Archival Report

Biological Psychiatry

MicroRNA-Mediated Rescue of Fear Extinction Memory by miR-144-3p in Extinction-Impaired Mice

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ABSTRACT

BACKGROUND: MicroRNA (miRNA)-mediated control of gene expression suggests that miRNAs are interesting targets and/or biomarkers in the treatment of anxiety- and trauma-related disorders, where often memory-associated gene expression is adversely affected.

METHODS: The role of miRNAs in the rescue of impaired fear extinction was assessed using the 129S1/SvImJ (S1) mouse model of impaired fear extinction. miRNA microarray analysis, reverse transcription polymerase chain reaction, fluorescent in situ hybridization, lentiviral overexpression, and Luciferase reporter assays were used to gain insight into the mechanisms underlying miRNA-mediated normalization of deficient fear extinction.

RESULTS: Rescuing impaired fear extinction via dietary zinc restriction was associated with differential expression of miRNAs in the amygdala. One candidate, miR-144-3p, robustly expressed in the basolateral amygdala, showed specific extinction-induced, but not fear-induced, increased expression in both extinction-rescued S1 mice and extinction-intact C57BL/6 (BL6) mice. miR-144-3p upregulation and effects on subsequent behavioral adaption was assessed in S1 and BL6 mice. miR-144-3p overexpression in the basolateral amygdala rescued impaired fear extinction in S1 mice, led to enhanced fear extinction acquisition in BL6 mice, and furthermore protected against fear renewal in BL6 mice. miR-144-3p targets a number of genes implicated in the control of plasticity-associated signaling cascades, including *Pten*, *Spred1*, and *Notch1*. In functional interaction studies, we revealed that the miR-144-3p target, PTEN, colocalized with miR-144-3p in the basolateral amygdala and showed functional down-regulation following successful fear extinction in S1 mice.

CONCLUSIONS: These findings identify a fundamental role of miR-144-3p in the rescue of impaired fear extinction and suggest this miRNA as a viable target in developing novel treatments for posttraumatic stress disorder and related disorders.

Keywords: Anxiety- and trauma-related disorders, Basolateral amygdala, Fear, MicroRNAs, PI3K/AKT, Signaling cascade modulation

http://dx.doi.org/10.1016/j.biopsych.2016.12.021

Over the past decade, microRNAs (miRNAs) have emerged to play an important role in central nervous system mechanisms, including synaptic plasticity (1) and learning and memory (2). Furthermore, aberrant expression of miRNAs has been associated with the pathogenesis of many common central nervous system disorders, including psychiatric disorders such as anxiety and depression (3). The functional role of miRNAs in controlling plasticity mechanisms in the adult brain (4) makes them desirable targets for the treatment of disorders where these mechanisms are adversely affected. The extinction of conditioned fear, a basic principle of exposure-based therapy, requires the formation of new fear-inhibitory memories (5,6). Recent studies have begun to elucidate the role of miRNAs in fear learning (7,8) and its extinction (9). In particular, a deeper understanding of the underlying miRNA-mediated mechanisms could provide important leads for the development of novel targets for exposure-based therapy. In line with this, a role for the brain-specific miR-128b in the extinction of fear was previously uncovered (9). The authors demonstrated increased miR-128b expression in the infralimbic prefrontal cortex following fear extinction training, which disrupted the stability of a number of plasticity-associated genes implicated in the retrieval of fear memory, including its host gene, RCS (regulator of calmodulin signaling). RCS is known to competitively inhibit calmodulin and increase the activation of the protein phosphatase calcineurin, which contributes to the strength of aversive memories. The authors hypothesized that increased miR-128b expression in the infralimbic prefrontal cortex may facilitate the transition from the retrieval of the original fear toward the formation of the new fear-inhibitory memory.

ISSN: 0006-3223

So far, these studies have been performed in extinction-intact C57BL/6 (BL6) mice; however, the role of miRNAs in relevant psychopathological models of dysfunctional extinction memory remains to be elucidated.

Mimicking anxiety patients who display deficits in extinction memory formation, we have previously shown that 129S1/SvImJ (S1) mice display an inability to extinguish conditioned fear, which is also characterized by aberrant immediate early gene expression in a corticolimbic circuit (10–13). Furthermore, deficits in both fear extinction acquisition and fear extinction consolidation are observed in these mice. We have previously reported that dietary zinc restriction (ZnR) can rescue fear extinction deficits in S1 mice and normalize the aberrant immediate early gene expression in the affected corticolimbic circuit (14,15). In a recent report, we revealed that not only can ZnR rescue impaired fear extinction, but it leads to the formation of enduring and context-independent fear extinction memory via protection against spontaneous recovery and fear renewal (16). Thus, ZnR can be used as an ideal experimental tool to study the underlying neurobiological mechanisms of impaired fear extinction rescue, including miRNA-mediated regulation of gene expression. Understanding the underlying mechanisms associated with successful fear extinction in such psychopathological models has the potential to reveal novel therapeutic targets for the treatment of debilitating disorders, including posttraumatic stress disorder.

Because successful fear extinction requires the expression of learning-associated genes in the amygdala (17), which are known to undergo regulation by miRNAs (18,19), we explored the significance of miRNA-mediated regulation within the amygdala during successful fear extinction. We hypothesized that extinction-associated gene expression in the amygdala following successful fear extinction is controlled by miRNA-mediated mechanisms. To assess this, an unbiased microarray screen was first used to examine miRNA expression following the rescue of impaired fear extinction in the amygdala. We then examined the functional implications of the most robustly regulated miRNA in the basolateral amygdala (BLA) of impaired S1 and normally extinguishing BL6 mice. Lastly, we explored the downstream target genes and their implications in successful fear extinction.

METHODS AND MATERIALS

Animals

Male 129S1/SvImJ mice (Jackson Laboratory, Bar Harbor, ME) and C57BL/6J mice (Charles River Laboratories, Wilmington, MA) were obtained between 8 and 9 weeks old and left to acclimate for 2 weeks before commencing any experiments. They were housed (4–5 per cage) in a temperature (21 ± 2°C)- and humidity (50%–60%)-controlled vivarium under a 12-hour light/dark cycle. All behavioral experiments were approved by the Austrian Animal Experimentation Ethics Board (Bundesministerium für Wissenschaft Forschung und Wirtschaft, Kommission für Tierversuchsangelegenheiten) and the University Laboratory Animal Resources of the University of California, Irvine.

Fear Conditioning and Extinction Procedure

Fear conditioning, extinction, and extinction retrieval were performed as previously described (14). The effect of miR-144-3p overexpression in the BLA was assessed during fear extinction and extinction retrieval (see Supplemental Methods and Materials for full details).

Tissue Collection, RNA Isolation for Messenger RNA, and miRNA Transcripts

Amygdala tissue punches were collected 2 hours following the start of extinction training. Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO) as per the manufacturer's guidelines. Complementary DNA and subsequent reverse transcription polymerase chain reaction were performed using the miScript II reverse transcription kit and the miScript II polymerase chain reaction kit (Qiagen, Hilden, Germany) for miRNAs and qScript (Quanta Biosciences, Beverly, MA) and Fast SYBR master mix (Applied Biosystems, Foster City, CA) for messenger RNAs (mRNAs) (see Supplemental Methods and Materials for full details).

miRNA Microarray Analysis

Microarray analysis was carried out by employing the miRCURY LNA (locked nucleic acid) miRNA Array 7th generation microarray from Exiqon (Woburn, MA). Experiments were performed in dye swap pairs with four biological replicates from ZnR S1 mice and age-matched S1 control mice, respectively (see Supplemental Methods and Materials for full details).

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) was performed as previously described in detail (20). Prelabeled LNA probes with 5′ and 3′ fluorescein tags were ordered from Exiqon for miR-144-3p and miR-scrambled probe. FISH was performed on frozen tissue sections from impaired and ZnR-rescued S1 mice (see Supplemental Methods and Materials for full details).

Lentiviral Surgery and Overexpression of miR-144-3p

Lentiviral plasmids were generated as previously described (9). Briefly, either miR-144-3p or scramble control fragments were inserted immediately downstream of the human H1 promoter in a modified FG12 vector, which was subsequently packaged into a lentivirus. Surgery was performed a minimum of 4 days before fear conditioning, where two single cannulas were bilaterally implanted into the BLA of S1 mice (−1.4 mm [anteroposterior], ±3.3 mm [mediolateral], −4.0 mm [dorsoventral]) and BL6 mice (−1.3 mm [anteroposterior], ±3.2 mm [mediolateral], −4.0 mm [dorsoventral]). A total volume of 0.8 μL of lentivirus was infused via two injections delivered within 48 hours (see Supplemental Methods and Materials for full details).

Statistical Analysis

Data are presented as mean ± SEM. The *n* numbers are given in figure legends. Statistical analysis was performed using Prism 6 (GraphPad Software, San Diego, CA). All experiments were analyzed with parametric tests (*t* test, all two tailed) or one/two-way analysis of variance (ANOVA) with repeated

measures for trial (Bonferroni or Fisher least significant difference is described). Variance was similar between all groups used. Main effects and interactions for significant ANOVAs are described. Throughout, $p < .05$ was considered significant.

RESULTS

Enhanced miR-144-3p Expression Is Observed in the Amygdala Following Impaired Fear Extinction Rescue and Following Fear Extinction in Extinction-Intact Mice

To gain insight into the regulation of miRNAs in the amygdala, we quantified changes in miRNA expression following behavioral rescue of impaired fear extinction (Figure 1A).

Both groups displayed similar freezing levels in response to fear (Figure 1B). Replicating previous findings on ZnR-mediated rescue (15), S1 mice fed a ZnR diet displayed significantly lower freezing levels during fear extinction training when compared with their control (Ctl)-fed counterparts (Figure 1B). Microarray analysis of amygdala tissue following fear extinction training revealed a select number of miRNAs that were regulated in S1 mice following the rescue of impaired fear extinction (Supplemental Figure S1A). These included the increased expression of plasticity-related miRNAs, miR-29a, miR-132, miR-219, and miR-144-3p (Supplemental Figure S1B). Of these candidate plasticity-associated miRNAs, miR-144-3p has recently emerged as an important miRNA in the brain and has been implicated in the response to naturalistic (exam) stress (21), in the response to treatments for antidepressant action (22) and mood stabilizers (23), and in Alzheimer's disease (24) and traumatic brain injury (25). Furthermore, miR-144-3p has been linked to the restoration of mitochondrial function (26), a mechanism that has been linked to learning and memory (27). In light of its robust regulation following the rescue of impaired fear extinction and the studies highlighting the importance of miR-144-3p expression in the brain, including memory- and emotion-related functions, we chose to further explore the role of this miRNA in successful fear extinction. The enhanced expression of miR-144-3p in the amygdala of S1 mice following rescue of impaired fear extinction was validated by quantitative reverse transcription polymerase chain reaction (Figure 1C). Next, to control for diet and fear conditioning effects on the enhanced expression of miR-144-3p in ZnR S1 mice, we quantified the expression of miR-144-3p in S1 mice fed Ctl and ZnR diets that were subjected to fear conditioning but not fear extinction training (Supplemental Figure S2A). All groups displayed similar freezing levels in response to fear conditioning (Supplemental Figure S2A). During fear extinction training, S1 mice fed a ZnR diet displayed lower freezing levels when compared with their Ctl-fed counterparts, and no differences in fear expression were observed (Supplemental Figure S2A). Dietary control groups that received fear conditioning but were not exposed to the tone (conditioned stimulus [CS]) in the extinction chamber displayed negligent freezing levels (Supplemental Figure S2A). miR-144-3p expression was increased only in ZnR-fed mice that received extinction training, indicating that the combination of diet and extinction training, but not the diet alone, was required to induce expression of miR-144-3p (Supplemental Figure S2B). These findings also demonstrated that increased miR-144-3p expression was induced by fear extinction and not by the previous fear conditioning. Taken together, our data reveal that rescuing impaired fear extinction in S1 mice via ZnR leads to increased expression of a select number of miRNAs, including in particular the selective extinction-induced, but not fear-induced, increased expression of miR-144-3p in the amygdala.

Next, we examined the role of miR-144-3p in normally extinguishing mice and assessed the expression levels of this miRNA following fear extinction in extinction-intact C57BL/6J (BL6) mice. We performed fear conditioning, extinction, and amygdala dissection after extinction training identical to the S1 experiments (Figure 1D). All BL6 groups increased freezing during fear conditioning in a similar manner (Figure 1E). Fear extinction was evident, with the BL6-CS+ Ext group exhibiting lower freezing levels from CS trials 8 to 16 (Figure 1E). Enhanced amygdala expression of miR-144-3p was observed following successful fear extinction but not in response to fear expression or in the control group (BL6-CS+ No-Ext) (Figure 1F). Building on our data for the role of miR-144-3p in the rescue of impaired fear extinction, these results suggest that enhanced expression of miR-144-3p is also implicated in successful fear extinction in extinction-intact mice. Together, these results highlight a more fundamental role of miR-144-3p in gating fear extinction in both normal and pathological conditions.

In Vivo Overexpression of miR-144-3p in the BLA Facilitated Fear Extinction in Impaired S1 and Normally Extinguishing BL6 Mice

Next, we aimed to assess whether the expression of miR-144-3p is merely a readout of reduced freezing induced by successful fear extinction training or whether miR-144-3p can drive fear extinction learning. Because successful extinction (ZnR-S1 or BL6) was associated with increased miR-144-3p expression, we overexpressed miR-144-3p using lentiviral-driven constructs (miR-144-Ox) in the BLA of both S1 and BL6 mice and assessed effects on extinction. FISH results revealed a robust expression of miR-144-3p in the BLA of S1 mice following the rescue of impaired fear extinction (Supplemental Figure S3C, D). Therefore, the BLA region of the amygdala was chosen to overexpress miR-144-3p. Evaluation of lentiviral spread (Supplemental Figure S4A) and cannula placement (Supplemental Figure S4B, C) confirmed specific BLA-restricted lentiviral expression of miR-144-3p in S1 and BL6 mice.

To selectively assess the effects of overexpression of miR-144-3p during fear extinction, mice received BLA infusions of lentivirus containing constructs for miR-144-3p overexpression or mutated miR-144-3p following fear conditioning and prior to extinction training (Figure 2A). In S1 mice, results revealed that miR-144-3p overexpression (miR-144-Ox) and mutated control groups did not differ in freezing during fear conditioning (Figure 2B). Reduced freezing in miR-144-Ox Ext mice, compared with control S1 mice (miR-144-Ctl Ext), was observed during extinction training (Figure 2B), suggesting that miR-144-Ox rescues impaired fear extinction in S1 mice. Moreover, miR-144-3p overexpression per se in S1 mice did not reduce freezing, as shown by similar fear expression at the start of the extinction session. During the extinction retrieval session, we observed that overexpression of miR-144-3p in S1 mice subjected to fear

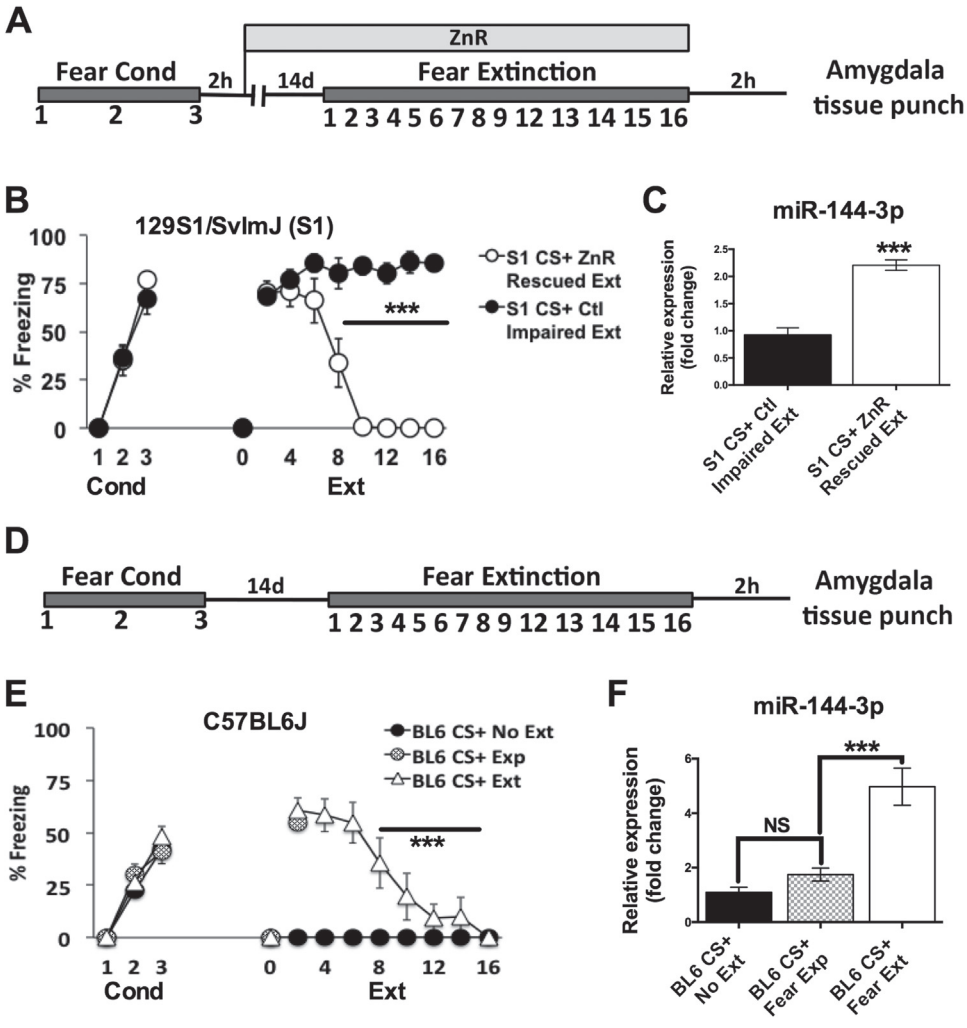

Figure 1. Rescuing impaired fear extinction with zinc restriction (ZnR) is associated with the increased expression of miR-144-3p in the amygdala, which also undergoes increased expression in the amygdala of extinction-intact C57BL/6J (BL6) mice. **(A)** Mice assigned to 129S1/SvImJ (S1) conditioned stimulus (CS+) control (Ctl) and S1 CS+ ZnR groups were subjected to fear conditioning (0.6 mA) and extinction (16 CS presentations) training (n = 4/group). Two hours following fear conditioning, the S1 CS+ ZnR (rescued) group was switched to a diet without zinc (ZnR) for 14 days. Two hours following the start of extinction training, the amygdala was dissected out and total RNA was isolated and used for Exiqon microRNA microarray analysis. **(B)** During fear conditioning, freezing in CS– unconditioned stimulus (US) paired mice increased across trials regardless of group assignment (analysis of variance [ANOVA] effect of trial [freezing to CS presentations]: $F_{2,18}$ = 34.89, $p < .001$, no effect of group or interaction). During fear extinction training, there was a significant trial × diet interaction for freezing ($F_{8,54}$ = 9.248, $p < .001$; n = 4/group), indicating that ZnR S1 mice display normal extinction learning compared with extinction-resistant Ctl-fed S1 mice. Post hoc tests revealed that freezing was significantly lower in ZnR than in Ctl-fed S1 mice during CS presentations 8 to 16. Data are presented as mean ± SEM; ***$p <$ .001. **(C)** miR-144-3p expressional changes were validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Data are expressed as mean ± SEM. Fold change ($2^{-\Delta\Delta Ct}$), normalized to control U6 small nuclear RNA levels. ***$p < .001$, two-tailed unpaired t test: miR-144-3p (t_{10} = 7.90, $p < .001$). **(D)** BL6 CS+ No Ext, BL6 CS+ Exp, and BL6 CS+ Ext mice were subjected to fear conditioning (0.6 mA) and extinction (16 CS presentations) training (n = 5–6/group). After 14 days extinction training was performed, and 2 hours following this the amygdala was dissected out and total RNA was isolated and used for qRT-PCR. **(E)** During fear conditioning, freezing in all CS+ paired groups increased similarly across trials regardless of group assignment (ANOVA effect of trial [freezing to CS presentations]: $F_{2,45}$ = 102.5, $p < .001$, no effect of group or interaction). During fear extinction training, there was a significant effect of trial (freezing to CS presentations: $F_{8,135}$ = 22.22, $p < .001$; n = 5–6/group, no difference between groups BL6 CS+ Exp [fear expression] and BL6 CS+ Ext [fear extinction] at initial CS presentations 1–2), revealing that freezing decreased in the BL6 CS+ Ext group from CS presentations 8 to 16. Data are presented as mean ± SEM; ***$p <$ .0001. **(F)** Changes in miR-144-3p expression levels were assessed by qRT-PCR. One-way ANOVA indicated a significant effect of group: $F_{2,13}$ = 20.11, $p <$ .001. Bonferroni's post hoc test: BL6 CS+ Exp vs. BL6 CS+ Ext (***), demonstrating that miR-144-3p expression is increased in an extinction-specific manner following fear extinction training. Data are expressed as mean ± SEM. Fold change ($2^{-\Delta\Delta Ct}$), normalized to control U6 small nuclear RNA levels. ***$p < .001$. Cond, fear conditioning.

conditioning but not extinction training exhibited no difference in freezing compared with control mice (which exhibited no fear extinction) (Figure 2B). However, mice that received miR-144-Ox and extinction training displayed reduced freezing levels when compared with the control miR-144-Ctl groups (Figure 2B), indicating extinction memory consolidation. Freezing levels were not different between groups during the fear renewal test (Figure 2B), suggesting that miR-144-Ox did not protect against the renewal of fear. Collectively, these results reveal that a combination of extinction training and enhanced miR-144-3p expression is required to induce fear extinction in extinction-resistant S1 mice.

To assess the effects of enhanced miR-144-3p expression on the formation of fear extinction memory in extinction-intact mice, we overexpressed miR-144-3p in the BLA of BL6 mice. miR-144-Ox in BL6 mice facilitated fear extinction acquisition, with reduced freezing being observed during fear extinction training in miR-144-Ox Ext mice when compared with control miR-144-Ctl Ext mice (Figure 2C). miR-144-Ox Ext displayed similar freezing levels during extinction retrieval to the miR-

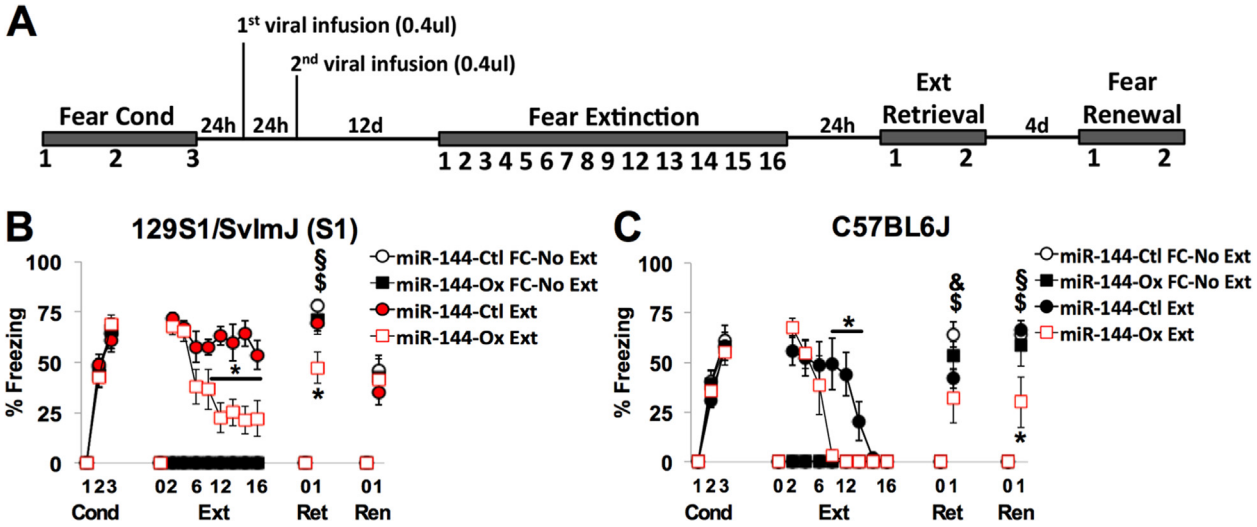

Figure 2. Overexpression of miR-144-3p in the basolateral amygdala of 129S1/SvImJ (S1) and C57BL/6J (BL6) mice leads to alterations in fear extinction memory formation. **(A)** Following bilateral implantation of single guide cannulas, mice were subjected to fear conditioning (Cond). During the following 48 hours after fear conditioning, mice received two infusions of lentiviral constructs containing either the miR-144-3p overexpression sequence (miR-144-Ox FC No-Ext; miR-144-Ox Ext) or a scrambled control (miR-144-Ctl FC No-Ext; miR-144-Ctl Ext). After a 12-day incubation period, all groups were subjected to fear extinction (miR-144-Ox Ext; miR-144-Ctl Ext) or a similar time exposure in context B (miR-144-Ox FC No-Ext; miR-144-Ctl FC No-Ext). Extinction retrieval was tested 24 hours following extinction training, and fear renewal was tested 4 days thereafter. **(B)** 129/SvImJ mice: During fear conditioning, freezing in conditioned stimulus–unconditioned stimulus (CS–US) paired mice increased similarly across trials regardless of group assignment (analysis of variance [ANOVA] effect of trial [freezing to CS presentations]: $F_{2,102} = 238.1$, $p < .0001$, no effect of group or interaction). During fear extinction training (Ext), there was a significant trial × viral interaction for freezing ($F_{24,288} = 6.015$, $^*p < .0001$; $n = 8$–11/group), indicating that miR-144-Ox Ext S1 mice display rescued fear extinction learning compared with miR-144-Ctl Ext S1 mice. Post hoc tests revealed that freezing was significantly lower in the miR-144-Ox Ext group than in miR-144-Ctl Ext mice during CS presentations 8 to 16. During extinction retrieval, miR-144-Ox Ext displayed significantly lower levels of freezing when compared with all other groups (miR-144-Ox FC No-Ext, miR-144-Ctl FC No-Ext, and miR-144-Ctl Ext), post hoc, Bonferroni for miR-144-Ctl Ext ($t_{54} = 3.763$; $^*p < .01$), miR-144-Ctl FC No-Ext ($t_{54} = 5.138$; §$p < .001$), and miR-144-Ox FC No-Ext ($t_{54} = 4.044$; $p < .01$). There was no effect of miR-144-3p overexpression on fear renewal in S1 mice. **(C)** C57BL/6J mice: During fear conditioning, freezing in CS–US paired mice increased similarly across trials regardless of group assignment (ANOVA effect of trial [freezing to CS presentations]: $F_{2,60} = 102.7$, $p < .0001$, no effect of group or interaction). During fear extinction training, there was a significant trial × viral interaction for freezing ($F_{24,180} = 10.41$, $^*p < .0001$; $n = 5$–7/group), indicating that miR-144-Ox Ext mice display enhanced acquisition of extinction learning compared with miR-144-Ctl Ext mice. Post hoc tests revealed that freezing was significantly lower in the miR-144-Ox Ext group than in miR-144-Ctl Ext mice during CS presentations 8 to 12. During extinction retrieval, miR-144-Ox Ext and miR-144-Ctl Ext displayed significantly lower levels of freezing when compared with their controls, miR-144-Ox FC No-Ext and miR-144-Ctl FC No-Ext mice, respectively (post hoc, Bonferroni for miR-144-Ox Ext [$t_{36} = 4.004$; $p < .01$] and miR-144-Ctl Ext [$t_{36} = 3.736$; &$p < .01$]). Protection against fear renewal was seen in miR-144-Ox Ext mice compared with all other groups (two-way ANOVA, trial vs. viral interaction for freezing: $F_{3,38} = 3.148$, $p < .0001$). Post hoc Bonferroni revealed significantly lower freezing in miR-144-Ox Ext mice when compared with all other groups (miR-144-Ctl FC-No Ext [$t_{38} = 3.147$; §$p < .05$], miR-144-Ctl Ext [$t_{38} = 3.803$; $^*p < .01$], and miR-144-Ox FC-No Ext [$t_{38} = 3.719$; $p < .01$]). Data are presented as mean ± SEM, (*) miR-144-Ox Ext vs. miR-144-Ctl Ext, (&) miR-144-Ctl Ext vs. miR-144-Ctl FC No-Ext, ($) miR-144-Ox Ext vs. miR-144-Ox FC No-Ext, (§) miR-144-Ox Ext vs. miR-144-Ctl FC No-Ext. Cond, fear conditioning; Ext, fear extinction; Ren, fear renewal; Ret, extinction retrieval.

144-Ctl Ext group (Figure 2C), suggesting that the enhanced rate at which fear extinction is acquired in the miR-144-Ox group does not further improve the expression of fear extinction memory. One reason for this could be that the extinction session employed here results in the formation of maximal extinction memory and further improvements are difficult to show. During fear renewal, however, miR-144-Ox mice displayed lower freezing levels compared with miR-144-Ctl mice, indicating that miR-144-Ox enhanced extinction memory to protect against fear renewal. miR-144-Ox alone had no effect on extinction retrieval or fear renewal, as indicated by no difference in freezing between miR-144-Ox FC-No Ext and miR-144-Ctl FC-No Ext during either fear retrieval or fear renewal (Figure 2C).

Taken together, these data suggest that BLA miR-144-Ox can rescue impaired fear extinction in S1 mice. In extinction-intact BL6 mice, miR-144-Ox facilitates fear extinction acquisition and protects against fear renewal. Moreover, these data reveal that the combination of extinction training plus miR-144-Ox is required to elicit effects on fear extinction.

miR-144-3p Expression Is Induced by Neuronal Activity and Is Implicated in the Control of Plasticity-Associated Signaling Cascades, Including PI3K/AKT, MAPK/ERK, and Notch Signaling

To gain insight into the expression inducibility of miR-144-3p, we performed high potassium chloride-induced depolarization in ex vivo cortical neurons and quantified miR-144-3p expression levels. miR-144-3p expression was increased 30 minutes to 1 hour following potassium chloride administration, suggesting that miR-144-3p is expressed in an activity-dependent manner (Figure 3A). To elucidate the downstream targets of miR-144-3p, we performed a bioinformatical analysis. Gene ontology analysis of predicted targets of miR-144-3p confirmed a high number of target genes implicated, for example, in the regulation of gene expression, stimulus response, and

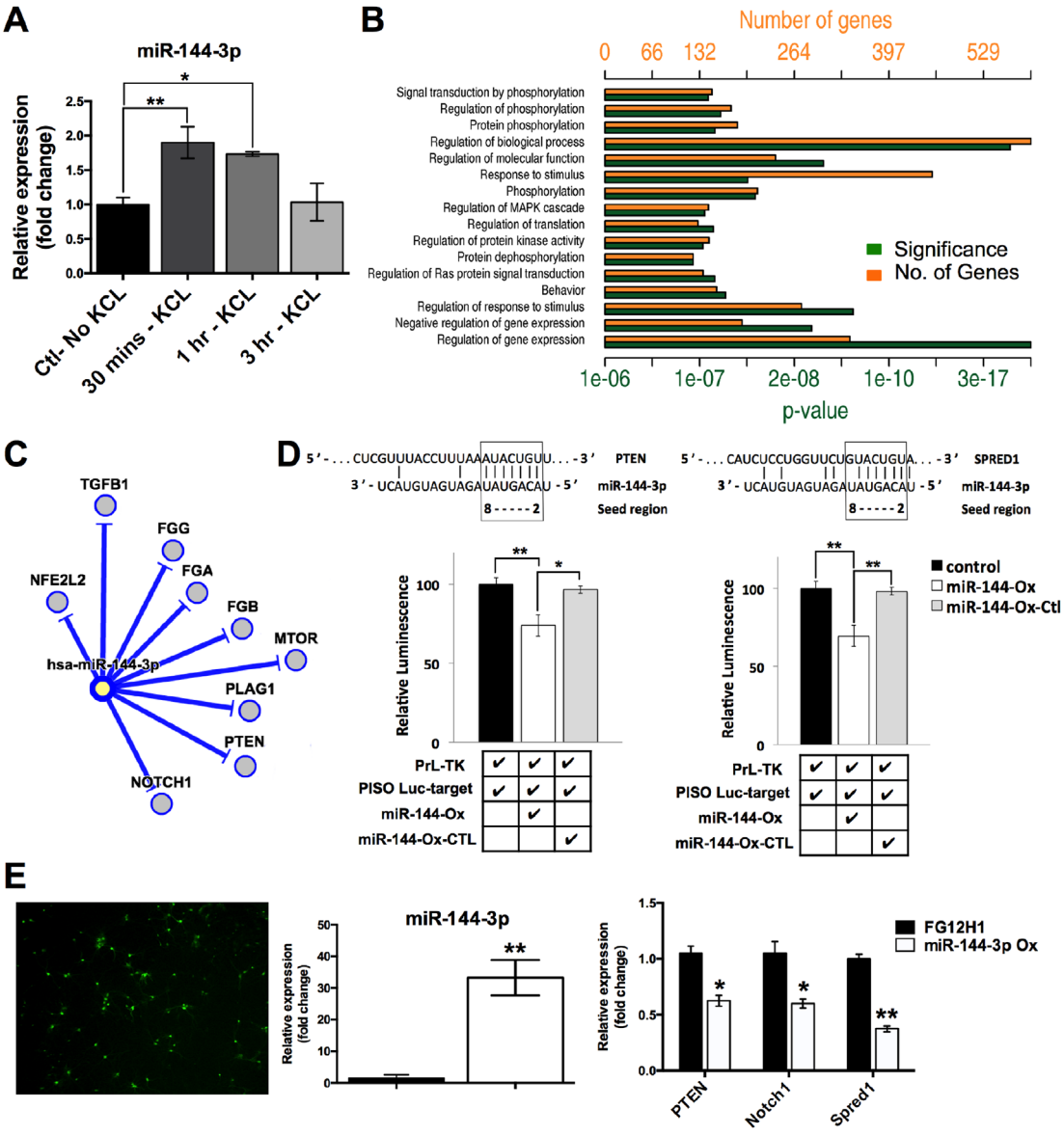

Figure 3. miR-144-3p is activity induced and targets genes involved in the control of plasticity-associated signaling cascade activation. **(A)** The activity dependency of miR-144-3p was assessed by high potassium chloride (KCL)-induced depolarization of primary cortical neurons. One-way analysis of variance (ANOVA) indicated a significant effect of group: $F_{3,8} = 6.283$, $p < .05$. Fisher least significant difference post hoc test: Ctl-No KCL vs. 30 minutes-KCL (**), Ctl-No KCL vs. 1 hour-KCL (*). Data are expressed as mean ± SEM. Fold change ($2^{-\Delta\Delta Ct}$), normalized to control U6 small nuclear RNA levels. **(B)** Histogram showing the biological processes in which miR-144-3p gene targets are implicated (orange (upper bars of pairs) indicates number of genes; green (lower bars of pairs) indicates their significance of interaction). Data were gathered by assessing the target genes of miR-144-3p in DAVID (Database for Annotation, Visualization, and Integrated Discovery). **(C)** Schematic of experimentally validated miR-144-3p target genes. Graph was obtained from miRTarBase (54). **(D)** Top row: Schematic displaying nucleotide alignments of miR-144-3p with target genes *Pten* (left) and *Spred1* (right). The seed region (miRNA targeting sequence) is indicated by a rectangle highlighting miR-144-3p nucleotides 2 to 8. Bottom: A luciferase reporter assay was used to validate miR-144-3p interaction with the 3′ untranslated region (UTR) of its target (left) *Pten* and predicted target (right) *Spred1*. Cotransfection of either *Pten* or *Spred1* 3′UTR with miR-144 overexpression (miR-144-Ox) led to reduced luciferase signal of both *Pten* (ANOVA: $F_{2,7} = 10.77$, $^{**}p < .01$; Bonferroni's post hoc test: Ctl vs. miR-144-Ox, $^{**}p < .01$, miR-144-Ox Ctl vs. miR-144-Ox, $^{*}p < .05$, $n = 3$–4) and *Spred1* (ANOVA: $F_{2,7} = 15.62$, $^{**}p < .01$; Bonferroni's post hoc test: Ctl vs. miR-144-Ox, $^{**}p < .01$, miR-144-Ox Ctl vs. miR-144-Ox, $^{**}p < .01$, $n = 3$–4). **(E)** Left: Image of primary cortical neurons following 6-day infection with a lentiviral vector containing the miR-144-3p construct. Middle: miR-144-3p overexpression in primary cortical neurons was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (unpaired *t* test: $t_6 = 5.578$, $p < .01$). Right: qRT-PCR for messenger RNA levels in primary cortical neurons after 6-day lentiviral infection with miR-144-3p revealed decreased expression levels of the putative target *Pten* (unpaired *t* test: $t_6 = 5.28945$, $p < .05$), *Notch1* (unpaired *t* test: $t_6 = 4.02492$, $p < .01$), and *Spred1* (unpaired *t* test: $t_6 = 13.0558$, $p < .001$). Data are presented as mean ± SEM, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Fold change (relative luminescence), normalized to control Renilla luminescence levels. $^{*}p < .05$, $^{**}p < .01$. Ctl, control.

phosphorylation of relevant signaling transduction molecules (Figure 3B). MiRTarBase, which displays experimentally validated targets of any given miRNA, revealed that a number of genes targeted by miR-144-3p are implicated in the regulation of plasticity-associated signaling cascades, including Notch (*Notch1*) and PI3K/AKT (*Pten*) (Figure 3C). Interestingly, miR-144-3p-mediated regulation of the candidate gene *Pten* has been extensively reported (28,29). miR-144-3p has a strong binding affinity for plasticity-associated signaling cascade target genes, including an 8-mer complementary overlap between miR-144-3p and *Pten* (Figure 3D, left) and a 7-mer overlap with *Spred1* (Figure 3D, right). *PTEN* has previously been validated as a target of human hsa-miR-144-3p (29) and rat rno-miR-144-3p (30). Here, we determined the functional relationship between mouse miR-144-3p and the 3′ untranslated region of *Pten* by luciferase assay. Overexpression of miR-144-3p led to decreased activity of firefly luciferase gene fused to the 3′ untranslated region of *Pten* (Figure 3D, left). Furthermore, enhanced miR-144-3p expression also decreased the activity of the firefly luciferase gene fused to the 3′ untranslated region of *Spred1* (Figure 3D, right). Overexpression of miR-144-3p in primary cortical neurons (Figure 3E, left), leading to increased transcript levels of miR-144-3p (Figure 3E, middle), was associated with decreased mRNA levels of the target genes *Pten*, *Notch1*, and *Spred1* (Figure 3E, right). Together, these results suggest that, in mice, miR-144-3p expression is important for the control of plasticity-associated signaling cascades, including PI3K/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK), and Notch pathways, through regulation of the target genes *Pten*, *Spred1*, and *Notch1*, respectively.

Increased miR-144-3p in the BLA After Rescue of Impaired Fear Extinction Is Associated With Decreased PTEN-Positive Neurons

Recently, it was reported that inhibition of PTEN rescued cognitive deficits in an animal model of Alzheimer's disease (31). In light of this interesting finding, we assessed whether decreases in PTEN expression are observed in S1 mice following ZnR-induced rescue of deficits in fear extinction learning. Furthermore, we also examined whether these PTEN-positive cells colocalized with miR-144-3p in the BLA. To achieve this in vivo, we used FISH for the detection of miRNAs (20) and stained for miR-144-3p transcript and PTEN protein levels in the BLA of impaired and rescued S1 mice. miR-144-3p staining was validated against a scrambled probe in all experiments (Supplemental Figure S3A–D). Following extinction training, an increased number of miR-144-3p-positive cells was observed in the BLA compared with the nonextinguishing Ctl diet group (Figure 4A–G). Furthermore, miR-144-3p colocalized with PTEN, and following extinction training a reduced number of PTEN-positive cells was observed in the BLA of extinguishing ZnR-treated mice compared with nonextinguishing Ctl diet control mice (Figure 4H–N). These results confirm, using a different method, that miR-144-3p levels are increased in the BLA following the rescue of impaired fear extinction in S1 mice and that miR-144-3p colocalizes with PTEN, which in turn displays decreased expression levels following extinction rescue.

DISCUSSION

The current findings identify the importance of miRNA-mediated regulation for the rescue of impaired fear extinction learning. Rescuing deficient fear extinction in S1 mice by dietary ZnR led to altered expression of a select number of miRNAs in the amygdala. We confirmed the extinction-specific regulation of one miRNA candidate, miR-144-3p, in S1 mice and found similar regulation in normally extinguishing BL6 mice. Virally enhanced expression of miR-144-3p in the BLA was sufficient to rescue impaired fear extinction (S1 mice), whereas in BL6 mice, overexpression of miR-144-3p could facilitate fear extinction acquisition and showed extinction-promoting effects by protection against fear renewal. Bioinformatical analysis of miR-144-3p targets revealed a number of genes implicated in the regulation of plasticity-associated signaling cascades, including PI3K/AKT, MAPK/ERK, and Notch. Finally, we revealed that extinction-rescued S1 mice displayed increased miR-144-3p expression, which colocalized with PTEN in the BLA. Furthermore, the functional relevance of miR-144-3p effects on PTEN were seen, with the number of PTEN-positive cells, and thus PTEN expression levels, being decreased in the BLA following this rescue.

Rescuing impaired fear extinction led to increased expression of a number of additional plasticity-associated miRNAs, including the synaptically enriched miR-29a (32), the cyclic adenosine monophosphate response element binding protein–regulated miR-132 (33), and the *N*-methyl-D-aspartate receptor-regulated miR-219 (34). Importantly, of these identified candidate miRNAs, miR-144-3p was shown to be extinction specific and exhibited increased expression in the amygdala of both extinction-rescued S1 and extinction-intact BL6 mice. Together, these data support the hypothesis that fear extinction is associated with mechanisms supporting increased synaptic plasticity in the amygdala (35).

miR-144-3p has emerged as an important miRNA implicated in a number of human central nervous system pathologies (21,24,25,36). Our current data show that expression of miR-144-3p is an early component of fear extinction learning, displaying enhanced expression following induction of new fear-inhibitory learning. Furthermore, miR-144-3p expression was not regulated by fear memory retrieval, which strongly suggests a specific role of this miRNA in fear extinction. Indeed, overexpression of miR-144-3p in S1 mice rescued impaired fear extinction revealing that increased expression of miR-144-3p can drive fear extinction. This finding was supported by the observation that BLA overexpression of miR-144-3p facilitated fear extinction acquisition and protected against fear renewal in normally extinguishing BL6 mice.

To gain further insight into how miR-144-3p could act as a cognitive enhancer for extinction learning, we performed detailed in vitro and bioinformatical analysis of miR-144-3p. Using potassium chloride–induced depolarization, we revealed that miR-144-3p is activity induced. This result suggests that neuronal activity induced by extinction training within the BLA (10,15) could mediate enhanced expression of miR-144-3p. Profiling the predicted and validated targets of miR-144-3p revealed a number of genes associated with the control of plasticity-associated signaling cascades, including genes

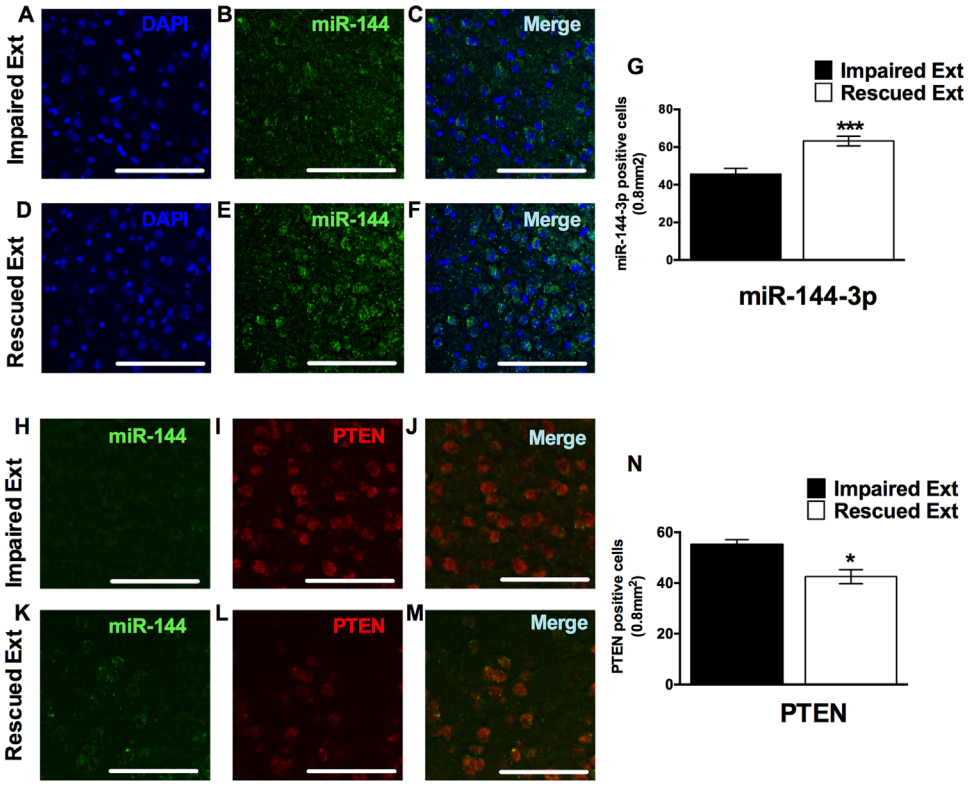

Figure 4. Rescue of impaired fear extinction is associated with increased miR-144-3p positive neurons in the basolateral amygdala (BLA) and colocalizes with PTEN. **(A–F)** Image series of fluorescent in situ hybridization with locked nucleic acid probes against miR-144-3p in the BLA of 129S1/SvImJ (S1) mice that display either impaired **(A–C)** or rescued **(D–F)** fear extinction. **(G)** Successful rescue of impaired fear extinction was associated with a 39% increase in miR-144-3p-positive cells in the BLA (two-tailed unpaired *t* test: $t_{14} = 4.398$, $^{***}p < .001$, $n = 8$/group). **(H–M)** Image series of fluorescent in situ hybridization adapted to include an immunostaining step with a monoclonal antibody against PTEN. miR-144-3p colocalizes with its target PTEN in the BLA of S1 mice (J+M). **(N)** Rescue of impaired fear extinction was associated with a 23% decrease in PTEN-positive cells in the BLA (two-tailed unpaired *t* test: $t_4 = 3.772$, $^{*}p < .05$, $n = 5$/group). Data are expressed as mean positively stained cells ± SEM, $^{*}p < .05$, $^{***}p < .001$. Scale bar = 100 μm. Ext, fear extinction.

involved in the regulation of PI3K/AKT, MAPK/ERK, and Notch signaling. The effects of miR-144-3p on the regulation of these signaling cascades has been reported previously. showing that miR-144-3p expression levels affect the activation of PI3K/AKT (28,29), Notch (37), and MAPK/ERK (38) signaling cascades. Interestingly, modulation of these signaling cascades in the BLA has been previously implicated in the regulation of fear and extinction learning (7,39,40). Dias *et al.* found that miR-34a decreased Notch signaling to facilitate the consolidation of fear conditioning in the BLA of BL6 mice (7). Interestingly, in ZnR-rescued S1 mice, we observed the increased expression of miR-34b, which belongs to the same miRNA precursor family as miR-34a [for a review, see (41)] and also targets Notch1 (42). Herry *et al.* revealed that the extinction of auditory fear conditioning requires the activation of MAPK/ERK signaling in the BLA (40). Here, we provide functional evidence that overexpression of miR-144-3p in primary cortical neurons decreased mRNA transcript levels of *Pten*, *Notch1*, and *Spred1*. A possible interaction of miR-144-3p with these target genes and resulting effects in extinction are depicted in Figure 5.

In impaired fear extinction, the lack of fear extinction training-induced miR-144-3p expression (1) results in higher levels of *Pten*, *Notch1*, *Mtor*, and *Spred1* (2) (Figure 5A). Subsequently, genes such as *Pten* and *Spred1* inhibit the activation of the plasticity-associated pathways PI3K/AKT and MAPK/ERK, respectively (3). In successful fear extinction, fear extinction-induced expression of miR-144-3p (1) inhibits the expression of its target genes, including *Pten*, *Spred1*, *Notch1*, and *Mtor* (2), which subsequently results in enhanced activation of PI3K/AKT and MAPK/ERK signaling and decreased activation of Notch and Mtor signaling (Figure 5B). The enhanced activation of PI3K/AKT and MAPK/ERK signaling cascades results in increased levels of phospho-AKT and -ERK (3), which subsequently translocate to the nucleus and induce the expression of transcription factors, immediate early genes, and other plasticity-associated genes (4). These genes in turn lead to a transcriptional ON state (5) and subsequent enhanced expression of plasticity-associated proteins (6) (Figure 5B). In summary, these findings demonstrate the importance of miR-144-3p expression in the regulation of plasticity-associated signaling cascades and shed light on how increased miR-144-3p expression could facilitate the extinction of fear.

To gain further insight into miR-144-3p-mediated target regulation during the rescue of impaired fear extinction, we looked at the putative target gene *Pten* (negative regulator of PI3K/AKT activation). We demonstrate for the first time that *Pten* is a target of the mouse mmu-miR-144-3p by showing that lentiviral-mediated miR-144-3p overexpression led to decreased transcript levels of *Pten*. Whereas *Pten* knockout leads to deficits in learning and memory (43), pharmacological inhibition of PTEN rescued cognitive deficits in an Alzheimer's mouse model (31). Furthermore, the synaptic accumulation of PTEN is associated with impairments in long-term potentiation (44,45). A recent study

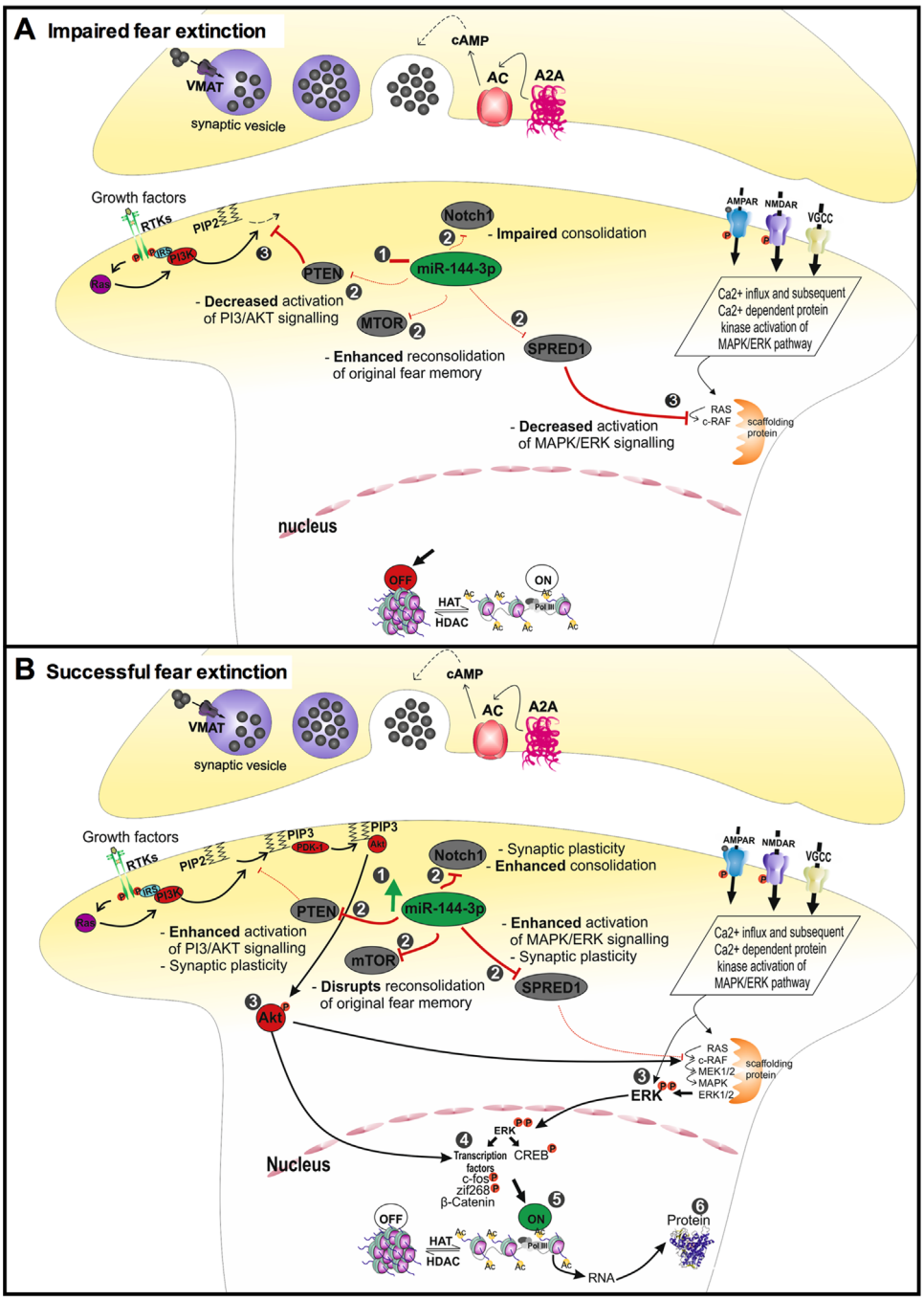

Figure 5. Cartoon depicting a working hypothesis of miR-144-3p expression in impaired and successful fear extinction. **(A)** In impaired fear extinction, there is no extinction-induced increase in miR-144-3p expression (1); therefore, there is no miRNA-mediated inhibition of the targets genes *Pten*, *Spred1*, *Notch1*, and *Mtor* (2). Subsequently, these targets elicit their effects, including PTEN's mediated inhibition of protein kinase B (AKT) phosphorylation and SPRED1's inhibition of c-RAF (RAF proto-oncogene serine/threonine-protein kinase) phosphorylation (3). The PTEN- and SPRED1-mediated inhibition of these targets leads to the inactivation of the PI3K/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) signaling cascades, respectively. Along with this, the lack of miR-144-3p-mediated inhibition of NOTCH1 and MTOR results in increased activation of these pathways. **(B)** In successful fear extinction, extinction-induced increased expression of miR-144-3p (1) leads to the subsequent inhibition of its target genes, including *Pten*, *Spred1*, *Notch1*, and *Mtor* (2). This inhibition facilitates the phosphorylation of AKT and ERK and the subsequent activation of the PI3K/AKT and MAPK/ERK signaling pathways, respectively (3). Phosphorylated AKT and ERK are transported to the nucleus, where they lead to enhanced expression of transcription factors and plasticity-related genes such as *c-fos*, *zif-268*, and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (4). In turn, these plasticity-related genes mediate an active chromatin state (transcriptional ON state) (5), leading to the expression of memory-associated proteins (6). AC, adenylyl cyclase; Ac, acetyl group; A2A, adenosine A2A receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; HAT, histone acetylase; HDAC, histone deacetylase; IRS, insulin receptor substrate; MEK1/2, MAPK/ERK kinase 1/2; NMDAR, *N*-methyl-D-aspartate receptor; PDK-1, pyruvate dehydrogenase kinase 1; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; Pol III, DNA polymerase III; Ras, retrovirus associated DNA sequences; RTK, receptor tyrosine kinase; VGCC, voltage gated calcium channels; VMAT, vesicular monoamine transporter.

demonstrated that inhibition of PTEN's downstream target pathway, PI3K/AKT, in the BLA led to impaired fear extinction and increased fear memory expression (46). To explore a possible functional association between miR-144-3p and PTEN following the rescue of impaired fear extinction, we showed that miR-144-3p colocalized with PTEN in the BLA and, furthermore, that PTEN expression was decreased in the BLA of ZnR-rescued S1 mice. Given the interaction of miR-144-3p and PTEN leading to increased PI3K/AKT activation, this finding is in line with the report that PI3K/AKT cascade activation in the BLA is required for fear extinction (39).

Taken together, the effects of miR-144-3p regulation in the BLA suggest that this miRNA plays a critical role in the rescue of impaired fear extinction as well as in normal extinction mechanisms. Furthermore, we provide evidence that this could be due to miR-144-3p-mediated regulation of plasticity-associated signaling cascades, including PI3K/AKT, MAPK/ERK, and Notch. To our knowledge, this is the first report describing the critical involvement of a specific miRNA in facilitating fear extinction in a relevant psychopathological model of deficient fear extinction. Studying the implications of experimentally reducing miR-144-3p expression in normally extinguishing mice may shed further light on the implications of miR-144-3p expression in successful fear extinction. However, there is a large body of studies demonstrating negative effects associated with decreased miR-144-3p expression, including promoting proinflammatory cytokine production (47), cancer (36,48–50), and tuberculosis (51) and in impaired ischemic preconditioning (52). On the other hand, the availability of drugs that can enhance miR-144-3p expression in the brain, such as valproate, highlights promising potential therapeutic strategies (53) for fear-, anxiety-, and trauma-related disorders that are often associated with deficient extinction. Furthermore, because changes in miR-144-3p expression can be detected in the periphery (21), this miRNA could be developed as a biomarker predicting treatment success.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the Austrian Science Fund (FWF) (Grant Nos. SFB F4410 [to NS], SFB F4411 [to AH], and P25375-B24 [to NS]), Signal Processing In Neurons (FWF Grant No. DK W-1206 [to NS]), European Molecular Biology Organization (ASTF Grant No. 459-2015 [to CPM]), the National Health and Medical Research Council (Grant No. 1069570 [to TWB]), and the National Institute of Mental Health (Grant No. R21MH103812 [to TWB]).

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

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Received Aug 19, 2016; revised Dec 6, 2016; accepted Dec 13, 2016.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2016.12.021.

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