1   Title Page

2   **Anterior cingulate cortex and ventral hippocampal inputs to the basolateral**

3   **amygdala selectively control generalized fear**

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8   **Abbreviated Title:** ACC AND VHPC INPUTS TO BLA IN FEAR GENERALIZATION

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26   **Abstract**

27   Nearly one third of Americans have been afflicted with an anxiety disorder. A common

28   symptom of anxiety disorders is the over generalization of fear across a broad range of

29   contextual cues. We previously found that the anterior cingulate cortex and ventral

30   hippocampus (vHPC) regulate generalized fear. Here, we investigate the functional

31   projections from the ACC and vHPC to the amygdala and their role in governing

32   generalized fear in a preclinical rodent model. A chemogenetic approach (DREADDs)

33   was used to inhibit glutamatergic projections from the ACC or vHPC that terminate

34   within the basolateral amygdala (BLA) at recent (1 day) or remote (28 days) time points

35   after contextually fear conditioning male mice. Inactivating ACC or vHPC projections to

36   the BLA significantly reduced generalized fear to a novel, nonthreatening context but

37   had no effect on fear to the training context. Further, our data indicate that the ACC-

38   BLA circuit supports generalization in a time-independent manner. We also identified for

39   the first time a strictly time-dependent role of the vHPC-BLA circuit in supporting remote

40   generalized contextual fear. Dysfunctional signaling to the amygdala from the ACC or

41   the hippocampus could underlie over-generalized fear responses that are associated

42   with anxiety disorders. Our findings demonstrate that the ACC and vHPC regulate fear

43   expressed in novel, nonthreatening environments via projections to the BLA but do so

44   as a result of training intensity or time, respectively.

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47   **Significance Statement**

48   Anxiety disorders are characterized by a common symptom that promotes

49   overgeneralization of fear in non-threatening environments. Dysregulation of the

50   amygdala, anterior cingulate cortex (ACC), or hippocampus has been hypothesized to

51   contribute to increased fear associated with anxiety disorders. Our findings show that

52   the ACC and HPC projections to the basolateral amygdala regulate generalized fear in

53   non-threatening, environments. However, descending ACC projections control fear

54   generalization independent of time, whereas HPC projections play a strictly time-

55   dependent role in regulating generalized fear. Thus, dysfunctional ACC/HPC signaling

56   to the BLA may be a predominant underlying mechanism of non-specific fear associated

57   with anxiety disorders. Our data have important implications for predictions made by

58   theories about aging memories and interactions between the hippocampus and cortical

59   regions.

60   **Introduction**

61   Exposure to stressful events can precipitate anxiety disorders, which afflict

62   approximately one third of the adult U.S. population (Kessler et al., 2012). A debilitating

63   symptom of many anxiety disorders is the overgeneralization of fear (Dymond et al.,

64   2015; Morey et al., 2015), manifesting as hyperarousal across a range of contexts that

65   are not associated with any aversive event (Lissek et al., 2005; 2010). Moreover,

66   people with anxiety disorders have hyperreactive amygdalae (Shin et al., 2004; 2006)

67   along with decreased anterior cingulate cortex (ACC) (Yamasue et al., 2003; Woodward

68   et al., 2006; Asami et al., 2008; Greenberg et al., 2013) and hippocampal volumes

69   (Gurvits et al., 1996; Shin et al., 2006; Chen and Etkin, 2013). Although these regions

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70   are associated with anxiety disorders, there is no evidence demonstrating how these

71   brain areas interact to support over-generalization of fear, leading to the maintenance of

72   anxiety symptomology. In this study, we explore generalized fear – fear occurring in

73   non-threatening contexts – using a preclinical rodent model to identify if glutamatergic

74   projections from the ACC and/or hippocampus to the amygdala regulate generalized

75   fear.

76   Rodent models of context fear learning have been used for decades to study the

77   underlying mechanisms of fear generalization (for review see Jasnow et al., 2012; 2016;

78   Asok et al., 2018). Twenty-four hours after training mice to fear a context with specific

79   cues, if placed back in the training context, mice display high levels of freezing – a

80   fundamental rodent fear response. If mice are instead placed in a novel context that is

81   different from the training context, they display low levels of freezing, indicating little fear

82   to the novel context. As the time interval between training and testing increases, mice

83   freeze in the novel context at similar levels to those in the training context, generalizing

84   fear to the novel, non-threatening context.

85   Time-dependent generalized fear is thought to rely on cortical regions (Frankland

86   et al., 2004b; Einarsson et al., 2015), independent of the hippocampus whereas fear

87   responses to specific contexts – *specific fear* – are reliant on the hippocampus (Zola-

88   Morgan and Squire, 1990; Frankland et al., 1998; 2004a; Teyler and Rudy, 2007;

89   Winocur et al., 2007; Wiltgen et al., 2010). We previously identified that generalized

90   fear is simultaneously dependent on the ACC and the ventral hippocampus (vHPC);

91   inactivation of either region reduced fear in a novel, non-threatening context, but left

92   fear to the training context unaltered (Cullen et al., 2015).

93   Although the ACC and hippocampus are implicated in anxiety disorders (see

94   above citations) and generalized fear (Einarsson and Nader, 2012; Cullen et al., 2015;

95   Zhou et al., 2017), little is known about the circuits through which they govern

96   generalized fear responses. A single study found that circuits connecting the ACC and

97   vHPC in the nucleus reunions are necessary for the learning of specific fear (Xu and

98   Südhof, 2013) – inactivating these circuits prior to training induces rapid fear

99   generalization. However, how the ACC and vHPC outputs govern temporally graded

100   generalized fear during recall is completely unknown. The ACC and vHPC each

101   communicate with the basolateral amygdala (BLA) (Maren and Fanselow, 1995;

102   Cenquizca and Swanson, 2007; Morozov et al., 2011) – a critical region for fear

103   acquisition and expression (Kim and Fanselow, 1992; Kim et al., 1993; Campeau and

104   Davis, 1995; Maren et al., 1996; Schafe et al., 2005; Do-Monte et al., 2016). Thus, we

105   hypothesize that ACC and vHPC projections converge within the BLA to regulate time-

106   dependent contextual generalization of fear.

107   To identify if ACC and vHPC projections to the BLA regulate generalized fear, we

108   used DREADDs (Armbruster et al., 2007), to selectively express the modified human

109   muscarinic acetylcholine receptor 4 (hM4D) within the ACC or vHPC. We found new

110   evidence that inactivation of ACC or vHPC projections in the BLA dramatically

111   attenuated generalized fear in time-independent and time-dependent processes,

112   respectively; specific fear was unaltered. Our findings suggest that over-generalization

113   of fear in people with anxiety disorders may result from hyperreactive amygdalae due to

114   dysfunctional signaling from the ACC or hippocampus.

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Materials and Methods

Subjects

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117   Experiments 1 (Fig. 1B), 2 (Fig. 1C-G), 3 (Fig. 2), 5 (Fig. 4), and 6 (Fig. 5) used

118   224 C57BL/6J male mice. Experiments 4 (Fig 3) and 7 (Fig 6) used 87 F1 male hybrids

119   generated from crossing C57BL/6 males and 129S1SvImJ females (Jackson). All mice

120   were generated from a breeding colony in the Department of Psychological Sciences at

121   Kent State University. Mice were five to seven weeks of age before they were used for

122   experimentation and were group housed (2-5 mice per cage) with free access to food

123   and water in a room maintained on a 12:12 light/dark cycle. All procedures were

124   conducted in a facility accredited by the AALAC, in accordance with the NIH guidelines,

125   and with approval by Kent State University IACUC guidelines.

Surgical Procedures

127   Mice were anesthetized with a subcutaneous injection of a Ketamine (75 mg/kg)

128   + Xylazine (10 mg/kg) + Acepromazine (2 mg/kg) cocktail. Following administration of

129   anesthesia, mice were mounted on a stereotaxic apparatus (David Kopf Instruments,

130   Tujunga, CA). The scalp of each mouse was retracted; the skull was adjusted so that

131   bregma and lambda were on the same horizontal plane (within .05mm of each other).

132   Two 0.33 gauge infusion needles were guided to the appropriate coordinates relative to

133   bregma and small bilateral burr holes were drilled. Coordinates for the following brain

134   regions were ACC: .08 mm AP, $\pm$.07 mm ML, -3.6 mm DV from bregma at a 14° angle;

135   vHPC: -3.2 mm AP, $\pm$3.3 mm ML, -4.25 mm DV from bregma. AAV8-CaMKIIa-

136   hM4D(Gi)-mCherry virus (hM4D) (Addgene) or a control virus under the same promoter,

137   AAV8-CamKIIα-EGFP (EGFP) (Addgene) was bilaterally infused at 0.1μL/minute to a

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138   total infusion volume of 0.25μL and the needle was left in place for five minutes after

139   completion of the infusion. Upon completion of the virus infusion, the anesthesia was

140   reversed with a subcutaneous injection of atipamezole (0.5 mg/kg).

141   All behavioral testing was completed seven weeks after viral infusions in order to

142   control for viral expression. Cannulations for the BLA were completed one week prior to

143   behavioral training procedures, controlling for recovery time between the final surgery

144   and the start of behavioral training. Mice were anesthetized and mounted on a

145   stereotaxic apparatus with the same surgical procedures as described above. Two

146   guide cannulae (Plastics One, Roanoke, VA) were surgically implanted bilaterally above

147   the basolateral amygdala (-1.6 mm AP, ±3.4 mm ML, -4.9 mm DV from bregma).

148   Dummy cannulae were inserted into the guide cannulae after surgery. For viral spread

149   analysis and drug targeting for each experiment see figures: 1E,F, and H, 2D-E, 3D-E,

150   4D, 5D-E, 6A-B.

151   **Fear Conditioning**

152   Fear conditioning was performed in four identical conditioning chambers (7" W x

153   7" D x 12"H) containing two Plexiglas walls, two aluminum sidewalls, and a stainless-

154   steel grid-shock floor (Coulbourn Instruments, Allentown, PA). The training context

155   consisted of the conditioning chamber with a polka-dot insert attached to the rear

156   Plexiglas wall, white noise (70db), dim illumination, and the stainless-steel grid floors

157   were cleaned with 70% ethanol. The novel context consisted of the conditioning

158   chamber with no visible illumination (illuminated only with an infrared light), fan, and flat

159   brown Plexiglas floors which were cleaned with 50% Quatricide.

160   Mice were pre-exposed to the context for five minutes on the two days prior to

161   fear conditioning. Fear conditioning occurred in the training context with five unsignaled

162   footshocks (1s, 1.0 mA), each separated by 90s, mice were removed from the

163   apparatus 30s after the last shock and returned to their home cage. After training, we

164   conducted a 5-minute test in either the training context or the novel context at 24 hours

165   or 28 days after training.

166   Mice were given 5mg/kg intraperitoneal (IP) injections of clozapine-n-oxide

167   (CNO) (Cayman Chemical) or saline 30 minutes prior to testing in the CNO control

168   experiments and systemic inactivation studies. The dose of 5mg/kg was selected due

169   to common IP injection doses used for DREADD experiments and has shown to have

170   reduced effects on behavior in naïve mice (MacLaren et al., 2016; Jendryka et al.,

171   2019). In experiments in which mice were given a localized infusion of CNO (0.2μL of

172   650μM at 0.1μL/min), a concentration within the range of those previously reported

173   (Mahler et al., 2014; Vazey and Aston-Jones, 2014; Scofield et al., 2015), the drug was

174   infused five minutes prior to testing in order to inactivate ACC or vHPC projections

175   terminating in the basolateral amygdala. The within-subject fear testing used F1 hybrids

176   in the same training procedures as described previously with counterbalanced testing.

177   F1 hybrids were tested in both the training and novel contexts for five minutes with 72-

178   hours between testing. Five minutes prior to each test, F1 hybrids were given intra-BLA

179   infusions of CNO as previously described.

180   **Histology**

181   Mice were deeply anaesthetized with pentobarbital sodium and perfused

182   transcardially with 0.9% saline followed by 4% paraformaldehyde. After perfusion,

183   0.2μL of 0.5% neutral red solution was infused into the guide cannulae for site

184   verification of BLA targets then the brains were extracted. After extraction, brains were

185   post-fixed in 4% paraformaldehyde for 24-hours then transferred to 30% sucrose

186   solution until sectioning. Coronal sections (40μm thick, taken every 120μm) were cut on

187   a freezing microtome, mounted on glass microscope slide, and cover slipped with

188   MOWIOL mounting medium containing 2.5% DABCO before visualization. All imaging

189   was completed on a Nikon Eclipse Ti-S using a Nikon Intensilight C-HGFIE mercury

190   lamp in conjunction with FITC, and Cy3 filters and analyzed using NIS Elements

191   Software. Exclusion criteria for experiments include: unilateral expression of hM4D

192   within the ACC or vHPC or no expression within the vCA1 of the hippocampus. One

193   mouse was excluded due to hM4D cell body expression that significantly exceeded the

194   boundaries of the ACC into the motor cortex. No expression outside of the vHPC was

195   observed.

196   **Statistical Analyses**

197   Mean freezing during contextual fear testing were analyzed using a 2x2 factorial

198   analysis of variance (ANOVA) on Prism Graphpad statistical software. Statistically

199   significant ANOVAs were followed up with Tukey HSD post hoc comparisons. BLA

200   target comparisons were analyzed using a non-parametric Mann-Whitney t-test on

201   Prism Graphpad. Effect sizes were calculated for completed experiments along with

202   post-hoc power analyses using G*Power 3. Refer to **Tables 1-6** for detailed statistical

203   results for each experiment.

204   **Results**

205   **Clozapine-n-oxide administration in naïve animals has no effect on context fear**

206   **generalization**

207   Prior to the start of neuronal manipulation with the DREADD system, we tested

208   for non-constitutive effects of CNO on fear generalization. Non-DREADD-infused mice

209   were context fear conditioned and tested in the training context or a distinct novel

210   context where they had not been previously exposed (Fig. 1A) either one or 28 days

211   after training; 30 minutes prior to testing mice were administered CNO or saline. CNO

212   and saline controls displayed high levels of freezing to the training context and

213   significantly lower freezing levels in the novel context at the immediate time point

214   indicating no effect of CNO on normal freezing in either context (Tables 1, 2; Fig. 1B).

215   Furthermore, CNO had no effect on freezing at the remote test; all mice displayed high

216   freezing levels in the training and novel context (Table 1; Fig. 1B). These data indicate

217   that CNO alone, or its potential reverse metabolism to clozapine (Gomez et al., 2017),

218   has no effect on freezing to a specific or generalized context. Thus, any effects

219   observed on fear generalization in the following experiments are due to hM4D receptor

220   inactivation in the targeted region.

221   **The anterior cingulate cortex – basolateral amygdala circuit controls time-**

222   **independent generalized fear**

223   Our initial finding that the ACC plays a critical role in the generalization of context

224   fear (Cullen et al., 2015) was upheld using hM4D inactivation. hM4D-mediated

225   inactivation of the ACC with a systemic injection of CNO eliminated generalized fear to

226   the novel context, but not specific fear to the training context (Tables 3 and 4; Fig. 1G).

227   Therefore, we used the hM4D system with intracranial infusions of CNO to identify the

228   precise ACC circuit that regulates fear generalization. The ACC is known to convey

229   sensory information to the BLA (Morozov et al., 2011; McCullough et al., 2016);

230   therefore, we targeted ACC projection terminals in the BLA.

231   Mice with hM4D or EGFP virus in the ACC were context fear conditioned; five

232   minutes prior to testing all mice were administered intracranial infusions of CNO via

233   guide cannulae into the BLA (Fig 2A-C). Inactivation of the hM4D-expressing terminals

234   from the ACC in the BLA did not affect freezing in the training or novel context during

235   the immediate test, both hM4D and EGFP groups displayed high freezing in the training

236   context and low freezing in the novel context (Tables 3 and 4; Fig 2F, left panel).

237   However, inactivating ACC terminals in the BLA significantly reduced freezing only in

238   the novel context 28 days after training (Fig. 2F, right panel), whereas EGFP mice

239   displayed equivalent freezing in the training and novel contexts – indicating generalized

240   fear. The reduction of fear generalization in hM4D mice was specific to terminal

241   inactivation within the BLA; hM4D mice with extra-BLA infusions froze significantly more

242   in the novel context than those with intra-BLA infusions (Table 3; Fig. 2G). Thus, we

243   established that projections from the ACC to the BLA are critical for promoting

244   generalized fear at remote testing points.

245   Are the ACC projections to the BLA that support generalized fear restricted solely

246   to remote tests? If generalization occurs rapidly, does the ACC-BLA circuit still control

247   generalization? Based on our previous findings (Cullen et al., 2015) and the

248   experiments above, we predicted that ACC projections to the BLA would only support

249   generalized fear that develops over time. In the third experiment we used the F1

250   hybrids of C57BL/6J crossed with 129S1/SvImJ – a hybrid mouse line used by several

251   laboratories to study mechanisms of contextual fear (Frankland et al., 2004b; Smith et

252   al., 2007; Wiltgen and Silva, 2007; Wiltgen et al., 2010; Tanaka et al., 2014) due to their

253   rapid learning and high reliability in fear learning. This gave us the advantage of

254   ensuring that our experimental results were not restricted to C57BL/6J mice, as there is

255   considerable variability in learning and behavior across mouse lines (Hefner et al.,

256   2008). We first performed behavioral parametrics with the F1 hybrid line and found a

257   significant effect of number of shocks on the timing of generalization (Table 1; Fig. 3A).

258   Hybrid mice displayed high levels of freezing in the novel context one day after training

259   if the mice received five footshocks, yet this was not observed if the mice received only

260   three footshocks (Table 1; Fig. 3A); thus, providing a novel opportunity to study the role

261   of the ACC-BLA-vHPC circuit in non-temporally graded generalization.

262   Experimental procedures were carried out as described in experiment two;

263   however, mice were tested a second time 72-hours after the first test in the opposite

264   context to reduce potential testing-order effects and allow for CNO to be completely

265   metabolized before the second test (Fig. 3B and C). Hybrid mice with EGFP virus

266   displayed increased freezing in the novel context during immediate and remote tests

267   (Fig 3F, left panel). Unexpectedly, hM4D inactivation of the projections from the ACC to

268   the BLA at both the immediate and remote tests reduced freezing in the novel context

269   but not in the training context (Table 3 and 4; Fig. 3F), indicating that projections from

270   the ACC to the BLA promote freezing to a novel context in a time-independent manner.

271   The ACC-BLA pathway controls generalized fear to the novel context but not specific

272   fear to the training context; this effect is upheld across mouse strains and experimental

273   testing designs.

The ventral hippocampus - basolateral amygdala circuit controls time-dependent

generalized fear

276   In addition to identifying the ACC as a critical locus supporting generalized

277   contextual fear, we previously identified that the vCA1 of the hippocampus also

278   underlies generalized contextual fear at remote time points (Cullen et al., 2015). This

279   finding was replicated by using hM4D to inactivate the vHPC. Inactivation of the vHPC

280   with a systemic injection of CNO significantly reduced generalized fear to the novel

281   context but not specific fear to the training context at a remote time point (Tables 5 and

282   6; Fig. 4C). As done with Experiment 2, we used intracranial infusions of CNO to

283   identify the vHPC circuit that regulates fear generalization. Given that the vCA1 of the

284   hippocampus has direct connections with the BLA (Cenquizca and Swanson, 2007;

285   Fanselow and Dong, 2010) and is thought to be crucial for conveying contextual

286   information to the BLA (Maren and Fanselow, 1995; Huff et al., 2016) we targeted vHPC

287   projections terminating in this region.

288   Mice with hM4D virus or EGFP control virus in the vHPC were context fear

289   conditioned; five minutes prior to testing all mice were given intracranial infusions of

290   CNO via guide cannulae into the BLA (Fig. 5A-C). Inactivation of hM4D terminals from

291   the vHPC in the BLA did not affect freezing in the training or novel context during the

292   immediate test; both hM4D and EGFP groups displayed high freezing in the training

293   context and low freezing in the novel context (Tables 5 and 6; Fig. 5F, left panel). When

294   mice were tested 28 days after training, EGFP-expressing mice displayed equivalent

295   freezing levels in the training and novel contexts (Tables 5 and 6; Fig. 5F, right panel) –

296   indicating generalized fear. hM4D inactivation of the vHPC terminals in the BLA

297   significantly reduced freezing in the novel context but did not alter freezing in the

298   training context. Again, this effect observed in hM4D-expressing mice was specific to

299   projections from the vHPC terminating in the BLA. HM4D mice with targets outside of

300   the BLA froze significantly more in the novel context at a remote time point than those

301   with correct target placement within the BLA even though they both expressed hM4D

302   and received intracranial CNO infusions (Table 5; Fig. 5G). These findings indicate that

303   activity of vHPC projections – likely via vCA1 outputs (Cenquizca and Swanson, 2007;

304   Cullen et al., 2015) – to the BLA promote generalized fear, but only at a remote time

305   point.

306   Are the vHPC projections to the BLA that support generalized fear restricted to

307   remote tests? As with Experiment 3, during the immediate test EGFP F1 hybrids

308   displayed increased freezing in the novel context (Tables 5 and 6; Fig. 6E, left panel) –

309   displaying immediate fear generalization. However, unlike the results from ACC-BLA

310   circuit, inactivation of vHPC terminals in the BLA at the immediate time point did not

311   reduce freezing in the novel context nor the training context; reduced generalization was

312   only observed at the remote time point (Tables 5 and 6; Fig. 6E). Given that our

313   previous tests in the novel context at the immediate time point had a floor effect, these

314   experiments identified for the first time a strictly time-dependent role of the vHPC-BLA

315   circuit in supporting generalized contextual fear. Conversely, the ACC governs

316   generalization at both recent and remote tests. Thus, our evidence supports a role for

317   the ACC in supporting generalized fear regardless of the passage of time, whereas the

318   vHPC is engaged in support of generalized fear only at a remote time point.

319   **Discussion**

320   Clinical studies implicate that the hyperreactive amygdalae observed in people

321   with anxiety disorders may be due to an inhibitory dysregulation caused by a

322   malfunctioning anterior cingulate cortex and hippocampus (Gurvits et al., 1996;

323   Yamasue et al., 2003; Shin et al., 2006; Woodward et al., 2006; Asami et al., 2008;

324   Chen and Etkin, 2013; Greenberg et al., 2013). These studies are limited in making

325   causal conclusions about connectivity, as they associate hyperactive amygdalae with

326   decreased volume and activity of the ACC or hippocampus. Here, we identified causal

327   relationships that fear to novel contexts is in fact regulated by the glutamatergic,

328   CamKIIα-expressing projection neurons from the ACC and vHPC to the BLA but via

329   separate training and time-dependent mechanisms. The regulation of generalized fear

330   by projections from the ACC to the BLA is a time-independent effect that may depend

331   on the strength of the training based on our finding that 5-shock, not 3-shock, training

332   induced immediate generalization. These findings support recent hypotheses that

333   propose that the ACC regulates generalized fear responses (Teyler and Rudy, 2007;

334   Winocur et al., 2007; Einarsson and Nader, 2012; Cullen et al., 2015), but not specific

335   fear responses. The time-independent mechanism of the ACC-BLA connection is in

336   contrast to what we observed with the vHPC. When we induced rapid generalization,

337   inactivation of projections from the vHPC to the BLA did not reduce freezing in the novel

338   context. Generalization was only eliminated when the vHPC-BLA circuit was inactivated

339   at a remote time point. Thus, the vHPC-BLA circuit plays a specific role in time-

340   *dependent* generalization of contextual fear.

341   We have consistently observed a role for the ACC that is specific to generalized

342   fear responding (Cullen et al., 2015), and this is supported by other recent work

343   (Einarsson et al., 2015). We note two prior studies which found that the ACC regulates

344   specific fear responses at remote time points after training (Frankland et al., 2004a;

345   Goshen et al., 2011). In one case, this discrepancy could be due to specific

346   methodological differences during testing; we performed local intracranial infusions of

347   CNO without anesthetizing mice prior to testing unlike the previous study (Frankland et

348   al., 2004a). In the other case, the authors performed tone-dependent fear training with

349   context as background and used multiple recall tests in the same context (Goshen et

350   al., 2011). Here, we used unsignaled shocks to train specifically for contextual fear and

351   mice were only tested in a single context once. This discrepancy provides evidence

352   that ACC regulation of fear responses is related to the strength – and type – of the fear

353   training. This was not the case for the role of the vHPC in generalized fear responding.

354   For decades, the focus of identifying neural mechanisms of fear responding has

355   been the dorsal hippocampus (dHPC), and much of the current theory is based on

356   experiments within this region (Squire and Alvarez, 1995a; Frankland et al., 1998;

357   Teyler and Rudy, 2007; Winocur et al., 2007; Wiltgen et al., 2010; Hardt et al., 2013;

358   Winocur et al., 2013). Notably, the experiments described here, and our previous study

359   (Cullen et al., 2015), are the only studies to date examining the role of vHPC in

360   generalized fear responses. Generalized, remote fear responses require the vHPC;

361   whereas the dHPC is crucial for maintaining specific fear responses (Frankland et al.,

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362   1998; Wiltgen et al., 2010; Winocur et al., 2013; Cullen et al., 2015). Over time, activity

363   of the vHPC and its projections to the BLA exert greater control over generalized fear

364   rather than maintaining control over specific fear, like the dHPC. Our vHPC results also

365   emphasize that there is a dissociation between the roles of the ventral and dorsal

366   hippocampus in the control of fear processing, an effect that has support from

367   neuroanatomical and connectivity studies (Fanselow and Dong, 2010), but limited

368   systems and behavioral support (Morris, 1981; Maren and Holt, 2004; Hunsaker and

369   Kesner, 2008). The present data also have important implications for predictions that

370   are made by theories about aging fear memories and interactions between the

371   hippocampus and cortical regions (Squire and Alvarez, 1995a; Teyler and Rudy, 2007;

372   Winocur et al., 2007; Hardt et al., 2013).

373   Systems consolidation hypothesizes that memories stored in the neocortex are

374   identical to those encoded by the hippocampus and does not address time-dependent

375   changes in memory specificity (Squire and Alvarez, 1995a). Our previous (Cullen et al.,

376   2015) and current findings challenge the view that neocortical stored memories are

377   identical to those stored in the hippocampus. In addition, our data suggest that aged

378   memories continue to be dependent on the hippocampus, albeit control shifts to the

379   ventral region. Another memory hypothesis suggests that specific memories are initially

380   dependent on the hippocampus and are transformed to schematic – generalized –

381   memories as they are stored in the neocortex, called the transformation hypothesis

382   (Winocur et al., 2007; 2013), which stems from Multiple Trace Theory (Nadel and

383   Moscovitch, 1997). In the transformation hypothesis, both the schematic memory and

384   the specific memory are continuously accessible; however, specific memories are

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385   *always* dependent on the hippocampus whereas generalized memories are dependent

386   on the neocortex as they are transformed over time– independent of the hippocampus.

387   Therefore, at remote time points there can be two memory traces and either can be

388   accessed depending on the situational requirements.

389   Our data challenge the transformation hypothesis' notion that neocortical regions

390   control generalized memories as a function of the training-to-testing interval; our data

391   here show that memories may be immediately stored in a generalized state within the

392   ACC. Experiments employing immediate post-training inactivation of the ACC followed

393   by a test for generalization within a novel context are needed in order to confirm the

394   immediate storage hypothesis. Thus, our current data support the neocortex's

395   involvement in generalized memories, but not that generalized memories are

396   transformed over time, or that they are independent of the hippocampus.

397   Studies in full support of the transformation hypothesis thus far have not found

398   evidence for a functional dissociation between the dorsal and ventral hippocampus on

399   generalization (Winocur et al., 2007; 2009), suggesting that the hippocampus - as a

400   whole - is not required for generalized memory recall. Here, we discovered that

401   immediately generalized memories do not require the vHPC, whereas remote

402   generalized memories do – showing an opposite role of that of the dHPC. Thus, our

403   data, in combination with recent findings (Lynch et al., 2017; Zhou et al., 2017), suggest

404   that transformation of a specific fear memory into a generalized form may actually

405   involve a shift in control over memory recall from the dHPC to the vHPC over time.

406   Utilizing chemogenetics, we reliably replicated the effects of the ACC and vHPC

407   regulating fear generalization via projections to the BLA; however, there have been

408   recent validity threats to the DREADD system. The DREADD activator – CNO – may

409   be reverse metabolized into clozapine with widespread effects and non-specific binding

410   of the DREADD receptor (MacLaren et al., 2016; Whissell et al., 2016; Gomez et al.,

411   2017; Manvich et al., 2018). To control for potential off-target effects of CNO, we fear

412   conditioned naïve mice and tested them 30 minutes after an injection of CNO or saline.

413   We found no effect of CNO on contextual fear or the generalization of contextual fear –

414   eliminating the potential confound of CNO specifically for our paradigm. Additionally,

415   intracranial infusions of CNO directly into the BLA replicated the systemic DREADD

416   inactivation findings, and mice expressing hM4D with targets outside the BLA displayed

417   normal freezing behavior in the novel context. Although one study reported off target

418   effects with lower a concentration of CNO when locally infused near the hypothalamus

419   (Stachniak et al., 2014), the small volume of the infusions used here (0.2µL), and the

420   lack of any behavioral effect when CNO was infused outside of the BLA strongly

421   suggests that our observed results were not due to off target effects of CNO – or its

422   reversal into clozapine – and that the effects were specific to inactivation of axonal

423   projections terminating in the BLA. A few reports suggest that CNO must be first

424   converted into clozapine in order to cross the blood brain barrier and exert its effects

425   (Bender et al., 1994; Gomez et al., 2017). Our intra-BLA infusions surpass the blood-

426   brain barrier, therefore CNO – not clozapine – in experiments 3, 4, 6, and 7 specifically

427   acted on the DREADD receptors in virally infused mice.

428   These findings help to uncover part of the neural connectome involved in both

429   specific and general fear responses, which is critical for understanding how humans and

430   non-humans alike express fearful responses in safe environments (pathological

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431   generalization). Clinical research hypothesizes that reduced volume of the ACC and

432   hippocampus restricts normal inhibitory function on the amygdala leading to increased

433   fear responding (Gurvits et al., 1996; Schuff et al., 2001; Yamasue et al., 2003; Shin et

434   al., 2006; Woodward et al., 2006; Asami et al., 2008; Chen and Etkin, 2013; Greenberg

435   et al., 2013). Our findings confirm that the ACC and hippocampus, specifically the

436   vHPC, regulate fear in novel, or non-threatening, environments through their outputs to

437   the amygdala. Furthermore, they do so in a functionally different manner. The ACC

438   time-independently controls generalization, whereas the vHPC plays a strictly time-

439   dependent role in regulating generalized fear. Thus, dysfunctional signaling to the BLA

440   from the ACC or hippocampus – in combination or alone – may be a predominant

441   underlying mechanism of non-specific fear responses associated with anxiety disorders

442   in clinical populations.

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646 **Figure Legends**

647 **Figure 1. Inactivation of the anterior cingulate cortex eliminates time-dependent**

648 **generalized context fear.**

649 **(A)** All mice underwent context fear conditioning which consisted five unsignaled

650 footshocks (1s, 1.0 mA), each separated by 90s, in the training context which

651 included the conditioning chamber with a polka-dot insert attached to the rear

652 Plexiglas wall, white noise (70db), dim illumination, and the stainless-steel grid

653 floors were cleaned with 70% ethanol. One day or 28 days after training mice

654 were either placed back in the training context or a distinct novel context which

655 included the conditioning chamber with a small exhaust fan, and flat brown

656 Plexiglas floors which were cleaned with 50% Quatricide. There was no visible

657 illumination (illuminated only with an infrared light), and no polka-dot wall insert.

658 **(B)** There was no effect of CNO alone on context dependent fear behavior. As a

659 CNO control experiment, naïve mice were context fear conditioned and given an

660 IP injection of CNO or saline 30 minutes prior to testing either 1 or 28 days after

661 training. Percent freezing levels of animals that received saline (filled symbols)

662 or CNO (open symbols) during immediate (circles) and remote (squares) tests in

663 the training or neutral context were analyzed ($\pm$ SEM). Two-way ANOVA

664 identified a significant main effect of context at the immediate time point, $F_{(1,12)}$ =

665 96.40, p< 0.001, but not at the remote time point; mice froze significantly more in

666 the training context than the novel context at 1 but not 28 days after training. ***,

667 significantly different from animals tested in training context, p < 0.001.

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668   **(C)** On the first day of the experimental procedures, pAAV-CaMKIIa-hM4D(Gi)-

669   mCherry virus (hM4D) or pAAV-CamKIIa-EGFP (EGFP) was bilaterally infused

670   into the anterior cingulate cortex (ACC). All behavioral tests were completed

671   seven weeks after viral infusions. For the immediate test, mice were tested 1

672   day after training,

673   **(D)** whereas mice tested at the remote time were tested 28 days after training. All

674   mice were given an IP injection of CNO 30 minutes prior to testing.

675   **(E)** Analysis of transgene expression in all hM4D infusions into the ACC for mice

676   tested with systemic injection of CNO. No expression was observed outside of

677   the ACC for systemic inactivation. Dark red: minimum spread observed and

678   included in analysis; red: representative spread observed; light red: maximum

679   spread observed and included in behavioral analysis.

680   **(F)** Representative image of pAAV-CaMKIIa-hM4D(Gi)-mCherry expression in the

681   ACC. Expression of mCherry was observed throughout the ACC and was typical

682   of a membrane bound fluorophore. White arrows indicate fiber tracts exiting the

683   ACC towards the corpus callosum.

684   **(G)** hM4D mice administered CNO froze significantly less than EGFP control mice in

685   the novel context only during the remote test, suggesting that inactivation of the

686   ACC eliminates generalized fear at a remote time point. Percent freezing levels

687   of EGFP (○) and hM4D (●) mice during immediate (left panel) and remote (right

688   panel) tests in the training or neutral context were analyzed ($\pm$ SEM). Two-way

689   ANOVA identified a significant main effect of context at the immediate time point,

690         $F_{(1,16)} = 64.2$, p< 0.001, and a the remote time point $F_{(1,17)} = 52.3$, p<.001; mice

691   froze more in the training context than the novel context. However, there was a

692   significant context x treatment interaction only at the remote time point, $F_{(1,17)}$ =

693   4.64, p< 0.05. *p < 0.05, **p < 0.01, *** < 0.001.

694   **(H)** Representative image of pAAV-CaMKIIa-hM4D(Gi)-mCherry expression in the

695   BLA in a mouse that had virus infused into the ACC. Robust expression of

696   mCherry was observed in the external capsule fibers entering the basolateral

697   amygdala.

698   **Figure 2. Inactivation of anterior cingulate cortex CamKIIα projections in the**

699   **basolateral amygdala eliminates time-dependent generalized fear.**

700   **(A)** To identify if the ACC regulates fear generalization via CamKIIα projections to

701   the BLA, pAAV-CaMKIIa-hM4D(Gi)-mCherry virus (hM4D) or pAAV-CamKIIa-

702   EGFP (EGFP) was bilaterally infused into the anterior cingulate cortex (ACC)

703   followed by cannulations targeting their axon terminals in the BLA.

704   **(B)** All behavioral tests were completed seven weeks after viral infusions.

705   Cannulations for the BLA were completed one week prior to behavioral training

706   procedures. Mice were tested 1 day or

707   **(C)** 28 days after training. All mice were given a local infusion of CNO into the BLA 5

708   minutes prior to testing to inactivate ACC CamKIIα projections.

709   **(D)** Analysis of transgene expression in all hM4D mice tested with inactivation of BLA

710   terminals. One mouse was excluded from analysis due to significant hM4D

711   expression in the motor cortex. Dark red: minimum spread observed and

712   included in analysis; red: representative spread observed; light red: maximum

713   spread observed and included in behavioral analysis.

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714   **(E)** Cannulation targets within the BLA; black dots indicate animals included in

715   behavioral analyses, red Xs indicate missed targets and used in a site specific

716   control analysis.

717   **(F)** hM4D mice with inactivated CamKIIα projections from the ACC to the BLA froze

718   significantly less than EGFP mice in the novel context, but not in the training

719   context only at the remote test. Percent freezing levels of EGFP (○) and hM4D

720   (●) mice during immediate (left panel) and remote (right panel) tests in the

721   training or neutral context 5 minutes after a microinfusion of CNO were analyzed

722   (± SEM). A two-way ANOVA identified a significant effect of context at the

723   immediate test $F_{(1,27)}$ = 47.1, p< 0.001, and remote test, $F_{(1,35)}$ = 15.6, p< 0.001.

724   As observed previously, there was a significant interaction only at the remote test

725            $F_{(1,35)}$ = 6.71, p< 0.05. Thus, inactivation of ACC CamKIIα projections to the BLA

726   eliminated time-dependent generalized fear.

727   **(G)** hM4D mice with extra-BLA infusions did not show a reduction in freezing in the

728   novel context. Percent freezing levels of hM4D mice tested in the neutral context

729   with missed BLA targeting compared to hM4D mice with specific targeting in the

730   BLA was analyzed (± SEM). A non-parametric Mann-Whitney t-test showed a

731   significant effect of CNO infusion target, p< 0.05. *p < 0.05, **p < 0.01, ***p <

732   0.001.

733   **Figure 3. Inactivation of anterior cingulate cortex to basolateral amygdala**

734   **CamKIIα projections eliminates time-independent generalized fear.**

735   **(A)** Hybrid B6S1 mice were tested for contextual fear after training with either 3, 1mA

736   shocks or 5, 1mA shocks. Percent freezing levels of 3 shock (○) and 5 shock (●)

737   trained mice in the training context were analyzed ($\pm$ SEM). A two-way ANOVA

738   identified significant shock x context interaction $F_{(1,19)}$ = 5.42, p< 0.05, showing

739   that 5-shock training, but not 3-shock training, significantly increased freezing in

740   the novel context at the 24h test.

741   **(B)** All behavioral tests were completed seven weeks after viral infusions.

742   Cannulations for the BLA were completed one week prior to behavioral training

743   procedures. In this experiment immediate generalization was induced using a

744   hybrid mouse line. Mice were tested once in each context at 1 day or

745   **(C)** 28 days after training with a 72-hour inter-test-interval. All mice were given a

746   local infusion of CNO into the BLA 5 minutes prior to testing to inactivate ACC

747   CamKIIα projections.

748   **(D)** As done previously, mice were infused with the hM4D or EGFP virus into the

749   ACC with cannulations targeting the BLA. Viral spread analysis of all hM4D mice

750   tested using a within subject design with inactivation of BLA terminals identified

751   no expression outside of the ACC. Dark red: minimum spread observed and

752   included in analysis; red: representative spread observed; light red: maximum

753   spread observed and included in behavioral analysis.

754   **(E)** Cannulation targets were analyzed to correct placement into the BLA. No mice

755   had targets localized outside of the BLA in this experiment.

756   **(F)** At recent and remote tests, inactivating CamKIIα projections from the ACC to the

757   BLA significantly reduced freezing to the novel context. Percent freezing levels

758   of EGFP ($\bigcirc$) and hM4D ($\bullet$) mice during within-subject immediate (left panel) or

759   remote (right panel) tests in the training and neutral context 5 minutes after a

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760   microinfusion of CNO were analyzed ($\pm$ SEM). A two-way ANOVA identified

761   significant main effects of context at the immediate $F_{(1,10)}$ = 64.8, p< 0.001 and

762   remote tests $F_{(1,13)}$ = 17.9, p< 0.001. However, for the first time, there was a

763   significant interaction at the immediate $F_{(1,10)}$ = 5.35, p< 0.05 and remote times

764    $F_{(1,13)}$ = 4.93, p< 0.05, suggesting that ACC CamKIIα projections to the BLA

765   control a time-independent form of generalization.

766   **Figure 4. Inactivation of the ventral hippocampus eliminates time-dependent**

767   **context fear generalization.**

768   **(A)** On the first day of the experimental procedures, pAAV-CaMKIIa-hM4D(Gi)-

769   mCherry virus (hM4D) or pAAV-CamKIIa-EGFP (EGFP) was bilaterally infused

770   into the ventral hippocampus (vHPC). All behavioral tests were completed seven

771   weeks after viral infusions. For the immediate test, mice were tested 1 day after

772   training,

773   **(B)** whereas mice tested at the remote time were tested 28 days after training. All

774   mice were given an IP injection of CNO 30 minutes prior to testing.

775   **(C)** Analysis of transgene expression in hM4D infusions into the vHPC for mice

776   tested with systemic injection of CNO. No expression was observed outside of

777   the vHPC. Dark red: minimum spread observed and included in analysis; red:

778   representative spread observed; light red: maximum spread observed and

779   included in behavioral analysis.

780   **(D)** hM4D mice administered CNO froze significantly less than EGFP control mice in

781   the novel context only. Percent freezing levels of EGFP ($\bigcirc$) and hM4D ($\bullet$) mice

782   during immediate (left panel) and remote (right panel) tests in the training or

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783   neutral context were analyzed ($\pm$ SEM). Two-way ANOVA identified a significant

784   main effect of context at the immediate time point, $F_{(1,21)} = 70$, $p < 0.001$, and a

785   the remote time point $F_{(1,16)} = 40.9$, $p<.001$; mice froze more in the training

786   context than the novel context. However, there was a significant context x

787   treatment interaction only at the remote time point $F_{(1,16)} = 15.9$, $p< 0.01$. *** $p <$

788   0.001, suggesting that the vHPC also regulates time-dependent generalized fear.

789   **(E)** Representative photomicrograph of pAAV-CaMKIIa-hM4D(Gi)-mCherry

790   expression in the vHPC. Robust transgene expression was observed throughout

791   the vHPC and typical of a membrane-bound fluorophore. Inset is 20x

792   magnification. White arrows indicate examples of somatic transgene expression.

793   **Figure 5. CamKIIα projections from the ventral hippocampus to the basolateral**

794   **amygdala regulate time-dependent generalized.**

795   **(A)** To identify if the vHPC regulates fear generalization via its CamKIIα projections

796   to the BLA, pAAV-CaMKIIa-hM4D(Gi)-mCherry virus (hM4D) or pAAV-CamKIIa-

797   EGFP (EGFP) was bilaterally infused at into the vHPC followed by cannulations

798   targeting the BLA.

799   **(B)** All behavioral tests were completed seven weeks after viral infusions.

800   Cannulations for the BLA were completed one week prior to behavioral training

801   procedures. Mice were tested 1 day or

802   **(C)** 28 days after training. All mice were given a local infusion of CNO into the BLA 5

803   minutes prior to testing.

804   **(D)** Viral spread analysis of all hM4D mice tested with inactivation of BLA terminals.

805   Dark red: minimum spread observed and included in analysis; red: representative

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806   spread observed; light red: maximum spread observed and included in

807   behavioral analysis.

808   **(E)** Cannulation targets within the BLA; black dots indicate animals included in

809   behavioral analyses, red Xs indicate missed targets and used in a site specific

810   control analysis.

811   **(F)** hM4D mice with inactivated CamKIIα projections from the vHPC to the BLA froze

812   significantly less than EGFP mice in the novel context, but not in the training

813   context. Percent freezing levels of EGFP (○) and hM4D (●) mice during

814   immediate (left panel) and remote (right panel) tests in the training or neutral

815   context 5 minutes after a microinfusion of CNO were analyzed ($\pm$ SEM). A two-

816   way ANOVA identified a significant effect of context at the immediate test $F_{(1,20)}$ =

817   68.6, p< 0.001, and remote test, $F_{(1,24)}$ = 13.3 p< 0.01. As observed previously,

818   there was a significant interaction only at the remote test $F_{(1,24)}$ = 4.34, p< 0.05.

819   **(G)** hM4D mice with off-target infusions did not show a reduction in freezing in the

820   novel context. Percent freezing levels of hM4D mice tested in the neutral context

821   with missed BLA targeting compared to hM4D mice with specific targeting in the

822   BLA was analyzed ($\pm$ SEM). A non-parametric Mann-Whitney t-test showed a

823   significant effect of CNO infusion target, p< 0.05. *p < 0.05, **p < 0.01, ***p <

824   0.001.

825   **Figure 6. The ventral hippocampus selectively controls time-dependent**

826   **generalization.**

827   **(A)** As done previously, mice were infused with hM4D or EGFP virus into the vHPC

828   with cannulations targeting the BLA. Viral spread analysis of all hM4D mice

829   tested using a within subject design with inactivation of BLA terminals identified

830   no expression outside of the vHPC. Dark red: minimum spread observed and

831   included in analysis; red: representative spread observed; light red: maximum

832   spread observed and included in behavioral analysis.

833   **(B)** Cannulation targets were again analyzed to correct placement into the BLA.

834   There were no missed targets outside of the BLA in this experiment.

835   **(C)** All behavioral tests were completed seven weeks after viral infusions.

836   Cannulations for the BLA were completed one week prior to behavioral training

837   procedures. In this experiment immediate generalization was induced using a

838   hybrid mouse line. Mice were tested once in each context at 1 day or

839   **(D)** 28 days after training with a 72-hour inter-test-interval. All mice were given a

840   microinfusion of CNO into the BLA 5 minutes prior to testing.

841   **(E)** Inactivating CamKIIα projections from the vHPC to the BLA significantly reduced

842   freezing to the novel context only at the remote test. These data suggest that

843   glutamatergic projections from the vHPC to the BLA selectively control time-

844   dependent generalized fear. Percent freezing levels of EGFP ($\bigcirc$) and hM4D ($\bullet$)

845   mice during within-subject immediate (left panel) or remote (right panel) tests in

846   the training and neutral context 5 minutes after a local infusion of CNO were

847   analyzed ($\pm$ SEM). A two-way ANOVA identified significant main effects of

848   context at the immediate $F_{(1,13)} = 19$, $p< 0.001$ and remote tests $F_{(1,9)} = 80.9$, $p<$

849   0.001. After induced generalization, there was a significant interaction only at the

850   remote test $F_{(1,9)} = 14.6$, $p< 0.01$. *** $p < 0.001$.

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Figure 1. Inactivation of the anterior cingulate cortex eliminates time-dependent generalized context fear.
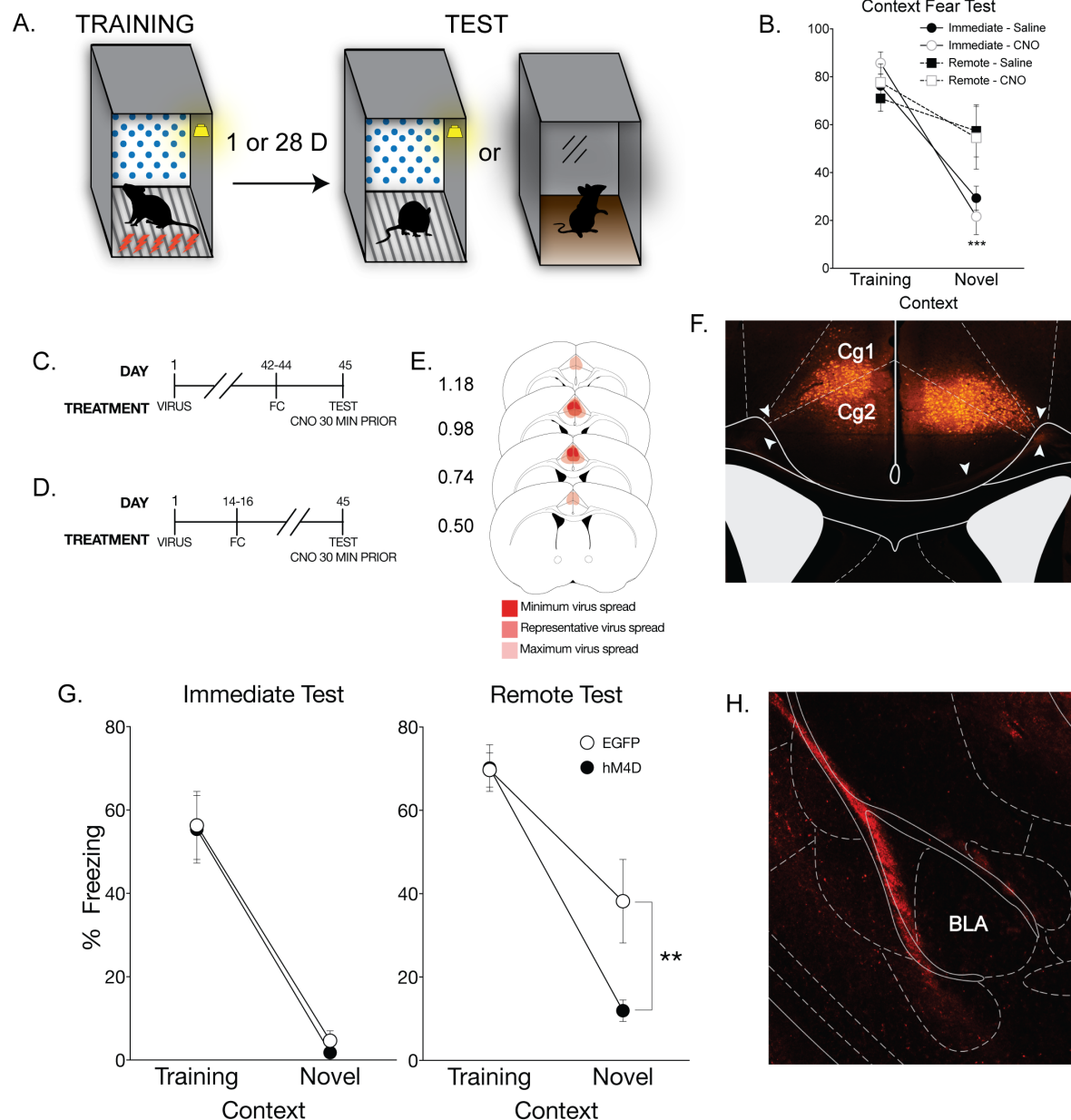
856 **Figure 2.** Inactivation of anterior cingulate cortex CamKIIα projections in the basolateral
857 amygdala eliminates time-dependent generalized fear.
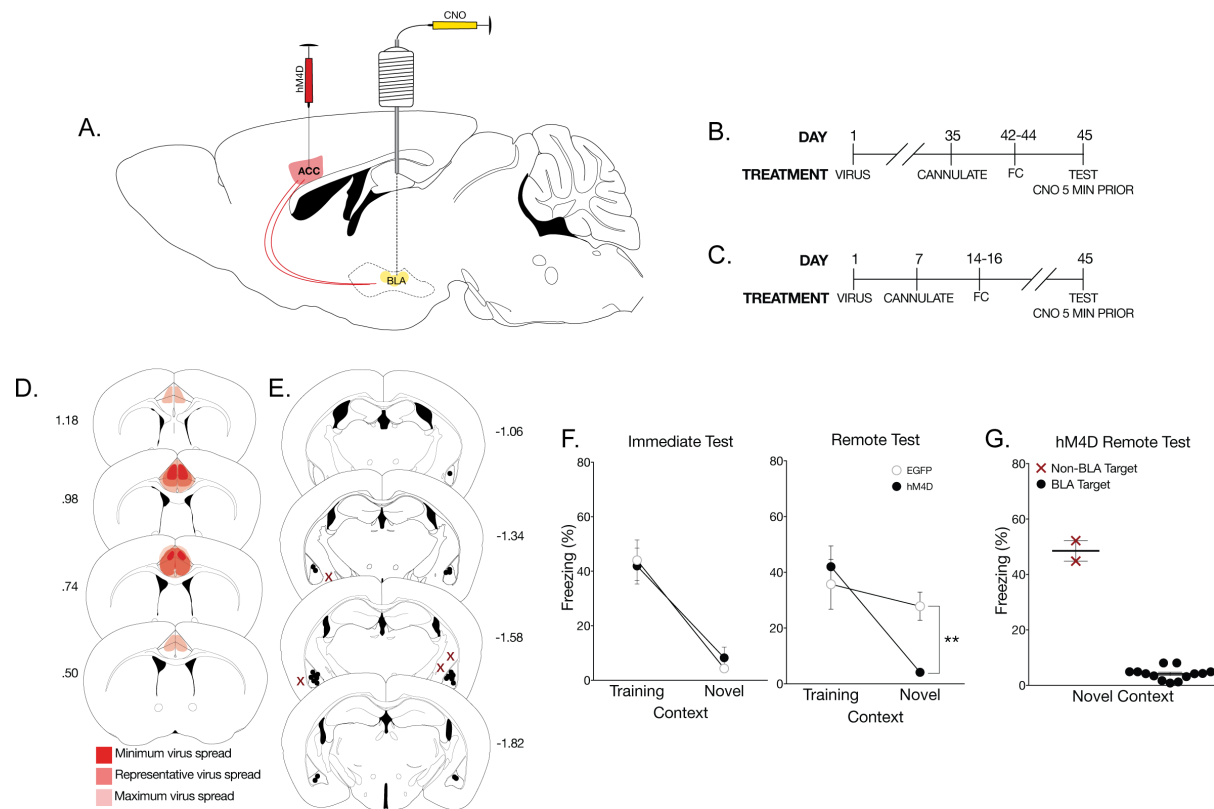
858

Figure 3. Inactivation of anterior cingulate cortex to basolateral amygdala CamKIIα projections eliminates time-independent generalized fear.

862 **Figure 4.** Inactivation of the ventral hippocampus eliminates time-dependent context
863 fear generalization.
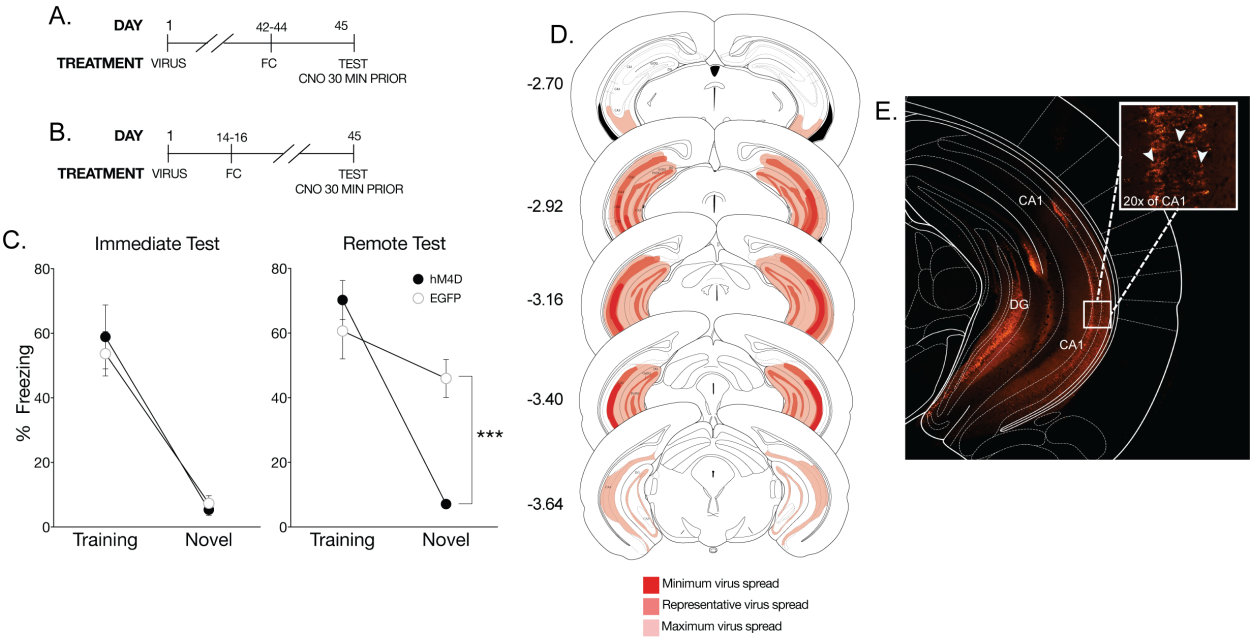
864

865 **Figure 5.** CamKIIα projections from the ventral hippocampus to the basolateral
866 amygdala regulate time-dependent generalized.

867
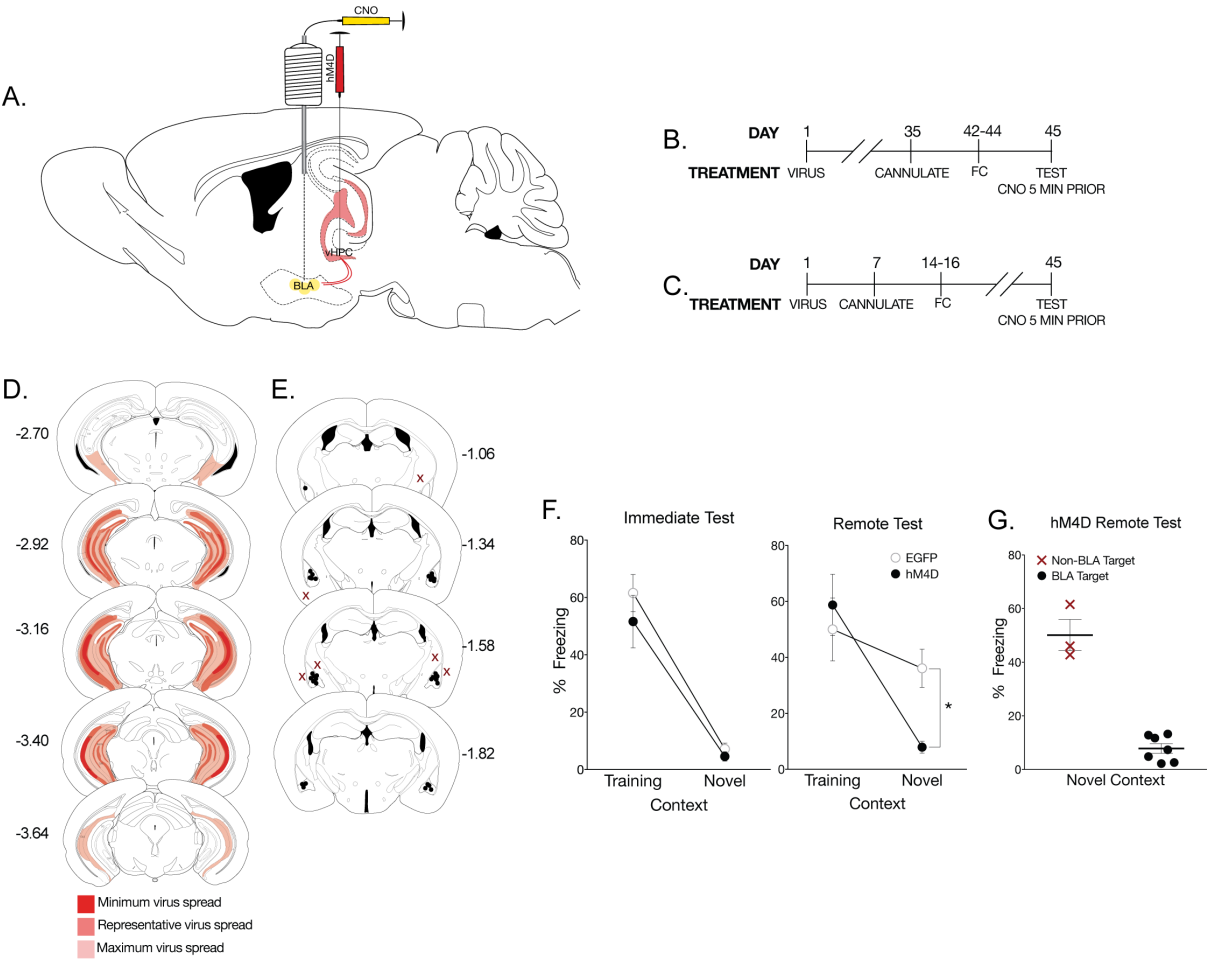
868   **Figure 6.** The ventral hippocampus selectively controls time-dependent generalization.

870 **Table 1.** Clozapine-N-Oxide & Hybrid B6S1 Behavior Statistical Summary

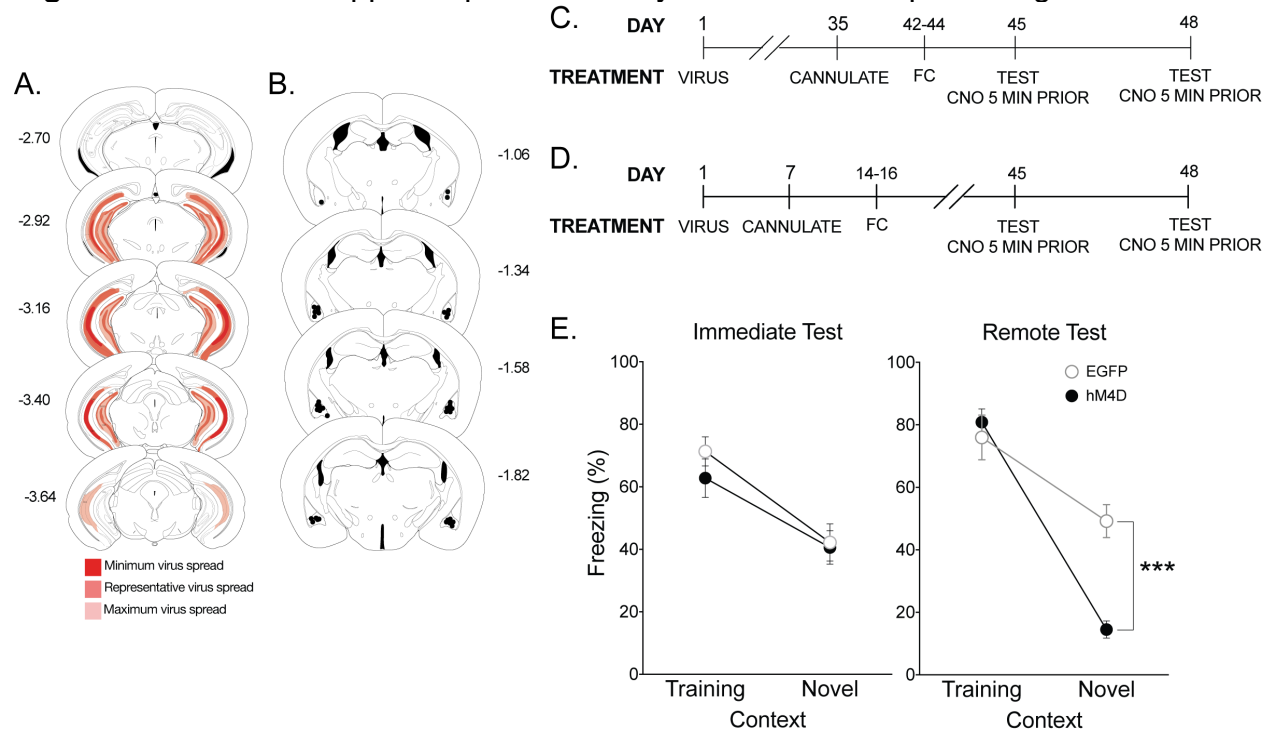| Mouse Strain	Manipulation	Statistical Test	Test Delay	Comparison	F/t Statistic	DF	% Total Variance	P	*	ηp^2	Effect Size	Power	Figure
C57BL/6	CNO vs Saline	Two-Way ANOVA	1 D	Context x Treatment	2.30	1,12	2.08	0.155	ns	0.160	0.43	0.36	1B
				Context	96.40	1,12	87.10	<.001	***	0.889	2.85	1.00	
				Viral Treatment	0.03	1,12	0.02	0.873	ns	0.002	0.05	0.05	
			28 D	Context x Treatment	0.32	1,11	2.23	0.584	ns	0.028	0.16	0.09	
				Context	2.61	1,11	18.7	0.131	ns	0.195	0.49	0.41	
				Viral Treatment	0.80	1,11	0.606	0.774	ns	0.007	0.08	0.06	
	Three vs Five Shock		1 D	Context x Treatment	5.42	1,19	4.01	0.03	*	0.222	0.53	0.68	
				Context	90.60	1,19	67.10	<.001	***	0.821	2.18	1.00	
				Shock Treatment	16.10	1,19	11.90	<.001	***	0.78	0.29	0.26	

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873 **Table 2.** Clozapine-N-Oxide & Hybrid B6S1 Behavior significant post-hoc comparisons summary.

Statistical Test	Test Delay	Significant Post-Hoc Comparisions Context:Treatment	Mean 1	Mean 2	N1	N2	t	DF	P	*	Figure
Pos-Hoc Comparison	1 D	Training:Saline vs. Novel:Saline	76.3	29.3	4	4	0.8	4	<0.001	***	1B
		Training:Saline vs. Novel:CNO	76.3	21.7	4	4	0.8	4	<0.001	***	
		Training:CNO vs. Novel:Saline	85.8	29.3	4	4	0.1	4	<0.001	***	
		Training:CNO vs. Novel:CNO	85.8	21.7	4	4	0.1	4	<0.001	***	
Pos-Hoc Comparison	1 D	Training:3-Shock vs. Novel:3-Shock	79.5	16.6	7	6	9	19	<.001	***	
		Training:3-Shock vs. Novel:5-Shock	79.5	50.2	7	5	4	19	<.001	***	
		Training:5-Shock vs. Novel:3-Shock	88.4	16.6	5	6	9.4	19	<.001	***	
		Training:5-Shock vs. Novel:5-Shock	88.4	50.2	5	5	4.8	19	<.001	***	
		Novel:3-Shock vs. Novel:5-Shock	16.6	50.2	6	5	4.4	19	<.001	***	

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875 **Table 3.** Anterior Cingulate Cortex Statistical Summary

Mouse Strain	Inactivation	Statistical Test	Test Delay	Comparison	F/t Statistic	DF	% Total Variance	P	*	ηp^2	Effect Size	Power	Figure
C57BL/6	Systemic	Two-Way ANOVA	1 D	Context x Treatment	0.02	1,16	0.03	0.886	-	0.001	0.04	0.05	1G
				Context	64.20	1,16	78.80	<.001	***	0.801	2.00	1.00	
				Viral Treatment	0.08	1,16	0.10	0.776	-	0.005	0.07	0.06	
			28 D	Context x Treatment	4.64	1,17	5.94	0.046	*	0.230	0.52	0.62	
				Context	52.30	1,17	66.9	<.001	***	0.770	1.75	1.00	
				Viral Treatment	4.34	1,17	5.55	0.053	-	0.219	0.50	0.59	
	BLA Terminals		1D	Context x Treatment	0.32	1,27	0.42	0.578	-	0.012	0.10	0.09	2F
				Context	47.10	1,27	63.00	<.001	***	0.636	1.32	0.99	
				Viral Treatment	0.03	1,27	0.04	0.867	-	0.001	0.03	0.05	
			28 D	Context x Treatment	6.71	1,35	10.3	0.014	*	0.161	0.44	0.76	
				Context	15.6	1,35	23.9	<.001	***	0.308	0.67	0.98	
				Viral Treatment	2.25	1,35	3.45	0.142	-	0.061	0.25	0.34	
		Mann-Whitney Test	28 D	Target Location				0.019	*	-	8.93	1.00	2G
C57BL/6 x 129S1vmJ		Repeated Measures Two-Way ANOVA	1D	Context x Treatment	5.35	1,10	5.04	0.043	*	0.333	0.71	0.97	3E
				Context	64.8	1,10	61	<.001	***	0.858	2.46	1.00	
				Viral Treatment	14.3	1,10	14.4	0.004	**	0.588	1.20	0.99	
			28D	Context x Treatment	4.93	1,13	6.28	0.045	*	0.128	0.39	0.71	
				Context	17.9	1,13	22.8	<.001	***	0.348	0.73	0.99	
				Viral Treatment	3.08	1,13	10.1	0.103	-	0.192	0.49	0.89	

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Table 4. Anterior cingulate cortex significant post-hoc comparisons summary.

Mouse Strain	Inactivation	Statistical Test	Test Delay	Significant Post-Hoc Comparisions Context:Treatment	Mean 1	Mean 2	N1	N2	t	DF	P	*	Figure
C57BL/6	Systemic	Two-Way ANOVA	1 D	Training:hM4D vs. Novel:hM4D	55.4	1.79	5	5	5.83	16	<.001	***	1G
				Training:hM4D vs. Novel:EGFP	55.4	4.65	5	4	5.2	16	<.001	***	
				Training:EGFP vs. Novel:hM4D	56.3	1.79	6	5	6.19	16	<.001	***	
				Training:EGFP vs. Novel:EGFP	56.3	4.65	6	4	5.51	16	<.001	***	
			28 D	Training:hM4D vs. Novel:hM4D	70.1	11.9	6	5	6.78	17	<.001	***	
				Training:hM4D vs. Novel:EGFP	70.1	38.2	6	5	3.72	17	<.01	**	
				Training:EGFP vs. Novel:hM4D	69.7	11.9	5	5	6.45	17	<.001	***	
				Training:EGFP vs. Novel:EGFP	69.7	38.2	5	5	3.51	17	<.01	**	
				Novel:hM4D vs. Novel:EGFP	11.9	38.2	5	5	2.93	17	<.001	**	
	BLA Terminals		1D	Training:hM4D vs. Novel:hM4D	41.9	8.26	7	8	4.38	27	<.001	***	2F
				Training:hM4D vs. Novel:EGFP	41.9	4.34	7	8	4.89	27	<.001	***	
				Training:EGFP vs. Novel:hM4D	44	8.26	8	8	4.82	27	<.001	***	
				Training:EGFP vs. Novel:EGFP	44	4.34	8	8	5.34	27	<.001	***	
			28 D	Training:hM4D vs. Novel:hM4D	42	4.13	10	13	5.08	35	<.001	***	
				Training:EGFP vs. Novel:hM4D	35.7	4.13	8	13	3.96	35	<.001	***	
				Novel:hM4D vs. Novel:EGFP	4.13	27.8	13	8	2.97	35	<.01	**	
C57BL/6 x 129S1vmJ		Repeated Measures Two-Way ANOVA	1D	hM4D: Training vs. Novel	71.3	16.7	6	6	7.33	10	<.001	***	3E
				EGFP: Training vs. Novel	79.7	49.5	6	6	4.05	10	<.01	**	
				Novel: hM4D vs. EGFP	16.7	49.5	6	6	4.32	20	<.001	***	
			28D	hM4D: Training vs. Novel	67.7	37.6	8	8	4.72	13	<.001	***	
				Novel: hM4D vs. EGFP	37.6	61.1	8	7	2.66	26	<.05	*	

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880   **Table 5.** Ventral hippocampus cortex statistical analysis summary**.**

Mouse Strain	Inactivation	Statistical Test	Test Dela	Comparison	F/t Statistic	DF	% Total Variance	P	*	ηp^2 Effect Size	Power	Figure
C57BL/6	Systemic	Two-Way ANOVA	1 D	Context x Treatment	0.36	1,21	0.40	0.553	-	0.13	0.09	4F
				Context	70.00	1,21	76.20	<.001	***	1.82	1.00	
				Viral Treatment	0.07	1,21	0.08	0.79	-	0.06	0.06	
			28 D	Context x Treatment	15.90	1,16	20.2	0.001	**	1.00	0.97	
				Context	40.90	1,16	52.1	<.001	***	1.60	0.99	
				Viral Treatment	5.79	1,16	7.38	0.029	*	0.60	0.71	
	BLA Terminals		1D	Context x Treatment	0.36	1,20	0.39	0.556	-	0.13	0.10	5F
				Context	68.60	1,20	75.10	<.001	***	1.85	1.00	
				Viral Treatment	1.05	1,20	1.15	0.318	-	0.22	0.19	
			28 D	Context x Treatment	4.34	1,24	10.5	0.048	*	0.43	0.51	
				Context	13.3	1,24	32.2	0.001	**	0.76	0.94	
				Viral Treatment	1.21	1,24	2.92	0.283	ns	0.22	0.18	
		Mann-Whitney Test	28 D	Target Location				0.017	*	4.02	0.99	5G
C57BL/6 x 129S1vmJ		Repeated Measures Two-Way ANOVA	1D	Context x Treatment	0.348	1,13	0.798	0.565	ns	0.16	0.19	6E
				Context	19	1,13	43.5	<.001	***	1.21	1.00	
				Viral Treatment	0.952	1,13	1.71	0.347	ns	0.24	0.35	
			28D	Context x Treatment	14.6	1,9	12.4	0.004	**	1.17	0.99	
				Context	80.9	1,9	68.6	<.001	***	2.75	1.00	
				Viral Treatment	6.95	1,9	7.02	0.027	*	0.88	0.99	

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883   **Table 6.** Ventral hippocampus significant post-hoc comparisons summary.

Mouse Strain	Inactivation	Statistical Test	Test Delay	Significant Post-Hoc Comparisions Context:Treatment	Mean 1	Mean 2	N1	N2	t	DF	P	*	Figure
C57BL/6	Systemic	Two-Way ANOVA	1 D	Training:hM4D vs. Novel:hM4D	58.9	5.36	6	7	6.5	21	<.001	***	4F
				Training:hM4D vs. Novel:EGFP	58.9	7.35	6	6	6	21	<.001	***	
				Training:EGFP vs. Novel:hM4D	53.7	5.36	6	7	5.8	21	<.001	***	
				Training:EGFP vs. Novel:EGFP	53.7	7.35	6	6	5.4	21	<.001	***	
			28 D	Training:hM4D vs. Novel:hM4D	70.2	7.1	5	5	7.3	16	<.001	***	
				Training:hM4D vs. Novel:EGFP	70.2	46	5	5	2.8	16	< 0.05	*	
				Training:EGFP vs. Novel:hM4D	60.7	7.1	5	5	6.2	16	<.001	***	
				Novel:hM4D vs. Novel:EGFP	7.1	46	5	5	4.5	16	<.001	***	
	BLA Terminals		1D	Training:hM4D vs. Novel:hM4D	51.6	4.57	6	5	5.2	20	<.001	***	5F
				Training:hM4D vs. Novel:EGFP	51.6	7.16	6	6	5.2	20	<.001	***	
				Training:EGFP vs. Novel:hM4D	61.6	4.57	7	5	6.5	20	<.001	***	
				Training:EGFP vs. Novel:EGFP	61.6	7.16	7	6	6.6	20	<.001	***	
			28 D	Training:hM4D vs. Novel:hM4D	58.7	7.9	7	6	3.9	24	< .001	***	
				Training:EGFP vs. Novel:hM4D	50	7.9	7	6	3.2	24	<.01	**	
				Novel:hM4D vs. Novel:EGFP	7.9	36.1	6	8	2.2	24	<.05	*	
C57BL/6 x 129S1vmJ		Repeated Measures Two-Way ANOVA	1D	hM4D: Training vs. Novel	62.8	40.6	7	7	2.6	13	<.05	*	6E
				EGFP: Training vs. Novel	71.3	42.2	8	8	3.6	13	<.01	**	
			28D	hM4D: Training vs. Novel	80.8	14.5	5	5	8.7	9	<.001	***	
				EGFP: Training vs. Novel	76	49.2	6	6	3.8	9	<.01	**	
				Novel:hM4D vs. Novel:EGFP	14.5	49.2	5	6	4.5	18	<.001	***	

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