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Amygdala-Dependent Fear Memory Consolidation via miR-34a and Notch Signaling

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SUMMARY

Using an array-based approach after auditory fear conditioning and microRNA (miRNA) sponge-mediated inhibition, we identified a role for miR-34a within the basolateral amygdala (BLA) in fear memory consolidation. Luciferase assays and bioinformatics suggested the Notch pathway as a target of miR-34a. mRNA and protein levels of Notch receptors and ligands are downregulated in a time- and learning-specific manner after fear conditioning in the amygdala. Systemic and stereotaxic manipulations of the Notch pathway indicated that Notch signaling in the BLA suppresses fear memory consolidation. Impairment of fear memory consolidation after inhibition of miR-34a within the BLA is rescued by inhibiting Notch signaling. Together, these data suggest that within the BLA, a transient decrease in Notch signaling, via miR-34a regulation, is important for the consolidation of fear memory. This work expands the idea that developmental molecules have roles in adult behavior and that existing interventions targeting them hold promise for treating neuropsychiatric disorders.

INTRODUCTION

Forming associations about events and then consolidating memories of those associations is an important strategy for navigating our environments. However, in traumatic situations, these associations sometimes become overly consolidated and then, potentially, are resistant to extinction over time, resulting in post-traumatic stress disorder (PTSD) and other fear-related disorders in some individuals (Parsons and Ressler, 2013; Steckler and Risbrough, 2012). Future therapeutic intervention strategies for PTSD might therefore target this enhanced consolidation and/or lack of extinction to salient cues in the environment (Dias et al., 2013; Fitzgerald et al., 2013; Vanelzakker et al., 2013). To accomplish such goals, we need knowledge of the genetic and molecular mechanisms occurring within specific brain regions that underlie specific memory consolidation processes.

Auditory fear conditioning presents a framework within which to study the consolidation of cued fear memory (Johansen et al., 2011). In this classical conditioning paradigm, animals are first trained to associate auditory cues with mild foot-shocks. By subsequently presenting animals with only the auditory cues, the consolidation of associative learning can be assessed. Within the brain, the amygdala has received considerable attention for its role in consolidating these learned associations (Maren, 2003). As a result, the molecular and genetic landscape within the amygdala during the period of consolidation after conditioning undoubtedly holds clues to how memories come to be consolidated.

Regulation of both gene expression and protein synthesis are crucial to the consolidation of memory (Dudai, 2004; Maguschak and Ressler, 2012; Maren et al., 2003; Silva and Giese, 1994). In recent times, the epigenetic regulation of gene expression has received considerable attention within the realm of neuropsychiatric disorders (Gräff and Mansuy, 2008; Zovkic and Sweatt, 2013). Among these epigenetic mechanisms, microRNA (miRNA)-mediated regulation of gene expression has been implicated in spatial memory, trace conditioning, extinction memory, and fear memory consolidation (Gao et al., 2010; Griggs et al., 2013; Konopka et al., 2010; Spadaro and Bredy, 2012; Wang et al., 2012; Zovkic et al., 2013). Of note, some of these studies uncovered miRNA that target specific genes whose functions are related to synaptic and structural plasticity (Griggs et al., 2013; Lin et al., 2011). Another molecular perspective suggests that signaling molecules canonically viewed as essential to development have functions in the adult that include synaptic plasticity (Ables et al., 2011; Pierfelice et al., 2011). In keeping with this perspective, we have previously uncovered a role for developmental signaling pathways (namely, Wnt signaling) within the amygdala in adult learning and memory (Maguschak and Ressler, 2011, 2012).

In this paper, we examined if activity of miR-34a, a target identified from an unbiased miRNA screening study, is required for cued fear memory consolidation. When we examined the targets of this miRNA, we found them to be components of the Notch signaling pathway normally involved in development, unearthing

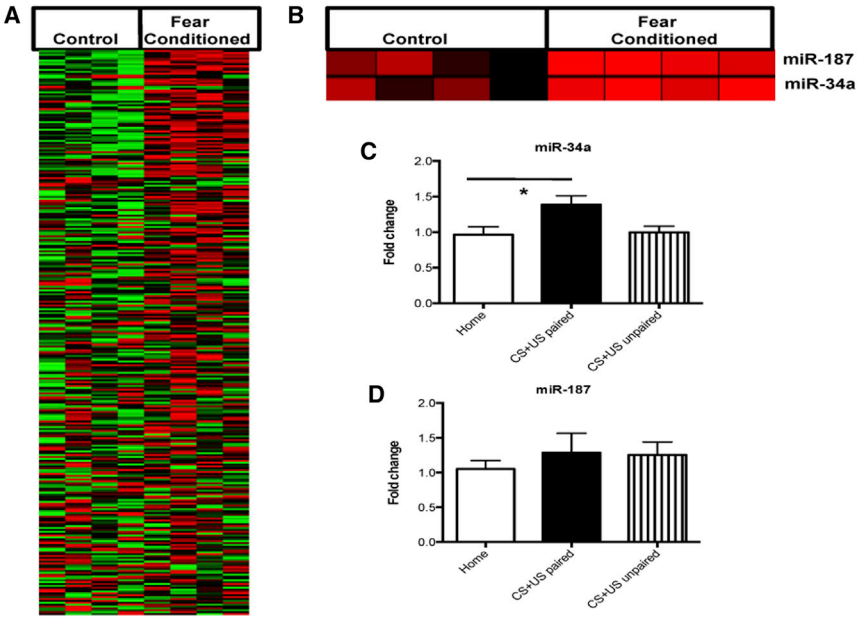

Figure 1. miRNA Expression Profiling and qPCR Confirmation Shows Upregulation of miR-34a in the Amygdala 30 min after Fear Conditioning

(A) miRNA levels in the amygdala of fear-conditioned adult male mice compared to Home cage controls were profiled by Exiqon using an array-based approach (Green-Black-Red: low to high miRNA levels).

(B–D) (B) Two miRNA (miR-34a and miR-187) met the fold change threshold ($p < 0.01$ and dLMR = 0.5) (n = 4/group). qPCR analysis on a completely independent set of samples (n = 8/group) indicated that miR-34a (C) but not miR-187 (D) was significantly upregulated in the amygdala 30 min after fear conditioning in a learning-dependent manner. Data are shown as mean +SEM. *$p < 0.05$.

a role for a developmental molecular cascade in the consolidation of cued fear memory in the adult.

To our knowledge, this is the first study that links a miRNA (miR-34a) and developmental signaling cascade (Notch signaling) to a role in memory consolidation. In addition, this work adds to the growing body of literature that classically considered developmental molecules function outside of a developmental context.

RESULTS

miR-34a Is Upregulated in the Amygdala 30 min after Auditory Fear Conditioning

A microarray was performed on miRNA extracted from amygdala of adult mice 30 min after fear conditioning. Results indicated a dynamic regulation of several noncoding RNA species during the period of fear memory consolidation (Figure 1A). Two miRNA (miR-34a and miR-187) met the a priori criteria ($p < 0.01$ and dLMR = 0.5) as being significantly upregulated in the amygdala of adult mice 30 min after fear conditioning (Figure 1B). To independently verify whether these miRNA were indeed regulated in a learning-dependent manner, we replicated the aforementioned behavioral protocol with a completely independent set of animals and used qPCR to quantify miRNA levels. We found that only miR-34a was significantly upregulated in the amygdala in a learning-dependent manner 30 min after fear conditioning (Figure 1C) (ANOVA: $F(2,19) = 4.42$, $p = 0.026$; Home versus CS+US Paired: $p < 0.05$). miR-187 levels in the amygdala were not significantly regulated 30 min after fear conditioning (Figure 1D).

Inhibiting miR-34a Function Suppresses Fear Memory Consolidation

We reasoned that an increase in miR-34a in the amygdala 30 min after fear conditioning would be permissive to fear memory consolidation. To test this idea, we infused viruses that expressed sponge sequences to inhibit miR-34a activity or scrambled controls into the BLA (Figure S1 available online). The sponge construct contained seed sequences to which the miR-34a would bind and thereby not be able to exert its effect. Using traditional cell counting methods, we estimate that on average our infusions infected 200,000 cells/mm^3 of tissue. Two weeks after infusion of the lentivirus into the BLA (Figure 2A), animals were tested in an elevated plus maze (EPM) to assay for anxiety-like behavior. We found no differences between groups in the EPM, implying no effect of blocking miR-34a action on anxiety-like metrics (Figure S2). Both groups were then subjected to auditory fear conditioning and acquired fear similarly on the day of training (Figure 2B). Twenty-four hours later, when tested with five CS presentations, miR-34a sponge-infused animals froze significantly less than miR-34a sponge-scrambled, control-infused animals, suggesting that blocking miR-34a from exerting its effect suppressed fear memory consolidation ($t = 2.27$, $df = 13$, $p = 0.040$). Freezing levels to individual CS presentations (CS 1, CS2, etc.) are shown in Figure S7A. It is important to note that this suppression of fear memory consolidation was replicated in an independent experiment, where we found it to be specific to the consolidation of long-term memory (tested 24 hr after training) (Figure 6), with no differences detected within the time frame of short-term memory (STM) (tested 1 hr after training) (Figure S2).

Components of the Notch Pathway Are Targets of miR-34a

Published work in the field of cancer biology has suggested that Notch1 is a bona fide target of miR-34a (Comery et al., 2005; Kashat et al., 2012). We wanted to extend our search for targets of miR-34a and turned to several algorithms (e.g., miRDB, TargetScan, and MicroCosm). While extremely useful, these algorithms generated many possible predicted targets. We decided instead to focus on a list of targets for miR-34a using a more directed approach that utilized the filter functions of Ingenuity Pathway Analysis (https://analysis.ingenuity.com/pa/). Given that our previous work has implicated the developmental

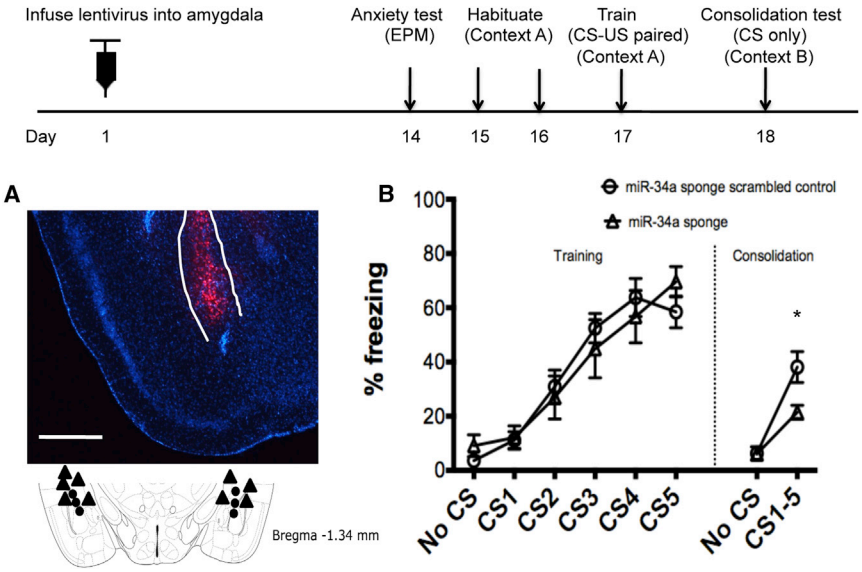

Figure 2. Blocking miR-34a Action in the BLA Using a Sponge-Based Strategy Impairs the Consolidation of Cued Fear Memory

Lentiviral particles that expressed either a miR-34a sponge or a scrambled control under the control of a Ubc promoter were injected into the BLA. Two weeks later, animals were fear conditioned using five tone-shock pairings (CS-US pairings) and 24 hr later tested for the consolidation of cued fear memory.

(A) Based on mouse stereotaxic atlas by Paxinos and Franklin (2004), representative expression of mCherry (also driven by the Ubc promoter) in the BLA indicates correct targeting of the injection (filled circles) versus incorrect targeting (solid triangles). Shown are only representative hits and misses. For final analysis, all LV-miR-34a sponge and LV-miR-34a sponge scrambled control hits targeting the BLA were included. Scale bar, 1 mm.

(B) No differences between groups were observed in the acquisition of fear responses as a function of CS presentation on training day. However, blocking miR-34a action using the sponge impaired the consolidation of the cued fear memory as observed by a diminished freezing response during the five CS presentations in the fear expression test compared to the scrambled control animals. Data are shown as mean ±SEM. *p < 0.05.

Wnt molecules in the formation of fear memory and our desire to maximize the probability that miR-34a actually targets some of the genes in our bioinformatics query, we filtered results in IPA using the "developmental molecules" and "observed targets" filter functions. It is our understanding that with this approach the Ingenuity Pathway software collates from a variety of algorithms literature that has demonstrated genes to be experimentally validated targets of specific miRNA. Thus, the gene list generated is not only the result of Ingenuity's own algorithm ("Ingenuity Expert Findings") but instead includes other commonly mined algorithms like TargetScan, TarBase, and miRecords. This approach gave us a list of 13 genes. On further examination, we noticed that a number of both ligands and receptors involved in Notch signaling predominated this list and so chose to focus on the Notch pathway moving forward (Figure 3A).

We then used cell culture and a luciferase-based system to ask whether *Notch1* is indeed a molecular target of miR-34a (Figure S3). To begin, the 3′ UTR of *Notch1* was cloned downstream of luciferase, and luciferase-mediated luminescence was normalized to 1. When HEK293T cells were cotransfected with a construct encoding miR-34a and the construct containing the 3′ UTR of *Notch1,* luciferase-mediated luminescence was decreased. This suggests that miR-34a targeted the 3′ UTR of *Notch1* to suppress luciferase activity (Figure 3B). This reduction was ameliorated when miR-34a activity was inhibited using the same sponge construct used for the in vivo experiment in Figure 2. (ANOVA: $F(3,11) = 5.414$, $p = 0.015$; post hoc tests: FF-Luc-Notch1/Ren-Luc versus FF-Luc-Notch1/Ren-Luc/miR-34a: $p = 0.006$, FF-Luc-Notch1/Ren-Luc versus FF-Luc-Notch1/Ren-Luc/miR-34a/mirR-34a sponge: $p = 0.09$, FF-Luc-Notch1/Ren-Luc versus FF-Luc-Notch1/Ren-Luc/mirR-34a sponge: $p = 0.09$.)

To ascertain that the miR-34a-sponge used was specific to miR-34a action and did not inhibit closely related miRNA activity, we turned to recent literature that comprehensively showed corticotrophin-releasing factor receptor 1 (*CRFR1*) to be a target of both miR-34c and miR-34a. As was shown by these authors, luciferase-mediated luminescence was decreased in our experiment when the 3′ UTR of CRFR1 was targeted by miR-34c and miR-34a. While miR-34a-sponge cotransfected with the miR-34c construct did not rescue this decrease, this decrease was rescued when the miR-34a activity was inhibited by miR-34a-sponge (Figure S4)

mRNA Levels of Components of the Notch Pathway Are Regulated in the Amygdala after Auditory Fear Conditioning

With miR-34a being regulated in the amygdala 30 min after auditory fear conditioning, and bioinformatics and luciferase analyses indicating that Notch pathway components are targets of miR-34a, we sought to examine whether Notch pathway mRNA levels are themselves regulated in the amygdala after fear conditioning. Included in this experiment were CS+US paired and CS-US unpaired groups, to control for unconditioned stress responses separate from associative memory formation. We then performed qPCR on mRNA isolated from the amygdala of mice that had been sacrificed 2, 6, and 12 hr after fear conditioning. More specifically, we chose to measure the mRNA levels of ligands (*Jag1* and *Dll1*) and receptors (*Notch1* and *Notch2*).

Analysis of the amygdala mRNA (Figure 4) demonstrated a significant reduction in Notch pathway mRNA levels at primarily 2 and 6 hr after fear conditioning. By the 12 hr time point, all groups showed similar mRNA levels of the components queried.

Ligands

Jag1 mRNA levels (Figure 4A) were significantly reduced at the 2 hr time point in both the paired and unpaired groups as compared to home cage controls ($F(2,19) = 42.06$, $p < 0.0001$, Home versus Paired: $p < 0.001$, Home versus Unpaired: $p < 0.001$). At 6 hr, *Jag1* levels in the paired group were significantly

A

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Source	Confidence	Symbol
TargetScan Human,miRecords	Experimentally Observed,Moderate (predicted)	AXIN2
Ingenuity Expert Findings,TarBase,TargetScan Human,miRecords	Experimentally Observed,High (predicted)	CCND1
Ingenuity Expert Findings	Experimentally Observed	CREB1
TarBase,TargetScan Human,miRecords	Experimentally Observed,High (predicted)	DLL1
Ingenuity Expert Findings,TargetScan Human	Experimentally Observed,Moderate (predicted)	HDAC1
TargetScan Human,miRecords	Experimentally Observed,High (predicted)	JAG1
TargetScan Human,miRecords	Experimentally Observed,High (predicted)	MAP2K1
Ingenuity Expert Findings,TargetScan Human,miRecords	Experimentally Observed,High (predicted)	MET
miRecords	Experimentally Observed	MYC
Ingenuity Expert Findings,TarBase,TargetScan Human,miRecords	Experimentally Observed,High (predicted)	NOTCH1
TargetScan Human,miRecords	Experimentally Observed,High (predicted)	NOTCH2
miRecords	Experimentally Observed	VEGFA
TargetScan Human,miRecords	Experimentally Observed,High (predicted)	WNT1

B

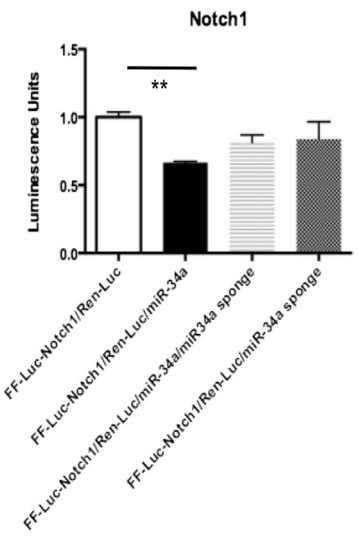

Figure 3. Notch1 Is a Target of miR-34a, and Components of the Notch Signaling Pathway Are Also Targets of miR-34a

(A) Ingenuity Pathway Analysis filtered for Developmental Molecules and Experimentally Observed revealed several components of the Notch pathway to be targets of miR-34a.

(B) A luciferase-based assay was used to demonstrate that Notch1 is a target of miR-34a. Co-transfection of HEK293T cells with a plasmid that contains the 3′ UTR of Notch1 cloned downstream of the Firefly-Luciferase gene (FF-Luc-Notch1) and one that expresses miR-34a decreased the luminescence relative to the FF-Luc-Notch1 control. Blocking miR-34a action using a sponge-based strategy (miR-34a sponge) prevents this decrease in luminescence. Data are shown as mean +SEM. $^{**}p < 0.01$.

downregulated as compared to those in the Home cage and unpaired group ($F(2,20) = 4.569$, $p = 0.023$, Paired versus Unpaired: $p < 0.05$). *Dll1* mRNA levels (Figure 4B) showed similar regulation at the 2 hr mark, as both the paired and unpaired groups were reduced as compared to home cage ($F(2,19) = 6.13$, $p = 0.008$, Home versus Paired: $p < 0.05$, Home versus Unpaired: $p < 0.01$). However, there was no significant difference in mRNA levels among the groups at 6 hr.

Receptors

Notch1 mRNA levels (Figure 4C) were also decreased in the paired and unpaired groups at the 2 hr time point as compared to home cage ($F(2,19) = 17.56$, $p < 0.0001$, Home versus Paired: $p < 0.001$, Home versus Unpaired: $p < 0.001$). At 6 hr, the paired group displayed lower mRNA levels as compared to the unpaired group ($F(2,19) = 3.67$, $p = 0.045$, Paired versus Unpaired: $p < 0.05$). No regulation was noted in *Notch2* mRNA levels (Figure 4D) between any of the groups. Together, these data suggest that the *Jag1* ligand and *Notch1* Receptor mRNAs are dynamically regulated during the consolidation period following fear conditioning in an associative learning-specific manner.

Systemic γ-Secretase Inhibition to Inhibit Notch Signaling Facilitates Fear Memory Consolidation

Our data so far suggest that mRNA levels of the Notch signaling components in the amygdala change in a time-dependent manner after fear conditioning, downstream of miR34a regulation. Next we chose to directly manipulate Notch signaling to test how consolidation of fear memory may be affected. Upon ligand binding to the Notch receptors, γ-secretase cleaves the Notch receptor so that the Notch intracellular domain can then translocate into the nucleus and exert its function (Sail and Hadden, 2012). DAPT, a γ-secretase inhibitor that crosses the blood-brain barrier, was administered intraperitoneally (i.p.) to animals 15 min after the last conditioning trial to block γ-secretase during consolidation. When tested 24 hr later for expression of consolidated fear memory, DAPT-injected animals froze more than vehicle (DMSO)-injected controls (Figure 5A) ($t = 2.41$, $df = 19$, $p = 0.026$). These data suggest that transiently inhibiting Notch signaling in adults creates a molecular environment that is permissive to the consolidation of fear memory. Freezing levels to individual CS presentations (CS1, CS2, etc.) are shown in Figure S7B.

Inhibiting Notch Signaling in the BLA Facilitates Fear Memory Consolidation

While the DAPT experiment demonstrates that γ-secretase inhibition and, by proxy inhibition of Notch signaling, facilitates fear memory formation, γ-secretase activity is also important for the processing of other peptides, including amyloid precursor protein (APP), which has been implicated in Alzheimer's disease (Comery et al., 2005). To more specifically determine what effect inhibition of Notch signaling within the BLA has on fear memory consolidation, we utilized a Notch-inhibiting antibody with stereotaxic intracranial manipulation. Cannulae were implanted into the BLA at least 1 week prior to fear conditioning. On the day of fear acquisition, 15 min after the last training trial, animals were injected with an antibody (Jag1-Fc) at a concentration that has been shown to inhibit Notch signaling (Conboy et al., 2003). When tested 24 hr later for expression of consolidated fear memory, animals that received the Jag1-Fc antibody infused into the BLA froze more than Control antibody-injected controls (Figure 5B) ($t = 2.36$, $df = 12$, $p = 0.036$). These data further corroborate the idea that inhibition of Notch signaling within the BLA facilitates the consolidation of fear memory. While we cannot definitively rule out the possibility that the antibody-based Notch inhibition extended beyond the BLA, infusion of these antibodies into the BLA followed by sacrifice 1 hr later and immunohistochemistry to detect the antibody infusion indicated localization to the BLA (Figure S5). Freezing levels to individual CS presentations (CS1, CS2, etc.) are shown in Figure S7C.

Gain-of-Function of Notch Signaling in the BLA Suppresses Fear Memory Consolidation

Having demonstrated that inhibiting Notch signaling is permissive to the consolidation of fear memory, we asked whether

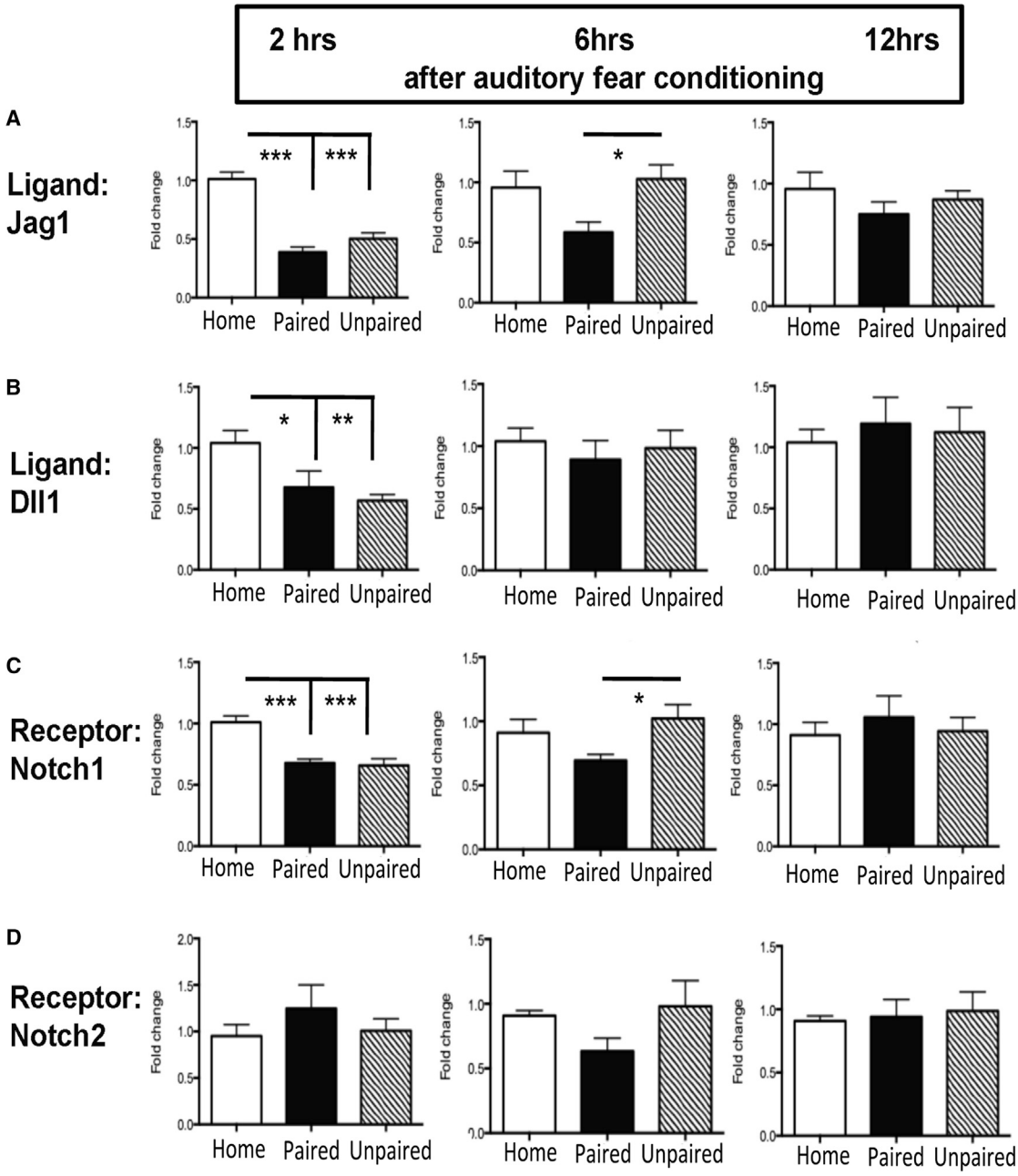

Figure 4. mRNA Levels of Components of the Notch Pathway in the Amygdala Are Altered 2 and 6 hr after Fear Conditioning

(A–C) After subjecting animals to Paired and Unpaired conditioning paradigms, amygdala mRNA levels of Notch pathway components were measured 2, 6, and 12 hr after fear conditioning relative to Home cage controls (n = 8/group). mRNA levels of the ligands, Jag1 (A), Dll1 (B), and receptor Notch1 (C) were significantly downregulated 2 hr after fear conditioning in both the Paired and Unpaired groups, suggestive of a cued- and contextual-learning-related regulation. In addition, Jag1 and Notch1 mRNA levels remained significantly downregulated in the Paired group at 6 hr after fear conditioning compared to the Unpaired condition. (D) Notch2 mRNA levels remained unchanged throughout. All mRNA levels returned to baseline levels 12 hr after the behavioral paradigms. Data are shown as mean +SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

overexpressing Hes1 in the BLA, and thereby increasing Notch signaling, might inhibit fear memory consolidation. While having functions outside of a Notch signaling context, Hes1 is one of the main effectors of Notch signaling and hence is used in our experiment to activate this pathway. Our desire to manipulate Notch signaling that encompasses a large family of receptors and ligands and prevent any compensatory effects from other Notch pathway components led us to utilize a broader approach via the targeting of Hes1 instead of merely overexpressing Notch1.

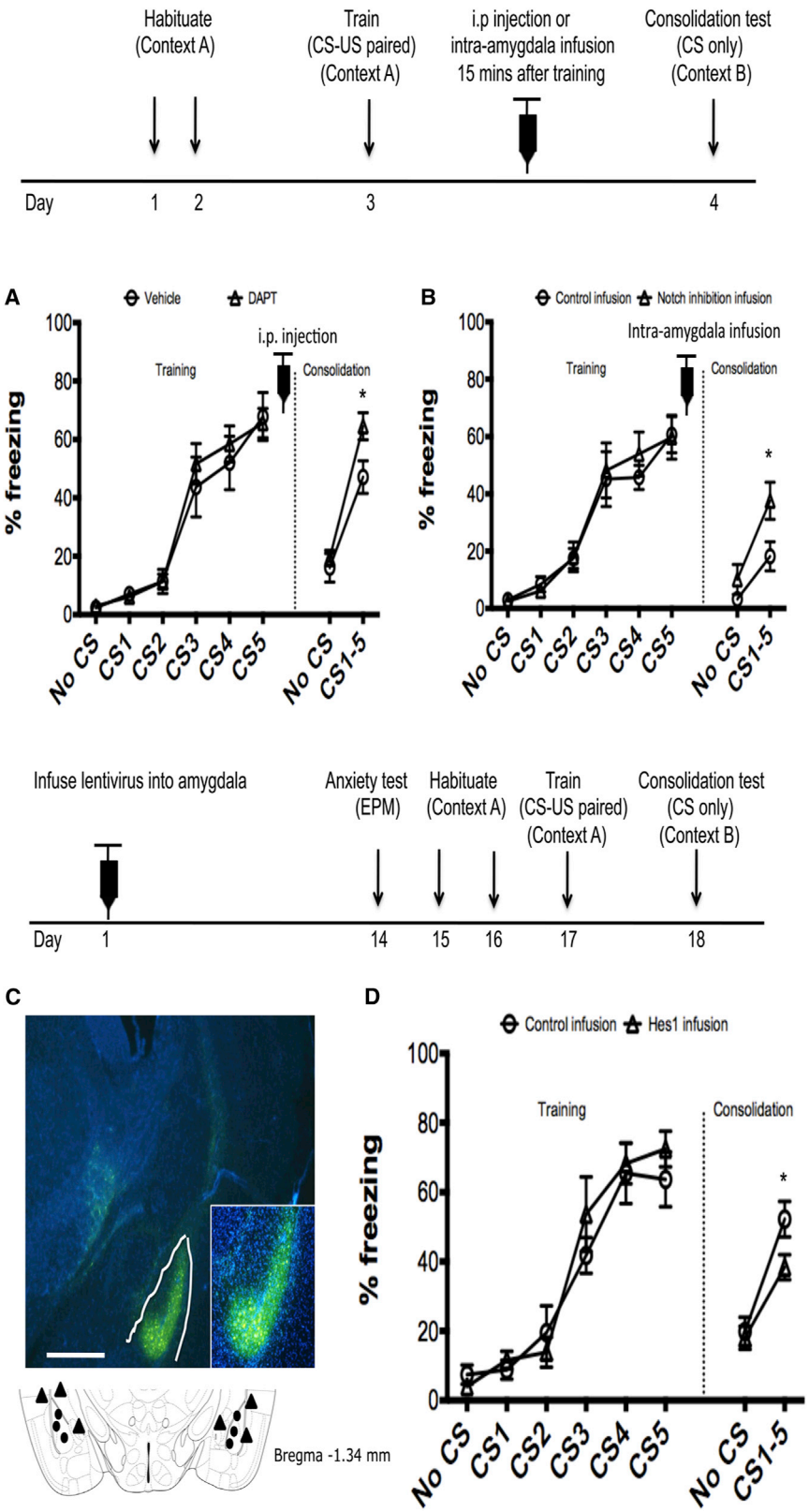

Figure 5. Manipulating Notch Activity Affects the Consolidation of Cued Fear Memory

(A and B) Inhibiting Notch activity facilitates the consolidation of cued fear memory.

(A) The γ-secretase inhibitor (DAPT) or Vehicle (100% DMSO) was injected i.p. 15 min after the last CS+US pairing. Systemic inhibition of Notch signaling enhanced the consolidation of cued fear memory when tested 24 hr later as seen by an increase in freezing behavior compared to the Vehicle group.

(B) Anti-Jag1 Fc at a concentration known to inhibit Notch signaling was injected into the BLA 15 min after the last CS+US pairing. Such local inhibition of Notch signaling also facilitated the consolidation of cued fear memory compared to the control infusion of anti-Human Fc when freezing responses to the tone were measured 24 hr later.

(C and D) Increasing Notch signaling in the BLA by overexpressing a downstream effector of Notch signaling (Hes1) impairs the consolidation of cued fear memory. Lentiviral particles that expressed Hes1 or control GFP under the control of a EF1a promoter were injected into the BLA. Two weeks later, animals were fear conditioned using five tone-shock pairings (CS-US pairings) and 24 hr later tested for the consolidation of cued fear memory.

(C) Representative expression of GFP (driven by the CMV promoter in the dual promoter Hes1 plasmid) in the BLA indicates correct targeting of the injection (filled circles) versus incorrect targeting (solid triangles). Scale bar, 1 mm.

(D) Both groups acquired similar fear responses as a function of CS presentation on training day. However, increasing Notch signaling via Hes1 overexpression impaired the consolidation of the cued fear memory as observed by a diminished freezing response compared to the GFP-control-infused animals. Data are shown as mean ±SEM. *$p < 0.05$.

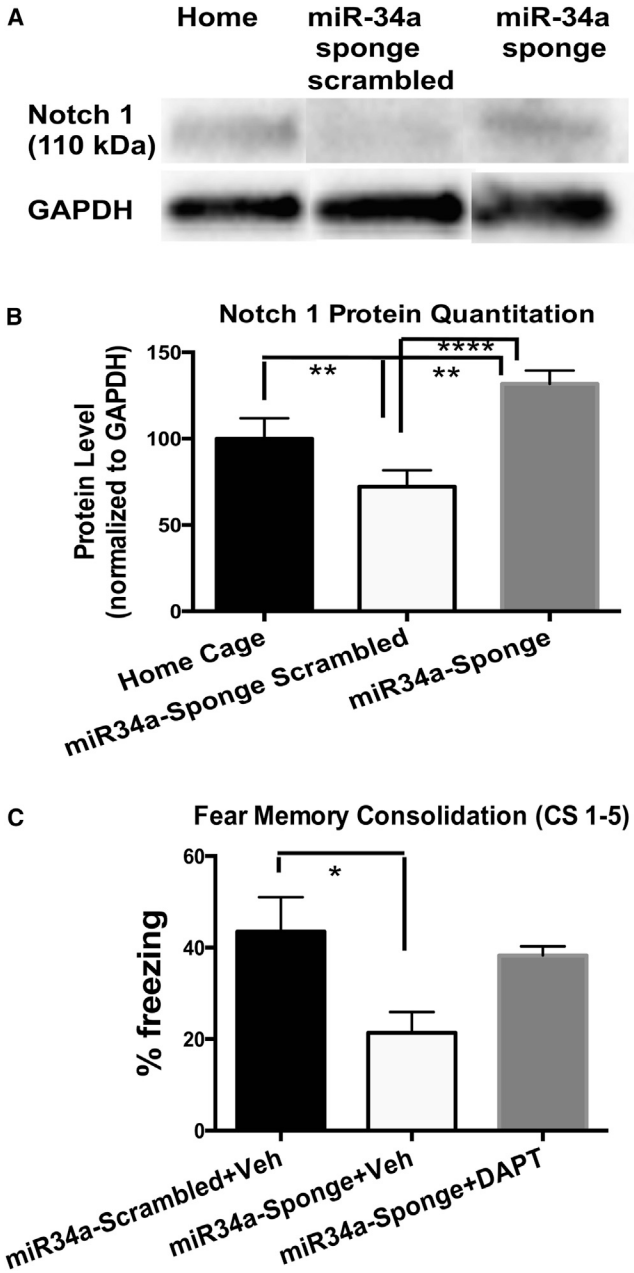

Figure 6. miR-34a Decreases Notch Signaling and Facilitates the Consolidation of Fear Memory

(A and B) Western blotting using an antibody against Notch1 revealed a decrease in Notch1 protein level in the amygdala 2 hr after auditory fear conditioning (Home cage versus miR-34a sponge scrambled control) suggestive of a decrease in Notch signaling that mirrors the decrease in mRNA levels (Figure 4). Infusion of the miR-34a sponge prevents this decrease emphasizing the in vivo functionality of the sponge construct used.

(C) The consolidation of fear memory that is impaired when animals are tested 24 hr after conditioning (Figure 2 and this figure) (miR-34a-Scrambled+Veh versus miR-34a Sponge+Veh) is no longer impaired when Notch signaling is decreased via administration of DAPT, a γ-secretase inhibitor. Data are shown as mean +SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

For this purpose, lentiviral particles that contained either LV-EF1a-mHes1-CMV-GFP (Hes1) or LV-GFP (GFP) were infused into the BLA, followed by a 2-week incubation period prior to behavior (Figure 5C). Animals were then tested in EPM to assay for anxiety-like behavior, and we again found no differences between groups (Figure S6). Both groups were then subjected to a five CS+US paired auditory fear conditioning paradigm and acquired fear similarly on the day of training (Figure 5D). However, 24 hr later, when tested with five CS presentations, Hes1-infused animals froze significantly less than GFP-infused control animals ($t = 2.16$ df $= 15$, $p = 0.043$) (Figure 5D). Freezing levels to individual CS presentations (CS1, CS2, etc.) are shown in Figure S7D. These data suggest that increasing Notch downstream signaling suppresses fear memory consolidation.

miR-34a in the BLA Decreases Notch Signaling to Facilitate Fear Memory Consolidation

Our data thus far have separately implicated BLA miR-34a and Notch function in fear memory consolidation. We chose to ask if miR-34a and Notch interact to influence this consolidation (Figure 6). In addition to the decrease in *Notch1* mRNA levels in the amygdala, we also detected a decrease in Notch1 protein levels in the amygdala 2 hr after auditory fear conditioning (Figures 6A and 6B) (ANOVA: $F(2,10) = 42.50$, $p < 0.0001$). Evidence of this downregulation after fear conditioning comes from comparing Notch1 protein levels in the amygdala of Home cage control animals to those in which miR-34a-sponge-scrambled control was infused into the amygdala (Home versus miR-34a-sponge scrambled control: $p < 0.01$). By infusing miR-34a-sponge into the amygdala, we could also verify the efficacy of sponge activity in vivo. We find that miR-34a-sponge activity within the amygdala prevents any decrease in Notch1 levels after fear conditioning (Home versus miR-34a-sponge: $p < 0.01$, miR-34a-sponge scrambled control versus miR-34a-sponge: $p < 0.0001$).

For behavior, we first infused either miR-34a-sponge or miR-34a-sponge-scrambled control into the BLA of animals. This allowed us to replicate our initial finding that inhibiting miR-34a activity in the BLA suppressed fear memory consolidation. The suppression of fear memory consolidation in miR-34a-sponge animals was rescued by administration of DAPT (Figure 6C). This suggests that it was the increase in Notch signaling (based on the western blot data) (Figures 6A and 6B) in the miR-34a-sponge animals that was responsible for the suppression, which is now overridden by administration of DAPT, a γ-secretase inhibitor, and consequently inhibitor of Notch activity (ANOVA: $F(2,12) = 4.886$, $p = 0.02$; post hoc tests: miR-34a-Scrambled + Veh versus miR-34a-Sponge+Veh: $p < 0.05$).

DISCUSSION

Recently, miRNA belonging to the noncoding RNA family have begun to be investigated for their role in neuropsychiatric disorders. For example, SNPs in several human miRNAs were found to be associated with panic disorder in humans, and a specific miRNA was found to be associated with dementia (Muiños-Gimeno et al., 2011; Zovoilis et al., 2011).

Animal models are being increasingly utilized to address how specific miRNA may be related to such disorders and the

mechanisms underlying the same. With specific brain regions known to be involved in anxiety that accompanies fear expression, as well as mechanisms underlying the consolidation and extinction of fear memories (Davis et al., 2010; Fanselow and Poulos, 2005), these studies have asked whether miRNA in these brain regions are involved in the behavioral endpoints. Of note are studies that have implicated miR-34c in the central amygdala in stress-induced anxiety (Haramati et al., 2011), miR-128b in prefrontal cortex in the formation of fear extinction memory (Lin et al., 2011), and miR-182 in the lateral amygdala in the consolidation of fear memory in rats (Griggs et al., 2013). By blocking miR-34a function in the mouse BLA using a sponge-based strategy, we demonstrated that this miRNA species is necessary for the consolidation of cued fear memory. Our luciferase assays indicated that the miR-34a sponge that we used was specific to miR-34a and did not inhibit the action of the closely related miR-34c. It must be noted that expression of the mCherry and sponge/sponge scrambled sequences are driven by the strong nonspecific Ubc promoter, and as such, we cannot make any claims about the cellular identity of the infected cells. Our future studies would benefit from manipulating miR34a activity in specific cellular populations to enable circuit level analysis of the role of amygdalar miR-34a in fear memory consolidation. Our array and sponge-based functional studies and previously cited data taken together indicate that amygdala miRNA regulation and action accompanies the consolidation of memory across species and present a framework to investigate how regulation of gene expression mediates this important behavioral process.

It is important to note that we observe an increase in miR-34a levels in the amygdala shortly (30 min) after auditory fear conditioning, and in a learning-specific manner. Our reasoning to choose this time point was based on the fact that consolidation typically occurs in the few hours following conditioning, and levels of regulatory miRNA should consequently be altered quickly so as to enable their action on target mRNA within the temporal window of consolidation. We do not observe an increase in miR-34a in a stressful but nonassociative context (CS+US unpaired), in contrast to the published report of a stress-induced increase in miR-34a (Haramati et al., 2011). Two parameters might explain this apparent discrepancy: the nature of the stressor being acute restraint stress in the prior study contrasted by multiple mild foot-shocks in ours and the arrays being performed 90 min after the acute restraint stress versus 30 min after in our paradigm.

While it is relatively straightforward to measure changes in the miRNA profile in specific brain regions and correlate them to a behavior of choice, understanding how a specific miRNA exerts its action is a complicated endeavor. Their short nature coupled with the fact that a very small seed region in the target mRNA is required for a miRNA to exert its effect mean that a single miRNA can target hundreds or thousands of genes (Lim et al., 2005). Using our previously described focused approach, it was reassuring and important that Wnt1 and CREB were on our list of miR-34a targets, with both having documented roles in the consolidation of fear memory (Cowansage et al., 2013; Frankland et al., 2004; Josselyn et al., 2001; Maguschak and Ressler, 2011). To our unexpected surprise, ligands and receptors of the Notch pathway dominated the list. It is worth noting that our report of miR-34a-mediated regulation of Notch1, Jag1, and Dll1 has been previously suggested by cancer researchers (de Antonellis et al., 2011; Pang et al., 2010). As such, this presented us with a compelling rationale to focus on not just one gene in a developmental signaling cascade, but rather on multiple members of a pathway. Per the accepted role of miRNAs decreasing target mRNA levels, we hypothesized that an increase in miR-34a would result in a decrease in mRNA of Notch pathway components. More importantly, we predicted that a decrease in Notch signaling would be permissive to fear memory consolidation.

We did indeed observe decreases in both ligand and receptor mRNA levels of Notch pathway components in the amygdala that coincided with the time frame of fear memory consolidation. At both the 2 and 6 hr time points, this decrease was observed in the paired versus control group, indicating learning-dependent gene regulation. It should be noted that 6 hr after fear conditioning we do not observe statistically significant differences in *Jag1* and *Notch1* mRNA levels of Home Cage and Paired groups, but we instead detect significant differences between the Paired and Unpaired groups. While we appreciate that the Home cage group is the most important control in this case, we have found in our hands that Unpaired groups show negligible cue-specific learning, and as such, we interpret our differences between Paired and Unpaired groups to be the result of learning-specific effects on mRNA levels. We also observed a decrease in mRNA levels of some components in our unpaired group at 2 hr after the unpaired protocol, and postulate that this could be related to consolidation of contextual fear memory that may occur in this unpaired condition. We know the amygdala to be involved in the consolidation of both cued (our main focus) and contextual fear memory (Davis, 1992; Sauerhöfer et al., 2012). Future studies would do well to investigate the role of Notch signaling in this dichotomy. However, we cannot rule out the possibility that foot-shocks (US) or tones (CS) alone might be responsible for these effects, and in future studies, we will incorporate shock controls in which unsignaled foot-shocks are administered immediately upon entering the training arena followed by CS presentations—a manipulation resulting in no context conditioning (Landeira-Fernandez et al., 2006). In support of our results (albeit in a different memory task), Notch mRNA levels have also been shown to be downregulated in the rat hippocampus during the window of memory consolidation in the hippocampus-dependent passive avoidance task (Conboy et al., 2007). Using western blotting, we were also able to show that Notch1 protein levels are decreased in the amygdala, 2 hr after fear conditioning, with this decrease prevented when miR-34a-sponge was infused into the BLA. Therefore mRNA and protein levels of components of the Notch pathway are regulated in the period that consolidation of fear memory occurs.

From a learning perspective, the role of Notch signaling in memory formation appears to be complicated. Consistent with our data, in the passive avoidance paradigm cited above, a transient attenuation of Notch signaling facilitates memory consolidation in rats and forced activation of the Notch pathway impairs consolidation (Conboy et al., 2007). In apparent contrast to this finding is the observation that mutant $Notch^{+/-}$ mice appear to have cognitive impairment, evidenced by poor performance on the hippocampal-dependent water maze task (Costa et al.,

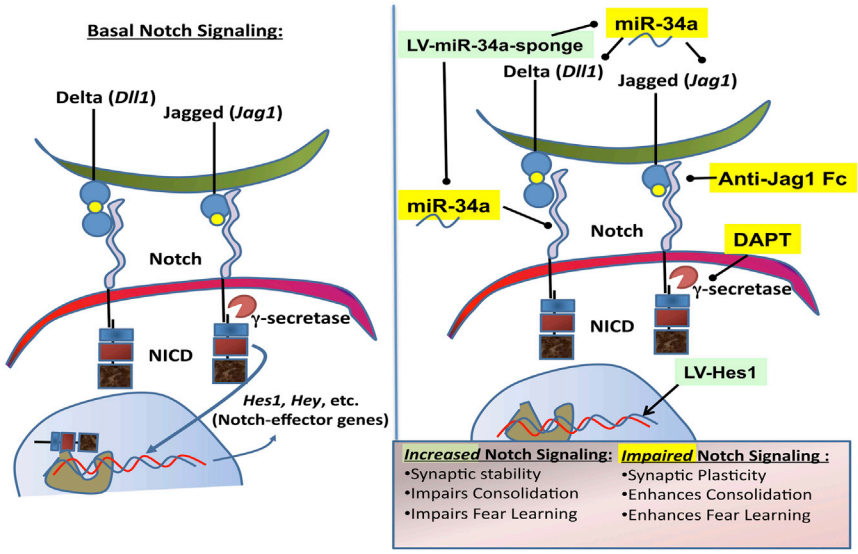

Figure 7. Working Model of miR-34a-Mediated Regulation of Notch Signaling as Being Permissive to Fear Memory Consolidation

(A) Baseline levels of Notch signaling in the BLA as a consequence of ligand (Jag1 and Dll1) and receptor (Notch 1 and Notch 2) interaction maintains status quo and the baseline state.

(B) After training, miR-34a upregulation in the amygdala results in downregulation of components of the Notch pathway and a decrease in Notch signaling, thereby creating a molecular and cellular environment that is permissive to the consolidation of cued fear memory. Green: Inhibiting miR-34a action in the BLA by expressing miR-34a sponges via lentiviruses impairs fear memory consolidation, as does overexpressing Hes1 in the BLA and consequently increasing Notch signaling. Yellow: In contrast, inhibiting Notch signaling by suppressing the activity of γ-secretase using systemic DAPT administration or using Anti-Jag1-Fc antibody in the BLA enhances fear memory consolidation.

2003), with cued fear memory being unaffected. We propose that this difference between the *Notch*$^{+/-}$ mice and our data might be a consequence of the constitutive deletion of Notch1 versus a more acute manipulation of Notch signaling. It also appears that the involvement of Notch in learning and memory may be task dependent. A most recent study using *Jag*$^{+/-}$ mice demonstrated that an impairment in Notch signaling affected spatial memory formation but not other forms of memory (Sargin et al., 2013). Using conditional inhibition of Notch signaling by deleting a downstream effector (RPBj) in a CamKII-Cre-dependent manner, another recent study found no evidence for any impairment in learning and memory (Sato et al., 2012).

Using loss-of-function studies, we have shown that decreasing cleavage of the Notch receptor by inhibiting γ-secretase facilitates cued fear memory consolidation. This is in keeping with the reports of such inhibition of γ-secretase facilitating the consolidation of contextual and spatial memory, which are both hippocampus dependent (Comery et al., 2005; Dash et al., 2005). However, an important caveat of our experiment is that γ-secretase is not only involved in Notch signaling but also plays a role in the processing of APP and consequently has a role in Alzheimer's disease. Despite this lack of specificity, we are confident that decreasing Notch signaling facilitates cued memory formation because intra-amygdala inhibition of Notch signaling using a previously published antibody-based strategy (Conboy et al., 2003) also facilitates memory consolidation in our design. Complementing this experiment is our finding that overexpressing Hes1, a key effector of Notch signaling, in the BLA suppressed the consolidation of cued fear memory. Utilizing an experimental design that is focused on cued fear conditioning and conditional approaches of manipulating Notch function at acute time scales, our data make a case for Notch signaling as being suppressive of the consolidation of cued fear memory. We suspect that this focused and conditional manipulation may explain some of the differences between our conclusions and the studies that suggest Notch signaling to be permissive of memory consolidation.

The impairment of fear memory consolidation that results from inhibiting miR-34a action in the BLA could be rescued by inhibiting γ-secretase activity and consequently Notch activity. Our data over bioinformatics, expression, biochemical, and behavioral levels of analyses viewed together lead us to postulate that Notch signaling at baseline conditions maintains a steady state of synaptic stability, repressing synaptic plasticity. A fear-conditioning-mediated, transient increase in miR-34a in the amygdala then decreases Notch signaling, thereby creating an environment that is transiently permissive of plasticity and consequently consolidation of the cued fear memory (Figure 7). While we contribute to the body of work that suggests that Notch signaling impairs the consolidation of cued fear memory, further investigation of the role of Notch signaling in learning and memory will need to be nuanced to determine its task-, manipulation- (constitutive versus conditional), and region-specific roles.

In keeping with the idea that gene expression and protein synthesis are involved in fear memory consolidation, our study makes two important contributions to our understanding of how this occurs and a candidate gene pathway that is regulated during consolidation. First, using an array-based approach followed by loss-of-function in vivo manipulation, we show that miR-34a function in the amygdala allows for memory of a fearful stimulus to be consolidated. Second, using a targeted bioinformatics approach, complemented with loss- and gain-of-function studies, we establish a link for miRNA-mediated regulation of the Notch pathway as being involved in this consolidation. With Notch pathway function being traditionally viewed through the lens of stem cell differentiation during development (Carlson and Conboy, 2007), this second contribution indicates that signaling pathways normatively used in development are also parsimoniously utilized for adult behavior. Finally, Notch signaling is now being targeted in the treatment of various cancers (Rizzo et al., 2008; Sail and Hadden, 2012), with numerous new therapeutic tools available. We would do well to co-opt relevant therapeutic agents that regulate Notch signaling and use them in the treatment or prevention of disorders that have a

substantial memory component, including those with declarative memory loss, such as dementia, or those with substantial over-consolidation of emotional memory such as PTSD, OCD, and substance abuse disorders.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted with 2-month-old C57Bl/6J male mice purchased from Jackson Laboratory (Bar Harbor). Animals were housed on a 12 hr light/dark cycle in standard group cages (≤5/cage) with ad libitum access to food and water. All experiments were conducted during the light half of the cycle. All procedures were approved by Emory University's IACUC and followed guidelines set by NIH.

Behavior

All behavior was performed in a double-blind manner, and data were acquired using automated computer software programs.

Elevated Plus Maze

Mice were placed in the center of the plus maze arms with the maze elevated about 2 ft from the floor. Animals were recorded while exploring the plus maze for 5 min in a dimly lit room. The amount of time spent in the closed versus open arms is viewed as a measure of anxiety-like behavior (Gafford et al., 2012). The recording and analysis were both carried out using Limelight Software (Actimetrics).

Auditory Fear Conditioning

Auditory fear conditioning was carried out as previously described (Dias and Ressler, 2014). Briefly, mice were pre-exposed to sound attenuated conditioning chambers (San Diego Instruments) (grid floors, room light on, cleaned with Quatricide: Context A) for 3 consecutive days before training. On the day of auditory fear conditioning in Context A, mice received five CS-US pairings (CS: 30 s, 6 kHz, 75 db tone) (US: 500 ms, 0.6 mA foot-shock) wherein the tone coterminated with the mild foot-shock with a 5 min intertrial interval (ITI). Where an unpaired condition was used, the same CS and US parameters were used with no cotermination and presented in a random sequence. The percentage of time spent freezing during fear acquisition was measured by SR-LAB software (San Diego Instruments). The consolidation of fear memory was tested 24 hr after fear conditioning in a novel context (modular test chambers; Med Associates Inc.) (plexiglass floor, room light off, red chamber lights on, cleaned with EtOH: Context B) when mice were exposed to five CS tones with a 2 min ITI. Freezing during the tone presentations was measured with FreezeView software (Coulbourn Instruments). All statistical analyses were conducted using a repeated-measure ANOVA design with Bonferroni correction.

miRNA Microarray and Data Analysis

Animals were either subjected to the auditory fear conditioning protocol mentioned above or left undisturbed in their home cages. Thirty minutes after conditioning, both groups of animals were decapitated, brains rapidly frozen on dry ice, and then stored at −80°C. Fresh-frozen brains were mounted on the Microm HM450 freezing microtome with Tissue-Tek O.C.T. compound with the tissue kept at −23.0°C. Using a 1.0 mm biopsy tool, bilateral punches of the brain were made to collect amygdala tissue (Bregma −0.94 mm to −2.3 mm) (Paxinos and Franklin, 2004). The punches were stored at −80°C until miRNA extraction. miRNA were extracted from this tissue using the miRNeasy kit (QIAGEN). The miRNA array (miRCURY LNA miRNA array, 6th gen) was conducted by Exiqon using previously published techniques (http://www.exiqon.com for more information). To examine miRNA that were upregulated after fear conditioning, we set a priori thresholds ($p < 0.01$ and dLMR = 0.5) for follow-up studies. We then proceeded to verify whether specific miRNA that met those criteria were indeed regulated after fear conditioning using a completely different cohort of animals.

Independent Verification of miRNA Microarray Hits Using Quantitative RT-PCR

After miRNA were extracted from amygdala tissue punches obtained from animals 30 min after auditory fear conditioning (Home Cage, Paired, and Unpaired groups), the Exiqon cDNA synthesis kit was used to reverse transcribe the miRNA to cDNA. Quantitative RT-PCR (qRT-PCR) was then performed using the above cDNA as template in a SYBRGreen MasterMix+primer mixture. Plates were run in the Applied Biosystems 7500 Fast Real-Time PCR System under the Standard 7500 run mode (one cycle 95°C, 10 min; 40 cycles 95.0°C, 10 s; and 60°C, 1 min with fluorescence measured during 60°C step; 1 cycle 95.0°C, 15 s, 60°C, 1 min, 95.0°C, 15 s, 60°C, 15 s). Data were then analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All collected data were normalized to the Home Cage group, and statistical analysis involved ANOVA on the fold change values with Bonferroni post hoc correction.

Molecular Cloning and Virus Production

The plasmids ED156, ED350, ED245, and LIB01 were generously gifted by Dr. Dieter Edbauer (Munich). Primers used for all subcloning experiments are listed in Figure S1. Schematic of constructs used for experiments are shown in Figure S1.

Notch1 3′ UTR Downstream of Firefly-Luciferase for Luciferase Assay

The 3′ UTR of *Notch1* and CRFR1 was subcloned into ED156 downstream of the firefly luciferase gene (FF-luc) with the FF-luc being driven by the Synapsin promoter (See Figure S1 for Notch 1 primer sequences; CRF-R1 primers were same as used in Haramati et al., 2011). Another construct (ED350) containing Renilla luciferase (R-luc) downstream of the Synapsin promoter was used as an internal control.

miR-34a

The miR-34a sequence was subcloned into ED245 downstream of the Synapsin promoter. In addition, miR-34c was also subcloned into ED245 with primers used by Haramati et al., 2011.

miR-34a Sponge and Scrambled Constructs

Seed sequences (four concatenated seeds) to which miR-34a can bind, thus inhibiting its activity, were subcloned into LIB01 downstream of the Synapsin promoter. As a control, these sequences were scrambled (miR-34a-sponge scrambled control).

Hes1 Overexpression

We obtained Plasmid #17625 from Addgene and packaged it to produce lentivirus to overexpress GFP (driven by Ubc promoter) and Hes1 (driven by Ef-1a promoter) in the mouse brain.

Lentiviral Production

The miR-34a-sponge, miR-34a-sponge-scrambled control, and Hes1 overexpression constructs were packaged as lentiviruses by the Emory Viral Vector Core using previously published protocols (Lois et al., 2002).

Luciferase Activity to Validate Notch1 as a Target of miR-34a

We used methodology referenced in Edbauer et al. (2010). Briefly, luciferase assays (Figure S3) were used to validate *Notch1* as a target of miR-34a. To validate *Notch1* as an actual target of miR-34a, HEK293T cells were cotransfected with (a) the FireFly-luc-Notch1 UTR and Renilla-luc constructs; (b) the FF-luc-Notch1 UTR, R-luc, and miR-34a constructs; (c) the FF-luc-Notch1 UTR, R-luc, miR-34a, and miR-34a sponge constructs; and (d) the FF-luc-Notch1 UTR, R-luc, and miR-34a sponge constructs. To determine the specificity of the miR-34a sponge, we also performed a luciferase assay wherein the 3′ UTR of CRFR1 was subcloned downstream of FireFly luciferase. Seeing that CRFR1 is a target of miR-34c and miR-34a (Haramati et al., 2011), HEK293T cells were cotransfected with (a) the FireFly-luc-CRFR1 UTR and Renilla-luc constructs; (b) the FF-luc-CRFR1 UTR, R-luc, and miR-34c constructs; (c) the FF-luc-CRFR1 UTR, R-luc, miR-34c, and miR-34a sponge constructs; (d) the FF-luc-CRFR1 UTR, R-luc, and miR-34a sponge constructs; and (e) the FF-luc-CRFR1 UTR, R-luc, miR-34a, and miR-34a sponge constructs. HEK293T cells were harvested 2 days after the transfection and luminescence as a function of both FF-luc and R-luc was measured using the Promega Stop-n-Glo system.

mRNA Quantification in the Amygdala after Fear Conditioning Using qRT-PCR

Male mice were subjected to auditory fear conditioning (Paired and Unpaired groups). Brains from these animals and Home Cage controls were collected 2, 6, and 12 hr after fear conditioning. After micropunching the amygdala as

outlined above, mRNA were extracted from the tissue punches using the RNeasy Kit (QIAGEN). The SABiosciences RT^2 First Strand Kit was used to reverse transcribe the mRNA to cDNA. qRT-PCR was then performed using the above cDNA as template in a TaqMan Universal PCR Master Mix mixture. The primers included Mouse *Gapdh* (GAPDH) as Endogenous Control, Mouse *Notch1* (Mm00435249_m1), Mouse *Notch2* (Mm00803077_m1), Mouse *Dll1* (Mm01279269_m1), and Mouse *Jag1* (Mm00496902_m1). The plate was run in the Applied Biosystems 7500 Fast Real-Time PCR System under the Standard 7500 run mode (one cycle 50.0°C, 2 min; one cycle 95.0°C, 10 min; 40 cycles 95.0°C, 15 s and 60°C, 1 min with fluorescence measured during 60°C step). Data were analyzed as noted previously.

Western Blot Analysis of Notch1 Protein Levels in BLA of miR-34a Sponge-Infused Animals

Notch1 protein levels in the amygdala were measured 2 hr after fear conditioning in Home cage controls versus LV-miR-34a-sponge, and LV-miR-34a-spongeSCR animals using western blotting. Briefly, 30 μg of protein was run in a 4%–20% MiniProtean TGX gel (BioRad) and then blotted on to nitrocellulose membrane. The membrane was then blocked with 1× TBST+5% milk and incubated overnight with 1:1,000 of Rbt anti-Notch1 (D1E11) (Cell Signaling Technology) in blocking solution. After washing, the membrane was then incubated with 1:2,000 Peroxidase anti-Rbt (Vector Laboratories), washed again, and bands detected using WestPico Chemiluminescent Substrate (Pierce Biotechnology) and a BioRad ChemiDoc system. Two bands were detected for Notch 1 with the higher band known to be nonspecific. The Notch 1 band at 110 kDa was quantified using ImageJ software and normalized to the GAPDH bands detected using Mouse anti-GAPDH (Fitzgerald Laboratories).

Manipulation of miR-34a and Memory Consolidation

Using stereotaxic surgery, 1ul of LV-miR-34a-sponge or LV-mmu-miR-34a-spongeSCR was infused into the basolateral amygdala (BLA) over a period of 8 min using a Hamilton syringe precoated with 10% BSA (From Bregma: −1.4 A/P, −5.0 D/V, ± 3.4 M/L). The needle was left in place for 10 min after infusion and gradually withdrawn over 2 min. Viral titer was at least 2 × 10^9 iu/ml. Two weeks later, mice were habituated and fear conditioned in Context A, as noted above. Consolidation of cued fear memory was then tested 24 hr later in Context B, as previously described. Following behavioral studies, brains were sectioned for histological confirmation of LV infection.

γ-Secretase Inhibition and Memory Consolidation

Mice were habituated and then fear conditioned in Context A as noted above. Ten minutes after training in Context A, mice were injected i.p. with either Vehicle (0.1 ml, 100% DMSO) or γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma-Aldrich) (75 mg/kg DAPT in 100% DMSO). The following day, consolidation of memory was tested in Context B, as previously described.

Manipulation of Notch Signaling and Memory Consolidation

Gain-of-Function

Using stereotaxic surgery, 1 μl of LV-Hes1 (Plasmid #17625 from Addgene) or a control LV-GFP was infused into the BLA as noted above (From Bregma: −1.4 A/P, −5.0 D/V, ±3.4 M/L). Two weeks after infection, mice were habituated and fear conditioned in Context A, as noted above. Consolidation of cued fear memory was then tested 24 hr later in Context B, as previously described.

Loss-of-Function

Using stereotaxic surgery, guide cannulae were implanted such that infusions could be made into the BLA at −1.4 A/P, −5.0 D/V, and ±3.4 M/L from Bregma. One week after cannulation, mice were habituated and fear conditioned in Context A, as noted above. Thirty minutes after conditioning, 0.5 μl of Notch-inhibiting antibody or control antibody was infused into the BLA and animals were returned to the vivarium. Consolidation of cued fear memory was then tested 24 hr later in Context B, as previously described. To inhibit the Notch pathway, a previously used antibody strategy was used. Briefly, 10 μg of Jagged-1 Fc (1277-JG: R&D systems) was incubated with anti-human Fc (i2136: Sigma) for 1 hr on ice before use, with the anti-human Fc being used as the control infusion. To visualize the localization of this antibody-based inhibition (Figure S5), we conducted immunohistochemistry on fresh-frozen brain tissue that was harvested 1 hr after infusion of the antibodies into the BLA. Briefly, sections were fixed in 4% paraformaldehyde for 10 min, washed with 1× PBS, and then incubated overnight with biotinylated anti-goat antibody (Vector laboratories, 1:500). After rinsing in 1× PBS, the sections were incubated for 2 hr with 1:1000 Streptavidin conjugated to AlexaFluor-568, coverslipped, and visualized using an epifluorescent microscope.

Interaction between miR-34a and Notch Signaling in Memory Consolidation

Using stereotaxic surgery as noted above, 1 μl of LV-miR-34a-sponge or LV-mmu-miR-34a-spongeSCR was infused into the BLA. Two weeks later, mice were habituated and fear conditioned in Context A, as noted above. Ten minutes later, animals were injected i.p. with either Vehicle (0.1 ml, 100% DMSO) or γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma-Aldrich) (75 mg/kg DAPT in 100% DMSO). Fifty minutes after the injection, these animals were exposed to two CS presentations in Context B to test for STM. Consolidation of cued fear memory was then tested 24 hr later in Context B, as previously described. Following behavioral studies, brains were sectioned for histological confirmation of LV infection.

ACCESSION NUMBERS

The GEO accession number for the miRNA array is GSE59072.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.07.019.

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REFERENCES

Ables, J.L., Breunig, J.J., Eisch, A.J., and Rakic, P. (2011). Not(ch) just development: Notch signalling in the adult brain. Nat. Rev. Neurosci. *12*, 269–283.

Carlson, M.E., and Conboy, I.M. (2007). Regulating the Notch pathway in embryonic, adult and old stem cells. Curr. Opin. Pharmacol. *7*, 303–309.

Comery, T.A., Martone, R.L., Aschmies, S., Atchison, K.P., Diamantidis, G., Gong, X., Zhou, H., Kreft, A.F., Pangalos, M.N., Sonnenberg-Reines, J., et al. (2005). Acute γ-secretase inhibition improves contextual fear conditioning in the Tg2576 mouse model of Alzheimer's disease. J. Neurosci. *25*, 8898–8902.

Conboy, I.M., Conboy, M.J., Smythe, G.M., and Rando, T.A. (2003). Notch-mediated restoration of regenerative potential to aged muscle. Science *302*, 1575–1577.

Conboy, L., Seymour, C.M., Monopoli, M.P., O'Sullivan, N.C., Murphy, K.J., and Regan, C.M. (2007). Notch signalling becomes transiently attenuated during long-term memory consolidation in adult Wistar rats. Neurobiol. Learn. Mem. *88*, 342–351.

Costa, R.M., Honjo, T., and Silva, A.J. (2003). Learning and memory deficits in Notch mutant mice. Curr. Biol. *13*, 1348–1354.

Cowansage, K.K., Bush, D.E.A., Josselyn, S.A., Klann, E., and Ledoux, J.E. (2013). Basal variability in CREB phosphorylation predicts trait-like differences in amygdala-dependent memory. Proc. Natl. Acad. Sci. USA *110*, 16645–16650.

Dash, P.K., Moore, A.N., and Orsi, S.A. (2005). Blockade of γ-secretase activity within the hippocampus enhances long-term memory. Biochem. Biophys. Res. Commun. *338*, 777–782.

Davis, M. (1992). The role of the amygdala in fear and anxiety. Annu. Rev. Neurosci. *15*, 353–375.

Davis, M., Walker, D.L., Miles, L., and Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacology *35*, 105–135.

de Antonellis, P., Medaglia, C., Cusanelli, E., Andolfo, I., Liguori, L., De Vita, G., Carotenuto, M., Bello, A., Formiggini, F., Galeone, A., et al. (2011). MiR-34a targeting of Notch ligand delta-like 1 impairs CD15+/CD133+ tumor-propagating cells and supports neural differentiation in medulloblastoma. PLoS ONE *6*, e24584.

Dias, B.G., and Ressler, K.J. (2014). Parental olfactory experience influences behavior and neural structure in subsequent generations. Nat. Neurosci. *17*, 89–96.

Dias, B.G., Banerjee, S.B., Goodman, J.V., and Ressler, K.J. (2013). Towards new approaches to disorders of fear and anxiety. Curr. Opin. Neurobiol. *23*, 346–352.

Dudai, Y. (2004). The neurobiology of consolidations, or, how stable is the engram? Annu. Rev. Psychol. *55*, 51–86.

Edbauer, D., Neilson, J.R., Foster, K.A., Wang, C.-F., Seeburg, D.P., Batterton, M.N., Tada, T., Dolan, B.M., Sharp, P.A., and Sheng, M. (2010). Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron *65*, 373–384.

Fanselow, M.S., and Poulos, A.M. (2005). The neuroscience of mammalian associative learning. Annu. Rev. Psychol. *56*, 207–234.

Fitzgerald, P., Seemann, J., and Maren, S. (2013). Can fear extinction be enhanced? A review of pharmacological and behavioral findings. Brain Res. Bull. *105*, 46–60.

Frankland, P.W., Josselyn, S.A., Anagnostaras, S.G., Kogan, J.H., Takahashi, E., and Silva, A.J. (2004). Consolidation of CS and US representations in associative fear conditioning. Hippocampus *14*, 557–569.

Gafford, G.M., Guo, J.D., Flandreau, E.I., Hazra, R., Rainnie, D.G., and Ressler, K.J. (2012). Cell-type specific deletion of GABA(A)α1 in corticotropin-releasing factor-containing neurons enhances anxiety and disrupts fear extinction. Proc. Natl. Acad. Sci. USA *109*, 16330–16335.

Gao, J., Wang, W.-Y., Mao, Y.-W., Gräff, J., Guan, J.-S., Pan, L., Mak, G., Kim, D., Su, S.C., and Tsai, L.-H. (2010). A novel pathway regulates memory and plasticity via SIRT1 and miR-134. Nature *466*, 1105–1109.

Gräff, J., and Mansuy, I.M. (2008). Epigenetic codes in cognition and behaviour. Behav. Brain Res. *192*, 70–87.

Griggs, E.M., Young, E.J., Rumbaugh, G., and Miller, C.A. (2013). MicroRNA-182 regulates amygdala-dependent memory formation. J. Neurosci. *33*, 1734–1740.

Haramati, S., Navon, I., Issler, O., Ezra-Nevo, G., Gil, S., Zwang, R., Hornstein, E., and Chen, A. (2011). MicroRNA as repressors of stress-induced anxiety: the case of amygdalar miR-34. J. Neurosci. *31*, 14191–14203.

Johansen, J.P., Cain, C.K., Ostroff, L.E., and LeDoux, J.E. (2011). Molecular mechanisms of fear learning and memory. Cell *147*, 509–524.

Josselyn, S.A., Shi, C., Carlezon, W.A., Jr., Neve, R.L., Nestler, E.J., and Davis, M. (2001). Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. J. Neurosci. *21*, 2404–2412.

Kashat, M., Azzouz, L., Sarkar, S.H., Kong, D., Li, Y., and Sarkar, F.H. (2012). Inactivation of AR and Notch-1 signaling by miR-34a attenuates prostate cancer aggressiveness. Am. J. Transl. Res. *4*, 432–442.

Konopka, W., Kiryk, A., Novak, M., Herwerth, M., Parkitna, J.R., Wawrzyniak, M., Kowarsch, A., Michaluk, P., Dzwonek, J., Arnsperger, T., et al. (2010). MicroRNA loss enhances learning and memory in mice. J. Neurosci. *30*, 14835–14842.

Landeira-Fernandez, J., DeCola, J.P., Kim, J.J., and Fanselow, M.S. (2006). Immediate shock deficit in fear conditioning: effects of shock manipulations. Behav. Neurosci. *120*, 873–879.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769–773.

Lin, Q., Wei, W., Coelho, C.M., Li, X., Baker-Andresen, D., Dudley, K., Ratnu, V.S., Boskovic, Z., Kobor, M.S., Sun, Y.E., and Bredy, T.W. (2011). The brain-specific microRNA miR-128b regulates the formation of fear-extinction memory. Nat. Neurosci. *14*, 1115–1117.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868–872.

Maguschak, K.A., and Ressler, K.J. (2011). Wnt signaling in amygdala-dependent learning and memory. J. Neurosci. *31*, 13057–13067.

Maguschak, K.A., and Ressler, K.J. (2012). A role for WNT/β-catenin signaling in the neural mechanisms of behavior. J. Neuroimmune Pharmacol. *7*, 763–773.

Maren, S. (2003). The amygdala, synaptic plasticity, and fear memory. Ann. N Y Acad. Sci. *985*, 106–113.

Maren, S., Ferrario, C.R., Corcoran, K.A., Desmond, T.J., and Frey, K.A. (2003). Protein synthesis in the amygdala, but not the auditory thalamus, is required for consolidation of Pavlovian fear conditioning in rats. Eur. J. Neurosci. *18*, 3080–3088.

Muiños-Gimeno, M., Espinosa-Parrilla, Y., Guidi, M., Kagerbauer, B., Sipilä, T., Maron, E., Pettai, K., Kananen, L., Navinés, R., Martín-Santos, R., et al. (2011). Human microRNAs miR-22, miR-138-2, miR-148a, and miR-488 are associated with panic disorder and regulate several anxiety candidate genes and related pathways. Biol. Psychiatry *69*, 526–533.

Pang, R.T.K., Leung, C.O.N., Ye, T.-M., Liu, W., Chiu, P.C.N., Lam, K.K.W., Lee, K.-F., and Yeung, W.S.B. (2010). MicroRNA-34a suppresses invasion through downregulation of Notch1 and Jagged1 in cervical carcinoma and choriocarcinoma cells. Carcinogenesis *31*, 1037–1044.

Parsons, R.G., and Ressler, K.J. (2013). Implications of memory modulation for post-traumatic stress and fear disorders. Nat. Neurosci. *16*, 146–153.

Paxinos, G., and Franklin, K. (2004). The Mouse Brain in Stereotaxic Coordinates: Compact, Second Edition. (San Diego, CA: Elsevier Academic Press).

Pierfelice, T., Alberi, L., and Gaiano, N. (2011). Notch in the vertebrate nervous system: an old dog with new tricks. Neuron *69*, 840–855.

Rizzo, P., Osipo, C., Foreman, K., Golde, T., Osborne, B., and Miele, L. (2008). Rational targeting of Notch signaling in cancer. Oncogene *27*, 5124–5131.

Sail, V., and Hadden, M.K. (2012). Notch Pathway Modulators as Anticancer Chemotherapeutics. Annu. Rep. Med. Chem. *47*, 267–280.

Sargin, D., Botly, L.C.P., Higgs, G., Marsolais, A., Frankland, P.W., Egan, S.E., and Josselyn, S.A. (2013). Disrupting Jagged1-Notch signaling impairs spatial memory formation in adult mice. Neurobiol. Learn. Mem. *103*, 39–49.

Sato, C., Turkoz, M., Dearborn, J.T., Wozniak, D.F., Kopan, R., and Hass, M.R. (2012). Loss of RBPj in postnatal excitatory neurons does not cause

neurodegeneration or memory impairments in aged mice. PLoS ONE *7*, e48180.

Sauerhöfer, E., Pamplona, F.A., Bedenk, B., Moll, G.H., Dawirs, R.R., von Hörsten, S., Wotjak, C.T., and Golub, Y. (2012). Generalization of contextual fear depends on associative rather than non-associative memory components. Behav. Brain Res. *233*, 483–493.

Silva, A.J., and Giese, K.P. (1994). Plastic genes are in! Curr. Opin. Neurobiol. *4*, 413–420.

Spadaro, P.A., and Bredy, T.W. (2012). Emerging role of non-coding RNA in neural plasticity, cognitive function, and neuropsychiatric disorders. Front Genet *3*, http://dx.doi.org/10.3389/fgene.2012.00132.

Steckler, T., and Risbrough, V. (2012). Pharmacological treatment of PTSD - established and new approaches. Neuropharmacology *62*, 617–627.

Vanelzakker, M., Dahlgren, K.M., Davis, F., Dubois, S., and Shin, L. (2013). From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. Neurobiol. Learn. Mem. *113*, 3–18.

Wang, W., Kwon, E.J., and Tsai, L.-H. (2012). MicroRNAs in learning, memory, and neurological diseases. Learn. Mem. *19*, 359–368.

Zovkic, I.B., and Sweatt, J.D. (2013). Epigenetic mechanisms in learned fear: implications for PTSD. Neuropsychopharmacology *38*, 77–93.

Zovkic, I.B., Guzman-Karlsson, M.C., and Sweatt, J.D. (2013). Epigenetic regulation of memory formation and maintenance. Learn. Mem. *20*, 61–74.

Zovoilis, A., Agbemenyah, H.Y., Agis-Balboa, R.C., Stilling, R.M., Edbauer, D., Rao, P., Farinelli, L., Delalle, I., Schmitt, A., Falkai, P., et al. (2011). microRNA-34c is a novel target to treat dementias. EMBO J. *30*, 4299–4308.