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Involvement of CRFR1 in the basolateral amygdala in the immediate fear extinction deficit

CRFR1 in immediate fear extinction deficit

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5 1. Manuscript Title:
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37 **Abstract**

38 Several animal and clinical studies have highlighted the ineffectiveness of fear extinction
39 sessions delivered shortly after trauma exposure. This phenomenon, termed the immediate
40 extinction deficit, refers to situations in which extinction programs applied shortly after fear
41 conditioning may result in the reduction of fear behaviors (in rodents frequently measured
42 as freezing responses to the conditioned cue/s) during extinction training, but failure to
43 consolidate this reduction in the long-term. The molecular mechanisms driving this
44 immediate extinction resistance remain unclear. Here we present evidence for the
45 involvement of the corticotropin releasing factor (CRF) system in the basolateral amygdala
46 (BLA) in male Wistar rats. Intra-BLA micro-infusion of the $CRFR_1$ antagonist NBI30775
47 enhances extinction recall, while administering the CRF agonist CRF_{6-33} before delayed
48 extinction disrupts recall of extinction. We link the immediate fear extinction deficit with
49 dephosphorylation of GluA1 glutamate receptors at Ser^{845} and enhanced activity of the
50 protein phosphatase calcineurin in the BLA. Their reversal following treatment with the
51 $CRFR_1$ antagonist indicates their dependency on $CRFR_1$ actions. These findings can have
52 important implications for the improvement of therapeutic approaches to trauma, as well as
53 furthering our understanding of the neurobiological mechanisms underlying fear-related
54 disorders.

55 **Significance Statement**

56 Trauma-related disorders are costly, highlighting the need to understand the reduction of
57 fear through extinction learning for the development of better therapies. When extinction
58 programs are applied too soon after the traumatic event, numerous studies have found it to
59 be ineffective, though the underlying mechanisms were unclear. Here we confirm that

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60 futility of immediate extinction and provide a mechanistic explanation. Using a
61 pharmacological approach, we show evidence for the involvement of the corticotropin
62 releasing factor (CRF) system in the basolateral amygdala in this extinction deficit. We link
63 this involvement with downstream molecular targets of the CRF system that are critical in
64 synaptic plasticity, thus explaining the futility of immediate extinction and providing further
65 insight into fear-related disorders.

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67 **Introduction**

68 Trauma-related disorders impose a high burden to both individuals and society (Kessler et
69 al., 2012), inspiring numerous studies of the mechanisms underlying fear extinction learning
70 (Pape and Pare, 2010; Milad and Quirk, 2012). Learning to reduce fear responses to
71 previously fearful stimuli through fear extinction learning is a complex process involving a
72 broad network of brain structures, including the basolateral nucleus of the amygdala (BLA)
73 (Sotres-bayon et al., 2004; Quirk and Mueller, 2008, Herry et al., 2010). Several molecular
74 targets have been identified for the development of therapeutic interventions for
75 posttraumatic stress disorders (PTSD) and other fear-related disorders (Milad and Quirk,
76 2012; Singewald et al., 2015). Among them, emerging evidence points to a key role for the
77 central corticotropin releasing factor (CRF) system, which is well-known for its role in the
78 regulation of stress, fear, stressful learning and anxiety responses (Bale, 2005; Nemeroff et
79 al., 2006; Regev and Baram, 2014). CRF, a 41 amino acid peptide involved in the activation of
80 the hypothalamic-pituitary-adrenal (HPA) axis, also exerts extra-hypothalamic actions in
81 different brain regions through activation of two G-protein coupled receptors, $CRFR_1$ and
82 $CRFR_2$. Both the BLA and the central nucleus of the amygdala contain CRF-expressing
83 neurons, and the BLA presents particularly high $CRFR_1$ densities (Korosi and Baram, 2008).

84 Recently, a key role for the CRF system in impaired extinction processes has been suggested.
85 In humans, enhanced CRF levels found in the cerebrospinal fluid of PTSD patients have
86 indicated a link between increased CRF concentrations and disrupted fear extinction
87 observed in PTSD and anxiety disorders (for reviews, see Gafford and Ressler, 2015;
88 Bangasser and Kawasumi, 2015). In rats, administration of CRF into the lateral amygdala
89 before fear recall testing in formerly fear conditioned animals induced enhanced freezing

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90 responses to the conditioned stimulus (Isogawa et al., 2013). Additionally, pharmacological
91 enhancement of CRF in the BLA impaired long-term retention of fear extinction, while $CRFR_1$
92 antagonism had the opposite effect (Abiri et al., 2014), supporting a detrimental role of CRF
93 in the BLA in the consolidation of cued fear extinction. Furthermore, cell-specific genetic
94 disruption of GABAAα1 within CRF-expressing neurons in mice was found to specifically
95 impair fear extinction (i.e., not affecting fear conditioning or retention) processes, while
96 systemic administration of a CRF antagonist partially rescued the fear extinction deficit in
97 these mice (Gafford et al., 2012). Accordingly, an overactive CRF system in the BLA seems to
98 interfere with extinction processes.

99 Importantly, it is not known whether the CRF system is involved in a particularly challenging
100 extinction case known as 'immediate fear extinction deficit' (Maren, 2014). This refers to the
101 ineffectiveness of fear extinction programs, frequently observed both in animals and
102 humans, when extinction training is administered soon (e.g., from minutes to a few hours)
103 after fear conditioning. Specifically, despite exhibiting decreased within-session freezing
104 during extinction training, subjects fail to maintain this response over long-term retention
105 intervals (Maren and Chang, 2006; Woods and Bouton, 2008; Schiller et al., 2008; Chang and
106 Maren 2009; Archbold et al., 2010; but see Myers et al., 2006). Given that, in addition to its
107 involvement in the acquisition and consolidation of fear conditioning (see Sah et al., 2008),
108 the BLA has been implicated in fear extinction (Sotres-Bayon et al., 2004; Quirk and Mueller,
109 2008, Herry et al., 2010), we hypothesized that mechanisms that contribute to fear
110 conditioning in the BLA might underlie the effectiveness of immediate extinction trials.
111 Converging lines of evidence support the involvement of the BLA CRF system in the
112 immediate extinction deficit. First, acute stress has been shown to rapidly induce CRF release

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113 in the amygdala (Pich et al., 1995; Merali et al., 1998) leading to activation of $CRFR_1$ in the
114 BLA in the consolidation of fear learning (Roozendaal et al., 2002; 2008; Hubbard et al.,
115 2007). Secondly, CRF increases excitability of $CRFR_1$-containing BLA projection neurons
116 (Rainnie et al., 1992) and induces long-lasting increases on the amplitude of field post-
117 synaptic potentials in the BLA (Ugolini et al., 2008; Sandi et al., 2008). These effects are
118 reversed by antagonizing $CRFR_1$, but not $CRFR_2$ (Rainnie et al., 1992; Ugolini et al., 2008).
119 Finally, whereas CRF infusions in the lateral amygdala before long-term memory testing
120 enhanced freezing responses to the conditioned stimulus (Isogawa et al., 2013), $CRFR_1$
121 antagonism in the BLA facilitated long-term retention of fear extinction (Abiri et al., 2014).
122 Therefore, we evaluated the involvement of the CRF system in the amygdala in the minimal
123 fear suppression induced by extinction training given shortly after fear conditioning and
124 explored the molecular machinery involved.

125 **Materials and Methods**

126 ***Subjects***

127 Male Wistar rats (Charles River, L'Arbresle, France) weighing 250-300g at the start of the
128 experimentation served as subjects and were singly-housed in polypropylene cages (34 x 29
129 x 17 cm) lined with abundant pine shavings. Animals had ad libitum access to food and
130 water, and were maintained in constant temperature (23°C) and lighting (0700-1900 hr)
131 conditions. All experiments were performed during the light phase. Animals were allowed to
132 habituate to the vivarium for one week and were then handled for 2 minutes on 3 days prior
133 to the beginning of all experiments.

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134 All procedures were conducted in conformity with the [Author University's] guidelines for
135 animal experimentation. All efforts were made to minimize suffering and reduce the number
136 of animals used.

137 ***Elevated plus maze***

138 Prior to experiments, anxiety-related behavior was measured using the Elevated Plus Maze
139 (EPM) according to the procedure described in Herrero et al., 2006. As previous reports
140 indicate that CRF antagonist NBI30775 affects subjects differently depending on their natural
141 anxiety level (Sandi et al., 2008), all groups were matched according to similar scores in this
142 test. EPM sessions for all experiments were conducted 4-7 days before the first fear
143 conditioning session.

144 ***Fear conditioning***

145 *Conditioning session.* The training cage (Context A) consisted of a Plexiglas transparent
146 chamber (30 × 37 × 25 cm; Panlab, Spain) that was positioned inside a sound-attenuating
147 chamber. This chamber was constructed of black stainless steel walls of smooth texture,
148 with a ceiling and door made of Plexiglas. The floor consisted of 20 steel rods wired to a
149 shock source and solid-state scrambler for the delivery of footshocks. Conditioning took
150 place in a single session. After 3-min of free exploration, rats received five pairings of a 2-sec
151 CS tone (80 db, 2000 Hz) and a 0.5-sec US footshock (0.6 mA). The inter-shock interval was
152 60-sec. Subjects were removed from the chambers 58-sec after the final shock presentation
153 (thus, the training session lasted 8-min) and left undisturbed in their home cage until the
154 extinction session.

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155 *Extinction session.* Extinction of cued fear learning took place in a different context (Context
156 B). The context shape was modified, the grid replaced by a plastic smooth floor, and visual
157 and odor cues were changed. Animals were free to explore the environment during the first
158 three minutes and then, 70 CS were presented every 40-sec. Depending on the protocol, the
159 extinction session took place 30-min, 3, or 24h after training. For each behavioral
160 experiment, separate groups of animals were placed in the extinction context without any
161 CS presentation as controls.

162 *Testing session.* 48h after training, extinction memory was assessed in Context B. After 3-min
163 of free exploration, the rats received five CS presentations with an inter-trial interval of 60-
164 sec. Rats were removed from the chambers 58-sec after the final CS presentation (8-min
165 total duration)

166 In all sessions, behavior was monitored with a camera connected to a video-recorder for
167 offline analysis which were performed by an experimenter blind to the animal's
168 experimental condition. Fear was assessed by measuring the percentage of time spent
169 freezing, characterized by a crouching posture and an absence of any visible movement
170 except for that due to breathing.

171 ***Surgery and amygdala microinfusions***

172 Animals were anaesthetized with i.p. ketamine (70 mg/kg) and xylazine (6 mg/kg). They were
173 then implanted with two 18-mm stainless steel guide cannulae (23-gauge, Plastic One,
174 Roanoke, VA) using a standard stereotaxic frame (Kopf Instruments, Bioseb, France).
175 Cannulae were bilaterally implanted at the BLA coordinates (antero-posterior, -2.8 mm
176 relative to Bregma; lateral, $\pm$ 5.1 mm from midline; ventral, -5.5 mm from dura). The tips of

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177 the cannulae were aimed 2 mm above the intended area. The cannulae were fixed to the
178 skull with dental acrylic cement. Stylets were inserted into the guide cannulae to prevent
179 clogging. Rats were given one week to recover from surgery, after which they received the
180 EPM and fear sessions as described above.

181 All animals were handled individually for approximately 1-2 min each day during the 2-3 days
182 preceding infusion to habituate them to the infusion procedure. Immediately after training,
183 rats were gently restrained, stylets removed and injection needles (30 gauges) inserted,
184 extending 2 mm from the tip of the guide cannula. The injection needles were connected via
185 polyethylene tubing to two 10 µl Hamilton microsyringes driven by an automated
186 microinfusion pump (Harvard Apparatus, Bioseb, France). The needles were then left in
187 position for an additional minute to enable diffusion of the solution into the tissue and to
188 minimize dragging of the liquid along the injection track.

189 ***Drugs***

190 NBI30775 (3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2, 5-dimethyl-N, N-dipropyl-pyrazolo
191 [2,3-a]pyrimidin-7-amine), a non-peptide $CRFR_1$ antagonist (also known as R121919) was a
192 generous gift from Dimitri Grigoriadis (Neurocrine Inc). It was dissolved in DMSO and
193 bilaterally infused in the BLA at different concentrations, 0.1, 0.3, 1, or 10µg in 0.5µl, at a
194 rate of 0.3µl/min immediately after fear conditioning. Vehicle infusions of DMSO were
195 administered in a similar manner. The infusion volume for NBI experiments was
196 0.5µl/hemisphere. Depending on the experiment, animals were subject to extinction 30min
197 thereafter or left undisturbed until the testing session 48hr and 7d after training.

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198 The CRF agonist $CRF_{6\text{-}33}$ (Sigma-Aldrich, Saint-Louis, MO, USA) was dissolved in saline and
199 infused bilaterally in the BLA at a concentration of 0.1µg in 0.2 µl, at a rate of 0.3µl/min just
200 prior to a delayed (24hr after training) extinction session. The infusion volume for agonist
201 experiments was 0.2µl/hemisphere. Animals were tested 48h after training. Vehicle
202 infusions of saline were administered in a similar manner.

203 After completion of behavioral experiments, animals were overdosed with sodium
204 pentobarbital (100 mg/kg i.p.). The brains were removed and immediately frozen at -50°C in
205 isopentane and stored at -20°C. Coronal sections (40µm thick) were stained with thionine for
206 histological checking. Out of the 84 implanted animals, 12 were discarded due to
207 misplacement of one or both cannulae.

208 ***Western blot***

209 *Tissue preparation.* Immediately after decapitation, the amygdala was rapidly dissected out
210 and frozen at -80° C until processing. Tissue was homogenized in ten volumes of ice-cold
211 sucrose (0.32 m) and HEPES (5 mm) buffer that contained a cocktail of protease inhibitors
212 (Complete TM, Roche, UK) with 16 strokes and centrifuged at 1000 x g for 5 min. The
213 resulting total fraction pellet was resuspended in Krebs buffer with 1% NP40, incubated at
214 4C for 40 minutes, and then centrifuged at 10000 x g for 20 minutes at 4C. Protein
215 concentration for each sample was estimated by BCA protein analysis (Bio-Rad).

216 *Quantification of phosphorylation of the AMPA GluA1 subunits.* Ten µg of protein were
217 loaded in each well and then separated on 10% (w ⁄ v) SDS-PAGE and transferred (70V, 1h30
218 minutes) to a Nitrocellulose membrane (Whatman). Membranes were incubated overnight
219 at 4°C with a rabbit anti-Phospho-Ser^{845} GluA1 polyclonal antibody (detects phosphorylation

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220 on GluA1 serine-845 only; 1:5000; PhosphoSolutions cat. P1160-845, RRID: AB_2492128), a
221 rabbit anti-Phospho- Ser^{831} GluA1 monoclonal antibody (detects phosphorylation on GluA1
222 serine-831 only; 1:5000; Merck-Millipore cat. 04-823, RRID: AB_1977218), and a rabbit anti-
223 GluA1 polyclonal antibody (detects GluA1 irrespective of modifications; 1:10000; Assay
224 Designs cat. ADI-905-416-1, RRID: AB_2039139). Monoclonal mouse anti-Actin (1:5000;
225 Sigma-Aldrich cat. A3853, RRID: AB_262137) and a mouse monoclonal anti-GAPDH
226 (1:40000; Abcam cat. Ab8245, RRID: AB_2107448) were incubated as loading controls. The
227 blots were washed with PBS-T, incubated for 1h with a secondary antibody, a goat anti-
228 rabbit IgG HRP conjugate (1:5000; ThermoFisher Scientific cat. G-21234, RRID: AB_1500696)
229 and a goat anti-mouse IgG peroxidase conjugate for loading controls (1:5000; Calbiochem
230 cat. 401215, RRID: AB_10682749) and developed using an enhanced chemiluminescence
231 system (Pierce). Bands were revealed with a ChemiDoc imaging system (Bio-Rad) for
232 optimum exposure time. Images were analyzed using QuantityOne software v4.6.3 (Bio-Rad)
233 where the adjusted volume was calculated for each band. For each group, the value of
234 pGluA1 Serine subunit was normalized to total GluA1 following normalization to loading
235 controls. In order to assess changes relative to the basal state, each experimental group is
236 reported as a percentage of Home cage group values.

237 ***Calcineurin activity ELISA assay***

238 *Tissue preparation.* Immediately following rapid decapitation, brains were dissected out and
239 flash frozen in ice-cold isopentane. The basolateral amygdala was tissue punched on a
240 freezing cryostat and stored at -80C for further processing. Samples were homogenized and
241 processed according to the manufacturer’s protocol for the Calcineurin Cellular Activity kit
242 (Enzo Life Sciences, BML-AK816, Switzerland), with the following adjustments. Punches were

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243 homogenized in 200uL lysis buffer with protease inhibitor using a motorized pestle and then
244 passed through a desalting column to remove excess phosphate. Calcineurin activity was
245 then measured according to manufacturer's specifications.

246 ***Data Analyses***

247 Intergroup comparisons were evaluated using Student's unpaired *t*-test, one- or two-way
248 ANOVA followed by the Fisher test for *post hoc* analysis where appropriate. Differences were
249 considered significant if $p<0.05$. Superscript letters listed with *p*-values correspond to the
250 statistical tests shown in Table 1. For western blot data and calcineurin activity, data are
251 shown as % of home cage controls.

252 **Results**

253 In all experiments, we verified that freezing levels during fear conditioning did not differ for
254 the different experimental groups included. For the sake of clarity, these analyses are
255 reported in Table 2.

256 ***Immediate extinction sessions result in inefficient extinction***

257 In order to identify appropriate experimental conditions to evaluate mechanisms underlying
258 the immediate fear extinction deficit phenomenon, we first examined the efficiency of
259 performing extinction training at several time points post-conditioning (Figure 1A). Here,
260 efficiency means that we examined whether animals exposed to extinction training at
261 particular post-conditioning intervals were able to demonstrate significantly reduced
262 freezing levels during a test, compared to corresponding non-extinguished (No-EXT) groups.
263 All groups were balanced for trait anxiety using the Elevated Plus Maze (EPM) such that

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there were no *a priori* significant differences in the time spent on the open arms ($F_{(1,38)}$=0.03; p^a=0.86; Figure 1B). While pre-tone freezing levels for the immediate extinction groups were high, we found this behavior to be common in the literature when extinction training sessions were given shortly following fear conditioning (Maren and Chang, 2006; Chang and Maren, 2009; Chan et al., 2010; Goode et al., 2015). A three-way repeated measures general linear model (extinction x interval x trial) for the percentage of time spent freezing during exposure to either the extinction training (EXT) or context B (No-Ext). EXT groups found significant, but equivalent changes in freezing behavior, as indicated by a significant main effect of trial ($F_{(2,36)}$=47.8; p^b<0.0001), extinction x trial interaction ($F_{(2,36)}$=8.47; p^c=0.001), but no significant interval x trial ($F_{(4,74)}$=1.53; p^d=0.21) or extinction x interval x trial interaction ($F_{(4,74)}$=0.322; p^e=0.86; Figure 1C). During the extinction test performed 48h after conditioning, a two-way ANOVA (extinction x interval) revealed a significant effect of extinction ($F_{(1,42)}$=18.85, p^f<0.001), interval ($F_{(2,42)}$=8.05, p^g=0.001) and extinction x interval interaction ($F_{(2,55)}$=3.22, p^h=0.026, Figure 1D). Fisher's post-hoc tests revealed that extinction applied 30min after conditioning was ineffective in suppressing CS-elicited fear responses since the level of freezing of animals with extinction was not significantly different than non-extinguished control animals (p^i=0.63). However, animals were able to extinguish fear responses when extinction training was given following a post-conditioning delay of 3h (p^j=0.01) and 24h (p^k<0.001). The extinction was more efficient for a delay of 24h than for 3h since animals extinguished 24h after conditioning exhibited significantly less freezing than animals of the 3h group (p^l<0.01) during the test.

Then, in order to examine the relationship between fear training and extinction, we performed Pearson's correlational analyses between the percentage of time spent freezing

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287 during fear conditioning and during the extinction test. We found a significant positive
288 correlation in animals who received extinction 30min after conditioning, such that those that
289 exhibited the greatest fear during conditioning also exhibited the greatest deficit (R^2=0.74,
290 p^m=0.006; Figure 1E). Conversely, in animals who received extinction 24h after conditioning,
291 we found the opposite correlation, with those who exhibited the least freezing during
292 conditioning exhibiting the highest levels of freezing during the extinction test (R^2=0.52,
293 p^n=0.044; Figure 1F). Animals who received extinction 3h after conditioning had no
294 significant correlation between freezing behaviors during the fear conditioning and the
295 extinction test (data not shown; R^2=0.28, p^o=0.17).

296 ***Intra-BLA infusion of a CRFR$_1$-antagonist post-conditioning facilitates immediate extinction***
297 ***without interfering with fear learning consolidation***

298 Given the above behavioral results, we chose to perform pharmacological experiments
299 (intra-BLA infusion of NBI30775 at doses of 0.1, 0.3, 1, and 10µg; Figure 2A-C) at a post-
300 conditioning interval of 30min with groups balanced for their *a priori* anxiety-like behavior
301 on the elevated plus maze ($F_{(4,33)}$=0.33; p^p=0.86; Figure 2B). During extinction sessions, all
302 groups significantly decreased their freezing across extinction training trials ($F_{(2,34)}$=294.8;
303 p^q<0.0001), with no significant differences in freezing between vehicle- and drug-treated
304 animals (F values <1; Figure 2D). For the extinction test performed 48h after fear
305 conditioning, a one-way ANOVA revealed a significant effect of the drug infusion ($F_{(4,34)}$=3.51,
306 p^r=0.01; Figure 2E). Fisher's post-hoc tests showed that infusion of the highest dose of
307 NBI30775, i.e. 10µg, promoted extinction efficiency since these animals exhibited
308 significantly less freezing than vehicle-treated control animals (p^s=0.009, Figure 2E). For the
309 second test performed 1 week after fear conditioning, a one-way ANOVA revealed a

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310 significant effect of the prior drug infusion ($F_{(4,34)}$=3.51, p^t=0.017). Fisher's post-hoc tests
311 showed that infusion of both 1µg/0.5µl and 10µg/0.5µl promoted long-term extinction
312 efficiency since these animals exhibited significantly less freezing than vehicle-treated
313 control animals (p^u=0.04 and p^v=0.004 respectively, Figure 2F).

314 Subsequently, we performed a follow up experiment to investigate whether the observed
315 effects of the $CRFR_1$ antagonist could have been due to an interference with the
316 consolidation of fear conditioning. In this experiment, animals were infused with 10µg of NBI
317 30775 into the BLA immediately after conditioning and did not receive any extinction
318 session. When they were tested 48h afterwards, NBI-treated animals did not differ in their
319 freezing levels from the vehicle-infused group (Student's t-test t=1.32, p^w=0.21, Figure 2G).

320 Taken together, these results reveal that blocking CRF activity in the BLA immediately after
321 fear conditioning facilitates an immediate extinction carried out 30min after training but
322 does not interfere with normal consolidation processes.

323 ***Intra-BLA infusion of a CRF-agonist immediately before a delayed extinction impairs***
324 ***extinction***

325 Given the above findings, we then wanted to investigate whether CRH activation was
326 sufficient to produce an extinction deficit. We focused on a delayed extinction protocol (24h
327 following fear conditioning) where we found no evidence of an extinction deficit (Figure 1).
328 We reasoned that if, in immediate extinction protocols, endogenously shock-induced
329 activation of CRH is sufficient for the extinction deficit, then enhancing CRH activation in the
330 BLA before a delayed extinction protocol should also induce a deficit. Thus, we infused a CRF
331 agonist into the BLA 30 min prior to a delayed extinction session given 24h after fear

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332 conditioning (Figure 3A) in groups balanced for their *a priori* anxiety-like behavior on the
333 EPM (t=1.7; p^x=0.12; Figure 3B). During extinction training, both groups exhibited significant
334 decreases in freezing behavior indicated by a main effect of trial ($F_{(2,8)}$=9.94; p^y=0.007) at a
335 similar level (interaction and treatment effect F values<1; Figure 3C). As hypothesized, pre-
336 extinction training infusion of 1µg/0.2µl of $CRF_{6\text{-}33}$ into the BLA altered extinction efficiency
337 as these animals exhibited significantly more freezing than vehicle-infused animals in the
338 extinction test given 24 h afterwards (Student's t-test t=2.90, p^z=0.028, Figure 3D). These
339 results indicate that infusion of a CRF agonist just before extinction training reduces
340 subsequent fear extinction efficiency.

341 ***Alteration of extinction is correlated with alteration of phosphorylation of the AMPA***
342 ***GluA1 subunits***

343 To uncover possible mechanisms underlying the involvement of the CRF system in the BLA in
344 impaired extinction learning, we examined phosphorylation of AMPA receptors at specific
345 serine residues as they have been previously linked with extinction learning (Monfils et al.,
346 2009). A new cohort of animals was conditioned and received an extinction session (CtxB CS)
347 either immediately (30min: CtxB CS 30min) or following a delay (24h: CtxB CS 24h) following
348 the fear conditioning session, and animals were sacrificed at the end of the session (Figure
349 4A) and western blots were performed against phosphorylated AMPA receptor subunits. In
350 order to compare our results with others who have examined phosphorylated AMPA
351 receptor subunit changes following 3 min of CS extinction exposure, we included an
352 additional group (CtxB 3 min CS) where animals were exposed to 3 minutes of extinction
353 following either 30min or 24h after training, and took brain samples immediately afterwards.
354 To control for possible effects of the new context, an additional group of animals was

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355 exposed to the context B without any CS presentations (CtxB no CS). All groups were
356 balanced for their *a priori* anxiety-like behavior on the EPM ($F_{(2,31)}$=.0002; p^{aa}=0.99; Figure
357 4B). During extinction training, all groups exhibited significant decreases in freezing
358 behaviors indicated by a main effect of trial ($F_{(1,31)}$=103.1, p^{bb}<0.0001), but no significant
359 effects of training interval ($F_{(1,31)}$=2.18; p^{cc}=0.15), group ($F_{(2,31)}$=2.37; p^{dd}=0.11), or interaction
360 ($F_{(2,31)}$=1.45; p^{ee}=0.25; Figure 4C). For the 30min post-conditioning interval, i.e. when animals
361 exhibited a deficit in fear extinction efficiency, a one-way ANOVA revealed a significant
362 effect of condition ($F_{(3,19)}$=4.35, p^{ff}=0.017). Fisher's post-hoc tests showed that the
363 percentage of phosphorylation of the AMPA GluA1 Ser^{845} subunit in Home cage was not
364 significantly different from the CtxtB no CS control group (p^{gg}=0.75) but was significantly
365 higher than CtxB CS 30min and CtxB 3minCS groups (p^{hh}=0.042, p^{ii}=0.019 respectively; Figure
366 4D), indicating that AMPA GluA1 Ser^{845} phosphorylation is decreased 30min following fear
367 conditioning. For the 24 h post-conditioning interval, i.e. when animals did not show any
368 deficit in fear extinction learning, a one-way ANOVA did not reveal any significant effect (F
369 values<1). Analysis of phosphorylation at another serine residue, GluA1 Ser^{831}, found no
370 significant alterations in phosphorylation compared to home cage controls in any condition
371 (F values<1; Figure 4E). Importantly, analysis of the total GluA1 receptors also revealed no
372 significant differences between groups (F values<1; Figure 4F).

373 ***Modulation of AMPA GluA1 Ser ^{845} phosphorylation by $CRFR_1$ antagonist in the BLA***

374 To determine whether AMPA GluA1 Ser^{845} phosphorylation levels were modulated by the
375 actions of CRF following fear conditioning, we examined phosphorylation in the amygdala in
376 animals infused with either vehicle or NBI30775 and sacrificed subsequent to an extinction
377 session delivered 30min post-conditioning (Figure 5A) in groups balanced for their *a priori*

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378 anxiety-like behavior on the EPM ($F_{(2,27)}$=0.004; p^{jj}=0.99; Figure 5B). During extinction
379 training, there was a significant main effect of trial ($F_{(2,26)}$=89.4; p^{kk}<0.0001) indicating that
380 both groups decreased their freezing over time. There was also a significant effect of
381 treatment (F(1,13)=7.26; p^{ll}=0.02) and a trend for an interaction ($F_{(1.6,\ 20.7)}$=3.06; p^{mm}=0.08;
382 Figure 5C), where NBI treatment reduced freezing during extinction training. Western blots
383 were performed against phosphorylated AMPA receptor subunits in samples from the
384 amygdala (Figure 5D). Vehicle-treated animals exhibited significantly reduced
385 phosphorylation on the Ser^{845} subunit compared to Home cage controls (Student's t-test,
386 *t*=4.68; p^{nn}<0.01; Figure 5D,E). Infusion of the $CRFR_1$ antagonist NBI30775 restored the
387 phosphorylation levels of this subunit to those of home cage levels (Student's t-test, *t*=0.18;
388 p^{oo}=0.86). Notably, there was no effect of either vehicle or NBI treatment on GluA1 Ser^{831}
389 phosphorylation (Figure 5D,F) nor an effect of NBI treatment alone on GluA1 Ser^{845}
390 phosphorylation after fear conditioning ($F_{(1,\ 56)}$=0.388; p^{pp}=0.54; Figure 5G).

391 ***Calcineurin modulates AMPA GluA1 Ser^{845} phosphorylation in the BLA***

392 We next investigated whether activity of the phosphatase calcineurin might be mediating
393 the actions of CRF on AMPA GluA1 Ser^{845} phosphorylation during extinction. In the 30min
394 post-conditioning interval, as both CtxB 3min CS and CtxB CS 30min groups exhibited
395 significantly decreased phosphorylation (see Figure 4D), we examined whether treatment
396 with NBI30775 reduced calcineurin activity, which would allow for the restoration of
397 phosphorylation levels. Animals were divided into groups (Figure 6A) balanced for their *a*
398 *priori* anxiety-like behavior on the EPM ($F_{(3,69)}$=0.07; p^{qq}=0.97; Figure 6B). Then, they were
399 treated with either NBI30775 or vehicle and sacrificed 30min post-conditioning following
400 either 3min (CtxB 3min CS) or a full (CtxB CS 30min) extinction exposure. A second group of

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401 animals received the same behavioral and pharmacological treatments but were sacrificed
402 from the home cage as controls. Treatment with NBI30775 significantly reduced calcineurin
403 activity in the BLA compared to vehicle-treated extinction (CtxB-CS 30min and CtxB-3minCS)
404 groups (two-way ANOVA, $F_{(1,67)}$ = 6.17; p^{rr}=0.015; Figure 6C), indicative of a link between CRF
405 levels and calcineurin activity. Post-hoc tests revealed a significant increase in calcineurin
406 activity in the vehicle-treated CtxB-CS 30min group (p^{ss}=0.02) that was blocked by NBI
407 treatment (p^{tt}=0.75) compared to vehicle-treated home cage controls.

408 **Discussion**

409 Previous work has highlighted a deficit for extinction programs that are delivered shortly
410 after fear conditioning, as opposed to a better efficiency of delayed extinction sessions (for a
411 review see Maren, 2014). Similarly, psychotherapeutic interventions provided soon after a
412 traumatic event have been reported to be rather ineffective in reducing long-term fear
413 responses (Rothbaum and Davis, 2003; Gray and Litz, 2005) though the underlying
414 mechanisms were unclear. Here, we provide strong evidence implicating the CRF system in
415 the BLA as a key mechanism mediating this immediate extinction deficit and identify the
416 phosphorylation of GluA1 and enhanced calcineurin activity as potential downstream
417 mechanisms for CRF actions.

418 First, in agreement with substantial work in the literature (Maren and Chang, 2006; Woods
419 and Bouton, 2008; Chang and Maren, 2009; reviewed in Maren 2014), we show here in rats
420 that long-term extinction efficiency is impaired when extinction training occurs shortly
421 (30min) after fear conditioning, but correctly retained when extinction training is given
422 following a longer delay period (24 h; note that some effectiveness of the extinction training
423 can be observed at the 3h time point). Furthermore, individuals exhibiting the greatest

424 amount of freezing during training also show the greatest extinction impairment when the
425 extinction training occurs shortly after fear conditioning, as opposed to the opposite
426 relationship between freezing at training extinction efficiency when extinction occurs.

427 In our effort to investigate underlying mechanisms, we reasoned that mechanisms
428 facilitating fear conditioning in the BLA might be at the core of the immediate extinction
429 deficit and postulated that a fear training-activated CRF system in the BLA interferes with the
430 immediate extinction process. This hypothesis was based on previous work implicating CRF
431 in delayed extinction deficits (Gafford et al., 2012) and evidence for a rapid activation of CRF
432 in the amygdala elicited by acute stress, including footshock (Merali et al., 1998; Yamano et
433 al., 2004) which facilitates fear consolidation processes (Roozendaal et al., 2008; Pitts and
434 Takahashi, 2011; but see Isogawa et al., 2013) through the activation of $CRFR_1$ in the BLA
435 (Roozendaal et al., 2002; Hubbard et al., 2007). In agreement with our hypothesis, we found
436 that intra-BLA post-training infusion of the $CRFR_1$ antagonist NBI30775 (0.1–10μg) given
437 shortly after fear conditioning promoted, at the higher doses tested, long-term extinction
438 learning in animals submitted to extinction training 30 min post-conditioning and tested for
439 extinction 48 hours and 7 days post-conditioning. Importantly, the reduced long-term
440 freezing exhibited by animals treated with the $CRFR_1$ antagonist is not simply the result of
441 disrupted fear consolidation, as animals just treated with NBI30775 post-training (i.e., not
442 submitted to extinction learning) show freezing levels comparable to vehicle-infused animals
443 when tested 48 hours afterwards. In supplementary experiments using delayed extinction
444 procedures, we obtained further evidence in support of a causal link between shock-
445 immediacy and/or increased CRF in the BLA and extinction deficits. Similarly to the
446 immediate extinction deficit, we found that the effectiveness of extinction training was

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447 impaired when an intra-BLA infusion of the CRF agonist $CRF_{6\text{-}33}$ (0.1μg) was applied just prior
448 to a delayed (i.e., 24 h post-conditioning) extinction training session. Although this evidence
449 supports our line of reasoning, our data do not allow us to exclude the alternative possibility
450 that CRF treatment could have acted as a CS on the extinction session, inducing some sort of
451 aversive conditioning to context B which would then be manifested as increased freezing
452 during the extinction testing session. Further experiments including extinction testing in a
453 different "C" context are warranted for unambiguously concluding the impact of increased
454 CRF on extinction efficiency.

455 Therefore, we identify here the activation of $CRFR_1$ in the BLA as a key mechanism
456 interfering with the effectiveness of immediate fear extinction training. This novel finding fits
457 with previous reports that had implicated the BLA CRF system in impaired extinction in
458 delayed extinction training paradigms (Gafford et al., 2012; Abiri et al., 2014), and fits with
459 the view that the degree of fear individuals experience just prior to the onset of an
460 extinction session might determine the efficacy of extinction learning (Maren and Chang,
461 2006; Maren, 2014). Although whether BLA $CRFR_1$ activation precisely reflects the CS-related
462 degree of amygdalar excitation remains to be established, intra-BLA CRF infusions were
463 shown to lead to robust increases in anxiety behaviors (Sajdyk et al., 1999) and to induce
464 long-lasting sensitization of noradrenergic substrates and PTSD-like symptoms (Rajbhandari
465 et al., 2015) in rodents. Moreover, in vivo release of CRF in the BLA has been found to
466 correlate with the level of freezing behavior in response to fear conditioning experiments
467 (Mountney et al., 2011).

468 In addition, we show evidence implicating a decrease in phosphorylation of the GluA1
469 glutamate receptors at Ser^{845}, but not Ser^{831}, as a downstream mechanism of BLA $CRFR_1$

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470 implication in the immediate fear extinction deficit. GluA1 membrane insertion was shown
471 to be required for fear conditioning-induced synaptic plasticity and consolidation (Rumpel et
472 al., 2005) and regulated by GluA1 phosphorylation at Ser^{845} (Blackstone et al., 1994).
473 Importantly, a transient up-regulation of GluA1 phosphorylation at Ser^{845} has been critically
474 implicated in the susceptibility of long-term expressed memories to fear erasure by
475 manipulations involving reconsolidation (Clem and Huganir, 2010) or extinction (Monfils et
476 al., 2009) protocols after a brief retrieval of the fear memory. Conversely, administration of
477 two retrieval sessions of a long-term established auditory fear memory close in time (i.e., the
478 second within 1 h after the first retrieval session) led to a rapid dephosphorylation of GluA1
479 at Ser^{845} that was associated with the inability to induce memory-impairing effects (i.e., fear
480 memory reconsolidation) by a protein synthesis inhibition (Jarome et al., 2012). These data
481 suggest that the second retrieval rapidly altered the phosphorylation state of GluA1. This fits
482 with our findings that, whereas fear conditioned animals placed in a novel context 30 min
483 post-training showed similar levels as home cage controls, those exposed to either a short (3
484 min) or long (30 min) extinction protocol displayed a dephosphorylation of GluA1 at Ser^{845} in
485 the BLA. Importantly, the same extinction treatments did not affect the phosphorylation rate
486 when they were given 24 hours after fear conditioning, further supporting a potential role of
487 dephosphorylation of GluA1 at Ser^{845} in the BLA in the immediate extinction deficit. As
488 observed in our study, the proximity of the CS application in the short and long extinction
489 sessions to fear conditioning in the immediate fear extinction deficit phenomenon mimics
490 mechanisms underlying the repetition of stimuli and conditions described by Jarome et al.
491 (2012) that make the fear memory resistant to erasure. The rapid dephosphorylation of
492 GluA1, as observed in our study, has been linked to AMPAR endocytosis, leading to
493 alterations in synaptic strength (Ehlers 2000) and shown to depend on increased activity of

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494 the protein phosphatase 2B or calcineurin (Ehlers 2000; Snyder et al. 2003). In full
495 agreement with these findings, we observed increased calcineurin activity in the BLA in the
496 groups submitted to short (3 min) or long (30 min) extinction protocols starting 30 min after
497 fear conditioning, the same time points which also display a dephosphorylation of $GluR_1$ at
498 Ser845. Calcineurin has been previously linked to the regulation of anxiety and fear
499 conditioning in the amygdala (Lin et al., 2003; Baumgärtel et al., 2008). Importantly, intra-
500 BLA infusion of NBI30775 immediately after fear training (at the dose of 10 µg that enables
501 efficient extinction in the immediate extinction protocol) in animals that were exposed to
502 extinction training 30 min after fear conditioning prevented: (1) the dephosphorylation
503 GluA1 at Ser^{845} and (2) the increase in calcineurin activity in the BLA observed in vehicle-
504 treated animals following extinction. Therefore, our findings suggest a possible mechanism
505 whereby fear conditioning-induced enhancement of CRF and activation of $CRFR_1$ in the BLA
506 may act to prevent immediate extinction learning by blocking GluA1 insertion into the
507 synapse via targeted dephosphorylated GluA1 AMPA subunits by enhanced calcineurin
508 activity.

509 While $CRFR_1$ is primarily associated with increased production of cyclic AMP (cAMP) through
510 adenyl cyclases, studies have shown that it can also interact and influence other g-protein
511 systems, such as those of protein phosphokinases, modifying the balance between several
512 signaling cascades rather than just one pathway, in a tissue-specific manner
513 (Grammatopoulos and Chrousos, 2002; Gallagher et al., 2008). Evidence from the literature
514 point to spiny pyramidal glutamatergic projection neurons in the BLA as particularly involved
515 in the $CRFR_1$-mediated effects reported in this study. For example, administration of CRF into
516 the BLA produced a specific dose-dependent increase in the expression of cFos-ir in

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517 pyramidal neurons (Rostkowski et al., 2013). In addition, calcineurin is predominantly found
518 in pyramidal neurons in the BLA (Leitermann et al., 2012). The involvement of BLA projection
519 neurons is particularly relevant in the context of extinction learning, as substantial work
520 shows that the BLA regulates the consolidation of fear extinction not only through local
521 mechanisms, but also through reciprocal projections to other brain regions, particularly the
522 medial prefrontal cortex (Akirav and Maroun, 2007; Quirk and Mueller, 2008; Herry et al.,
523 2010; Pape and Pare, 2010). In fact, the medial prefrontal cortex has been critically
524 implicated in the encoding and retrieval of extinction (reviewed in Maren, 2014) and with
525 stress-induced morphological changes associated with impaired extinction (Izquierdo et al.,
526 2006; Miracle et al., 2006; Wilber et al., 2011). Furthermore, studies have identified links
527 between BLA activity and mPFC function (Dilgen et al., 2013). Thus, in the case of immediate
528 extinction deficit, it has been proposed that amygdalar hyperexcitability may inhibit mPFC
529 circuitry and interfere with extinction retrieval. Given the ability of CRF to render the BLA
530 excitable for long periods of time (Rainnie et al., 1992; Sandi et al., 2008), our data here
531 support this hypothesis via a CRF-mediated mechanism.

532 Immediate extinction therapies have been offered as a potential solution to combat the
533 development of PTSD in individuals exposed to traumatic events. Studies from animal
534 research have demonstrated that these kinds of therapies may not be effective. Here we go
535 beyond the behavioral level and identify the activation of $CRFR_1$ in the BLA as a critical
536 mechanism underlying this phenomenon. Our findings highlight the treatment with $CRFR_1$
537 antagonists as a potential adjuvant capable to improve the effectiveness of behavioral
538 therapies given shortly after exposure to trauma.

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546

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547 **References**

548 Abiri D, Douglas CE, Calakos KC, Barbayannis G, Roberts A, Bauer EP (2014) Fear extinction
549 learning can be impaired or enhanced by modulation of the CRF system in the basolateral
550 nucleus of the amygdala. *Behav Brain Res 271:*234-239.
551
552 Akirav I, Maroun M (2007) The role of the medial prefrontal cortex-amygdala circuit in stress
553 effects on the extinction of fear. *Neural Plast 2007*:30873.
554
555 Archbold GE, Bouton ME, Nader K (2010) Evidence for the persistence of contextual fear
556 memories following immediate extinction. *Eur J Neurosci 31*(7):1303-1311.
557
558 Bale TL (2005) Sensitivity to stress: dysregulation of CRF pathways and disease development.
559 *Horm Behav 48*(1):1-10.
560
561 Bangasser DA, Kawasumi Y (2015) Cognitive disruptions in Stress-Related psychiatric
562 disorders: A role for corticotropin releasing factor (CRF). *Horm Behav 76:*125-135.
563
564 Baumgärtel K, Genoux D, Welzl H, Tweedie-Cullen RY, Koshibu K, Livingstone-Zatchej M et al.
565 (2008) Control of the establishment of aversive memory by calcineurin and Zif268. *Nat*
566 *Neurosci 11*(5):572-578.
567

eNeuro Accepted Manuscript

568 Blackstone C, Murphy TH, Moss SJ, Baraban JM, Huganir RL (1994) Cyclic AMP and synaptic
569 activity-dependent phosphorylation of AMPA-preferring glutamate receptors. *J Neurosci*
570 *14*(12):7585-7593.
571
572 Chan, W. Y. M., Leung, H. T., Westbrook, R. F., & McNally, G. P. (2010). Effects of recent
573 exposure to a conditioned stimulus on extinction of Pavlovian fear conditioning. *Learn Mem*
574 *17*(10):512-521.
575
576 Chang CH, Maren S (2009) Early extinction after fear conditioning yields a context-
577 independent and short-term suppression of conditional freezing in rats. *Learn Mem*
578 *16*(1):62-68.
579
580 Clem RL, Huganir RL (2010) Calcium-permeable AMPA receptor dynamics mediate fear
581 memory erasure. *Science 330*:1108-1112.
582
583 Dilgen J, Tejeda HA, O'Donnell P (2013) Amygdala inputs drive feedforward inhibition in the
584 medial prefrontal cortex. *J Neurophysiol 110*(1):221-9.
585
586 Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-
587 dependent endocytic sorting. *Neuron 28*(2):511-525.
588
589 Gafford GM, Ressler KJ (2015) Mouse models of fear-related disorders: cell-type-specific
590 manipulations in amygdala. *Neuroscience 321*:108-20.
591

eNeuro Accepted Manuscript

592 Gafford GM, Guo JD, Flandreau EI, Hazra R, Rainnie DG, Ressler KJ (2012) Cell-type specific
593 deletion of GABA (A) α1 in corticotropin-releasing factor-containing neurons enhances
594 anxiety and disrupts fear extinction. *Proc Natl Acad Sci USA 109*(40):16330-16335.
595
596 Gallagher JP, Orozco-Cabal LF, Liu J, Shinnick-Gallagher P (2008) Synaptic physiology of
597 central CRH system. *Eur J Pharmacol 583*(2-3):215-25.
598
599 Goode, T. D., Kim, J. J., & Maren, S. (2015). Relapse of extinguished fear after exposure to a
600 dangerous context is mitigated by testing in a safe context. *Learn Mem 22*(3):170-178.
601
602 Grammatopoulos DK, Chrousos GP (2002) Functional characteristics of CRH receptors and
603 potential clinical applications of CRH-receptor antagonists. *Trends Endocrinol Metab*
604 *13*(10):436-44.
605
606 Gray MJ, Litz BT (2005) Behavioral Interventions for Recent Trauma Empirically Informed
607 Practice Guidelines. *Behav Modif 29*(1):189-215.
608
609 Herrero AI, Sandi C, Venero C (2006) Individual differences in anxiety trait are related to
610 spatial learning abilities and hippocampal expression of mineralocorticoid receptors.
611 *Neurobiol Learn Mem 86*(2):150-159.
612
613 Herry C, Ferraguti F, Singewald N, Letzkus JJ, Ehrlich I, Lüthi A (2010) Neuronal circuits of fear
614 extinction. *Eur J Neurosci 31*(4):599-612.
615

eNeuro Accepted Manuscript

616 Hubbard DT, Nakashima BR, Lee I, Takahashi LK (2007) Activation of basolateral amygdala
617 corticotropin-releasing factor 1 receptors modulates the consolidation of contextual fear.
618 *Neuroscience 150*(4) 818-828.
619
620 Isogawa K, Bush DE, LeDoux JE (2013) Contrasting effects of pretraining posttraining and
621 pretesting infusions of corticotropin-releasing factor into the lateral amygdala: attenuation
622 of fear memory formation but facilitation of its expression. *Biol Psychiatry 73*(4):353-359.
623
624 Izquierdo A, Wellman CL, Holmes A (2006) Brief uncontrollable stress causes dendritic
625 retraction in infralimbic cortex and resistance to fear extinction in mice. *J Neurosci*
626 *26*(21):5733-8.
627
628 Jarome TJ, Kwapis JL, Werner CT, Parsons RG, Gafford GM, Helmstetter FJ (2012) The timing
629 of multiple retrieval events can alter GluR1 phosphorylation and the requirement for protein
630 synthesis in fear memory reconsolidation. *Learn Mem 19*(7):300-306.
631
632 Kessler RC, Avenevoli S, Costello EJ, Georgiades K, Green JG, Gruber MJ et al (2012)
633 Prevalence persistence and sociodemographic correlates of DSM-IV disorders in the National
634 Comorbidity Survey Replication Adolescent Supplement. *Arch Gen Psychiatry 69*(4):372-380.
635
636 Korosi A, Baram TZ (2008) The central corticotropin releasing factor system during
637 development and adulthood. *Eur J Pharmacol 583*(2):204-214.
638

eNeuro Accepted Manuscript

639 Leitermann RJ, Sajdyk TJ, Urban JH (2012) Cell-specific expression of calcineurin
640 immunoreactivity within the rat basolateral amygdala complex and colocalization with the
641 neuropeptide Y Y1 receptor. *J Chem Neuroanat 45*(1):50-56.
642
643 Lin CH, Yeh SH, Leu TH, Chang WC, Wang ST, Gean PW (2003) Identification of calcineurin as
644 a key signal in the extinction of fear memory. *J Neurosci 23*(5):1574-1579.
645
646 Maren S (2014) Nature and causes of the immediate extinction deficit: A brief review.
647 *Neurobiol Learn Mem 113:*19-24.
648
649 Maren S, Chang CH (2006) Recent fear is resistant to extinction. *Proc Natl Acad Sci USA*
650 *103*(47):18020-18025.
651
652 Merali Z, McIntosh J, Kent P, Michaud D, Anisman H (1998) Aversive and appetitive events
653 evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the
654 central nucleus of the amygdala. *J Neurosci 18*(12):4758-4766.
655
656 Milad MR, Quirk GJ (2012) Fear extinction as a model for translational neuroscience: ten
657 years of progress. *Annu Rev Psychol 63:*129-151.
658
659 Miracle AD, Brace MF, Huyck KD, Singler SA, Wellman CL (2006) Chronic stress impairs recall
660 of extinction of conditioned fear. *Neurobiol Learn Mem 85*(3):213-8.
661

eNeuro Accepted Manuscript

662 Monfils MH, Cowansage KK, Klann E, LeDoux JE (2009) Extinction-reconsolidation
663 boundaries: key to persistent attenuation of fear memories. *Science 324*:951-955.
664

665 Mountney C, Anisman H, Merali Z (2011) In vivo levels of corticotropin-releasing hormone
666 and gastrin-releasing peptide at the basolateral amygdala and medial prefrontal cortex in
667 response to conditioned fear in the rat. *Neuropharmacology 60*(2-3):410-7.
668

669 Myers KM, Ressler KJ, Davis M (2006) Different mechanisms of fear extinction dependent on
670 length of time since fear acquisition. *Learn Mem 13*(2):216-223.
671

672 Nemeroff CB, Bremner JD, Foa EB, Mayberg HS, North CS, Stein MB (2006) Posttraumatic
673 stress disorder: a state-of-the-science review. *J Psychiatr Res 40*(1):1-21.
674

675 Pape HC, Pare D (2010) Plastic synaptic networks of the amygdala for the acquisition
676 expression and extinction of conditioned fear. *Physiol Rev 90*(2):419-463.
677

678 Pich EM, Lorang M, Yeganeh M, De Fonseca FR, Raber J, Koob GF, Weiss F (1995) Increase of
679 extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of
680 awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. *J*
681 *Neurosci 15*(8):5439-5447.
682

683 Pitts MW, Takahashi LK (2011) The central amygdala nucleus via corticotropin-releasing
684 factor is necessary for time-limited consolidation processing but not storage of contextual
685 fear memory. *Neurobiol Learn Mem 95*(1):86-91.

eNeuro Accepted Manuscript

686

687 Quirk GJ, Mueller D (2008) Neural mechanisms of extinction learning and retrieval.
688 *Neuropsychopharmacology 33*(1):56-72.

689

690 Rainnie DG, Fernhout BJ, Shinnick-Gallagher P (1992) Differential actions of corticotropin
691 releasing factor on basolateral and central amygdaloid neurones in vitro. *J Pharmacol Exp*
692 *Ther 263*(2):846-858.

693

694 Rajbhandari AK, Baldo BA, Bakshi VP (2015) Predator Stress-Induced CRF Release Causes
695 Enduring Sensitization of Basolateral Amygdala Norepinephrine Systems that Promote PTSD-
696 Like Startle Abnormalities. *J Neurosci 35*(42):14270-14285.

697

698 Regev L, Baram TZ (2014) Corticotropin releasing factor in neuroplasticity. *Front*
699 *Neuroendocrinol 35*(2):171-179.

700

701 Roozendaal B, Schelling G, McGaugh JL (2008) Corticotropin-releasing factor in the
702 basolateral amygdala enhances memory consolidation via an interaction with the β-
703 adrenoceptor–cAMP pathway: dependence on glucocorticoid receptor activation. *J Neurosci*
704 *28*(26):6642-6651.

705

706 Roozendaal B, Brunson KL, Holloway BL, McGaugh JL, Baram TZ (2002) Involvement of stress-
707 released corticotropin-releasing hormone in the basolateral amygdala in regulating memory
708 consolidation. *Proc Natl Acad Sci USA 99*(21):13908-13913.

709

eNeuro Accepted Manuscript

710 Rostkowski AB, Leitermann RJ, Urban JH (2013) Differential activation of neuronal cell types
711 in the basolateral amygdala by corticotropin releasing factor. *Neuropeptides 47*(4):273-280.
712
713 Rothbaum BO, Davis M (2003) Applying learning principles to the treatment of Post-Trauma
714 reactions. *Ann N Y Acad Sci 1008*(1):112-121.
715
716 Rumpel S, LeDoux JE, Zador A, Malinow R (2005) Postsynaptic receptor trafficking underlying
717 a form of associative learning. *Science 308*:83-88.
718
719 Sah P, Westbrook RF, Lüthi A (2008) Fear Conditioning and Long-term Potentiation in the
720 Amygdala. *Ann N Y Acad Sci 1129*(1):88-95.
721
722 Sajdyk TJ, Schober DA, Gehlert DR, Shekhar A (1999) Role of corticotropin-releasing factor
723 and urocortin within the basolateral amygdala of rats in anxiety and panic responses. *Behav*
724 *Brain Res 100*(1):207-215.
725
726 Sandi C, Cordero MI, Ugolini A, Varea E, Caberlotto L, Large CH (2008) Chronic stress-induced
727 alterations in amygdala responsiveness and behavior–modulation by trait anxiety and
728 corticotropin-releasing factor systems. *Eur J Neurosci 28*(9):1836-1848.
729
730 Schiller D, Cain CK, Curley NG, Schwartz JS, Stern SA, LeDoux JE, Phelps EA (2008) Evidence
731 for recovery of fear following immediate extinction in rats and humans. *Learn Mem*
732 *15*(6):394-402.
733

734 Singewald N, Schmuckermair C, Whittle N, Holmes A, Ressler KJ (2015) Pharmacology of
735 cognitive enhancers for exposure-based therapy of fear anxiety and trauma-related
736 disorders. *Pharmacol Ther 149*:150-190.
737
738 Snyder GL, Galdi S, Fienberg AA, Allen P, Nairn AC, Greengard P (2003) Regulation of AMPA
739 receptor dephosphorylation by glutamate receptor agonists. *Neuropharmacology 45*(6):703-
740 713.
741
742 Sotres-Bayon F, Bush DE, LeDoux JE (2004) Emotional perseveration: an update on
743 prefrontal-amygdala interactions in fear extinction. *Learn Mem 11*(5):525-535.
744
745 Ugolini A, Sokal DM, Arban R, Large CH (2008) CRF1 receptor activation increases the
746 response of neurons in the basolateral nucleus of the amygdala to afferent stimulation.
747 *Front Behav Neurosci 2:*2.
748
749 Wilber AA, Walker AG, Southwood CJ, Farrell MR, Lin GL, Rebec GV, Wellman CL (2011)
750 Chronic stress alters neural activity in medial prefrontal cortex during retrieval of extinction.
751 *Neuroscience 174*:115-31.
752
753 Woods AM, Bouton ME (2008) Immediate extinction causes a less durable loss of
754 performance than delayed extinction following either fear or appetitive conditioning. *Learn*
755 *Mem 15*(12):909-920.
756

eNeuro Accepted Manuscript

eNeuro Accepted Manuscript

757 Yamano Y, Yoshioka M, Toda Y, Oshida Y, Chaki S, Hamamoto K, Morishima I (2004)
758 Regulation of CRF POMC and MC4R gene expression after electrical foot shock stress in the
759 rat amygdala and hypothalamus. *J Vet Med Sci 66*(11):1323-1327.

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762 **Figure Legends**

763 **Figure 1:** Efficient fear extinction is dependent on the interval between training and
764 extinction sessions. Animals were separated into extinction (EXT) and no-extinction (No-EXT;
765 NE) groups (A), balanced for anxiety-like behavior on the elevated plus maze (B) and trained
766 to similarly associate a footshock with a tone assessed by levels of freezing (see also Table
767 2). Animals in the EXT group were then exposed to an extinction session either 30min 3h or
768 24h after training with all interval groups showing similar patterns of extinction (C, open
769 symbols). Animals in the No-EXT group were exposed to the context B arena without tones
770 and each subgroup shows similar levels of freezing during this period (C, filled symbols).
771 Animals exposed to extinction sessions immediately after training (30min) exhibited similar
772 levels of freezing as the No-EXT group during the extinction test (D). Animals exposed to
773 delayed (3h 24h) extinction sessions exhibited significantly reduced freezing levels. "BL" =
774 baseline freezing during the first 3 minutes pre-tone. The freezing behavior during the
775 extinction test in animals exposed to an extinction session 30min after training was
776 positively and significantly correlated with the amount of fear shown during the initial fear
777 training sessions (E), while those receiving an extinction session 24h after training had a
778 significant negative correlation between freezing behavior in the extinction test versus fear
779 training (F). Data depicted as mean percentage of time spent freezing ± SEM; *****: Significant
780 difference ($p<0.05$) with the corresponding No-EXT group; n=8/group.

781 **Figure 2:** Impaired extinction efficiency due to immediate post-training interval is restored
782 by a post-training bilateral infusion of $CRFR_1$ antagonist NBI30775 in the BLA. Animals were
783 separated into groups (A), balanced for anxiety-like behavior on the elevated plus maze (B)
784 and following successful training received a bilateral infusion (C) of NBI30775 or vehicle

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785 immediately at the end of the session and given an extinction training session 30min later
786 (D). Animals infused with the 10ug dose of NBI30775 showed significantly reduced freezing
787 levels during the first testing session 48h post-training (E). Both 1ug and 10ug doses showed
788 significantly reduced freezing levels when tested one week later (F). A separate group of
789 animals were trained and received NBI or vehicle afterwards and left undisturbed until
790 testing 48h later. Treated animals showed similar levels of freezing in the test suggesting
791 that infusion of NBI does not affect memory consolidation (G). "BL" = baseline freezing
792 during the first 3 minutes pre-tone. Data depicted as mean percentage of time spent
793 freezing ± SEM; *****: Significant difference ($p<0.05$) with the DMSO vehicle group; n= 6-
794 10/group.

795 **Figure 3:** Infusion of CRF agonist $CRF_{6\text{-}33}$ in the BLA immediately before delayed extinction
796 session alters extinction efficacy. Animals were separated into groups (A) balanced for
797 anxiety-like behavior on the elevated plus maze (B), successfully fear conditioned to a similar
798 level (C) and infused with $CRF_{6\text{-}33}$ or saline 30min prior to a delayed (24h) extinction session.
799 Treated animals showed significantly increased freezing levels compared to vehicle (D). "BL"
800 = baseline freezing during the first 3 minutes pre-tone. Data depicted as percentage of time
801 spent freezing ± SEM; *****: Significant difference ($p<0.05$) with the CRF agonist $CRF_{6\text{-}33}$ group
802 (0.1µg/0.2µl); n=5-6/group.

803 **Figure 4:** Immediate extinction impairment is associated with reduced phosphorylation of
804 the AMPA GluA1 Ser^{845} subunit. Animals were separated into groups (A), balanced for
805 anxiety-like behavior on the elevated plus maze (B), trained and sacrificed (denoted as "X")
806 immediately following various context B (CtxB) exposures either 30min or 24h after training,
807 with each group showing similar levels of freezing during CtxB exposure (C). Phosphorylation

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808 of AMPA GluA1 Ser^{845} was significantly decreased in animals exposed to CS presentations
809 30min post-training but not in those with a delayed (24h) exposure (D) compared to the
810 home cage controls (HC). There were no significant differences in the phosphorylation levels
811 of AMPA GluA1 Ser^{831} (E) or in total GluA1 receptor protein (F). Data depicted as percentage
812 of control group ± SEM; *****: Significant difference ($p<0.05$) with the home cage (HC) group;
813 n=6/group.

814 **Figure 5:** GluA1 Ser^{845} phosphorylation levels are reversed with administration of NBI.
815 Animals were separated into groups (A), balanced for anxiety-like behavior on the elevated
816 plus maze (B), and received a post-training infusion of either the $CRFR_1$ antagonist NBI30775
817 or vehicle and an immediate extinction session 30min later (C; open box denote NBI-treated
818 animals; filled box denotes vehicle-treated animals). Treatment with NBI30775 restored
819 GluA1 Ser^{845} phosphorylation to vehicle home cage (HC) levels (D-E) but had no effect on
820 GluA1 Ser^{831} (D,F) or on its own (G). Data depicted as percentage of vehicle home cage
821 control group (hatched bar and also dotted line) ± SEM; *****: Significant difference ($p<0.05$)
822 with the vehicle-treated home cage group; n=8-12/group.

823 **Figure 6:** Enhanced calcineurin activity following extinction is reversed by NBI
824 administration. Animals were trained and infused post-training with either NBI30775 or
825 vehicle and separated into groups (A) balanced for anxiety-like behavior on the elevated plus
826 maze (B) and then sacrificed (denoted as "X") for calcineurin activity assessment after either
827 3min or a full extinction session 30min after training. Calcineurin activity tended to increase
828 following the extinction session in vehicle-treated animals (CtxB CS group) but was blocked
829 by post-training treatment of NBI30775 (C). Data depicted as percentage of vehicle home

830 cage control group (hatched bar and also dotted line) ± SEM; *: Significant difference

831 (p<0.05) with the vehicle-treated home cage group; n=8-10/group.

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833 **Tables**

834 **Table 1:** Summary of statistics for all experiments.

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	Figure	Description	Data structure	Type of test	Power
a	1B	EPM: time spent on the open arms	Normal Distribution	2-way ANOVA	1
b	1C	Extinction training: effect of "trial"	Normal Distribution	3-way repeated ANOVA	1
c	1C	Extinction training: "extinction" x "trial" interaction	Normal Distribution	3-way repeated ANOVA	0.95
d	1C	Extinction training: interval x trial interaction	Normal Distribution	3-way repeated ANOVA	0.45
e	1C	Extinction training: extinction x interval x trial	Normal Distribution	3-way repeated ANOVA	0.12
f	1D	Extinction test: effect of extinction	Normal Distribution	2-way ANOVA	0.99
g	1D	Extinction test: effect of interval	Normal Distribution	2-way ANOVA	0.94
h	1D	Extinction test: extinction x interval interaction	Normal Distribution	2-way ANOVA	0.69
i	1D	Extinction test: freezing (30min EXT vs 30min No-EXT)	Normal Distribution	Fisher's post hoc test	0.24
j	1D	Extinction test: freezing (3h EXT vs 3h No-EXT)	Normal Distribution	Fisher's post hoc test	1
k	1D	Extinction test:freezing (24h EXT vs 24h No-EXT)	Normal Distribution	Fisher's post hoc test	1
l	1D	Extinction test:freezing (3h EXT vs 24h EXT)	Normal Distribution	Fisher's post hoc test	1
m	1E	Correlation: 30m post-conditioning train vs test	Normal Distribution	Linear correlation	R^2=0.74
n	1F	Correlation: 24h post-conditioning train vs test	Normal Distribution	Linear correlation	R^2=0.52
o		Correlation: 3h post-conditioning train vs test, not shown	Normal Distribution	Linear correlation	R^2=0.28
p	2B	EPM: time spent on the open arms	Normal Distribution	1-way ANOVA	1
q	2D	Extinction training: effect of "trial"	Normal Distribution	2-way repeated ANOVA	1
r	2E	Extinction test: effect of drug	Normal Distribution	1-way ANOVA	0.81
s	2E	Extinction test: freezing (VEH vs 10ug)	Normal Distribution	Fisher's post hoc test	1
t	2F	Extinction test-7d: effect of drug	Normal Distribution	1-way ANOVA	0.81
u	2F	Extinction test-7d: freezing (VEH vs 1ug)	Normal Distribution	Fisher's post hoc test	1
v	2F	Extinction test-7d: freezing (VEH vs 10ug)	Normal Distribution	Fisher's post hoc test	1
w	2G	Consolidation control: freezing (VEH vs 10ug)	Normal Distribution	Student's 2-tailed t-test	0.96
x	3B	EPM: time spent on the open arms	Normal Distribution	Student's 2-tailed t-test	0.8
y	3C	Extinction training: effect of "trial"	Normal Distribution	2-way repeated ANOVA	0.91
z	3D	Extinction test: effect	Normal Distribution	Student's 2-tailed t-test	0.83
aa	4B	EPM: time spent on the open arms	Normal Distribution	2-way ANOVA	1
bb	4C	Protein: Extinction training effect of "trial"	Normal Distribution	2-way repeated ANOVA	1
cc	4C	Protein: Extinction training effect of "interval"	Normal Distribution	2-way repeated ANOVA	0.3
dd	4C	Protein: Extinction training: effect of "group"	Normal Distribution	2-way repeated ANOVA	0.44
ee	4C	Protein: Extinction training: interaction	Normal Distribution	2-way repeated ANOVA	0.29
ff	4D	Protein: 30min interval	Normal Distribution	1-way ANOVA	0.79
gg	4D	Protein: 30min (HOME vs CtxB no CS)	Normal Distribution	Fisher's post hoc test	0.12
hh	4D	Protein: 3min (HOME vs CtxB CS)	Normal Distribution	Fisher's post hoc test	1
ii	4D	Protein: 30min (HOME vs CtxB-3minCS	Normal Distribution	Fisher's post hoc test	1
jj	5B	EPM: time spent on the open arms	Normal Distribution	1-way ANOVA	1
kk	5C	Protein: Extinction training: effect of "trial"	Normal Distribution	2-way repeated ANOVA	1
ll	5C	Protein: Extinction training: effect of "treatment"	Normal Distribution	2-way repeated ANOVA	0.7
mm	5C	Protein: Extinction training: interaction	Normal Distribution	2-way repeated ANOVA	0.47
nn	5E	Protein (VEH vs HOME)	Normal Distribution	Student's 2-tailed t-test	1
oo	5E	Protein (NBI vs HOME)	Normal Distribution	Student's 2-tailed t-test	0.06
pp	5G	Protein: effect of treatment	Normal Distribution	2-way ANOVA	0.11
qq	6B	EPM: time spent on the open arms	Normal Distribution	2-way ANOVA	1
rr	6C	Calcineurin activity: effect of "NBI"	Normal Distribution	2-way ANOVA	0.79
ss	6C	Calcineurin activity (VEH CtxB-CS vs home cage)	Normal Distribution	Fisher's post hoc test	0.99
tt	6C	Calcineurin activity (NBI CtxB-CS vs home cage)	Normal Distribution	Fisher's post hoc test	0.07

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839 **Table 2:** Summary of statistics for fear conditioning sessions.

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Experiment	Group	MEAN	SEM	Test	*p* value
Figure 1	No-Ext 30min	80.154	3.378	Three-way repeated ANOVA	
	Ext 30min	77.576	2.659		Time: <0.0001
	No-Ext 3h	100.147	12.016		TimexExtinct: 0.51
	Ext 3h	91.933	10.933		TimexInterval: 0.51
	No-Ext 24h	88.575	1.827		Interaction: 0.17
	Ext 24h	78.4	2.728		
Figure 2	VEH	81.8	3.872	One-way repeated ANOVA	
	0.1ug	85.033	2.789		Time: <0.0001
	0.3ug	83.571	3.521		Group: 0.21
	1ug	69.486	8.752		
	10ug	77.28	3.238		
Figure 3	VEH	75.566	7.053	One-way repeated ANOVA	Time: <0.0001
	0.1ug	84.853	3.149		Group: 0.89

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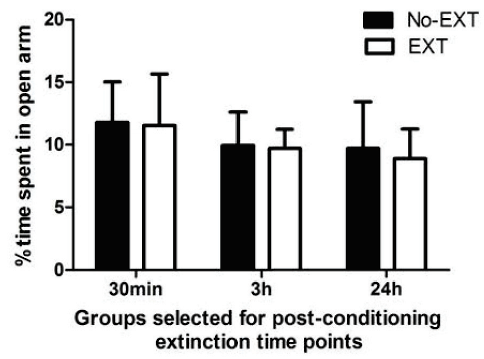
B



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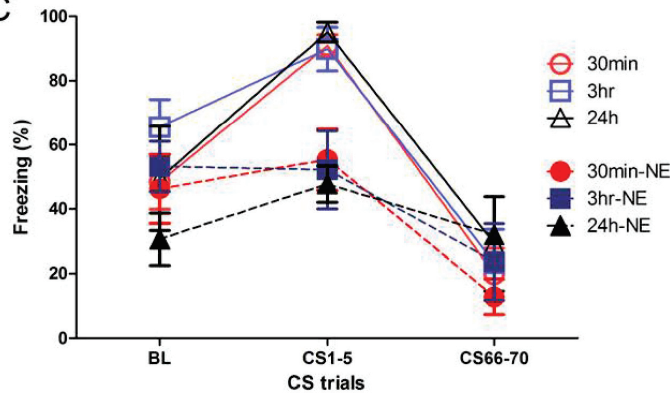

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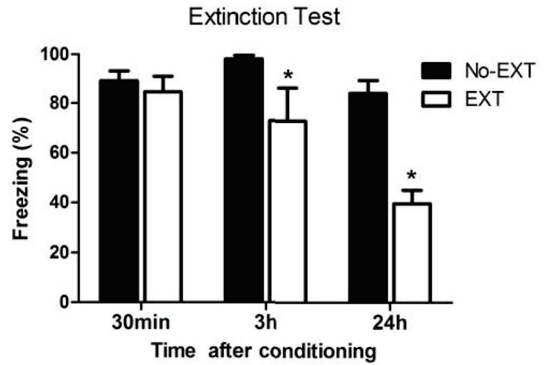

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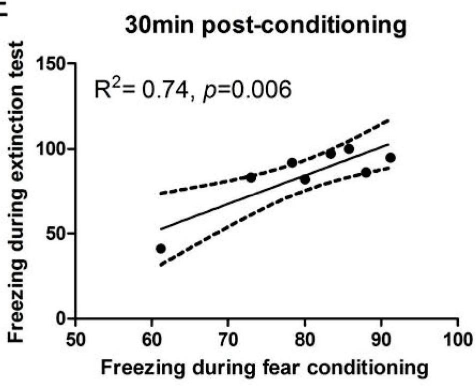

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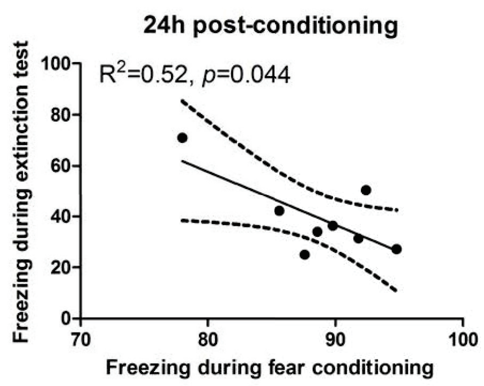

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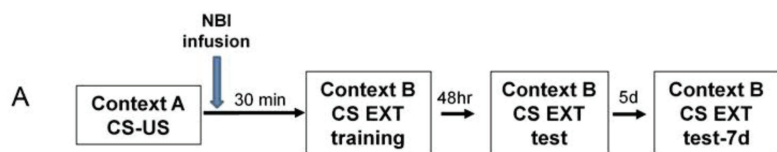

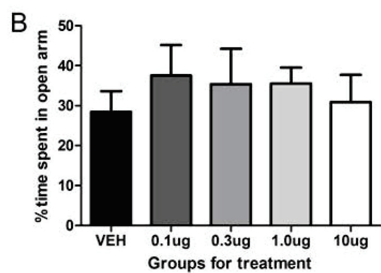

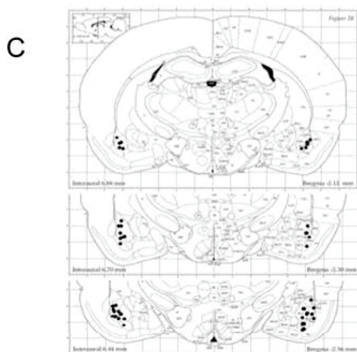

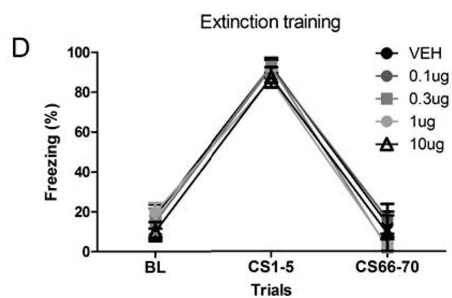

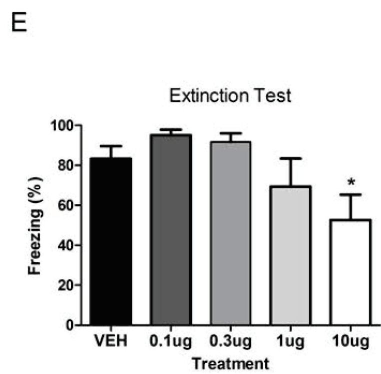

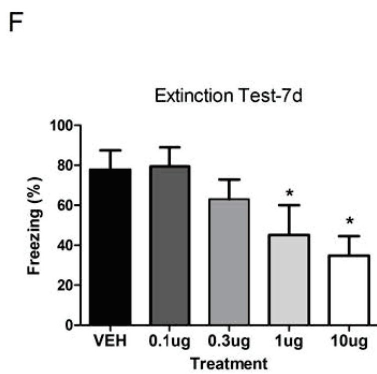

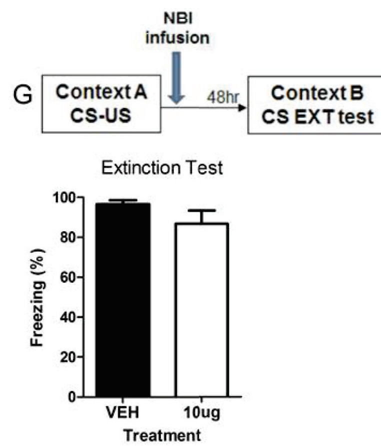

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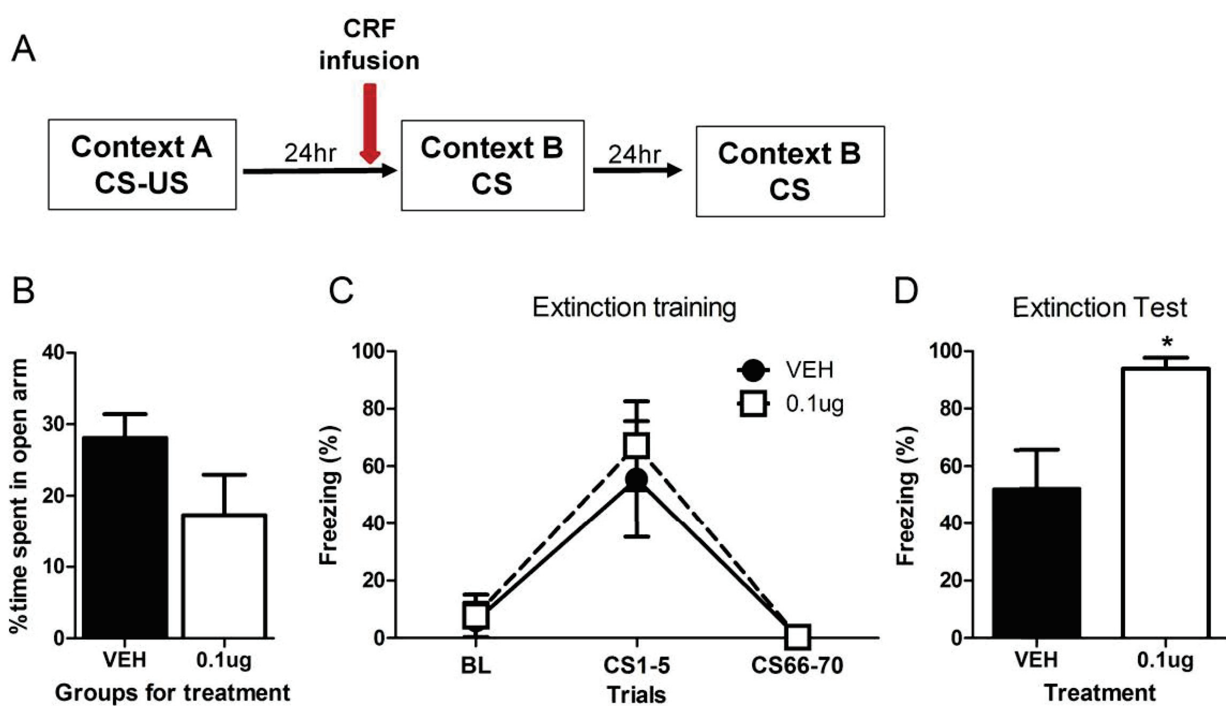

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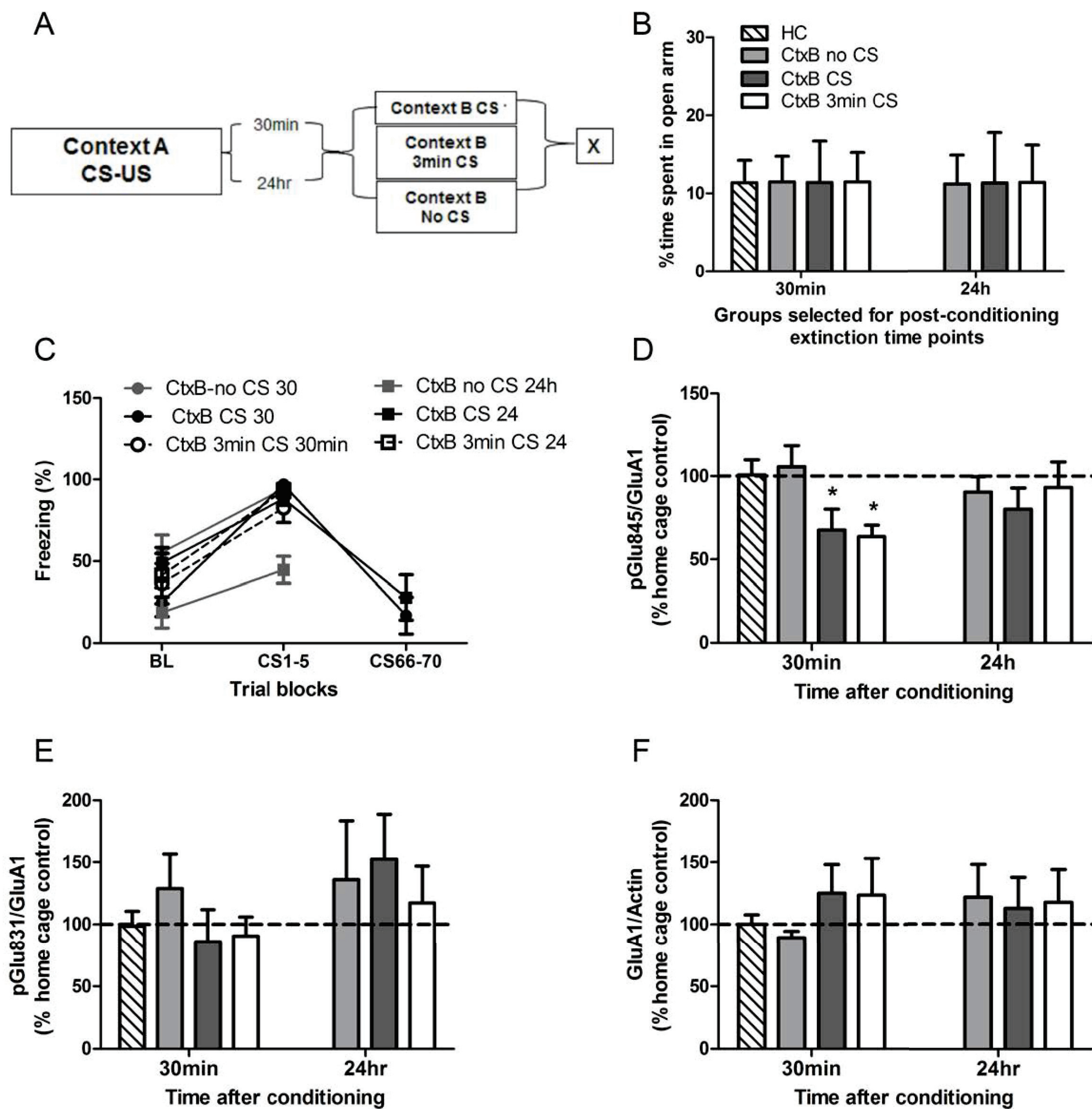

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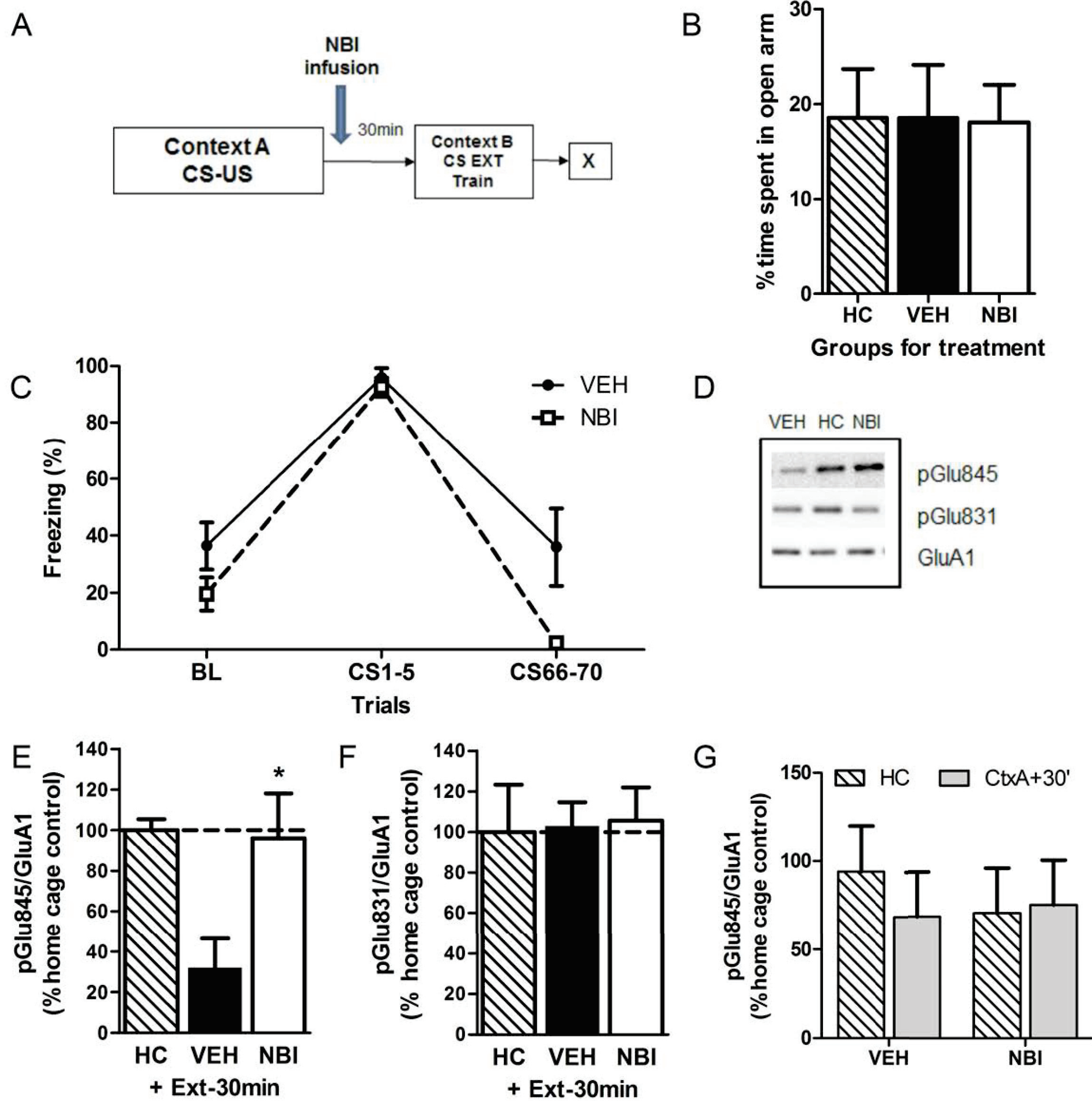

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