INHIBITION OF HISTONE DEACETYLASE 3 VIA RGFP966 FACILITATES EXCEPTIONALLY SPECIFIC AND ENDURING MEMORY FOR EXCITATORY AND INHIBITORY SOUND-SIGNAL ASSOCIATIONS

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ABSTRACT OF THE THESIS

Inhibition of Histone Deacetylase 3 via RGFP966 Facilitates Exceptionally Specific and Enduring Memory for Excitatory and Inhibitory Sound-Signal Associations

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Epigenetic mechanisms that modulate gene expression are key to regulating long-term memory (LTM) formation, and are known to exert control on memory formation in multiple systems of the adult brain, including the sensory cortex. Chromatin modifications have been shown to have powerful effects on experience-dependent transcription for neuroplasticity underlying memory processes. One mechanism for chromatin modification is histone acetylation. Histone acetyltransferases (HATs) generally facilitate LTM formation by promoting gene expression, while histone deacetylases (HDACs) tend to have gene silencing effects, and negatively regulate LTM. Thus, blocking the action of HDACs has been shown to facilitate LTM formation. Because sensory cortex undergoes learning-induced remodeling over a lifetime, here we aimed to identify the ways in which HDAC-inhibition acts to facilitate LTM using a standard model of auditory memory and cortical plasticity. Auditory cortical plasticity in

particular has been extensively studied in learning and memory processes. Representational plasticity in primary auditory cortex (A1) is known to reflect the formation of strong and sound-selective associative memory for behaviorally relevant sound features. In this present study, we used RGFP966, a class I HDAC inhibitor with selectivity for HDAC3 that has been shown to modulate associative learning-induced A1 plasticity (Bieszczad et al., 2015), to facilitate memory consolidation in rats learning a 2-tone sound frequency discrimination (2TD) task. We found that systemic treatments of the HDAC3-inhibitor early in 2TD task training facilitated associative learning for both excitatory (CS+) and inhibitory (CS-) sound signals, and altered the LTM formed in two ways that were independent of the final performance level achieved, which was equivalent between groups. We found that HDAC3-inhibition enhanced memory specificity for the sound-frequency of the two pure-tone CS cues, and strengthened memory for the excitatory and inhibitory sound-specific associations. Moreover, the behavioral effects of an initial, limited bout of HDAC3-inhibitor were long-lasting, enduring for at least weeks following the last administration of RGFP966. The present results support a role for HDAC3 during auditory memory consolidation by regulating the specificity and strength of newly learned sound-signal associations. This conclusion complements existing research on the effects of HDAC-inhibitors by providing a potential behavioral explanation for long-term memory enhancements.

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Table of Contents

I. Title Page……..……...…i

II. Abstract……...…….....ii

III. Acknowledgement……...……iv

IV. Table of Contents…….…..…..v

V. List of Figures……...…...…vi

VI. Introduction…….....…..……1

VII. Methods and Materials…….....……6

VIII. Results…….……12

IX. Discussion……...……24

X. References……...……34

List of Figures

Figure 1. Schematic for sound frequency representation in A1 with associative learning.....37

Figure 2. Protocol for auditory learning and behavioral memory testing…….38

Figure 3. Two-tone discrimination task acquisition over sessions 1-9……..…40

Figure 4. Bar-pressing under sound control across tasks…….....41

Figure 5. CS specific learning over 2TD training sessions 1-9……...….42

Figure 6. Bar-presses to SGT test tones……43

Figure 7. Behavioral stimulus generalization gradients for number of bar-presses…….44

Figure 8. Frequency generalization gradients for bar-press latency…….....45

Figure 9. Frequency-specificity of memory assessed by comparing behavioral gradients to test tones neighboring the CS frequency.....46

Figure 10. Bar-presses to new CS- and CS+ on the reversal test…….47

Introduction

Behavioral psychology has largely focused on learning and memory processes since they are essential to adapting behavior in a changing environment across a lifetime. The neural substrates of memory are thought to rely on experience-dependent neuroplasticity. Therefore, a key open question is to link learning-induced neuroplasticity and its neurobiological mechanisms to its behavioral functions.

In the adult brain, the sensory cortex undergoes continual remodeling as we experience and learn throughout a lifetime. The most widely-studied sensory system in the adult brain for associative learning-induced neuroplasticity is the auditory cortex, and in particular the *primary* auditory cortex (A1), which is a known neural substrate of the learned significance of sound. Since the initial discovery for learning-induced plasticity in primary auditory cortex (A1) by Galambos et al. (1956), neurophysiological studies have demonstrated a role for primary sensory cortices as more than just stimulus analyzers. Rather, learning-induced plasticity in A1 functions to instruct behavior associated with sound.

Extensive research in the auditory domain has shown that associative learning, in which an animal learns to associate a sensory stimulus with a behaviorally meaningful stimulus e.g., a reward in appetitive learning paradigms or a mild footshock in aversive learning paradigms (e.g. Bakin & Weinberger, 1990), is accompanied by representational plasticity (RP) in A1 (reviewed in Weinberger & Diamond, 1987; Weinberger, 2007). Auditory cortical learning-induced RP is selective for the specific acoustic features of meaningful sounds (e.g. frequency, Recanzone et al., 1993; sound level, Schreiner & Malone, 2015; repetition rate, Bao et al., 2004; sound level or acoustic frequency, Polley

et al., 2006), especially when that feature is learned and remembered to be relevant for predicting a significant outcome. Therefore, A1-RP tracks both learned acoustic features and their learned associative significance.

Primary sensory cortices are particularly well-suited for representing the specific sensory "content" of cues in associative memory. Furthermore, sensory cortical organization is a useful experimental tool to investigate this kind of content in memory since the sensory receptive fields of cells and cortical columns are organized in a topographic manner (**Fig. 1A**). This spatial organization—like for acoustic frequency in the *tonotopy* of A1—allows access to the neural representation of the particular stimulus that is being associated with a significant behavioral outcome, as well as to the neural representation of the frequency dimension as a whole. Thus, the investigation of representational plasticity in A1 is to determine the relative sound-specificity of learning-induced effects for one acoustic frequency versus another by revealing changes in tonotopic organization (**Fig. 1B**). RP is known to induce lasting changes in neural activity that remodel the representation of sound in A1(reviewed in Weinberger, 2005; 2015). Frequency-specific auditory cortical remodeling is related to the formation of memory for specific sounds (e.g. Bieszczad & Weinberger, 2010; 2012). Indeed, accumulating evidence suggests that RP in A1 is a likely neural substrate of specific and long-lasting auditory memory (Scheich & Ohl 2005; Weinberger 2015; McGann 2015) (**Fig. 1C**).

Learning and memory research has long been focused on investigations of the mechanisms controlling the strength of memory formation over time and interference. In this domain, research findings support that gene expression is necessary for the formation of long-term memory (LTM) defined as that which lasts beyond ~24 hours (Alberini,

2009). Thus, molecular mechanisms that control gene expression have been investigated as a molecular-level requisite for successful gene expression underlying experience-dependent plasticity, memory consolidation and LTM.

Recent research in the neurobiology of learning and memory has highlighted epigenetic control on gene expression in the adult brain. Chromatin remodeling occurs by various mechanisms including, but not limited to, DNA methylation, histone acetylation and nucleosome remodeling (Sweatt, 2013). Here the focus is on histone acetylation, which regulates gene expression via enzymes called histone acetyltransferases (HATs) and histone deacetylases (HDACs) that together regulate the epigenetic control on gene expression required for LTM (reviewed in Barrett & Wood, 2008). HATs tend to relax chromatin structure, enabling gene expression, whereas HDAC enzymes that oppose acetylation restrict chromatin. Therefore, promoting HAT function while blocking the action of HDACs will remodel chromatin to permit an "open" state of euchromatin that is permissive to transcription and subsequent LTM formation.

In this respect, HDACs have been shown to be powerful negative regulators of LTM formation. In particular, the inhibition of HDACs is known to facilitate memory processes for leaning events that would otherwise not have been later remembered. Strikingly, LTM enabled by HDAC inhibition persists beyond the time point at which normal memory fails (Stefanko et al., 2009). This suggests that LTM enabled by HDAC inhibition (subsequently referred to as LTM*) is different to long-term memory formed under normal learning conditions – the memories that form outlast LTM that forms naturally. Initial interpretations of the surprising discovery of LTM* was that the memory formed was stronger than normal, with strength defined as an increased durability to the

passage of time. An alternative, but complementary interpretation of LTM* is that HDAC-inhibition enables memory that is not only longer-lasting, but also stronger due to the amount of sensory information encoded. Thus, LTM*s might contain more detailed cues that could later be used for recall. This implies that LTM* formed with HDAC-inhibition contains more sensory information from the learning experience in a way that alters the *contents* of the memory. This potential explanation is formally proposed as the "informational capture" hypothesis (Phan & Bieszczad, 2016). The idea is based on the "molecular brake pad hypothesis" set forth by McQuown & Wood (2011), which states that HDACs usually act as molecular "brakes" on gene expression required for memory formation. Together these hypotheses propose that HDAC-inhibition releases the brakes in cells within systems-level representation that have recently become active during learning events to subsequently enable plasticity in these cells and representations that might not otherwise have formed. Indeed, not all experiences induce plasticity, and not all learning becomes LTM, as is the case for non-salient or behaviorally insignificant experiences. Thus, HDAC-inhibition may permit LTM and enable LTM* by increasing the amount and detail of sensory features that are encoded from a learning experience into memory.

Whether epigenetic mechanisms regulate memory processes that enable more and more detailed sensory information about learning experiences to be encoded in LTM (and LTM*) remains an open question. Studies in the auditory system are uniquely positioned to address this potential role of epigenetic mechanisms in regulating not only the strength of memory, but also the specific contents of memory, and neurobiological correlates in auditory cortical RP. Prior work in the auditory cortex has implicated HDAC3 as a critical negative regulator of auditory memory formation (Bieszczad et al., 2015). Rats treated with

RGFP966 (a class I HDAC-inhibitor with selectivity for HDAC3) while learning a sound-reward association formed a more specific memory for the behaviorally-significant sound signals. Moreover, RP in A1 was unusually "tuned-in" to the specific sound cues and their acoustic features could be associated with reward (compared to vehicle-treated rats). This supports a strong link between HDACs, A1-RP and auditory memory. Additionally, recently published findings using the same RGFP966 inhibition of HDAC3 in an avian model confirmed the importance of HDAC3 for neurophysiologically encoding complex auditory stimuli with exceptionally detailed acoustic features (Phan et al., 2017). That auditory memory in both mammals and birds is susceptible to HDAC-inhibition suggests an evolutionarily conserved role for HDAC3 in learning and memory processes.

The goal of this study was to identify the behaviorally functional roles that HDAC3 plays in a standard model of auditory associative learning and memory known to induce A1-RP. In order to determine the influence of HDAC3 *on memory processes*, we designed a behavioral instrumental conditioning and discrimination task that challenged rats to learn and remember two sound-frequency associations for reward outcome. We used systemic injections of RGFP966 to manipulate levels of HDAC3-inhibtion during consolidation (immediate post-training injections) in early phases of learning. If HDAC3-inhibition enhances the LTM formed by so-called informational capture, then behavioral evidence of memory for sound-signal associations will show increased strength (i.e. resistance to interference), as well as increased sound-specificity for the behaviorally relevant acoustic-frequencies in rats treated with the RGFP966 (compared to vehicle-treated controls). We found behavioral evidence for both of these enhancements. The results support a role for

HDAC3 during auditory memory consolidation by regulating both the specificity and the strength of newly learned sound-signal associations.

Methods and Materials

Subjects

A total of 24 adult male Sprague-Dawley rats (275-300g on arrival; Charles River) were used for this experiment. All animals were individually housed in a temperature-controlled (24°C) colony room on a 12-hour light/dark cycle. Subjects had *ad libitum* access to food and water prior to behavioral training. Animals weights were monitored daily when access to water was restricted (see Water Restriction), with supplements and *ad libitum* access on weekends given as necessary to maintain weight. All procedures were approved and conducted according to guidelines by the Institutional Animal Care and Use Committee (IACUC) at Rutgers University.

Water Restriction

The behavioral experiments used operant conditioning paradigms to train rats to associate new information about auditory stimuli with water reward. To motivate animals to perform the instrumental tasks, rats were placed on a schedule of restricted water to reach 85-90% of their *ad libitum* water access body weight. Prior to the start of water restriction, rats are weighed daily for at least 3 days to establish individual baseline weights. While on a schedule of restricted water access, rats were weighed daily and were given water supplements in their home cage as needed to maintain 85-90% of their baseline weight.

Behavioral Apparatus and Stimuli

All behavioral sessions were conducted in two identical instrumental conditioning chambers (H10-11R-TC; Colbourn Instruments, Holliston, MA) within a sound-attenuated box. Daily training sessions were counterbalanced to ensure equal exposure to both chambers. Each chamber (12" W x 10" D x 12" H; wire mesh floor, H10-11R-TC-NSF) was fitted with a response lever (H21-03R), house light (H11-01R), infrared lights (H27-91R), a speaker (H12-01R), and a water delivery system (H14-05R). During training phases, depressing the response lever ("bar-pressing") triggered the presentation of a water cup (~0.02cc) in the reward port (1.25" W x1.625" H). In early bar-press (BP) shaping sessions, a hand switch (H21-01) was also used to trigger presentations of the water cup. Behavioral responses were recorded using Graphic State 4 software (Colbourn Instruments) for offline analysis.

All auditory stimuli were generated using Tucker-Davis Technologies (TDT, Alachua, FL) and RPvdsEx software, and presented via the operant chamber's wall-mounted speaker. Sound levels were calibrated daily (Larson Davis SoundTrack LxT1). White noise (during early instrumental training only) was presented for 7 or 9 seconds in duration (75 dB SPL). Pure tones were always presented for 8 seconds (70 dB SPL). All tone frequencies were cosine-squared gated with rise/fall (10-90%) of 20ms.

Behavioral Training

Initial handling and bar-press shaping. After 1 day of acclimating to the vivarium, rats were handled daily for a minimum of 3 days to familiarize them to the transportation to the laboratory and the weighing process. Several days prior to beginning

behavioral training, rats were placed on a schedule of restricted water until they reached 85% of their non-deprived weight.

Water-restricted rats were trained to manipulate the wall-mounted lever for water reward. Bar-presses (BPs) resulted in the availability of water reward on a 1:1 ratio. Rewards were available for 5 days for 5 seconds (first two sessions, which were always consecutive), then for 3 seconds. The first BP shaping session lasted ~90 minutes or until the animal reached satiety. Remaining sessions were limited to 45 minutes each. Rats were weighed before and after each session. All subjects learned to bar-press for water reward within 1-2 sessions, as indicated by a substantial increase in pre- vs. post-training weight.

Noise training. After 5 days of BP shaping, bar-pressing behavior was placed under sound control. Animals learned to bar-press only during presentations of an auditory stimulus (CS) for water reward. BPs made in the presence of the CS (white noise; 75 dB SPL) resulted in the availability of water reward; Bar-presses made during the silent inter-trial intervals (ITI; 5-25s, randomized) resulted in an error signal (flashing house light) and a "time-out" (an additional 6s lengthening of the ITI). To prevent the animal from making associations between the duration of the CS and reward, the presentation of sound varied (either 7 or 9s, randomized). Animals could receive a maximum of 2 or 3 rewards per trial (**Fig. 2B**).

Performance was monitored on a daily basis, and calculated for each session as follows: performance = (# BPs to white noise/ Total # BPs). Criterion for moving onto the next stage of training was defined as 2 consecutive days of performance >90% or asymptotic performance (cv <0.1) for 3 consecutive days. Subjects were "noise trained"

daily (5 days a week) for 45 minute sessions until performance criterion was attained. On average, 9 training sessions were required (M = 8.67, SD = 1.99). All animals (n=24) successfully learned to associate sound with reward.

2 Tone frequency discrimination task (2TD). To examine the overarching question of whether HDAC3 inhibition during consolidation of associative learning for behaviorally-relevant acoustic information enhances the specificity of memory formed, we trained rats on a two-tone frequency discrimination task (2TD). Water-restricted rats were trained to discriminate between two spectrally distinct sound-frequencies. Bar-presses to the CS+ (5.0 kHz pure tone) in the presentation of water reward, while bar-presses to the CS- (11.5 kHz pure tone) were unreinforced and triggered an error signal (flashing house light) and a "time-out" (an extended interval until the start of the next trial). CS+ and CS- trials were randomized, and all tones were presented at 70 dB SPL for 8 seconds. Inter-trial intervals (ITIs) were 5-25 seconds (randomized). Bar-presses to the CS- had a 70% change of causing a time-out, a 6 second extension of the ITI. Sessions were on average 50 minutes in length (**Fig. 2C**).

All animals (n = 24) acquired similar levels of task performance. Daily 2TD performance was calculated as: number of bar-presses to the CS+ divided by the number of bar-presses to both the CS+ and CS- ($\#BP^{CS+}/(\#BP^{CS+} + \#BP^{CS-})$]. Performance criterion was defined as 2 consecutive days of performance ≥90% or 3 days of asymptotic performance (c.v. ≤0.1; coefficient of variation = standard deviation/mean). Upon reaching performance criterion, rats were trained for several additional days to insure stable performance.

Behavioral assays for associative memory. Following successful 2TD task acquisition (24 hours after the last training session), all rats underwent behavioral memory testing to determine effects of HDAC3 inhibition on sound frequency learning. Memory for the associative sound-signals was assessed through a stimulus generalization test (SGT) or a reversal test (reverse 2TD).

Stimulus Generalization Test (SGT). To determine memory specificity from the 2TD task, animals (n = 18) were presented with a range of sound frequencies, including the CS+ and CS- frequencies. 10 different tones were tested: 3.6, 4.2, 5.0 (CS+), 5.9, 7.0, 8.3, 9.7, 11.5 (CS-), 13.6, and 16.0 kHz (all pure tones at 70 dB SPL). Test tones neighbored the CS+ or CS- at a distance of~1/4 octave. The SGT session began with 15 2TD trials (BP to the CS+ are rewarded; BPs to the CS- are not) to confirm stable performance. Test frequencies were then presented in pseudorandom order over 120 unrewarded trials (to yield 12 presentations of each test frequency) (**Fig. 2E**). Behavioral responses to test tones are used to determine frequency generalization gradients.

The number of BPs and the latency to the first BP for each of the frequencies were used to determine behavioral frequency generalization gradients. The number of BPs were expressed as a proportion of the total number of BPs for individual animals, and averaged for HDAC3i and VEH groups. Latency was measured as the seconds to first BP after tone onset. Analysis for memory specificity was assessed separately for the CS+ and CS- frequencies.

Reverse 2TD test. A subset of animals (n=6) underwent a "reversal" test to assess the strength of frequency-specific memory from the 2TD task. Tone-reward pairings were reversed: 11.5 kHz became the *new* CS+, and 5.0 kHz became the *new* CS-. With the

exception of the tone-reversal, all other conditions were identical to the 2TD task (**Fig. 2D**). Behavioral responses (number of BPs) was assessed for the first 45 minutes of the test session, a duration comparable to the average length of the SGT.

Pharmacological Inhibition of HDAC3

A pharmacological class I HDAC inhibitor with enhanced selectivity for HDAC3, RGFP966 (10 mg/kg; *s.c.*), was used to manipulate levels of gene expression. Systemic injections of RGFP966 have been shown to penetrate the blood-brain barrier in rodents and are effective in promoting histone acetylation linked to gene expression in the rat A1 (Malvaez et al., 2013; Bieszczad et al., 2015, Phan et al., 2017). RGPF966 reaches C_{max} 30 to 80 minutes post-injection, and remains high for at least 4 hours (Bieszczad et al., 2015). Therefore, RGFP966 delivery immediately post-training can reveal effects of HDAC3-inhibition on memory formation processes in A1.

The pharmacological manipulation began concurrently with 2TD training. Within each performance-matched pair (based on Noise training acquisition), rats were assigned to receive either RGPF966 (n=12) or vehicle (n=12). Treatment was counterbalanced across performance: in half the pairs, the higher performing animal received RGFP966 and the lower performing animal received vehicle (VEH), and vice versa.

Injections were given immediately after the end of each 2TD training session. Animals received 3 days of RGFP966/VEH treatment, and saline injections after the subsequent training sessions.

Statistical Analysis

Differences between HDAC3i and VEH groups were analyzed using ANOVA (α = 0.05) and independent samples t-tests. Analyses across training sessions (for noise

training and the 2TD task acquisition) were performed using 2-way repeated measures ANOVAs, with treatment and session as the between-subjects factors. Data from the single reversal test session (rev2TD) was analyzed similarly, but across time bins (referring the first 15 minute blocks within the single session). The Bonferroni procedure was used to correct for multiple comparisons. SGT data were analyzed separately for the CS+ and CS- frequencies (also using ANOVA). One-sample t-tests were used to compare responses frequency-specific responding to generalized responding. One-way ANOVA were used to analyze differences between HDAC3i and VEH groups in responding to frequencies neighboring the CS.

Results

HDAC3-inhibition by RGFP966 facilitates associative sound discrimination learning.

To test the hypothesis that HDAC3 inhibition has a key function in specific information encoding, we used the class I HDAC inhibitor RGFP966 in rats learning a sound discrimination task. A total of 24 animals (HDAC3i, N = 12; VEH, N = 12) were trained to asymptotic, high performance on a 2-tone discrimination (2TD) task. Successful performance on the 2TD task requires the animal to learn and remember two sound-frequency associations and instrumental responses: barpresses (BPs) to the CS+ (5.0 kHz, 70 dB SPL) result in reward, while BPs to the CS- (11.5 kHz; 70 dB SPL) do not result in reward delivery and instead trigger an error signal (flashing house light) and a time-out period that extends the time until the next trial. Sound discrimination performance was calculated using the proportion of BPs to the CS+ tone relative to BPs

to either tone. Bar-presses during the silent ITIs are excluded from this performance metric to attribute high performance values to successful sound-*frequency* discriminations. Thus, the daily performance value is a metric of the animal's ability to discriminate between the CS+ and CS- to instruct the appropriate associated behavior. Performance (%) was calculated as:

$$Performance = \frac{BP^{CS+}}{BP^{CS+} + BP^{CS-}} \times 100\%$$

To assess differences between HDAC3i- and Vehicle-treated groups of animals on early acquisition of the 2TD task (i.e. before reaching asymptotic levels of performance), we compared performance over the first 8 daily training sessions after the first injection of either RGFP966 (in the HDAC3i group) or vehicle (in the VEH group). Post-training injections of RGFP966 or vehicle only occurred after the first three daily sessions; animals received saline injections of similar volume following all other sessions.

Overall, all animals were able to acquire the task, regardless of treatment. Performance increased significantly over training sessions ($F_{(7,154)} = 59.65$, $p<0.001$). However, the HDAC3i group's overall performance was higher ($F_{(1,22)} = 6.89$, $p = 0.015$). Though the interaction between session and treatment was not statistically significant ($F_{(7,154)} = 1.424$, $p = 0.199$), multiple comparisons across training sessions show that a significant difference between groups emerges on day 3, after the second RGFP966 (or vehicle) treatment (HDA3i, 81.06%; VEH, 62.55%; $t_{(176)} = 3.755$, $p = 0.002$). Thereafter, all animals reach asymptotic levels of performance by the eighth session. Performance was not different between groups on all other days ($p>0.05$) (**Fig. 3A**).

These findings are interpreted to indicate that the effects of HDAC3-inhibition by RGFP966 is to enable frequency-specific associative learning *per se*, rather than simply associative learning about the task rules or instrumental response. Prior to 2TD training, all animals were trained in a noise-dependent instrumental task that required them to only bar-press in the presence of a simple auditory stimulus (white noise burst; 7-9 seconds in duration; 75 dB SPL) for the same water reward. All animals achieved the performance criterion (for Noise Training, this was defined as 2 consecutive days of performance ≥90% [performance = number of BPs to sound/total number of BPs] or 3 consecutive days of asymptotic performance [c.v. ≤0.10]). All subjects reached criterion for sound control with 5 to 15 days of noise training (M = 9.67 ± 0.44 days) (**Fig. 4A**). Thus, all animals were equally well-learned in the instrumental response of bar-pressing and also in placing their bar-pressing behavior under sound (i.e. noise) control.

Since the effect of HDAC3-inhibition appears to dictate frequency-specific learning, we next examined more closely the pattern of responses to the rewarded (CS+) and unrewarded (CS- or silent ITI) periods that may have accounted for overall difference in 2TD performance between the HDAC3i and VEH groups. Successful performance in the 2TD task also requires animals to inhibit instrumental responses (i.e. bar-presses, BPs) during silent ITI periods. Importantly, BPs made during the ITI triggers an error signal and a "time-out" period that extends the time before the next trial. Therefore, fewer ITI BPs will increase the number of available trials (and opportunities for reward) in a 50-minute training session (average session time: = 49.12 ± 1.51 min). Thus, to determine whether the better performance in the HDAC3i group might be explained by fewer ITI barpresses that generated more opportunities for CS+ or CS-

trials, a new performance measure for maintained "Sound Control" in 2TD was calculated to include ITI BPs. Sound Control on the 2TD task was calculated as:

$$Sound\ Control = \frac{BP^{CS+} + BP^{CS-}}{BP^{CS+} + BP^{CS-} + BP^{ITI}} \times 100\%$$

All animals retained a high level of sound control from the Noise Training into 2TD task (session 1: HDAC3i, 62.98 ± 4.92%; VEH, 63.80 ± 4.59%). Sound Control in the 2TD task increased across sessions for both groups (2-Way RM ANOVA: Session, $F_{(7,154)}$ = 16.67, p<0.0001), however there was neither a main effect of treatment ($F_{(1,22)}$ = 0.123, p = 0.729), nor an interaction between treatment and session ($F_{(7,154)}$ = 0.443, p = 0.874) were significant (**Fig. 4B**). Thus, the groups were equally able to limit their responding to the CS sounds during 2TD and difference in performance between HDAC3i and VEH groups are likely due to frequency-specific associative learning.

HDAC3-inhibition by RGFP966 facilitates both excitatory and inhibitory associative learning specific to sound frequency cues.

If HDAC3-inhibition effects are driven by frequency-specific learning alone, then the effects should be similar between learning to respond to the CS+ (i.e., by increasing BPs) and learning to *not* respond to the CS- (i.e., by decreasing BPs). Thus, group differences in learning about each of the two frequencies (CS+, 5.0 kHz; CS-, 11.5 kHz) were determined separately as the tones signal two different outcomes (reward vs. no reward). To determine CS+ learning in 2TD, performance was assessed using a ratio of CS+ BPs to all CS BPs, and to determine CS- learning in 2TD, performance was assessed using a ratio of CS- BPs to all CS BPs. Both learning for the CS+ and CS- frequencies

were compared between groups. Frequency-specific learning (i.e. CS+ and CS- associations) was calculated independently for the CS+ and CS- as:

$$CS\ Learning = \frac{BP^{CS+/-}}{Total\ BP}$$

Over daily training sessions, bar-pressing to the CS+ increased significantly in both HDAC3i and VEH groups, from an initial performance value of 33.44 ± 3.78% (HDAC3i) and 27.58 ± 4.36% (VEH) on session 1 (2-Way ANOVA: Sessions, $F_{(7,154)}$ = 47.17, p<0.001; **Fig. 5A**) which is consistent with the initial reported overall performance measure (**Fig 3A**). There was no difference between group performance (Treatment, $F_{(1,22)}$ = 2.29, p = 0.144), and no significant interaction between session and treatment(Session x Treatment, $F_{(7,154)}$ = 1.026, p = 0.415).

Over daily training sessions, bar-pressing to the CS- decreased, which also corresponds to overall 2TD performance improvements reported in Figure 1 (2-Way RM ANOVA: Sessions, $F_{(7,154)}$ = 54.97, p<0.001) (**Fig 5B**). Furthermore, consistent with overall 2TD performance, this CS- focused analysis revealed that BP decreases to the CS- across session differed significantly between HDAC3i and VEH groups (Treatment, $F_{(1,22)}$ = 12.91, p = 0.002), though the interaction between treatment and session was not significant (Treatment x Session, $F_{(7,154)}$ = 1.678, p = 0.118). However, further analysis of daily sessions (Bonferroni-corrected pairwise comparisons) showed that HDAC3i-treated rats bar-pressed significantly less to CS- tones on session 3, which is the training session immediately following two sessions with post-training RGFP966 treatment (HDAC3i, 13.35 ± 1.78%; VEH, 26.57 ± 3.00%; $t_{(176)}$ = 2.343, p<0.001; all other sessions: p>0.05).

Thus, after just 2 bouts of HDCA3i-inhibition during 2TD consolidation, early learning about associative outcome of frequencies is enhanced (relative to vehicle treatment). Over additional training sessions, however, both groups of animals are able to reach equivalent levels of 2TD task performance. HDAC3i does not affect overall acquisition or level of performance. Together these findings indicate that HDAC3i operates at the level of enabling rapid (sound-frequency) stimulus specific associative memory during new learning.

HDAC3-inhibition by RGFP966 Facilitates Frequency-Specific Memory for Associative Sound Signals

Behavioral frequency generalization gradients reveal differences in the frequency-specificity of memory for the learned sound signals between the HDAC3i and VEH groups. To further test the hypothesis that HDAC3 inhibition regulates the stimulus-specificity of memory encoded for learned associative signals, we tested animals on a stimulus generalization test (SGT) 24 hours after the last 2TD training session. The SGT determined the stimulus-specificity of memory for sound frequencies to within approximately ¼ octave. Over a single test session, animals were presented with ten test tone frequencies, including the CS+ and CS- frequencies: 3.6, 4.2, 5.0 (CS+), 5.9, 7.0, 8.3, 9.7, 11.5 (CS-), 13.6, and 16.0 kHz (each tone played for 8 seconds; 70 dB SPL). The order of tone presentations was pseudorandomized for a total of 120 test trials (12 presentations of each frequency). Barpresses to the CS+ and CS- tones were not reinforced and all other test tone trials are likewise unrewarded. Behavioral responses to each tone (number of BPs and the latency to BP from each tone onset) were recorded and used to construct behavioral

generalization gradients to reveal the degree of memory specificity for the learned sound-signals.

As the SGT was conducted under extinction conditions the number of barpresses itself may not be indicative of behavioral specificity (e.g. the number of BPs decrease over the course of a test administered without rewards so barpress number also reflects the general extinction process to respond to tones in general; Bieszczad & Weinberger, 2010). Therefore, barpresses for each test frequency were calculated as the proportion of each individual animal's total number of BPs to tones during the SGT session. The CS+ and CS- frequencies were associated with two opposite instrumental response (go vs. no-go, respectively) and outcomes (i.e. for reward vs. to avoid time-outs, respectively) that are differently susceptible to extinction processes. Therefore, since an analysis across all ten test tones may not be accurate to quantify memory specificity and strength for the two signal tones together, memory specificity for the CS+ and CS- was determined separately. Responses to the CS tone and its immediate neighboring frequencies determined the CS-specific SGT gradient (i.e. for CS+: 3.6, 4.2, 5.0 (CS+), 5.9, 7.0 kHz; and for CS-: 8.3, 9.7, 11.5 (CS-), 13.6, 16.0 kHz).

If animals did not have specific memory for the stimulus frequencies, bar-press responses would be equally distributed across all test frequencies (i.e. 10% of Total BPs for ten test tones; grey dashed line, **Fig. 6A and 6B**). Behavioral frequency generalization gradients show that all animals tested (N = 18 out of the 24 trained; 6 animals underwent a reverse 2TD test to assess for memory strength) learned about frequency (2-Way ANOVA: Frequency, $F_{(9,160)} = 31.74$, $p < 0.001$). Responses to the CS+ and neighboring tones were all significantly higher than a generalized response (i.e. 10%; independent-

samples t-test: p =0.006 for 5.9 and 7.0 kHz; p < 0.0001 for all other frequencies; Holm-Bonferroni corrected) (**Fig. 6A**). The generalization gradient for CS- also indicates memory for associative sound-signals: bar-pressing to 11.5 kHz and the neighboring frequencies are all below 10%. Multiple comparisons at each tone to a generalized response show that responses to 9.7, 11.5, 13.6 and 16.0 kHz are all significantly below a generalized response (**Fig. 6B**). Overall, this initial analysis supports the 2TD performance findings that all animals learned to discriminate between the two sounds and perform the appropriate instrumental responses associated with each CS.

To examine the effects of HDAC3 inhibition on the specificity of memory for stimulus frequency, behavioral responses between HDAC3i and VEH groups to each of the test tones were compared. If HDAC3 inhibition by RGFP966 facilitates the specificity of information encoded, animals treated with the HDAC3i should exhibit responses that indicate enhanced memory for the CS+ and CS- tones (5.0 and 11.5 kHz respectively) relative to vehicle-treated animals. That is, difference gradients (HDAC3i gradient – VEH gradient) should peak at 5.0 kHz and reverse-peak (dip) at 11.5 kHz. We first examined frequency-specificity for the CS+. As all trials on the SGT were unreinforced, bar-pressing behavior directed at the remembered CS+ was sensitive to the effects of extinction over repeated trials. Both HDAC3i and VEH groups bar-pressed significantly less to tones during the second half of the SGT session (1-Way RM ANOVA: Time, $F_{(1,16)} = 17.05$, p = 0.001; Treatment, $F_{(1,16)} = 0.795$, p = 0.386; Time x Treatment, $F_{(1,16)} = 0.795$, p = 0.386). To reduce confounding effects of general behavioral extinction, analysis of CS+ memory was restricted to the first 60 SGT trials. More barpresses to the 5.0 kHz tone relative to the neighboring frequencies (4.2 and 5.9 kHz) reflects specific memory for the CS+ tone.

Frequency generalization gradients for the HDAC3i group (**Fig. 7B**; red**)** show a peak in bar-presses to 5.0 kHz (21.13 ± 2.70%). Although VEH also bar-pressed more to 5.0 kHz (19.35 ±1.82%), the two lower test frequencies (4.2 and 3.6 kHz) both also received similar numbers of BPs (4.2 kHz: 20.52 ±1.64%; 3.6 kHz: 19.42 ± 3.32%) (**Fig. 7B**; blue). This suggested a generalization effect in vehicle-treated animals across all lower test frequencies. However, while there was an effect of frequency ($F_{(4,80)} = 3.76$, $p = 0.007$), there was no difference between HDAC3i and VEH groups ($F_{(1,80)} = 1.078$, $p = 0.302$) and no significant interaction between treatment and frequency ($F_{(4,80)} = 0.626$, $p = 0.645$) (**Fig. 7A and 7B**).

We next examined frequency-specificity for the CS- tone. Again, if HDAC3 inhibition acts as a regulator on the specificity of any signal information encoded, then memory for the CS- tone (11.5 kHz) might also be encoded with more frequency specificity. Because bar-presses to the CS- frequency were never rewarded in any of the prior training, the number of BPs to the 11.5 kHz (relative to neighboring tones) is not sensitive to the same effects of overall behavioral extinction as was the CS+ tone described above since lack of reward does not violate prior expectations. Rather, strong and specific memory for the CS- would manifest as a nadir (i.e., reversed peak) in generalization gradients. All 120 SGT trials were included in this analysis since we aimed to observe *failures* to bar-press to the 11.5 kHz. Indeed, the frequency generalization gradient for the HDAC3i group show that animals bar-pressed least to the 11.5 kHz tone (1.53 ± 0.60%), and more to neighboring test frequencies (9.7 kHz: 2.64 ± 0.74%; 13.6: 3.29 ± 1.19%) (**Fig. 7D**; red). Consistent with the frequency generalization gradients for the CS+ frequency, VEH animals also showed signal-specific memory to the CS- tone, with decreased BPs to

11.5 kHz (3.65 ± 1.72%), but extended this to behavior across several high-frequency test tones (13.6 kHz: 2.239 ± 0.74%; 16.0 kHz: 1.44 ± 0.74%) (**Fig .7D**; blue). There was a significant main effect for frequency (2-Way ANOVA: $F_{(4,80)} = 7.219$, $p < 0.0001$), but neither the effects of treatment ($F_{(1,80)} = 2.46$, $p = 0.1207$), nor the interaction between frequency and treatment were significant (Treatment x Frequency, $F_{(4,80)} = 1.857$, $p = 0.126$).

The lack of absolute differences between the mean frequency generalization gradients by bar-pressing to the CS+ and CS- frequencies provide some support that frequency-specific memory for the two signal tones are similar between HDAC3i and VEH groups. However, the VEH group exhibited more generalized behavioral responses to low/high test tones and a lack of CS-specific "peaks' in behavioral gradients, which indicates differences in the specificity of sound frequency memory.

Latency to bar-press reveals effects of HDAC3 inhibition on forming robust and highly specific associative memory. We next compared bar-press latency to test tones between HDCA3i and VEH groups. Using barpress latency to presentations of the test frequencies as a metric for the specificity of sound frequency signals is complementary to the analyses using number of barpresses. Behavioral frequency generalization gradients using latency measures show whether animals respond faster (or slower) to certain test tones relative to their neighbors. Thus, if HDAC3i exerts its influence on the specificity of information encoded, this normalized latency to barpress to the CS+ tone (5.0 kHz) should be shorter (i.e., faster) than BPs to nearby test frequencies. As with the analyses for number of BPs, examination of the CS+ signal memory was confined to the first 60 SGT trials, and all 120 SGT trials were included for the CS- signal memory assessment.

Analysis of barpress latency for CS+ signal-specificity did not reveal differences between HDAC3i and VEH groups in frequency-specific memory (2-Way ANOVA: Frequency, ($F_{(4,80)}$ = 0.889, p = 0.475; Treatment, $F_{(1,80)}$ = 0.096, p = 0.758; Frequency x Treatment, F(4,80) = 0.957, p = 0.436). BP latency was similar across test frequencies for both groups (**Fig. 8A**). In contrast, analysis of BP latency for CS- signal-specificity revealed a significant interaction between treatment and frequency (2-Way ANOVA: Interaction, $F_{(4,52)}$ = 3.579, p = 0.0119) (**Fig. 8B**). However, multiple comparisons showed that HDAC3i and VEH groups had similar latencies at each of the CS- relevant frequencies ($p>0.05$).

Behavioral gradients were next assessed for peaks. A "peak" was defined as a response to a test tone at least one standard error (across all test frequencies) away from its neighbors (Bieszczad et al., 2015). Frequency generalization gradients for the HDAC3i group show a specific "peak" in BP latency at 11.5 kHz. Consistent with prior associative training (i.e. bar-presses to the CS- were unrewarded), HDAC3i-treated rats were *slower* to bar-press to the 11.5 kHz test tone. Analysis for peaks in the VEH-group gradient also reveal a "peak" in latency to the CS- tone, however animals were in fact *faster* to bar-press to the 11.5 kHz frequency. This was an unexpected finding that may indicate other cognitive processes that may have been present in VEH vs. HDAC3i animals and will be discussed below.

For memory to be highly specific, it needs to also be precise. If an animal acquired a highly frequency-specific memory for the CS- tone, we would expect to see a similar latency to bar-press to all neighboring test tones (**Fig. 9A)**. That is, *only* the specific test tone corresponding to the associative memory (i.e. 11.5 kHz) should elicit a change in

response. To examine this, we compared BP latency to "near neighbors" (i.e. test tones directly flanking the CS- frequency; 9.7 and 13.6 kHz) to "far neighbors" (i.e. 8.3 kHz and 16.0 kHz). The HDAC3i group responded similarly to both near and far neighbors (t-test: $t(27) = 0.17$, $p>0.99$) (**Fig. 9B**). The VEH group was significantly slower to bar-press to near-neighbor tones than far-neighbor tones ($t(27) = 3.714) = 0.0056$) (**Fig. 9C)**.

Taken together, these analyses of bar-press latency provide some support for differences in frequency-specific memory for learned sound-signals between HDAC3i and VEH animals. More strikingly, differences in the accuracy of *associative* memory for the CS- frequency combined with the lack of frequency-specificity for the CS+ sound-signal suggest the effects of HDAC3 inhibition by RGFP966 on memory encoding are modulated by associative memory for behaviorally relevant outcomes.

HDAC3 inhibition by RGFP966 facilitates robust memory for sound-signal outcome contingencies

If HDAC3 inhibition enhances memory specificity for associative sound frequency signals, memory for stimulus-specific outcomes should also be stronger in the HDAC3i treated animals. A subset of animals (HDAC3, $n = 3$; VEH, $n = 3$) were tested on a reversal test after 2TD acquisition (same time-point in training as animals tested on the SGT). This assessment is conducted under conditions identical to the 2TD task, except that the tone-outcome contingencies were reversed: bar-presses to 11.5 kHz were rewarded (*new* CS+), while bar-presses to 5.0 kHz resulted in an error signal and time-out (*new* CS-). The number of bar-presses to each tone were compared across 3 fifteen-minute blocks (for a total of 45 minutes) to examine differences between treatment groups. Bar-presses were calculated as

a ratio of BPs to CS trials. A score of 100% (grey dashed line, **Fig. 10A and 10B)** indicates that animals bar-pressed once per trial.

HDAC3i rats showed a marked decrease to bar-press to 5.0 kHz (**Fig. 10A**) over the 45-minute session, whereas the VEH group does not show the same effects of behavioral extinction. Bar-presses to the 11.5 kHz (now reinforced) also reveals differences between HDAC3i and VEH groups. Rats that had previously been treated with the HDAC3i perseverate in inhibiting responses to 11.5 kHz, and eventually stop responding to the new CS+ tone altogether (block 3, **Fig. 10B**). In comparison, VEH animals generalize their responses to both tones, and bar-press in similar proportions to both the 5.0 kHz and 11.5 kHz in similar proportions (blocks 2-3). Together, these behavioral results further support the long-lasting effects (>2 weeks later) of a limited, early bout of RGFP966 on enduring memory specificity for associative sound frequency signals.

Discussion

Summary of Findings

There were three main findings. First, HDAC3-inhibition facilitates associative auditory discrimination learning for both excitatory (CS+) and inhibitory (CS-) sound associations. Second, memory tests conducted after all animals reached equivalent levels of asymptotic performance on the 2TD task show differences between HDAC3i and VEH groups in the specificity of memory for sound-frequency, which was especially evident for the CS- sound frequency. Third, memory tests also revealed that sound-specific associations were stronger in animals that had received the HDAC3 inhibitor than in vehicle-treated animals, which was evident in perseverating behavioral responses to

previously learned CS contingencies despite a reversal test that would have rewarded a response to the former CS- sound. Finally, it is important to note the long-lasting effect of the HDAC3-inhibitor to facilitate learning and alter memory strength and specificity weeks after only a limited treatment schedule (after just the first three 2TD sessions). Taken together, these results support a role for HDAC3 during memory consolidation for modulating the strength and specificity of newly learned auditory associations.

Validity of Findings

Vehicle-treated animals are an appropriate control to reveal HDAC3i effects. HDAC3i and VEH groups were treated identically, with the exception of drug treatment itself (i.e. RGFP966 or Vehicle solution during the first 3 days of 2TD task training). In particular, the training and testing chambers were shared between the two groups, who had equal exposure to the chambers, handling, weighing, and water. Consistent with identical experiences prior to drug (or vehicle) treatment, the groups performed the 2TD task identically on the first day of training, which was immediately prior to RGFP966 or vehicle injections. Therefore, differences in 2TD performance and subsequent memory between HDAC3i and VEH groups could not have resulted from baseline differences in learning abilities or overall levels of sound-control induced by noise training. Furthermore, individual acquisition curves for the noise training task were used to establish performance-matched pairs of animals, one of which was randomly assigned to be treated with the HDAC3 inhibitor, and the other with vehicle in the 2TD task. Moreover, treatment groups did not differ in the level of absolute performance attained by the end of 2TD training. Therefore, it is unlikely that the 2TD task acquisition and

memory effects are due to differences between individual animals in their procedural experiences or their prior learning abilities.

Performance differences are not due to motivation or state-dependent effects. Motivational level is a known factor in modulating performance on instrumental conditioning paradigms that can impact the level of absolute task performance (Bieszczad & Weinberger, 2010) as well as the degree of associative neural plasticity (Rutkowski & Weinberger, 2005).

Differences in behavioral memory between treatment groups in this experiment are predicated on the effects of HDCA3-inhibition by RGFP966 to facilitate specific representational A1 plasticity for the behaviorally relevant sound-signal frequencies. Though water-restriction was used to motivate animals to perform the appetitive instrumental response (to "bar-press for water reward"), all animals were under the same degree of water restriction, which was monitored daily (85-90% of their *ad lib* weight), a level of water deprivation consistent with research using similar appetitive paradigms (e.g. Berlau & Weinberger, 2008; Elias et al., 2015). Therefore, differences in 2TD task acquisition and behavioral memory cannot be explained by motivational differences between individual animals and/or treatment groups.

Furthermore, HDAC3i/VEH treatment occurs post-training. Injections of RGFP966 or vehicle are given immediately *after* 2TD training (sessions 1-3). Effects of HDAC3i are, thus, during memory consolidation, which excludes the possibility of the effect to be attributed to acute attentional, motivational, perceptual, or motor processes because the agent is not present during learning or behavioral testing.

Magnitude of HDAC3i effects on discrimination performance and subsequent memory are subtle but significant. A potential explanation for the subtle effects of HDAC3-inhibition on facilitating learning on a discrimination task (and subsequent memory) is that the task could be acquired too easily. Effects of HDAC3 inhibition on associative sound frequency learning may have been constrained by a ceiling effect – that is, vehicle-treated animals acquired the discrimination quickly. Indeed, there was no difference between HDAC3i and VEH groups in the number of training sessions needed to reach performance criterion.

Future studies utilizing a 2TD paradigm can be modified to delay task acquisition by increasing task difficulty. This strategy may increase sensitivity to the effects of HDAC3i on sound-frequency learning and associative memory. One approach would be to increase the difficulty of the discrimination by using sound signals that are more spectrally similar. This would challenge animals to quickly develop a *highly frequency-specific* memory (above requirements for the current task version of 1.20 octaves between the CS+ and CS-) in order to achieve high levels of performance. A previous study found that associative plasticity (receptive field tuning shifts) occurred with easy and difficult frequency discrimination training, although animals were not able to behaviorally perform the difficult discrimination (Edeline & Weinberger, 1993). Therefore, performance differences in animals treated with the HDAC3-inhibitor (relative to vehicle-treatment) on a difficult frequency-discrimination task would provide further behavioral-level evidence of HDAC3-inhibition on enhancing the specificity of information encoding.

An alternative strategy would be to modify the 2TD task difficulty along a non-acoustic dimension. This strategy would also provide opportunities to assess the influence of HDAC3-inhibtion on encoding the associative value of sound-signals for both CS+ and CS- signals. This approach might involve limiting the "reward window" after tone onset in which animals must BP for reward so that animals must learn to respond quickly after tone onset in order to achieve high levels of performance. Shortening the reward window for a response from tone onset has been shown to increase task difficulty in instrumental auditory associative learning in a way that places greater demands on behavioral performance and also on auditory system plasticity. Interestingly, instead of gains in A1 area, some individual animals that are not able to acquire a "short reward window" task because they fail to respond in time for reward actually *lose* A1 representational area for the tone-cue frequency (Bieszczad & Weinberger, 2010).

In the current study, evidence for the HDAC3-inhibitor enhancements on memory specificity was only seen for the CS- tone. A preliminary interpretation might be that HDAC3i had no effect on frequency-specificity for reinforced CS+ sound signals. However, this interpretation is inconsistent with previous findings on a single-tone associative learning task. Animals treated with the HDAC3i showed enhanced memory, specifically for the sound-cues that had previously been associated with reward (Bieszczad et al., 2015). More likely is the alternative explanation that the two-phase training methods used in the present study (first noise-training, and second the 2TD task) produced more profound group differences for the CS- sound. This is explained by considering the new information being learned by animals in 2TD. Prior to 2TD, all animals learn to respond broadly to "noise," which is a broad-band stimulus containing

power across a range of frequencies that span all sound frequencies used in this study (CS+, CS-, and all test tones). Therefore, animals that enter into the 2TD are challenged to mostly learn when to *inhibit* bar-press responses, namely to the CS- tone frequency. Responses to all other frequencies can apply the same strategy learned in the noise-training phase: respond to all sounds. This new CS- association is the basis of the 2TD task challenge, for which learning was presumably enabled by treatment with the HDAC3-inhibitor. Future studies using a modified version of the 2TD task, could omit the preceding noise-training phase. Overall, these protocol modifications may both slow the rate of acquisition and challenge animals with new learning for both the CS+ and CS- associations that could potentially reveal larger magnitude effects of HDAC3i on learning rate in 2TD and subsequent memory for the CS+ and CS- sound signals.

Memory enhancements from HDAC3i are long-lasting. It is significant to note that memory testing was conducted at remote time-point (up to 10 days) from the last bout of HDAC3i/VEH, at which point concentrations of RGFP966 in the brain from systemic injections would have returned to a zero baseline (Bowers et al., 2015; Malvaez et al., 2013, Bieszczad et al., 2015). The findings from this study therefore demonstrate that the effects of HDAC3-inhibition administered during early phases of associative learning for sound-frequency are long-lasting. These results also establish that just three bouts of immediate post-training treatment via systemic injections of RGFP966 (10mg/kg) is sufficient to induce behaviorally detectable and long-lasting differences in long-term auditory associative memory.

A1 Representational Plasticity is Predicted to Underlie the Behavioral Effects of HDAC3-inhibition on Frequency-Specific Memory

A1 Representational Plasticity is Predicted to Underlie the Behavioral Effects of HDAC3-inhibition on Frequency-Specific Memory

Experience-dependent A1 remodeling has been extensively studied as a neural substrate of learning and memory. The current behavioral results provide a framework for guiding predictions as to how HDAC3-inhibtion operates at a systems level in adult sensory cortices to facilitate learning and memory. Previous work found that auditory memory for signal-specific cues was exceptionally larger and more specific in animals treated with HDAC3i, compared to those treated with vehicle. HDAC3i enabled larger A1 map expansion for two sound signals associated with reward in (at least) two acoustic dimensions (frequency and sound level) for those acoustic cues. Additionally, HDCA3i-treatment also enabled an increase in the specificity (by reduction of bandwidth) in the individual receptive fields recorded from A1 that were tuned to the behaviorally relevant frequency (Bieszczad et al., 2015). By an extension of prior findings, we here might also expect similar frequency-specific changes in A1 tonotopy. Namely, prior findings support the prediction that HDAC3i animals would show expanded map representation for the CS tones and narrower frequency tuning. However, it is also important consider ways in which this present study differed from prior work, which will impact our predictions.

HDAC3i enhances memory specificity for the CS- sound signal. An unexpected finding from this study was the frequency-specific memory to the CS- tone. That is, behavioral responses showed that memory formed with HDAC3i treatment was more specific for the *inhibitory* sound signal. The ability to detect this inhibitory behavioral

gradient is unusual, especially since the memory tests are conducted under extinction conditions, which further reduce the likelihood of behavioral responses. Nevertheless, the tactic used here to test memory using frequency generalization gradients for bar-press number and latency across 10 distinguishable tone cues permitted detection of inhibitory *behavioral* differences to reveal memory for a non-reinforced CS- tone. Thus, the findings were able to show increased specificity in an inhibitory behavioral gradient peaked at the CS- frequency.

One possible explanation for the surprising evidence of inhibitory stimulus generalization gradients is from research that combines auditory representational plasticity with Pavlovian conditioning and instrumental extinction processes to explain the effect of HDAC3i on memory specificity. Learning for the CS- on the 2TD requires animals to learn an inhibitory association (i.e. "do not bar-press"). One form of inihibtory learning is *habituation* that occurs as the CS- tone is presented repeatedly (over training trials). Habituation is a form of non-associative learning that, like associative learning, has been shown to induce frequency-specific receptive field plasticity (Condon & Weinberger, 1991) in A1. This provides feasibility for frequency-specific inhibitory processes in the auditory system. Thus, HDAC3i may act to alter A1 representations of the CS- tone and thereby, enhance frequency-specific inhibitory associative memory (evidenced by behavioral gradients on the Stimulus Generalization Test). One form of inhibitory A1 representational plasticity that HDAC3i could control might be narrow tuning bandwidth at the CS- frequency (here, 11.5 kHz). This effect was shown to occur by RGFP966 administration in Bieszczad et al. (2015) for tones associated with reward; the same could occur of tones specifically *not* associated with reward. This is a

particularly important potential finding because it would imply that this form of plasticity for tuning bandwidth in A1 is inconsequent to the direction of the contingency between sound and reward. Rather, the change in bandwidth could be a neural trace of the frequency-specificity of memory *per se*.

Additionally, the current data can also be explained by a "below zero" extinction (Pavlov, 1927) approach to understanding the effects of HDAC3i on CS- memory. Extinction training that does not result in spontaneous recovery is known to occur with a decrease in representational area of the extinguished signal-sound in A1. Moreover, the size of the reduction in A1 area (or the failure to reduce A1 area at all after extinction training) corresponds to the altered strength of the original memory (Bieszczad & Weinberger, 2012). Behavioral data from animals in this experiment on the reversal test provide two compelling pieces of preliminary support for a potential "sub-zero" effect. First, HDAC3i-treated rats show an inhibitory behavioral generalization gradient around the CS- frequency. Second, HDAC3i-treated rats fail to initiate responding to the former CS- frequency (11.5 kHz) despite the fact that responses would have been rewarded in the reversal test when the former CS+ is no longer rewarded. Together, these findings provide evidencing for a frequency-specific and robust inhibitory memory revealed by inflexible CS- behavior that would delay the formation of new excitatory associations for the frequency of the CS-. Therefore, HDAC3-inhibition appears to control the ability to form highly robust and frequency-specific excitatory and also inhibitory sound-reward associations.

Conclusion

An overarching goal of this research has been to address the effects of epigenetic control by HDAC3 inhibition on memory specificity and strength by examining its role in an auditory discrimination paradigm. Collectively, these findings show that that inhibition of HDAC3 by systemic injections of RGFP966 early in learning is sufficient to facilitate acquisition of a sound frequency discrimination task, and alter memory strength and sound-specificity. These results provide additional support for the growing body of work that HDAC3 is a key negative regulator of long-term memory formation. Critical future investigations to examine HDAC3-inhibition function to modulate associative auditory memory on a neural systems level will be to understand how the behavioral effects are linked to representational plasticity for sound in A1, and further to identify the molecular links between HDAC3 modulation and cortical plasticity.

References

Ahmad Ganai, S., Ramadoss, M., & Mahadevan, V. (2016). Histone Deacetylase (HDAC) Inhibitors - emerging roles in neuronal memory, learning, synaptic plasticity and neural regeneration. *Current Neuropharmacology*, *14*(1), 55–71. http://doi.org/10.2174/1570159X13666151021111609

Alberini, C. M. (2009). Transcription Factors in Long-Term Memory and Synaptic Plasticity. *Physiological Reviews*, *89*(1), 121–145. http://doi.org/10.1152/physrev.00017.2008

Bakin, J. S., & Weinberger, N. M. (1990). Classical conditioning induces CS-specific receptive field plasticity in the auditory cortex of the guinea pig. *Brain Research*, *536*(1-2), 271–286. http://doi.org/10.1016/0006-8993(90)90035-a

Bao, S., Chan, V. T., Zhang, L. I., & Merzenich, M. M. (2003). Suppression of cortical representation through backward conditioning (Vol. 100, pp. 1405–1408). Presented at the Proceedings of the National Academy of Sciences of the United States of America, National Acad Sciences. http://doi.org/10.1073/pnas.0337527100

Bao, S., Chang, E. F., Woods, J., & Merzenich, M. M. (2004). Temporal plasticity in the primary auditory cortex induced by operant perceptual learning. *Nature Neuroscience*, *7*(9), 974–981. http://doi.org/10.1038/nn1293

Barrett, R. M., & Wood, M. A. (2008). Beyond transcription factors: The role of chromatin modifying enzymes in regulating transcription required for memory. *Learning & Memory*, *15*(7), 460–467. http://doi.org/10.1101/lm.917508

Berlau, K. M., & Weinberger, N. M. (2008). Learning strategy determines auditory cortical plasticity. *Neurobiology of Learning and Memory*, *89*(2), 153–166. http://doi.org/10.1016/j.nlm.2007.07.004

Bieszczad, K. M., Bechay, K., Rusche, J. R., Jacques, V., Kudugunti, S., Miao, W., et al. (2015). Histone Deacetylase Inhibition via RGFP966 Releases the Brakes on Sensory Cortical Plasticity and the Specificity of Memory Formation. *Journal of Neuroscience*, *35*(38), 13124–13132. http://doi.org/10.1523/JNEUROSCI.0914-15.2015

Blake, D. T., Strata, F., Churchland, A. K., & Merzenich, M. M. (2002). Neural correlates of instrumental learning in primary auditory cortex (Vol. 99, pp. 10114–10119). Presented at the Proceedings of the National Academy of Sciences of the United States of America, National Acad Sciences. http://doi.org/10.1073/pnas.092278099

Chavez, C. M., McGaugh, J. L., & Weinberger, N. M. (2013). Activation of the basolateral amygdala induces long-term enhancement of specific memory representations in the cerebral cortex. *Neurobiology of Learning and Memory*, *101*, 8–18. http://doi.org/10.1016/j.nlm.2012.12.013

Day, J. J., & Sweatt, J. D. (2011a). Cognitive neuroepigenetics: A role for epigenetic mechanisms in learning and memory. *Neurobiology of Learning and Memory*, *96*(1), 2–12. http://doi.org/10.1016/j.nlm.2010.12.008

Day, J. J., & Sweatt, J. D. (2011b). Epigenetic Mechanisms in Cognition. *Neuron*, *70*(5), 813–829. http://doi.org/10.1016/j.neuron.2011.05.019

Edeline, J.-M., & Weinberger, N. M. (1992). Associative retuning in the thalamic source of input to the amygdala and auditory cortex: Receptive field plasticity in the medial

division of the medial geniculate body. *Behavioral Neuroscience*, *106*(1), 81–105. http://doi.org/10.1037//0735-7044.106.1.81

Edeline, J.-M., Neuenschwander-El Massioui, N., & Dutriex, G. (1990). Frequency-specific cellular changes in the auditory system during acquisition and reversal of discriminative conditioning. *Psychobiology*, *18*(4), 382–393.

Elias, G. A., Bieszczad, K. M., & Weinberger, N. M. (2015). Learning strategy refinement reverses early sensory cortical map expansion but not behavior: Support for a theory of directed cortical substrates of learning and memory. *Neurobiology of Learning and Memory*, (126), 39–55. http://doi.org/10.1016/j.nlm.2015.10.006

Hawk, J. D., Florian, C., & Abel, T. (2011). Post-training intrahippocampal inhibition of class I histone deacetylases enhances long-term object-location memory. *Learning & Memory*, *18*(6), 367–370. http://doi.org/10.1101/lm.2097411

Hemstedt, T. J., Lattal, K. M., & Wood, M. A. (2017). Reconsolidation and extinction: Using epigenetic signatures to challenge conventional wisdom. *Neurobiology of Learning and Memory*, *142*, 55–65. http://doi.org/10.1016/j.nlm.2017.01.007

Kilgard, M. P., & Merzenich, M. M. (1999). Distributed representation of spectral and temporal information in rat primary auditory cortex. *Hearing Research*, *134*(1-2), 16–28. http://doi.org/10.1016/s0378-5955(99)00061-1

Lattal, K. M., & Wood, M. A. (2013a). Epigenetics and persistent memory: implications for reconsolidation and silent extinction beyond the zero. *Nature Neuroscience*, *16*(2), 124–129. http://doi.org/10.1038/nn.3302

Lattal, K. M., & Wood, M. A. (2013b). Epigenetics and persistent memory: implications for reconsolidation and silent extinction beyond the zero. *Nature Neuroscience*, *16*(2), 124–129. http://doi.org/10.1038/nn.3302

Leon, M. I., Miasnikov, A. A., Wright, E. J., III, & Weinberger, N. M. (2017). CS-specific modifications of auditory evoked potentials in the behaviorally conditioned rat. *Brain Research*, *1670*, 235–247. http://doi.org/10.1016/j.brainres.2017.06.030

McGann, J. P. (2015). Associative learning and sensory neuroplasticity: how does it happen and what is it good for? *Learning & Memory*, *22*(11), 567–576. http://doi.org/10.1101/lm.039636.115

Migler, B., & Millenson, J. R. (1969). Analysis of response rates during stimulus generalization. *Journal of the Experimental Analysis of Behavior*, *12*, 81–87.

Ohl, F. W., & Scheich, H. (2005). Learning-induced plasticity in animal and human auditory cortex. *Current Opinion in Neurobiology*, *15*(4), 470–477. http://doi.org/10.1016/j.conb.2005.07.002

Phan, M. L., & Bieszczad, K. M. (2016). Sensory Cortical Plasticity Participates in the Epigenetic Regulation of Robust Memory Formation. *Neural Plasticity*, *2016*(3), 1–12. http://doi.org/10.1155/2016/7254297

Phan, M. L., Gergues, M. M., Mahidadia, S., Jimenez-Castillo, J., Vicario, D. S., & Bieszczad, K. M. (2017). HDAC3 Inhibitor RGFP966 Modulates Neuronal Memory for Vocal Communication Signals in a Songbird Model. *Frontiers in Systems Neuroscience*, *11*, 121–12. http://doi.org/10.3389/fnsys.2017.00065

Polley, D. B. (2006). Perceptual Learning Directs Auditory Cortical Map Reorganization through Top-Down Influences. *Journal of Neuroscience*, *26*(18), 4970–4982. http://doi.org/10.1523/JNEUROSCI.3771-05.2006

Recanzone, G. H., Schreiner, C. E., & Merzenich, M. M. (1993). Plasticity in the Frequency Representation of Primary Auditory Cortex following Discrimination Training in Adult Owl Monkeys. *The Journal of Neuroscience*, *13*, 87–103.

Rescorla, R. A. (1988). Pavlovian conditioning: It's not what you think it is. *American Psychologist*, *43*(3), 151–160. http://doi.org/10.1037//0003-066x.43.3.151

Rutkowski, R. G., & Weinberger, N. M. (2005). Encoding of learned importance of sound by magnitude of representational area in primary auditory cortex (Vol. 102, pp. 13664–13669). Presented at the Proceedings of the National Academy of Sciences of the United States of America, National Acad Sciences. http://doi.org/10.1073/pnas.0506838102

Schreiner, C. E., & Malone, B. J. (2015). Representation of loudness in the auditory cortex. *The Human Auditory System: Fundamental Organization and Clinical Disorders* (1st ed., Vol. 129, pp. 73–84). Elsevier B.V. http://doi.org/10.1016/B978-0-444-62630-1.00004-4

Sweatt, J. D. (2013). The Emerging Field of Neuroepigenetics. *Neuron*, *80*(3), 624–632. http://doi.org/10.1016/j.neuron.2013.10.023

Talwar, S. K., & Gerstein, G. L. Auditory frequency discrimination in the white rat. Weinberger, N. M. (2004). Specific long-term memory traces in primary auditory cortex. *Nature Reviews Neuroscience*, *5*(4), 279–290. http://doi.org/10.1038/nrn1366

Weinberger, N. M. (2007a). Associative representational plasticity in the auditory cortex: A synthesis of two disciplines. *Learning & Memory*, *14*(1-2), 1–16. http://doi.org/10.1101/lm.421807

Weinberger, N. M. (2007b). Associative representational plasticity in the auditory cortex: resolving conceptual and empirical problems. *Debates in Neuroscience*, *1*(2-4), 85–98. http://doi.org/10.1007/s11559-007-9011-9

Weinberger, N. M. (2007c). Auditory associative memory and representational plasticity in the primary auditory cortex. *Hearing Research*, *229*(1-2), 54–68. http://doi.org/10.1016/j.heares.2007.01.004

Weinberger, N. M. (2010). Plasticity in the Primary Auditory Cortex: Substrate of Specific Long-Term Memory Traces. *Encyclopedia of Behavioral Neuroscience* (pp. 79–86). Elsevier. http://doi.org/10.1016/b978-0-08-045396-5.00151-2

Zovkic, I. B., Guzman-Karlsson, M. C., & Sweatt, J. D. (2013). Epigenetic regulation of memory formation and maintenance. *Learning & Memory*, *20*(2), 61–74. http://doi.org/10.1101/lm.026575.112

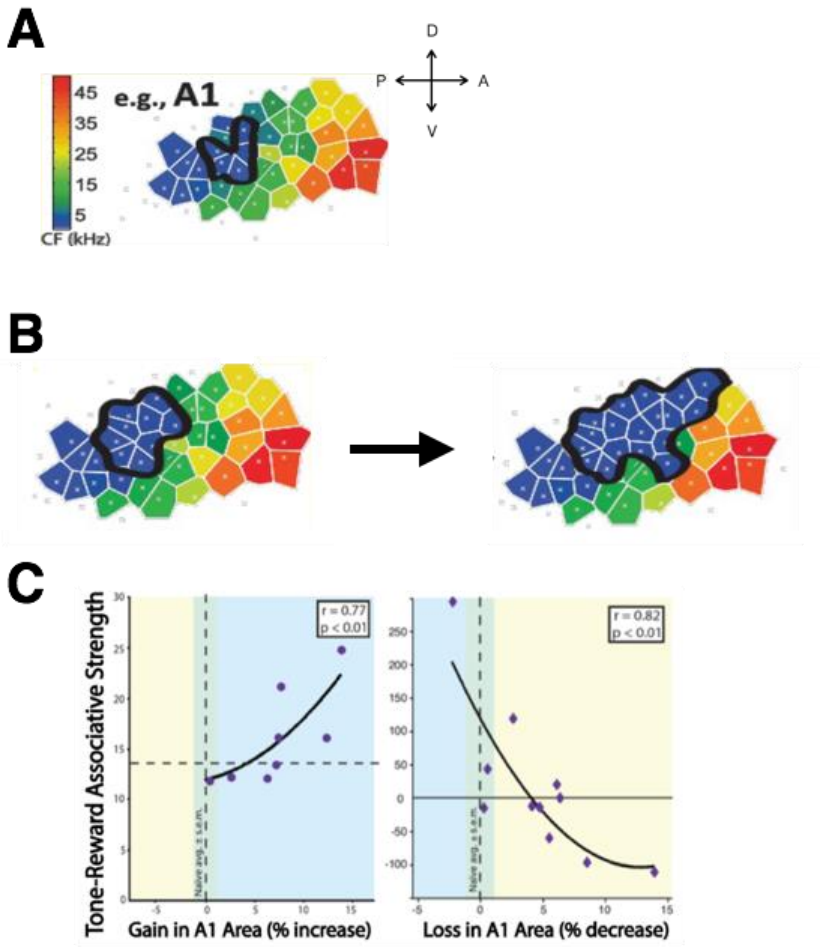

Figure 1. Schematic for sound frequency representation in A1 with associative learning. (A) Exemplar map of A1 shows topographic organization for sound frequency. Auditory cortical cells are organized in a tonotopic manner. **(B) Remodeling with associative learning.** Representation for the learned sound-frequency signal (outlined) increases with learning. **(C) Representational plasticity in A1 a likely neural substrate of learning and memory (from Bieszczad & Weinberger, 2010; 2012).** Remodeling of A1 is strongly correlated with learning experiences. Signal-specific area gains in A1 with acquisition of a tone-reward association (left); signal-specific area loss in A1 following behavioral extinction of the tone-reward signal (right).

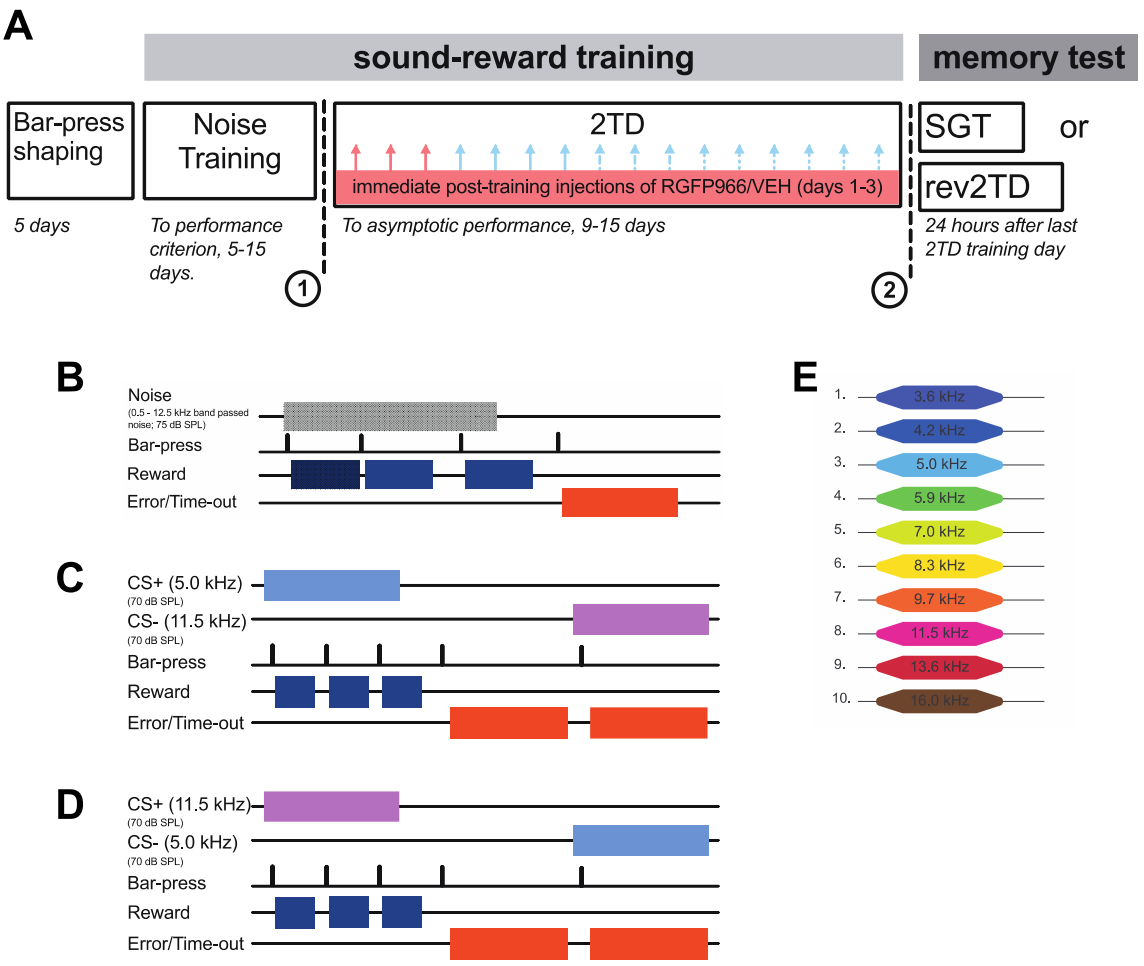

Figure 2. Protocol for associative auditory learning and behavioral memory testing. (A) Timeline for behavioral procedures. Animals are bar-press shaped for 5 days, followed by Noise Training to performance criterion. Animals are performance-matched based on acquisition curves (1). Performance-matched pairs of animals then proceed onto 2TD task training, and are trained daily to performance criterion (7-12 days), with an additional 2-3 days to insure stable performance (9-15 days total). Animals received immediate post-training injections of RGFP966/Vehicle after sessions 1-3 (pink arrows), and saline injections after subsequent training sessions (blue arrows). Pairs of animals are trained until both animals reach asymptotic performance; memory tests occur on the same day for both animals (2). 24 hours after the last 2TD training session, memory for the associative sound-signals is tested on a Stimulus Generalization Test (SGT; n = 18) or a reversal test (rev2TD; n = 6). **(B) Protocol for Noise Training.** All animals were trained to BP to a simple auditory stimulus (white noise, 75 dB) for water reward. Each noise presentation lasted 7 or 9 seconds. Up to 3 rewards (water cup presented for 3 seconds each) could be attained per trial. Bar-presses during the silent inter-trial periods resulted in an error signal/time-out. **(C) Protocol for 2TD Training.** Bar-presses to the CS+ (5.0 kHz pure tone, 70 dB SPL) are rewarded (3 second presentation of water reward). Bar-presses to the CS- (11.5 kHz pure tone, 70 dB SPL) or silent ITIs result in an error signal

(flashing house light) and a time-out. Trials are randomized; all tones are presented for 8 seconds. A maximum of 3 rewards/trial can be attained. **(D) Stimulus Generalization Test.** Ten test tones (including the CS+ and CS- frequencies) are presented in a pseudorandom order over 120 trials. All trials are unrewarded. **(E) Reversal Test.** All parameters are identical to the 2TD, except for the reversal of the tone-outcome contingencies. BPs to the 11.5 kHz tones are rewarded, while BPs to the 5.0 kHz trigger an error signal/time-out.

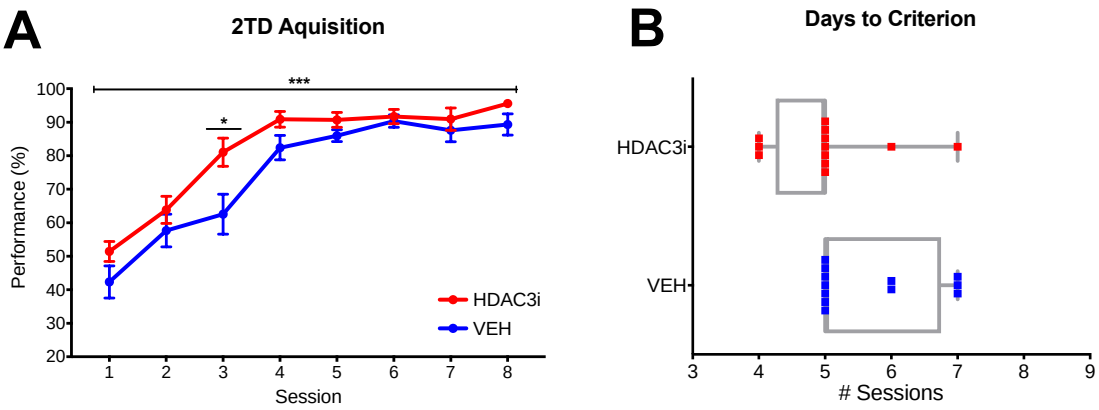

Figure 3. Two-tone discrimination task acquisition over days 1-9. (A) Acquisition was calculated as the number of BPs to the CS+ tone divided by the total number of BPs to the CS+ or CS-tone (#CS+ BPs/[#CS+ BPs & #CS- BPs]). Performance increased for both groups over daily training sessions ($p<0.001$). The HDAC3i group's overall performance higher than the VEH group ($p = 0.015$). Groups were significantly different on session 3: HDAC3i, 81.06 ± 4.22%; VEH, 62.55 ± 5.96%, $p = 0.002$ (corrected for multiple comparisons; all other days, $p > 0.05$). **(B)** Days to reach performance criterion. HDAC3i: M = 5.33 ± 0.40 sessions, min = 4, max = 8; VEH: M = 5.58 ± 0.34, min = 3, max = 7. Data for (A) show mean ± SEM. ***$p<0.001$; *$p<0.05$; (B) show mean, range, and individual animal's days to criterion.

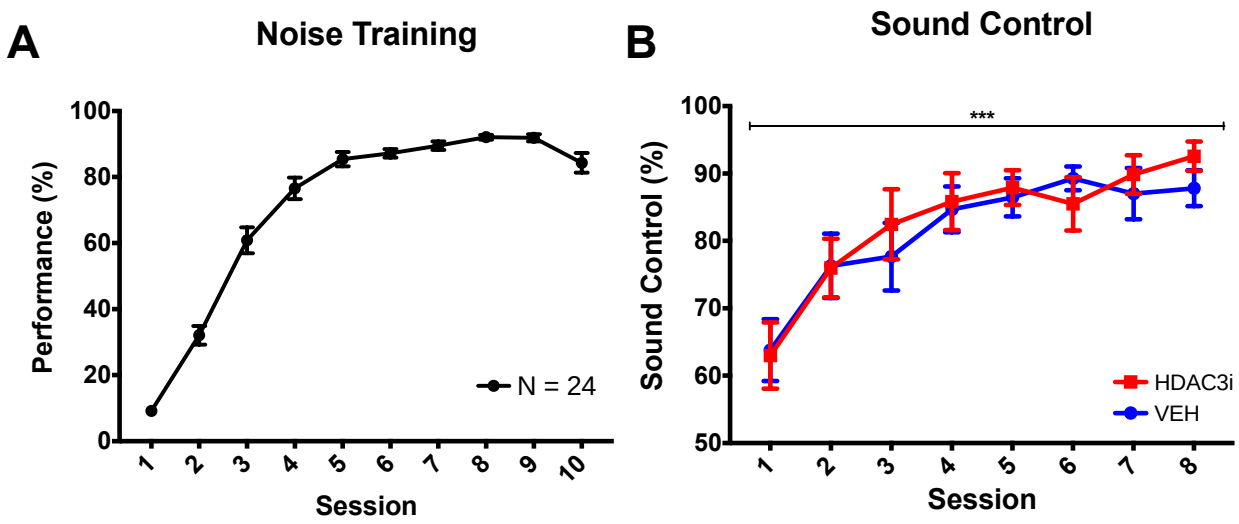

Figure 4. Bar-pressing under sound control across tasks. (A) Animals (learn to bar-press only to sound for reward. Performance = #BP to sound/#Total BP. Performance criterion (is achieved after 5 to 15 training sessions (session 1-10 shown; $M = 9.67 \pm 0.44$ sessions; session 1-5, n=24; session 6-7, n=23; session 8, n=20; session 9, n=10; session 10, n=5). **(B)** Sound controlled bar-pressing behavior on the 2TD (sessions 1-8). Sound control = (#BPs to the CS+ and CS-)/#Total BPs. Both HDAC3i (red; n=12) and VEH (blue; n=12) groups selectively bar-press to tones (note: y-axis starts at 50%). Sound control significantly increased over sessions ($p<0.001$). There is no effect of treatment or an interaction between treatment and session on sound control. All data represent mean ± sem. *** $p<0.001$.

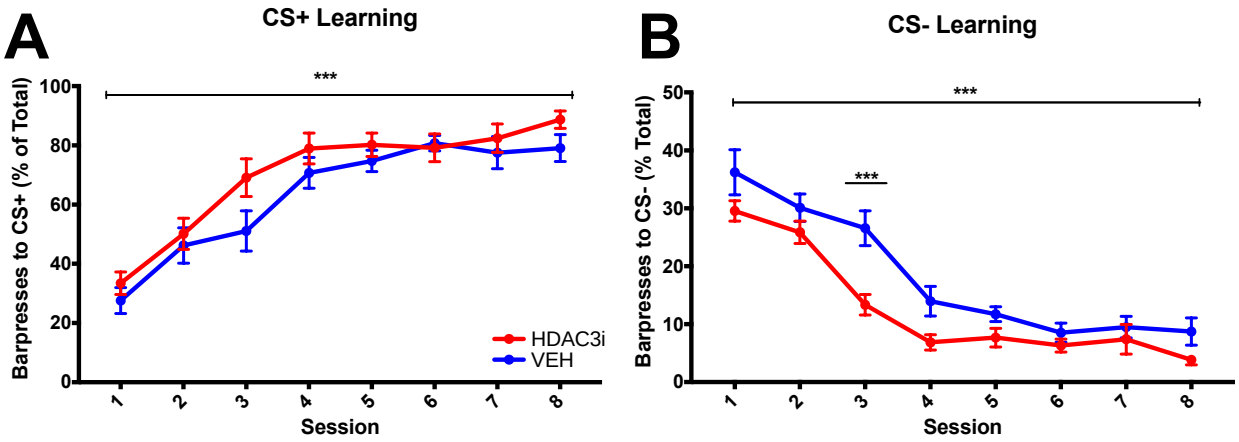

Figure 5. CS specific learning over 2TD training sessions 1-9. (**A)** CS+ barpresses significantly increased over daily training sessions ($p<0.001$). Session 1: HDAC3i, 33.44 ±2.78%; VEH, 27.58 ± 4.36%. Day 8: HDAC3i, 88.72 ± 2.91%; VEH, 79.01 ± 4.56%. There was no difference between treatment groups ($p = 0.144$). (**B)** CS- barpresses significantly decreased over sessions ($p<0.001$). Session 1: HDAC3i, 28.54 ± 1.75%; VEH, 36.21 ± 3.89%. Day 8: HDAC3i, 3.84 ± 0.87%; VEH, 8.73 ± 2.34%. The HDAC3i group bar-pressed significantly less to the CS- on session 3 than the VEH group ($p<0.001$). N=12 for both HDAC3i and VEH groups across all sessions. Data represent mean ± SEM. *** $p<0.001$.

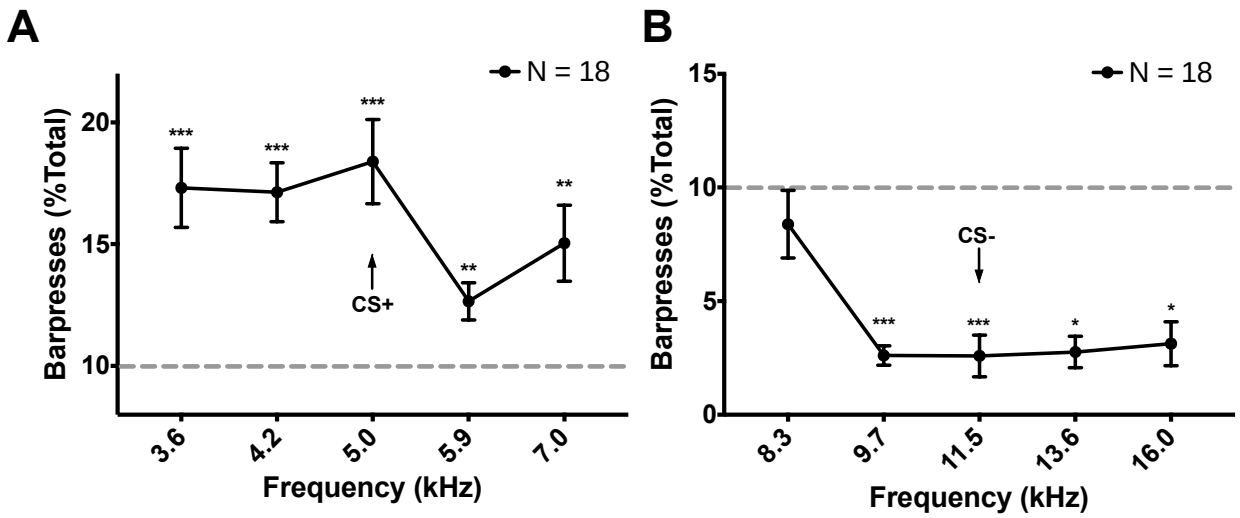

Figure 6. Bar-presses to SGT test tones. (A). Barpresses to the CS+ frequency (5.0 kHz) and neighboring tones. BPs peak at 5.0 kHz, the CS+ (18.39 ± 1.73%). Responses to tones are all significantly higher than a generalized response distribution (10%; grey dashed line): 3.6 kHz, $p<0.0001$; 4.2 kHz, $p<0.0001$; 5.0 kHz, $p<0.0001$; 5.9 kHz, $p = 0.006$; 7.0 kHz $p = 0.006$. **(B)** Barpresses to the CS- frequency and neighboring tones. Rats barpress the least to 11.5 kHz (M = 2.59 ± 0.92%). Responses to 9.7 ($p <0.0001$), 11.5 ($p<0.0001$), 13.6 ($p = 0.012$), and 16.0 kHz ($p = 0.036$) are significantly lower than a generalized response. Barpresses to 8.3 kHz are not significantly different ($p = 0.890$). ***$p<0.0001$, **$p<0.01$ * $p<0.05$ relative to generalized response, Holm-Bonferroni corrected for multiple comparisons.

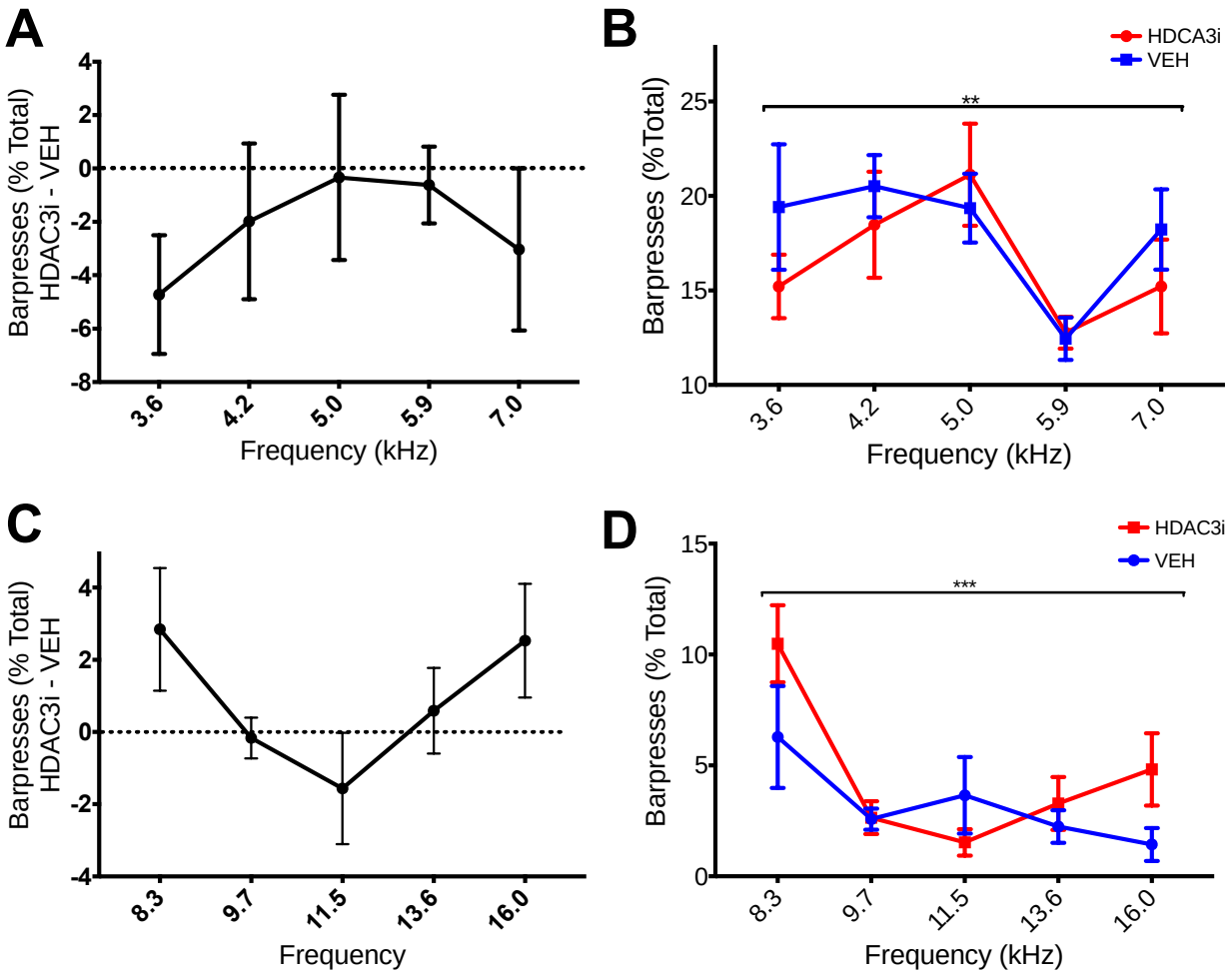

Figure 7. Behavioral stimulus generalization gradients for number of bar-presses. **(A**) Differences between performance-matched pairs of HDAC3i and VEH treated animals on the SGT. Data show number of barpresses to CS+ and neighboring test frequencies over the first 60 SGT trials. Dashed line indicates no difference between HDAC3i and VEH pairs. **(B).** Barpresses at the CS+ and neighboring test frequencies on the SGT (trials 1-60). Data show unpaired mean BPs for the HDAC3i and VEH group. The number of BPs differs significantly across test frequencies ($p = 0.007$), but HDAC3i and VEH groups did not statistically differ in their BP 4responses ($p>0.05$). **(C)** Differences between performance-matched pairs of HDAC3i and VEH treated animals on the SGT. Data show number of BPs to the CS- and neighboring test frequencies over all SGT trials (120 trials). Dashed line indicates no differences between HDAC3i and VEH groups. **(D)** Barpresses to the CS- (11.5 kHz) and neighboring frequencies. Data show unpaired group means. Barpresses to test tones was significantly different across frequencies ($p <0.0001$), but behavioral response gradients for HDAC3i and VEH groups were not statistically different ($p>0.05$). **$p<0.01$, ***$p<0.0001$.

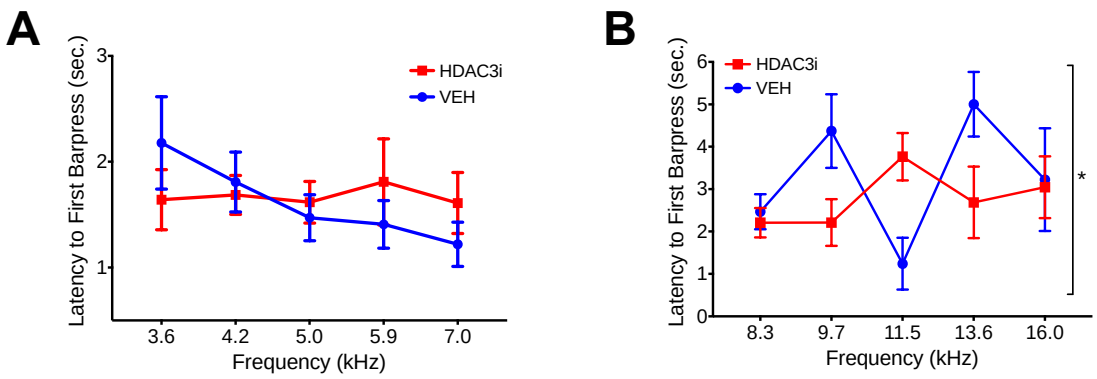

Figure 8. **Frequency generalization gradients for bar-press latency. (A)** Latency to bar-press to CS+ and neighboring frequencies. Latency to bar-press is similar across frequencies for HDAC3i and VEH groups ($p>0.05$). **(B)** Latencies to bar-press to CS- and neighboring frequencies. ANOVA reveled a significant interaction between treatment and frequency ($p = 0.0119$), however, multiple-comparisons at each of the test frequencies showed no difference between the two groups.

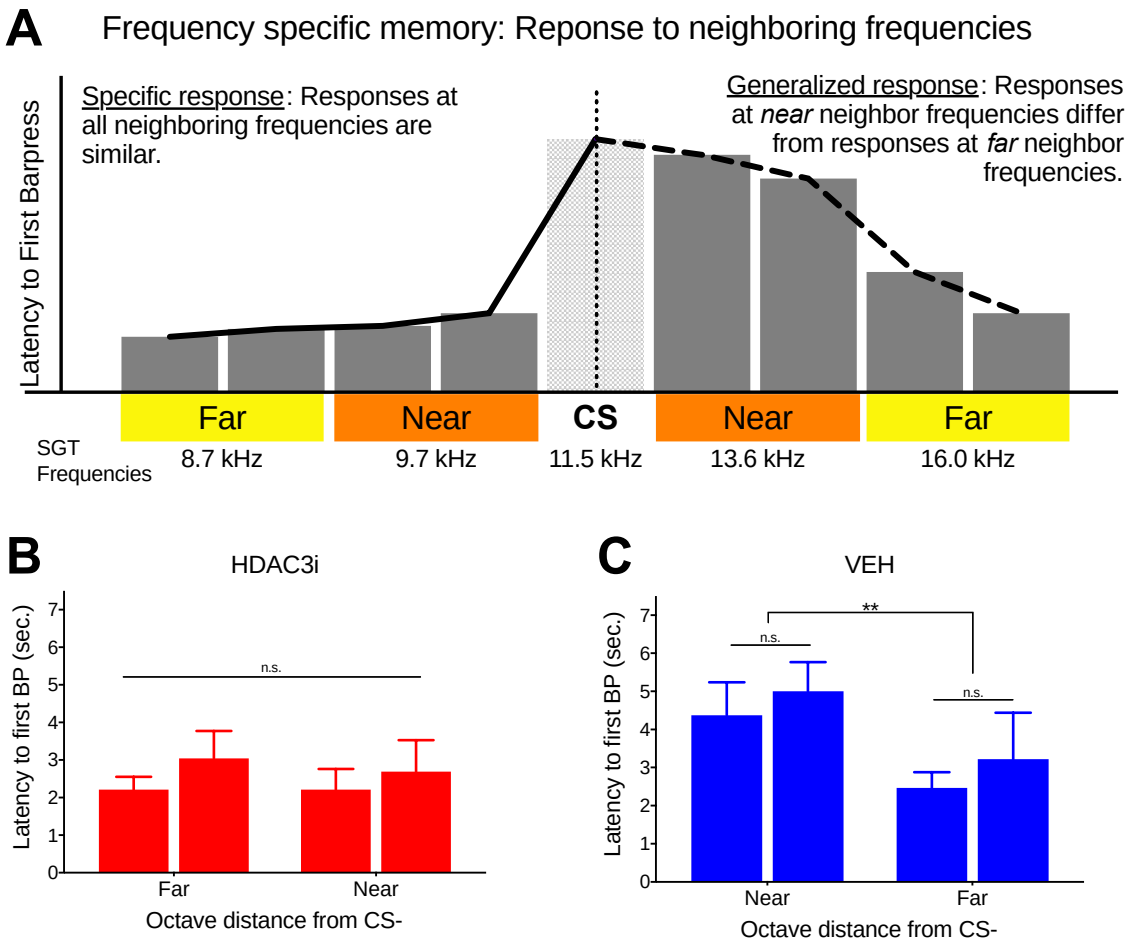

Figure 9. Frequency-specificity of memory assessed by comparing behavioral gradients to test tones neighboring the CS frequency. (A) Model for specific vs. generalized memory for the CS- frequency (11.5 kHz). Left: Behavioral response peaks (here, latency to bar-press) occur only to the CS- test tone when memory for acoustic frequency is highly specific. Responses to stimuli that are near and far neighbors (i.e. closer and farther from the CS in sound frequency) of the CS are similar. Right: Near and far neighbor tones elicit different behavioral responses. Failure to generalize responses to *non-CS signals* indicate less precise frequency-specific memory. **(B)** HDAC3i group means for bar-press latency are similar for near and far neighbor test tones around the CS- frequency. **(C).** VEH group means show that animals are slower to bar-press to tones close to the CS- frequency than to more distance neighbors ($p<0.001$).

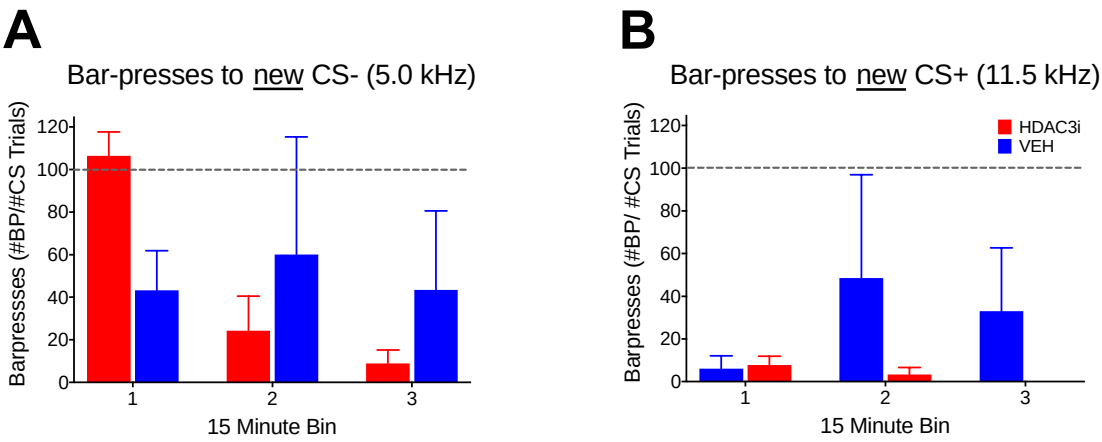

Figure 10. Bar-presses to new CS- and CS+ on the reversal test. Bar-presses are calculated as: number of bar-presses to tone/number of CS tone trials. A score of 100% indicates an average of 1 BP per CS- trial. **(A)** Bar-presses to 5.0 kHz, the new CS-, are not rewarded. **(B)** Responses to the 11.5 kHz tone.