Behavioral/Systems/Cognitive

Rescue of Impaired Fear Extinction and Normalization of Cortico-Amygdala Circuit Dysfunction in a Genetic Mouse Model by Dietary Zinc Restriction

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Fear extinction is impaired in neuropsychiatric disorders, including posttraumatic stress disorder. Identifying drugs that facilitate fear extinction in animal models provides leads for novel pharmacological treatments for these disorders. Zinc (Zn) is expressed in neurons in a cortico-amygdala circuit mediating fear extinction, and modulates neurotransmitter systems regulating extinction. We previously found that the 129S1/SvImJ mouse strain (S1) exhibited a profound impairment in fear extinction, coupled with abnormalities in the activation of the extinction circuit. Here, we tested the role of Zn in fear extinction in S1 and C57BL/6N reference strain (B6) by feeding the mice a Zn-restricted diet (ZnR) and testing for fear extinction, as well as neuronal activation of the extinction circuit via quantification of the immediate-early genes c-Fos and Zif268. Results showed that (preconditioning or postconditioning) ZnR completely rescued deficient extinction learning and long-term extinction retrieval in S1 and expedited extinction learning in B6, without affecting fear acquisition or fear expression. The extinction-facilitating effects of ZnR were associated with the normalization of Zif268 and/or c-Fos expression in cortico-amygdala regions of S1. Specifically, ZnR increased activity in infralimbic cortex, lateral and basolateral amygdala nuclei, and lateral central amygdala nucleus, and decreased activity in prelimbic and insular cortices and medial central amygdala nucleus. ZnR also increased activation in the main intercalated nucleus and decreased activation of the medial paracapsular intercalated mass in S1. Our findings reveal a novel role for Zn in fear extinction and further support the utility of the S1 model for identifying extinction facilitating drugs.

Introduction

Anxiety disorders, including posttraumatic stress disorder (PTSD) and phobias, are associated with an inability to extinguish learned fear responses (Myers and Davis, 2007). A substantial proportion of anxiety patients do not respond effectively to existing treatments, namely cognitive behavioral therapy and/or pharmacotherapy (Yehuda and LeDoux, 2007). Animal models of impaired fear extinction can provide insight into the etiology of persistent fear memory and identify novel targets for extinction-facilitating treatments (Holmes and Quirk, 2010).

Various rodent models of impaired extinction have been developed. For example, rats bred for high anxiety-like behavior (Muigg et al., 2008) or learned helplessness (Wrubel et al., 2007) exhibit impaired extinction learning. In mice, we recently found that the 129S1/SvImJ (S1) inbred mouse strain displays a profound impairment in fear extinction (Hefner et al., 2008). Neither extended extinction training nor D-cycloserine improved extinction in this strain, indicating strong resistance to extinction. Moreover, impaired S1 extinction was correlated with abnormal brain activation in a cortico-amygdala circuit mediating fear extinction (Quirk and Mueller, 2008; Herry et al., 2010). Specifically, S1 showed low expression of the immediate-early genes (IEG) c-Fos and/or Zif268 in the infralimbic cortex (IL), lateral and basolateral amygdala (La and BA, respectively), and high IEG expression in the central amygdala (CeA) and medial paracapsular intercalated cell mass (Imp), relative to the well extinguishing C57BL/6 (B6) mouse strain (Hefner et al., 2008). The S1 mouse provides a genetic model for identifying novel extinction-facilitating drugs and their effects on underlying neural circuitry.

Previous studies have identified multiple fear extinction-facilitating molecular and neurochemical targets. These include NMDA receptor (NMDAR) partial agonism (Walker and Davis, 2002; Ledgerwood et al., 2003, 2005; Davis et al., 2006; Sotres-Bayon et al., 2007), AMPA receptor (AMPAR) potentiation (Zushida et al., 2007), metabotropic glutamate receptor mGluR7 activation (Fendt et al., 2008), noradrenaline agonism (Ouyang and Thomas, 2005; Berlau and McGaugh, 2006), α-2-adrenoreceptor antagonism (Cain et al., 2004; Morris and Bouton, 2007; Hefner et al., 2008), dopamine D_2 receptor antagonism (Ponnusamy et al., 2005), and increased $GABA_A$ receptor ($GABA_AR$) activity

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(Harris and Westbrook, 1998; Chhatwal et al., 2005; Akirav et al., 2006; Lin et al., 2009).

Zinc (Zn)-containing neurons are highly expressed in the cortico-amygdala extinction circuit (Brown and Dyck, 2004). Zn has neuromodulatory effects on many of the aforementioned extinction-related molecular systems; for example, Zn exerts antagonistic action on NMDAR (Westbrook and Mayer, 1987; Christine and Choi, 1990; Williams, 1996; Choi and Lipton, 1999) via a high-affinity binding site on NR2A and a low-affinity NR2B binding site (Choi and Lipton, 1999), and is an antagonist at AMPAR (Bresink et al., 1996), $GABA_AR$ (Westbrook and Mayer, 1987; Hosie et al., 2003; Ruiz et al., 2004), and neuronal nitric oxide synthase (Persechini et al., 1995). Here, we examined the effects of depleting Zn by feeding a Zn-restricted diet (ZnR) on fear extinction in the S1 model of impaired extinction. To elucidate the neural basis of these effects, we mapped cortico-amygdala neuronal activation by quantifying the IEGs c-Fos and Zif268.

Materials and Methods

Subjects. Subjects were 12- to 13-week-old male 129S1/SvImJ (S1) and C57BL/6N (B6) mice (Charles River) housed (4–5 per cage) side-by-side in a temperature- (22 ± 2°C) and humidity- (50–60%) controlled vivarium under a 12 h light/dark cycle (lights on at 7:00 A.M.). All experimental procedures were approved by the Austrian Animal Experimentation Ethics Board (Bundesministerium für Wissenschaft und Verkehr, Kommission für Tierversuchsangelegenheiten).

Behavioral testing. Fear conditioning and extinction was performed as previously described (Hefner et al., 2008). Mice were fear conditioned in a 26 × 30 × 32 cm chamber with transparent walls and a metal rod floor (context A). After a 120 s acclimation period, there were five pairings (120 s interpairing interval) between a 120 s, 80 dB white noise [conditioned stimulus (CS)] and a 2 s, 0.7 mA scrambled footshock, in which the shock was presented during the last 2 s of the CS. There was a 120 s no-stimulus consolidation period after the final pairing before mice were returned to the home cage.

Twenty-four hours later, mice received extinction training in a novel context (context B) (26 × 20 × 13 cm cage, cleaned with a 100% ethanol, illuminated to 10 lux). After a 120 s acclimation period, there were 15 CS presentations (5 s no-stimulus interval).

Twenty-four hours later, extinction retrieval was tested in context B. After a 120 s acclimation period, mice either received one or 15 CS presentations.

Freezing was measured as an index of fear (Blanchard and Blanchard, 1969), manually scored based on DVD recordings of the duration of the CS (120 s), defined as no visible movement except that required for respiration, and converted to a percentage [(duration of freezing within the CS/total time of the CS) × 100] by a trained observer blind to the animals' treatment.

IEG quantification. Mice were killed 2 h after the start of the extinction retrieval session [time interval according to postextinction c-Fos and Zif268 data obtained previously (Herry and Mons, 2004)]. Mice were deeply anesthetized with an overdose of sodium pentobarbital (200 mg/kg) and transcardially perfused with 20 ml of 0.9% saline followed by 20 ml of 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Brains were then removed and postfixed at 4°C overnight in 4% paraformaldehyde in phosphate buffer. Brains were sectioned in the coronal plane at 50 μm thickness on a vibratome (VT1000S, Leica Microsystems) and collected in immunobuffer. The free-floating sections were processed for c-Fos immunoreactivity as described previously (Singewald et al., 2003), via incubation with a polyclonal primary antibody (1:10,000; sc-52; Santa Cruz Biotechnology), and for Zif268-like immunoreactivity as described previously (Hefner et al., 2008), via incubation with a polyclonal primary antibody (1:5000; sc-189; Santa Cruz Biotechnology) and a biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories).

The anatomical localization of c-Fos-positive or Zif268-positive cells was aided by using the illustrations in a stereotaxic atlas (Paxinos and Franklin, 2001). Zif268-positive neurons in intercalated cell masses (ITCs) were identified with reference to published studies in the rat and mouse (Millhouse, 1986; Berretta et al., 2005; Marowsky et al., 2005; Geracitano et al., 2007; Hefner et al., 2008). Specifically, one mass of ITC cells situated along the external capsule at the junction of the La and the BA was labeled as lateral paracapsular ITC neurons (Ilp). A second mass of ITC cells was observed along the intermediate capsule at the junction of La and BA and lateral to the CeA and was defined as the Imp. The main ITC nucleus was defined as described previously (Paxinos and Franklin, 2001). Since recent evidence (Busti et al., 2010) points to differential connectivity of these three cell groups, we quantified IEG expression separately in these groups. Unless otherwise stated, all c-Fos-positive or Zif268-positive cells that were distinguishable from background staining were bilaterally counted in each region of interest within a defined area (0.01 mm^2). Counts were averaged from two to four sections per mouse (depending on the brain area under investigation) and presented as cells/0.01 mm^2.

Experiment 1: effects of Zn restriction on extinction. S1 and B6 were fed commercially prepared food pellets (ssniff Spezialdiäten) containing low Zn [12.3 mg/kg; 40% of the recommended daily intake requirement (Reeves et al., 1993)] or standard food pellets containing normal quantities of Zn (65 mg/kg) for 3 weeks before fear conditioning and throughout the completion of testing. No seizures or other adverse reactions were observed in animals on this diet. Fear conditioning (five tone-shock pairings in context A), extinction training (15 CS in context B), and extinction retrieval (15 CS in context B) was conducted over 3 consecutive days as previously described (Hefner et al., 2008). Mice were killed for IEG analysis 2 h after the start of the extinction-retrieval session. To provide an IEG control group, mice were subjected to the same conditioning and extinction procedures as the 15 CS group with the exception that there was no unconditioned stimulus (US) during conditioning.

Experiment 2: effects of Zn restriction on extinction (short-retrieval session). Mice were treated and tested as above, but the extinction retrieval session was reduced (one CS) to examine effects of extinction retrieval separate from any additional extinction learning. As with experiment 1, mice were killed for IEG analysis 2 h after the start of the extinction retrieval session.

Experiment 3: effects of Zn restriction on weak conditioning. Mice were treated and tested for fear conditioning and fear expression (one CS), but not extinction, as in experiment 1, with the exception that fear conditioning was designed to be weaker by reducing the number of pairings (from five to three) and footshock intensity (from 0.7 to 0.3 mA).

Experiment 4: effects of postconditioning Zn restriction on extinction. Mice were treated and tested as in experiment 2, with the exception that Zn restriction did not begin until after fear conditioning was conducted, which continued for 2 weeks until (and through) extinction training and retrieval testing.

Statistical analysis. All data were first examined for equal variances using Levene's test before performing ANOVA. The effects of Zn-restriction and trial on freezing during the conditioning, extinction, and (for the 15 CS group) extinction-retrieval phases were analyzed in each strain using multiple-factor ANOVA, with repeated measures for trial, followed by Fisher LSD *post hoc* analysis in the presence of significant interaction effects. The effects of Zn-restriction on freezing during extinction retrieval in the one CS group was analyzed in each strain using Bonferroni-corrected Student's *t* test.

Results

Experiment 1: ZnR facilitates extinction

The first objective of this experiment was to test whether dietary Zn restriction affected fear extinction in (extinction-intact) B6 and (extinction-impaired) S1 mice. Mice were fed a Zn-deficient diet and then subjected to fear conditioning, extinction training, and a relatively long (15 CS) extinction-retrieval session.

During conditioning, all experimental groups showed an increase in freezing across conditioning trials, which did not differ between groups (Fig. 1). This was ascertained from ANOVA results, which revealed a significant effect of trial (freezing to CS

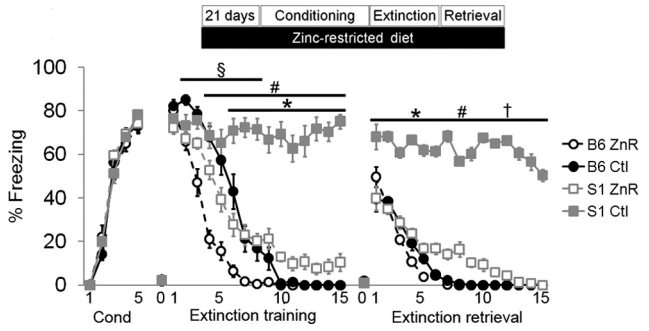

Figure 1. Effect of ZnR on fear extinction using a paradigm consisting of 15 CS presentations during extinction retrieval. ZnR beginning 21 d before conditioning (Cond) rescued impaired extinction learning and retrieval in S1 mice and facilitated extinction learning in B6 mice, relative to control diet (Ctl), but did not lead to savings in extinction (re)learning during extinction retrieval (15 CS presentations) ($n = 8$ per group). Data are presented as means ± SEM. $^{*}p < 0.05$, S1 control diet versus B6 control diet; $^{\#}p < 0.05$, S1 control diet versus S1 ZnR; $^{\dagger}p < 0.05$, B6 ZnR versus S1 control diet; $^{\S}p < 0.05$, B6 control diet versus B6 ZnR.

presentations; $F_{(4,160)} = 182.03$, $p < 0.01$) and conditioning protocol (conditioned vs unconditioned mice; $F_{(1,40)} = 607.50$, $p < 0.01$), but not strain (S1 vs B6) or diet (control-diet-fed mice vs ZnR-fed mice) or interactions, on freezing during conditioning.

During extinction training, there was a significant trial–strain–diet–protocol interaction for freezing ($F_{(14,616)} = 7.03$, $p < 0.01$). Impaired extinction learning was evident in S1 as freezing was higher in control-diet S1 than in control-diet B6 during CS presentations 6–15. ZnR led to induction of extinction learning in S1 as freezing was lower in ZnR S1 than control-diet S1 on CS presentations 4–15 (Fig. 1). The rate of extinction learning induced by ZnR in S1 was similar to the rate of extinction learning in B6, as no differences in freezing was observed between ZnR S1 and control-diet B6 during any CS presentation ($p > 0.05$). Furthermore, ZnR facilitated extinction learning in B6, as reduced freezing was observed in ZnR B6 compared with control-diet B6 on CS presentations 2–8 (Fig. 1). Freezing in nonconditioned mice was negligible (<5% in all groups), regardless of trial or diet (protocol effect: $F_{(1,40)} = 261.99$, $p < 0.01$).

During extinction retrieval, there was a significant trial–strain–diet–protocol interaction for freezing ($F_{(14,616)} = 1.92$, $p < 0.05$). In control-diet groups, impaired extinction (re)learning was observed as freezing was higher in S1 than B6 throughout all CS presentations (Fig. 1). Freezing was lower in ZnR S1 than control-diet S1 throughout all CS presentations (Fig. 1). Freezing in nonconditioned mice was again negligible (<5% in all groups; protocol effect: $F_{(1,40)} = 92.44$, $p < 0.01$).

ZnR affects cortico-amygdala c-Fos activation

We next asked whether ZnR-induced changes in extinction behavior were associated with altered patterns of cortico-amygdala activation of the IEG c-Fos.

There was a significant strain–diet–conditioning protocol interaction for the number of c-Fos-positive cells in the IL (1.98 mm from bregma, $F_{(1,40)} = 8.54$, $p < 0.01$; 1.78 mm from bregma, $F_{(1,40)} = 9.10$, $p < 0.01$; 1.54 mm, $F_{(1,40)} = 7.73$, $p < 0.01$), BA ($F_{(1,40)} = 9.77$, $p < 0.01$), and medial division of the CeA (CeM; $F_{(1,40)} = 15.33$, $p < 0.01$) (Table 1). *Post hoc* testing revealed that fear-related CS-exposure induced c-Fos expression in the BA in all groups, except for control-fed S1 mice, after extinction retrieval relative to unconditioned control (i.e., CS-only) groups (Table 1). Furthermore, nonextinguishing control-fed S1 mice exhibited fewer numbers of c-Fos-positive cells in the IL and BA regions and increased numbers of c-Fos-positive cells in the CeM compared with extinguishing-control-fed B6 mice (Table 1). These strain differences in prefrontal cortex (PFC) and amygdala regions were absent in ZnR groups due to normalization of S1 activation to B6-control levels (Table 1).

All of the following brain areas expressed significantly more c-Fos-positive cells after extinction retrieval in conditioned groups than in unconditioned groups, but did not differ between strain or diet (Table 1): prelimbic cortex (PrL; 1.98 mm from bregma, $F_{(1,40)} = 270.63$, $p < 0.01$; 1.78 mm from bregma, $F_{(1,40)} = 564.11$, $p < 0.01$; 1.54 mm from bregma, $F_{(1,40)} = 245.63$, $p < 0.01$), cingulate cortex area 1 (1.78 mm from bregma, $F_{(1,40)} = 232.84$, $p < 0.01$; 1.54 mm from bregma, $F_{(1,40)} = 319.92$, $p < 0.01$), cingulate cortex area 2 (1.10 mm from bregma, $F_{(1,40)} = 503.98$, $p < 0.01$), dorsal division of the lateral amygdaloid nucleus (Lad; $F_{(1,40)} = 18.37$, $p < 0.01$), lateral division of the CeA (CeL; $F_{(1,40)} = 27.56$, $p < 0.01$), capsular division of the CeA ($F_{(1,40)} = 10.86$, $p < 0.01$), posterodorsal division of the medial amygdaloid nucleus ($F_{(1,40)} = 82.92$, $p < 0.01$), posteroventral division of the medial amygdaloid nucleus ($F_{(1,40)} = 59.35$, $p < 0.01$), anterior cortical amygdaloid area ($F_{(1,40)} = 73.80$, $p < 0.01$), and posterolateral cortical amygdaloid area ($F_{(1,40)} = 29.07$, $p < 0.01$).

In a control group that received equivalent CS and context exposure, but not US during fear conditioning, c-Fos expression after the extinction-retrieval session was unaffected by strain or diet for all brain regions examined (Table 1 and supplemental Table S1, available at www.jneurosci.org as supplemental material).

ZnR affects cortico-amygdala Zif268 activation

We also asked whether ZnR-induced changes in extinction behavior were associated with altered patterns of cortico-amygdala Zif268activation.

There was a significant strain–diet–conditioning protocol interaction for the number of Zif268-positive cells in the IL (1.98 mm from bregma, $F_{(1,40)} = 9.61$, $p < 0.01$; 1.78 mm bregma, $F_{(1,40)} = 18.11$, $p < 0.01$; 1.54 mm bregma, $F_{(1,40)} = 16.99$, $p < 0.01$), Lad ($F_{(1,40)} = 10.23$, $p < 0.01$), ventral division of the lateral amygdaloid nucleus (Lav; $F_{(1,40)} = 5.78$, $p = 0.02$), BA ($F_{(1,40)} = 13.05$, $p < 0.01$), CeM ($F_{(1,40)} = 17.48$, $p < 0.01$), CeL ($F_{(1,40)} = 3.64$, $p = 0.04$), Imp ($F_{(1,40)} = 18.19$, $p < 0.01$) and intercalated nucleus (In; $F_{(1,40)} = 5.90$, $p = 0.02$) (Table 2). *Post hoc* testing revealed that fear-related CS-exposure induced Zif268 expression in all groups, except for control-fed S1 mice in the IL (1.98 mm from bregma), Lad, and In, after extinction retrieval relative to unconditioned control (i.e., CS-only) groups (Table 2). Furthermore, nonextinguishing control-fed S1 mice exhibited fewer numbers of Zif268-positive cells in the IL, Lad, Lav, BA, CeL, and In regions and increased numbers of Zif268-positive cells in the CeM and Imp compared with extinguishing-control-fed B6 mice (Table 2). These strain differences in PFC and amygdala regions were absent in ZnR groups due to normalization of S1 activation to B6-control levels (Table 2).

All of the following brain areas expressed significantly more Zif268-positive cells after extinction retrieval in conditioned groups than in unconditioned groups, but did not differ between strain or diet (Table 2): PrL (1.98 mm from bregma, $F_{(1,40)} = 73.52$, $p < 0.01$; 1.78 mm from bregma, $F_{(1,40)} = 72.56$, $p < 0.01$; 1.54 mm from bregma, $F_{(1,40)} = 118.88$, $p < 0.01$), insular cortex (1.98 mm from bregma, $F_{(1,40)} = 39.80$, $p < 0.01$), ventral division of the insular cortex (1.78 mm from bregma, $F_{(1,40)} = 25.63$, $p < 0.01$; 1.54 mm from bregma, $F_{(1,40)} = 162.00$, $p < 0.01$), dorsal division of the insular cortex (1.78 mm from bregma, $F_{(1,40)} = 16.79$, $p < 0.01$; 1.54 mm from bregma, $F_{(1,40)} = 101.17$,

Table 1. c-Fos expression after (15 CS presentations) extinction retrieval in mice fed control or ZnR diets 3 weeks prior to conditioning

	Unconditioned				Conditioned				
	S1		B6		S1		B6		
Region (distance from bregma)	Ctl	ZnR	Ctl	ZnR	Ctl	ZnR	Ctl	ZnR	Strain–diet–protocol interaction
Cortical regions									
M1, 1.78 mm	0.5 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.3	0.4 ± 0.2	$F_{(1, 40)} = 0.01, p = 0.98$
M2, 1.78 mm	0.8 ± 0.4	0.8 ± 0.3	0.3 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.5	0.5 ± 0.2	$F_{(1, 40)} < 0.01, p = 0.97$
Cg1, 1.78 mm [§]	1.4 ± 0.2	1.4 ± 0.3	0.8 ± 0.3	0.8 ± 0.4	9.8 ± 1.1	9.9 ± 0.7	8.9 ± 0.5	9.7 ± 1.0	$F_{(1, 40)} = 0.08, p = 0.77$
Cg1, 1.54 mm [§]	0.9 ± 0.1	1.1 ± 0.3	1.0 ± 0.2	1.1 ± 0.3	9.7 ± 0.7	9.0 ± 0.7	9.9 ± 0.6	9.5 ± 0.4	$F_{(1, 40)} = 0.20, p = 0.65$
Cg2, 1.10 mm [§]	1.1 ± 0.2	1.5 ± 0.4	1.1 ± 0.3	1.4 ± 0.2	12.3 ± 1.0	11.4 ± 0.8	11.7 ± 0.6	11.4 ± 0.4	$F_{(1, 40)} = 0.17, p = 0.68$
PrL, 1.98 mm [§]	2.8 ± 0.4	2.1 ± 04	2.7 ± 0.2	3.0 ± 0.4	12.4 ± 0.7	12.0 ± 0.7	11.1 ± 0.7	11.7 ± 0.5	$F_{(1, 40)} = 0.02, p = 0.90$
PrL, 1.78 mm [§]	2.1 ± 0.3	2.1 ± 0.4	2.5 ± 0.3	2.3 ± 0.4	9.3 ± 0.8	10.7 ± 0.5	9.9 ± 0.4	9.8 ± 0.8	$F_{(1, 40)} = 0.46, p = 0.50$
PrL, 1.54 mm [§]	2.1 ± 0.3	1.8 ± 0.4	2.1 ± 0.3	1.9 ± 0.2	10.9 ± 0.8	11.2 ± 0.7	10.9 ± 0.6	11.0 ± 0.5	$F_{(1, 40)} = 0.03, p = 0.85$
IL, 1.98 mm [§]	2.3 ± 0.2 [‡‡]	2.3 ± 0.1 [‡‡]	2.4 ± 0.2 [‡‡]	2.7 ± 0.1 [‡‡]	5.0 ± 0.5	9.5 ± 0.5 [##]	9.4 ± 0.7**	9.0 ± 0.7 [††]	**$F_{(1, 40)} = 8.54, p < 0.01$**
IL, 1.78 mm [§]	1.6 ± 0.3 [‡‡]	2.3 ± 0.4 [‡‡]	2.3 ± 0.2 [‡‡]	1.9 ± 0.2 [‡‡]	6.8 ± 0.4	12.2 ± 0.5 [##]	11.3 ± 0.4**	11.5 ± 0.6 [††]	**$F_{(1, 40)} = 9.10, p < 0.01$**
IL, 1.54 mm [§]	1.6 ± 0.3 [‡‡]	1.8 ± 0.4 [‡‡]	1.5 ± 0.2 [‡‡]	1.3 ± 0.3 [‡‡]	6.0 ± 0.4	10.3 ± 0.6 [##]	10.1 ± 0.4**	10.3 ± 0.6 [††]	**$F_{(1, 40)} = 7.73, p < 0.01$**
AI, 1.98 mm	No detectable expression				No detectable expression				
AID, 1.78 mm	No detectable expression				No detectable expression				
AID, 1.54 mm	No detectable expression				No detectable expression				
AIV, 1.78 mm	No detectable expression				No detectable expression				
AIV, 1.54 mm	No detectable expression				No detectable expression				
PRh, −1.58 mm	No detectable expression				No detectable expression				
Ect, −1.58 mm	No detectable expression				No detectable expression				
Amygdala nuclei (all −1.58 mm)									
Lad [§]	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	2.3 ± 0.6	2.6 ± 0.4	2.6 ± 0.3	2.7 ± 0.3	$F_{(1, 40)} = 0.06, p = 0.81$
Lav	No detectable expression				No detectable expression				
BA [§]	3.6 ± 0.3	3.3 ± 0.3 [‡‡]	3.3 ± 0.3 [‡‡]	3.7 ± 0.3 [‡‡]	2.5 ± 0.4	7.8 ± 0.9 [##]	7.6 ± 0.4**	8.1 ± 0.8 [††]	**$F_{(1, 40)} = 9.77, p < 0.01$**
CeM [a§]	6.0 ± 0.7 [‡‡]	6.8 ± 0.7 [‡]	7.0 ± 1.2 [‡]	7.5 ± 0.5 [‡]	19.6 ± 1.0	9.9 ± 0.7 [##]	10.0 ± 0.8**	10.8 ± 0.9 [††]	**$F_{(1, 40)} = 15.33, p < 0.01$**
CeL [a§]	7.3 ± 2.0	8.3 ± 1.6	7.8 ± 1.6	8.0 ± 1.9	15.4 ± 1.7	16.1 ± 1.1	15.1 ± 1.0	15.4 ± 1.0	$F_{(1, 40)} = 0.01, p = 0.93$
CeC [a§]	6.5 ± 1.6	7.5 ± 1.5	7.3 ± 1.5	7.0 ± 1.6	10.8 ± 1.2	11.4 ± 0.9	10.1 ± 0.6	10.2 ± 1.1	$F_{(1, 40)} = 0.03, p = 0.86$
Imp	No detectable expression				No detectable expression				
Ilp	No detectable expression				No detectable expression				
In	No detectable expression				No detectable expression				
MePD [§]	2.8 ± 0.5	2.5 ± 0.4	3.1 ± 0.3	2.6 ± 0.5	5.6 ± 0.5	6.8 ± 0.8	6.4 ± 0.4	6.8 ± 0.5	$F_{(1, 40)} = 0.12, p = 0.73$
MePV [§]	5.8 ± 0.5	5.3 ± 0.4	5.1 ± 0.7	6.0 ± 0.7	8.3 ± 0.5	8.3 ± 0.4	8.2 ± 0.3	8.3 ± 0.3	$F_{(1, 40)} = 0.72, p = 0.40$
ACo [§]	5.8 ± 0.4	6.3 ± 0.4	5.5 ± 1.1	6.5 ± 0.5	10.6 ± 0.6	11.8 ± 0.5	9.6 ± 0.5	9.8 ± 0.5	$F_{(1, 40)} = 0.63, p = 0.43$
PLCo [§]	5.5 ± 0.5	5.0 ± 0.3	5.3 ± 0.7	6.3 ± 0.4	8.1 ± 0.7	7.8 ± 0.7	7.4 ± 0.3	7.8 ± 0.6	$F_{(1, 40)} = 0.18, p = 0.68$

[a]Whole (sub)nucleus counted.

**$p < 0.01$, S1 control diet versus B6 control diet; [##]$p < 0.01$, S1 control diet versus ZnR S1; [††]$p < 0.01$, S1 control diet versus ZnR B6; [‡]$p < 0.05$, [‡‡]$p < 0.01$, within-strain and -diet conditioned versus unconditioned; [§]$p < 0.05$, conditioned versus non-conditioned (significant effect of protocol).

Bold type indicates significant strain–diet–protocol ANOVA interactions. Data are mean ± SEM cell numbers. $n = 8$ per group for conditioned and $n = 4$ per group for unconditioned mice. ACo, Anterior cortical; AI, insular cortex; AIV, insular cortex, ventral; CeC, central, capsular; Cg1, cingulate area 1; Cg2, cingulate area 2; Ctl, control diet; Ect, ectorhinal cortex; M1, primary motor; M2, secondary motor; MePD, medial, posterodorsal; MePV, medial, posteroventral; PLCo, posterolateral cortical; PRh, perirhinal cortex.

$p < 0.01$), secondary motor cortex ($F_{(1,40)} = 55.91$, $p < 0.01$), cingulate cortex area 1 (1.78 mm from bregma, $F_{(1,40)} = 142.71$, $p < 0.01$), cingulate cortex area 2 (1.10 mm from bregma, $F_{(1,40)} = 64.61$, $p < 0.01$), perirhinal cortex ($F_{(1,40)} = 85.22$, $p < 0.01$), ectorhinal cortex ($F_{(1,40)} = 6.44$, $p = 0.02$), capsular division of the CeA ($F_{(1,40)} = 232.05$, $p < 0.01$), Ilp ($F_{(1,40)} = 10.26$, $p < 0.01$), posterodorsal division of the medial amygdaloid nucleus ($F_{(1,40)} = 20.80$, $p < 0.01$), posteroventral division of the medial amygdaloid nucleus ($F_{(1,40)} = 14.93$, $p < 0.01$), anterior cortical amygdaloid area ($F_{(1,40)} = 12.27$, $p < 0.01$), and posterolateral cortical amygdaloid area ($F_{(1,40)} = 76.04$, $p < 0.01$).

Control mice receiving equivalent CS and context exposure, but not US during fear conditioning, showed no effects of strain or Zn diet on Zif268 expression in any brain region examined (Table 2 and supplemental Table S1, available at www.jneurosci.org as supplemental material).

Experiment 2: confirmation that ZnR facilitates extinction—effects on IEG expression

The purpose of this experiment was to confirm that ZnR facilitated long-term fear extinction by testing extinction retrieval with just one CS, and thereby avoiding a possible effect on extinction (re)learning that could occur with multiple CS exposures.

As above, experimental groups showed a similar increase in freezing across conditioning trials (Fig. 2). This was ascertained from ANOVA results that revealed there was a significant effect of trial ($F_{(4,80)} = 318.70$, $p < 0.01$), but not strain or diet, and no interactions, on freezing during conditioning.

During extinction training, there was a significant trial–strain–diet interaction for freezing ($F_{(14,280)} = 19.94$, $p < 0.01$). Impaired extinction learning was observed in S1 as freezing was higher in control-diet S1 than in control-diet B6 during CS presentations 6–15 (Fig. 2). ZnR induced extinction learning in S1, as freezing was less in ZnR-fed S1 mice than control-diet S1 on CS presentations 5–15 (Fig. 2). The rate of extinction learning induced by ZnR in S1 was similar to the rate of extinction learning in B6 (no differences in freezing during any CS presentation, $p > 0.05$). Furthermore, ZnR facilitated extinction learning in B6 as less freezing was observed in ZnR B6 than in control-diet B6 on CS presentations 3–8 (Fig. 2).

Most importantly, during extinction retrieval there was a significant strain–diet interaction for freezing ($F_{(1,20)} = 13.77$, $p <$

Table 2. Zif268 expression after (15 CS presentations) extinction retrieval in mice fed control or ZnR diets 3 weeks prior to conditioning

	Unconditioned				Conditioned				
	S1		B6		S1		B6		
Region (distance from bregma)	Ctl	ZnR	Ctl	ZnR	Ctl	ZnR	Ctl	ZnR	Strain–diet–protocol interaction
Cortical regions									
M1, 1.78 mm	12.4 ± 0.9	13.3 ± 0.8	11.6 ± 0.7	13.1 ± 0.6	14.1 ± 0.5	13.8 ± 0.7	13.1 ± 0.7	13.0 ± 1.0	$F_{(1, 40)} = 0.02, p = 0.88$
M2, 1.78 mm [§]	14.8 ± 0.7	14.1 ± 0.8	14.3 ± 0.9	14.6 ± 0.9	22.8 ± 0.6	22.5 ± 0.9	22.4 ± 0.6	22.5 ± 0.5	$F_{(1, 40)} = 0.07, p = 0.79$
Cg1, 1.78 mm [§]	16.5 ± 0.3	17.6 ± 0.6	14.1 ± 1.0	15.9 ± 0.3	22.0 ± 0.8	22.7 ± 0.7	23.4 ± 0.9	23.4 ± 0.7	$F_{(1, 40)} = 0.31, p = 0.58$
Cg1, 1.54 mm [§]	11.6 ± 0.4	12.1 ± 0.6	13.3 ± 0.6	12.1 ± 0.1	13.8 ± 0.6	14.3 ± 0.8	13.8 ± 0.4	13.2 ± 0.3	$F_{(1, 40)} = 0.26, p = 0.62$
Cg2, 1.10 mm [§]	13.9 ± 0.4	13.9 ± 0.2	13.9 ± 0.3	14.1 ± 0.3	20.7 ± 0.8	20.0 ± 0.9	19.6 ± 0.6	19.9 ± 0.6	$F_{(1, 40)} = 0.09, p = 0.77$
PrL, 1.98 mm [§]	10.3 ± 0.1	10.5 ± 0.3	10.7 ± 0.6	9.6 ± 0.7	15.8 ± 1.0	15.5 ± 0.8	15.4 ± 0.9	15.3 ± 1.0	$F_{(1, 40)} = 0.29, p = 0.60$
PrL, 1.78 mm [§]	12.9 ± 0.6	12.8 ± 0.7	10.8 ± 0.6	12.6 ± 0.5	21.2 ± 1.2	22.0 ± 0.6	21.7 ± 0.4	21.3 ± 0.8	$F_{(1, 40)} = 1.66, p = 0.21$
PrL, 1.54 mm [§]	14.3 ± 0.2	13.5 ± 0.7	12.0 ± 1.1	11.7 ± 0.4	23.1 ± 1.1	22.7 ± 0.9	22.8 ± 0.7	22.2 ± 0.8	$F_{(1, 40)} = 0.02, p = 0.90$
IL, 1.98 mm [§]	7.8 ± 0.3	8.0 ± 0.3 [‡‡]	8.0 ± 0.3 [‡‡]	7.6 ± 0.4 [‡‡]	9.4 ± 0.5	14.8 ± 0.7 [##]	15.3 ± 0.6**	14.3 ± 0.7 [††]	$\mathbf{F_{(1, 40)} = 9.61, p < 0.01}$
IL, 1.78 mm [§]	6.8 ± 0.5 [‡‡]	6.3 ± 0.6 [‡‡]	7.3 ± 0.2 [‡‡]	7.2 ± 0.7 [‡‡]	9.7 ± 0.6	17.7 ± 0.4 [##]	16.4 ± 0.9**	16.3 ± 0.4 [††]	$\mathbf{F_{(1, 40)} = 18.11, p < 0.01}$
IL, 1.54 mm [§]	9.3 ± 0.9 [‡]	8.4 ± 0.4 [‡‡]	8.3 ± 0.7 [‡‡]	8.0 ± 0.6 [‡‡]	12.9 ± 0.9	25.1 ± 1.0 [##]	21.1 ± 0.9**	22.2 ± 1.4 [††]	$\mathbf{F_{(1, 40)} = 16.99, p < 0.01}$
AI, 1.98 mm [§]	9.6 ± 0.6	9.9 ± 0.4	9.4 ± 0.5	9.0 ± 0.5	15.3 ± 0.9	14.4 ± 0.6	14.6 ± 0.7	14.9 ± 0.8	$F_{(1, 40)} = 0.54, p = 0.47$
AID, 1.78 mm [§]	6.5 ± 0.3	6.8 ± 0.5	6.4 ± 0.4	6.9 ± 0.4	11.6 ± 0.9	11.9 ± 1.1	11.4 ± 1.0	11.7 ± 0.7	$F_{(1, 40)} = 0.13, p = 0.72$
AID, 1.54 mm [§]	5.8 ± 0.7	5.9 ± 0.4	5.2 ± 0.3	5.8 ± 0.5	12.8 ± 0.8	12.5 ± 0.8	12.7 ± 0.7	13.3 ± 0.6	$F_{(1, 40)} = < 0.01, p = 0.96$
AIV, 1.78 mm [§]	11.8 ± 0.4	11.4 ± 0.9	11.4 ± 0.9	11.7 ± 0.5	16.5 ± 1.0	14.8 ± 0.7	15.2 ± 0.8	14.7 ± 0.6	$F_{(1, 40)} = 0.19, p = 0.73$
AIV, 1.54 mm [§]	10.1 ± 0.5	9.0 ± 0.4	9.9 ± 0.3	9.8 ± 0.5	14.4 ± 1.0	15.0 ± 1.0	14.6 ± 0.6	14.4 ± 0.8	$F_{(1, 40)} = 0.21, p = 0.65$
PRh, −1.58 mm [§]	7.9 ± 0.3	6.5 ± 0.5	7.3 ± 0.7	7.3 ± 0.5	18.7 ± 1.7	18.1 ± 1.1	18.2 ± 1.0	18.4 ± 0.9	$F_{(1, 40)} = 0.03, p = 0.87$
Ect, −1.58 mm [§]	16.8 ± 0.6	16.1 ± 0.8	16.1 ± 0.5	15.8 ± 0.8	18.4 ± 1.1	18.1 ± 0.4	18.3 ± 0.3	17.4 ± 1.0	$F_{(1, 40)} = 0.20, p = 0.66$
Amygdala nuclei (all −1.58 mm)									
Lad [§]	16.2 ± 0.5	16.4 ± 1.6 [‡‡]	15.6 ± 0.5 [‡‡]	15.9 ± 0.8 [‡‡]	13.6 ± 0.9	22.4 ± 1.3 [##]	20.6 ± 1.0**	20.5 ± 0.7 [††]	$\mathbf{F_{(1, 40)} = 10.23, p < 0.01}$
Lav [§]	6.8 ± 0.4 [‡]	7.5 ± 0.4 [‡‡]	7.1 ± 0.3 [‡‡]	6.9 ± 0.3 [‡‡]	9.6 ± 0.8	16.4 ± 1.0 [##]	15.9 ± 0.6**	16.1 ± 1.0 [††]	$\mathbf{F_{(1, 40)} = 5.78, p = 0.02}$
BA [§]	3.5 ± 0.1 [‡‡]	3.3 ± 0.3 [‡‡]	3.7 ± 0.4 [‡‡]	4.2 ± 0.3 [‡‡]	7.6 ± 1.3	13.5 ± 0.7 [##]	13.3 ± 0.3**	12.8 ± 0.7 [††]	$\mathbf{F_{(1, 40)} = 13.05, p < 0.01}$
CeM [a§]	33.1 ± 0.8 [‡‡]	35.6 ± 1.5 [‡‡]	34.1 ± 2.2 [‡‡]	34.5 ± 1.3 [‡‡]	74.3 ± 1.3	45.6 ± 1.4 [##]	49.8 ± 4.0**	49.1 ± 2.5 [††]	$\mathbf{F_{(1, 40)} = 17.48, p < 0.01}$
CeL [a§]	49.5 ± 2.5 [‡‡]	48.3 ± 3.3 [‡‡]	46.5 ± 3.0 [‡‡]	48.8 ± 3.2 [‡‡]	71.5 ± 3.5	90.6 ± 1.9 [##]	89.0 ± 3.1**	94.6 ± 4.0 [††]	$\mathbf{F_{(1, 40)} = 3.642, p = 0.04}$
CeC [a§]	40.8 ± 2.3	40.3 ± 2.8	41.8 ± 2.1	42.0 ± 2.3	46.7 ± 0.8	45.6 ± 1.6	45.6 ± 1.6	47.0 ± 2.0	$F_{(1, 40)} = 0.08, p = 0.78$
Imp [a§]	38.1 ± 1.8 [‡‡]	40.0 ± 2.1 [‡‡]	40.8 ± 1.4 [‡‡]	38.5 ± 2.2 [‡‡]	75.9 ± 3.9	40.8 ± 2.8 [##]	44.9 ± 2.0**	42.3 ± 2.3 [††]	$\mathbf{F_{(1, 40)} = 18.19, p < 0.01}$
Ilp [a§]	29.0 ± 4.4	28.8 ± 3.8	29.3 ± 1.8	30.0 ± 2.3	41.3 ± 3.9	40.9 ± 5.3	39.9 ± 1.7	37.9 ± 3.5	$F_{(1, 40)} = 0.05, p = 0.83$
In [a§]	51.6 ± 6.2	52.5 ± 5.7 [‡‡]	50.8 ± 4.0 [‡‡]	51.0 ± 2.8 [‡‡]	52.9 ± 7.6	90.8 ± 2.4 [##]	91.1 ± 3.6**	93.1 ± 2.1 [††]	$\mathbf{F_{(1, 40)} = 5.90, p = 0.02}$
MePD [§]	6.9 ± 0.6	7.3 ± 0.7	6.8 ± 0.4	6.6 ± 0.3	9.8 ± 0.6	10.6 ± 0.7	10.8 ± 0.8	10.3 ± 0.8	$F_{(1, 40)} = 0.03, p = 0.87$
MePV [§]	11.2 ± 0.5	10.0 ± 0.5	10.8 ± 0.5	10.6 ± 0.6	13.8 ± 1.2	14.4 ± 0.7	13.4 ± 0.8	13.6 ± 0.6	$F_{(1, 40)} = 0.29, p = 0.60$
ACo [§]	17.9 ± 2.0	17.9 ± 1.8	17.1 ± 1.1	17.7 ± 0.7	19.3 ± 0.9	19.4 ± 0.6	18.6 ± 1.2	18.8 ± 1.1	$F_{(1, 40)} = 0.01, p = 0.93$
PLCo [§]	10.0 ± 0.7	9.8 ± 0.9	9.6 ± 0.2	9.9 ± 0.5	16.8 ± 0.6	17.9 ± 1.0	17.8 ± 0.9	18.5 ± 0.6	$F_{(1, 40)} = 0.10, p = 0.75$

[a]Whole (sub)nucleus counted.

**$p < 0.01$, S1 control diet versus B6 control diet; [##]$p < 0.01$, S1 control diet versus ZnR S1; [††]$p < 0.01$, S1 control diet versus ZnR B6; [‡]$p < 0.05$, [‡‡]$p < 0.01$, within-strain and -diet conditioned versus unconditioned; [§]$p < 0.05$, conditioned versus non-conditioned (significant effect of protocol).

Bold indicates significant strain–diet ANOVA interactions. Data are mean ± SEM cell numbers. $n = 8$ per group for conditioned and $n = 4$ per group for unconditioned mice. ACo, Anterior cortical; AI, insular cortex; AIV, insular cortex, ventral; CeC, central, capsular; Cg1, cingulate area 1; Cg2, cingulate area 2; Ctl, control diet; Ect, ectorhinal cortex; M1, primary motor; M2, secondary motor; MePD, medial, posterodorsal; MePV, medial, posteroventral; PLCo, posterolateral cortical; PRh, perirhinal cortex.

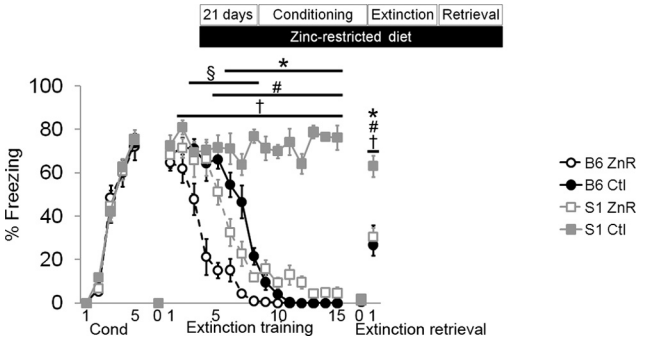

Figure 2. Effect of ZnR on fear extinction using a paradigm consisting of one CS presentation during extinction retrieval. ZnR beginning 21 d before conditioning (Cond) rescued impaired extinction learning and retrieval in S1 mice and facilitated extinction learning in B6 mice, relative to control diet (Ctl) ($n = 6$ per group). Data are presented as means ± SEM. *$p < 0.05$, S1 control diet versus B6 control diet; [#]$p < 0.05$, S1 control diet versus S1 ZnR; [†]$p < 0.05$, B6 ZnR versus S1 control diet; [§]$p < 0.05$, B6 control diet versus B6 ZnR.

0.01). In control-diet groups, impaired extinction retrieval was observed as freezing was higher in S1 than B6 (Fig. 2). Freezing was less in ZnR S1 than control-diet S1 (Fig. 2).

ZnR affects cortico-amygdala c-Fos and Zif268 activation

As in experiment 1, we quantified patterns of cortico-amygdala c-Fos and Zif268 expression associated with the ZnR rescue of S1 extinction retrieval.

There was a significant strain–diet interaction for c-Fos-positive cells in the IL (1.98 mm, $F_{(1,20)} = 23.04$, $p < 0.01$; 1.78 mm, $F_{(1,20)} = 46.44$, $p < 0.01$), PrL (1.98 mm, $F_{(1,20)} = 17.96$, $p < 0.01$; 1.78 mm, $F_{(1,20)} = 35.35$, $p < 0.01$), and CeM ($F_{(1,20)} = 8.47$, $p = 0.01$) (Table 3). In control-diet groups, PrL and CeM were more and IL and BA were less activated in S1 than B6 after extinction retrieval. These strain differences in cortical (Fig. 3) and amygdala (Fig. 4) regions were absent in ZnR groups due to normalization of S1 activation to B6-control levels. No other brain region examined was affected by strain or ZnR (Table 3).

There was a significant strain–diet–conditioning protocol interaction for Zif268-positive cells in IL (1.98 mm, $F_{(1,20)} = 33.28$, $p < 0.01$; 1.78 mm, $F_{(1,20)} = 22.12$, $p < 0.01$), PrL (1.98 mm,

Table 3. IEG expression after (1 CS presentation) extinction retrieval in mice fed control or ZnR diets 3 weeks prior to conditioning

Region (distance from bregma)	c-Fos					Zif268				
	S1		B6			S1		B6		
	Ctl	ZnR	Ctl	ZnR	Strain– diet interaction	Ctl	ZnR	Ctl	ZnR	Strain– diet interaction
Cortical regions										
M1, 1.78 mm	0.7 ± 0.2	0.6 ± 0.3	0.6 ± 0.2	0.8 ± 0.3	$F_{(1,20)} = 0.44, p = 0.51$	14.0 ± 0.6	13.6 ± 0.5	14.2 ± 0.9	14.3 ± 1.0	$F_{(1,20)} = 0.10, p = 0.75$
M2, 1.78 mm	1.8 ± 0.9	1.8 ± 0.6	1.4 ± 0.2	1.5 ± 0.3	$F_{(1,20)} = 0.01, p = 0.94$	17.3 ± 0.9	18.3 ± 1.0	17.3 ± 1.1	17.8 ± 0.7	$F_{(1,20)} = 0.10, p = 0.76$
Cg1, 1.78 mm	1.7 ± 0.2	2.2 ± 0.5	2.4 ± 0.5	2.6 ± 1.0	$F_{(1,20)} = 0.08, p = 0.78$	17.9 ± 1.4	17.7 ± 0.4	18.0 ± 0.4	18.8 ± 0.9	$F_{(1,20)} = 0.17, p = 0.69$
Cg1, 1.54 mm	1.2 ± 0.4	1.7 ± 0.3	1.5 ± 0.4	1.4 ± 0.4	$F_{(1,20)} = 0.61, p = 0.45$	14.0 ± 0.5	13.4 ± 0.8	13.3 ± 0.2	14.8 ± 0.5	$F_{(1,20)} = 3.87, p = 0.06$
Cg2 , 1.10 mm	1.4 ± 0.2	1.1 ± 0.3	1.9 ± 0.5	1.5 ± 0.3	$F_{(1,20)} = 0.02, p = 0.89$	14.1 ± 0.4	14.6 ± 0.5	13.9 ± 0.3	13.7 ± 0.3	$F_{(1,20)} = 0.66, p = 0.43$
PrL, 1.98 mm	5.3 ± 0.3	2.9 ± 0.2$^{\#\#}$	3.0 ± 0.1**	2.8 ± 0.2††	**$F_{(1,20)} = 23.04, p < 0.01$**	15.2 ± 0.5	10.7 ± 0.3$^{\#\#}$	10.8 ± 0.3**	11.2 ± 0.5††	**$F_{(1,20)} = 33.28, p < 0.01$**
PrL, 1.78 mm	5.5 ± 0.4	2.5 ± 0.1$^{\#\#}$	2.9 ± 0.1**	3.3 ± 0.2††	**$F_{(1,20)} = 46.44, p < 0.01$**	23.4 ± 1.7	13.5 ± 0.7$^{\#\#}$	13.1 ± 1.0**	13.5 ± 0.5††	**$F_{(1,20)} = 22.12, p < 0.01$**
PrL, 1.54 mm	2.9 ± 0.4	2.3 ± 0.4	3.0 ± 0.4	2.5 ± 0.5	$F_{(1,20)} = 0.032, p = 0.86$	14.2 ± 0.4	14.5 ± 0.7	13.5 ± 0.8	14.6 ± 0.7	$F_{(1,20)} = 0.38, p = 0.54$
IL, 1.98 mm	2.1 ± 0.2	4.5 ± 0.2$^{\#\#}$	4.4 ± 0.5**	4.2 ± 0.2††	**$F_{(1,20)} = 17.96, p < 0.01$**	8.3 ± 0.5	11.7 ± 0.4$^{\#\#}$	10.8 ± 0.3**	11.6 ± 0.3††	**$F_{(1,20)} = 12.43, p = 0.01$**
IL, 1.78 mm	2.3 ± 0.3	8.4 ± 0.5$^{\#\#}$	8.1 ± 0.4**	8.6 ± 0.7†	**$F_{(1,20)} = 35.35, p < 0.01$**	8.2 ± 0.5	16.0 ± 0.5$^{\#\#}$	16.2 ± 0.6**	16.0 ± 0.9††	**$F_{(1,20)} = 41.29, p < 0.01$**
IL, 1.54 mm	2.2 ± 0.3	2.8 ± 0.4	1.8 ± 0.2	2.3 ± 0.3	$F_{(1,20)} = 0.00, p = 1.00$	10.6 ± 0.5	11.9 ± 0.3	11.9 ± 0.3	11.8 ± 0.8	$F_{(1,20)} = 1.65, p = 0.21$
AI, 1.98 mm	No detectable expression					15.1 ± 1.2	14.0 ± 0.7	14.8 ± 0.3	14.4 ± 0.4	$F_{(1,20)} = 0.30, p = 0.59$
AID, 1.78 mm	No detectable expression					11.7 ± 0.4	7.5 ± 0.5$^{\#\#}$	6.5 ± 0.4**	6.2 ± 0.5††	**$F_{(1,20)} = 25.64, p < 0.01$**
AID, 1.54 mm	No detectable expression					10.5 ± 0.4	7.3 ± 0.3$^{\#\#}$	6.4 ± 0.7**	6.9 ± 0.7††	**$F_{(1,20)} = 12.29, p = 0.02$**
AIV, 1.78 mm	No detectable expression					14.0 ± 0.9	14.2 ± 0.4	13.5 ± 0.5	13.6 ± 0.6	$F_{(1,20)} = 0.01, p = 0.97$
AIV, 1.54 mm	No detectable expression					13.7 ± 0.6	13.7 ± 0.4	11.8 ± 0.6	11.3 ± 0.9	$F_{(1,20)} = 0.22, p = 0.65$
PRh, −1.58 mm	No detectable expression					8.4 ± 0.4	8.1 ± 0.2	8.7 ± 0.4	9.3 ± 0.5	$F_{(1,20)} = 1.19, p = 0.29$
Ect, −1.58 mm	No detectable expression					18.8 ± 0.6	18.3 ± 0.7	19.3 ± 0.5	19.7 ± 0.8	$F_{(1,20)} = 0.42, p = 0.53$
Amygdala nuclei (all −1.58 mm)										
Lad	1.7 ± 0.2	1.9 ± 0.3	1.8 ± 0.2	1.6 ± 0.2	$F_{(1,20)} = 0.95, p = 0.341$	17.4 ± 0.6	23.2 ± 0.6$^{\#\#}$	23.0 ± 0.7**	23.4 ± 0.3††	**$F_{(1,20)} = 22.50, p < 0.01$**
Lav	No detectable expression					9.2 ± 0.8	15.9 ± 0.7$^{\#\#}$	15.0 ± 1.0**	14.5 ± 1.1††	**$F_{(1,20)} = 16.05, p < 0.01$**
BA	3.2 ± 0.3	5.9 ± 0.6$^{\#\#}$	5.4 ± 0.2**	5.8 ± 0.4††	**$F_{(1,20)} = 4.60, p = 0.05$**	7.3 ± 0.5	10.6 ± 0.7$^{\#\#}$	10.4 ± 0.2**	9.9 ± 0.4††	**$F_{(1,20)} = 14.20, p < 0.01$**
CeM[a]	15.3 ± 1.0	10.4 ± 1.3$^{\#\#}$	8.8 ± 1.0***	10.2 ± 0.9††	**$F_{(1,20)} = 8.47, p = 0.01$**	71.3 ± 1.7	40.3 ± 3.5$^{\#\#}$	38.9 ± 2.1**	38.2 ± 1.2††	**$F_{(1,20)} = 42.68, p < 0.01$**
CeL[a]	14.6 ± 1.4	15.2 ± 1.4	14.4 ± 1.1	15.6 ± 1.4	$F_{(1,20)} = 0.05, p = 0.83$	63.4 ± 2.9	91.3 ± 4.3$^{\#\#}$	89.2 ± 3.1**	94.4 ± 3.3††	**$F_{(1,20)} = 10.66, p < 0.01$**
CeC[a]	9.8 ± 1.4	9.7 ± 1.4	8.8 ± 1.4	8.2 ± 1.2	$F_{(1,20)} = 0.04, p = 0.84$	51.2 ± 1.1	47.2 ± 2.0	45.3 ± 4.1	47.0 ± 2.9	$F_{(1,20)} = 1.06, p = 0.32$
Imp[a]	No detectable expression					75.8 ± 2.9	41.8 ± 3.3$^{\#\#}$	40.0 ± 3.0**	41.8 ± 2.6††	**$F_{(1,20)} = 36.77, p < 0.01$**
Ilp[a]	No detectable expression					34.5 ± 3.8	33.2 ± 4.8	33.8 ± 3.7	31.7 ± 4.0	$F_{(1,20)} = 0.01, p = 0.92$
In[a]	No detectable expression					57.7 ± 5.8	93.4 ± 3.1$^{\#\#}$	89.3 ± 3.0**	93.0 ± 2.6††	**$F_{(1,20)} = 17.33, p < 0.01$**
MePD	4.8 ± 0.5	4.6 ± 0.6	4.5 ± 0.4	4.0 ± 0.6	$F_{(1,20)} = 0.05, p = 0.82$	11.3 ± 0.7	12.1 ± 0.4	10.5 ± 0.5	11.0 ± 0.4	$F_{(1,20)} = 0.07, p = 0.79$
MePV	8.7 ± 1.0	8.8 ± 0.9	7.2 ± 1.3	7.8 ± 0.6	$F_{(1,20)} = 0.06, p = 0.80$	12.8 ± 0.7	13.0 ± 0.4	13.4 ± 0.6	12.5 ± 0.8	$F_{(1,20)} = 0.73, p = 0.40$
ACo	8.1 ± 0.9	7.8 ± 1.3	8.0 ± 0.6	7.2 ± 0.9	$F_{(1,20)} = 0.07, p = 0.80$	18.6 ± 0.8	18.2 ± 1.6	16.0 ± 1.4	18.8 ± 2.1	$F_{(1,20)} = 1.02, p = 0.32$
PLCo	8.0 ± 0.9	7.4 ± 0.8	7.2 ± 0.2	7.7 ± 0.6	$F_{(1,20)} = 0.64, p = 0.43$	13.4 ± 1.4	11.8 ± 0.9	12.1 ± 0.7	11.2 ± 1.1	$F_{(1,20)} = 0.08, p = 0.78$

[a]Whole (sub)nucleus counted.

$n = 6$ per group. **$p < 0.01$, S1 control diet versus B6 control diet; $^{\#\#}p < 0.01$, S1 control diet versus ZnR S1; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, S1 control diet versus ZnR B6.

Bold indicates significant strain– diet ANOVA interactions. Data are mean ± SEM cell numbers. ACo, Anterior cortical; AI, insular cortex; AIV, insular cortex, ventral; CeC, central, capsular; Cg1, cingulate area 1; Cg2, cingulate area 2; Ctl, control diet; Ect, ectorhinal cortex; M1, primary motor; M2, secondary motor; MePD, medial, posterodorsal; MePV, medial, posteroventral; PLCo, posterolateral cortical; PRh, perirhinal cortex.

$F_{(1,20)} = 12.43$, $p = 0.01$; 1.78 mm, $F_{(1,20)} = 41.29$, $p < 0.01$), dorsal insular cortex (AID; 1.78 mm, $F_{(1,20)} = 25.64$, $p < 0.01$; 1.54 mm, $F_{(1,20)} = 12.29$, $p = 0.02$), Lad ($F_{(1,20)} = 22.50$, $p < 0.01$), Lav ($F_{(1,20)} = 16.05$, $p < 0.01$), BA ($F_{(1,20)} = 14.20$, $p < 0.01$), CeM ($F_{(1,20)} = 42.68$, $p < 0.01$), CeL ($F_{(1,20)} = 10.66$, $p < 0.01$), Imp ($F_{(1,20)} = 36.77$, $p < 0.01$), and In ($F_{(1,20)} = 17.33$, $p < 0.01$) (Table 3). In control-diet groups, PrL, AID, CeM, and Imp were more highly activated, and IL, Lad, Lav, BA, CeL, and In less activated, in S1 than in B6 after extinction retrieval. These strain differences in PFC (Fig. 3), amygdala (Fig. 4), and ITCs (Fig. 5) were absent in ZnR groups due to normalization of S1 activation to B6-control levels (Table 3).

Experiment 3: ZnR does not affect weak fear conditioning

The results of experiments 1 and 2 demonstrate selective effects of ZnR on extinction learning and subsequent retrieval, but not the acquisition and expression of fear itself. However, enhancing Zn can promote fear in rats (Railey et al., 2010) and it remains possible that fear-reducing effects of ZnR in our study were obscured by the strength of the conditioning protocol used. We therefore examined the effects of ZnR on the acquisition and expression of relative weak conditioned fear.

ZnR did not affect fear acquisition in either S1 or B6. As ANOVA results revealed, there was a significant effect of trial (freezing to CS presentations; $F_{(2,40)} = 100.13$, $p < 0.01$), but not of strain (S1 vs B6), diet (control-diet fed mice vs ZnR fed mice), or interactions, on freezing during conditioning (Fig. 6*A*). ZnR also failed to alter fear expression in S1 or B6, assessed 24 h after fear conditioning, as ANOVA revealed no significant strain– diet interaction for freezing during fear expression (Fig. 6*A*).

Experiment 4: postconditioning ZnR facilitates extinction

To further rule out the possibility that the effects of ZnR on fear extinction were an artifact of unknown effects on fear conditioning, we replicated the design of experiment 2 but did not begin ZnR until after conditioning.

Before conditioning, all experimental groups showed an increase in freezing across fear-conditioning trials, which did not differ between groups. There was a significant effect of trial ($F_{(4,108)} = 280.72$, $p < 0.01$) but not strain, and no interaction, for freezing (Fig. 6*B*).

During extinction training, there was a significant trial–strain–diet interaction for freezing ($F_{(14,378)} = 2.77$, $p < 0.01$). In control-diet groups, freezing was higher in S1 than B6 during CS

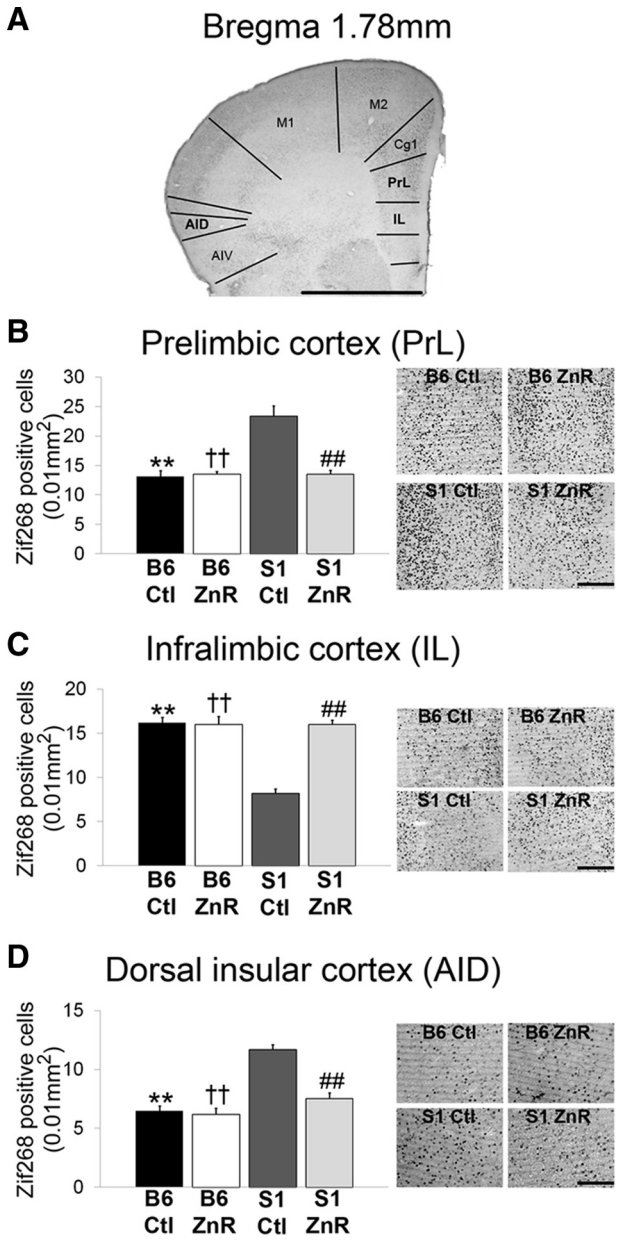

Figure 3. Effect of ZnR on Zif268 expression in PFC following extinction retrieval. ***A***, Representative coronal section from a control-fed S1 mouse stained for Zif268 immunoreactivity showing delineation of the prefrontal cortex regions quantified [delineation of regions was aided by use of a mouse atlas (Paxinos and Franklin, 2001)]. ***B–D***, Control-diet (Ctl) S1 mice showed increased Zif268 expression in PrL (***B***), decreased expression in IL (***C***), and increased expression in AID (***D***), compared with ZnR S1 or B6 fed either control or ZnR diets. Scale bars, 500 μm (***A***), 200 μm (***B***, ***C***), 100 μm (***D***). $n = 6$ per group. Data are presented as means ± SEM. AIV, Ventral insular cortex; Cg1, Cingulate cortex area 1; M1, primary motor cortex; M2, secondary motor cortex. $^{**}p < 0.01$, B6 control diet versus S1 control diet; $^{††}p < 0.01$, B6 ZnR versus S1 control diet; $^{\#\#}p < 0.01$, S1 ZnR versus S1 control diet.

presentations 2–15. Freezing was less in ZnR S1 than in control-diet S1 on CS presentations 2–15 (Fig. 6*B*). There was also a significant strain–diet interaction for freezing during extinction retrieval ($F_{(1,27)} = 14.237$, $p < 0.01$). In control-diet groups, freezing was higher in S1 than B6. Freezing was lower in ZnR S1 than control-diet S1 (Fig. 6*B*).

These data confirm that ZnR postconditioning is sufficient to facilitate subsequent extinction. We noticed, however, that the rate of extinction learning in control-diet B6 was faster than in experiments 1 and 2. The main difference between experiment 4 and experiments 1 and 2, other than when ZnR was applied, was the interval between conditioning and extinction, which, by necessity of the treatment regimen, was 14 d rather than 1 d in experiment 4. To more formally examine this, we statistically compared freezing during extinction training in experiment 4 versus averaged freezing during experiments 1 and 2. There was a significant trial–protocol (day 1 vs day 14) interaction for control-diet B6 ($F_{(1,294)} = 14.73$, $p < 0.001$), ZnR S1 ($F_{(1,252)} = 3.25$, $p < 0.001$), and ZnR B6 ($F_{(1,238)} = 1.81$, $p = 0.038$), but not for control-diet S1. Freezing during extinction training performed 14 d after fear conditioning was less in control-fed B6 (CS presentations 2–8), ZnR S1 (CS presentations 2–6), and ZnR B6 (CS presentations 2–3) than during extinction training in the same experimental groups performed 1 d after conditioning (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). This tentatively suggests there may be savings in extinction learning when the interval between conditioning and extinction training is longer; although additional experiments will be needed to more directly test this.

Discussion

The two major novel findings from the current study were, first, that ZnR rescued impaired fear extinction in S1 mice without affecting fear acquisition or fear expression and, second, that this rescue of extinction was associated with the normalization of aberrant cortico-amygdala activation.

Zn-restricted diet facilitates fear extinction

Replicating our recent observation (Hefner et al., 2008; Camp et al., 2009), current data demonstrated a profound impairment in Pavlovian fear extinction in S1 mice. Feeding S1 mice a ZnR diet for 3 weeks before fear conditioning (and through extinction) effectively restored short-term extinction learning to untreated B6 levels. The ZnR also facilitated short-term extinction in B6, but did not further improve extinction retrieval in this strain, probably because extinction was at asymptote in control-diet B6. Importantly, the proextinction effects of ZnR appear to be unrelated to locomotor effects, as no alterations in locomotor activity were observed in the home cage or open field (Whittle et al., 2009). Furthermore, although enhancing Zn can promote fear in rats (Railey et al., 2010), the effects of ZnR under our test conditions were independent of alterations in fear itself. We found no differences in fear learning or expression, even when a weak conditioning protocol was used to circumvent possible ceiling effects, and we also demonstrated that ZnR facilitated extinction when restriction occurred after fear conditioning. Collectively, these data provide a compelling demonstration of a potent and selective proextinction effect of a Zn-restricted diet.

The exact pharmacological mechanism underlying these effects remains to be determined. For example, NMDARs are involved in fear learning, expression, and extinction (Quirk and Mueller, 2008). As Zn antagonizes NMDARs (see Introduction), we expected enhanced fear learning and/or fear expression, in addition to induction of extinction learning, within ZnR groups, but did not find this effect. Zn also exerts antagonistic action on $GABA_A$Rs and thus Zn-restriction may promote extinction learning via disinhibition of $GABA_A$Rs (see Introduction). Along these lines are studies showing that reduction in extracellular Zn enhances extracellular amygdala GABA concentration (Minami et al., 2002) and synaptically released Zn in the La is found to suppress feedforward GABAergic inhibition of principal neurons (Kodirov et al., 2006). In addition, Zn-restriction may promote

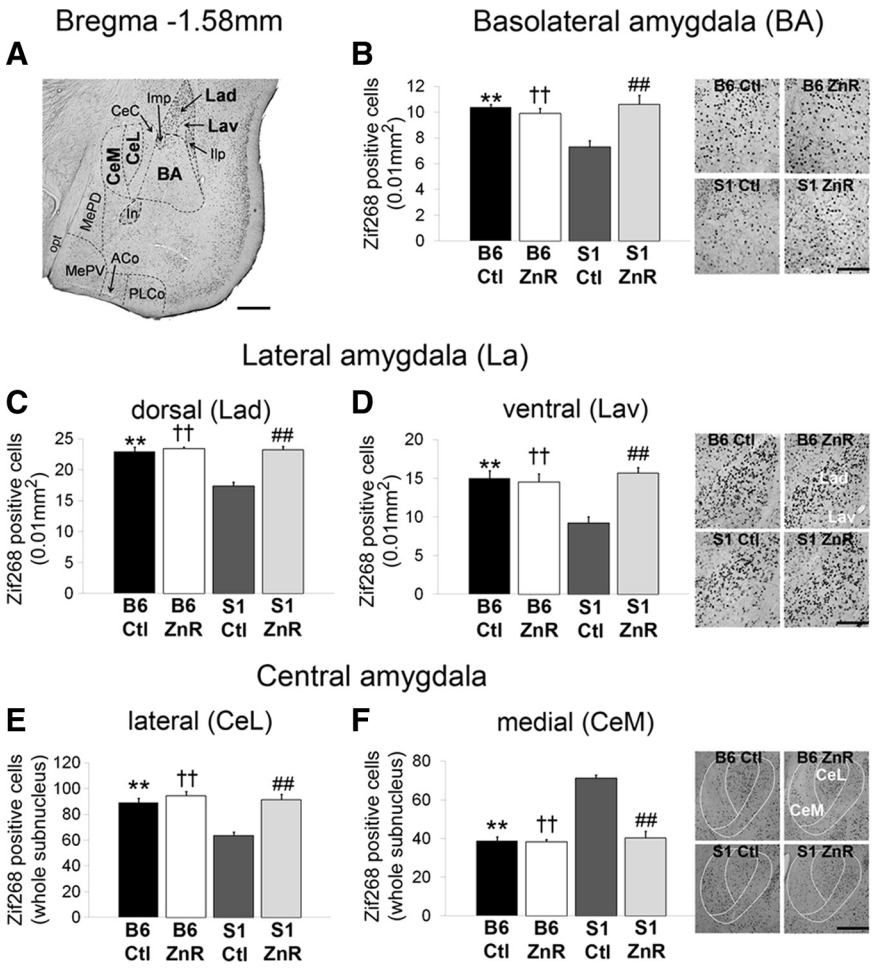

Figure 4. Effect of ZnR on Zif268 expression in amygdala following extinction retrieval. ***A***, Representative coronal section from a control-fed S1 mouse stained for Zif268 immunoreactivity showing delineation of amygdala regions quantified [delineation of regions was aided by use of a mouse atlas (Paxinos and Franklin, 2001)]. ***B–F***, Control-diet (Ctl) S1 mice showed decreased Zif268 expression in BA (***B***), Lad (***C***), Lav (***D***), and CeL (***E***), and increased expression in CeM (***F***), compared with ZnR S1 or B6 fed either control or ZnR diets. Scale bar, 500 μm (***A***), 50 μm (***B***, ***D***), 200 μm (***F***). $n = 6$ per group. ACo, Anterior cortical amygdala; CeC, capsular subdivision of the central amygdala; MePD, posterodorsal division of the amygdala; MePV, posteroventral subdivision of the medial amygdala; PLCo, posterolateral cortical amygdala; opt, optic tract. Data are presented as means ± SEM. $^{**}p < 0.01$, B6 control diet versus S1 control diet; $^{\dagger\dagger}p < 0.01$, B6 ZnR versus S1 control diet; $^{\#\#}p < 0.01$, S1 ZnR versus S1 control diet.

extinction learning by reducing the activity of Zn-dependent HDAC isoforms (Ficner, 2009) since HDAC inhibitors have been shown to facilitate extinction learning in rodents (for review, see Bredy et al., 2010). Further studies will be needed to parse the contribution of these and possibly other mechanisms to the effects of Zn-restriction on extinction.

Cortical dysfunction in S1, normalization via ZnR

Current data show that impaired extinction in S1 was associated with functional abnormalities in a cortico-amygdala circuit regulating fear extinction (Quirk and Mueller, 2008; Herry et al., 2010) and is dysfunctional in PTSD (Milad et al., 2009). This was determined by mapping the expression of the IEGs c-Fos and Zif268, which are transcription factors used as surrogate markers for neuronal activation (Colombo, 2004; Herry and Mons, 2004; Knapska and Kaczmarek, 2004; Singewald, 2007). Replicating our earlier finding (Hefner et al., 2008), impaired extinction was associated with reduced IL activation in control-diet S1. In unconditioned mice, there were no differences between strains in the number of c-Fos- or Zif268-positive cells in IL, or in any other brain region examined. These findings agree with data showing that IL activity in rodents (Quirk et al., 2000, 2003; Herry and Garcia, 2002; Milad and Quirk, 2002; Barrett et al., 2003) and ventromedial PFC activity in humans (Phelps et al., 2004; Milad et al., 2007a, 2009), via connections to the amygdala, is a critical gate in the expression of extinction.

A novel finding was that impaired S1 extinction was also associated with increased activation of the AID and PrL, suggesting a role in fear mechanisms. Indeed, fMRI studies of patients with anxiety disorders show increased insular activity (Paulus and Stein, 2006; Reinhardt et al., 2010). AID could possibly influence fear via its projection to the output nucleus of the amygdala (CeM) (Mcdonald et al., 1996).

The finding that impaired extinction was associated with PrL hyperactivity in S1 is intriguing given emerging evidence that PrL promotes fear. For example, increased fear and resistance to extinction correlates with single unit activity in the rat PrL (Vidal-Gonzalez et al., 2006; Corcoran and Quirk, 2007; Burgos-Robles et al., 2009) and blood oxygenation level-dependent signal in the homologous human region (Milad et al., 2007b), whereas PrL inactivation reduces fear expression (Corcoran and Quirk, 2007; Laurent and Westbrook, 2009). PrL hyperactivity could, therefore, contribute to the maintenance of fear in S1.

In contrast to the other cortical and amygdala regions examined, PrL and AID activity in S1 differed from B6 extinction retrieval after one CS presentation, but not 15 CS presentations. This appears to be due to increased PrL and AID activation after the longer test in all groups, which prevented the detection of strain differences due to a ceiling effect. This could also explain why PrL and AID activation were not associated with extinction differences in earlier studies using a multi-CS retrieval test (Hefner et al., 2008; Knapska and Maren, 2009).

Supporting the functional relationship between PrL/AID hyperactivity and IL hypoactivity in S1 and impaired extinction, the rescue of extinction by ZnR was associated with the normalization to B6 control levels of activity patterns in all three cortical regions. Moreover, ZnR did not alter basal (i.e., unconditioned) IEG expression in any region examined or affect IEG expression after extinction in any cortical region not recruited during extinction, consistent with modulation of extinction-driven functional activation rather than a nonspecific change in neuronal activity.

Amygdala dysfunction in S1, normalization via ZnR

We found lower La activation in S1 indicated by Zif268 but not c-Fos; the reason for this is not clear, but a similar observation was made in subpopulations of good and poor extinguishing mice (Herry and Mons, 2004; Hefner et al., 2008). Notwithstanding this observation, Zn-restriction increased Zif268 expression

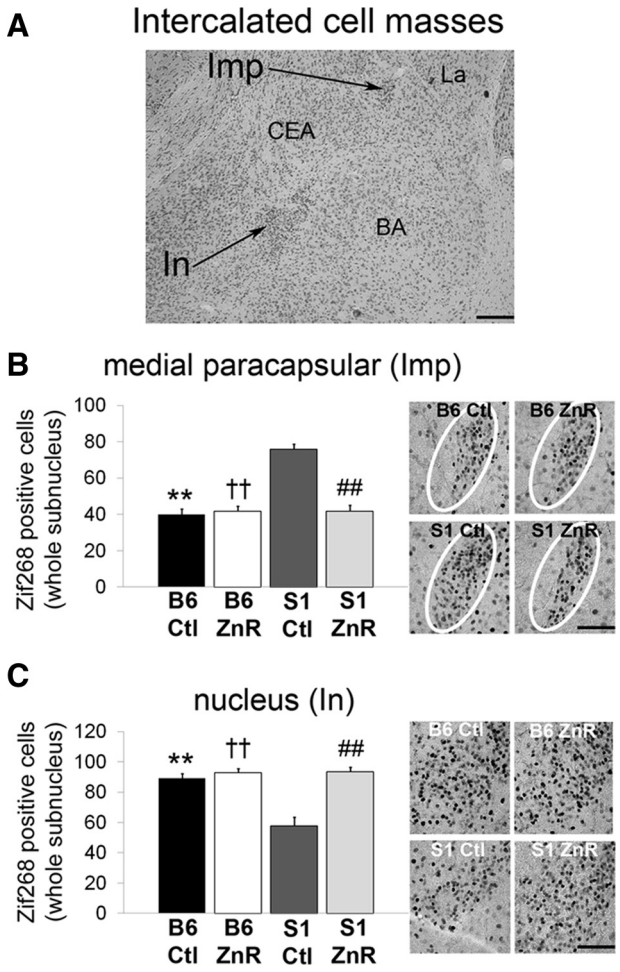

Figure 5. Effect of ZnR on Zif268 expression in the ITC masses following extinction retrieval. ***A***, Nissl stain of a control-fed S1 brain depicting ITC regions at bregma −1.58 mm. Scale bar, 100 μm. ***B***, ***C***, Control-diet (Ctl) S1 mice showed increased Zif268 expression in Imp (***B***) and decreased expression in the In (***C***) compared with ZnR S1 or B6 fed either control or ZnR diets. Scale bar, 25 μm. $n = 6$ per group. Data are presented as means ± SEM. $^{**}p < 0.01$, B6 control diet versus S1 control diet; $^{\dagger\dagger}p < 0.01$ B6 ZnR versus S1 control diet; $^{\#\#}p < 0.01$, S1 ZnR versus S1 control diet.

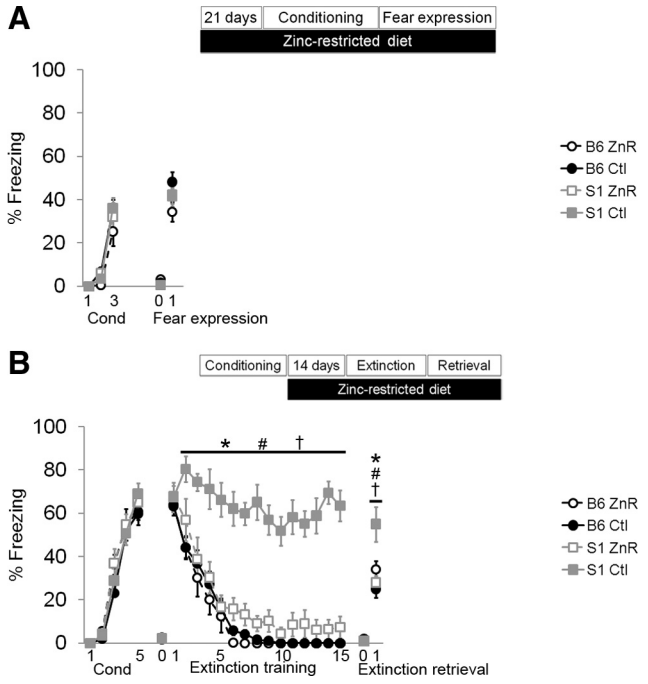

Figure 6. ***A***, Effect of ZnR on fear conditioning (Cond) and fear expression using mild fear conditioning. ZnR beginning 21 d prior to fear conditioning did not enhance fear conditioning or fear expression in either S1 or B6 ($n = 6$ per group). Data are presented as means ± SEM. ***B***, Effect of postconditioning ZnR on fear extinction. ZnR beginning after conditioning rescued impaired extinction learning and retrieval in S1, relative to control diet (Ctl) ($n = 6$–9 per group). Data are presented as means ± SEM. $^{*}p < 0.05$, S1 control diet versus B6 control diet; $^{\#}p < 0.05$, S1 control diet versus S1 ZnR; $^{\dagger}p < 0.05$, B6 ZnR versus S1 control diet.

in S1 in tandem with extinction rescue. As NMDAR agonism facilitates extinction and extinction consolidation when microinjected into La before and immediately after extinction training, respectively (Myers and Davis, 2007), the enhanced Zif268 activation in the La following Zn-restriction potentially reflects enhanced NMDA receptor activation, contributing to extinction rescue (although see Discussion, above).

Activation of the BA is necessary for the gating of fear via projections to CeM (Quirk et al., 2003); however, BA hypoactivity is also associated with poor extinction (Herry and Mons, 2004; Hefner et al., 2008; Knapska and Maren, 2009). We found a similar relationship in S1 under control-diet conditions and its reversal by ZnR. The two functions of BA are parsed by separate subpopulations of fear- and extinction-associated BA neurons (Herry et al., 2008). The net reduction in BA activation in control-fed S1 could reflect a failure to specifically recruit the latter population. The activity of the extant population of BA fear neurons could then drive the increased CeM activation seen in S1, leading to sustained amygdala output. This sustained fear could also stem from a failure of other inhibitory inputs to CeM. In this context, a subpopulation of neurons in the CeL was recently found to inhibit CeM output (Huber et al., 2005). Consistent with a failure to engage this inhibitory pathway to reduce fear, high CeM activation in S1 was coupled with low CeL activation. Zn-restriction, which rescued impaired extinction in S1, normalized IEG expression in the BA, CeL, and CeM.

Functional dissociation of ITC masses

ITC masses represent a key node within extinction circuitry receiving input from basolateral amygdaloid complex and PFC subnuclei to exert inhibitory control over CeA output (Royer et al., 1999). Functional involvement in extinction circuitry has been shown previously, as postextinction lesion of ITCs impairs extinction retrieval (Likhtik et al., 2008). Somewhat unexpectedly, we previously found that impaired extinction in S1 was associated with increased, rather than decreased, activation in the Imp ITC mass (Hefner et al., 2008). Current data extend and potentially resolve this finding by again demonstrating that, although Imp was hyperactive in S1 relative to B6 under control-diet conditions, the more ventrally located In mass was hypoactive in S1. Hypoactive In was normalized (i.e., activated) by rescue of extinction via ZnR, which is in line with recent data in rats recalling extinction (Knapska and Maren, 2009) and may point to a possible causal relationship between these events (Amano et al., 2010).

The contrasting patterns of ITC mass activation raise important issues regarding possible functional heterogeneity of the different masses, suggesting that Imp and In may occupy differing and even opposing positions within the extinction circuit. Electrophysiological studies provide precedent to this hypothesis, as stimulation of guinea pig Imp neurons elicits inhibitory responses in In (Royer et al., 2000), suggesting that axonal projec-

tions from Imp to In that are known to exist (Royer et al., 2000; Geracitano et al., 2007) might form an inhibitory pathway counteracting proficient extinction by reducing In-driven feedforward inhibition of CeM output (Amano et al., 2010). Indeed, a hypoactive S1 In and resultant failure of CeM inhibition would be congruent with existing models of impaired extinction. Clearly, however, additional work will be needed to elucidate the efferent and afferent connections of Imp and In to amygdala and PFC subnuclei and how these might map onto functional differences in regulation of fear extinction.

Conclusions

In summary, current data demonstrate that dietary depletion of Zn expedited fear extinction in B6 and rescued impaired extinction in S1. Deficient extinction in S1 was associated with aberrant recruitment of the cortico-amygdala extinction circuit and these functional abnormalities were resolved by feeding S1 mice a Zn-restricted diet. These findings identify a novel mechanism underlying fear extinction and support the utility of the S1 model of impaired extinction as a tool to screen for innovative extinction-facilitating drugs and elucidate the neural basis of their effects.

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