

ORIGINAL ARTICLE

Enhancing dopaminergic signaling and histone acetylation promotes long-term rescue of deficient fear extinction

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Extinction-based exposure therapy is used to treat anxiety- and trauma-related disorders; however, there is the need to improve its limited efficacy in individuals with impaired fear extinction learning and to promote greater protection against return-of-fear phenomena. Here, using 129S1/SvImJ mice, which display impaired fear extinction acquisition and extinction consolidation, we revealed that persistent and context-independent rescue of deficient fear extinction in these mice was associated with enhanced expression of dopamine-related genes, such as dopamine D1 (*Drd1a*) and -D2 (*Drd2*) receptor genes in the medial prefrontal cortex (mPFC) and amygdala, but not hippocampus. Moreover, enhanced histone acetylation was observed in the promoter of the extinction-regulated *Drd2* gene in the mPFC, revealing a potential gene-regulatory mechanism. Although enhancing histone acetylation, via administering the histone deacetylase (HDAC) inhibitor MS-275, does not induce fear reduction during extinction training, it promoted enduring and context-independent rescue of deficient fear extinction consolidation/retrieval once extinction learning was initiated as shown following a mild conditioning protocol. This was associated with enhanced histone acetylation in neurons of the mPFC and amygdala. Finally, as a proof-of-principle, mimicking enhanced dopaminergic signaling by L-dopa treatment rescued deficient fear extinction and co-administration of MS-275 rendered this effect enduring and context-independent. In summary, current data reveal that combining dopaminergic and epigenetic mechanisms is a promising strategy to improve exposure-based behavior therapy in extinction-impaired individuals by initiating the formation of an enduring and context-independent fear-inhibitory memory.

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INTRODUCTION

Fear-, anxiety- and trauma-related disorders are highly prevalent today.^{1–3} Psychotherapeutic interventions such as exposure-based cognitive behavioral therapy are effective treatments;^{4–6} however, many patients retain symptoms after initial treatment^{7–9} and return-of-fear is often reported.^{10,11} Recent research has expanded understanding of the neural¹² and molecular^{13–15} mechanisms underlying fear extinction, which is the basis of exposure-based cognitive behavioral therapy. However, there is a paucity of information regarding the critical neurobiological mechanisms required to overcome treatment resistance (extinction resistance) in exposure-based cognitive behavioral therapy and to protect against return-of-fear phenomena such as spontaneous fear recovery and fear renewal.¹⁴

To gain insight into molecular pathways that support long-lasting rescue of deficient fear extinction in a context-independent manner, we used 129S1/SvImJ (S1) mice that exhibit normal fear learning, but deficient fear extinction acquisition^{16–22} and impaired fear extinction consolidation/retrieval.²³ This deficit in fear extinction is associated with a failure to properly engage corticolimbic extinction circuitry, including, the medial prefrontal cortex (mPFC) and amygdala.^{20,22} Using these mice, we aimed to identify mechanisms that are associated with persistent and

context-independent rescue of deficient fear extinction, using dietary zinc restriction (ZnR) as an experimental tool that we have previously revealed to rescue impaired fear extinction.²² We assessed gene expression changes in extinction-relevant brain regions after the ZnR-induced rescue of impaired fear extinction in S1 mice, given that long-lasting fear extinction memories require the coordinated transcription of specific genes coding for learning-associated transcription factors, neurotransmitter receptors, cytoskeletal proteins and other cellular substrates.^{15,24,25} To confirm the functional significance of the identified molecular pathways for long-term extinction rescue, we performed behavioral proof-of-principle experiments by administering pharmacological adjuncts that target the identified pathways and revealed the effects on rescue of extinction acquisition, spontaneous recovery and/or fear renewal.

MATERIALS AND METHODS

Animals and husbandry

Male 3-month-old S1 mice (Charles River, Sulzfeld, Germany) were housed (four to five per cage) in a temperature- (22 ± 2 °C) and humidity- (50–60%) controlled vivarium under a 12 h light/dark cycle. The Austrian Animal Experimentation Ethics Board (Bundesministerium für Wissenschaft

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Forschung und Wirtschaft, Kommission für Tierversuchsangelegenheiten) approved all experimental procedures.

Fear conditioning and extinction procedure

Fear conditioning, extinction and extinction retrieval was carried out as previously described.²³ Persistence and context independence of fear extinction memories was assessed using spontaneous fear recovery tests in the extinction context²⁶ and fear renewal tests in a novel context²⁷ (see Supplementary Materials and Methods for full details).

Drug treatments and experimental manipulations

Dietary ZnR. Animals were fed food pellets (ssniff Spezialdiäten, Soest, Germany) containing low Zn (ZnR; 12.3 mg kg⁻¹ or 40% of the recommended daily intake requirement²⁸) or standard food pellets containing normal quantities of Zn (Ctl; 65 mg kg⁻¹) in control mice as previously described.²² Mice were randomly selected to be subjected to either the Ctl diet or ZnR diet. The duration of each experiment's diet regimen is provided in the respective figure.

Drug administration. MS-275 (Entinostat, Selleck Chemicals, Vienna, Austria; 10 mg kg⁻¹ dissolved in saline+25% dimethylsulfoxide vehicle) was administered immediately (< 1 min) following an extinction training session and L-dopa (Sigma-Aldrich, Vienna, Austria; 20 mg kg⁻¹ dissolved in saline) was administered either 1 h before or immediately following an extinction training session. All drugs were administered intraperitoneally in a volume of 10 ml kg⁻¹ body weight. Control animals received the respective vehicle. Mice were randomly selected to be administered either vehicle or pharmacological compound.

Immunofluorescence staining

Mice were perfused 2 h after the extinction training session started. Coronal sections were incubated with primary antibodies raised against either Zif268, acLYS, ac-H4K5,8,12,16 (acH4) and/or NeuN, followed by incubation with fluorescent-labeled secondary antibodies (see Supplementary Materials and Methods for full details).

Genome-wide expression profiling

We extracted RNA (Qiagen, Hilden, Germany) from mPFC, amygdala, dorsal hippocampus and ventral hippocampus punches 2 h after the extinction training session started. The RNA was quality-controlled (Bioanalyzer, Agilent, Vienna, Austria) and analyzed using Affymetrix Mouse Genome 430 2.0 gene chip arrays (Affymetrix UK, High Wycombe, UK). Genes exhibiting a fold change of ≥ 1.6 and a significance of 0.3 false-discovery rate were considered differentially expressed. We used quantitative reverse transcriptase-PCR (qRT-PCR) following standard procedures (SYBR green) with exon-specific primers to validate gene expression. Gene ontology analysis was performed on genes exhibiting differential expression (see Supplementary Materials and Methods for full details).

Chromatin immunoprecipitation

mPFC and amygdala tissue punches were formaldehyde crosslinked and chromatin immunoprecipitation was performed following a modification of the Active Motif ChIP-IT Express kit protocol with an acH4 antibody. Quantitative qRT-PCR was performed using standard procedures (SYBR green) and DNA expression was normalized to input with promoter-specific DNA primers (see Supplementary Materials and Methods for full details).

Statistical analysis

Data were assessed for normal distribution before performing parametric tests. All behavioral, immunohistochemistry, qRT-PCR and ChIP experiments were analyzed using parametric tests (*t*-test or one-way analysis of variance (ANOVA) or multiple-way ANOVA with repeated measures for trial (behavior)). Main effects and interactions for significant ANOVAs are described. Fisher least significant difference *post hoc* tests are listed for each condition examined. All *t*-tests were two-tailed. Throughout, $P < 0.05$ was considered significant. Sample size for behavior and molecular analysis was decided on the basis of our previous experience in the field and published studies (for molecular analysis) and was not pre-determined by a sample size calculation. Data are presented as mean \pm s.e.

m. Detailed listing of statistical results are presented in Supplementary Table S1.

RESULTS

Enduring and context-independent rescue of impaired fear extinction by dietary ZnR

We have previously shown that dietary ZnR can rescue extinction deficits in S1 mice and restore the aberrant recruitment of extinction-related brain areas while leaving fear acquisition and fear expression unaffected.²² Given that long-term effects of this intervention has not been studied, we now assessed spontaneous fear recovery and fear renewal in a novel context (Figure 1a) to determine whether this ZnR-induced rescued extinction memory is enduring and context-independent. Replicating our previous finding of deficient fear extinction rescue,²² present data revealed lower freezing in ZnR-fed mice compared with control diet-fed counterparts during extinction training and extinction retrieval sessions (Figure 1b). Importantly, we now found that this induced fear extinction memory was long-lasting and context-independent as, compared with control-diet-fed mice, ZnR-fed mice exhibited lower freezing during spontaneous fear recovery and fear renewal tests (Figure 1b). These effects were specific to extinction induced by the combination of ZnR and extinction training as spontaneous fear recovery and fear renewal has been observed following successful fear extinction and extinction retrieval induced by other pharmacological means in S1 mice.²⁹ To reveal whether memory update mechanisms³⁰ influenced the ZnR-induced rescue of impaired fear extinction, we subjected an additional group of ZnR-treated mice to fear expression only and assessed freezing during subsequent retrieval tests (Figure 1a). Following fear re-activation, ZnR-fed mice did not differ in freezing responses compared to control diet-fed mice during subsequent fear expression sessions (Figure 1b), suggesting that interference with memory update mechanisms does not significantly contribute to ZnR-induced rescue of impaired fear extinction. Taken together,

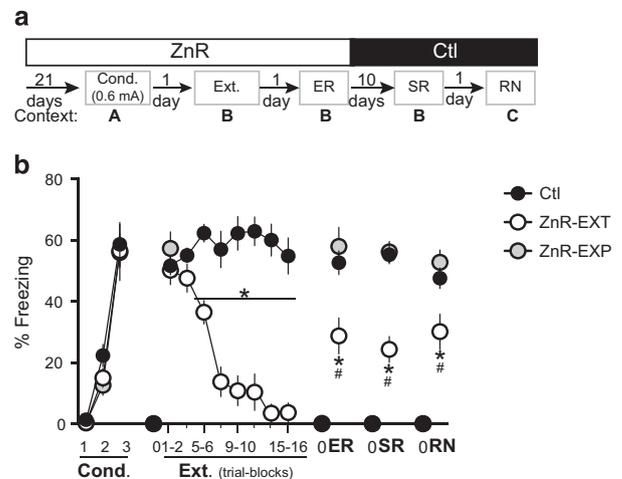


Figure 1. Dietary Zn restriction rescues impaired fear extinction in an enduring and context-independent manner. (a) Scheme illustrating experimental paradigm. Cond., 'normal' 0.6 mA fear conditioning in context A; Ext., extinction training in context B; ER, extinction retrieval in context B; SR, spontaneous recovery in context B; RN, fear renewal in context C; Ctl, Control diet; ZnR, zinc-restricted diet. (b) Freezing during Cond., Ext., ER, SR and RN in Ctl, ZnR-Ext and ZnR-Exp groups. 0, pre-tone freezing. During fear extinction training (trial blocks), ER, SR and RN individual freezing data are presented as the average freezing during two conditioned stimulus (CS) presentations. $n = 10$ per group. * $P < 0.05$ *post hoc* testing Ctl-Ext versus ZnR-Ext, # $P < 0.05$ ZnR-Exp versus ZnR-Ext.

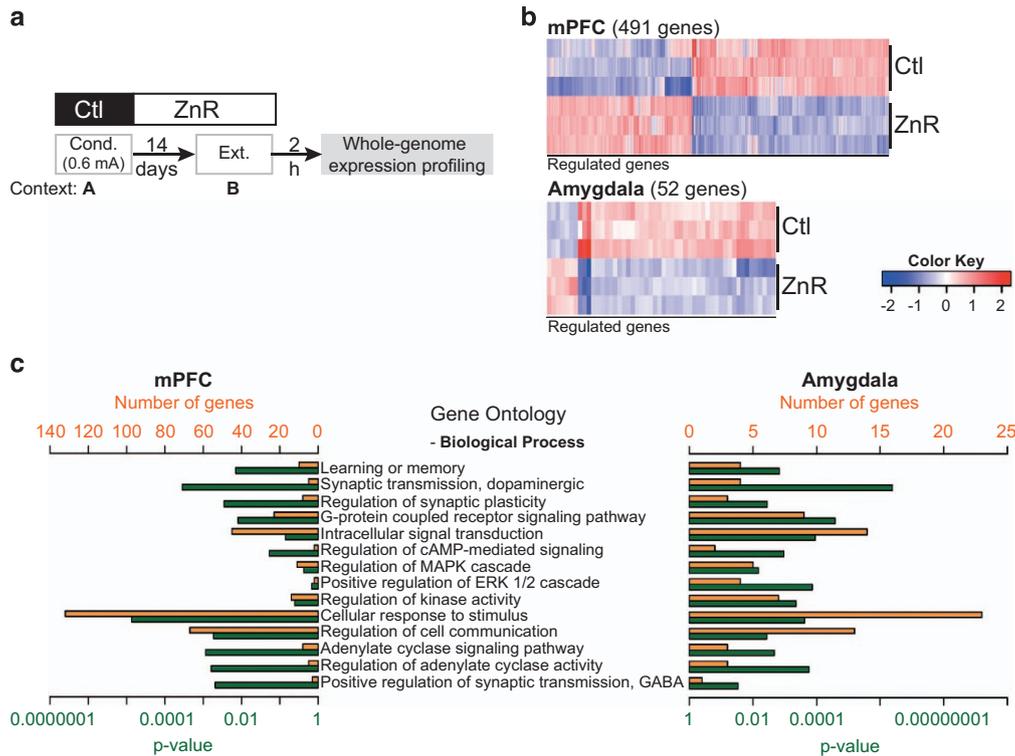


Figure 2. Rescue of impaired fear extinction induces a select transcriptional response in the medial prefrontal cortex (mPFC) and amygdala. **(a)** Scheme illustrating experimental paradigm. Mice were subjected to ‘normal’ 0.6 mA fear conditioning (Cond.) in context A and fear extinction (Ext training in context B). All groups were conditioned on control diet (Ctl) before mice were subjected to dietary zinc restriction (ZnR) for 14 days till Ext. **(b)** Heatmap depicting differentially expressed genes in the mPFC (491) and amygdala (52) determined using microarray analysis between non-extinguishing Ctl diet-fed mice and extinguishing ZnR-fed mice. Each line depicts a differentially regulated gene; each row depicts the gene expression in each animal. Blue and red indicate low and high levels of expression, respectively. **(c)** Histogram showing the biological process to which the differentially regulated genes belong (orange, number of differentially regulated genes per biological process; green, significance of enrichment).

current data suggest that ZnR is an ‘extinction inducer’ that durably rescues deficient fear extinction in a context-independent manner and is therefore considered an ideal model tool to study mechanisms supporting long-term context-independent rescue of impaired fear extinction.

Rescue of impaired fear extinction initiates select gene expression changes, including, in particular, genes of dopamine signaling

We next sought to reveal the molecular mechanisms that are associated with ZnR-induced rescue of impaired fear extinction. Following extinction acquisition, a period of neuronal plasticity is initiated, which is thought to transform the newly formed labile fear extinction memory into a stable long-term memory. Changes in gene expression in extinction-relevant brain regions are essential mechanisms governing neuronal plasticity.¹⁴ To gain insight into the genomic response initiated with successful rescue of impaired fear extinction, we used whole-genome mRNA expression to identify differentially expressed RNAs in extinguishing ZnR mice compared with non-extinguishing control-diet-fed mice in the mPFC, amygdala and hippocampus (Figure 2a). Extinction-relevant brain regions³¹ were chosen based on findings showing enhanced neuronal activity (assessed via Zif268 immediate-early gene mapping^{32,33}) in infralimbic (IL) and prelimbic (PL) areas of the mPFC, in the basal amygdala (BA) and in the dorsal and ventral hippocampal CA1 area following successful rescue of deficient fear extinction acquisition (Supplementary Figures S1–S6 and Supplementary Tables S2–S5). Rescue of impaired fear extinction was associated with a restricted transcriptional response, involving 491 differentially regulated genes in the mPFC and 52 in the amygdala (Figure 2b,

Supplementary Tables S6 and S7). No changes in gene expression were observed in dorsal and ventral hippocampal regions. Pathway and gene ontology analysis revealed the differentially regulated genes’ participation in biological processes related to neuronal plasticity, including learning- or memory-related processes, and regulation of extinction-relevant downstream signaling pathways including cAMP, MAPK and ERK1/2 cascades, among others (Figure 2c). Strikingly, genes coding for dopamine receptors D1 (*Drd1a*) and D2 (*Drd2*) and the dopamine receptor downstream target dopamine- and cAMP-regulated phosphoprotein DARPP-32 (*Ppp1r1b*) exerted the greatest statistical contribution to the transcriptional response in mPFC and amygdala regions of extinguishing ZnR S1 mice (Figure 2c). qRT-PCR confirmation in independent experiments (Figure 3a) confirmed the enhanced gene expression of *Drd1a*, *Drd2* in the mPFC and the amygdala (Figure 3b). Moreover, extinguishing mice showed a significant increase of *Ppp1r1b* in the amygdala and a nonsignificant increase in the mPFC.

To control for possible dietary effects of ZnR and reveal the extinction specificity of the observed gene changes following fear expression, which are components in early extinction training, we quantified gene expression changes in control diet-fed and ZnR-fed mice also following fear expression (Figure 3a). Results revealed that within the mPFC the observed increases in *Drd1a* and *Drd2* in ZnR-treated mice were extinction-specific and not observed in ZnR-fed mice subjected to fear expression only (Figure 3c). In contrast, enhanced expression of *Ppp1r1b* was also revealed in the mPFC of ZnR-fed mice following fear expression (Figure 3c). In the amygdala, only the increase in *Drd1a* expression was specifically related to the rescue of impaired fear extinction.

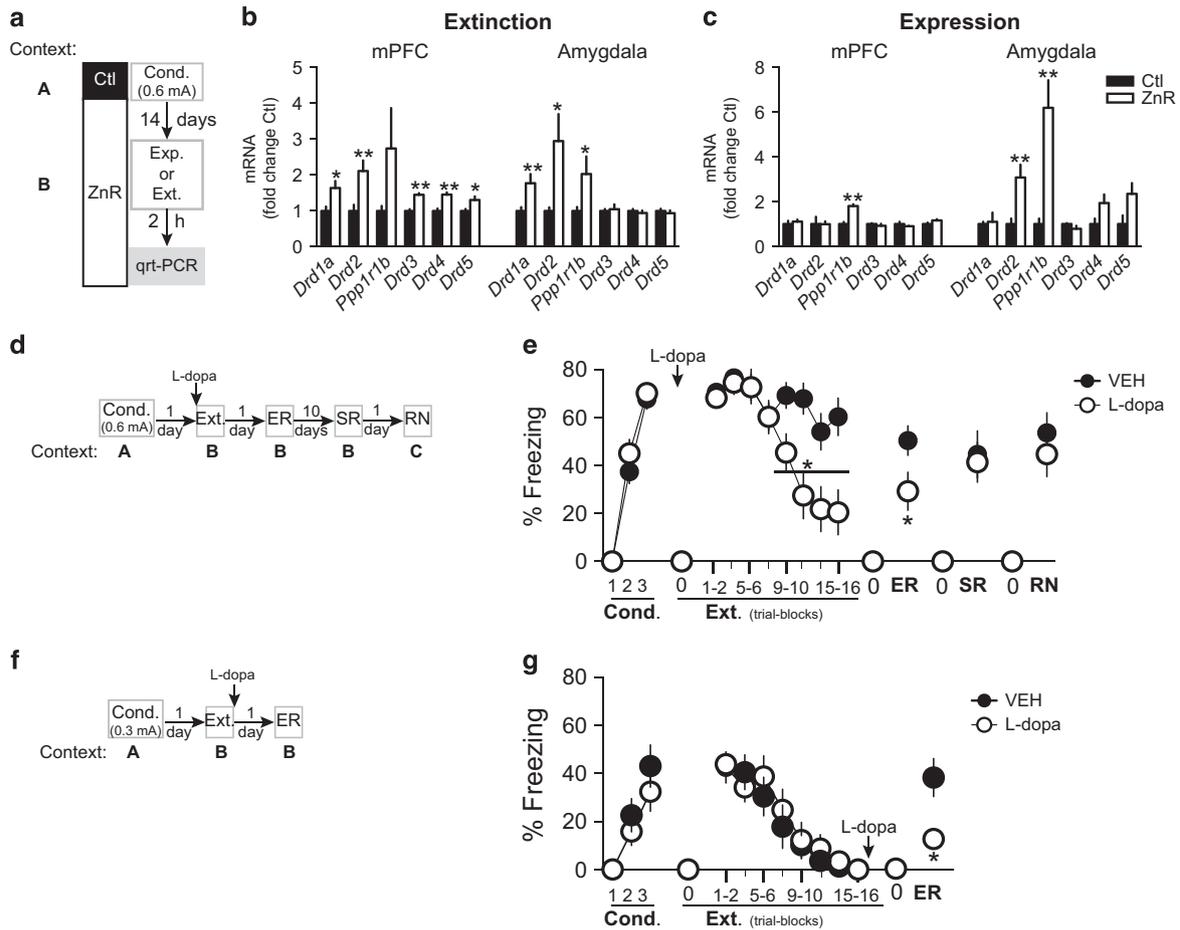


Figure 3. Rescue of impaired fear extinction enhances expression of genes coding for dopamine D1 (*Drd1a*) and D2 (*Drd2*) receptors. **(a)** Scheme illustrating experimental paradigm for **b**, **c**. Cond., 'normal' 0.6 mA fear conditioning; Ext, extinction training; Exp, fear expression; Ctl, control diet; ZnR, dietary zinc restriction. Expression of dopaminergic genes in the medial prefrontal cortex (mPFC) and amygdala following successful rescue of impaired fear extinction (**b**) and following fear expression (**c**). * $P < 0.05$ Ctl versus ZnR, ** $P < 0.01$ Ctl versus ZnR. $n = 3-8$ per group. **(d)** Scheme illustrating experimental paradigm for **e**. Cond., 'normal' (0.6 mA) conditioning; ER, extinction retrieval; SR, spontaneous recovery; RN, fear renewal. **(e)** Lower freezing was observed in L-dopa-treated mice when compared with VEH-treated during Ext. trial blocks 7-8 through to 15-16 and also during ER. $n = 10$ per group. * $P < 0.05$ post hoc testing L-dopa versus VEH. **(f)** Scheme illustrating experimental paradigm for **g**. Cond., 'weak' (0.3 mA) conditioning. **(g)** Lower freezing was observed in L-dopa-treated mice when compared with VEH-treated mice during ER. $n = 7-8$ per group. * $P < 0.05$ post hoc testing L-dopa versus VEH.

Compared with control diet-fed mice, ZnR-fed mice exhibited enhanced expression of *Drd2* and *Ppp1r1b* following fear expression (Figure 3c).

Dopamine receptors are clustered in two families: the D1-like family composed of D1 and D5 receptors, and the D2-like family composed of D2, D3 and D4 receptors.³⁴ To ascertain whether the gene expression profiles of *Drd1a* and *Drd2* reflect their family, we performed a comprehensive expression analysis of dopamine D3, D4 and D5 receptors after the rescue of impaired fear extinction and fear expression (Figure 3a): we observed enhanced mPFC expression of *Drd3*, *Drd4* and *Drd5* in ZnR-fed mice, compared with the control group (Figure 3b), whereas no changes were observed following fear expression (Figure 3c). No changes in amygdala *Drd3*, *Drd4* or *Drd5* expression were observed in any experimental group after rescue of impaired fear extinction (Figure 3b) or after fear expression (Figure 3c), underscoring the specificity of the observed gene changes. Taken together, these results suggest that rescue of deficient fear extinction, leading to the formation of an enduring and context-independent fear extinction memory, is associated with a general enhancement in genes coding for D1-like and D2-like families of dopaminergic

receptors in the mPFC. In contrast, only a select enhancement in the gene coding for dopamine D1 receptor is elicited in the amygdala.

Enhancing dopaminergic signaling rescues impaired fear extinction but does not protect from long-term fear recovery phenomena

To prove functional relevance of these findings, we next investigated whether drug-induced activation of dopaminergic signaling pathways indeed affects the rescue of impaired fear extinction. To do so, we administered L-dopa, which enhances central dopaminergic signaling,³⁵ before extinction training (Figure 3d) and observed lower freezing in L-dopa-treated mice compared with vehicle controls during extinction training and retrieval sessions (Figure 3e), revealing that enhancing dopaminergic signaling can rescue extinction acquisition and consolidation/retrieval deficits in S1 mice. No difference in spontaneous locomotor activity was detected between L-dopa-treated S1 mice and their vehicle-treated counterparts during the pre-tone period during extinction training (Distance traveled (L-dopa, 97.7 ± 11.7 cm; VEH, 104.8 ± 17.0 cm; $n = 10$ per group)) and thus

negated the possibility that the reduced freezing during the extinction training session was due to L-dopa *per se*. To gain greater insight into L-dopa's ability to overcome impaired extinction consolidation/retrieval in S1 mice, we used the recent finding from a 'weak' fear-conditioning paradigm where S1 mice show an extinction training effect but are unable to consolidate or retrieve the extinction memory.²³ We therefore administered L-dopa following extinction training to boost the impaired extinction consolidation (Figure 3f). Results revealed that lower freezing was observed in L-dopa-treated mice compared with fear extinction-deficient vehicle-treated controls during the extinction retrieval session 24 h later (Figure 3g), demonstrating that L-dopa can rescue extinction consolidation/retrieval deficits in S1 mice. It is unlikely that L-dopa itself influenced freezing during the extinction retrieval session, given that the half-life of L-dopa in rodents is less than an hour³⁶ and central levels of L-dopa peak 1–2 h after peripheral administration in mice.³⁷ However, during the spontaneous fear recovery and fear renewal tests, no differences in freezing was observed in L-dopa-treated mice, compared with vehicle (Figure 3e). Collectively, these results behaviorally indicate that enhancing dopaminergic signaling rescues impaired extinction acquisition and improves deficient consolidation/retrieval; however, it is not sufficient to promote enduring protection from return-of-fear in extinction-impaired individuals.

HDAC inhibition facilitates formation of an enduring and context-independent fear extinction memory

We assessed whether enhanced histone acetylation is associated with the ZnR-induced long-term rescue of impaired fear extinction, given the evidence that enhancing histone acetylation can induce enduring and context-independent fear extinction memories in normal extinguishing rodents^{23,38–47} and that zinc deprivation inhibits zinc-dependent histone deacetylases (HDACs) and increases acetylation of lysine residues on histone proteins.⁴⁸ We therefore quantified lysine acetylation in the IL, PL and BA and hippocampus 2 h after the rescue of impaired fear extinction and revealed enhanced lysine acetylation in extinction-activated (Zif268 positive) cell populations in the IL, PL, BA and the dorsal and ventral CA1 hippocampal regions (Supplementary Figures S1–S6 and Supplementary Tables S2–S5). No alterations in lysine acetylation were observed in cells not activated after fear extinction rescue, highlighting the extinction specificity of these changes (Supplementary Figures S1–S5 and Supplementary Tables S2–S5). To unequivocally demonstrate that ZnR-induced fear extinction rescue stimulates enhanced acetylation on histone proteins and contributes to the differential expression of extinction-regulated genes, we quantified the abundance of acetylated histone H4 in the promoter region of *Drd1a* and *Drd2* in the mPFC and the amygdala (Figure 4a). We chose this specific histone mark based on a study in normal extinguishing mice, showing that histone H4 acetylation is enhanced in the promoter region of the *BDNF* gene in the mPFC.⁴⁵ Results revealed that compared with non-extinguishing control-fed mice, extinguishing ZnR-fed mice displayed enhanced mPFC histone H4 acetylation in the promoter region of the *Drd2* gene (Figure 4b) and a trend of increase around the *Drd1a* promoter (Figure 4b). Importantly, histone H4 acetylation was not altered at the *Drd2* promoter in ZnR mice that underwent fear expression only (Figure 4c), indicating a selective role of *Drd2* in extinction learning rescue. Following fear extinction or expression, no alterations in *Drd1a* or *Drd2* histone H4 acetylation levels were observed in the amygdala (Figure 4b and c). These results suggest that histone H4 acetylation is an important, but not exclusive mechanism contributing to enhanced dopamine receptor gene expression following ZnR-induced fear extinction in S1 mice.

To gain functional (behavioral) evidence that enhancing histone acetylation contributes to the enduring and context-independent

rescue of fear extinction, we built on our previous finding showing that the HDAC inhibitor MS-275, targeting primarily HDAC1, HDAC2 and HDAC3 isoforms,⁴⁹ can improve fear extinction consolidation/retrieval in 'weak' conditioned S1 mice²³ and assessed now the long-term consequence of this strategy (Figure 4d). Results revealed that, compared with vehicle-treated controls, MS-275-treated mice exhibited lower freezing rates during extinction retrieval, spontaneous fear recovery and fear renewal tests (Figure 4e). This finding demonstrates that MS-275 can facilitate the formation of an enduring and context-independent rescue of impaired extinction consolidation/retrieval in a paradigm where S1 mice reduce freezing during extinction training.

Combination of HDAC inhibition and successful fear extinction acquisition is required to enhance histone acetylation in the mPFC and amygdala

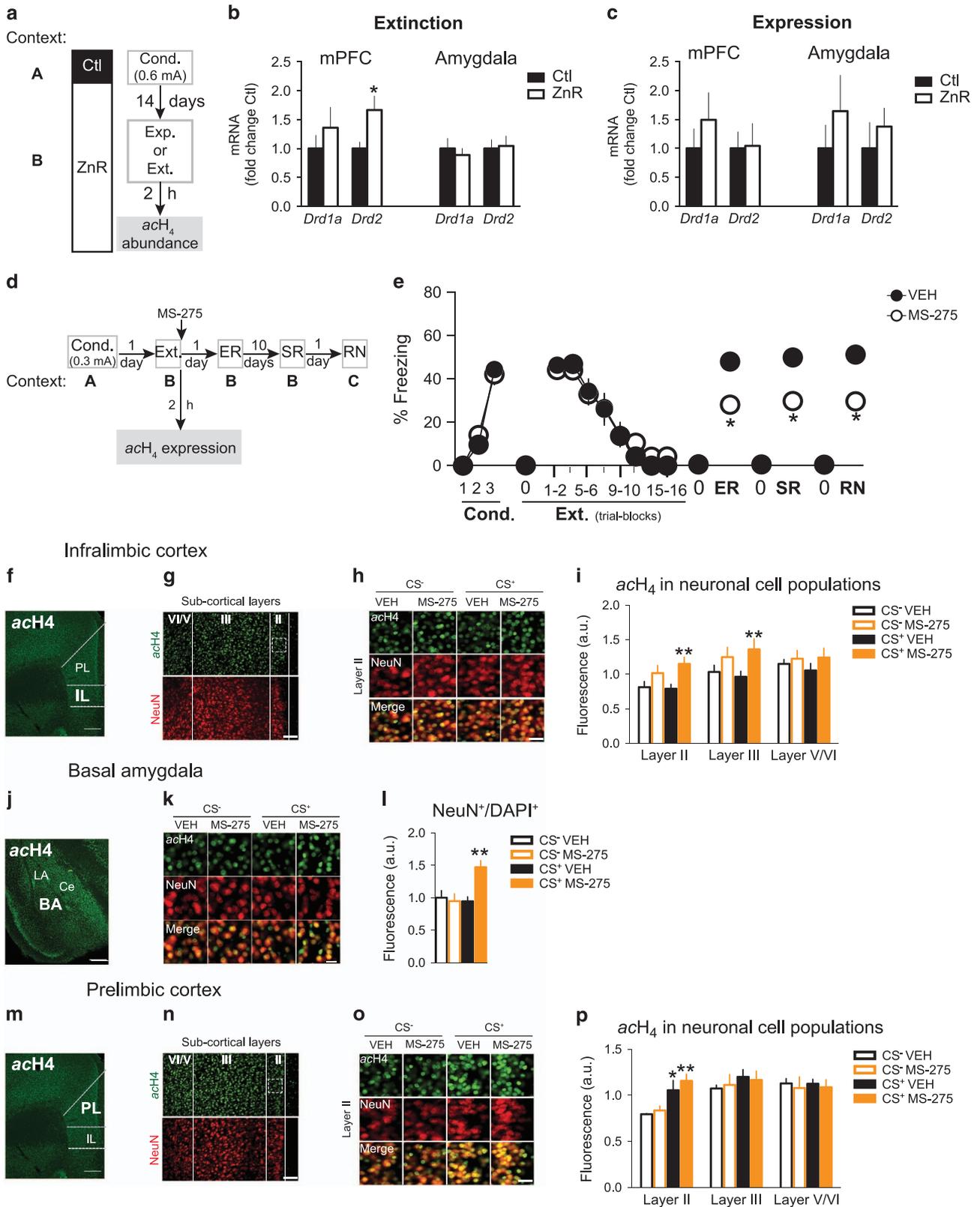
We next focused on revealing in greater detail the brain regions that show enhanced histone acetylation after MS-275-induced rescue of impaired fear extinction consolidation in the weak fear-conditioning paradigm (Figure 4d and e). To this end, we compared histone acetylation levels in neuronal (NeuN+) and non-neuronal (NeuN–) cell populations in the IL, PL and BA following MS-275 administration in conditioned mice (CS+) and in basal non-fearful non-conditioned mice (CS–). Results revealed that only a combination of extinction acquisition and MS-275, but not MS-275 treatment alone, enhanced histone H4 acetylation in neuronal (NeuN-positive) cell populations in layers II and III of the IL (Figure 4f–i) and in the BA (Figure 4j–l). No such combination effects were observed in the PL (Figure 4m–p), revealing brain region specificity of this response. Interestingly, compared with basal non-conditioned control mice, enhanced neuronal histone H4 acetylation was observed in layer II of the PL in conditioned mice following fear extinction acquisition, independent of whether mice were administered MS-275 or vehicle (Figure 4m–p). Given that there is evidence showing that activity in the PL supports fear memories,^{50–53} our finding of enhanced PL histone H4 acetylation (Figure 4m–p) may point to a potential molecular 'fear' correlate. This idea, however, needs further confirmation. We observed no changes in histone H4 acetylation in non-neuronal (NeuN–) cell populations within any experimental group in any brain region examined (Supplementary Figure S7).

Combination of treatments that enhance dopaminergic signaling and HDAC inhibition promotes an enduring, context-independent rescue of impaired fear extinction

We lastly assessed whether the transient L-dopa-induced extinction rescue can be made enduring and context-independent by co-administering of the HDAC inhibitor MS-275, similar to its effect in the 'weak' conditioning paradigm (see above). To assess this, we administered L-dopa before an extinction training session in 'normal' conditioned mice, and, additionally, administered MS-275 immediately following this extinction training session (Figure 5a). Replicating our earlier finding (see Figure 3d and e), L-dopa-treated mice displayed reduced freezing rates during extinction training and retrieval (Figure 5b). Importantly, in contrast to L-Dopa/vehicle-treated mice, L-dopa/MS-275-treated mice showed lower freezing rates during spontaneous recovery and fear renewal tests (Figure 5b). These results reveal that co-treatment with the HDAC inhibitor MS-275 transforms the labile L-Dopa-induced fear extinction memory into an enduring and context-independent fear extinction memory.

DISCUSSION

The present findings provide novel insights into mechanisms supporting long-term rescue of deficient fear extinction. This was



achieved by identifying molecular pathways associated with the behavioral rescue of impaired fear extinction in S1 mice using dietary ZnR, an experimental multitarget tool that induces normalization of aberrant activation in extinction-related brain

areas²² and as revealed here elicits long-lasting and context-independent fear extinction in extinction-impaired mice. We observed enhanced dopaminergic gene expression and histone acetylation in the mPFC and amygdala of extinction-rescued mice,

Figure 4. Enhancing histone acetylation promotes enduring and context-independent rescue of newly formed extinction memories. **(a)** Scheme illustrating experimental paradigm for **b, c**. Cond., 'normal' fear conditioning; Exp, fear extinction; Exp, fear expression; Ctl, control diet; ZnR, zinc-restricted diet. $n = 3-8$ per group. **(b)** Enhanced abundance of acH4 was observed in the promoter region of *Drd2* following fear extinction in the medial prefrontal cortex (mPFC). **(c)** No changes in any group were observed following fear expression. **(d)** Scheme illustrating experimental paradigm for **e-p**. ER, extinction retrieval; SR, spontaneous recovery; RN, renewal. **(e)** Freezing was lower in MS-275 administered mice during ER, SR and RN. $n = 9-10$ per group. $*P < 0.05$ *post hoc* testing MS-275 versus VEH. **(f)** Representative epifluorescent photomicrograph of a coronal section (Bregma +1.78 mm) stained for acH4 immunoreactivity. IL, infralimbic cortex; PL, prelimbic cortex. Scale bar = 500 μm . **(g)** Representative epifluorescent photomicrographs delineating the cortical layers within the IL. Scale bar = 100 μm . **(h, i)** Enhanced acH4 epifluorescence was observed in NeuN-positive (NeuN+) cell populations following MS-275 administration and following successful fear extinction (CS+) in layer II and in layer III, but not layer V/VI, compared with vehicle-treated counterparts (VEH). $**P < 0.01$ *post hoc* testing MS-275 versus VEH in conditioned (CS+) groups. $n = 4-6$ /group. Scale bar = 20 μm . **(j)** Representative epifluorescent photomicrograph of a coronal section (Bregma -1.56 mm) delineating the basal amygdala region quantified. BA, basal amygdala; Ce, central amygdala; LA, lateral amygdala. Scale bar = 250 μm . **(k, l)** Enhanced acH4 epifluorescence was observed in NeuN-positive nuclei (NeuN+) only successful fear extinction acquisition and MS-275 administration. Scale bar = 20 μm . $**P < 0.01$ *post hoc* testing MS-275 versus VEH in conditioned (CS+) groups. $n = 4-6$ /group. **(m)** Representative epifluorescent photomicrograph of a coronal section (Bregma +1.78 mm) stained for acH4 immunoreactivity. IL, infralimbic cortex; PL, prelimbic cortex. Scale bar = 500 μm . **(n)** Representative epifluorescent photomicrographs delineating the cortical layers within the PL. Scale bar = 100 μm . **(o,p)** Enhanced acH4 epifluorescence was observed in NeuN-positive (NeuN+) cell populations following successful fear extinction (CS+) in mice administered VEH and MS-275 to a similar level in layer II, but not layers II or V/VI, compared to vehicle-treated counterparts (VEH). Scale bar = 20 μm . $*P < 0.05$, $**P < 0.01$ *post hoc* testing MS-275 versus VEH in conditioned (CS+) groups. $n = 4-6$ per group.

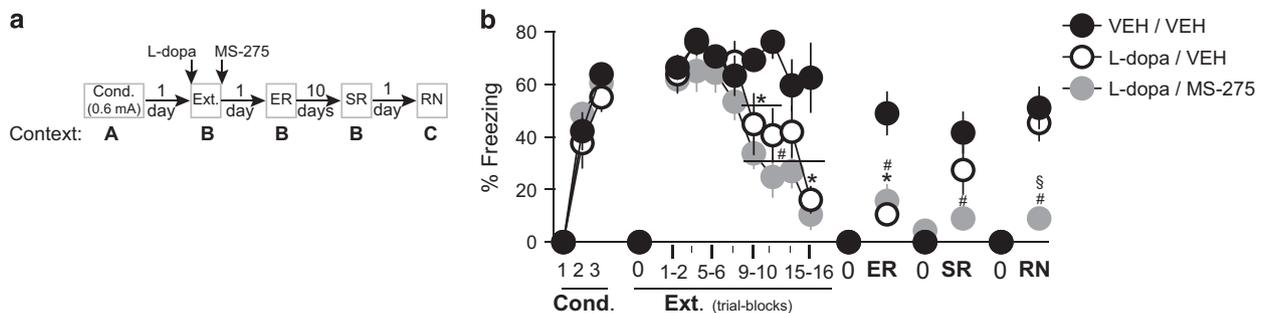


Figure 5. Combined treatments that enhance dopaminergic signaling and histone deacetylase inhibition are necessary to rescue impaired fear extinction in an enduring and context-independent manner. **(a)** Scheme illustrating experimental paradigm. Cond., 'normal' fear conditioning; Ext., fear extinction; ER, extinction retrieval; SR, spontaneous recovery; RN, fear renewal. **(b)** During extinction training and compared with VEH/VEH control group, reduced freezing was observed in L-dopa/VEH-treated mice during extinction trial blocks 9–10, 11–12 and 15–16 and in L-dopa/MS-275 during extinction trial blocks 9–10 through to 15–16. During ER reduced freezing was observed in L-dopa/VEH and L-dopa/MS-275 compared with VEH/VEH. L-dopa/MS-275-treated mice exhibited lower freezing compared with VEH/VEH during SR and VEH/VEH and L-dopa/VEH during RN. $*P < 0.05$ *post hoc* testing L-dopa/VEH versus L-dopa/MS-275, $\#P < 0.05$ *post hoc* testing L-dopa/VEH versus L-dopa/MS-275. $n = 8-10$ per group.

using a combination of genome-wide expression analysis, immunohistochemistry and chromatin immunoprecipitation, among other methods. Functional (behavioral) validation of these identified mechanisms revealed that boosting dopaminergic signaling, via single L-dopa administration, rescued impaired extinction acquisition and temporarily rescued extinction consolidation deficits, whereas enhancing histone acetylation via single administration of the HDAC inhibitor MS-275 failed to correct deficient fear extinction acquisition during extinction training but facilitated enduring and context-independent fear inhibition once extinction learning was initiated. Finally, as proof-of-principle, we found that a combined treatment approach administering L-dopa and MS-275 around extinction training indeed rescued deficient extinction and rendered this effect enduring and context-independent, mimicking the extinction-related effects of ZnR.

Using genome-wide transcriptome analysis, we showed the association between enduring and context-independent ZnR-induced rescue of impaired fear extinction and the differential expression of a select number of genes in the mPFC and amygdala. Our molecular analysis focused on the mPFC, amygdala and hippocampus because these interconnected brain regions, which display aberrant neuronal activation in extinction-deficient

S1 mice,^{18,20,22} exhibited enhanced neuronal activity after ZnR-induced fear extinction acquisition. This result extends findings in extinction-intact rodents^{20,22,54-61} and healthy humans⁶²⁻⁶⁴ to show that activity in these regions is associated with fear extinction. We found that successful fear extinction acquisition led to the regulation of a restricted number of neuroplasticity-associated genes, which is reminiscent of a recent finding revealing a specific, restricted transcriptional response after fear extinction.⁴⁷ In the current study, we observed alterations in gene expression in the mPFC and amygdala, but not hippocampus. The lack of hippocampal gene expression changes seems surprising, given our finding showing that ZnR S1 mice show no renewal of fear. One possibility to reconcile the lack of hippocampal gene expression changes with the formation of a context-independent extinction memory in ZnR S1 mice could be the differential involvement of hippocampal subregions to the context dependency of extinction.⁶⁵ Using immunohistochemistry experiments we demonstrated that rescue of impaired fear extinction in ZnR S1 mice was associated with increased activation of the CA1 subfield of the hippocampus only, which is in line with recent studies showing the importance of this region in the context specificity of extinction.^{65,66} Hence, we propose that the absence of extinction-related hippocampal gene expression changes in the present

study might be because of limitations in resolution, as whole hippocampus tissue was used for gene array experiments. Future experiments should therefore aim at investigating gene expression in hippocampal subregions to clarify the possible involvement of hippocampal gene expression changes in the context-independent rescue of impaired fear extinction in ZnR S1 mice. Among the identified differentially regulated genes in the mPFC and amygdala following successful ZnR-induced fear extinction in S1 mice, extinction-induced alterations in dopaminergic gene expression, which contributed most to the overall observed transcriptional response, were of particular interest, given the proposed critical role for dopamine in fear and extinction learning.¹⁵ Rescue of impaired fear extinction was specifically associated with enhanced expression of genes encoding the dopamine D1 class (D1 and D5) and D2 class (D2, D2 and D4) receptors in the mPFC. This result extends findings in normal extinguishing rodents, showing that enhanced mPFC dopamine release is observed during and following successful fear extinction training⁶⁷ and activity of dopamine D1- and D2 class signaling,^{68–70} is necessary for fear extinction. In the amygdala, expression of the gene coding for the dopamine D1 receptor (*Drd1a*) was selectively enhanced after rescue of impaired fear extinction, pointing to the contribution of amygdala dopamine D1 receptor-mediated activity. This hypothesis is supported by evidence showing that antagonizing Dopamine D1 class (D1 and D5) receptors in the basolateral amygdala during an extinction training session can antagonize fear extinction acquisition and subsequent extinction memory retrieval in normally extinguishing rats.⁶⁸

To confirm dopaminergic signalings' contribution in the rescue of deficient fear extinction, we administered L-dopa before extinction training, which resulted in an extinction-facilitating effect during a subsequent retrieval test. This extinction-inducing effect is likely not due to L-dopa *per se* as class I and II dopaminergic receptor agonists facilitate fear extinction only when combined with an extinction training session.^{70,71} This current finding extends data from extinction-intact rodents showing that dopaminergic signaling can influence fear extinction consolidation mechanisms.¹⁵ However, we could not demonstrate in S1 mice enduring rescue of impaired fear extinction, which contrasts with a recent finding showing that L-dopa can induce enduring fear extinction and protection from return-of-fear phenomena in extinction-intact mice and healthy humans.⁷² From a clinical perspective, these results reveal a distinction between extinction augmented in a non-pathological system with intact fear extinction, and extinction induced in pathological systems with deficient fear extinction.

The finding that the L-dopa-induced fear extinction memory was only temporary suggests that other mechanisms are involved after ZnR-induced rescue of impaired fear extinction to promote the observed long-term effects. For example, epigenetic priming of extinction-regulated genes^{73,74} is associated with formation of long-lasting fear extinction memories.⁴⁷ Indeed, quantifying histone abundance in the promoter region of the extinction-regulated dopaminergic genes revealed that increased mPFC histone H4 acetylation abundance accompanied the gene-transcription program initiated after the rescue of impaired fear extinction. We believe, to our knowledge, that this is the first report demonstrating an extinction-related acetylated histone abundance in genes coding for dopaminergic receptors. Future studies will be necessary to identify enhanced histone acetylation in additional genes whose activity can promote long-term fear extinction rescue. Our finding that treatment with the HDAC inhibitor MS-275, following extinction acquisition, facilitated enduring and context-independent rescue of deficient extinction retrieval also underscores the behavioral significance of histone acetylation mechanisms on long-term context-independent fear inhibition. Moreover, this current result adds to a growing

body of literature showing that HDAC inhibitors can facilitate formation of enduring and context-independent fear extinction memories^{43,46,47} even when extinction consolidation/retrieval is deficient.

Given the potential therapeutic use of MS-275 as adjunct therapy,¹⁴ we investigated the underlying mechanisms in HDAC-inhibitor-induced rescue of deficient extinction consolidation/retrieval. A striking finding was that a combination of successful fear extinction acquisition and HDAC inhibition was necessary to enhance histone H4 acetylation levels in layers II and III of the IL and within the BA. The specific dynamics and circuit organization, particularly of output neurons within cortical layers of the IL, remain largely unknown;⁷⁵ however, our results reveal novel insight into circuits mediating the rescue of impaired fear extinction. By quantifying epigenetic changes after the rescue of impaired fear extinction we have demonstrated enhanced histone H4 acetylation in neurons within layers II and III of the IL, which may project to the basolateral amygdala.⁷⁵ Given circuit-level evidence showing that fear extinction is associated with IL top-down control of the BA,^{18,53,76–78} our present finding of enhanced histone acetylation in the IL layers that specifically project to the BA, as opposed to other IL layers that project to other brain structures (for example, layer V IL neurons project to the periaqueductal gray),⁷⁵ raises the possibility that fear extinction memories are stored within discrete IL layers that project to the amygdala.

Finally, using a two-step pharmacological intervention, we showed that prior extinction training administration of L-dopa can initiate extinction acquisition in extinction-impaired mice, and this new memory can be made enduring and context-independent using MS-275. This finding suggests that dual strategy approaches are necessary to overcome high resilience to inhibit learned fear by targeting extinction (present findings) or reconsolidation mechanisms.^{29,79}

Collectively, these data have provided novel insight into molecular mechanisms that rescue deficient fear extinction in an enduring and context-independent manner. These results reveal that discrete signaling pathways can modulate different phases of disturbed fear extinction learning, and that therapeutically targeting dopaminergic and epigenetic mechanisms is a promising strategy to improve exposure-based cognitive behavior therapy in extinction-impaired individuals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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