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Absence of VGLUT3 expression leads to impaired fear memory in mice

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Manuscript Title Page

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53

54 **Abstract**

55 Fear is an emotional mechanism that helps to cope with potential hazards. However, when fear is generalized
56 it becomes maladaptive and represents a core symptom of Post-Traumatic Stress Disorder (PTSD). Converging
57 lines of research show that dysfunction of glutamatergic neurotransmission is a cardinal feature of trauma
58 and stress related disorders such as PTSD. However, the involvement of glutamatergic co-transmission in
59 fear is less well understood. Glutamate is accumulated into synaptic vesicles by vesicular glutamate trans-
60 porters (VGLUTs). The atypical subtype, VGLUT3 is responsible for the co-transmission of glutamate with
61 acetylcholine (ACh), serotonin (5-HT) or GABA.

62 To understand the involvement of VGLUT3-dependent cotransmission in aversive memories, we used a Pav-
63 lovian fear conditioning paradigm in VGLUT3^{-/-} mice. Our results revealed a higher contextual fear memory
64 in these mice, despite a facilitation of extinction. In addition, the absence of VGLUT3 leads to fear generali-
65 zation, probably due to a pattern separation deficit. Our study suggests that the VGLUT3 network plays a
66 crucial role in regulating emotional memories. Hence, VGLUT3 is a key player in the processing of aversive
67 memories and therefore a potential therapeutic target in stress-related disorders.

68

69

70 **Introduction**

71

72 Fear is an emotion in response to a threat that is essential for survival. However, generalization of fear is a
73 core symptom of major psychiatric disorders such as anxiety disorders, phobia, panic disorder and post-
74 traumatic stress disorder (PTSD) (Lissek and van Meurs, 2015; Besnard and Sahay, 2016). Major progress
75 has been made thanks to animal studies of aversive memories through the Pavlovian fear conditioning para-
76 digm (LeDoux, 2012). This test consists of associating an initially neutral stimulus (such as a tone, a light or
77 a context) to an aversive event (such as a footshock) (Maren et al., 2013). This paradigm is widely used to
78 dissect mechanisms underlying fear learning and memory, and to better understand fear-related disorders.

79 Fear generalization is defined by the fact that a distinct, but perceived by the animal as similar, context elicits
80 fear responses. The balance between contextual discrimination and generalization is a crucial aspect of the
81 expression of fear. Fear generalization is currently considered a central feature of generalized anxiety and
82 PTSD (Lissek, 2012; Mahan and Ressler, 2012).

83 An effective treatment for fear generalization is based on extinction training to reduce acquired fear (Craske
84 et al., 2008; Rothbaum and Davis, 2003). Fear extinction consists of new inhibitory learning after repeated
85 presentations of fear-associated stimulus, in the absence of the aversive event, leading to a gradual decrease
86 in the magnitude of the fear response (Myers and Davis, 2007). However, after extinction fear memory is not
87 erased, but inhibited, as it can reappear in spontaneous recovery, external disinhibition, renewal, and rein-
88 statement (Maren and Holmes, 2016). Therefore, it is important to better characterize neural circuits underly-
89 ing the formation and maintenance of aversive memories if we want to understand and treat generalized fear
90 more efficiently.

91 The neuronal circuits and the neuromodulators regulating emotional memories are well characterized. Emo-
92 tional memories rely on a complex network including the amygdala, the hippocampus and the prefrontal cor-
93 tex (Tovote et al., 2015). The amygdala is necessary for fear processing from acquisition to expression,
94 whereas the hippocampus is mainly involved in contextual memory processing (Myers and Davis, 2007; Si-
95 erra-Mercado et al., 2011; Fanselow, 2000; Marek et al., 2018). Finally, the infra-limbic (IL) and the prelim-
96 bic areas of the prefrontal cortex are essential for fear extinction (Sierra-Mercado et al., 2011; Marek et al.,
97 2019).

98 Several studies have highlighted the involvement of neurotransmitters including glutamate, GABA, acetyl-
99 choline and serotonin signaling in fear processing (Ballinger et al., 2016; Baratta et al., 2016; Christianson et
100 al., 2010; Craske et al., 2008; Jiang et al., 2016; Johnson et al., 2015; Knox, 2016; Krabbe et al., 2018; Wil-
101 son and Fadel, 2017). Interestingly several subpopulations of neurons and fibers of the amygdala, the hippo-
102 campus or the prefrontal cortex release more than one neurotransmitter (for review, see El Mestikawy et al.,
103 2011; Trudeau and El Mestikawy, 2018). Most of these bilingual neurons in the fear circuit express the atyp-
104 ical vesicular glutamate transporter type 3 (VGLUT3) (Rovira-Esteban et al., 2017; Sengupta and Holmes,
105 2019; Amilhon et al., 2010; Fasano et al., 2017; Herzog et al., 2004; Omiya et al., 2015). Studies have illus-
106 trated the involvement of VGLUT3 neurons in psychiatric disorders (Sakae et al., 2015; Favier et al., 2020).
107 Several studies have demonstrated that the absence of VGLUT3 in VGLUT3 neurons led to the abolishment
108 of glutamatergic currents mediated by mGlu receptors in the striatum or the hippocampus (Sakae et al, 2005,
109 Fasano et al, 2017, Favier et al, 2020) whereas others showed the abolition of a glutamatergic ionotropic cur-
110 rents (Higley et al., 2011; Varga et al., 2009). Interestingly, VGLUT3^{-/-} mice show a persistent hyper-
111 reactivity to stress (Amilhon et al., 2010) and a dysregulation of their HPA-axis (Balázsfi et al., 2018), but
112 only a few studies focused on the role of VGLUT3 in the regulation of emotion and fear. A couple of studies
113 previously showed that VGLUT3 deficient mice have a higher contextual fear memory and tend to general-
114 ize their fear to unrelated situations (Balázsfi et al., 2018) with no major other memory deficits (Fazekas et
115 al., 2019).

116 In this context, our aim was to confirm the role of VGLUT3 in aversive memories and to deepen our under-
117 standing of it by using a combination of behavioural paradigms. Using a Pavlovian fear conditioning paradigm,
118 we report that VGLUT3^{-/-} mice express more stable and generalized contextual memories associated with a
119 deficit of pattern separation. Interestingly, VGLUT3^{-/-} mice have no deficit in non-aversive learning or in
120 working memory, spatial reference memory, or in recognition memory. These results highlight the specific
121 role of the VGLUT3-positive network in the establishment and maintenance of aversive memories and most
122 notably in the generalization of fear. They also provide evidence that VGLUT3 could be considered as a po-
123 tential target for the treatment of stress-related disorders.

124

125 **Materials and methods**

126

127 ***Animals***

128 Animal care and experiments were conducted in accordance with the European Communities Council Di-
129 rective for the Care and the Use of Laboratory Animals (86/809/EEC) and in compliance with the French
130 Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale. All ef-
131 forts were made to minimize the number of animals and to ensure their well-being. Animals were group
132 caged and housed in a temperature-controlled room (20±2°C) with free access to water and food under a
133 light/dark cycle of 12h (light 7:30am – 7:30pm).

134 VGLUT3^{-/-} mice (Gras et al., 2008) were on a C57BL6/J background. Heterozygous mice were bred to ob-
135 tain VGLUT3^{-/-} mice and wildtype (VGLUT3^{+/+}) littermates. Pups were weaned around 22 days old, marked
136 by ear punch and genotyped using the ear sample. Experiments were performed with 2–4-month-old mice
137 (159 males and 50 females). Animals were randomly allocated to experimental groups and investigators
138 were blinded for experimental procedures. Total animal number used in each paradigm is presented in Table
139 1.

140

141 ***Behavioural paradigms***

142 **The Watermaze task**

143 The WM test was performed as described previously (Daumas et al., 2008). The mice were monitored with a
144 video tracking system (AnyMaze). First mice went through a 4 days cuetask protocol where the 1.8m diame-
145 ter pool is surrounded with curtains, and a cue placed on the platform (60sec trials, 4 trials a day,
146 ITI=20min). For the spatial reference memory (SRM) task, the platform was centered in one of the four
147 quadrants and kept stable throughout the task (without any cue on it). The protocol lasted 5 days (90sec tri-
148 als, 4 trials a day, ITI=10min). Ten minutes after the last trial on day 5, a 60-sec probe test (SRM-10 min)
149 was conducted during which the platform was removed. In order to avoid extinction, an additional trial with
150 the platform was done immediately after each probe test. A second probe test was performed 72 hrs after
151 assessing the long-term memory of the mice (SRM-72 hrs). For the SRM Reversal (SRM-R) task, which was

152 conducted immediately after the second probe test, learning flexibility was assessed by moving the platform
153 to the opposite quadrant used for the SRM task. The animals were trained for 3 days (90sec trials, 4 trials a
154 day, ITI=10min) and spatial memory was assessed at 10 min (SRM-R-10 min) and 72 hrs (SRM-R-72 hrs)
155 after the last SRM-R trial. Data for the following parameters were collected: latency to reach the platform
156 location, path length, swim speed, thigmotactic behavior and the percentage of time spent in the quadrant
157 zones.

158 **Novel place recognition (NPR) / Novel object recognition (NOR)**

159 The NPR/NOR task was performed in a square open-field (25cm) with sawdust on the floor and cues on the
160 walls. Habituation consisted of 1) a 10-min exploration period of the open-field with cagemates (Day1), 2)
161 two 5-min periods during which each mouse was placed individually in the empty open-field on two consec-
162 utive days (Day2-3), and 3) a 5-min period during which the mice were placed in the open-field with two
163 identical objects (Day4). On the training day (Day5), mice were allowed to explore two new identical objects
164 until they had accumulated 15 sec then 10 sec of total inspection time during the first and second training
165 session, respectively. Since *VGLUT3^{-/-}* mice are more anxious, the protocol was adapted in this way rather
166 than a fixed 10-min training session, to ensure that all animals explore the objects sufficiently to establish
167 memory formation. Therefore, the length of the session was different between animals, but the exploration of
168 the objects was identical. On Day6, the mice were tested for the NOR paradigm (10 min) during which one
169 of the original objects was replaced with a new object. On Day7 we started the NPR paradigm during which
170 two new objects were placed in the open-field. As for NOR, 2 sessions of training were run and consisted in
171 accumulating 15 sec and 10 sec of total exploration time. Twenty- four hours later (Day8), the mice were
172 tested in the NPR paradigm (10 min): the same pair of familiar objects was used but one of the objects was
173 displaced in another corner of the open- field. The percentage of time exploring the new object was calculat-
174 ed as a discrimination index: $[\text{novel} / (\text{novel} + \text{familiar})]$.

175 **Y Maze**

176 Working memory was assessed with a Y maze apparatus (Imetronic, Pessac, France). Mice freely explored
177 the maze for 10 min. The total number of entries was counted as well as the spontaneous alternation. Sponta-
178 neous alternation occurs when a mouse enters a different arm of the maze 3 consecutive times. The percent-

179 age of spontaneous alternation was calculated by dividing the number of spontaneous alternations by the to-
180 tal number of arm entries minus 2 and multiplied by 100.

181

182 **Fear Conditioning Experiments**

183 The Fear Conditioning Apparatus (BIOSEB) is made of black methacrylate walls, a grid floor and transpar-
184 ent ceiling and front door. Panlab software (BIOSEB) was used to carry out the experiments and record
185 freezing behaviour. A video recording system (Multimedia Video Record) allowed manual scoring of freez-
186 ing levels to validate the automatic counts.

187 **Shock sensitivity paradigm.** Because VGLUT3 is present in peripheral sensory neurons and contributes to
188 mechanical pain (Seal et al, 2009), we assessed the sensitivity to electric footshocks in VGLUT3^{-/-} mice. A
189 train of electric footshocks (ES, 1-sec duration) was delivered starting from 0.1 mA and gradually increasing
190 by 0.05 mA every 30 sec. Shock delivery was stopped when all expected behavioural responses were ob-
191 served: increased locomotor activity (movement), vocalization, running and jumping. The intensity of the
192 electric shock that first triggered each of these behaviours was recorded.

193 The **fear conditioning paradigm** was used to study learning and memory of aversive stimuli as previously
194 reported (LeDoux, 2003; Daumas, 2005). Since VGLUT3^{-/-} mice are deaf (Ruel et al., 2008; Seal et al.,
195 2008), a flashing light was used as the conditioned stimulus (CS: 20 sec, 2 sec ON / 2 sec OFF, 80 lux) and a
196 0.25 mA electric footshock as the unconditioned stimulus (US, 2 sec).

197 After 3 days of habituation (6 min per day), the conditioning session took place on day 4. After 2 min in the
198 chamber, the CS was triggered and its final 2 sec coincided with the US. After a 30 sec interval, a second
199 CS-US pairing was presented. Memory tests were done on day 5. Contextual memory was assessed with the
200 contextual test, and cue memory was assessed by the cue test 2 hours later. For the contextual test, mice were
201 placed in the conditioning context for 6 min without CS (light) or US. The cue test consisted of 3 min of ex-
202 ploration of a modified context (color, shape, light and odor), followed by 4 CS presentations with an
203 intertrial interval of 30 sec.

204 A **Pattern Separation** protocol was conducted for 11 days in two highly similar contexts: the shock associ-
205 ated context A and the safe context B as described by (Sahay et al., 2011). On day 0 mice were introduced

206 into context A and after 185 sec received a 0.75 mA US for 2 sec. During the following ten days, mice were
207 exposed to the US-associated context A (183 sec exploration - 2 sec US - 15 sec exploration, before being
208 removed to home cage) and one hour later to the safe context B (180 sec exploration) in a defined order.
209 Freezing behaviour was assessed during the first 180 sec for each context.

210 **Immediate shock** procedure. Mice were submitted to a no-shock (NS) or an immediate shock (IS) proce-
211 dure. For the NS, mice were free to explore the conditioning cage for 30sec. In the IS procedure, mice re-
212 ceived an immediate shock (0.25mA, 2sec) immediately after their placement in the conditioning chamber
213 and were removed after 30sec. Generalized fear was evaluated 24h later by placing the animals in the condi-
214 tioning chamber (same context; SC) or in a novel box (novel context; NC) for 5 min.

215 **Fear extinction learning** and memory were studied for 15 days. Mice were habituated to the conditioning
216 chamber for 2 min before ten CS-US were delivered at 75 sec intervals. From day 2 to day 8, extinction took
217 place in the modified context. Mice were exposed to 10 presentations of CS with an interval of 85 sec under
218 red light illumination. A learning index (LI) was calculated daily. This index is used to ascertain the daily
219 extinction rate by calculating the difference between the first and last CS-induced freezing. On day 15, mice
220 were re-exposed to the conditioning context with ten CS presentations to assess fear recall. On day 18, they
221 were placed in a new context and ten CS were once again presented in order to evaluate renewal in a new
222 context.

223

224 **Statistics**

225 Statistical comparisons were performed with Prism 9 (GraphPad software Inc. USA for Mac OS, La Jolla,
226 CA). Each statistical test was appropriately chosen for the relevant experimental design. Sidak's multiple
227 comparisons test was performed for post-hoc analysis when required unless otherwise indicated. All data are
228 presented as the mean \pm SEM, with differences considered significant at $p < 0.05$. Complete analysis and sta-
229 tistics are presented in Extended Tables.

230 **Results**

231 Fear conditioning is based on learning/memory and on the propensity of mice to feel and react to electric
232 footshock. VGLUT3 is expressed in the hippocampus where it contributes to hippocampal plasticity and
233 network properties (Fasano et al., 2017). On the other hand, VGLUT3 is also found in subsets of neurons in
234 pain circuits (Sakai et al., 2020; Larsson and Broman, 2019; Peirs et al., 2015; Draxler et al., 2014; Seal et
235 al., 2009; Landry et al., 2004). Therefore, prior to using the fear conditioning paradigm, we assessed learn-
236 ing, spatial memory and pain threshold (i.e., response to foot shock) in VGLUT3^{-/-} mice.

237

238 **The absence of VGLUT3 does not impair learning and memory in mice**

239 To explore the consequences of VGLUT3 deletion on spatial and non-spatial memories, we first used the
240 watermaze task (WM) (Fig.1; statistics details can be found in extended figure 1-1). Relative to wildtype
241 littermates, VGLUT3^{-/-} mice displayed no impairment of learning in either the non-spatial (Fig.1D) or the
242 spatial (Fig.1E) task. We observed a main effect of time but no main effect of genotypes or interaction be-
243 tween time and genotype. Therefore, both genotypes improved their learning during the training days
244 (Fig.1D-E $p < 0.0001$). To challenge them and assess their learning flexibility, a 3-day reversal task was per-
245 formed immediately after the spatial reference learning task (Fig.1E: R1-R3). On day 1 of reversal learning
246 (R1 in Fig.1E) both groups increased their latency to reach the new platform location, and then similarly im-
247 proved their performance (Fig.1E; time: $p < 0.0001$, genotype: $p = 0.882$). Spatial memory was assessed 10
248 min and 72h after training completion for SRM and SRM-R. In all tested conditions, control littermates as
249 well as VGLUT3^{-/-} mice spent significantly more than 25% of probe trial time in the targeted quadrant, indi-
250 cating intact spatial reference memory (group performance vs. 25% $p < 0.05$; Fig. 1F). However, during the
251 long-term memory test, VGLUT3^{-/-} mice showed better performances (SRM-PT2) than controls (Fig.1F).
252 Since VGLUT3^{-/-} mice are more vulnerable to anxiety than WT mice (Amilhon et al, 2010), we explored the
253 contribution of anxiety to memory formation and learning in VGLUT3^{-/-} mice in a more stressful condition,
254 when the water temperature was lowered to 19°C (Sandi et al., 1997). At 19°C, we observed no main effect
255 of genotype or interaction between genotype and time, but a main effect of time for both cuetask and
256 SRM/SRM-R (Fig.1G-H). A 3 way ANOVA revealed no main effect of genotype, tests or water temperature
257 and no interactions between these parameters except for the temperature x genotype ($p = 0.04$; see Extended

258 Figure 1-1 for statistical details). Moreover, in all tested conditions, VGLUT3^{-/-} and control mice show simi-
259 lar performances and spent more than 25% of their time in the correct quadrant (Fig.1I). These data show no
260 deficit of learning and memory in VGLUT3^{-/-} mice in the WM paradigm.

261

262 **Figure 1 AROUND HERE**

263

264 We then studied spontaneous learning and memory using the object recognition paradigm. We observed for
265 both genotypes a significant difference from chance level (score 0.5) revealing long-term recognition
266 memory for objects (Fig.2A) and position (Fig.2B) in all animals. VGLUT3^{-/-} mice show higher perfor-
267 mances than control littermates in the object recognition task (Fig.2A). Since VGLUT3^{-/-} mice spent signifi-
268 cantly more time in the open field, we wondered whether the time spent during training was correlated with
269 the memory score obtained in the object recognition test. The correlation curve (Fig.2C) and the linear re-
270 gression revealed no correlation between memory score and the length of the session in VGLUT3^{-/-} mice
271 ($R^2=0.03$, $F(1,11)=0.31$, $P>0.05$; equation: $Y=-0.001889*X+0.6951$). These experiments do not reveal major
272 learning or memory impairment in VGLUT3^{-/-} mice.

273

274 **Figure 2 AROUND HERE**

275

276 **Footshock sensitivity is not altered by VGLUT3 deletion**

277 Deletion of VGLUT3 did not affect the behavioral responses (movement, vocalization, running, jump) elicited
278 by footshock stimuli of varying intensity (Fig. 3A and statistics in in extended figure 3-1). This result
279 shows that pain sensitivity to electric footshocks is unaffected in VGLUT3^{-/-} mice.

280

281 **Visual and Contextual Fear Conditioning are altered in the absence of VGLUT3**

282 During conditioning we observed no main effect of genotype, no interaction between genotype and time,
283 only a main effect of time (Fig.3B /Table4). We then assessed contextual memory 24h after conditioning

284 (Fig.3C-D). The time-course analyses of the freezing rate during the 6 min test shows an interaction and a
285 time effect, but no main effect of genotype (Fig.3C/Table4). When we analyzed the test by 3 min bins
286 (Fig.3D), a clear genotype difference arises. Post-hoc analysis revealed higher freezing rate in VGLUT3^{-/-}
287 than in VGLUT3^{+/+} mice in the last 3-min of the test (Fig.3D: 0-3 min: $t(44)=0.4722$, $p=0.87$; 3-6 min:
288 $t(44)=2.464$, $p=0.03$; Sidak's multiple comparisons test). The cue test was then done by exposing mice to the
289 flashing light in a novel environment. Mice were free to explore the new context for three minutes before the
290 light (CS) was triggered (Fig.3E-F). The global analysis reveals only a main effect of time but no main effect
291 of genotype or interaction between genotype and time (Fig.3E/Table4). Remarkably, the freezing rate signif-
292 icantly increased in VGLUT3^{+/+} mice but not in VGLUT3^{-/-} mice after CS presentation in the new context
293 (Fig.3F: respectively $t(22)=2.541$, $p=0.03$; and $t(22)=1.395$, $p=0.32$; Sidak's multiple comparisons test). One
294 possible explanation of the higher fear expression observed in VGLUT3^{-/-} mice in the new context could be
295 that once conditioned, they show a higher fear response to a new context with either no specific freezing re-
296 sponses associated with the US or too low to be observed.

297

298 **Figure 3 AROUND HERE**

299

300 **The absence of VGLUT3 leads to a deficit in pattern separation**

301 Cued memory alterations in VGLUT3^{-/-} mice (Fig.3E-F) might be caused by a deficit to discriminate between
302 the two contexts, that associated with an US versus the safe one, a process governed by pattern separation.
303 To examine this possibility, we submitted a group of mice to a pattern separation protocol (Fig.4A) where
304 context A is always associated with an electric shock (ES), whereas context B is safe and free of ES. In
305 VGLUT3^{+/+} mice, we observed no main effect of context, but a main effect of time and an interaction be-
306 tween context and time (Fig.4B and extended figure 4-1) Over time VGLUT3^{+/+} mice learn to dissociate the
307 2 contexts since they significantly freeze less from Day 7 to Day 10 (Fig.4B; Day7, $t(11)=3.031$, $p=0.02$;
308 Day8, $t(11)=2.933$, $p=0.03$; Day9, $t(11)=2.917$, $p=0.03$; Day10, $t(11)=5.038$, $p<0.0001$; Sidak's multiple
309 comparisons test). Strikingly, in VGLUT3^{-/-} mice we observed no main effect of context, or interaction be-
310 tween context and time but a main effect of time (Fig.4C and extended figure 4-1). VGLUT3^{-/-} mice did not
311 learn to discriminate the two contexts as high freezing levels were maintained over the 10 days of the test

312 (Fig.4C). Furthermore, VGLUT3^{-/-} mice showed comparable levels of spontaneous freezing on Day 0 before
313 the occurrence of the first ES (Fig.4D).

314

315

Figure 4 AROUND HERE

316

317 However, on Day1, after conditioning, we observed a main effect of genotype, but no main effect of context
318 or interaction between context and genotype (Fig.4E and extended figure 4-1). On Day7 and 10, we observed
319 no main effect of genotype, but a main effect of context and an interaction between context and genotype
320 (Fig.4F-G and extended figure 4-1). VGLUT3^{+/+} mice clearly dissociated context A from B (Fig.4F; Day7,
321 $t(11)=4.04$, $p=0.001$; Fig.4G, Day10, $t(11)=7.934$, $p<0.0001$; Sidak's multiple comparisons test). This was
322 not the case with VGLUT3^{-/-} mice. Altogether, these results illustrate a deficit in pattern separation in
323 VGLUT3^{-/-} mice.

324

325 **The absence of VGLUT3 leads to generalized fear after aversive experiences**

326 The observed deficit in pattern separation could also represent generalized fear in VGLUT3^{-/-} mice. To in-
327 vestigate this point, we submitted a group of VGLUT3^{+/+} mice and VGLUT3^{-/-} mice to an immediate shock
328 paradigm (Fig.5). On day 1 mice were introduced to a context and either immediately received a footshock
329 (immediate shock (IS) condition) or nothing (no shock (NS) condition). The next day, they were tested in the
330 same context (SC) or in a new context (NC). As expected the immediate shock (IS) did not elicit freezing
331 behaviour on day 2 in VGLUT3^{+/+} mice, in either context (Fig.5 IS-SC or IS-NC). VGLUT3^{-/-} mice showed
332 no freezing when they were not shocked (Fig 5, NS), however significant higher freezing levels were ob-
333 served after the IS procedure in both contexts (Fig.5, IS-SC & IS-NC). These results revealed increased
334 freezing levels in VGLUT3^{-/-} mice after experiencing an aversive stimulus.

335

336

Figure 5 AROUND HERE

337

338 **Visual Fear Extinction is altered in the absence of VGLUT3**

339 Because of the impairment described in the cue-test (Fig.3.E-F), we wondered if VGLUT3^{-/-} mice were not
340 fully conditioned with a discrete CS such as a light. To answer this question, a cue fear conditioning extinc-
341 tion protocol was performed (Fig.6). On Day 1, mice were exposed to 10 CS-US presentations in a square
342 context, followed from Day 2 to Day 8 to a daily session of 10 CS-only presentations in a round context, to
343 assess cue extinction (Fig.6A). The overall analysis suggested a tendency for a main effect of genotype with
344 a clear main effect of time and an interaction between time and genotype (Table7). On Day 3, both groups
345 started the test with an equivalent high level of freezing that progressively decreased, reaching significance
346 on the 10th CS presentation (Fig.6A, $t(12)=3.77$, $p=0.01$; Sidak's multiple comparisons test).

347 To determine the extinction of learning performances of mice, we calculated a learning index (LI, Fig.6B-C).
348 We observed a main effect of genotype with no effect of time or interaction between time and genotype (Ta-
349 ble7). VGLUT3^{-/-} mice demonstrated a higher LI than VGLUT3^{+/+} mice during the first 2 days of the test,
350 followed by a similar pattern for the two genotypes during day 4-8 (Fig.6B: Day2: $t(12)=2.922$, $p=0.02$;
351 Day3, $t(12)=2.761$, $p=0.04$; Sidak's multiple comparisons test). Cumulative analysis showed that overall,
352 VGLUT3^{-/-} mice have a higher LI than VGLUT3^{+/+} mice but that both groups show significant positive LI
353 (Fig.6C). These findings suggest that VGLUT3^{-/-} mice properly learn to extinguish their fear, with an initial
354 higher performance than VGLUT3^{+/+} mice.

355 On day 15, mice were re-exposed to the original square context and their fear memory was examined
356 (Fig.6D-E, Recall 1). We observed a main effect of time and an interaction between time and genotype but
357 no main effect of genotype (Table7). Post-hoc analysis revealed a significant difference between the freezing
358 level of VGLUT3^{-/-} mice and VGLUT3^{+/+} mice for the first CS presentations (Fig.6D, L2 $t(12)=2.971$,
359 $p=0.03$; L3 $t(12)=3.773$, $p=0.002$ and L4 $t(12)=2.859$, $p=0.04$; Sidak's multiple comparisons test). This ef-
360 fect was confirmed when the first 5 recall sessions were analyzed separately from the last 5 sessions (Fig.6E;
361 L1-5, $t(12)=4.076$, $p=0.0004$; L6-10, $t(44)=1.292$, $p=0.36$; Sidak's multiple comparisons test).

362

363 **Figure 6 AROUND HERE**

364

365 To establish that the freezing behaviour observed during recall 1 was specific and was due to the occurrence
366 of the light in the conditioning context, half of the animals were tested on day 18 in a completely new envi-
367 ronment (Fig.6F; Recall 2). As can be seen from Fig.6F, we observed no main effect of genotype or time and
368 no interaction between time and genotype (Table7). Freezing levels were similar ($\approx 20\text{-}25\%$) for both
369 groups, showing no evidence of generalized freezing behaviour after extinction. These data sug-
370 gest that after an extinction procedure, $VGLUT3^{-/-}$ mice may have stronger original memory recall, with no
371 generalized freezing responses to a new context.

372

373 **Working memory is intact in the absence of VGLUT3**

374 The accelerated extinction observed in $VGLUT3^{-/-}$ mice during the first days of extinction (Fig.6A-B) could
375 reflect altered working memory (WM). Hence, we compared WM of WT mice and $VGLUT3^{-/-}$ mice using
376 the Y-Maze paradigm. Mice were free to explore the Y-Maze for 10 min and spontaneous alternation was
377 quantified. In line with their anxiety phenotype, $VGLUT3^{-/-}$ mice made significantly fewer arm entries than
378 controls (Fig.7A/Table8). However, both groups showed similar levels of spontaneous alternation, both
379 above chance level (Fig.7B). Overall, $VGLUT3^{-/-}$ mice show normal working memory despite a lower ex-
380 ploration activity.

381

Figure 7 AROUND HERE

382

383 **Discussion**

384 The involvement of the VGLUT3 network in cognition and related psychiatric pathologies has been investi-
385 gated in a few studies (Amilhon et al., 2010; Balázsfi et al., 2018; Favier et al., 2020; Fazekas et al., 2019;
386 Sakae et al., 2015). For example, Balázsfi (2018) and Fazekas (2019) who focused on the study of learning
387 and memory, concluded that the deficits in VGLUT3-deficient mice were very limited and mainly related to
388 stress. Understanding how aversive memories are processed in the brain will help to decipher its dysfunction
389 in trauma- and stress-related disorders. In the present study we explored the establishment and maintenance
390 of fear-related memories in mice lacking VGLUT3. Using a Pavlovian fear conditioning paradigm, we report

391 that VGLUT3^{-/-} mice express more stable and generalized contextual memories associated with a deficit of
392 pattern separation. Interestingly, VGLUT3^{-/-} mice have no deficit in non-aversive learning and memory, in-
393 cluding working memory, spatial reference memory, and cue-based extinction learning. Our results partly
394 confirm previous findings (Balázsfi et al., 2018; Fazekas et al., 2019) while deepening our understanding of
395 the involvement of VGLUT3-dependent cotransmission in aversive memories.

396

397 Before studying aversive memories, we first assessed the consequences of the lack of VGLUT3 in learning,
398 memory processing and cognitive flexibility in spatial and non-spatial tasks. No deficit was found in
399 VGLUT3-deficient mice. Our results are in agreement with data obtained by Fazekas et al., (2019), who also
400 found comparable spatial learning capacities in VGLUT3^{-/-} mice (although they trained only male mice, in a
401 pool that was half the size of ours), supporting the robustness of the observed phenotypes. However, our ap-
402 proach of systematically testing spatial memories has revealed improved long-term spatial memory perfor-
403 mance in VGLUT3^{-/-} mice compared to control mice at 22°C in the watermaze task. Since memory perfor-
404 mances of VGLUT3^{-/-} mice were comparable to controls when the water temperature was dropped to 19°C,
405 we hypothesized that the improved memory performance of VGLUT3^{-/-} mice could be related to their anxie-
406 ty trait (Amilhon et al. 2010) as well as to their hypothalamic-pituitary-adrenal axis dysfunctions (Balázsfi et
407 al. 2018) in less-stressful watermaze conditions (i.e. at 22°C). This is in agreement with the literature in both
408 humans and animals, highlighting that mild stress could have facilitating effects on memory consolidation
409 (Sandi, Loscertales, et Guaza 1997; Sandi et Pinelo-Nava 2007; Cahill et McGaugh 1998).

410 Nevertheless, depending on the behavioral paradigm used, this anxiety trait could interfere with appropriate
411 data interpretation. In order to overcome this and accurately assess recognition memory (object and spatial)
412 using an open-field, we had to adapt the protocol to ensure sufficient exploration of objects for recognition
413 memory to occur. By using a fixed exploration time per session rather than a fixed session duration, we were
414 able to circumvent the confounding effect of anxiety and ensure an unbiased assessment of recognition
415 memory in VGLUT3^{-/-} mice. We observed no deficit of recognition or spatial memories in VGLUT3^{-/-}
416 mice. In conclusion, using different protocols or paradigms, we confirmed that the absence of VGLUT3 does
417 not impair spatial reference, non-spatial memory or associative-learning processes.

418

419 We next explored fear-related memories in VGLUT3^{-/-} mice using a Pavlovian fear conditioning paradigm.
420 Because VGLUT3 is present in peripheral sensory neurons and contributes to mechanical pain detection
421 (Seal et al, 2009), we assessed sensitivity to electric footshocks in VGLUT3^{-/-} mice and found unchanged
422 sensitivity to electric foot shock in VGLUT3^{+/+} mice. This result confirmed previous findings by Balazsfi et
423 al (2018) using flinch and jump threshold as the readout. In the Pavlovian fear conditioning protocols used,
424 the deletion of VGLUT3 led to normal fear learning but a higher and persistent contextual memory, which is
425 consistent with the observations of Balazsfi et al (2018). However, our study highlighted an absence of cue
426 memory, which could be explained by a contextual generalization deficit. To express fear when it is relevant,
427 present and past associations have to be compared. This is adaptive, since it allows individuals to anticipate a
428 threat by discerning pertinent cues in the environment. Increased interference between past and new memo-
429 ries could promote reactivation of traumatic memories and lead to overgeneralization of fear. Considerable
430 evidence from the literature suggests the involvement of the hippocampal CA3-Dentate Gyrus (DG) circuit
431 in contextual discrimination (McHugh et al. 2007; Besnard et Sahay 2016; Cravens et al. 2006). CA3 plays
432 a major role in a process called pattern completion, which allows retrieval of a stored representation based on
433 sparse cues in the environment. In contrast, the DG is also involved in pattern separation, to minimize the
434 overlap between two similar representations. Precise memory requires remembering details with high speci-
435 ficity, so that memories can be discriminated from other similar memories to avoid interference. Pattern sep-
436 aration facilitates this discrimination by reducing the degree of similarities between overlapping experiences.
437 The pattern separation paradigm (Sahay et al. 2011) was used to study the propensity of mice to discriminate
438 among similar experiences (Yassa & Stark, 2011). At day 1, freezing levels were comparable between con-
439 texts A and B for both control and VGLUT3^{-/-} mice, suggesting the degree of similarity between the two
440 contexts was high enough to evoke generalization of contextual fear in both groups. However, control mice
441 learned to discriminate the shocked context from the safe context as early as day 7, whereas VGLUT3-
442 deficient mice were unable to discriminate between the two contexts within the 10-day protocol used. These
443 results highlight a significant deficit in pattern separation in VGLUT3^{-/-} mice (Fig.4 A2-3). Considerable
444 evidence supports a role for the hippocampus in pattern separation to constrain the overgeneralization of
445 fear. Previous work studied the hippocampal plasticity in VGLUT3 deficient mice (Fasano et al, 2017) and
446 found that the absence of glutamate released by VGLUT3 hippocampal interneurons led to increased GA-
447 BAergic transmission, altering the oscillatory activity of synchronized networks and inducing a metaplastic

448 shift of synaptic plasticity in the ventral hippocampus. As hippocampal long-term plasticity is currently
449 thought to underlie the cellular basis of such learning and memory processes, we cannot exclude that they
450 might cause the observed contextual overgeneralization in VGLUT3^{-/-} mice.

451

452 To better understand this discrimination deficit, we performed an immediate shock (IS) test. According to
453 Fanselow (2000), in the IS test, animals do not have enough time to form an integrated memory representa-
454 tion of context features in order to associate it to the electric shock. In line with this hypothesis, wildtype
455 mice do not form a contextual fear memory and show no freezing behaviour during retrieval tests. In con-
456 trast, when they were immediately shocked, VGLUT3^{-/-} mice increased their level of freezing whatever the
457 context used in the retrieval test (Fig.5). This observation suggests that in VGLUT3^{-/-} mice, the mere occur-
458 rence of the traumatic event (i.e., the foot shock) elicited impaired fear expression. In our view, this in-
459 creased fear expression reflects more than innate fear impairment in VGLUT3^{-/-} mice, since innate fear ex-
460 periences to natural threats need to be harmless (Silva, Gross, et Gräff 2016). When the animal experiences
461 pain such as a foot shock as in our experiment, it is a conditioned response and a learned experience.
462

463 One major treatment of fear-related disorders, called exposure therapy in clinics or extinction fear learning in
464 laboratory, involves repeatedly re-exposure of animals to the CS (the flashing light) previously associated
465 with the aversive US (the foot shock) in a different context. With time, the animals learn that the CS is no
466 longer associated with the US in this new context and thus the mice form a new « safer » memory (Myers et
467 Davis 2007; Perusini et Fanselow 2015). Surprisingly, during the initial steps of this extinction learning,
468 VGLUT3^{-/-} mice show improved performance (Fig.6 A-C). This is particularly surprising since the processes
469 governing extinction and generalization are thought to be similar (see Lopresto et al., 2016). The brain struc-
470 tures mainly involved are the prefrontal cortex (especially its infra limbic (IL-PFC) part) and the hippocam-
471 pus as previously discussed regarding pattern separation. However, extinction mostly relies on the interac-
472 tion of the IL-PFC with the basal-lateral amygdala. Those projections do not express VGLUT3 and might
473 effectively control the amygdala activity as observed. One hypothesis is that context generalization (or lack
474 of pattern separation) could be due to the dysfunction of the hippocampal network due to the absence of
475 VGLUT3, whereas the cue-based extinction may depend on the IL-PFC projections to the amygdala.

476 Original memory was assessed at day 15 in the conditioning context (Fig.6D-E). Results confirm that the
477 extinction procedure did not alter the original memory since both groups still displayed a high level of freez-
478 ing (significantly higher in VGLUT3^{-/-} than in control mice) to the context where they were originally
479 shocked. Surprisingly, when tested in a third context on D18 VGLUT3^{-/-} mice did not show fear generaliza-
480 tion, indicating that the animals might have associated the aversive value of the CS only to the original con-
481 text. This observation suggests that VGLUT3^{-/-} mice could show an associative cue learning that can proper-
482 ly be recalled and specific to a context.

483

484 In regards to the initial facilitation of the extinction, we cannot exclude that this could be due to increased
485 attention related to the anxiety trait in VGLUT3^{-/-} mice, or in their working memory. Attentional processes
486 are difficult to test in VGLUT3^{-/-} mice, since those experiments classically require the use of sound (e.g.,
487 prepulse inhibition, fear startle tests...) and these mutants are deaf (Ruel et al, 2008). To rule out any work-
488 ing memory modification that could explain this initial extinction improvement, we subjected our mice to a
489 Y-maze alternation protocol. Unlike Fazekas et al. (2019) we observed no alteration of working memory in
490 VGLUT3^{-/-} mice. Since mice lacking VGLUT3 tend to explore less due to their anxious phenotype, we in-
491 creased the test duration from 5 to 10 min to have substantial exploration levels in VGLUT3^{-/-} mice and WT
492 mice (>100 entries). This might explain the different findings, since poor exploration can directly affect be-
493 havioral performances. Therefore, in our hands, VGLUT3^{-/-} mice show no deficit or facilitation of their
494 working memory that could explain their better initial performance in fear extinction.

495 Some studies found VGLUT3-amacrine cells in mouse retina (Kim et al., 2015.; Lee et al., 2021, 2016) co-
496 releasing glutamate and glycine at glycinergic synapses. How the absence of VGLUT3 could impact the
497 function of these synapses in these animals, and therefore their ability to see properly, has yet to be deter-
498 mined. What seems to be accepted is the lack of VGLUT3 impacting the vision of movement (Kim et al.,
499 2015.; Lee et al., 2016). However, based on our results, it is unlikely that the observed initial lack of cue
500 conditioning can be due to visual impairment. First, we use a flashing light as a cue, that is a major visual
501 information. Then, VGLUT3 deficient mice have intact performances in the spatial reference memory task in
502 the watermaze, and in the object recognition tasks, both of which mainly rely on visual cues. Overall, we

503 cannot rule out differences in visual detection between controls and VGLUT3-deficient mice, but this alone
504 cannot explain the initial lack of cue conditioning observed.

505 Our findings on impaired fear-related memories in mice lacking VGLUT3 are in good agreement with the
506 electrophysiological reports (Fasano et al, 2017). However, this interpretation should be taken with care,
507 since a constitutive VGLUT3 deletion was used in the present study. Cholinergic fibers from the basal fore-
508 brain projecting to the basolateral amygdala are crucial in reinforcing learning and consolidating aversive
509 memories (Jiang et al. 2016; Crouse et al. 2020; Aitta-aho et al. 2018). Interestingly, a subset of those fibers
510 does express VGLUT3 (Nickerson Poulin et al. 2006). It is possible that this cholinergic pathway could also
511 be involved in fear-related disorders. A thorough description of the involvement of these different pathways
512 would require the deletion of VGLUT3 in specific subpopulation of neurons.

513 In conclusion, the present study suggests an important role of VGLUT3 in aversive memory processing such
514 as contextual generalization of fear memory which could be crucial in trauma- and stress-related disorders.

515

516

517 **Table 1.** Cohorts used.

Experiment (Figure)	Sex	N of VGLUT3 ^{+/+} (WT)	N of VGLUT3 ^{-/-} (KO)
Watermaze 22°C (Fig1D-F)	F	15	11
Watermaze 19°C (Fig1G-I)	F	13	11
Object recognition (Fig2)	M	13	12
Shock sensitivity (Fig3A)	M	8	6
Fear conditioning (Fig3B-F)	M	12	12
Pattern separation (Fig4)	M	11	10
Immediate shock (Fig5)	M	14	20
Fear extinction (Fig6)	M	12	12
Y Maze (Fig7)	M	9	8
<i>TOTAL</i>		<i>107</i>	<i>102</i>

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751

752 **Figure Legends**

753 **Figure 1. Cue and Spatial Reference Memory in VGLUT3^{-/-} mice.** (A-C) Watermaze experimental design: A) Cuetask, B) SRM task and C) SRM-Reversal task. (D-F) Mice were trained in 22°C water. 754
755 VGLUT3^{-/-} mice showed no deficit of learning either in the Cue- (D), or the SRM and reversal (E) tasks. (F) 756
757 Memory assessment was performed 10min (PT1 for SRM and SRM-R), 72h (PT2 for SRM) and 48h (PT2 758
759 for SRM-R) post training, and VGLUT3^{-/-} mice showed better performance at 72h post training, and in the 760
761 PT average. (G-I) Mice were trained in water at 19°C. VGLUT3^{-/-} mice show no deficit of learning either in 762
763 the Cue (G), or the SRM and reversal (H) tasks. (I) No differences were observed in memory tests done at 764
765 different times or on average. Data are mean ± SEM. Differences between genotypes: * p<0.05. PT: probe 766
767 test; R: reversal. All corresponding statistics are presented in Figure 1-1

768
769
770 **Figure 2. Object and Spatial Recognition in VGLUT3^{-/-} mice.** (A) Object Recognition (OR): both groups 771
772 show OR memory, with VGLUT3^{-/-} mice having higher scores. (B) Spatial Recognition (SR): both groups 773
774 show comparable SR memory level. (C) There is no correlation between learning sessions duration and 775
776 VGLUT3^{-/-} mice performances in OR. Slopes are -0.01563 for WT and -0.001889 for VGLUT3^{-/-} mice. 777
778 Data are mean ± SEM. Differences between genotypes: * p<0.05; Differences to chance level: ## p<0.01, 779
p<0.001. All corresponding statistics are presented in Figure 2-1

780
781
782 **Figure 3. Contextual and Cued Fear memories of VGLUT3^{-/-} mice.** (A) Shock sensitivity assessment, ie. 783
784 the intensity for which the mice express a given behaviour (movement, vocalization, running or jump). (B-F) 785
786 Fear memories in VGLUT3^{-/-} mice. (B) Freezing levels during fear conditioning consisting of 2 CS-US pair- 787
788 ings. (C-D) Contextual memory was tested 24h after conditioning and revealed a more stable memory in 789
790 VGLUT3^{-/-} mice. (E-F) Cued memory test revealed high level of freezing to new context for VGLUT3^{-/-} 791
792 mice. Data are mean ± SEM. post-hoc comparisons: * p<0.05. All corresponding statistics are presented in 793
794 Figure 3-1

779 **Figure 4. Pattern separation of VGLUT3^{-/-} mice.** (A) Behavioral protocol; (B) VGLUT3^{+/+} mice perfor-
780 mances; (C) VGLUT3^{-/-} mice performances. (D-G) Freezing levels on different days: (D) Day 0, before
781 conditioning, (E) Day 1, VGLUT3^{-/-} mice already show a higher freezing level, (F) Day7, VGLUT3^{+/+} mice
782 start to discriminate the different contexts, (G) On Day10, VGLUT3^{-/-} mice still do not discriminate the dif-
783 ferent contexts. Data are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. All correspon-
784 ding statistics are presented in Figure 4-1

785

786 **Figure 5. Immediate shock in VGLUT3^{-/-} mice.** Mice were subjected to a no shock (NS) or immediate
787 shock (IS) protocol to assess their levels of spontaneous freezing after experiencing an electric footshock.
788 They were either tested in the same context (IS-SC) or in a novel context (IS-NC). WT mice did not show
789 any freezing after either procedure, whereas VGLUT3^{-/-} mice expressed a significant increase of freezing
790 behaviour only after the IS, in either context. Data are mean ± SEM. post-hoc comparisons: *** p<0.001.
791 All corresponding statistics are presented in Figure 5-1

792 **Figure 6. Extinction Fear in VGLUT3^{-/-} mice.** (A) Extinction learning over a 7-day period. Filled black
793 and red circles represent the freezing levels of VGLUT3^{+/+} mice and VGLUT3^{-/-} mice (respectively) before
794 the presentation of the first CS. Open circles are used for the 10 subsequent CS. (B-E) Fear memory in
795 VGLUT3^{-/-} mice. (B-C) The Learning Index (LI) was calculated to illustrate learning efficacy over time (B)
796 and on average (C). (D-E) Original memory was recalled on Day15. (F) On D18, freezing to CS was asses-
797 sed in a new hexagonal context. Data are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001. L: light (CS). All
798 corresponding statistics are presented in Figure 6-1

799 **Figure 7. Working memory in VGLUT3^{-/-} mice.** (A) Number of entries in the Y-Maze arms for the first 5
800 min of the test (0-5min) or the total 10 min test (0-10min). (B) Percentage of alternation. Data are mean ±
801 SEM. Differences between genotype: * p<0.05, *** p<0.001; Differences compared to chance level: #
802 p<0.05, ## p<0.01. All corresponding statistics are presented in Figure 7-1

803

804

805 **Extended data Legends**

806 **Extended Figure 1-1. Statistics for Watermaze experiments.** ¹: SRM 10min ; ²: SRM 72h ; ³:

807 SRM-R 10min ; ⁴: SRM-R 48h ; ⁵: PTs average

808 **Extended Figure 2-1. Statistics for Object Recognition experiments.**

809 **Extended Figure 3-1. Statistics for Fear conditioning experiments.**

810 **Extended Figure 4-1. Statistics for Pattern separation experiment.**

811 **Extended Figure 5-1. Statistics for Immediate shock experiments.** NS: no shock; IS: immediate

812 shock; SC: same context; NC: new context

813 **Extended Figure 6-1. Statistics for Fear extinction experiments.**

814 **Extended Figure 7-1. Statistics for the Y-Maze experiment.**

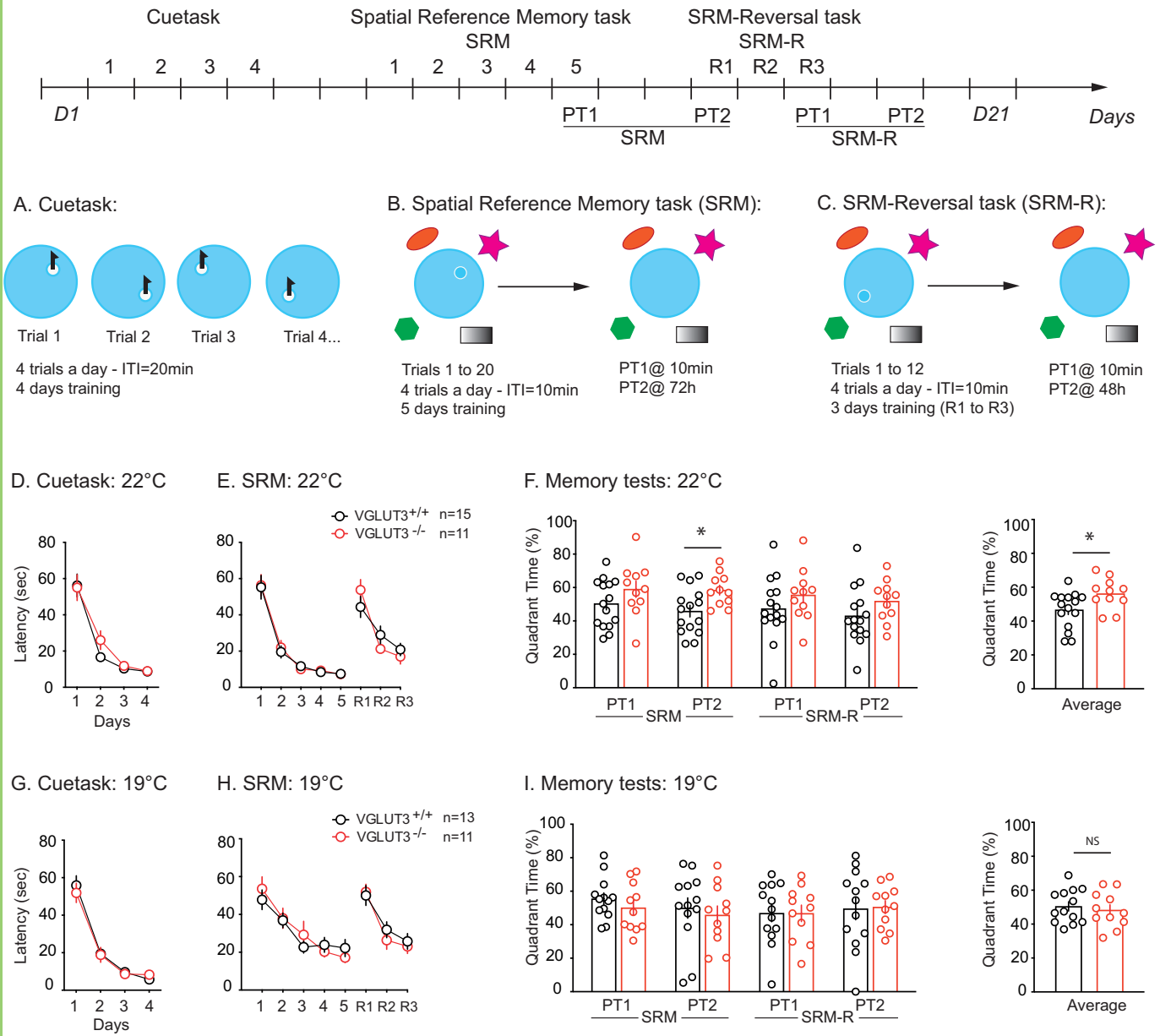
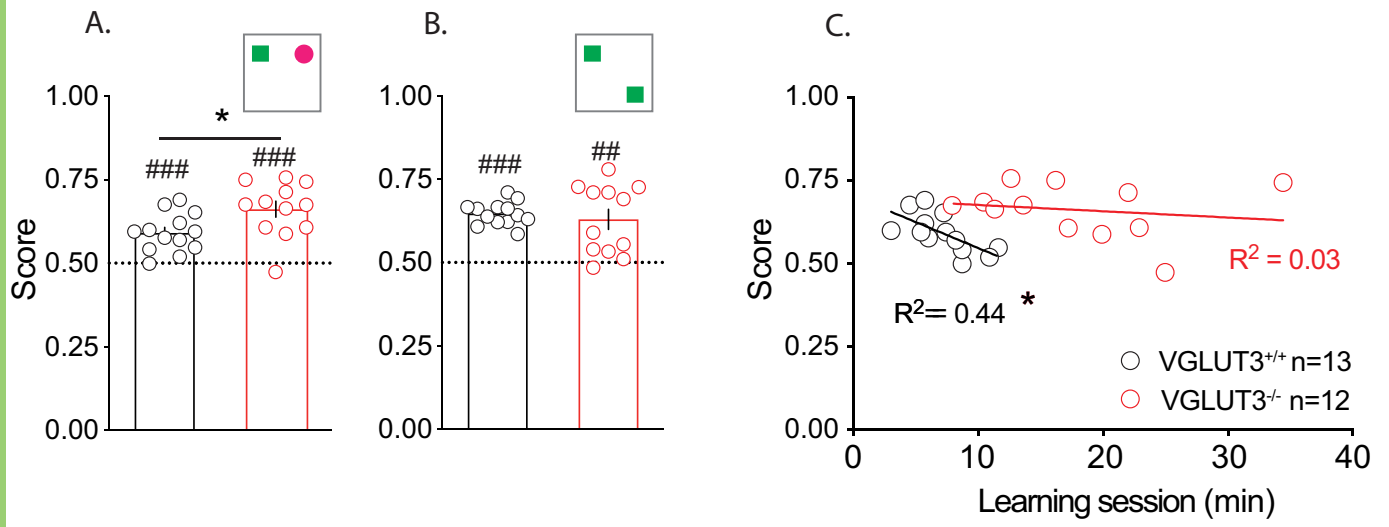
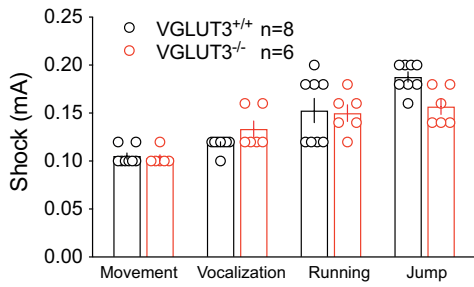


Figure 1

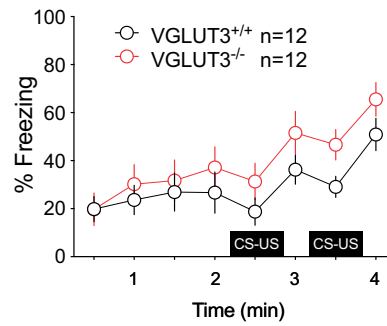
Figure 2



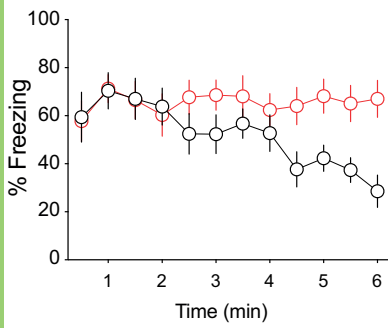
A. Shock sensitivity



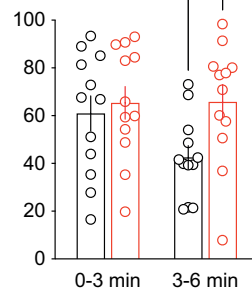
B. Conditioning



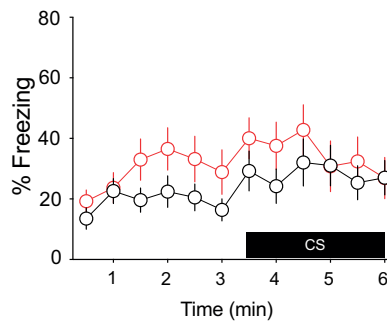
C. Context Test Kinetics



D. Context Test



E. Cue Test Kinetics



F. Cue Test

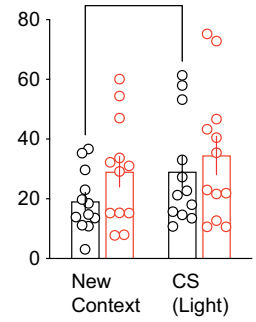


Figure 3

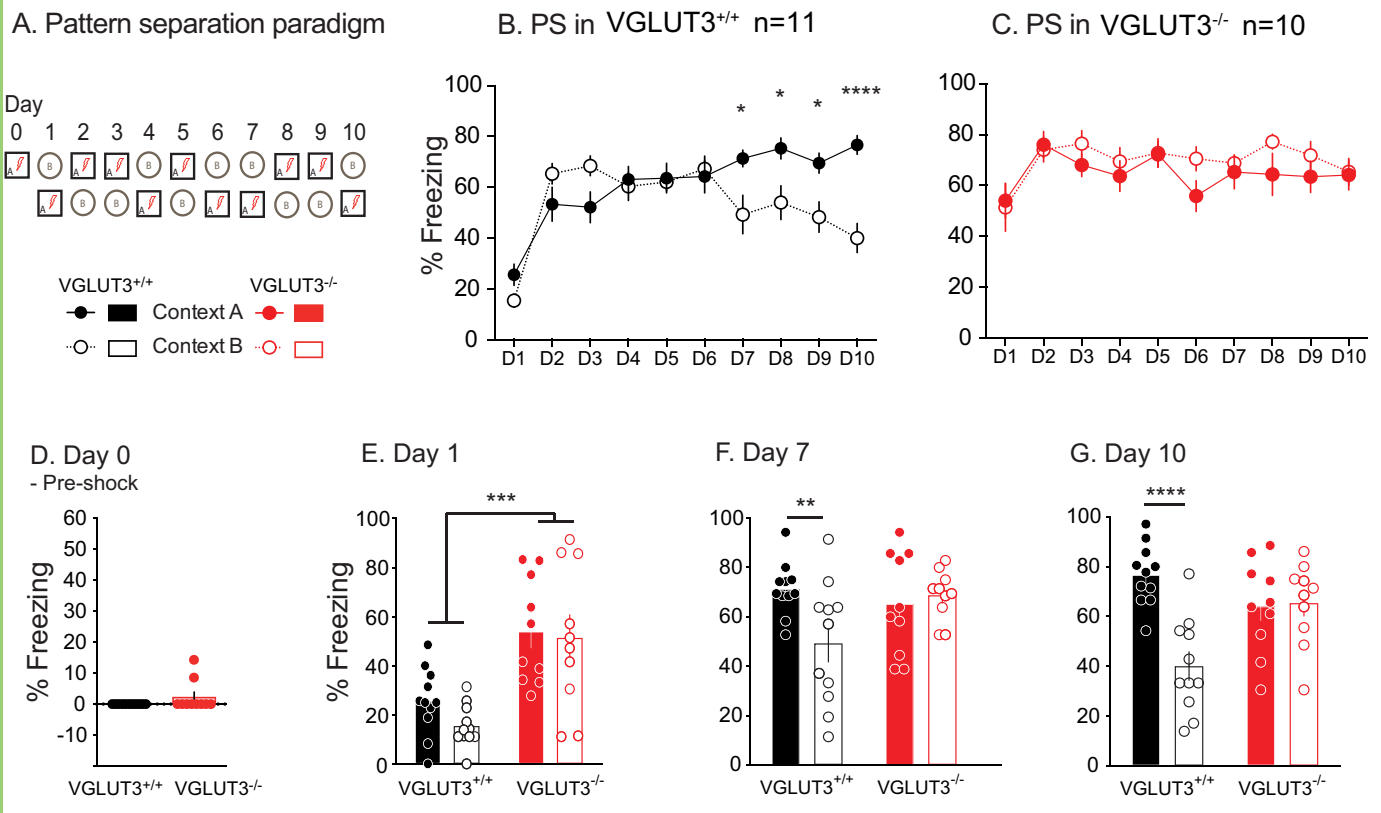
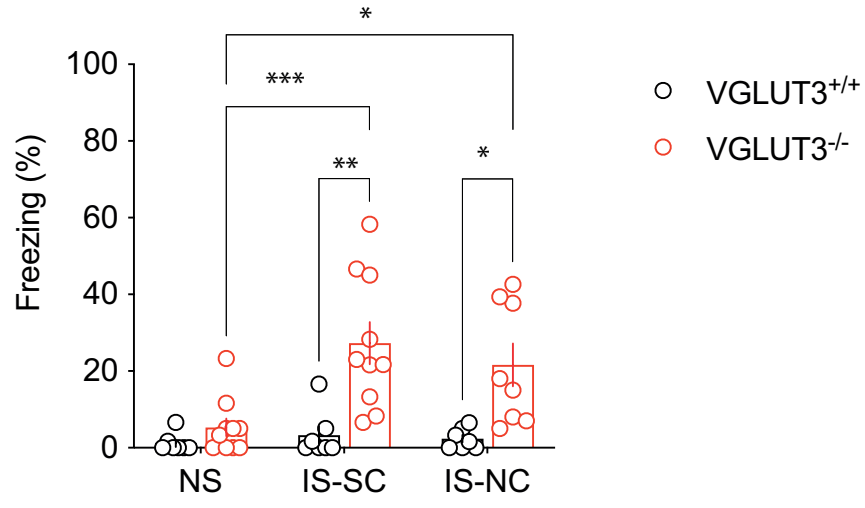


Figure 4



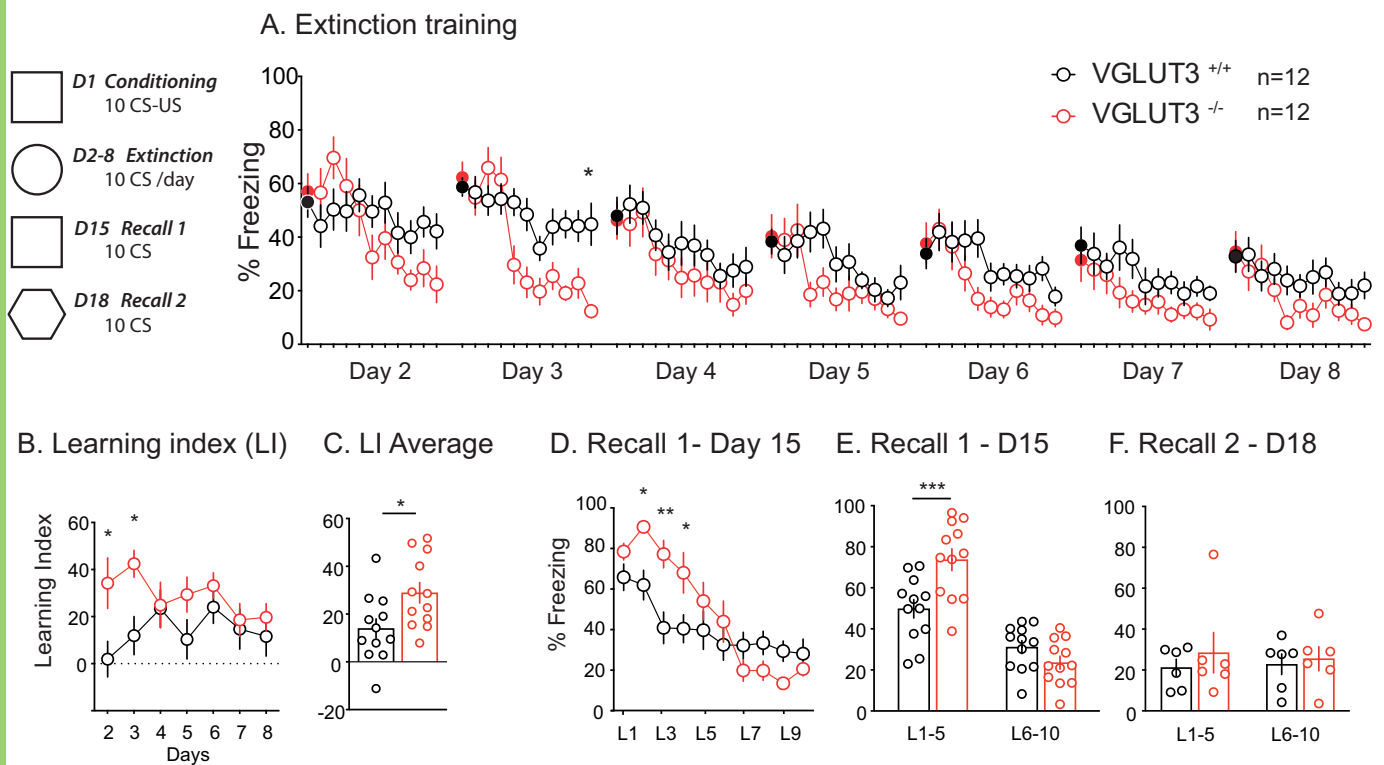


Figure 4

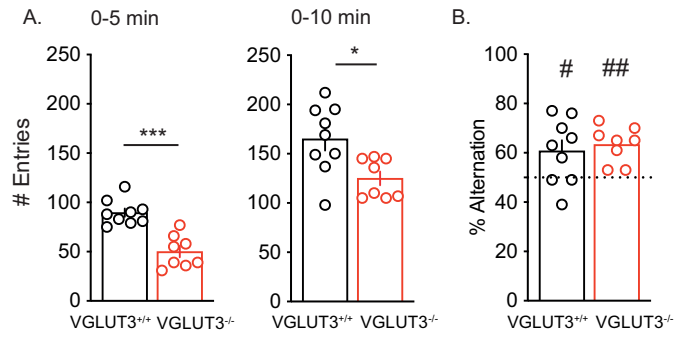


Figure 7