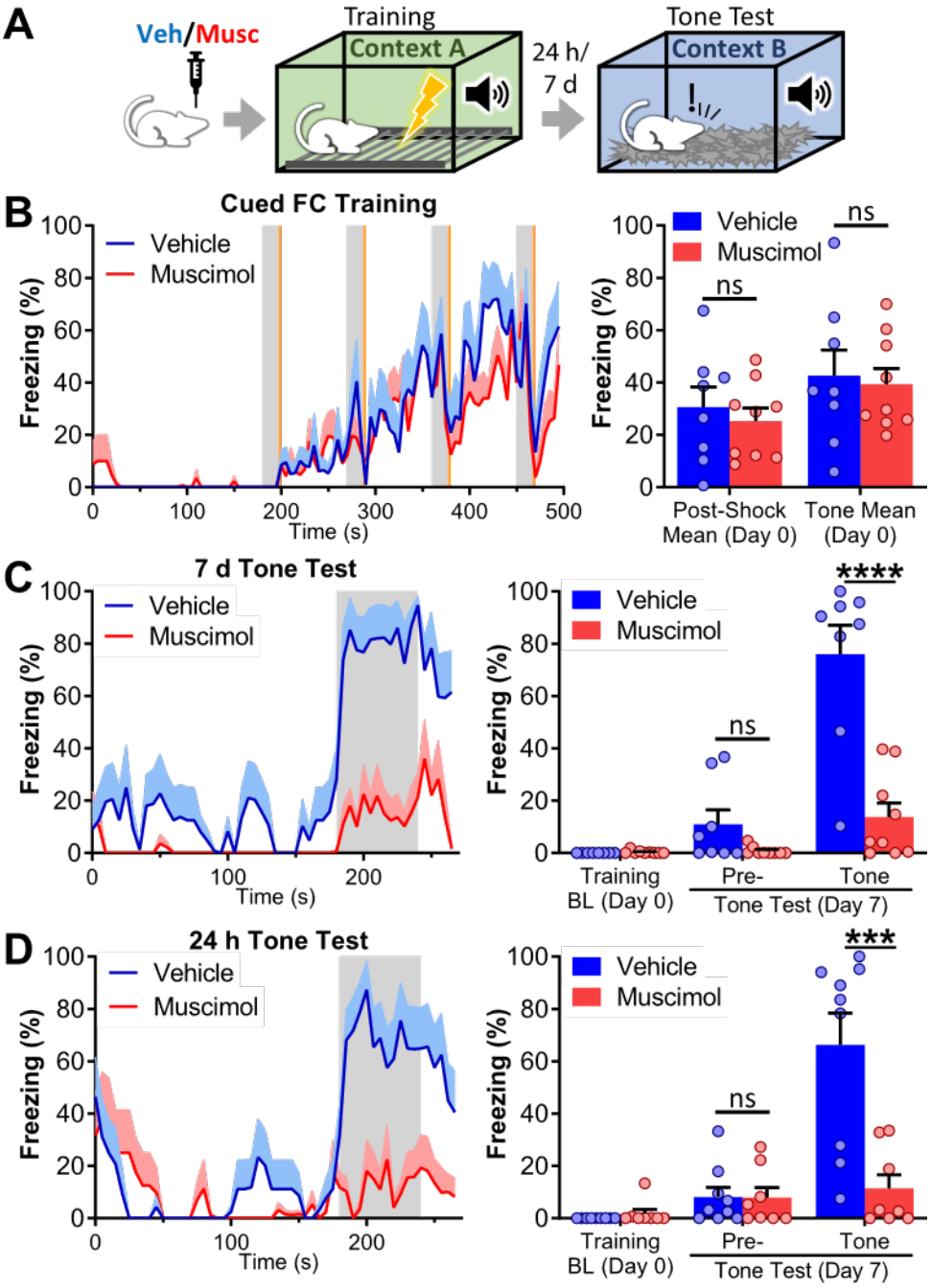
544 **C, D)** Seven days and 24 hours context tests. Left panels: freezing over time for groups tested at 7 days
545 (C) and 24 hours (D). Right panels: average freezing. In both experiments freezing in the muscimol group
546 was lower than in the control group (GzLMM t test for 7 days context test: $t_{(20)} = 4.294$, $p = 0.0004$, $n_{vehicle}$
547 $= 11$, $n_{muscimol} = 12$; GzLMM t test for 24 hours context test: $t_{(16)} = 3.964$, $p = 0.0011$; $n_{vehicle} = 9$, $n_{muscimol} =$
548 10.

549 In freezing over time plots, lines represent intersubjects' mean, and shaded area represents +SEM. In
550 bar plots, each dot represents a subject and bars represent mean and errors bars +SEM. ns $p > 0.05$, ** p
551 < 0.01, *** $p < 0.001$.

552

553

Fig. 2



554

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556

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

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

563 **B)** Freezing during cued FC training for the group tested 7 days after training. Left panel: freezing over
564 time. Tones were presented during the times indicated by the gray shaded areas. Shocks were
565 presented at times indicated by the orange shaded areas. Right panel: mean freezing during tone and
566 post-shock period. Freezing in both groups was equivalent. GzLMM Two-way repeated measures ANOVA
567 of treatment, training stage, and interaction: χ^2 treatment $_{(1)}$ = 0.185, p = 0.6668; χ^2 stage $_{(1)}$ = 6.308, p =
568 0.0120; χ^2 interaction $_{(1)}$ = 0.028, p = 0.8661. Sidak's multiple comparisons test: Post-shock freezing:
569 vehicle vs. muscimol: $t_{(28)}$ = 0.430, p = 0.8912; Tone freezing: vehicle vs. muscimol: $t_{(28)}$ = 0.297, p =
570 0.9463; $n_{vehicle}$ = 8, $n_{muscimol}$ = 9.

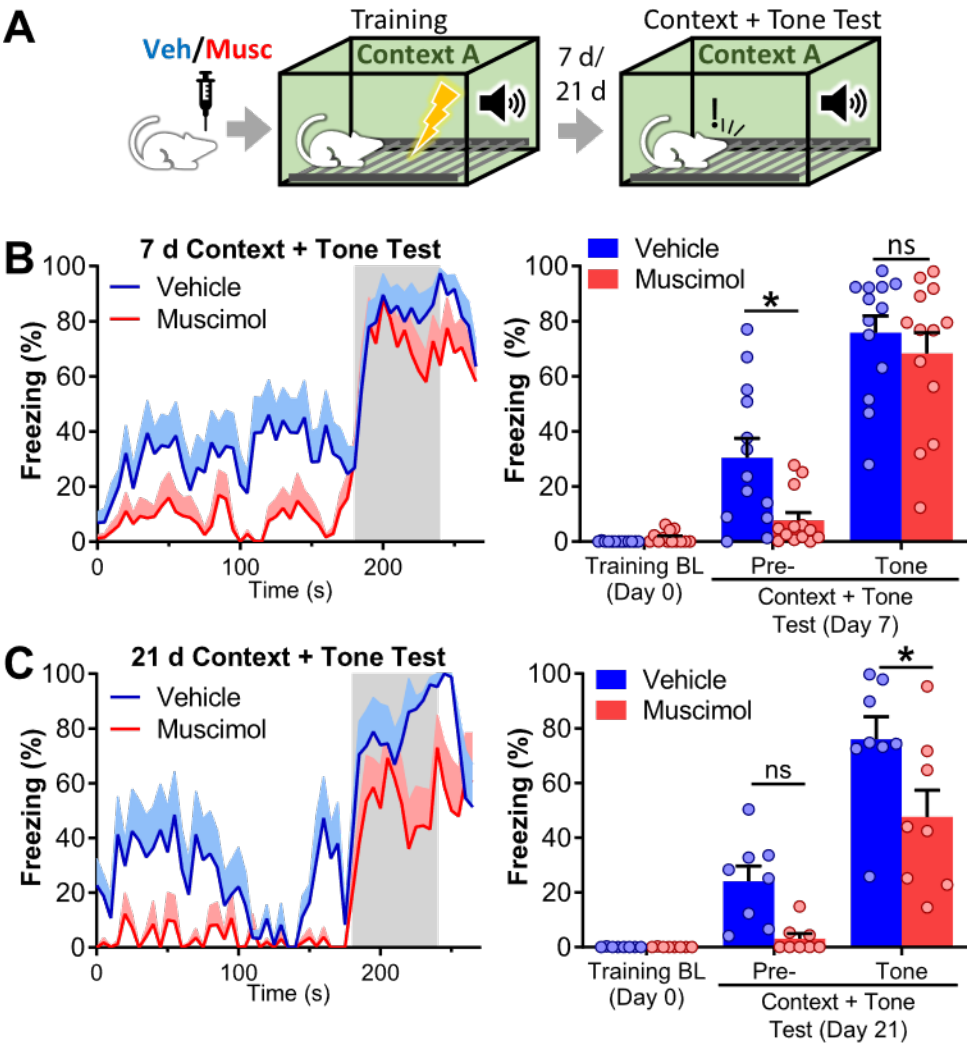
571 **C, D)** Seven days and 24 hours cued FC tests. Left panels: freezing over time for groups tested 7 days (C)
572 and 24 hours (D) after training. Gray areas indicate tone presentation. Right panels: average freezing for
573 pre- and tone period. Freezing during pre-tone period was not different between vehicle and muscimol
574 groups in either experiment. In contrast, during tone presentation, a highly significant reduction in
575 freezing was observed in the muscimol group in both experiments. GzLMM Two-way repeated measures
576 ANOVA of treatment, test stage, and interaction for 7 days experiment: χ^2 treatment $_{(1)}$ = 2.157, p =
577 0.1420; χ^2 stage $_{(1)}$ = 19.100, p < 0.0001; χ^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: χ^2 treatment $_{(1)} = 0.024$, $p = 0.8781$; χ^2 stage $_{(1)} = 0.108$, $p = 0.7428$; χ^2 interaction $_{(1)} = 12.447$, $p = 0.0004$. 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 $p > 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

588

Fig. 3



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592 Figure 3. FC memory formed under inactivation of the LHb could be retrieved in context + tone

593 conditions and showed decreased temporal stability.

594 **A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes

595 before training. Animals were trained in cued FC. Test was performed 7 or 21 days later in the same

596 context used for training (Context A). The tone was presented for 60 s after a pre-tone period of 180 s.

597 **B)** Freezing during context + tone test 7 days after training. Left panel: freezing over time. Gray area

598 indicates tone presentation. Right panel: average freezing for pre- and tone period. Freezing to the

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

600 reached values equivalent to the vehicle group. GzLMM Two-way repeated measures ANOVA of

601 treatment, test stage, and interaction: χ^2 treatment $_{(1)}$ = 6.830, p = 0.0090; χ^2 stage $_{(1)}$ = 44.992, p <

602 0.0001; χ^2 interaction $_{(1)}$ = 3.161, p = 0.0754. Sidak's multiple comparisons test: Pre-tone freezing: vehicle

603 vs. muscimol: $t_{(46)}$ = 2.613, p = 0.0474; Tone freezing: vehicle vs. muscimol: $t_{(46)}$ = 0.899, p = 0.8460;

604 Vehicle: pre-tone vs. tone freezing: $t_{(46)}$ = 6.014, p < 0.0001; muscimol: pre-tone vs. tone freezing: $t_{(46)}$ =

605 6.708, p < 0.0001; $n_{vehicle}$ = 13, $n_{muscimol}$ = 13.

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

607 indicates tone presentation. Right panel: average freezing for pre- and tone period. 21 days after

608 training freezing in context + tone condition in the muscimol group was lower than in the vehicle group

609 indicating a diminished temporal stability of context + tone memory. GzLMM Two-way repeated

610 measures ANOVA of treatment, test stage, and interaction: χ^2 treatment $_{(1)}$ = 7.123, p = 0.0076; χ^2 stage

611 $_{(1)}$ = 41.364, p < 0.0001; χ^2 interaction $_{(1)}$ = 0.176, p = 0.6751. Sidak's multiple comparisons test: Pre-tone

612 freezing: vehicle vs. muscimol: $t_{(26)}$ = 2.669, p = 0.0508; Tone freezing: vehicle vs. muscimol: $t_{(26)}$ = 2.683,

613 $p = 0.0491$; Vehicle: pre-tone vs. tone freezing: $t_{(26)} = 7.354$, $p < 0.0001$; muscimol: pre-tone vs. tone

614 freezing: $t_{(26)} = 6.431$, $p < 0.0001$; $n_{vehicle} = 8$, $n_{muscimol} = 8$.

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

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

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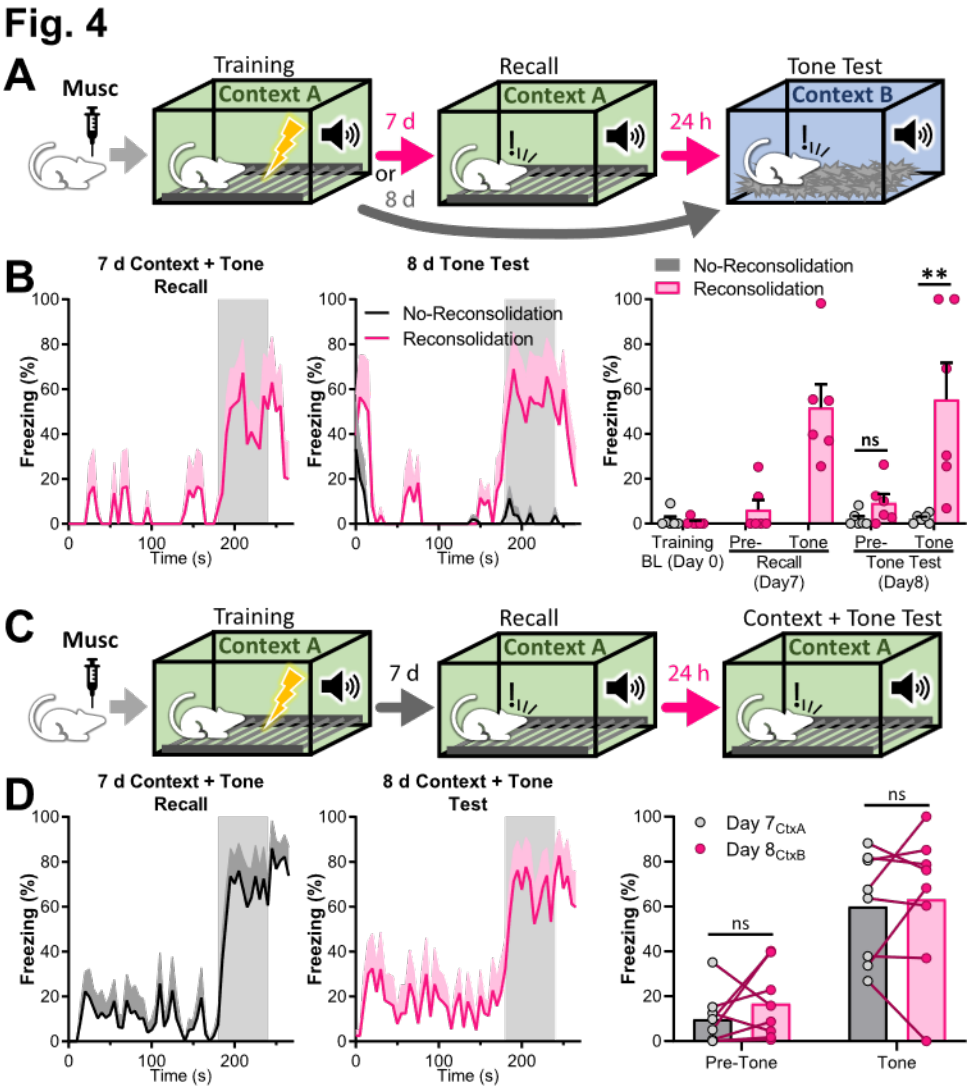
Fig. 4
A
Musc
Training
Context A
7 d
or
8 d
Recall
Context A
24 h
Tone Test
Context B
B
7 d Context + Tone Recall
8 d Tone Test
No-Reconsolidation
Reconsolidation
Freezing (%)
Time (s)
Training BL (Day 0)
Pre-
Tone
Recall (Day7)
Tone Test (Day8)
ns
**
C
Musc
Training
Context A
7 d
Recall
Context A
24 h
Context + Tone Test
Context A
D
7 d Context + Tone Recall
8 d Context + Tone Test
Day 7CtxA
Day 8CtxB
Pre-Tone
Tone

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

623 memories.

624 **A)** Diagram of reconsolidation experiment of cued FC memory: bilateral intra LHb muscimol infusions

625 were performed 30 minutes before cued FC training as described previously. Seven days later, animals

626 of the reconsolidation group were subjected to a recall session in which the cue was presented in the

627 training context. The following day, reconsolidation of cued memory was tested in context "B". The no-

628 reconsolidation group was not exposed to the recall session and was tested for cued memory in context

629 "B" 8 days after training.

630 **B)** Retrieval of FC memory in context + tone conditions restores cued FC memory. Left and center:

631 freezing over time for the recall and the cued memory test sessions, respectively. Animals of the no-

632 reconsolidation group showed very low levels of freezing, confirming the impairment in cued memory

633 induced by inactivation of the LHb during training. In contrast animals of the reconsolidation group

634 presented a marked freezing to the tone during the test session, reaching levels of freezing equivalent to

635 context + tone condition during the recall session. Right: quantification of freezing. Freezing to the tone

636 in the test session was significantly higher in the reconsolidation group. GzLMM Two-way repeated

637 measures ANOVA of reconsolidation, test stage, and interaction during tone test session: χ^2

638 reconsolidation $_{(1)}$ = 0.207, p = 0.6489; χ^2 stage $_{(1)}$ = 0.009, p = 0.9232; χ^2 interaction $_{(1)}$ = 9.170, p =

639 0.0025. Sidak's multiple comparisons test: Pre-tone freezing: reconsolidation vs. no-reconsolidation: $t_{(18)}$

640 = 0.455, p = 0.8805; Tone freezing: reconsolidation vs. no-reconsolidation: $t_{(18)}$ = 4.219, p = 0.0010;

641 $n_{reconsolidation}$ = 6, $n_{NO\text{-}reconsolidation}$ = 6.

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

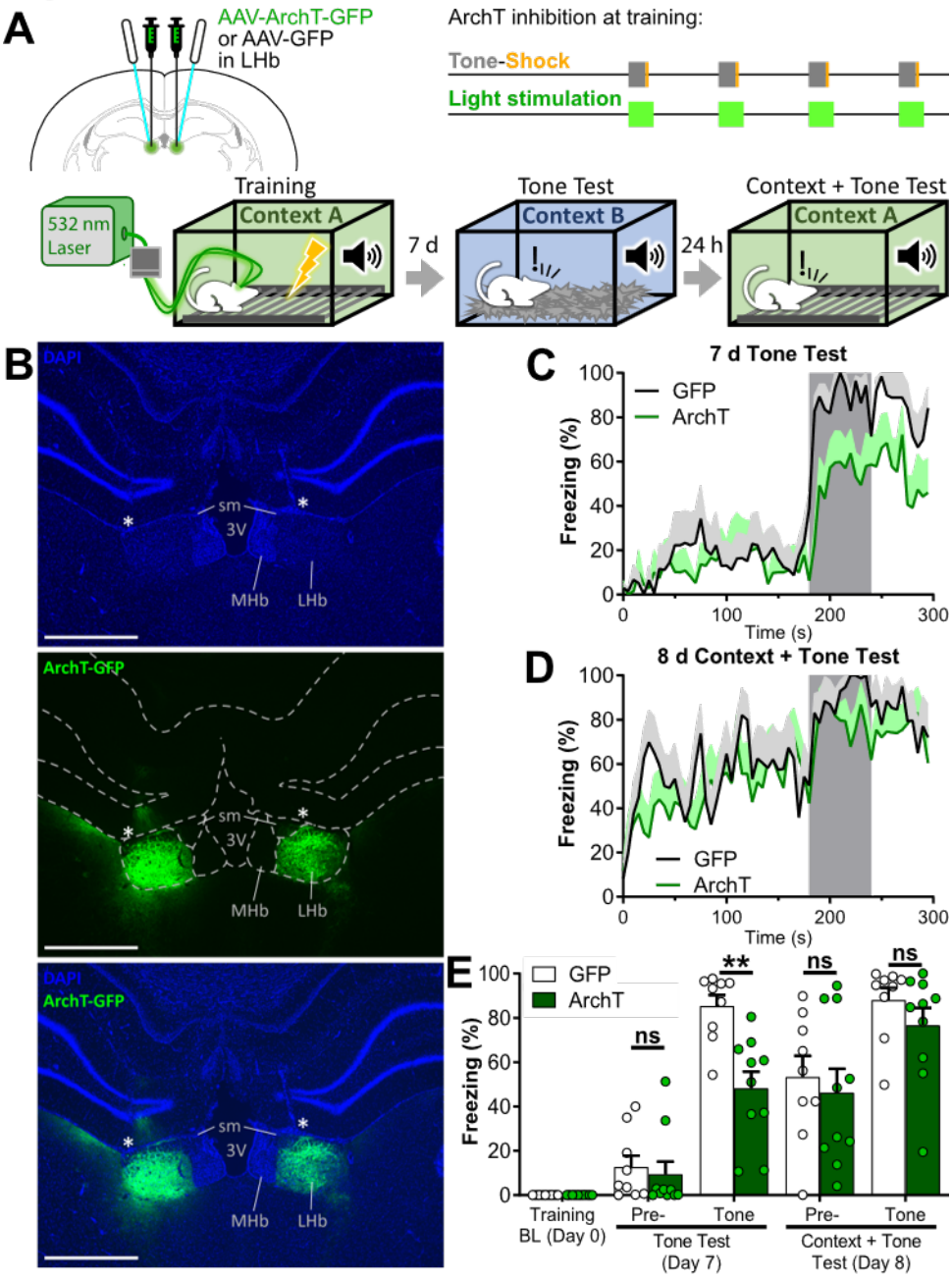
646 **D)** Retrieval of FC memory in context + tone conditions does not restore contextual FC memory. Left and
647 center: freezing over time for the recall and the test sessions, respectively. Right: paired comparison of
648 freezing for each subject on the recall and the test session. No differences in freezing were observed
649 between sessions indicating that retrieval of FC memory during the recall session did not restore
650 contextual FC memory, as it did with cued memory. GzLMM Two-way repeated measures ANOVA of
651 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
652 interaction $_{(1)}$ = 0.240, p = 0.6240. Sidak's multiple comparisons test: Pre-tone freezing: recall vs. test
653 day: $t_{(26)}$ = 0.717, p = 0.7292; Tone freezing: recall vs. test day: $t_{(26)}$ = 0.088, p = 0.9952; n = 6.

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

657

658

Fig. 5



659

660

661

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

663 memories.

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

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

666 training, optogenetic light stimulation was delivered starting with the tone and stopping 5 seconds after

667 the shock (tone and shock presentations were as previously described for cued FC). Bottom: diagram of

668 training and tests. Cued memory was tested 7 days after training in Context B. The same animals were

669 tested the following day in context + tone condition to evaluate contextual FC memory and freezing in

670 context + tone condition.

671 **B)** Microphotographs of the AAV-ArchT-GFP infection (top, middle, bottom: DAPI, GFP, and merge

672 respectively). Dashed white lines in the middle panel delimitates brain structures. * indicates the optic

673 fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third ventricle. Scale bars: 1 mm.

674 **C, D)** Freezing over time during tone test at day 7 (C) and during context + tone test at day 8 (D). Gray

675 area indicates tone presentation. Line represents intersubjects' mean, and shaded area represents

676 +SEM.

677 **E)** Average freezing on tone test, and context + tone test sessions. During tone test ArchT group

678 displayed lower levels of freezing to the tone than GFP group. The following day, during context + tone

679 test, freezing levels of the ArchT group to both to the context and to the tone were equivalent to those

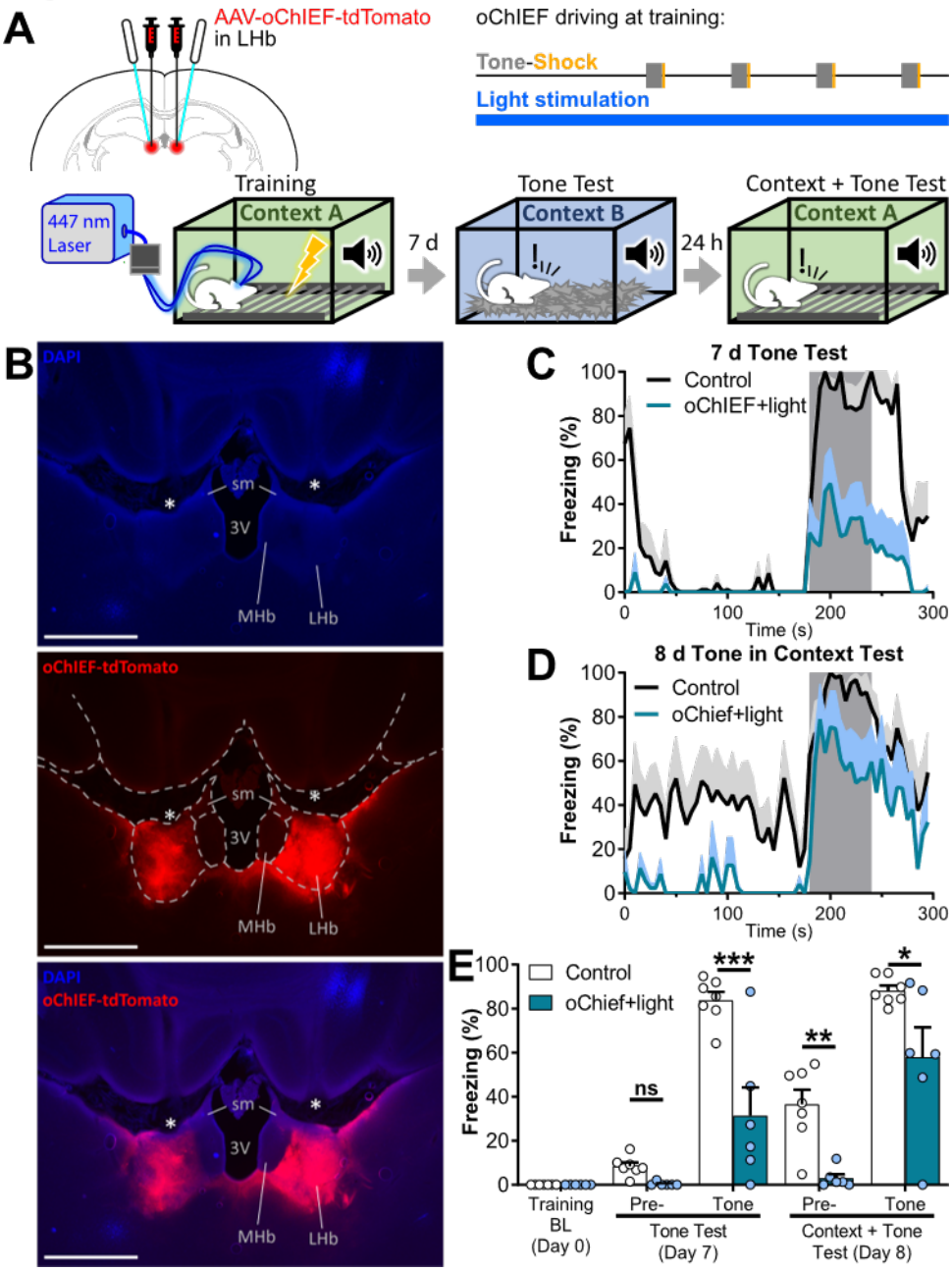
680 of the GFP group.

681 GzLMM Three-way repeated measures ANOVA of test day, test stage, optogenetic inhibition and
682 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 =
683 0.4555; χ^2 interaction(Day×Stage) $_{(1)}$ = 1.659, p = 0.1978; χ^2 interaction(Day×Inhibition) $_{(1)}$ = 0.294, p =
684 0.5879; χ^2 interaction(Stage×Inhibition) $_{(1)}$ = 5.006, p = 0.0253; χ^2 interaction(Day×Stage×Inhibition) $_{(1)}$ =
685 1.088, p = 0.2970. Sidak's multiple comparisons test: Day7: Pre-tone freezing: GFP vs. ArchT: $t_{(66)}$ = 0.746,
686 p = 0.9747; Day 7: Tone freezing: GFP vs. ArchT: $t_{(66)}$ = 3.410, p = 0.0067; Day 8: Pre-tone freezing: GFP
687 vs. ArchT: $t_{(66)}$ = 0.178, p = 1.0000; Day 8: Tone freezing: GFP vs. ArchT: $t_{(66)}$ = 1.145, p = 0.8308; ArchT:
688 Tone freezing: Day 7 vs. Day 8: $t_{(66)}$ = 3.366, p = 0.0076; Day 8: ArchT: Pre-tone vs. Tone freezing: $t_{(66)}$ =
689 3.475, p = 0.0054; n_{GFP} = 9, n_{ArchT} = 10. In bar plots each dot represents a subject and bars represent
690 mean + SEM. ns p > 0.05, ** p < 0.01.

691

692

Fig. 6



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694

695

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

697 memories.

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

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

700 training session the LHb was stimulated with 5 ms light pulses at 20 Hz to disrupt endogenous neuronal

701 activity. Bottom: diagram of training and tests. Cued memory was tested 7 days after training in Context

702 B. The same animals were tested the following day in context + tone condition to evaluate contextual FC

703 memory and freezing in context + tone condition.

704 **B)** Microphotographs of the AAV-oChIEF-tdTomato infection (top, middle, bottom: DAPI, tdTomato

705 fluorescence, and merge respectively). Dashed white lines in the middle panel delimitates brain

706 structures. * indicates the optic fiber tract. MHb: medial habenula, sm: stria medullaris, 3V: third

707 ventricle. Scale bars: 1 mm.

708 **C, D)** Freezing over time during cued test at day 7 (C) and during context + tone test at day 8 (D). Gray

709 area indicates tone presentation. Line represents intersubjects' mean, and shaded area represents +

710 SEM.

711 **E)** Average freezing of tone test and context + tone test sessions. Disruption of endogenous LHb activity

712 during training by optogenetic stimulation induced deficits in cued and contextual memories:

713 GzLMM Three-way repeated measures ANOVA of test day, test stage, optogenetic stimulation and

714 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

715 = 0.0234; χ^2 interaction(Day×Stage) $_{(1)}$ = 5.805, p = 0.0160; χ^2 interaction(Day×stimulation) $_{(1)}$ = 2.048, p =

716 0.15237; χ^2 interaction(Stage×stimulation) $_{(1)}$ = 2.489, p = 0.1146; χ^2 interaction(Day×Stage×stimulation)

717 $_{(1)}$ = 4.905, p = 0.0268. Sidak's multiple comparisons test: Day 7: Pre-tone freezing: Control vs. oChIEF:

718 $t_{(42)}$ = 2.266, p = 0.1601; Day 7: Tone freezing: Control vs. oChIEF: $t_{(42)}$ = 4.454, p = 0.0004; Day 8: Pre-

719 tone freezing: Control vs. oChIEF: $t_{(42)}$ = 3.874, p = 0.0022; Day 8: Tone freezing: Control vs. oChIEF: $t_{(42)}$ =

720 2.995, p = 0.0272; oChIEF: Tone freezing: Day 7 vs. Day 8: $t_{(42)}$ = 3.810, p = 0.0027; Day 8: oChIEF: Pre-

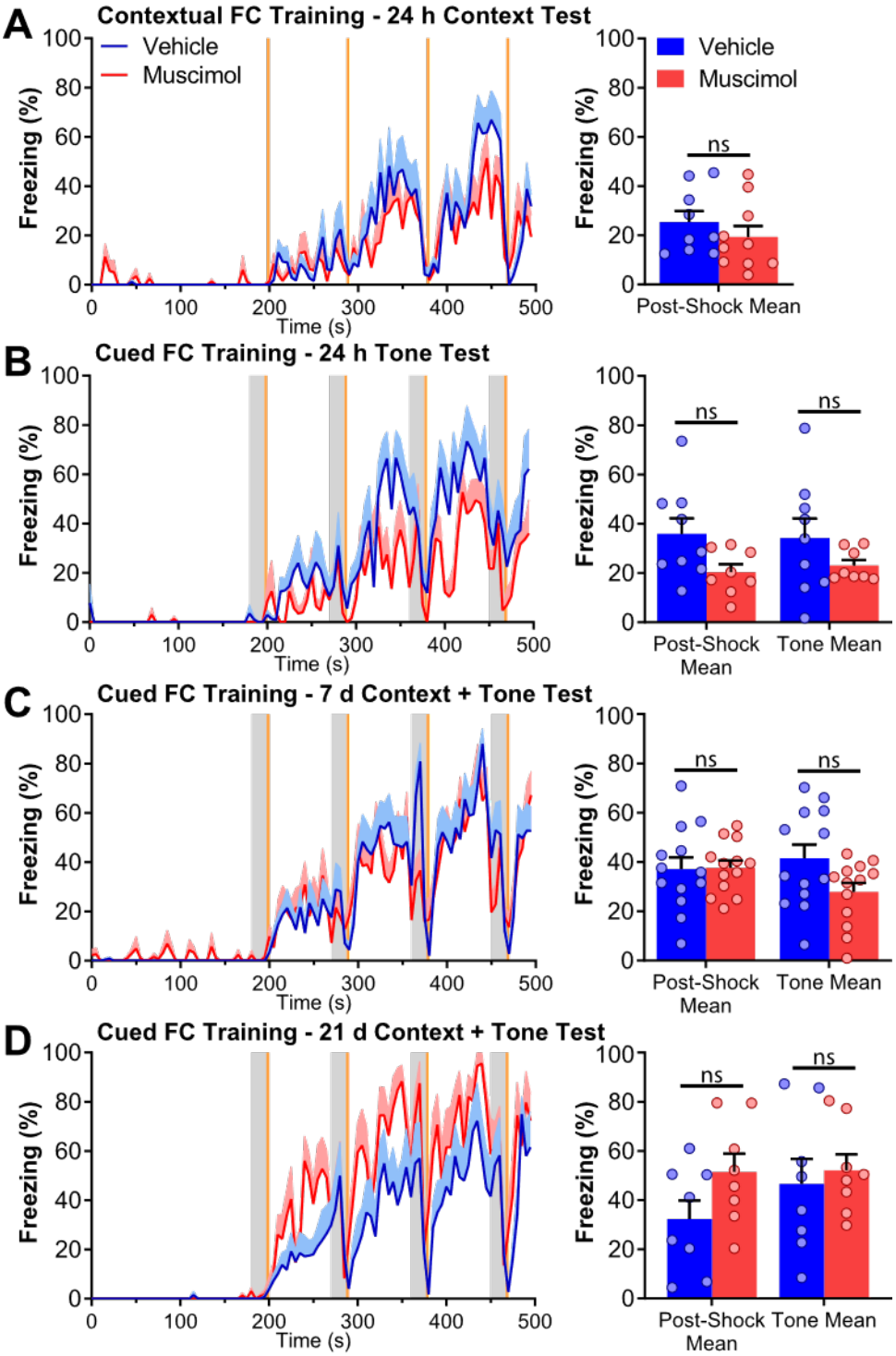
721 tone vs. Tone freezing: $t_{(42)}$ = 7.283, p < 0.0001; $n_{Control}$ = 7, n_{oChIEF} = 6. In bar plots, each dot represents a

722 subject and bars represent mean + SEM. ns p > 0.05, * p < 0.05, ** p < 0.01 *** p < 0.001.

723

Supplementary Material

Suppl. Fig. 1



726

727

728

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

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

736 Statistics:

737 - Contextual FC training – 24 hours test (A): GzLMM t test: $t_{(16)} = 1.217$, $p = 0.2414$. $n_{vehicle} = 9$, $n_{muscimol}$
738 $= 10$.
739 - Cued FC training – 24 hours test (B): GzLMM two-way repeated measures ANOVA of treatment,
740 training stage, and interaction: χ^2 treatment $_{(1)} = 3.796$, $p = 0.0514$; χ^2 stage $_{(1)} = 0.550$, $p = 0.4584$; χ^2
741 interaction $_{(1)} = 0.852$, $p = 0.3560$. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs.
742 muscimol: $t_{(28)} = 1.948$, $p = 0.1192$; Tone freezing: vehicle vs. muscimol: $t_{(28)} = 1.245$, $p = 0.3972$.
743 $n_{vehicle} = 9$, $n_{muscimol} = 8$. Stage factor refers to freezing between shock and tone, or during tone (post-
744 shock or tone respectively).
745 - Cued FC training - 7 days context + tone test (C): GzLMM two-way repeated measures ANOVA of
746 treatment, training stage, and interaction: χ^2 treatment $_{(1)} = 0.037$, $p = 0.8471$; χ^2 stage $_{(1)} = 8.741$, p
747 $= 0.0031$; χ^2 interaction $_{(1)} = 8.674$, $p = 0.0032$. Sidak's multiple comparisons test: Post-shock
748 freezing: vehicle vs. muscimol: $t_{(46)} = 0.193$, $p = 0.9769$; Tone freezing: vehicle vs. muscimol: $t_{(46)} =$

749 2.216, $p = 0.0624$. $n_{vehicle} = 13$, $n_{muscimol} = 13$. Stage factor with above described post-shock or tone

750 levels.

751 - Cued FC training - 21 days context + tone test (D): GzLMMtwo-way repeated measures ANOVA of

752 treatment, training stage, and interaction: χ^2 treatment $_{(1)} = 3.723$, $p = 0.0537$; χ^2 stage $_{(1)} = 0.023$, $p =$

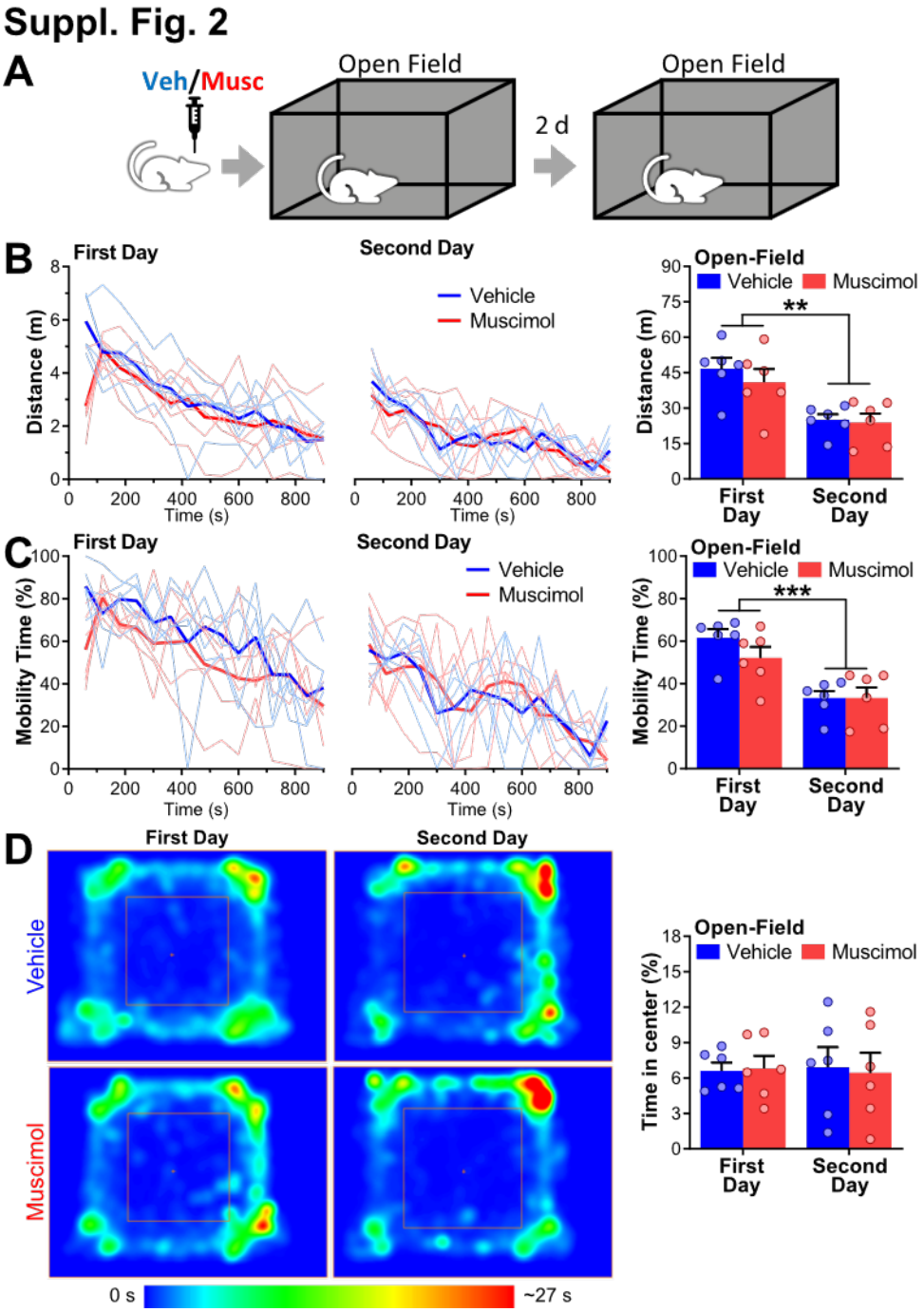
753 0.8802; χ^2 interaction $_{(1)} = 4.535$, $p = 0.0332$. Sidak's multiple comparisons test: Post-shock freezing:

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

755 0.8454. $n_{vehicle} = 8$, $n_{muscimol} = 8$. Stage factor with above described post-shock or tone levels.

756

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

760

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

762 to an Open Field.

763 **A)** Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed 30 minutes

764 before exposing the animals to an OF for 15 minutes. 48 hours later animals were exposed to the same

765 OF for a second time.

766 **B)** Distance traveled over time (60 s bins) during the first day (left) and the second day (right) in the OF.

767 Thin lines represent individuals, thick lines represent the average. Right: total distance traveled.

768 Distance traveled by both groups diminished the second day. No differences between groups were

769 observed (two-way repeated measures ANOVA of treatment, day, and interaction: $F_{treatment\ (1,\ 10)}$ =

770 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

771 multiple comparisons test: Day 1: vehicle vs. muscimol: t = 0.9534, p = 0.5798; Day 2: vehicle vs.

772 muscimol: t = 0.1807, p = 0.9800).

773 **C)** Percentage of mobility time in the Open Field in each day. Left: percentage of mobility over time (60 s

774 bins) during the first day (left) and the second day (right). Thin lines represent individuals, thick lines

775 represent the average. Right: percentage of mobility time during whole OF assay. Mobility of both

776 groups diminished the second day. No differences between groups were observed (two-way repeated

777 measures ANOVA of treatment, day, and interaction: $F_{treatment\ (1,\ 10)}$ = 1.670, p = 0.2253; $F_{day\ (1,\ 10)}$ =

778 21.48, p = 0.0009; $F_{interaction\ (1,\ 10)}$ = 0.8727, p = 0.3722. Sidak's multiple comparisons test: First day:

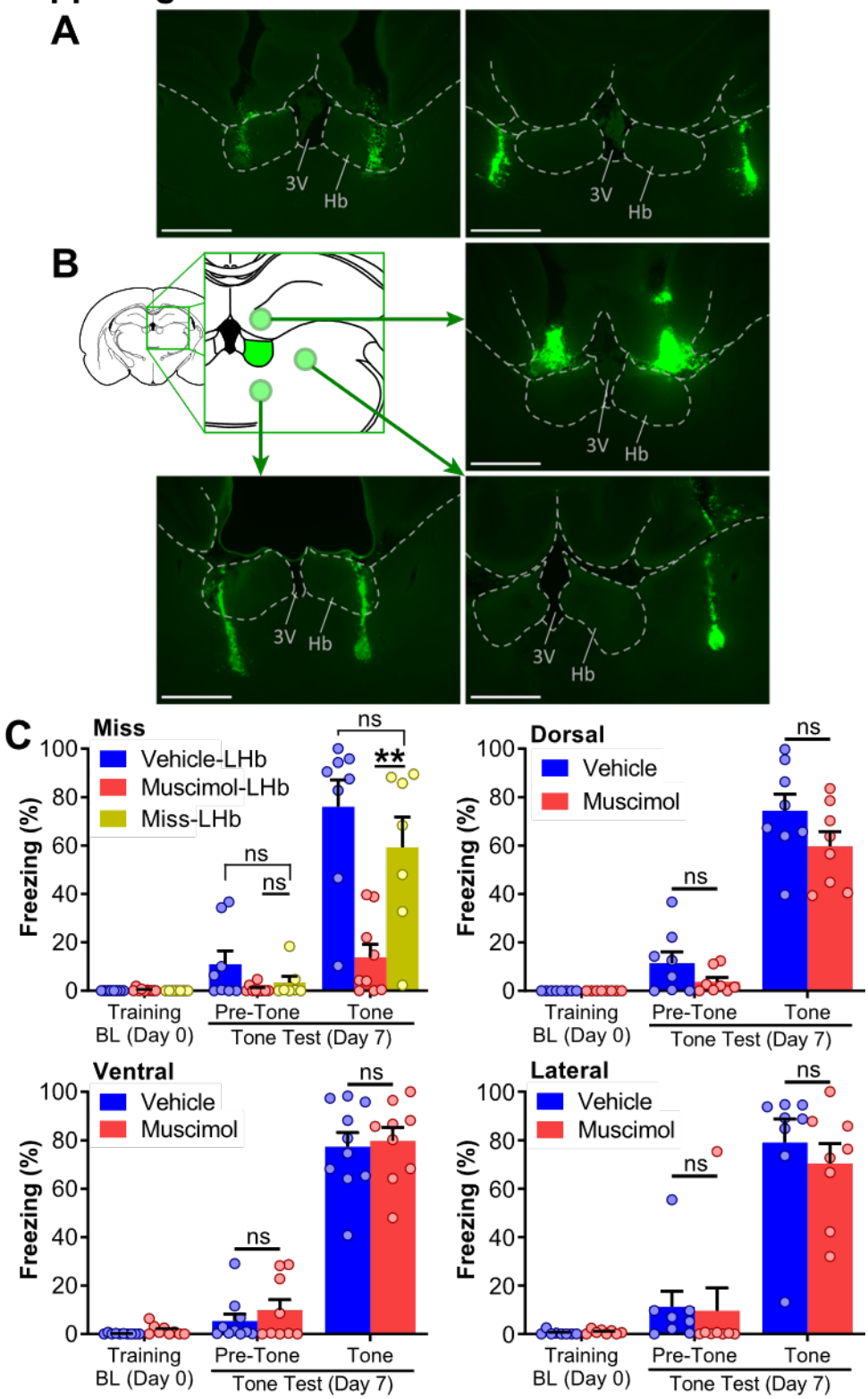
779 vehicle vs. muscimol: t = 1.512, p = 0.2708; Second day: vehicle vs. muscimol: t = 0.005307, p > 0.9999).

780 **D)** Heat map representation of spatial occupancy during OF. Left: average heat maps vehicle (top) and
781 muscimol (bottom) groups on the first day (left), and the second day (right). The center zone is indicated
782 by the orange square. Right: percentage of time in the center zone in both groups at each day. Time in
783 the center did not change between days and was equivalent among groups (two-way repeated
784 measures ANOVA of treatment, day, and interaction: $F_{\text{treatment}\,(1, 10)} = 0.01082$, $p = 0.9192$; $F_{\text{day}\,(1, 10)}$
785 $= 0.0003566$, $p = 0.9853$; $F_{\text{interaction}\,(1, 10)} = 0.05192$, $p = 0.8244$. Sidak's multiple comparisons test: Day
786 1: vehicle vs. muscimol: $t = 0.1082$, $p = 0.9928$; Day 2: vehicle vs. muscimol: $t = 0.2416$, $p = 0.9645$).

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

788

Suppl. Fig. 3



789

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

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

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

796 C) Freezing during cued memory testing in miss-LHb group, dorsal, ventral, and lateral controls. Miss-
797 LHb group was generated by selecting animals infused with muscimol in which both cannulae did not
798 target the LHb from the same cohorts included in Figure 2C. Dorsal, ventral, and lateral groups were
799 done as separate experiments. As in figure 2, infusions were done 30 minutes before training and test
800 was performed 7 days later. Miss-LHb group showed higher freezing to the tone than muscimol-LHb
801 group and equal freezing to the tone than the vehicle-LHb group. Muscimol infusion dorsal, ventral, or
802 lateral to the LHb did not affect tone evoked freezing. In bar plots each dot represents a subject and
803 bars represent mean + SEM. Muscimol-LHb and vehicle-LHb groups are taken from Figure 2C. ns $p >$
804 0.05, ** $p < 0.001$. Scale bars: 1 mm.

805 Statistics:

806 - GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for miss
807 graph: χ^2 treatment $_{(2)}$ = 2.5079, $p = 0.2854$; χ^2 stage $_{(1)}$ = 85.1604, $p < 0.0001$; χ^2 interaction $_{(2)}$ =
808 33.459, $p < 0.0001$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(40)}$ =
809 1.457, $p = 0.6306$; Pre-tone freezing: vehicle vs. miss: $t_{(40)}$ = 1.228, $p = 0.7863$; Pre-tone freezing:

810 muscimol vs. miss: $t_{(40)} = 0.168$, $p = 1.0000$; Tone freezing: vehicle vs. muscimol: $t_{(40)} = 5.191$, $p <$
811 0.0001; Tone freezing: vehicle vs. miss: $t_{(40)} = 1.550$, $p = 0.5636$; Tone freezing: muscimol vs. miss:
812 $t_{(40)} = 3.559$, $p = 0.0058$; $n_{vehicle} = 8$, $n_{muscimol} = 9$, $n_{miss} = 7$.

813 - GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for dorsal
814 graph: χ^2 treatment $_{(1)} = 1.7632$, $p = 0.1842$; χ^2 stage $_{(1)} = 102.565$, $p < 0.0001$; χ^2 interaction $_{(1)} =$
815 0.133, $p = 0.7153$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(26)} =$
816 1.328, $p = 0.3532$; Tone freezing: vehicle vs. muscimol: $t_{(26)} = 1.916$, $p = 0.1283$; $n_{vehicle} = 8$, $n_{muscimol} =$
817 8.

818 - GzLMM Two-way repeated measures ANOVA of treatment, test stage, and interaction for ventral
819 graph: χ^2 treatment $_{(1)} = 0.525$, $p = 0.4689$; χ^2 stage $_{(1)} = 165.9408$, $p < 0.0001$; χ^2 interaction $_{(1)} =$
820 0.259, $p = 0.6109$. Sidak's multiple comparisons test: Pre-tone freezing: vehicle vs. muscimol: $t_{(32)} =$
821 0.724, $p = 0.7235$; Tone freezing: vehicle vs. muscimol: $t_{(32)} = 0.360$, $p = 0.9224$; $n_{vehicle} = 10$, $n_{muscimol} =$
822 9.

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

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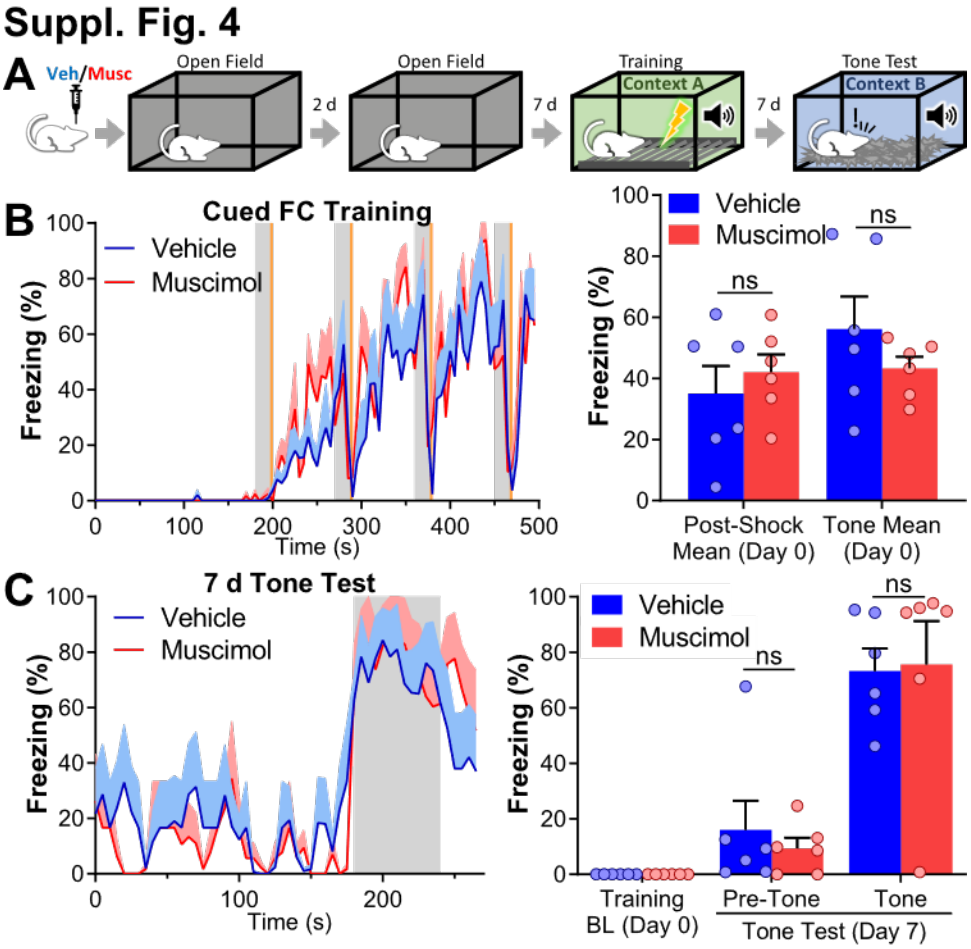

Suppl. Fig. 4
A
Veh/Musc
Open Field
2 d
Open Field
7 d
Training
Context A
7 d
Tone Test
Context B
B
Cued FC Training
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
ns
ns
Post-Shock Mean (Day 0)
Tone Mean (Day 0)
C
7 d Tone Test
Vehicle
Muscimol
Freezing (%)
Time (s)
Vehicle
Muscimol
ns
ns
Training BL (Day 0)
Pre-Tone
Tone
Tone Test (Day 7)

829

830

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

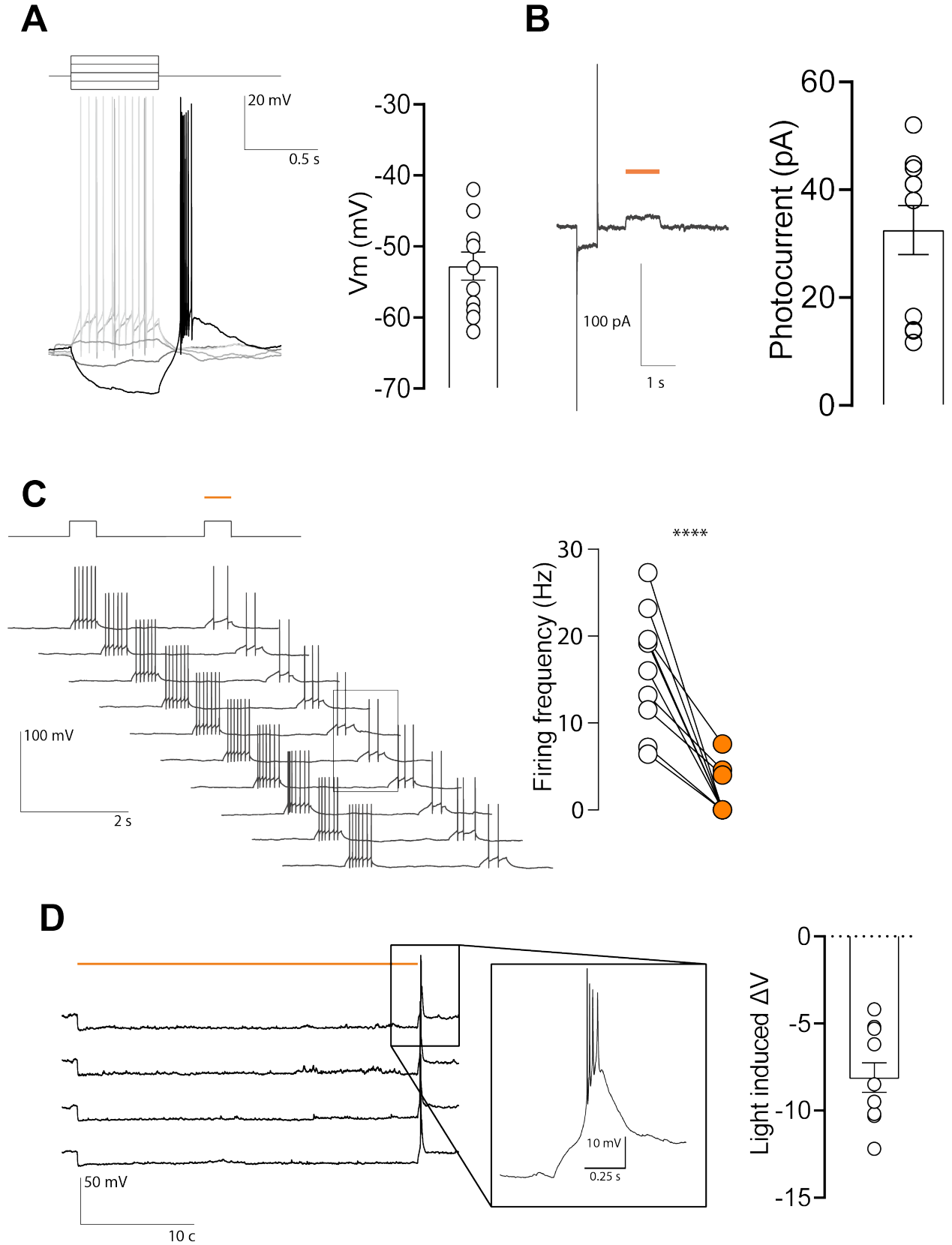
A) Experiment diagram: bilateral intra LHb infusions of vehicle/muscimol were performed in the animals that went through the OF (Supplementary Figure 2). Seven days after the second exposure to the OF animals were trained in cued FC. Memory was tested 7 days later.

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: χ^2 treatment $_{(1)}$ = 0.685, p = 0.4077; χ^2 stage $_{(1)}$ = 0.050, p = 0.8231; χ^2 interaction $_{(1)}$ = 7.700, p = 0.0055. Sidak's multiple comparisons test: Post-shock freezing: vehicle vs. muscimol: $t_{(18)}$ = 0.828, p = 0.6620; Tone freezing: vehicle vs. muscimol: $t_{(18)}$ = 1.265, p = 0.3945. $n_{vehicle}$ = 6, $n_{muscimol}$ = 6.

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

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.

Suppl. Fig. 5



853

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

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

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

861 **C)** Light induced action potential suppression in ArchT expressing LHb neurons. Light pulse (590 nm,
862 orange line) was applied every second time neurons were depolarized with positive current injection.
863 Left, representative recordings. Right, data summary. Light activation of ArchT significantly decreases
864 the number of action potentials evoked by current steps in LHb neurons (paired t test, $t_{(9)} = 6.25$, $p <$
865 0.001); n = 9 neurons from 3 rats.

866 **D)** Membrane potential response to a 30 s light pulse (orange line) in LHb neurons. Rebound spiking was
867 observed after termination of light pulse, a representative trace is expanded in the middle traces. This
868 behavior confirms the reported rebound responses of the LHb neurons following long periods of
869 optogenetic inhibition reported previously (13). Right: quantification of the stationary light induced ΔVm
870 during the last 5 s of the 30 s pulse; n = 10 neurons from 3 rats.

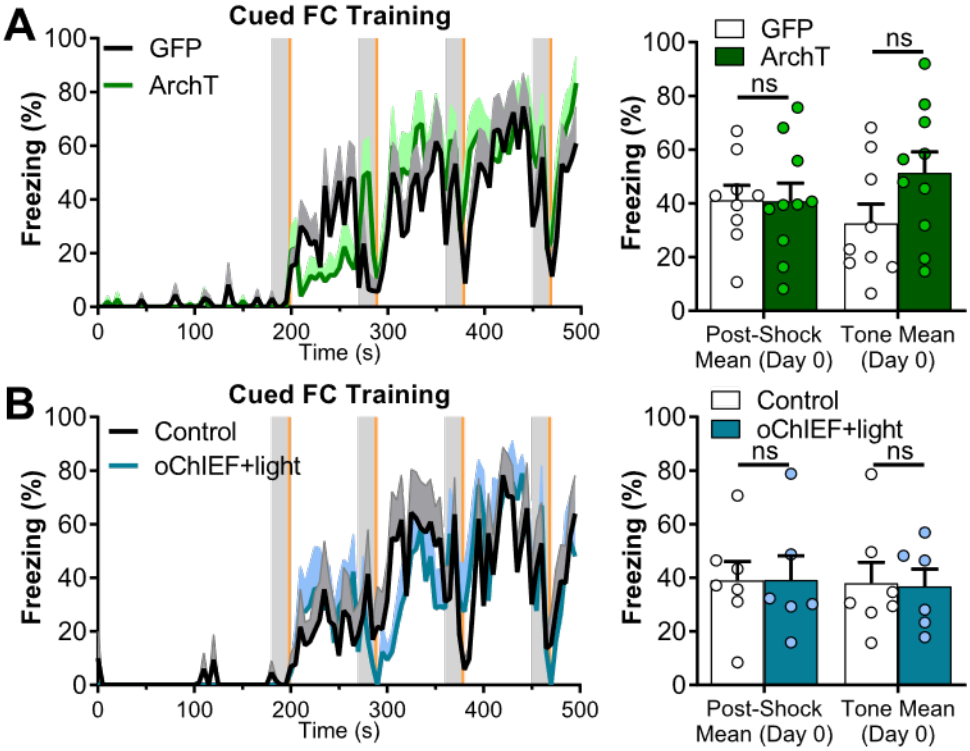
871 Methods details for this experiment: Surgery was similar to the optogenetic manipulation experiments
872 with the following differences: 3 weeks old rats were employed, the viral vector injection sites were
873 adjusted according to Bregma-Lambda distance (Bλ) of each rat (AP = -2.9 × Bλ ÷ 8.5 mm, ML = ± 0.7 × Bλ
874 ÷ 8.5 mm from Bregma and DV -4.3 mm from dura mater), no fiber optic implants, nor screws or acrylic

875 was used, and the skin was stitched at the end of surgery. Recordings were obtained from ArchT
876 expressing LHb neurons in acute brain slices prepared 3 weeks after infection. General methods for slice
877 preparation and recordings were replicated from previous publications of the group (*39*).

878

879

Suppl. Fig. 6



880

881

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

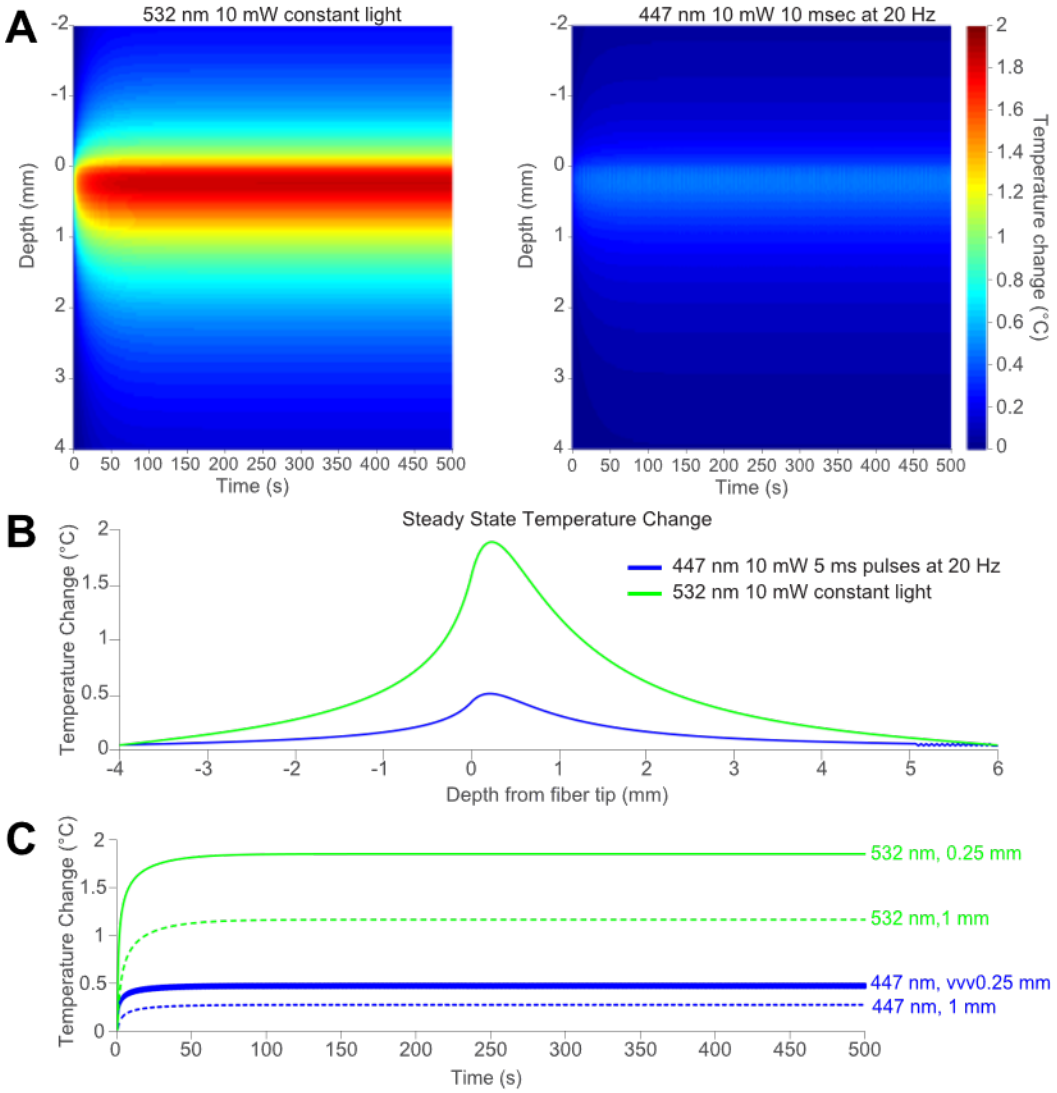
883 **A-B)** Left: temporal course of freezing during training in the ArchT (A) and oChIEF (B) stimulation
884 experiments. Right: average freezing on the same experiments. In freezing over time plots vertical
885 orange shaded areas indicate shock presentation, gray shaded areas indicate tone presentation, line
886 plots represent intersubjects' mean and shaded area over line plots represents +SEM. In either condition
887 no significant differences were observed between control and optogenetic stimulated groups. Stage
888 factor refers to freezing between shock and tone, or during tone (post-shock or tone respectively).

889 Statistics:

890 - For ArchT experiment: GzLMM two-way repeated measures ANOVA of optogenetic inhibition,
891 training stage, and interaction: χ^2 inhibition $_{(1)}$ = 0.002, p = 0.9631; χ^2 stage $_{(1)}$ = 3.173, p = 0.0749; χ^2
892 interaction $_{(1)}$ = 5.000, p = 0.0254. Sidak's multiple comparisons test: Post-shock freezing: GFP vs.
893 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} =
894 10.
895 - For oChIEF experiment: GzLMM two-way repeated measures ANOVA of optogenetic disruption,
896 training stage, and interaction: χ^2 disruption $_{(1)}$ = 0.007, p = 0.9358; χ^2 stage $_{(1)}$ = 0.018, p = 0.8946; χ^2
897 interaction $_{(1)}$ = 0.0650, p = 0.7988. Sidak's multiple comparisons test: Post-shock freezing: Control
898 vs. oChIEF: $t_{(20)}$ = 0.081, p = 0.9960; Tone freezing: Control vs. oChIEF: $t_{(20)}$ = 0.113, p = 0.9921. $n_{Control}$
899 = 7, n_{oChIEF} = 6.
900

901

Suppl. Fig. 7



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903

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

905 Simulation was implemented using the toolbox developed by Stujenske, Spellman and Gordon, (2015)
906 using parameters similar to the one used in our real experiments: optic fiber: radius = 0.2 mm, NA =
907 0.45, wavelength: 532 nm for ArchT and 447 nm for oChIEF, laser intensity 10 mW. Additional
908 parameters were taken from (*15*) (montecarlolight_function: modelnumber = 1; HeatDiffusionLight:
909 U_STEP = 0.03 mm, T_SAVE = 500, R_AVG = 0.25 mm).

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

914 **B**) Depth profile of temperature change in the steady state (calculated as the mean in the 250-300 s
915 interval). Constant light stimulation with 532 nm light would induce a significant temperature increase
916 that would extend over more than 1 mm and would induce non-controllable out of target changes in
917 neuronal activity. In contrast, pulsed activation of oChIEF would not induce physiologically relevant
918 temperature changes at any depth (*15*).

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