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Plasticity of NMDA Receptors at Ventral Hippocampal Synapses in the Infralimbic Cortex Regulates Cued Fear

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1 **Plasticity of NMDA Receptors at Ventral Hippocampal Synapses in the Infralimbic Cortex**
2 **Regulates Cued Fear**

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7
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11

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21 A.H. performed research; O.SC., O.TR., F.B., L.M., A.H., and J.T.P. analyzed data; O.SC. and

22 J.T.P. wrote the paper

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24

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32

33 **Abstract**

34 The medial prefrontal cortex (mPFC) processes contextual information from the hippocampus to
35 generate appropriate fear responses. In rodents, one path for sending contextual information to
36 the mPFC is via the direct projections from the ventral hippocampus (vHC) to the infralimbic
37 cortex (IL). Plasticity in the synaptic communication from the vHC to the IL could contribute to
38 the behavioral changes produced by the acquisition and extinction of conditioned fear. To
39 examine this possibility, we used optogenetic stimulation of vHC synapses in brain slices from
40 trained rats. We found that fear acquisition reduced NMDA receptor (NMDAR) currents at vHC
41 synapses onto IL pyramidal neurons. The depression of NMDAR currents reversed more
42 efficiently after extinction in the conditioning context than extinction in a novel context.
43 Moreover, a cohort of animals that exhibited poor extinction retrieval failed to reverse the
44 plasticity induced by fear conditioning. In addition, ex vivo application of brain-derived
45 neurotrophic factor, which is known to simulate extinction in IL, reversed this conditioning-
46 induced plasticity mimicking extinction. Therefore, we have identified a novel mechanism that
47 modulates conditioned fear via changes in NMDAR current at vHC synapses onto IL pyramidal
48 neurons. Disruption of this mechanism could contribute to the abnormal contextual modulation
49 of fear seen in posttraumatic stress disorder (PTSD).

50
51

52 **Significance Statement**

53 Contextual information processing by the medial prefrontal cortex is critical for appropriate
54 behavioral responses. Using optogenetics in brain slices, we found that acquisition of
55 conditioned fear depressed NMDA receptor currents at ventral hippocampal synapses in the
56 infralimbic cortex (IL) and extinction reversed the depression. BDNF could also reverse the

57 conditioning-induced depression mimicking extinction. In contrast, animals with impaired fear
58 extinction recall, a PTSD-like phenotype, failed to reverse the conditioning-induced changes.
59 Our findings suggest that conditioned fear responses are modulated by changes in NMDAR
60 current at ventral hippocampal synapses in IL and suggest a novel mechanism that could
61 contribute to impaired contextual modulation of fear seen in patients with PTSD.

62

63 **Introduction**

64 Contextual information is crucial to the generation of appropriate responses to environmental
65 cues. Inappropriate contextual information contributes to various psychopathological conditions.
66 For example, patients with PTSD show deficits in discriminating safe and dangerous contexts
67 (Rougemont-Bücking et al., 2011; Garfinkel et al., 2014). Functional imaging studies show that
68 patients with PTSD have abnormal activity in the ventromedial prefrontal cortex (vmPFC),
69 amygdala, and hippocampus during Pavlovian fear conditioning and extinction (Milad et al.,
70 2009; Rougemont-Bücking et al., 2011). These brain structures are closely implicated in
71 contextual processing (Maren et al., 2013) suggesting that deficits in activation of these
72 structures likely contribute to the discrimination deficits seen in these patients (Garfinkel et al.,
73 2014).

74

75 Fear extinction is context-dependent (Bouton and Bolles, 1979; Bouton, 2004; Kalisch et al.,
76 2006; Langton and Richardson, 2009) and involves an interconnected neural circuit composed of
77 the mPFC, hippocampus, and amygdala (Kalisch et al., 2006; Milad et al., 2007). The ventral
78 hippocampus (vHC) conveys contextual information and synaptically excites neurons in the
79 infralimbic mPFC (IL) (Maren et al., 2013; Kim and Cho, 2017), the homologous region to the
80 vmPFC in rodents. Since the stimulation of IL pyramidal neurons inhibits conditioned fear

81 (Milad et al., 2004; Bukalo et al., 2015; Do-Monte et al., 2015), synaptic communication
82 between the vHC and IL could mediate the context-dependency of extinction (Sierra-Mercado et
83 al., 2011; Orsini et al., 2013; Kim and Cho, 2017; Marek et al., 2018). However, whether vHC
84 synapses in IL are altered by fear conditioning or extinction remains unknown.

85

86 In this study, we investigated whether fear conditioning or extinction alter the synaptic efficacy
87 of vHC synapses onto IL pyramidal neurons. Additionally, we examined whether extinction
88 given in the conditioning context induces similar synaptic plasticity as extinction in a novel
89 context. To address these possibilities, we combined whole-cell patch-clamp electrophysiology
90 and optogenetics in brain slices with Pavlovian fear conditioning and extinction to specifically
91 examine vHC synapses onto IL pyramidal neurons. In contrast to previous studies that did not
92 find any plasticity of globally-activated synapses in IL after fear conditioning (Pattwell et al.,
93 2012; Sepulveda-Orengo et al., 2013), we found that fear conditioning induced plasticity in vHC
94 synapses in IL. Fear conditioning increased the ratio of AMPAR to NMDAR-mediated currents
95 by reducing NMDAR currents at the vHC synapses in IL. This postsynaptic plasticity was
96 reversed more efficiently when extinction was conducted in the conditioning context than in a
97 novel context. Furthermore, animals that showed poor extinction memory, a PTSD-like
98 phenotype, failed to reverse the conditioning-induced plasticity, whereas the plasticity could be
99 reversed by the ex vivo application of brain derived neurotrophic factor (BDNF), which is
100 known to simulate extinction when infused into IL (Peters et al., 2010; Rosas-Vidal et al., 2014).

101

102 **Methods**

103 *Animal subjects.*

104 All animal procedures were approved by the Institutional Animal Care and Use Committee in
105 compliance with NIH guidelines for the care and use of laboratory animals. Male Sprague
106 Dawley rats were transported from the institutional colony to a satellite facility nearby where
107 they were individually housed on a 12/12 h light/dark schedule with free access to food and
108 water.

109

110 *Stereotaxic surgery.*

111 Rats (between 90 to 120 g of weight) received bilateral injections of an AAV5 vector (1.0 μ l)
112 expressing channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (EYFP) driven
113 by the neuron-specific CaMKII promoter (AAV-CaMKIIa-hChR2(H134R)-EYFP; University of
114 North Carolina at Chapel Hill Vector Core Services) into the vHC via a 5 μ L Hamilton syringe
115 using stereotaxic coordinates (Sierra-Mercado et al., 2011). After waiting a period of 8 to 12
116 weeks for recovery and adequate ChR2 expression, rats received behavioral training. After
117 completing behavioral training, animals were euthanized for acute brain slice preparation. In
118 addition, brain sections containing the hippocampus were fixed and prepared for further
119 confirmation of EYFP expression in the ventral hippocampus.

120

121 *Behavioral apparatus.*

122 The fear conditioning context (context A) was a chamber of 25 x 29 x 28 cm with aluminum and
123 Plexiglass walls (Coulbourn Inst., Allentown, PA). The floor consisted of stainless steel bars that
124 could be electrified to deliver a mild shock and a single overhead light provided illumination.
125 Context B, which was used to give extinction and recall test to the DIFF group, consisted of a
126 hexagonal chamber with a flat floor, citric scent, and different illumination color. Context A and

127 B had speakers mounted on the outside wall and were situated inside a sound-attenuating box
128 (Med Associates, Burlington, VT) with a ventilating fan that produced an ambient noise level of
129 60 dB. The conditioned stimulus (CS) was a 4 kHz tone with duration of 30 s and an intensity of
130 80 dB. The inter-tone interval for successive tone presentations in the conditioning, extinction,
131 and test phases was an average of 2 min. The unconditioned stimulus (US) was a 0.50 mA
132 scrambled foot shock, 0.5 s in duration, that co-terminated with the tone during the conditioning
133 phase. Behavior was recorded with digital video cameras (Micro Video Products, Ontario,
134 Canada).

135

136 *Fear conditioning and extinction.*

137 Animals were randomly assigned to one of the following experimental groups: pseudo-
138 conditioned (PSEUDO), fear conditioned (COND), fear extinguished in context A (SAME), or
139 fear extinguished in context B (DIFF). On day 1, animals from the COND, SAME, and DIFF
140 groups received auditory fear conditioning, which consisted of one habituation tone followed by
141 five tone-shock pairings. Animals from the PSEUDO group received the same amount of tones
142 and shocks but in a non-paired manner. On day 2, PSEUDO and COND animals remained in
143 their home cages while SAME and DIFF animals received two sessions of 15 tone-alone
144 presentations, with one hour between sessions, in context A and context B, respectively. On day
145 3, rats from the PSEUDO, COND, and SAME groups received two tone-alone presentations in
146 context A, whereas rats from the DIFF group received two tone-alone presentations in context B.
147 For the BDNF experiments, animals received fear conditioning on day 1 and recall test on day 2.

148

149 *Whole-cell recordings.*

150 Immediately after the test on day 3, rats were deeply anesthetized with pentobarbital (65 mg/kg)
151 and perfused transcardially with ice-cold N-Methyl-D-glucamine (NMDG) based artificial
152 cerebrospinal fluid (ACSF) and decapitated. Brains were quickly removed and placed in ice-cold
153 NMDG ACSF. Then, 300 μ m coronal slices of the mPFC were cut with a Vibratome 1000 Plus
154 (Vibratome, St. Louis, MO). We used a modified NMDG-based ACSF to obtain healthy brain
155 slices from adult animals. The composition of the NMDG-based ACSF (Ting et al., 2014) was
156 the following: 93 mM NMDG, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 30 mM NaHCO_3 , 20 mM
157 HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10
158 mM MgSO_4 , and 0.5 mM CaCl_2 . The mPFC slices were initially incubated at 33° C in NMDG
159 ACSF for 12 min before being transferred to an additional 1 hr incubation in modified HEPES
160 ACSF at room temperature (21–23°C). The composition of the modified HEPES ACSF was the
161 following: 92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 30 mM NaHCO_3 , 20 mM HEPES, 25
162 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 2 mM MgSO_4 ,
163 and 2 mM CaCl_2 . Then, mPFC slices were transferred and submerged in the recording chamber
164 and perfused at 2–3 ml/min with room temperature ACSF with 1 μ M tetrodotoxin (TTX) and
165 100 μ M 4-aminopyridine (4-AP) to assess monosynaptic postsynaptic currents. In addition, 10
166 μ M picrotoxin was added to block GABA_A postsynaptic currents. The composition of the
167 recording ACSF was the following: 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4 , 1 mM
168 MgSO_4 , 26 mM NaHCO_3 , 20 mM glucose, and 2 mM CaCl_2 and bubbled with 95% O_2 and 5%
169 CO_2 . The neurons were visualized with infrared video microscopy using a 40x water-immersion
170 objective on an upright E600FN microscope (Nikon Instruments). Whole-cell recordings were
171 done with glass pipettes with a resistance of 2.5 – 4 M Ω and filled with cesium gluconate
172 internal solution containing the following (in mM): 12 TEA-Cl, 140 CsOH, 10 HEPES, 140

173 gluconic acid, 10 biocytin, 2 adenosine triphosphate, 3 guanosine triphosphate, and 0.4 cesium-
174 ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid (Cs-EGTA, 0.4). pH was
175 adjusted to 7.3 with CsOH (300 mOsm). After establishing a whole-cell voltage-clamp
176 recording, the membrane resistance, membrane capacitance, and access resistance were
177 measured. Recordings were filtered at 4 kHz, digitized at 10 kHz, and saved to a computer using
178 pCLAMP9 (Molecular Devices).

179

180 *AMPA and NMDAR currents.*

181 AMPA and NMDAR-mediated excitatory postsynaptic currents (EPSCs) in mPFC neurons were
182 measured in response to the optical stimulation of ChR2-expressing vHC axons innervating layer
183 II/III & V with a 470 nm light-emitting diode (LED) (M470L2, Thorlabs) through the 40x
184 objective centered at the soma of the patched neuron with light intensity of 1.5 mW. A total of
185 106 neurons were recorded from IL (89% in layer V; 11% in layer II/III). Picrotoxin was added
186 to the bath to block GABA_A-mediated currents. TTX and 4-AP were added to ensure
187 monosynaptic measurements. To evoke synaptic responses in the mPFC by photostimulation of
188 vHC fibers, we illuminated slices every 10 s with light pulses of 5–20 ms duration. AMPAR-
189 mediated EPSCs were measured as the peak of the EPSCs recorded at -70 mV. NMDAR-
190 mediated EPSCs were measured as the amplitude of the EPSC at +40 mV, 70 milliseconds after
191 the light stimulus. Initial experiments with NMDAR blockers showed that EPSCs contained
192 minimal contamination with AMPAR EPSCs at this time point. Measurements of AMPAR and
193 NMDAR EPSCs were also taken at 0 mV to calculate ion conductance from the current (I)
194 versus voltage (V) plot and peak values were taken similarly to at -70 and +40 mV. AMPAR
195 conductance values were calculated by taking the slope of the I/V plot of AMPA EPSCs

196 measured at holding potentials of -70 and 0 mV, whereas NMDAR conductance values were
197 calculated from the I/V plot values of NMDAR EPSCs measured at holding potentials of 0 and
198 +40 mV.

199

200 *BDNF experiments*

201 We prepared mPFC slices from rats that received fear conditioned on day 1 and a fear recall test
202 on day 2. The slices underwent the same recovery process as described above in the whole-cell
203 recordings section. After the slice recovery period, mPFC slice hemispheres were separated and
204 one hemisphere was randomly selected for an additional 1 hour incubation in room temperature
205 HEPES-ACSF containing 2 nM BDNF (Invitrogen). The other hemisphere was incubated an
206 additional hour in control HEPES-ACSF. After the 1 hour incubation with BDNF or control
207 HEPES-ACSF, slices were transferred to the recording chamber and AMPAR- and NMDAR-
208 mediated EPSCs evoked by optical stimulation of vHC axons were recorded in IL pyramidal
209 neurons.

210

211 *Histology.*

212 In all experiments, 10 mM biocytin was included in the recording solution to label the neurons
213 for post hoc morphological identification of IL pyramidal neurons. After completing
214 electrophysiological recordings, the slices were fixed overnight in 4% paraformaldehyde.
215 Recorded IL neurons were stained with a standard avidin-biotin peroxidase procedure
216 (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and visualized with bright-field
217 microscopy. Neurons that were not located in the IL or that were not pyramidal-shaped with
218 obvious apical dendrites were excluded from the analysis.

219

220 *Statistical analysis.*

221 Freezing behavior was used as an indicator of fear and was assessed uniformly using computer-
222 based analysis program (FreezeScan, Clever Systems). All behavioral data were compared with
223 repeated measures ANOVA followed by Tukey HSD post hoc test (IBM SPSS Statistics, IBM
224 Corp., Armonk, N.Y., USA). The electrophysiological data were analyzed using Clampfit (Axon
225 Instruments, Union City, CA) and were compared with non-parametric Kruskal–Wallis test or
226 Mann-Whitney test due to skewness of the data in its distribution (IBM SPSS Statistics, IBM
227 Corp., Armonk, N.Y., USA). Significant main effect with Kruskal–Wallis test was followed by
228 Dunn post hoc test. Effect sizes between means were assessed using Cohen’s d (Cohen, 1988;
229 Lenhard and Lenhard, 2016). Values are reported as the mean \pm the standard error of the mean
230 (S.E.M.). All animals in which we were able to obtain stable optically-evoked EPSCs were
231 included in the behavioral analysis. Since we wanted to correlate synaptic changes with
232 behavioral changes, we analyzed rats from the extinction groups that showed less than 50%
233 freezing during the extinction recall tones on day 3 (successful extinction) separate from those
234 rats that failed to recall extinction (unsuccessful) and froze more than 50% during the recall
235 tones on day 3. Seven of the 13 rats that were extinguished in the fear conditioning context
236 showed successful extinction recall in the conditioning context. All seven rats that received
237 extinction in the novel context showed successful extinction recall in the novel context.

238 **Results**

239 First, AAV vectors expressing channelrhodopsin-2 and enhanced yellow fluorescent protein
240 were injected into the ventral hippocampus of rats. Two to three months later, robust expression
241 of the viral proteins could be seen in the ventral hippocampus and in the axons in the mPFC

242 (Figure 1A-B). Optical stimulation of ChR2-expressing vHC axons evoked EPSCs in IL
243 pyramidal neurons. Whole-cell recordings at -70 mV allowed the measurement of AMPA
244 receptor-mediated EPSCs from the peak current (Figure 1C). Optical stimulation at +40 mV
245 evoked both AMPA and NMDAR currents. Initial experiments with NMDAR blockers showed
246 that 70 ms after stimulation the NMDAR EPSCs contained minimal contamination with AMPAR
247 EPSCs (Figure 1C). Next, we designed four experimental groups to test whether fear
248 conditioning or extinction induces changes in vHC synapses in IL, and whether extinction in the
249 same context (SAME) will induce different synaptic changes in vHC synapses than extinction in
250 a novel context (DIFF, Figure 1D). On day 1, the group that received conditioning but no
251 extinction (COND, n = 10) and the SAME (n = 7) and DIFF (n = 7) groups acquired similar
252 levels of fear after receiving auditory fear conditioning in context A (Figure 2A). The PSEUDO
253 (n = 7) group received unpaired tone and shock presentations in context A to avoid association of
254 the tone with the shock. Then, on days 2 and 3, the SAME group received fear extinction and
255 extinction retrieval test in context A whereas the DIFF group received extinction and retrieval
256 test in context B. On day 3, we tested all animals for cued fear and euthanized animals
257 immediately after the test. A cohort of rats in the SAME group were removed from the analysis
258 due to poor extinction memory recall on day 3 and were analyzed separately in Figure 4. As
259 expected (Figure 2A), a repeated measures ANOVA showed a significant main effect ($F(2,12) =$
260 40.63 , $p < 0.001$) and post hoc analysis confirmed that rats from the COND group had
261 significantly higher levels of freezing to the tone on day 3 compared to rats from the SAME,
262 DIFF, and PSEUDO groups ($p < 0.001$). The difference in fear expression among groups
263 indicates that all animals were successful in learning their respective behaviors. It is important to
264 point out that the DIFF group received extinction and extinction recall in a novel context.

265 Therefore, the DIFF group did not receive extinction of the conditioning context. Given the
266 abundant evidence of fear renewal (Bouton et al., 2006), the DIFF group would likely show
267 higher freezing in the conditioning context.

268

269 *Fear conditioning and extinction induce plasticity in vHC-IL synapses.*

270 After recall on day 3, we prepared acute brain slices and used whole-cell voltage-clamp
271 recordings to assess AMPA to NMDA ratios in mPFC pyramidal neurons after selective optical
272 stimulation of vHC axons (Figure 2B-C). The following number of cells was evaluated in each
273 group: PSEUDO (n = 22 cells from 7 rats), COND (n = 25 cells from 10 rats), SAME (n = 23
274 cells from 7 rats), and DIFF (n = 20 cells from 7 rats). Kruskal-Wallis test showed a significant
275 main effect in AMPA to NMDA ratios of vHC inputs in IL ($H(3,90) = 14.70$, $p = 0.002$). Dunn's
276 post hoc analysis revealed a significant increase in AMPA to NMDA ratios in the COND ($p =$
277 0.012) and DIFF ($p = 0.018$) groups compared to the PSEUDO group but not the SAME group
278 (Figure 2B; PSEUDO vs. SAME, $p = 1.00$; COND vs. SAME, $p = 0.12$; COND vs. DIFF, $p =$
279 1.00 ; SAME vs. DIFF, $p = 0.15$). Moreover, large effect sizes in AMPA to NMDA ratios were
280 found between the COND ($d = 0.8$) and the DIFF ($d = 1.1$) groups compared to the PSEUDO
281 group. SAME vs. COND ($d = 0.6$) and SAME vs. DIFF ($d = 0.5$) showed intermediate effect
282 sizes. Small effect sizes were found between PSEUDO vs. SAME ($d = 0.3$) and COND vs. DIFF
283 ($d = 0.3$). Therefore, a significant reversal of the AMPA to NMDA ratio increase found in the
284 COND group was observed only in the SAME group. These findings suggest that fear
285 conditioning induces postsynaptic plasticity of vHC inputs in IL that is more effectively reversed
286 when extinction occurs in the same context as conditioning.

287

288 *Fear conditioning and extinction alter NMDAR currents in vHC-IL synapses.*

289 Since changes in either AMPAR or NMDAR-mediated EPSCs could produce a change in the
290 AMPA to NMDA ratios, we evaluated the AMPAR and NMDAR components separately to
291 determine which one was responsible for the changes observed in IL neurons (Figure 3). First
292 we examined the AMPA component and found no significant differences in AMPAR EPSCs
293 ($H(3,90) = 5.48$, $p = 0.14$) or conductance ($H(3,90) = 1.95$, $p = 0.58$) (Figure 3A, B). In contrast,
294 we found that fear conditioning significantly reduced NMDAR EPSCs and extinction reversed
295 the reduction (Figure 3C). Kruskal-Wallis test showed a significant main effect in NMDAR
296 EPSCs ($H(3,90) = 15.98$, $p = 0.001$) and post hoc analysis revealed that NMDAR EPSCs were
297 smaller in the COND group compared to PSEUDO ($p = 0.002$) and SAME ($p = 0.023$) groups
298 (COND vs. DIFF, $p = 1.00$; PSEUDO vs SAME, $p = 1.00$; PSEUDO vs. DIFF, $p = 0.12$; SAME
299 vs. DIFF, $p = 0.65$). Cohen's d found intermediate effect sizes in NMDAR EPSCs in PSEUDO
300 vs. COND ($d = 0.7$), PSEUDO vs. DIFF ($d = 0.5$), COND vs. SAME ($d = 0.6$) and SAME vs.
301 DIFF ($d = 0.5$). Small effect sizes were found in COND vs. DIFF ($d = 0.3$), and no effect in
302 PSEUDO vs. SAME ($d = 0.01$). In addition, we found that fear conditioning decreased NMDAR
303 conductance and extinction reversed the decrease in NMDAR conductance (Figure 3D). Kruskal-
304 Wallis test showed a significant main effect in NMDAR conductance ($H(3,90) = 14.26$, $p =$
305 0.003) and post hoc analysis revealed that NMDAR conductance was smaller in the COND
306 group compared to PSEUDO ($p = 0.003$) and SAME ($p = 0.023$) groups (COND vs. DIFF, $p =$
307 0.53 ; PSEUDO vs. SAME, $p = 1.00$; PSEUDO vs. DIFF, $p = 0.60$; SAME vs. DIFF, $p = 1.00$).
308 Cohen's d found intermediate effect sizes in NMDAR conductance in COND vs. PSEUDO ($d =$
309 0.7) and COND vs. SAME ($d = 0.7$). Small effect sizes were found in PSEUDO vs. DIFF ($d =$
310 0.4), COND vs. DIFF ($d = 0.4$) and SAME vs. DIFF ($d = 0.4$). These results suggest that the

311 changes observed in AMPA to NMDA ratios were caused by alterations in NMDAR mediated
312 currents induced by fear conditioning and extinction. Acquisition of fear reduced the NMDAR-
313 mediated currents at vHC synapses onto IL neurons and extinction in the same context reversed
314 this depression. Extinction in a novel context failed to reverse the depressed NMDAR currents.

315

316 *Failure to recall extinction correlates with failure to reverse conditioning-induced depression of*
317 *NMDAR currents in vHC-IL synapses.*

318 As found previously by others (Burgos-Robles et al., 2007; Peters et al., 2010; Gruene et al.,
319 2015), we found that a cohort of animals that received extinction in the fear conditioning context
320 (SAME, from Figure 2A) that showed poor extinction memory retrieval (UNSUCCESSFUL;
321 Figure 4A). Thus, although these animals received the same behavioral treatment as the SAME
322 group in Figure 2A, they showed poor extinction memory with more than 50% freezing during
323 the extinction recall test (UNSUCCESSFUL group; Figure 4A). In fact, the UNSUCCESSFUL
324 group behaved as though it never received extinction and froze as much as the COND group at
325 recall ($t = 0.07634$, $df = 14$, $p = 0.9402$). In comparison, the SAME group from Figure 2A
326 showed less than 50% freezing during recall (SUCCESSFUL group; Figure 4A). If the reversal
327 of NMDAR currents by extinction is important for extinction memory, then animals that failed to
328 remember extinction should show smaller NMDAR currents. Consistent with this, AMPA to
329 NMDA ratios of vHC inputs in IL of the UNSUCCESSFUL ($n = 16$ cells from 6 rats) group
330 were greater than those from rats with successful extinction retrieval (SUCCESSFUL, $n = 23$
331 cells from 7 rats; Mann-Whitney U test, $U = 108$, $p = 0.030$). In addition, a large effect size was
332 found in AMPA to NMDA ratios between the groups ($d = 0.8$). As observed in COND animals
333 (Figure 3D), the larger AMPA to NMDA ratios in the UNSUCCESSFUL group was caused by

334 smaller NMDAR EPSCs (Figure 4D-E). Mann-Whitney U test showed that the
335 UNSUCCESSFUL group had smaller NMDAR EPSCs ($U = 266$, $p = 0.019$) with an
336 intermediate effect size ($d = 0.6$). Also, NMDAR conductance in UNSUCCESSFUL animals
337 showed a tendency towards a reduction ($U = 248$, $p = 0.069$) with an intermediate effect size ($d =$
338 0.6). Once again, no differences were found in the AMPAR current ($U = 216$, $p = 0.37$) or
339 conductance ($U = 192$, $p = 0.83$) (Figure 4F-G). These findings suggest that the observed
340 changes in NMDAR currents at vHC synapses in IL are important for successful extinction
341 memory retrieval.

342

343 During auditory fear conditioning, the rats learn to associate the tone and context with the
344 aversive foot shock. Therefore, differences in contextual fear among the behavioral groups may
345 also contribute to the synaptic differences. To address this issue, we calculated the percent of
346 time the rats froze during the minute prior to the first tone on day 3 which is a measure of fear to
347 the context A for the PSEUDO ($n = 7$), COND ($n = 10$), SUCCESSFUL ($n = 7$) and
348 UNSUCCESSFUL ($n = 6$) groups. The DIFF group that received extinction in a novel context B
349 were not included since their recall was given in context B and not in the fear conditioning
350 context A. Although the contextual fear varied among the groups (Figure 5A), there were no
351 significant differences among them ($F(3, 25) = 2.394$, $p = 0.092$). However, the small sample
352 size likely affected this statistical analysis; therefore, we also calculated effect size. When we
353 evaluated the effect size, we found a large overall effect in contextual freezing among groups (d
354 $= 1.0$). A large effect in contextual freezing was observed in the COND group compared to the
355 PSEUDO ($d = 0.9$) and SAME ($d = 0.8$) groups. An intermediate effect in contextual freezing
356 between the PSEUDO ($d = 0.6$) and SAME ($d = 0.5$) groups compared to the UNSUCCESSFUL

357 group. A small effect between the COND group compared to the UNSUCCESSFUL group ($d =$
358 0.2) and no effect between the PSEUDO and SAME groups ($d = 0.1$). Therefore, as expected the
359 COND and UNSUCCESSFUL groups showed more contextual fear than the PSEUDO and
360 SAME groups. Furthermore, the contextual fear shown on day 3 strongly correlated with the
361 cued fear to the tones (Figure 5B).

362

363 *BDNF mimicked fear extinction by reversing fear conditioning-induced increase in*
364 *AMPA/NMDA ratios and decrease in NMDAR currents in vHC – IL synapses.*

365 Previous studies have nicely demonstrated that in vivo infusion of BDNF into IL prior to
366 extinction is sufficient to decrease fear expression and induce extinction (Peters et al., 2010;
367 Rosas-Vidal et al., 2014). Therefore, if the changes we observed in the NMDAR currents are
368 important for extinction, then BDNF should produce similar changes in the NMDAR currents at
369 the vHC to IL synapses as fear extinction. As shown in Figure 6, we found that BDNF-treated
370 IL neurons ($n = 11$ cells from 4 rats) from fear conditioned animals showed lower
371 AMPA/NMDA ratios and larger NMDAR EPSCs than non-treated IL neurons ($n = 9$ cells from
372 4 rats) from the same animals. Mann-Whitney U test showed a significant mean difference in
373 AMPA/NMDA ratios ($p = 0.022$) and NMDAR EPSCs ($p = 0.033$). Cohen's d found large
374 effects in AMPA/NMDA ratios ($d = 1.2$) and NMDAR EPSCs ($d = 0.8$) between groups. Thus,
375 ex vivo incubation with BDNF decreased the AMPA/NMDA ratio and increased the NMDAR
376 currents in vHC-IL synapses mimicking the synaptic effects produced by fear extinction. These
377 findings further substantiate that the observed changes in NMDAR currents at vHC-IL synapses
378 contribute to the behavioral changes.

379 **Discussion**

380 By using optogenetic stimulation, we found that auditory fear conditioning induces postsynaptic
381 plasticity at vHC synapses onto IL pyramidal neurons that involves an increase in the ratio of
382 AMPA to NMDAR currents. Further examination revealed that fear acquisition reduced the
383 NMDAR currents without altering the AMPA currents. Previous studies using electrical
384 stimulation of all axons within the vicinity of the electrode did not observe synaptic plasticity
385 after fear conditioning in IL (Pattwell et al., 2012; Sepulveda-Orengo et al., 2013). This suggests
386 that the depression of NMDAR currents at vHC-IL synapses does not occur at the majority of
387 synapses onto IL neurons and could be a unique feature of vHC synapses. Although we used
388 established coordinates for infusion of the viral constructs into the rat ventral hippocampus
389 (Sierra-Mercado et al., 2011) and we confirmed expression of viral proteins in the ventral
390 hippocampus (Figure 1A), it is not possible to completely rule out the possibility that some fibers
391 from viral expression in areas near the ventral hippocampus also contribute to the inputs
392 recorded in IL. However, based on the relatively robust projections from the ventral
393 hippocampus to IL (Hoover and Vertes, 2007), the majority of inputs activated in IL likely come
394 from the ventral hippocampus.

395

396 Exposure to extinction reversed the fear conditioning-induced plasticity leading to an increase in
397 NMDAR currents and a corresponding decrease in AMPA/NMDA ratio. In support of our
398 findings, a recent study in mice also found increased NMDA currents at vHC-IL synapses after
399 fear extinction (Wang et al., 2018). In our experiments, extinction induced a more complete
400 reversal of conditioning-induced plasticity when the extinction was conducted in the fear
401 conditioning context. The fact that the group that received cued fear extinction in the novel

402 context showed intermediary changes in the NMDAR EPSCs compared to the group that
403 received extinction in the same context as fear conditioning suggests that extinction of fear to the
404 cue and extinction of fear to the context both contribute to the synaptic modifications of the
405 vHC-IL synapses. Exposure to extinction in the novel context likely left remnants of the
406 contextual fear which produced NMDAR EPSCs in between those of the COND group and the
407 group that received extinction in the fear conditioning context. This suggests that contextual and
408 cued fear modulate vHC-IL synapses independently. Whether the same or independent
409 populations of ventral hippocampal neurons are modulated remains to be determined.

410

411 The observed changes in AMPA/NMDA ratio, NMDAR EPSC, and NMDAR conductance at the
412 vHC-IL synapse varied with the levels of fear expression suggesting that these changes
413 contribute to the behavioral outcome. The fact that animals that failed to recall extinction
414 memory did not show a reversal of the conditioning-induced changes suggests that the changes
415 in NMDAR requires the behavioral change rather than simple exposure to extinction training.
416 Moreover, the extinction-induced changes were mimicked by incubating slices with BDNF.
417 Therefore, plasticity of NMDAR currents at vHC to IL synapses appears to modulate cued fear
418 expression and represents a novel mechanism for modulating conditioned fear.

419

420 Our findings suggest that learning-induced changes in NMDAR currents occur in response to the
421 acquisition and extinction of conditioned fear. Consistent with our findings, physiologically
422 relevant stimuli in brain slices can induce selective changes in NMDAR currents without altering
423 AMPA receptor currents (Kwon and Castillo, 2008; Rebola et al., 2008). Furthermore, NMDAR
424 show bidirectional plasticity in response to synaptic stimulation in slices (Rebola et al., 2010;

425 Hunt and Castillo, 2012). Similar to our findings, fear learning decreased NMDAR currents in
426 the amygdala by reduced phosphorylation of the GluN1 subunit of NMDAR (Zinebi et al.,
427 2003). Although acute stress can depress NMDAR currents (Kuzmiski et al., 2010), it is
428 unlikely that the changes in NMDAR currents we observed were mediated by stress, since our
429 results were compared to the pseudoconditioned group that should have similar stress exposure.

430

431 Previous studies demonstrated that infusion of BDNF into IL is sufficient to simulate extinction
432 and reduce fear to a conditioned stimulus (Peters et al., 2010; Rosas-Vidal et al., 2014). This
433 effect of BDNF was prevented by systemic blockade of NMDA receptors (Peters et al., 2010)
434 suggesting that BDNF may increase NMDA currents to reduce conditioned fear. Consistent with
435 this possibility, we found that ex vivo incubation of prefrontal slices with BDNF induced similar
436 synaptic changes in the vHC inputs in IL as extinction. Another recent study in mice also showed
437 that incubation with BDNF increases the NMDA currents at vHC synapses in IL (Wang et al.,
438 2018). Therefore, one synaptic mechanism by which BDNF simulates extinction may be by
439 increasing NMDAR currents at vHC inputs in IL pyramidal neurons. However, an infusion of
440 BDNF in vivo may also induce additional cellular and circuit effects that contribute to the
441 reduction in conditioned fear.

442

443 Inputs from vHC to the mPFC provide spatial representations that guide aversive behaviors
444 (Padilla-Coreano et al., 2016). Our findings suggest that the behavioral outcome of vHC
445 stimulation of IL is altered by fear conditioning and extinction. Consistent with IL's proposed
446 role of inhibiting freezing in response to aversive cues (Milad and Quirk, 2002; Sierra-Mercado
447 et al., 2011; Moscarello and LeDoux, 2013), weakened vHC activation of IL pyramidal neurons

448 after fear conditioning would increase freezing behavior. This reduced synaptic activation of IL
449 excitatory neurons combined with the strong feedforward inhibition produced by vHC inputs in
450 IL (Marek et al., 2018) and depressed intrinsic excitability of IL pyramidal neurons after fear
451 conditioning (Santini et al., 2008; Soler-Cedeño et al., 2016) would reduce IL activation of
452 downstream targets such as the amygdala (Quirk et al., 2003; Cho et al., 2013) and enhance
453 acquisition of conditioned fear. In support of this model, stimulation of IL excitatory neurons
454 impairs the acquisition of conditioned fear (Yizhar et al., 2011) as does increased glucocorticoid
455 signaling in IL (Criado-Marrero et al., 2017).

456

457 After fear extinction, the increase in vHC activation of NMDAR currents on IL neurons would
458 increase the activation of IL pyramidal neurons (Orsini et al., 2011; Kim and Cho, 2017). The
459 slower kinetics of NMDA receptors allow them to drive neuronal burst firing (Polsky et al.,
460 2009; Grienberger et al., 2014). Therefore, the relative increase in NMDAR currents after
461 extinction in the conditioning context likely contributes to the increase in NMDAR-dependent
462 burst firing seen in IL neurons shortly after acquisition of fear extinction which correlated with
463 good extinction memory (Burgos-Robles et al., 2007). The resulting increased activation of the
464 amygdala by IL projections would produce a context-dependent modulation of conditioned fear
465 (Orsini et al., 2011; Maren et al., 2013).

466

467 In conclusion, we have found that fear conditioning and extinction induce bidirectional changes
468 in NMDAR currents at vHC synapses in IL. Acquisition of conditioned fear reduced NMDAR
469 currents, while extinction enhanced the NMDAR currents. Failure to reverse the conditioning-
470 induced depression of NMDAR currents led to poor extinction memory and a PTSD-like

471 phenotype. Medications designed to activate BDNF receptors may be useful for enhancing
472 NMDAR currents at vHC synapses in IL and treating PTSD.

473

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597

598 **Figure 1. Experimental design to measure vHC EPSCs in IL neurons after fear**

599 **conditioning and extinction.** (A, B) Chr2 expression in the vHC and mPFC. (C) AMPAR-

600 mediated EPSCs were measured at the peak of the -70 mV current. NMDAR-mediated EPSCs

601 were measured 70 ms after stimulation to minimize the possibility of contamination with

602 AMPAR EPSC. (D) Behavioral groups. (E) Scheme of the general timeline for the experiments.

603

604 **Figure 2. Fear conditioning and extinction induce postsynaptic plasticity in vHC synapses**

605 **onto IL pyramidal neurons.** (A) Behavioral responses of all groups. (B) Mean AMPA/NMDA

606 ratios measured in IL pyramidal neurons from each group. (C) Example traces of vHC EPSCs

607 recorded in IL pyramidal neurons of each group. (A; *** $p < 0.001$, Repeated measures

608 ANOVA, COND vs PSEUDO, SAME, and DIFF) (B; * $p < 0.05$, Dunn's post hoc after
609 significant Kruskal-Wallis test).

610

611 **Figure 3. Fear conditioning and extinction induced bidirectional changes in NMDAR**
612 **currents at vHC synapses onto IL pyramidal neurons.** (A, B) Average AMPAR-mediated
613 EPSCs and conductance in each group. (C, D) Average NMDAR-mediated EPSCs and
614 conductance in each group. (** $p < 0.01$, * $p < 0.05$; Dunn's post hoc after significant Kruskal-
615 Wallis test).

616

617 **Figure 4. IL neurons from animals that failed to recall extinction showed greater**
618 **AMPA/NMDA ratios and reduced NMDAR-mediated currents.** (A) Behavioral data from
619 same context extinction animals that showed successful or failed extinction recall. (B) Example
620 vHC EPSCs recorded in IL pyramidal neurons. (C) Average AMPA/NMDA ratio in each group.
621 (D, E) Average NMDAR-mediated EPSC and conductance in each group. (F, G) Average
622 AMPAR-mediated EPSC and conductance in each group. (* $p < 0.05$; Mann-Whitney's test).

623

624 **Figure 5. Contextual fear to context A varies with fear to tones.** (A) Average freezing during
625 the minute prior to giving the first test tone on day 3 in the different groups. (B) Correlation
626 between freezing prior to the tones and during the tones on day 3. Rats from the PSEUDO,
627 COND, SUCCESSFUL, and UNSUCCESSFUL groups are plotted.

628

629 **Figure 6. *In vitro* BDNF treatment mimicked fear extinction reversal of conditioning-**
630 **induced changes in AMPA/NMDA ratio and NMDAR currents.** (A) Behavioral data. (B)

- 631 Example trace for each group. (C) Average AMPA/NMDA ratio for each group. (D) Average
632 NMDAR EPSC for each group. (E) Average AMPAR EPSCs. ($p < 0.05$, Mann-Whitney's test).











