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Jared Goodman April 17th, 2013

A Role for Notch Pathway Signaling in Amygdala-Dependent Fear Learning

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Abstract

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Fear and anxiety are evolutionarily conserved mental states that are caused by cues that predict aversive stimuli. Each is mediated by the amygdala, a region in the medial temporal lobe that has been implicated in both the storage of emotional memories and the output of their stereotyped behavioral and physiological responses. Previous literature has implicated the developmentally crucial Notch pathway in hippocampal-dependent memory tasks (Conboy et al., 2007). Our work used an auditory fear conditioning paradigm to implicate Notch signaling in amygdala-dependent fear consolidation wherein an auditory cue (CS) is paired with a mild foot-shock (US). The present study shows that ligand (Jag1, Dll1), receptor (Notch1), and effector (Hes5) amygdala mRNA levels are transiently decreased 2 hours after fear conditioning in the CS/US paired group. At 6 hours, Jag1 and Notch1 mRNA levels remained reduced in the paired group. Interestingly, there also is downregulation of each of these genes at the 2-hour time point in a behavioral group that does not result in any associative learning wherein the CS and US are unpaired. The hippocampus also shows decreased Jag1 and Dll1 expression 2 hours after fear conditioning in this same unpaired group, suggestive of an already published role for Notch in contextual fear memory consolidation. Furthermore, a single intraperitoneal injection of γ-secretase inhibitor DAPT (an inhibitor of Notch signaling) after fear training enhances cued and contextual fear memory consolidation. These data suggest a role for Notch pathway signaling in the amygdala during memory consolidation. Specifically, we find that Notch signaling is transiently decreased during fear consolidation and that pharmacological inhibition of the pathway enhances fear learning. We hypothesize that Notch signaling may serve to inhibit synaptic plasticity at baseline, and that transient decreases in Notch signaling serve to permit neural plasticity required for learning. These data provide a convincing base for further study of Notch in amygdala-dependent memory formation.

A Role for Notch Pathway Signaling in Amygdala-Dependent Fear Learning

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INTRODUCTION

Anxiety Disorders and PTSD

Anxiety and fear are evolutionarily conserved mental states that can be beneficial for survival. Each are similar in terms of their physiological and behavioral responses, as each elicit increased heart rate, vigilance, arousal, startle response, freezing, corticosteroid release, etc. (Davis, 1992). Even with these overlapping behavioral reactions, anxiety and fear are distinct mental states. Anxiety elicits these effects persistently and without any necessary cued or contextual stimulus, while fear produces this same state in a more temporary manner and in particular response to a cued or contextual trigger (Dias, Banerjee, Goodman, & Ressler, 2013). Although these states are natural and helpful in specific situations, many individuals suffer from an anxiety disorder, in which these responses are inappropriately augmented. The standing estimate states that 18.1% of the United States population should be diagnosed with an anxiety disorder over any 12-month period, yet only 42.2% of these individuals receive any sort of treatment for their condition (Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Wang et al., 2005). There are many types of anxiety disorders – general anxiety disorder, agoraphobia (social phobia), specific phobia, panic disorder, etc. – that each lie along the fear-anxiety spectrum. Post-traumatic stress disorder (PTSD), though, has apparent characteristics of both, which make it a prime candidate for research in fear and anxiety (*Diagnostic and statistical manual of mental disorders: DSM-IV-TR*, 2000)

. To be diagnosed with PTSD, an individual must have experienced or been witness to a traumatic event that involved intense fear, helplessness, or horror; the individual must have symptoms of vigilance and avoidance; these symptoms must be persistent and

problematic in social and occupational settings; there must be recurring recollections or dreams of the event; and each of these post-trauma symptoms must be present for more than 1 month (*Diagnostic and statistical manual of mental disorders: DSM-IV-TR*, 2000). It is therefore apparent that those who suffer from PTSD may do so because of a possible over-consolidation of the memory of the traumatic event, so much so that even in the absence (anxiety) and almost always in the presence (fear) of a reminder cue or context, the individual suffers from debilitating symptoms. Therefore, to study PTSD pathology, research must focus on brain structures that are critical for fear, anxiety, and memory formation. This recognition has guided investigators to the 'extended amygdala.'

Emotion Regulation, the 'Extended Amygdala,' and Fear (Fig. 1)

Fear and anxiety are modulated by a variety of brain regions, including the 'extended amygdala' – composed of the amygdala and basal nucleus of the stria terminalis (BNST) – prefrontal cortex (PFC), hippocampus, and hypothalamus. Each region of this fear circuit serves a different function in the production of the behavioral and physiological states associated with fear and anxiety. The sensory cortices (and thalami) send information, through axonal projections, to the hippocampus, PFC, and amygdala for stimulus processing. The hippocampus processes contextual and spatial stimuli, while the amygdala processes both contextual and cued stimuli; the prefrontal cortex is believed to repress fear memory recall in the hippocampus and amygdala, both of which are adjacently positioned in the medial temporal lobe; and, the BNST and hypothalamus serve as the highways for the stereotyped anxiety and fear responses (LeDoux, 2000; Choi et al., 2010; Dias et al., 2013). The amygdala, however, is a focal

point of this fear circuit, so it is of crucial importance for studying fear memory formation.

Because the amygdala is an evolutionarily conserved structure, researchers use animal and human models in a behavioral paradigm, called fear conditioning, to elucidate its functional role during fear memory formation. Much like Pavlov's classical conditioning method, cued fear conditioning pairs a formerly neutral conditioned stimulus (CS), such as a tone, with an aversive unconditioned stimulus (US), usually a mild foot-shock. Typically mice would only display a fear response (freezing, startle) in the presence of the US, but after mice are exposed to this CS/US pairing through classical conditioning, mice then learn to respond to the CS (cue) alone with a stereotyped fear response (Sah, Marek, Strobel, & Bredy, 2013; Johansen, Cain, Ostroff, & LeDoux, 2011). Controls for this paradigm include a CS/US unpaired protocol, during which separate mice are exposed to a neutral CS multiple times without a co-terminating aversive US. Instead, mice are given a US randomly and therefore should not associate the CS with the US (Schafe et al., 2000).

This classical associative-learning behavioral paradigm has been critical for the understanding of the amygdala's role in the stereotyped stress response. The current literature suggests that glutamatergic inputs from the sensory thalamic nuclei converge onto the basolateral amygdala (BLA), which then project their own glutamatergic efferents onto the central amygdala (CeA). The CeA, composed of mainly inhibitory GABAergic neurons, then projects to the hypothalamus, midbrain, pons, and BNST (Cassell, Gray, & Kiss, 1986; Ehrlich et al., 2009; McDonald, 1982; Sah et al., 2013). This then allows for the acute behavioral responses that one would expect during a fearful

situation – freezing, increased heart rate, pupil dilation, activation of hypothalamic-pituitary-adrenal axis, etc. (Davis, Rainnie, & Cassell, 1994).

Simultaneous processing of sensory information within the BLA is crucial for fear memory formation, and this process seems to be dependent upon intracellular depolarization-mediated signaling pathways. It has been shown that lesions of the BLA after training block the expression of conditioned fear (Anglada-Fingueroa & Quirk, 2005). Also, pre-training infusions of N-methyl-D-aspartate (NMDA) receptor antagonists directly into the BLA through cannulation specifically block the acquisition of fear memory in the fear-potentiated startle memory paradigm without disrupting the responsiveness to the foot-shock (Miserendino, Sananes, Melia, & Davis, 1990), indicating that classic Hebbian synaptic strengthening (Johansen et al., 2011) is necessary in the BLA for fear memory formation. Other than NMDA activation, transcriptional regulators such as Brain-Derived Neurotrophic Factor (BDNF), Ca2+/Calmodulin (Cam) Dependent Protein Kinase II (CaMKII), and cAMP Response Element Binding-Protein (CREB) have also been implicated in amygdala-dependent learning (Rodrigues, Schafe, & LeDoux, 2004; Josselyn et al., 2001; Andero et al., 2011; Johansen et al, 2011).

Interestingly though, there is a recently broadening literature base that has begun to elucidate the role of molecules crucial for development in fear memory formation during adulthood. Wnt (Wingless-Related MMTV Integration Site) signaling, which naturally inhibits the transcription of activators of axonal branching, growth cone enlargement, etc. during development, has been shown to be transiently downregulated after fear conditioning through degradation of β-catenin, a molecule important for synaptic stabilization (Maguschak & Ressler, 2011). We thus hope to continue this

exploration in the amygdala by studying the Notch pathway, another molecular mechanism that is crucial for the development of the nervous system.

The Notch Pathway (Fig. 2)

The Notch pathway has a long history of being implicated in the processes of both general and neuronal development. In 1919, one of the first studies of Notch showed that reduced expression (via haploinsufficiency) of the Notch receptor in Drosophila resulted in a 'notch' on the wing (Louvi & Artavanis-Tsakonas, 2006). In 1937, Poulson demonstrated its neuronal potential as complete deletion of the Notch receptor resulted in the 'neurogenic' phenotype, in which Drosophila contained vast amounts of neural tissue at the expense of epidermis. This lethal phenotype resulted from a 'failure of communication' between ectodermal stem cells, which sheds light onto Notch's role: cell-to-cell communication (Louvi & Artavanis-Tsakonas, 2006).

Notch's canonical mechanism of action begins with ligands Delta and Jagged on a cell attaching to the transmembrane Notch receptor of a neighboring cell. Notch is then cleaved by γ-secretase's presenilin-1 site to allow for the transport of the Notch intracellular domain (NICD) into the nucleus. The NICD then binds to mastermind-like protein 1 (MAML1), MAML2, or MAML3 to activate the normally inhibiting recombining binding protein suppressor of hairless (RBPJ) to enhance the transcription of various effectors (Bray, 2006; Ables, Breunig, Eisch, & Rakic, 2011). Most notable of these are the basic helix-loop-helix (bHLH) hairy enhancer of split (Hes) and Hes-related with YRPF motif (Hey), which both inhibit the transcription of various genes implicated in differentiation (Kageyama, Ishibashi, Takebayashi, & Tomita, 1997; Zanotti & Canalis, 2010). Outside the cell, the extracellular domain of Notch (NECD) is taken up

by the cell that expressed the Delta or Jagged, but the function of this is unknown (Ables et al., 2011). Thus, one of the overall results of Notch pathway activation is the prevention of differentiation, or lateral inhibition of cell growth (Lowell, Benchoua, Heavey, & Smith, 2006).

While Notch has traditionally been investigated in developmental contexts, recent studies have begun to shed light on Notch's post-developmental role, with particular interest in learning and memory. It has been shown that Notch1 is expressed in various tissues, including the hippocampus and amygdala. Mutant mice with reduced Notch1 expression showed an impaired long-term potentiation and enhanced long-term depression in hippocampal CA1 synapses (Wang et al., 2004). Also, increased Notch1 activity in cultured neurons disrupted neurite growth but increased axonal branching (Redmond, Oh, Hicks, Weinmaster, & Ghosh, 2000). This *in vitro* effect, however, can be rescued by the inclusion of Numb-like (NUMBL) – a promoter of neurite growth – into the media (Berezovska et al., 1999). Furthermore, Notch is a down-regulator of Neurogenin 3 (NGN3) – another promoter of neurite growth (Salama-Cohen, Arévalo, Grantyn, & Rodríguez-Tébar, 2006). Behaviorally, it has also been shown that mRNA of Notch signaling components decreases in the hippocampus of Wistar rats 12 hours after passive avoidance training (Conboy et al., 2007), and mutant Notch$^{+/-}$ mice appear to have cognitive impairment, evidenced by poor performance on the hippocampal-dependent water maze task (Costa, Honjo, & Silva, 2003). Yet, no study to our knowledge has specifically assessed the role of Notch signaling in the amygdala during fear conditioning.

To address these questions, we employed TaqMan® Real-Time PCR (RT-PCR) to determine the mRNA landscape of different genes along the Notch pathway during memory consolidation, at 2, 6, and 12 hours after cued fear conditioning training, in both CS/US paired and unpaired behavioral programs. Having observed dynamic changes in mRNA levels of Notch signaling components, we next manipulated Notch signaling by blocking the function of γ-secretase through acute intraperitoneal (i.p.) injection of a γ-secretase inhibitor (DAPT) after cued fear conditioning. We then assessed the changes in consolidation of cued and contextual fear memories as a result of this manipulation. *This study provides the first known evidence that the Notch pathway is regulated during fear memory consolidation and that Notch pathway inhibition with γ-secretase inhibitors enhances cued and contextual fear memory formation.*

MATERIALS METHODS

Cued Fear Conditioning (Fig. 3A, 3B)

C57BL/6J (Jackson Laboratories, Inc.) mice were split into three groups for cued fear conditioning: Paired, Unpaired, and Home cage. Both the paired and unpaired groups were habituated in the SR-LAB startle response system sound attenuated chambers (SR-LAB, San Diego Instruments, San Diego, California, USA) for 5 minutes each for two days prior to fear conditioning. On the third day, the Paired group was placed in the SR-LAB system for fear conditioning, and they were exposed to 5 conditioned stimuli (70-80 dB, 30 sec, 6 kHz tone), each co-terminating with an aversive unconditioned stimulus (0.6 mA, 0.5 sec shock) with an average inter-trial interval (ITI) of 2 mins. The Unpaired group also was placed in the SR-LAB system but was exposed to 5 conditioned stimuli (70-80 dB, 30 sec, 6kHz tone) and 5 randomly programmed aversive unconditioned stimuli (0.6 mA, 30 sec shock). The Home cage group was handled in the vivarium and not exposed to the chambers, tones, or foot-shocks. All animal procedures were in accordance with guidelines prescribed by IACUC.

mRNA Extraction and cDNA Synthesis (Fig. 3A, 3C)

Mice from each group were then sacrificed at 2 hours ($N_{HomeCage}$=8, N_{Paired}=7, $N_{Unpaired}$=7), 6 hours ($N_{HomeCage}$=8, N_{Paired}=8, $N_{Unpaired}$=8), and 12 hours ($N_{HomeCage}$=8, N_{Paired}=8, $N_{Unpaired}$=8) after exposure to the fear conditioning protocol. Brains were extracted and frozen immediately on dry ice and 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 was kept at -23.0°C. Using a 1.0 mm biopsy tool, bilateral punches of the brain were made to collect amygdala (Bregma -0.94 mm to -2.3 mm) and

hippocampus (Bregma -1.94 to -2.54) tissue (Franklin & Paxinos, 2001). The mRNA from these tissues was then extracted using the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Cat: 80004). Specifically, samples were homogenized with Fisher Scientific Sonic Dismembrator Model 100 on level 2 in Buffer RLT without β-mercaptoethanol. The Buffer RW1 washing step was not performed. All other procedures were carried out according to the included manual. mRNA concentration was detected using the Thermo Scientific Nanodrop 1000. cDNA synthesis from these mRNA samples was performed using the SABiosciences™ RT^2 First Strand Kit (Cat: 330401).

mRNA Quantification

TaqMan® Quantiative RT-PCR was performed using the TaqMan® Universal PCR Master Mix, 70-90 ng cDNA synthesized from the SABiosciences™ RT^2 First Strand Kit, and primers Applied Biosystems® Mouse GAPD (GAPDH) Endogenous Control (FAM™/MGB Probe, Non-Primer Limited), Mouse Notch1 (Mm00435249_m1), Mouse Notch2 (Mm00803077_m1), Mouse Dll1 (Mm01279269_m1), Mouse Jag1 (Mm00496902_m1), Mouse Hes1 (Mm01342805_m1), Mouse Hes5 (Mm00439311_g1), or Mouse Hey1 (Mm00468865_m1). Plate was run in the Applied Biosystems 7500 Fast Real-Time PCR System under the Standard 7500 run mode (1 cycle 50.0°C, 2 min; 1 cycle 95.0°C, 10 min; 40 cycles 95.0°C, 15 sec and 60°C, 1 min with fluorescence measured during 60°C step). Data were then analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Behavioral Testing with Gamma Secretase Inhibitor (Fig. 12)

Mice were habituated to the fear chambers (grid floors, room light on, cleaned with quatricide: Context A) for five minutes each for two consecutive days. The following day, mice were exposed to a 5-trial fear conditioning training paradigm in which 5 conditioned stimuli (70-80 dB, 6 kHz, 30 sec tone) were co-terminated each with an unconditioned stimulus (0.6 mA, 500 ms electrical shock), with a 3 minute acclimation period before the first tone and a 2 minute ITI. Ten minutes after training, mice were injected i.p. with either Vehicle (0.1 mL, 100% DMSO) or gamma 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, mice were placed in a different context (flat floors, room light off, red light on, cleaned with ethanol: Context B) to test for consolidation of cued fear memory, during which 30 conditioned stimuli (70-80 dB, 6 kHz, 30 sec tone) were played. 48 hours later, mice were placed in Context A for 10 min to determine consolidation of contextual fear memory. All data were analyzed with Freezeframe software as previously described (Maguschak & Ressler, 2008).

Statistical Analysis

We determined significance of mRNA expression levels across groups using One-Way Randomized ANOVA and post-hoc comparisons through the Tukey's Honestly Significant Difference (Tukey's HSD) test. For all behavioral analyses, the Student's t-test measured all significance levels. Significance was set at $p < 0.05$.

RESULTS

Transient Decrease in mRNA Levels of Genes along the Notch Pathway After Cued Fear Conditioning in the Amygdala and Hippocampus (Table 1, 2, 3, 4)

To determine the mRNA landscape of Notch signaling after cued fear conditioning, animals were habituated to startle chambers to limit contextual fear association and then tested on either a CS/US paired or CS/US unpaired protocol. The brains from these mice were then harvested at 2, 6, or 12 hours after the termination of their training for mRNA extraction, cDNA synthesis, and analysis using quantitative RT-PCR from both hippocampal and amygdala tissue punches (Fig. 3). As cued fear conditioning is a primarily amygdala-dependent process (Phillips & LeDoux, 1992; LeDoux, 1995; Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Anagnostaras, Maren, & Fanselow, 1999; Marschner, Kalisch, Vervliet, Vansteenwegen, & Büchel, 2008), the hippocampus was intended to serve as an anatomical control (Fig. 3).

Analysis of the amygdala punches demonstrated a significant reduction in Notch pathway mRNA levels at primarily 2 and 6 hours after fear conditioning. Jag1 mRNA levels were significantly reduced at the 2 hour time point [$F(2,19) = 42.058$, $p<0.001$**] in both the paired and unpaired groups as compared to home cage. At 6 hours [$F(2,20) = 4.569$, $p = 0.023$*], the paired group was downregulated as compared to the unpaired group. There was no significant differential effect among groups at the 12-hour mark [$F(2,19) = 1.701$, $p = 0.209$] (Fig. 4). Dll1 mRNA levels showed similar regulation at the 2-hour mark [$F(2,19) = 27.863$, $p<0.001$**], as both the paired and unpaired groups were reduced as compared to home cage. There, however, was no significant difference in mRNA levels among the groups at 6 hours [$F(2,20) = 0.175$, $p = 0.841$] or 12 hours

[F(2,20) = 0.182, p = 0.835] (Fig. 5). Notch1 mRNA levels also were also decreased in the paired and unpaired groups at the 2-hour time point [F(2,19) = 17.562, p < 0.001**] as compared to home cage. At 6 hours [F(2,19) = 3.669, p = 0.045*], the paired group displayed lower mRNA levels as compared to the unpaired group. No significant dissimilarities were present between groups at 12 hours after fear conditioning [F(2,20) = 0.243, p = 0.786] (Fig. 6). There was no significant regulation among any group at the 2-, 6-, and 12-hour mark in Notch2 [F(2,19) = 0.369, p = 0.696; F(2,20) = 1.856, p = 0.182; F(2,20) = 0.139, p = 0.871] (Fig. 7) or Hes1 [F(2,19) = 1.120, p = 0.347; F(2,20) = 1.003, p = 0.384; F(2,20) = 0.004, p = 0.996] (Fig. 8). Hes5 mRNA levels were reduced at 2 hours [F(2,19) = 11.885, p<0.001**] after fear conditioning in both the paired and unpaired groups as compared to home cage. Yet, there was no significant difference among groups at the 6- and 12-hour time points [F(2,16) = 0.825, p = 0.453; F(2,20) = 1.323, p = 0.289] (Fig. 9). Hey1 showed no significant effects 2, 6, or 12 hours after fear conditioning [F(2,19) = 0.383, p = 0.687; F(2,20) = 0.533, p = 0.597; F(2,19) = 0.849, p = 0.443] (Fig. 10).

Within the hippocampus, RT-PCR was performed on the samples from the home cage, paired, and unpaired groups at the 2-hour mark. For Jag1 [F(2,19) = 4.202, p = 0.031*], unpaired was significantly lower than home cage. Results were similar for Dll1 [F(2,19) = 5.465, p = 0.013*]: unpaired was significantly reduced when compared to home cage (Fig. 11A). There were no regulation differences between any group for Notch1 [F(2,19) = 0.017, p = 0.983], Notch2 [F(2,19) = 1.803, p = 0.192], Hes1 [F(2,19) = 1.075, p = 0.361], Hes5 [F(2,19) = 0.442, p = 0.649], or Hey1 [F(2,19) = 0.079, p = 0.925)] (Fig. 11B, 11C).

A Single Systemic DAPT Injection Enhances Consolidation of Cued and Contextual Fear Memories

To examine the functional role of the Notch signaling pathway in memory consolidation, mice were injected with a known γ-secretase inhibitor, DAPT, or a vehicle i.p. 10 minutes after fear training. Mice were then tested the next day in a different context to test freezing behavior in response to the tone alone (consolidation of cued fear memory). 48 hours later, mice were then re-exposed to the original context for 10 minutes in the absence of tone or shock to determine consolidation of contextual fear memory, using total freezing behavior (Fig. 12).

During fear conditioning, mice to be injected with DAPT and those to be injected with vehicle showed no significant difference between their abilities to acquire the cued fear memory [Pre-CS, $t(21) = 0.34$; CS, $t(21) = 0.71$] (Fig. 13). 24 hours later though, there was a significant enhancement in freezing behavior in the DAPT-injected group as compared to the vehicle-injected group when exposed to the tone alone [$t(21) < 0.001$**]. Before the exposure to the CS, the groups showed no significant difference in freezing behavior [$t(21) = 0.68$] (Fig. 14). When returned to the original context 48 hours later in the absence of tone or shock, DAPT-injected mice froze more in both the first and second five minutes of the 10 minute trial [$t(21) < 0.001$**; $t(21) < 0.001$**] as compared to those injected with vehicle (Fig. 15). Together, these data indicate that the DAPT-injected group had augmented consolidation of cued and contextual fear memory.

DISCUSSION

Notch Pathway mRNA Levels are Attenuated in the Amygdala and Hippocampus After Cued Fear Conditioning

We first found that the mRNA levels of different genes along the Notch signaling pathway are transiently downregulated in the amygdala after cued fear conditioning. Notch1 in the paired and unpaired groups were decreased as compared to home cage, respectively, at the 2-hour mark. At 6 hours, the unpaired group's mRNA levels had returned to non-significant levels as compared to home cage, but the paired group remained downregulated as compared to the unpaired group. Each group had returned to similar levels at the 12-hour mark. Hes5 showed a similar pattern of downregulation at the 2-hour mark, as both the paired and unpaired groups were reduced when compared to home cage. This effect in Hes5 was not apparent 6 hours after fear conditioning or the 12-hour time point. Jag1 and Dll1 also showed downregulation in the unpaired and paired groups at 2 hours after fear conditioning as compared to home cage. Jag1 levels were also reduced at the 6-hour mark as compared to the unpaired group, but Dll1 levels returned to non-significant levels 6 hours after fear conditioning. All groups in both ligands were returned to baseline levels at 12-hours after fear conditioning. Notch2, Hes1, and Hey1 showed no significant change at any time point tested.

Differences between the paired and unpaired groups indicate a learning-specific result. 6 hours after our paired learning paradigm, there was an alteration in the mRNA expression of genes at different time points on each part of the pathway: ligands (Jag1, Dll1) and receptor (Notch1). Moreover, each of these regulated genes had no differential expression at the 12-hour mark. As this time course is consistent with other molecules associated with memory consolidation within the amygdala (Rattiner, Davis, French, &

Ressler, 2004; Johansen et al., 2011), the regulation of these genes within the amygdala may be necessary for the consolidation of fear memory. Although the time course of these data are different from current literature on post-developmental Notch signaling, in which Notch pathway mRNA levels have been shown to be decreased in the hippocampus 12 hours after a hippocampal-dependent passive avoidance task (Conboy et al., 2007), the window of fear memory formation may be different between auditory fear conditioning and passive avoidance.

To our surprise, at the 2-hour mark, there also was a downregulation of the same genes (Jag1, Dll1, Notch1, Hes5) in both the paired and unpaired groups. There are two possible explanations for this result. First, this similar regulation that seems independent of learning indicates that the results from the 2-hour time point may be part of a 'stress response.' As both groups were shocked, it is possible that this downregulation is not learning-dependent but rather shock-dependent. The amygdala is a primary output center for the stereotyped behavioral and physiological fear responses (Cassell et al., 1986; Ehrlich et al., 2009; McDonald, 1982; Sah, Faber, Lopez De Armentia, & Power, 2003; Davis et al., 1994), so the shock during each group's behavioral paradigm could be triggering these reactions that would be potentially affecting Notch pathway mRNA levels.

Another plausible reason for this change in mRNA expression, though, is that the unpaired group was not a 'non-associative fear-learning' control, as ideally planned, but rather, in part, a contextual conditioning control. This possibility exists because the context is the only consistent element for the animals during multiple episodes of shock. 2 hours after fear conditioning, there was a significant decrease in the unpaired group in

the ligands only, Jag1 and Dll1within the hippocampus. There was no significant decrease in the paired group of these same genes, nor was there any other significant regulation in any other gene tested in either group. The fact that the only genes that displayed downregulation in the hippocampus also had a similar decrease in the amygdala indicates that this may be a context-dependent process, especially because there is considerable evidence that the hippocampus underlies only contextual fear memory formation, whereas the amygdala is functional in both cued and contextual fear memory formation (Phillips & Ledoux, 1992; LeDoux, 1995; Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Anagnostaras et al., 1999; Marschner et al., 2008). Moreover, this effect in Jag1 matches previous literature that has demonstrated a transient downregulation in Jag1 mRNA levels in the hippocampus after passive avoidance training in rats (Conboy et al., 2007).

Interestingly though, Conboy et al. also demonstrated a significant decrease in Notch1 mRNA levels in the hippocampus after the passive avoidance paradigm, yet our results indicate a significant decrease in the unpaired group only within the amygdala. This could either imply that Notch1 regulation is specific to the passive avoidance mechanisms of consolidation, which is also known to be hippocampal-dependent. Alternatively, the shock-response and context conditioning hypotheses are not mutually exclusive, in that Notch1 may be regulated in the unpaired group in the amygdala as a result of the shock alone, and Jag1 and Dll1 show decreases in the amygdala and hippocampus because of unknown contextual conditioning consolidation mechanisms. Because Hes5 shows similar regulatory effects as Notch1 in the amygdala and

hippocampus, yet it has not been previously examined, these same conclusions apply to the effector as well.

To investigate these possibilities, a future study might incorporate another mRNA correlational analysis using an unpaired, paired, and newly added tone-alone group. These animals would be habituated in the same manner as before, yet during the fear training, they would be exposed to 5 CS without any US. These mice would be a truly amygdala-dependent 'non-associative learning' control for the unpaired and paired learning groups because there are no aversive stimuli present to cause any significant amygdala activation.

The specific regulation of solely Hes5 and not Hes1 in the amygdala also suggests the possibility of a Notch-specific role in amygdala-dependent fear memory formation. Hes1 has been reported to function through both Notch and non-Notch signaling, while Hes5 transcription is solely dependent on Notch signaling (Kageyama et al., 1997). So, there is a possibility that the lack of change in Hes1 is a result of compensatory mechanisms through other pathways, and the regulation of Hes5 mRNA is an indicator of decreased Notch signaling at the protein level as well.

It is important to note another limitation of our study – that mRNA levels can only serve as proxies for signaling. A decrease in mRNA could be a faithful read-out of Notch signaling, but it could also serve as a compensatory mechanism for increased Notch signaling. It is therefore necessary to perform protein analysis through western blot and immunohistochemistry to determine the landscape of the functional network of Notch signaling. Through these tests, it would be possible to elucidate if there is a marked increase in NICD and effector protein levels, which would confirm that

downstream mechanisms are being modulated by this system. mRNA results alone only give the probability of these mechanisms to take place rather than a direct functional analysis. Immunohistochemistry, in combination with quantitative immunoblotting, would give spatial and functional representation of each of these signaling pathways, elucidating the regional specificity of these signaling effects.

DAPT Enhances Cued and Contextual Fear Memory Consolidation

To begin to functionally dissect the role of Notch signaling in fear memory consolidation, Notch signaling blockade using the γ-secretase inhibitor, DAPT, also showed significant changes in fear memory consolidation behavioral tests. Before I.P. injection, acquisition of fear memory was similar between animals to be injected with DAPT and those to be injected with vehicle. During testing for consolidation of cued fear memory, there was a significant increase in freezing behavior in the DAPT-injected group as compared to the vehicle-injected group. Also, during testing for the consolidation of contextual fear memory, the DAPT-injected animals showed increased freezing behavior as compared to the vehicle-injected group.

Although γ-secretase is necessary for Notch signaling (Ables et al., 2011; Bray, 2006; Louvi & Artavanis-Tsakonas, 2006), its activity is not specific to Notch receptor cleavage. Most prevalent of these non-Notch signaling functions is its role in the cleavage of amyloid precursor protein (APP) to form β-amyloid, which is heavily implicated in Alzheimer's Disease (Kimberly et al., 2003; Yu et al., 2000; Lee et al., 2002; Steiner et al., 2002; Dash, Moore, & Orsi, 2005). Previous literature has in fact shown that treatment of familial Alzheimer's mutant mice with oral DAPT rescues contextual fear conditioning deficits (Comery et al., 2005). Thus, the cued and contextual memory

enhancement that we find may occur through a combination of the effects of many different signaling blockades. However, the γ-secretase inhibitors in the Alzheimer's field are injected consecutively over several days, while we injected the animals only once. Furthermore, because the animals were injected i.p., the DAPT effect is occurring systemically, not just within the amygdala. Although habituation prior to CS/US pairing should limit the contextual conditioning to the chamber, the DAPT animals show a marked increase in consolidation of contextual fear memory, indicating recruitment of the hippocampus or amygdala via DAPT. Despite these potential confounds, our results remain consistent with previous literature that has shown that an intra-hippocampal infusion of DAPT enhances contextual and spatial memory tasks (Dash et al., 2005), demonstrating that γ-secretase plays a distinct role in the process of memory consolidation. We hypothesize that its role in memory consolidation may be primarily through its effects on inhibiting Notch1 signaling.

To establish a definitive role for Notch signaling in amygdala-dependent memory formation, it will be necessary to manipulate the pathway specifically within the amygdala. This could be done through infusions of a genetically modified lentiviral vector containing a constitutively active promoter for Hes1, Hes5, or Hey1 into the amygdala to enhance Notch activity; or via cannula implants into the amygdala for site-specific injections of DAPT or Notch antibodies (Ables et al., 2011).

Conclusions

Fear and anxiety are evolutionarily conserved mechanisms that can be beneficial for survival. These mental states are mediated by a group of connected brain structures called the 'extended amygdala' and its regulatory regions, with its namesake, the

amygdala, as the functional focal point of the system. Previous literature indicate that various protein signaling pathways are necessary for memory formation within the amygdala, and there is a broadening mechanistic focus on pathways crucial for development, including Notch receptor signaling. Our results are the first known to demonstrate Notch pathway mRNA decrease in the amygdala after cued fear conditioning. These correlative data are further supported by an enhancement in consolidation of cued and contextual fear memory when mice are treated with the γ-secretase inhibitor, DAPT, which is a potent inhibitor of Notch signaling. Although these studies are not yet comprehensive in establishing the hypothesized role, our results provide convincing support for further analysis of Notch signaling in amygdala-dependent fear memory formation.

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FIGURES

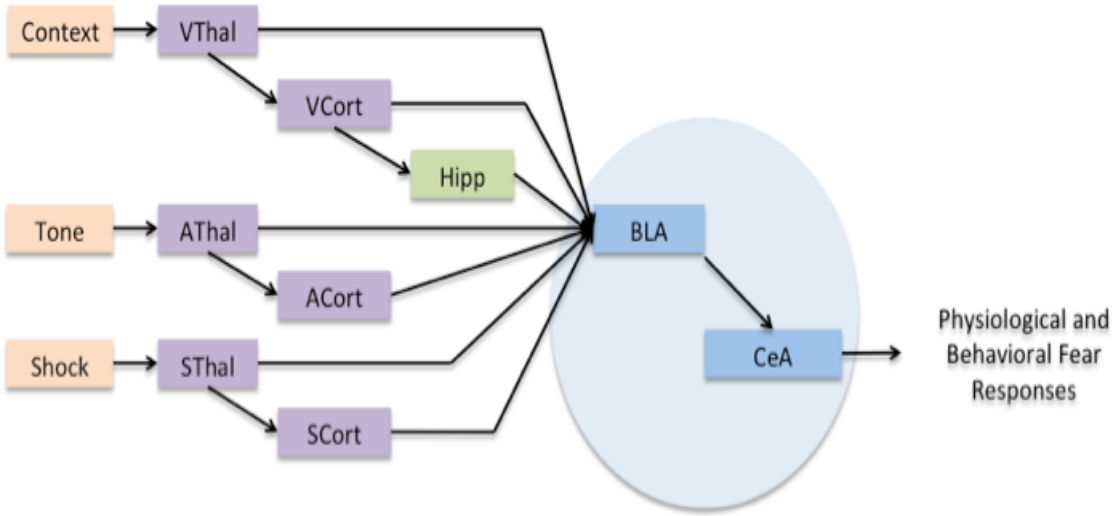

Figure 1: The amygdala is a coincidence detector for cued and contextual fear inputs. *A.* Visually cued and contextual sensory input flows through the visual thalamus (VThal) to the visual cortex (Vcort) and then to the hippocampus (Hipp). Each of these regions also project directly to the lateral amygdala (BLA). *B.* Auditory sensory input flows through the auditory thalamus (AThal) and then to the auditory cortex (ACort). Each of these regions also project directly to the LA. *C.* Somatosensory input flows through the somatosensory thalamus (SThal) to the somatosensory cortex (SCort). Each of these regions also project directly to the BLA. *D.* When the BLA receives coincidental inputs from different sensory circuits, information flows to the central amygdala (CeA), which then controls the different stereotyped physiological and behavioral responses associated with fear.

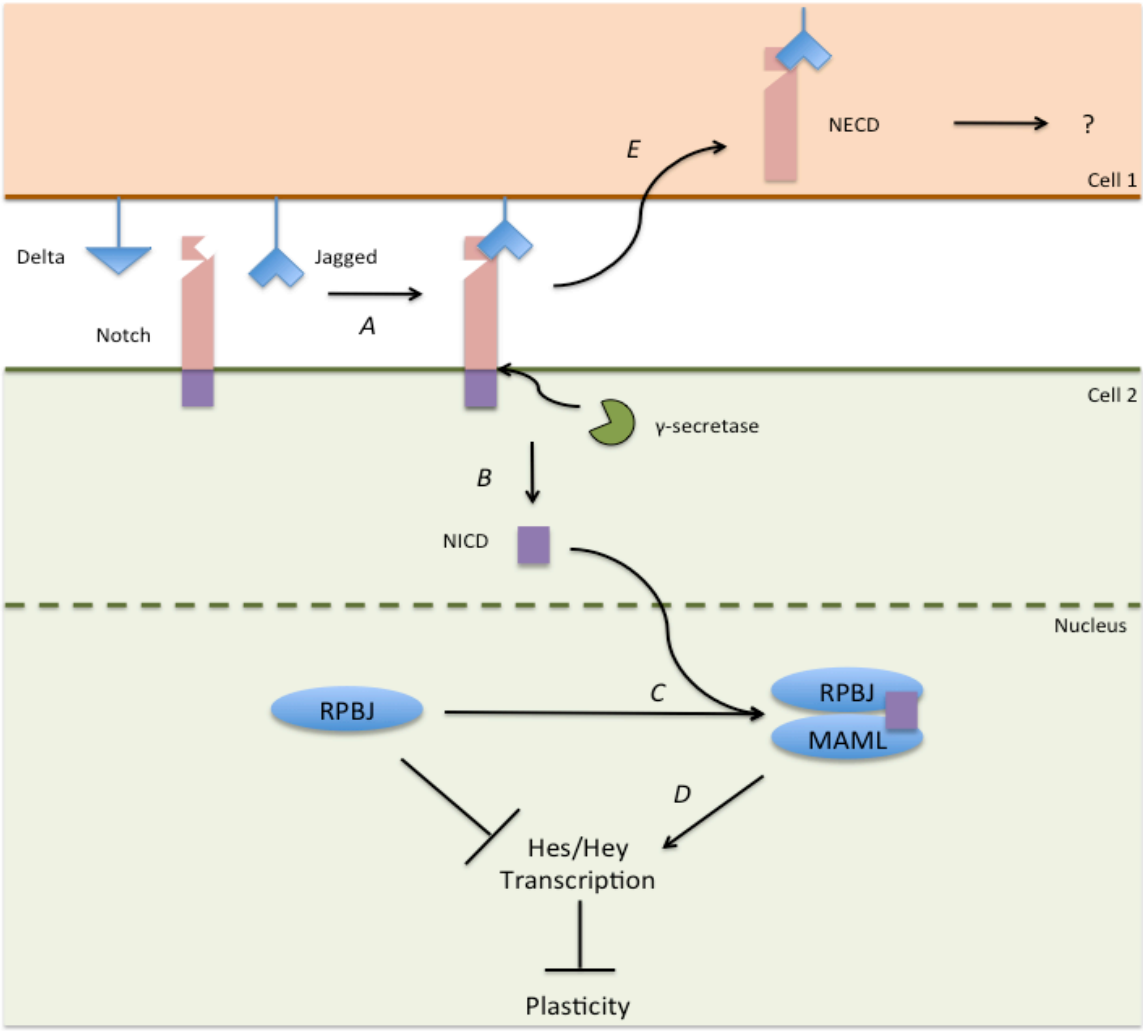

Figure 2: The Notch signaling pathway. *A.* Ligands, Delta and Jagged (Jag), bind to their respective sites on the Notch receptor's extracellular domain (NECD) on a neighboring cell. *B*. The bound ligand causes a conformational change on the intracellular domain (NICD) to cause cleavage of the Notch receptor. *C.* The NICD transits into the nucleus to bind to normally repressing recombining binding protein suppressor of hairless (RPBJ) and mastermind-like protein (MAML). *D.* This protein complex then causes the transcription of hairy enhancer of split (Hes) and Hes-related with YRPF motif (Hey), which each prevent the transcription of proteins that coordinate synaptic and structural plasticity. *E.* The NECD localizes into the ligand-expressing cell with unknown downstream effects.

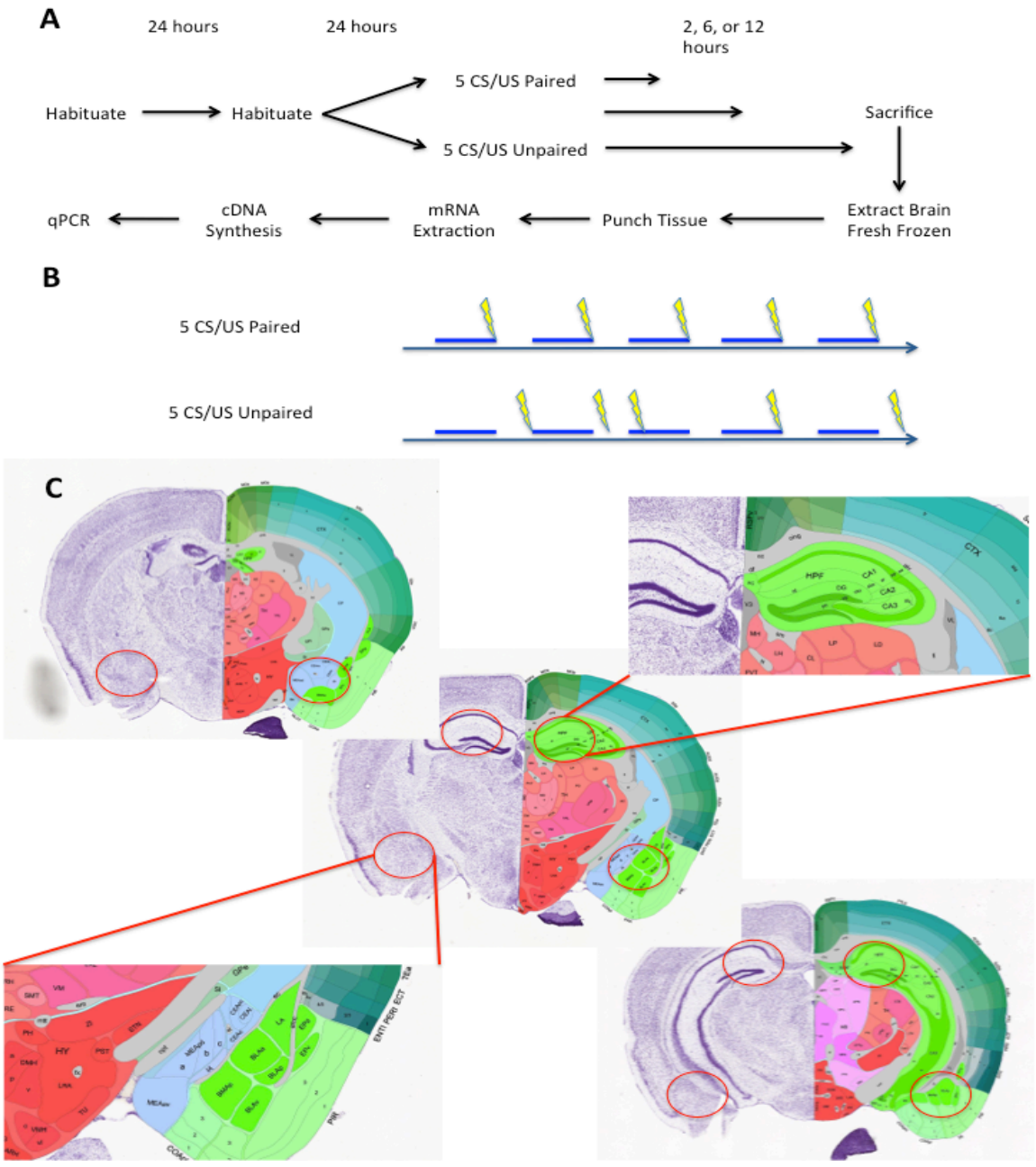

Figure 3: Experimental design to determine mRNA regulation in hippocampus and amygdala after fear conditioning. *A.* Mice were habituated to fear conditioning chambers for 2 days prior to exposure to the 5 CS/US paired or unpaired cued fear conditioning programs. Each group had mice sacrificed at either 2, 6, or 12 hours after termination of behavior, with brains harvested and immediately put onto dry ice. Follow-up was non-time-sensitive and composed of punching the tissue bilaterally at the hippocampus and amygdala, extracting mRNA, synthesizing cDNA, and then a quantitative Real-Time PCR. *B.* Programing of tones (blue) and shocks (bolt) in the 5 CS/US paired and 5 CS/US unpaired fear conditioning protocols. *C.* Allen Mouse Brain Atlas references for the amygdala and hippocampus sites of biopsy punching.

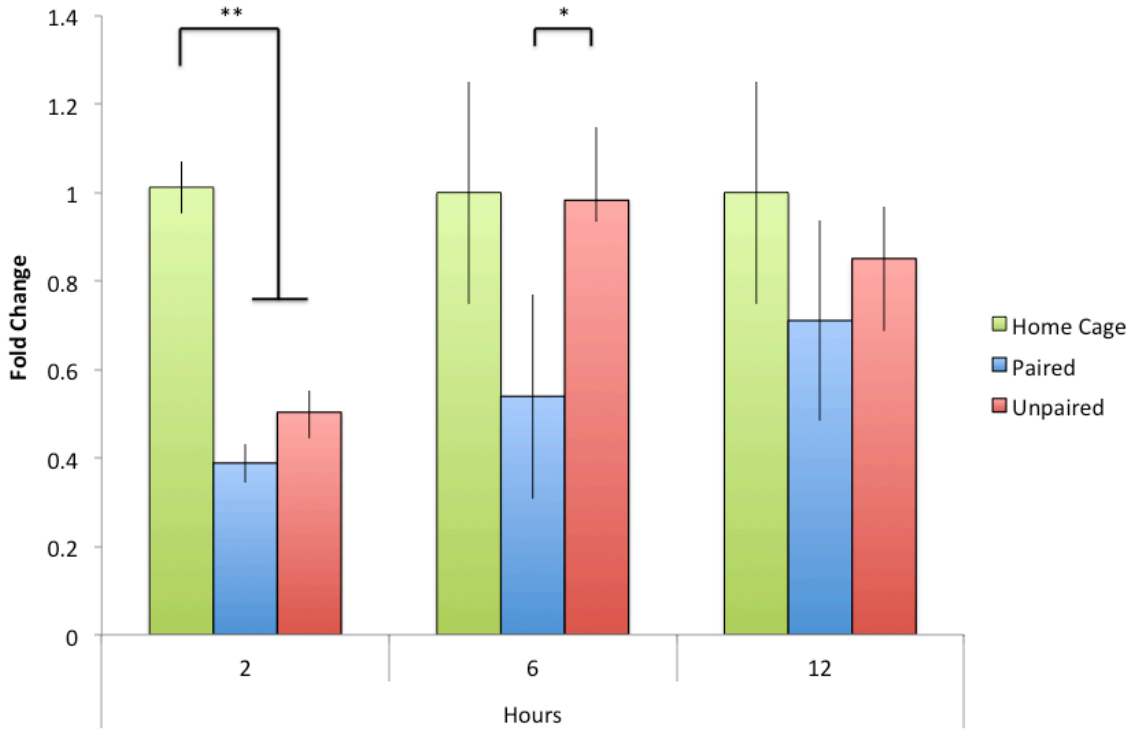

Figure 4: mRNA of ligand Jag1 is transiently downregulated in the amygdala after cued fear conditioning. Jag1 mRNA levels are significantly reduced in the unpaired and paired groups 2 hours after fear conditioning as compared to home cage. At 6 hours, Jag1 is downregulated in only the paired group when compared to unpaired, which displays similar mRNA levels as home cage. There is no significant difference among groups at 12 hours after fear conditioning. Columns and error bars represent mean ± S.E.M. fold change (*P<0.05, **P<0.01).

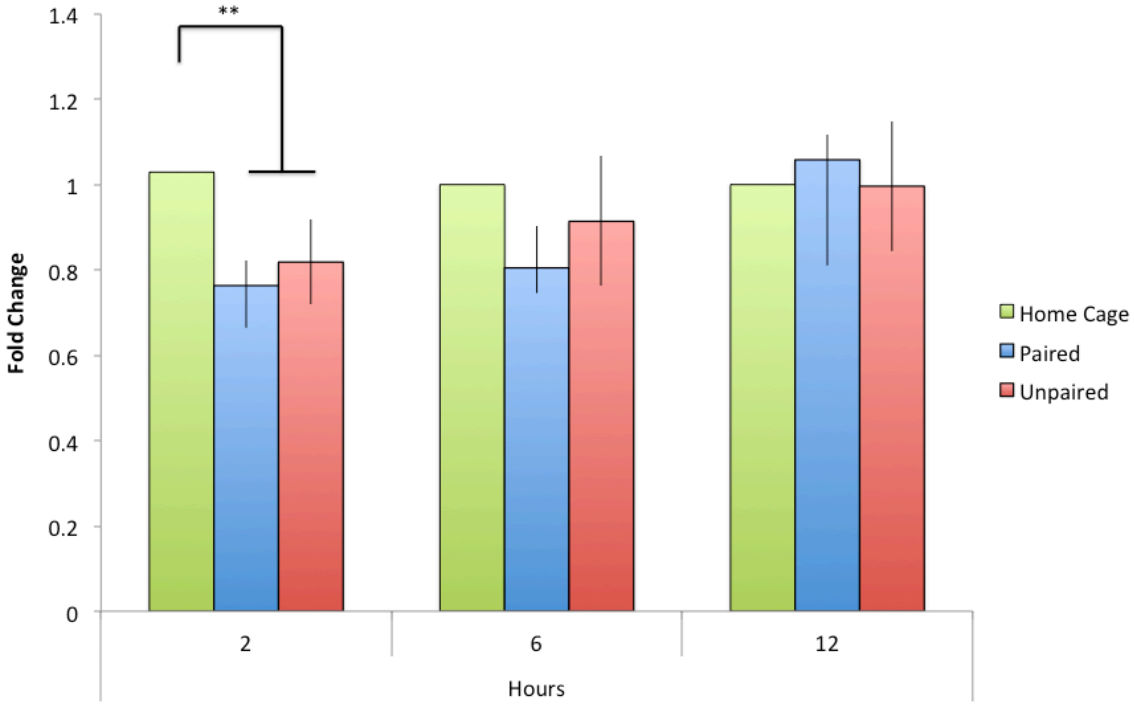

Figure 5: mRNA of ligand Dll1 is transiently downregulated in the amygdala after cued fear conditioning. Dll1 mRNA levels are reduced in both the unpaired and paired groups as compared to home cage at 2 hours after fear conditioning. There are no apparent mRNA changes in any group as compared to home cage at the 6- and 12-hour marks. Columns and error bars represent mean ± S.E.M. fold change (*P<0.05, **P<0.01).

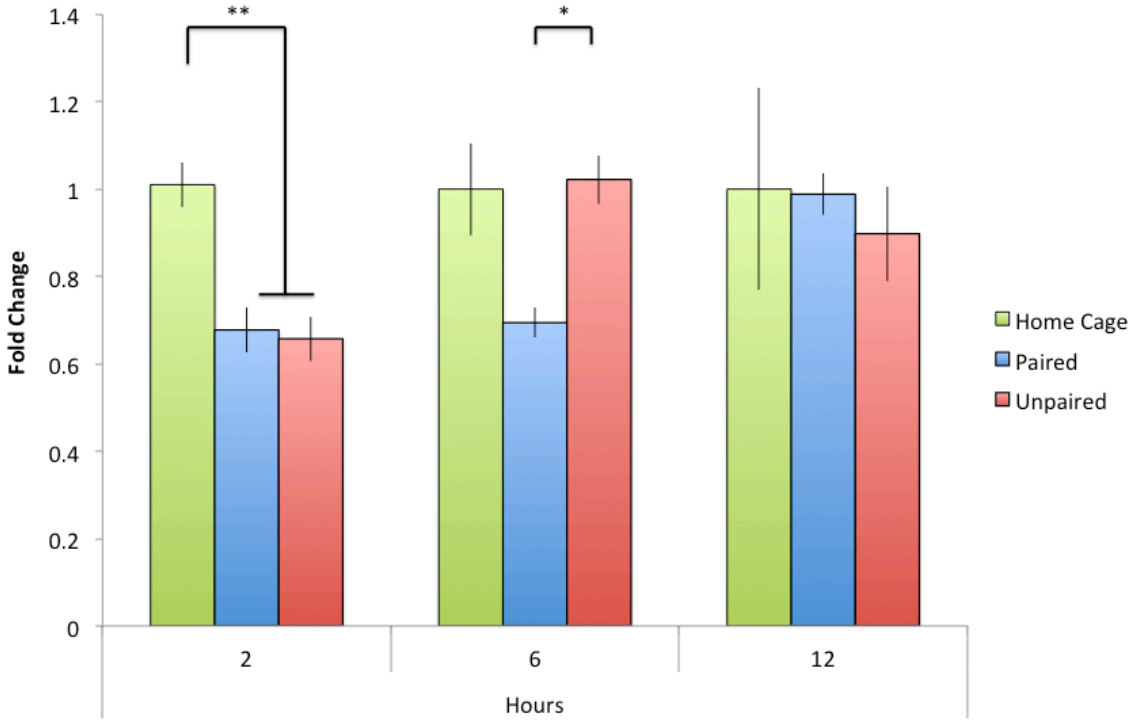

Figure 6: mRNA of receptor Notch1 is transiently downregulated in the amygdala after cued fear conditioning. Notch1 mRNA levels are reduced in the paired and unpaired groups at 2 hours after fear conditioning as compared to home cage. At 6 hours, mRNA levels in the paired group are decreased as compared to the unpaired group, with each showing no significant difference as compared to home cage. There is no significant effect at the 12-hour mark. Columns and error bars represent mean ± S.E.M. fold change (*P<0.05, **P<0.01).

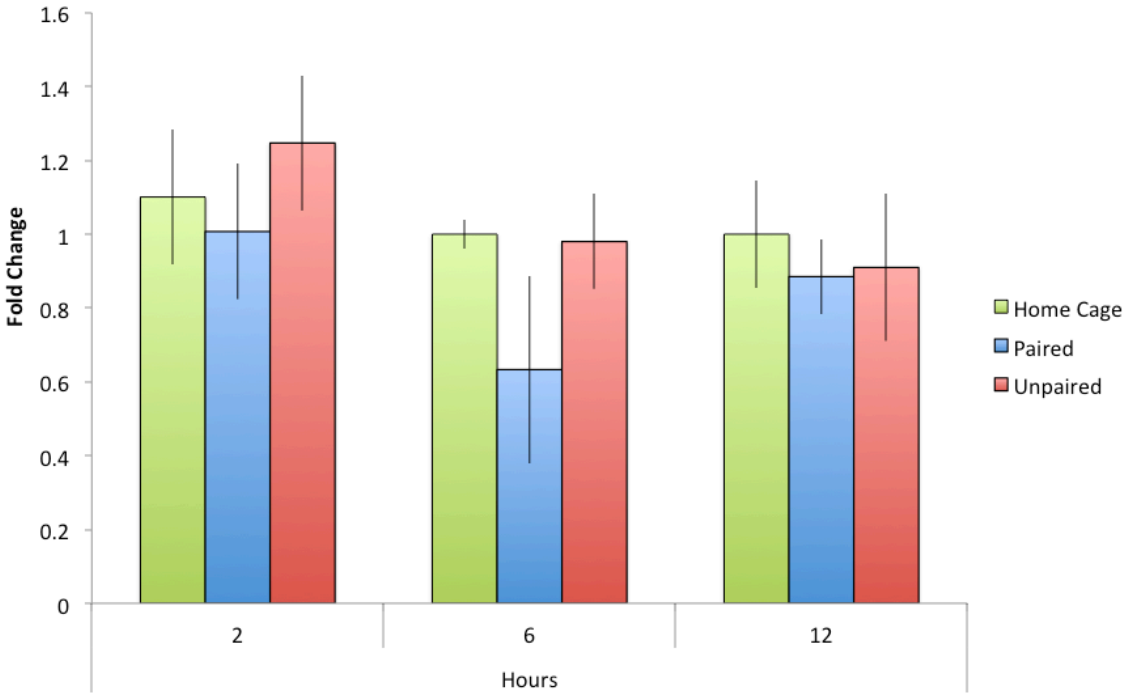

Figure 7: mRNA of receptor Notch2 is unchanged in the amygdala after cued fear conditioning. There are no significant changes in Notch2 mRNA expression among groups at any time point after fear conditioning. Columns and error bars represent mean ± S.E.M. fold change.

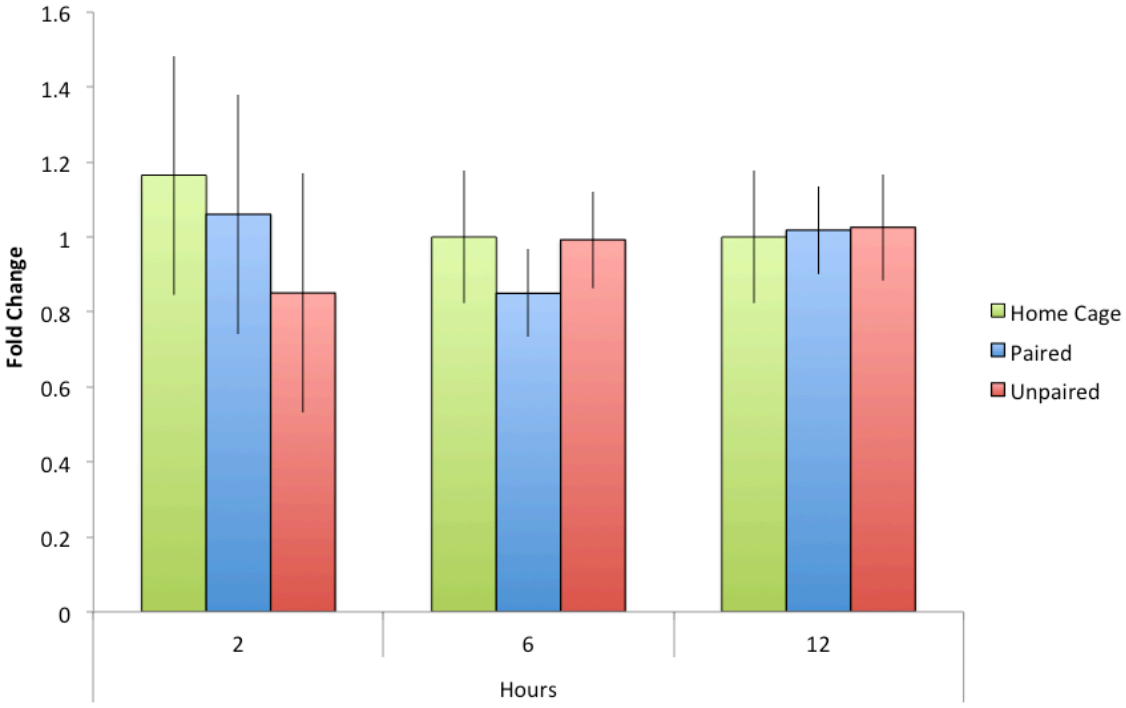

Figure 8: mRNA of receptor Hes1 is unchanged in the amygdala after cued fear conditioning. There are no significant differences in Hes1 mRNA levels among groups at any time point after fear conditioning. Columns and error bars represent mean ± S.E.M. fold change.

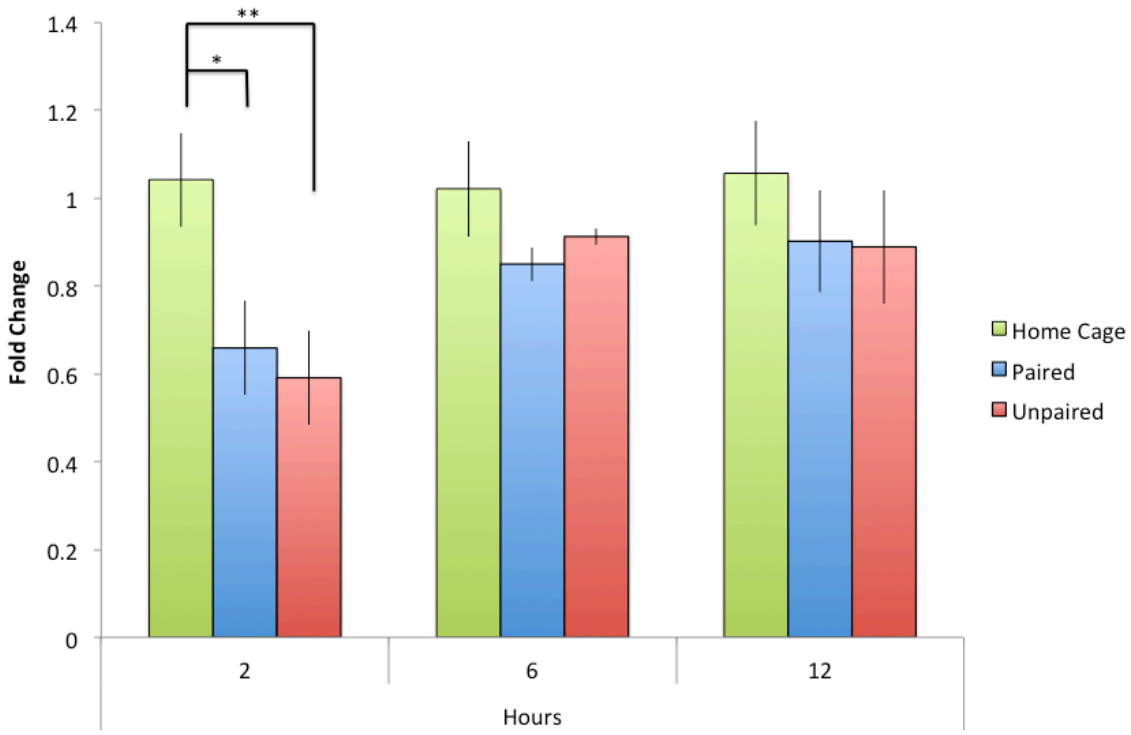

Figure 9: mRNA of receptor Hes5 is transiently downregulated in the amygdala after cued fear conditioning. Hes5 mRNA levels are reduced in both paired and unpaired groups 2 hours after fear conditioning. There are no significant differences at the 6- or 12-hour marks. Columns and error bars represent mean ± S.E.M. fold change (*P<0.05, **P<0.01).

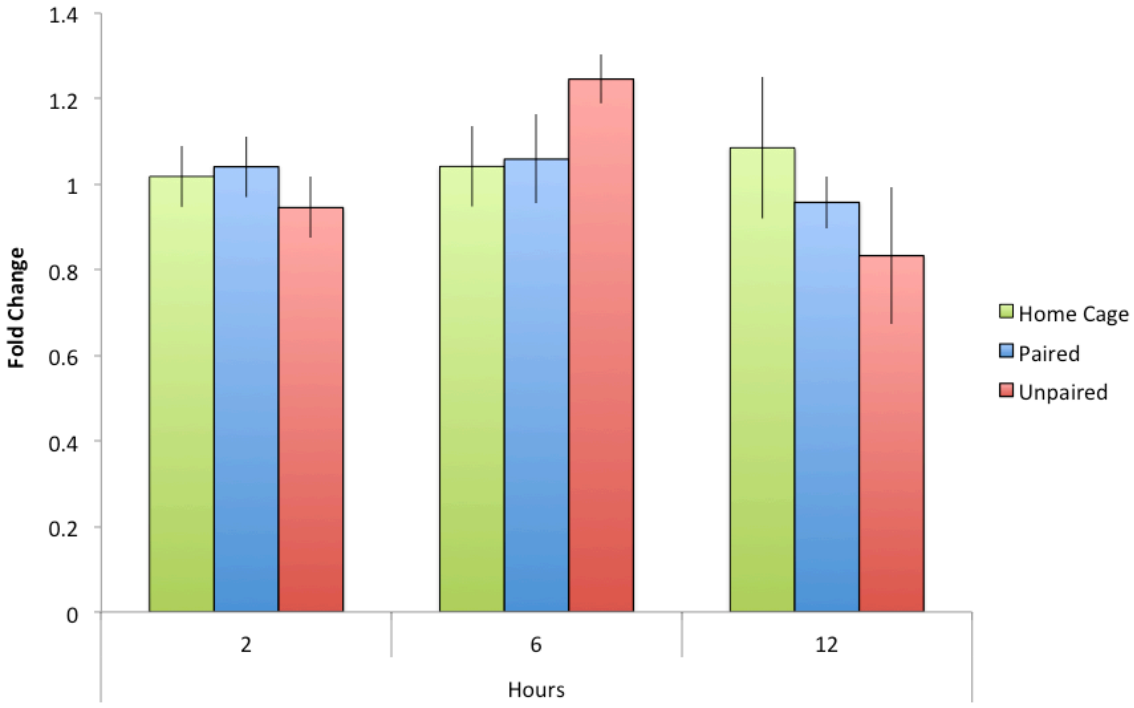

Figure 10: mRNA of receptor Hey1 is unchanged in the amygdala after cued fear conditioning. There are no significant differences in Hey1 mRNA levels at 2, 6, or 12 hours after fear conditioning. Columns and error bars represent mean ± S.E.M. fold change.

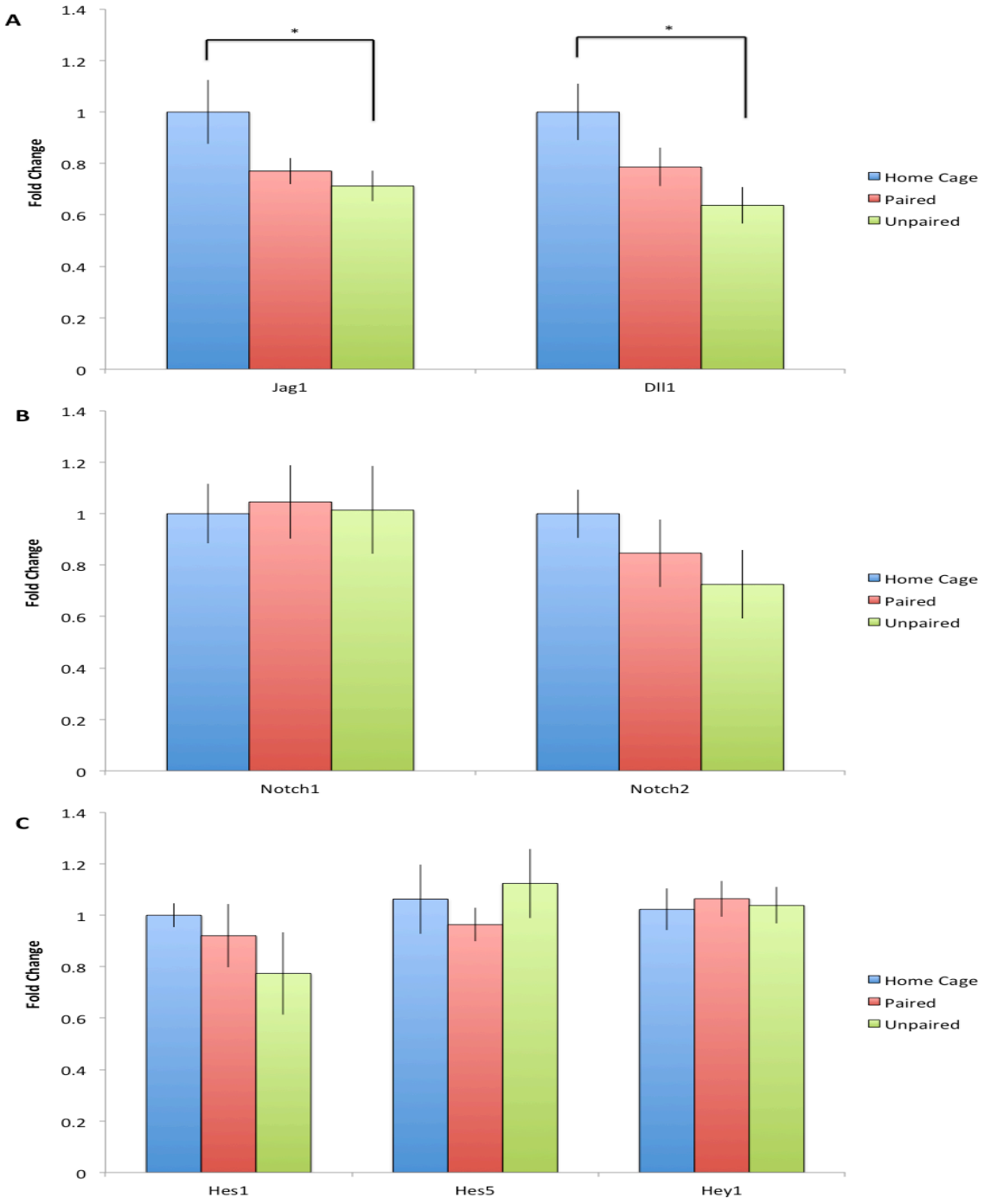

Figure 11: Jag1 and Dll1 mRNA levels are reduced in the hippocampus 2 hours after cued fear conditioning only in the unpaired group. *A.* Ligands Jag1 and Dll1 show reduced mRNA levels in the unpaired group as compared to home cage 2 hours after fear conditioning. *B.* Receptors Notch1 and Notch2 are unregulated at 2 hours after fear conditioning. *C.* Effectors Hes1, Hes5, and Hey1 show no mRNA level alterations 2 hours after fear conditioning. Columns and error bars represent mean ± S.E.M. fold change (*P<0.05, **P<0.01).

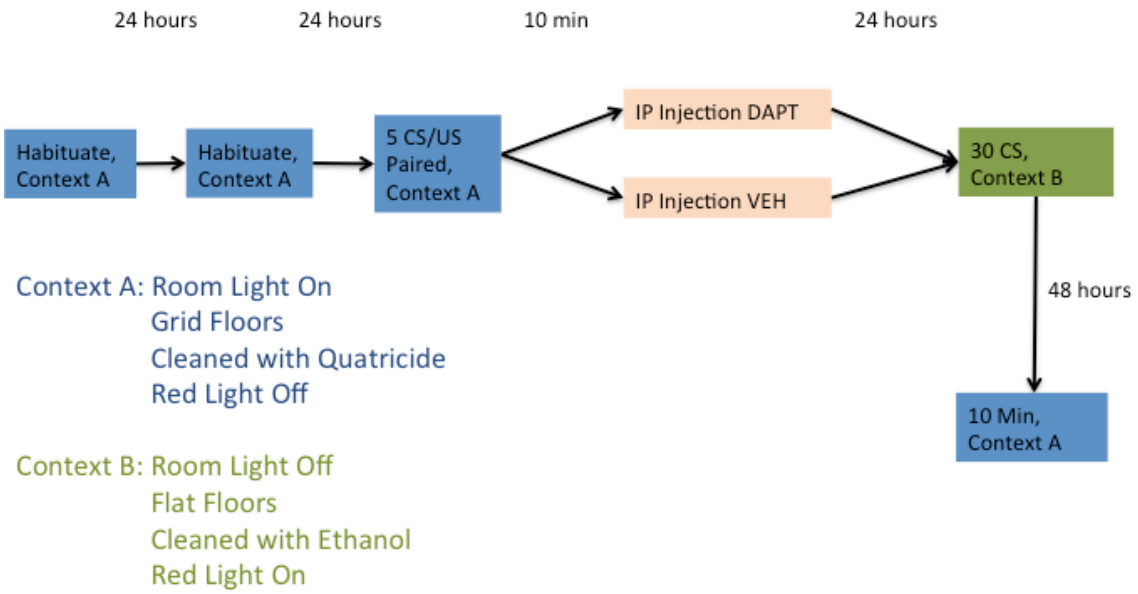

Figure 12: Experimental design for behavioral testing of animals injected with γ-secretase inhibitor, DAPT, against vehicle. Mice were habituated to context A (room lights on, grid floors, chambers cleaned with quatricide, red light off) for 2 days and then exposed to 5 CS/US pairings in the same context. 10 minutes after training, animals were either injected with DAPT or vehicle. 24 hours later, mice were exposed to context B (room lights off, flat floors, cleaned with ethanol, red light on) and exposed to 30 CS for cued fear memory consolidation testing. 48 hours later, animals were re-exposed to context A for 10 minutes each for testing of contextual fear memory consolidation.

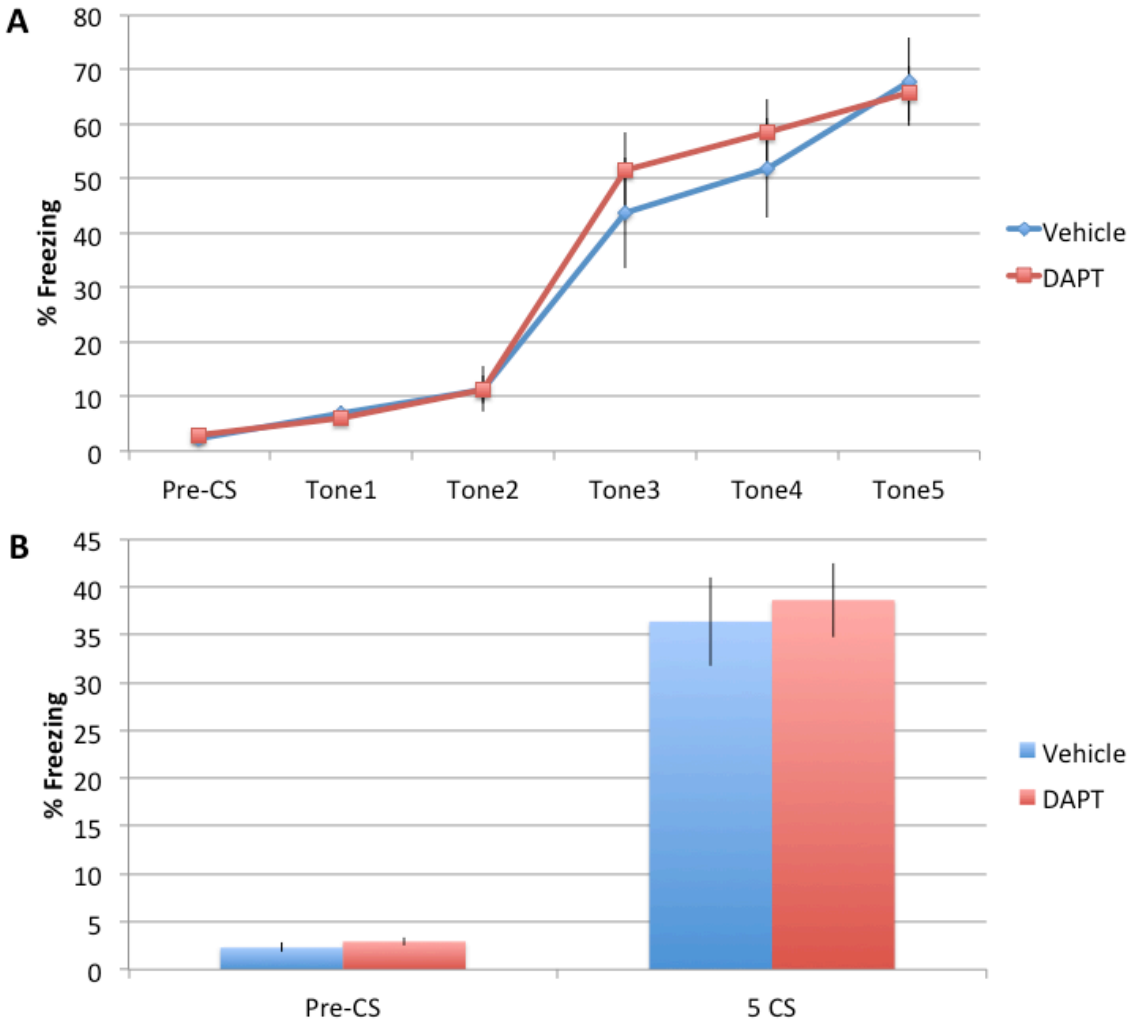

Figure 13: The acquisition of fear memory is similar in vehicle and DAPT groups. *A.* Percent freezing before the initiation of the CS and during each tone are similar in animals to be injected with DAPT and those to be injected with vehicle. *B.* Average percent freezing before the first CS and during all CS is not different in both groups. Symbols and columns with error bars represent mean ± S.E.M. percent freezing.

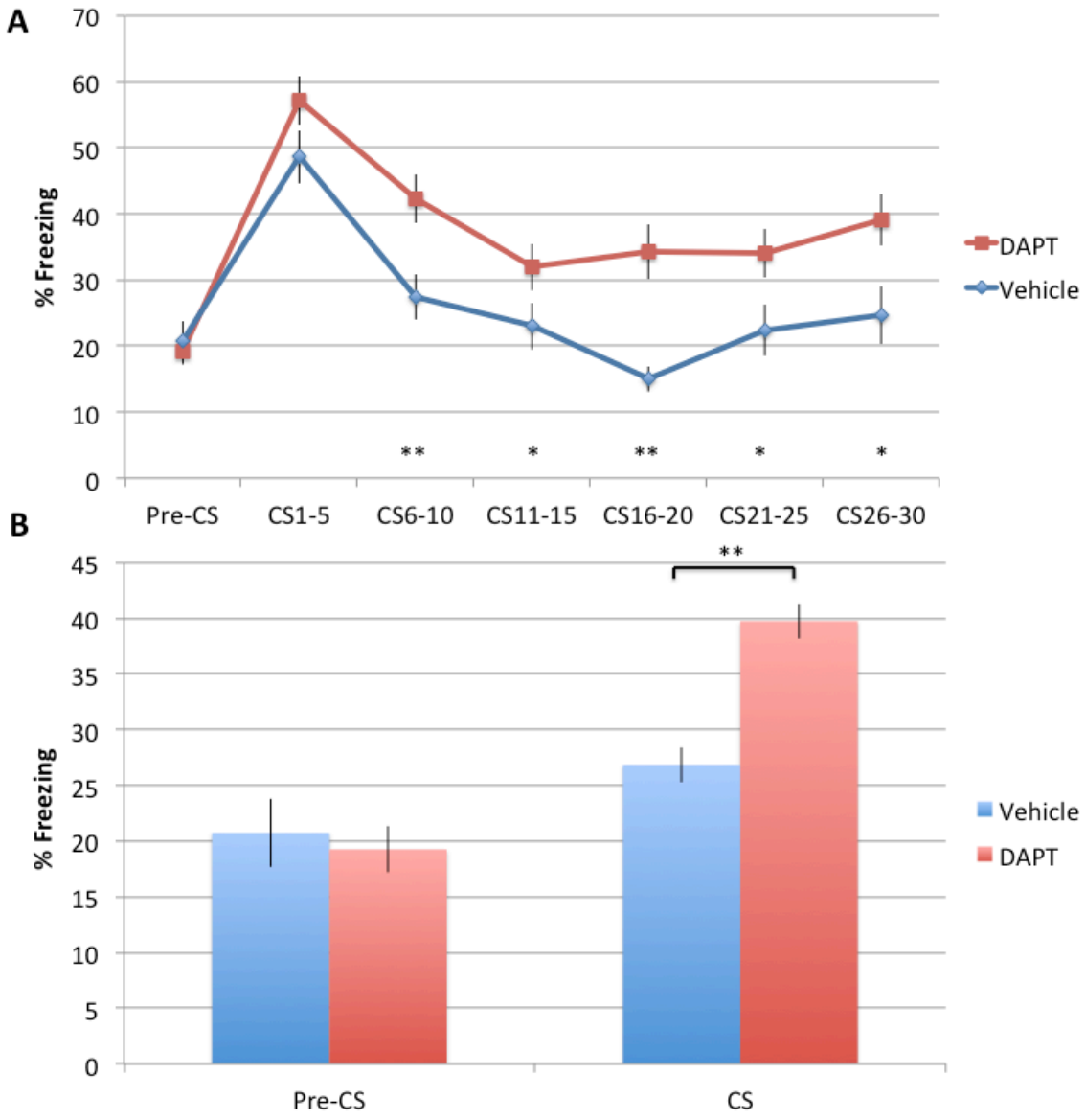

Figure 14: DAPT-injected animals show enhanced cued fear memory. *A.* Mice injected with DAPT show a higher percent freezing when exposed to CS alone as compared to vehicle-injected groups. *B.* Average percent freezing shows a significant increase in freezing when exposed to CS and no differences before the onset of any tone. Symbols and columns with error bars represent mean ± S.E.M. percent freezing (*P<0.05, **P<0.01).

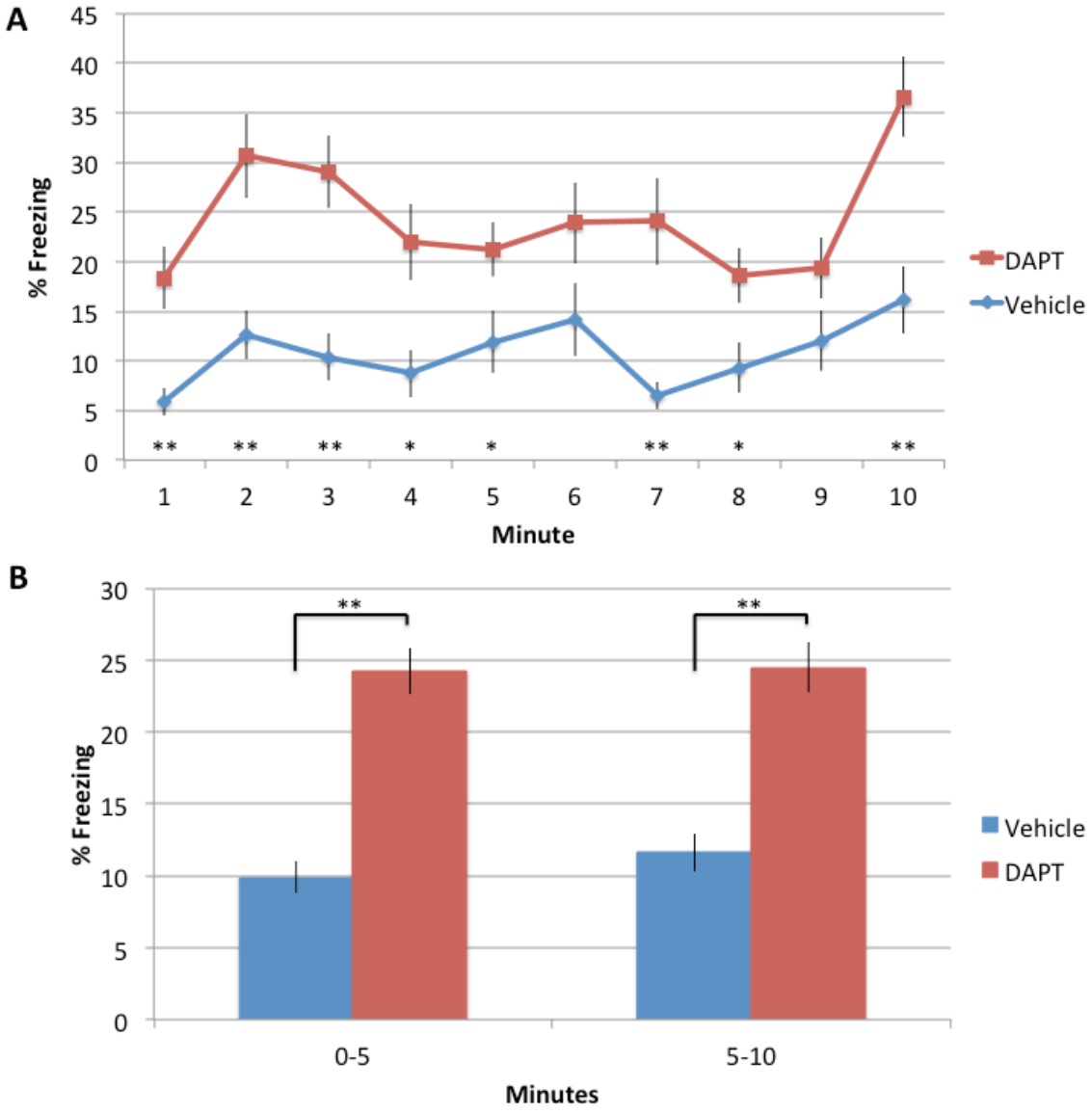

Figure 15: DAPT-injected animals show enhanced contextual fear memory. *A.* DAPT-injected mice show higher percent freezing when exposed only to the initial training context as compared to vehicle-injected animals. *B.* A significant similar is present when grouped in 5 minute intervals. Symbols and columns with error bars represent mean ± S.E.M. percent freezing (*P<0.05, **P<0.01).

Gene	2				6				12			
	Condition		ANOVA		Condition		ANOVA		Condition		ANOVA	
	Paired	Unpaired	F	p	Paired	Unpaired	F	p	Paired	Unpaired	F	p
Jag1	0.39±0.12[a]	0.5±0.13[a]	42.06	<0.001	0.53±0.65[b]	0.98±0.46	4.57	0.023	0.71±0.55	0.85±0.36	1.7	0.21
Dll1	0.76±0.15[a]	0.81±0.33[a]	27.86	<0.001	0.8±0.70	0.91±0.60	0.17	0.84	1.05±0.81	0.99±0.77	0.18	0.83
Notch1	0.67±0.09[a]	0.65±0.15[a]	27.56	<0.001	0.69±0.13[b]	1.02±0.04	3.7	0.045	0.98±0.54	0.89±0.49	0.24	0.79
Notch2	1±0.67	1.24±0.34	0.37	0.7	0.63±0.29	0.98±0.56	1.85	0.18	0.88±0.53	0.9±0.62	0.24	0.88
Hes1	1.06±0.31	0.85±0.34	1.12	0.34	0.84±0.33	0.99±0.40	1	0.38	1.01±0.58	1.02±0.42	0.004	0.99
Hes5	0.65±0.10[a]	0.59±0.05[a]	11.89	<0.001	0.84±0.32	0.91±0.34	0.82	0.45	0.9±0.29	0.88±0.18	1.32	0.29
Hey1	1.04±0.28	0.94±0.15	0.38	0.69	1.05±0.17	1.24±0.17	0.53	0.6	0.95±0.22	0.83±0.12	0.85	0.44

Table 1: Mean, standard deviation, and ANOVA values for time points 2, 6, and 12 hours after fear conditioning in the paired and unpaired groups in each gene in the amygdala (a, Tukey's HSD $P<0.05$ compared to home cage; b, Tukey's HSD $P<0.05$ compared to unpaired).

	2 Hours Amygdala		6 Hours Amygdala		12 Hours Amygdala	
Gene	Paired	Unpaired	Paired	Unpaired	Paired	Unpaired
Jag1	↓	↓	↓	—	—	—
Dll1	↓	↓	—	—	—	—
Notch1	↓	↓	↓	—	—	—
Notch2	—	—	—	—	—	—
Hes1	—	—	—	—	—	—
Hes5	↓	↓	—	—	—	—
Hey1	—	—	—	—	—	—

Table 2: Significant regulation of ligands, receptors, and effectors in the amygdala at 2, 6, and 12 hours after fear conditioning. At 2 hours, down arrows refer to significant reduction in mRNA levels as compared to home cage; at 6 hours, down arrows refer to significant decrease in mRNA levels as compared to unpaired. Horizontal lines indicate to no significant reduction as compared to home cage (for paired and unpaired) or unpaired (for paired).

Gene	Condition		ANOVA	
	Paired	Unpaired	F	p
Jag1	0.77±0.13	0.71±0.16[a]	4.2	0.031
Dll1	0.78±0.19	0.63±0.18[a]	5.47	0.013
Notch1	1.04±0.38	1.01±0.45	0.017	0.98
Notch2	0.84±0.35	0.72±0.35	1.8	0.19
Hes1	0.92±0.32	0.77±0.42	1.08	0.36
Hes5	0.96±0.17	1.12±0.36	0.44	0.65
Hey1	1.06±0.19	1.03±0.19	0.08	0.93

Table 3: Mean, standard deviation, and ANOVA values for time points 2 hours after fear conditioning in the paired and unpaired groups in each gene in the hippocampus (a, Tukey's HSD $P<0.05$ compared to home cage).

	2 Hours Hippocampus	
Gene	Paired	Unpaired
Jag1	—	↓
Dll1	—	↓
Notch1	—	—
Notch2	—	—
Hes1	—	—
Hes5	—	—
Hey1	—	—

Table 4: Significant regulation of ligands, receptors, and effectors in the hippocampus at 2 hours after fear conditioning. Down arrows refer to significant reduction in mRNA levels as compared to home cage. Horizontal lines indicate to no significant reduction as compared to home cage (for paired and unpaired) or unpaired (for paired).