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Distinct roles for the deacetylase domain of HDAC3 in the hippocampus and medial prefrontal cortex in the formation and extinction of memory

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ABSTRACT

Histone deacetylases (HDACs) are chromatin modifying enzymes that have been implicated as powerful negative regulators of memory processes. HDAC3 has been shown to play a pivotal role in long-term memory for object location as well as the extinction of cocaine-associated memory, but it is unclear whether this function depends on the deacetylase domain of HDAC3. Here, we tested whether the deacetylase domain of HDAC3 has a role in object location memory formation as well as the formation and extinction of cocaine-associated memories. Using a deacetylase-dead point mutant of HDAC3, we found that selectively blocking HDAC3 deacetylase activity in the dorsal hippocampus enhanced long-term memory for object location, but had no effect on the formation of cocaine-associated memory. When this same point mutant virus of HDAC3 was infused into the prelimbic cortex, it failed to affect cocaine-associated memory formation. With regards to extinction, impairing the HDAC3 deacetylase domain in the infralimbic cortex had no effect on extinction, but a facilitated extinction effect was observed when the point mutant virus was delivered to the dorsal hippocampus. These results suggest that the deacetylase domain of HDAC3 plays a selective role in specific brain regions underlying long-term memory formation of object location as well as cocaine-associated memory formation and extinction.

1. Introduction

Histone acetylation is a well-studied chromatin modifying mechanism involved in the regulation of gene expression required for memory. Numerous studies have shown that histone acetylation is involved in long-term memory formation (*e.g.* reviewed in Gräff & Tsai, 2013; Levenson & Sweatt, 2006; McQuown & Wood, 2011; Peixoto & Abel, 2013; Penney & Tsai, 2014). Histone acetylation is carried out by histone acetyltransferases and histone deacetylases (HDACs), which in general facilitate and repress gene expression, respectively (Jenuwein & Allis, 2001; Kouzarides, 2007). Histone deacetylase 3 (HDAC3) is the most highly expressed Class I HDAC in the brain, and this specific HDAC is a powerful negative regulator of learning and memory processes (Kwapis et al., 2017; Malvaez et al., 2013; McQuown et al., 2011; Rogge, Singh, Dang, & Wood, 2013). Our laboratory has shown that HDAC3 plays a critical role in object location and fear related memory formation (Kwapis et al., 2017; McQuown et al., 2011) using hippocampus and amygdala specific HDAC3 manipulations. We have also shown that HDAC3 inhibition facilitates extinction of drug-seeking behavior in a manner that blocks reinstatement (Malvaez et al., 2013). The extinction experiments in Malvaez et al. (2013) used a selective HDAC3 inhibitor given systemically, thus we were unable to identify which brain regions are most important for HDAC3-dependent modulation of extinction.

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HDAC3 itself has potent deacetylase activity (Guenther, Yu, Kao, Yen, & Lazar, 2002; Lahm et al., 2007; Zhang, Kalkum, Chait, & Roeder, 2002), and it associates with HDAC4/HDAC5 via interactions with the co-repressor nuclear receptor corepressor (NCoR) to form a functional, multi-protein repressor complex (Alenghat et al., 2008; Fischle et al., 2002; Guenther, Barak, & Lazar, 2001). One idea is that these multi-protein complexes may be required for deacetylation because HDAC4 has little to no catalytic activity on canonical acetyl lysine substrates (Lahm et al., 2007). HDAC4 has been shown to modulate memory independent of its deacetylase domain (Sando et al., 2012). Outside the field of learning and memory, HDAC3-mediated gene expression in other tissues does not necessarily require HDAC3 enzymatic function (Sun et al., 2013), suggesting that perhaps the deacetylase activity of HDAC3 may not be required for memory formation. Until recently, the deacetylase activity of HDAC3 in memory formation had not been directly tested. Kwapis et al. (2017) demonstrated that indeed the enzymatic activity of HDAC3 is required for amygdala-dependent forms of memory formation.

Previous studies had demonstrated that systemic administration of an HDAC3 inhibitor could facilitate both the formation of object location memory (OLM) as well as the extinction of cocaine-context associated memory (Malvaez et al., 2013). However, whether the deacetylase activity of HDAC3 is required for object location memory OLM in the hippocampus, and extinction of cocaine-context associated memory in the infralimbic cortex remain unclear. In the current study, we specifically targeted the deacetylase activity of HDAC3 in hippocampus-dependent memory formation as well as in the dorsal hippocampus (DH), prelimbic cortex (PrL), and infralimbic cortex (IL) with regard to the acquisition and extinction of cocaine-conditioned place preference, or cocaine-context associated memory processes.

2. Materials and methods

2.1. Subjects

All procedures were approved by the University of California, Irvine's Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health guidelines. Mice were 8–12 weeks old and had access to food and water ad libitum in their home cages with lights maintained on a 12 h light/dark cycle. Behavioral testing was performed during the light portion of the cycle. Subjects were adult male C57BL/6J mice for the AAV-HDAC3(Y298H)-v5 experiments. For the HDAC3 flox deletion experiment, $Hdac3^{flox/flox}$ and wild-type $Hdac3^{+/+}$ littermate mice were maintained on a C57BL/6J background (Mullican et al., 2011). Briefly, these mice were generated at the laboratory of Dr. Mitch Lazar at the University of Pennsylvania (Philadelphia, PA) with loxP sites flanking exon 4 through exon 7 of the *Hdac3* gene, a region required for the catalytic activity of the enzyme (Mullican et al., 2011).

2.2. Drugs

Cocaine-HCl was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in saline (0.9% NaCl). Cocaine-HCl is expressed as the weight of the salt. For cocaine-CPP training in the acquisition experiments, cocaine-HCl was dissolved to a final concentration of 0.5 mg/ml and administered in a volume of 10 ml/kg body weight, resulting in a final dose of 5 mg/kg. For cocaine-CPP training in the extinction experiment, cocaine-HCl was dissolved to a final concentration of 2 mg/ml and administered in a volume of 10 ml/kg body weight, resulting in a final dose of 20 mg/kg. Cocaine-HCl and saline were administered intraperitoneally (i.p).

2.3. Surgery

Mice were induced with 4% isoflurane in oxygen and maintained at 1.5–2.0% for the duration of surgery. Animals were injected with either AAV-HDAC3(Y298H)-v5 or AAV-EV (Empty Vector) (Kwapis et al., 2017). For the DH experiments, 1 µl of virus was infused bilaterally. For the prelimbic and infralimbic experiments, 0.3 µl of virus was infused bilaterally. Immunofluorescence was used to confirm expression of HDAC3(Y298H). Injection needles were lowered to the desired coordinates at a rate of 0.2 mm/15 s. 2 min after reaching the target depth, virus was injected at a rate of 6 µl/h. After infusion, injection needles were left in place for 2 min to allow the virus to diffuse. The injectors were then raised 0.1 mm and allowed to sit for another minute before being slowly removed (0.1 mm/15 s). The incision was sutured and 2 weeks were allowed for full viral expression before behavior. Viruses were infused with dual 28 gauge infusers (DH: 3 mm center-to-center; PrL/IL: 0.8 mm center-to-center) attached to PE50 tubing and connected to Hamilton Syringes mounted on infusion pumps. Coordinates for the DH were: AP, −2.0 mm; ML, ± 1.5 mm; DV, −1.5 mm relative to Bregma. Coordinates for the PrL were: AP, +1.9 mm; ML, ± 0.4 mm; DV, −2.2 mm relative to Bregma. Coordinates for the IL were: AP, +1.5; ML, ± 0.4 mm; DV, −3.2 mm relative to Bregma.

2.4. AAV production

Wild-type HDAC3 was amplified from mouse hippocampal cDNA and cloned into a modified pAAV-IRES-hrGFP (Agilent), under control of the CMV promoter and β-globin intron. To create the point mutation, a single nucleotide substitution in exon 11 to direct production of a histidine residue in place of tyrosine at amino acid 298 was created (plasmid MW92). For the Empty Vector control, the HDAC3 coding sequence was not present, but all other elements remain (plasmid MW87). Adeno-associated virus (AAV) was made by the Penn Vector Core (University of Pennsylvania) from the above described plasmids and was serotyped with AAV 2.1. The final titer of AAV-HDAC3(Y298H) was 6.48×10^{12} GC/ml and the final titer of AAV-EV was 1.35×10^{13} GC/ml.

2.5. Immunofluorescence

In behavioral experiments shown in Figs. 2–7, every animal included in the behavior analyses had viral infusion confirmed by immunohistochemistry. Mice were euthanized by cervical dislocation and their brains were removed and flash-frozen in ice-cold isopentane. 20 µm slices were collected throughout the IL/PrL or DH, thaw-mounted on slides, and stored at −80 °C until use. For immunofluorescence analysis, slides were fixed with 4% paraformaldehyde for 10 min and permeablized in 0.01% Triton X-100 in 0.1 M PBS for 15 min. Slides were then blocked for 1 h at room temperature in 8% normal goat serum (Jackson), and incubated overnight at 4 °C in primary antibody (HDAC3 clone Y415 antibody: 1:250; Abcam). The following day, slides were incubated for 1 h at room temperature with goat anti-rabbit Alexa 488 (1:1000 dilution, Invitrogen) in the dark followed by either a 15 min DAPI incubation (1:10,000, Invitrogen; DH). For the experiments in which red fluorescent nissl stain NeuroTrace (1:50 NeuroTrace 530/615; Life Technologies) was used, slides were incubated for 50 min at room temperature followed by two washes in 0.01% Triton-X-100 for 5 min and then two washes in PBS for 5 min. Slides were coverslipped using VectaShield Antifade mounting medium (Vector Laboratories).

All images were acquired with an Olympus Scanner VS110 with a 20× apochromatic objective (numerical aperture 0.75) with VS110 scanner software. All treatment groups were represented on each slide and all images on a slide were captured with the same exposure time. Immunolabeling intensity was quantified with ImageJ by sampling the optical density in the cell layer of CA1 normalized to background.

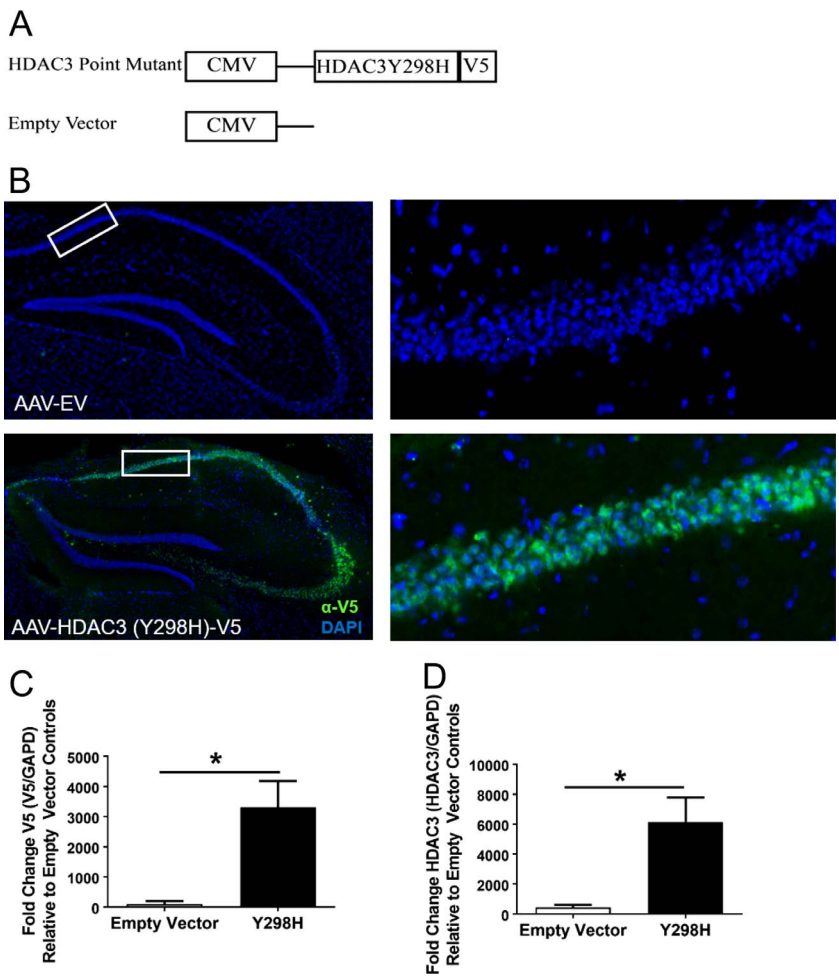


Fig. 1. Dominant negative point mutant virus (HDAC3(Y298H)-v5 expresses effectively in the dorsal hippocampus. (A) V5 epitope was added to the point mutant virus. (B) Representative immunofluorescence image showing expression of the V5 epitope tag (green) in the dorsal hippocampus (DH) after infusion of AAV-HDAC3(Y298H)-v5. No V5 staining was observed with the AAV-EV control virus. Cells were counterstained with DAPI (blue). (C) V5 mRNA was significantly increased in the DH of mice infused with AAV-HDAC3(Y298H)-v5, AAV-EV n = 5, HDAC3(Y298H)-v5 n = 4. (D) HDAC3 was also significantly increased in the DH of mice infused with AAV-HDAC(Y298H)-v5 compared to AAV-EV controls, AV-EV n = 6, HDAC3(Y298H)-v5 n = 6. $^{*}p < 0.05$.

2.6. Quantitative RT-PCR

Quantitative real-time RT-PCR was performed to verify *V5* expression and upregulation of wildtype *Hdac3* mRNA expression following AAV-HDAC3(Y298H) infusion (experiment 1) as previously described (López et al., 2016; White et al., 2016). For experiment 1, 1 mm punches in the DH were collected from 500 µm slices in the area of viral expression (or an equivalent region in EV mice) as confirmed by immunofluorescence. All tissue was stored at −80 °C until processing.

RNA was isolated using an RNeasy Minikit (Qiagen) and cDNA was created using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). The following primers were used, derived from the Roche Universal ProbeLibrary: *Hdac3* right primer, 5′-ttcaacgtgggtgatgactg-3′; *Hdac3* left primer, 5′- ttagctgtgttgctccttgc-3′; probe, ctgctccc; *Hdac3-v5* right primer, 5′-tggagattctcgagggtaagc-3′; *Hdac3-v5* left primer, 5′-atgccacccgtagatctgg-3′; probe, ctctcctc. Each of the probes for these target genes were conjugated to the dye FAM. Glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) was used as a reference gene for all RT-qPCR assays. For *Gapd*, we used the following primers: left primer, 5′atggtgaaggtcggtgtga-3′; right primer, 5-aatctccactttgccactgc-3′; probe, tggcggtattgg. The *Gapd* probe was conjugated to LightCycler Yellow 555. The non-overlapping dyes and quencher on the reference gene allow for multiplexing in the Roche LightCycle 480 II machine (Roche Applied Sciences). All values were normalized to *Gapd* expression levels. Analysis and statistics were performed using the Roche proprietary algorithms and REST 2009 software based on the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002).

2.7. OLM and ORM tasks

For OLM and novel object recognition memory (ORM), habituation data (distance traveled during individual habituation sessions), training, and testing videos were collected using ANY-maze behavioral analysis software. Training and testing for OLM and ORM were performed as described previously (López et al., 2016; McQuown et al., 2011; Vogel-Ciernia & Wood, 2014; Vogel-Ciernia et al., 2013). Before training, mice were handled 1–2 min for 4 d and were habituated to the experimental apparatus 5 min to the OLM chamber for 6 consecutive days in the absence of objects. During the training trial, mice were placed in the experimental apparatus with two identical objects (OLM: 100 ml beakers, 2.5 cm diameter, 4 cm height; ORM: spice tins and glass candle holders) and were allowed to explore these objects for 3 min, which does not result in short or long term memory. We have previously shown that a 3 min training period is insufficient to generate LTM tested at 24 h (Haettig, Sun, Wood, & Xu, 2013; Haettig et al., 2011; López et al., 2016; McQuown et al., 2011; Stefanko, Barrett, Ly, Reolon, & Wood, 2009). Twenty-four hours later, animals' retention was tested for 5 min. For OLM, one copy of the familiar object was placed in the same location as during the training trial, and one copy of the familiar object was placed in the middle of the box. For ORM, one copy of the familiar object and a new object were placed in the same location as during the training trial. All training and testing trials were video recorded and hand scored by individuals blind to animal treatments. Videos were analyzed for total exploration of objects in addition to the discrimination index (DI) [(time spent exploring novel object – time spent exploring familiar object)/(total time exploring both objects) × 100%]. All combinations and locations of objects were used in

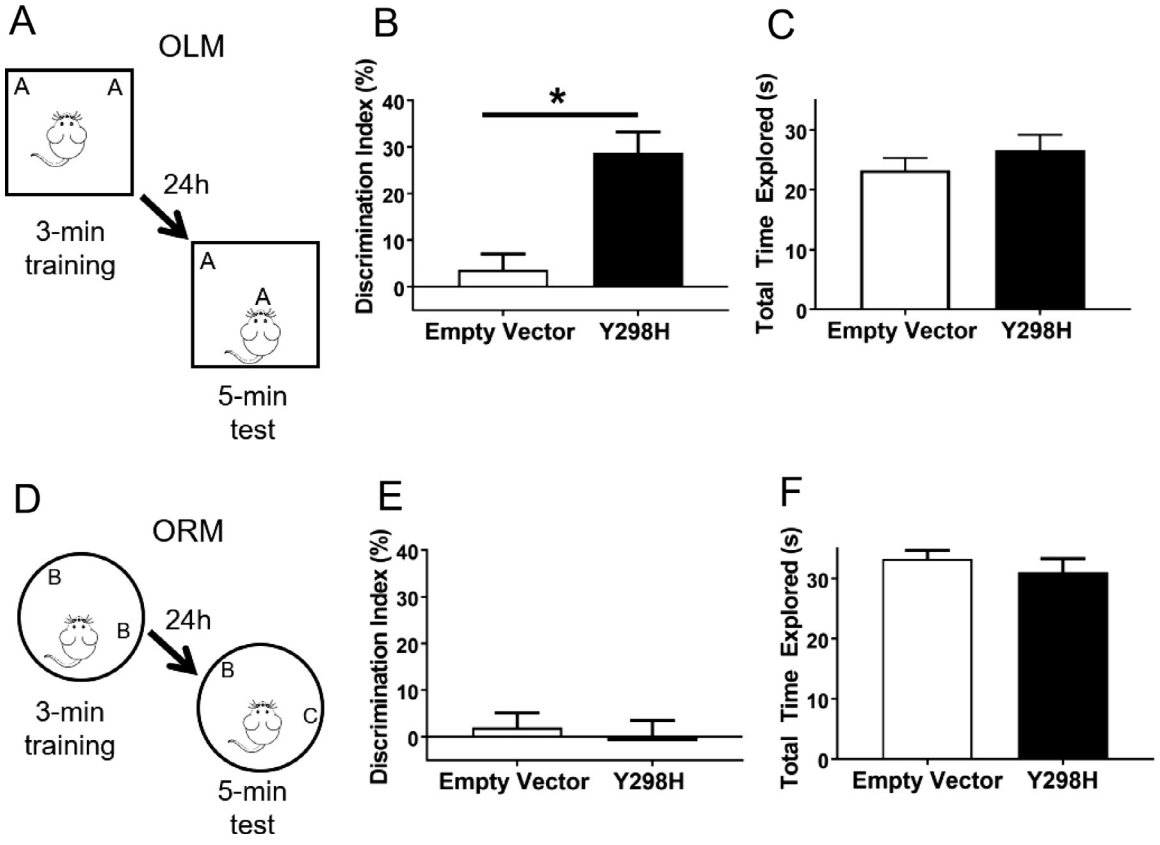
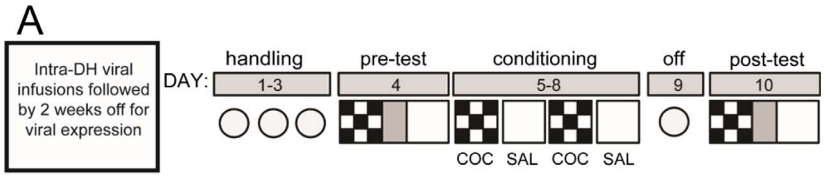


Fig. 2. Blocking HDAC3 activity in the DH enhanced memory for object location (OLM), but not for object recognition (ORM). (A) Mice received subthreshold training (3 min) in an environment with two identical objects and received a retention test 24 h later in which one object is moved to a new location. Schematic describes methods for B and C. (B) Mice given AAV-HDAC3(Y298H)-v5 showed a significant preference for the novel object location 24 h after training compared with EV controls, AAV-EV n = 8, HDAC3(Y298H)-v5 n = 6. (C) Groups did not differ in total exploration time of the two objects. (D) Mice received subthreshold training (3 min) in an environment with two identical objects and received a retention test 24 h later in which one object was replaced with a novel one (ORM), AAV-EV n = 9, HDAC3(Y298H)-v5 n = 7. Schematic describes methods for E and F. (E) Neither AAV-EV control or AAV-HDAC3(Y298H)-v5 mice exhibited significant preference for the novel object. (F) Groups did not differ in total exploration time of the two objects. $^{*}p < 0.05$.

a balanced manner to reduce potential biases attributable to preference for particular locations or objects.

2.8. Conditioned place preference apparatus

Place preference conditioning was performed as described previously in our studies (Malvaez, Mhillaj, Matheos, Palmery, & Wood, 2011; Rogge et al., 2013; White et al., 2016). Briefly, mice were



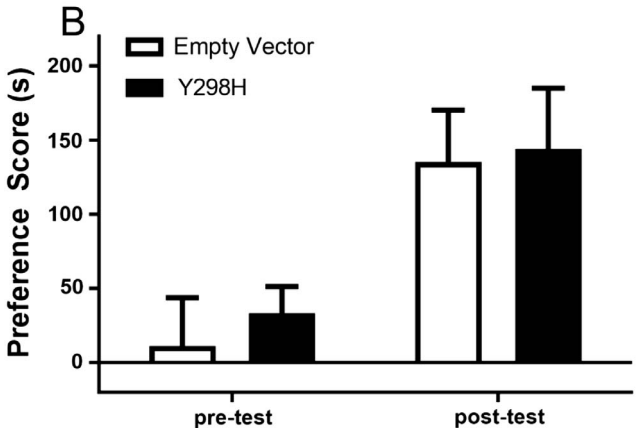


Fig. 3. Blocking HDAC3 activity in the dorsal hippocampus has no effect on the formation of cocaine-induced CPP memory. (A) Schematic of the cocaine-CPP procedure. (B) Cocaine-CPP expression indicated by mean CPP score (time spent in cocaine-paired (CS+) minus saline-paired (CS−) ± s.e.m). At 5 mg/kg cocaine-HCl conditioning dose, AAV-HDAC3(Y298H)-v5 mice exhibited similar CPP score to EV controls during the post-test, AAV-EV n = 15, HDAC3(Y298H)-v5 n = 15.

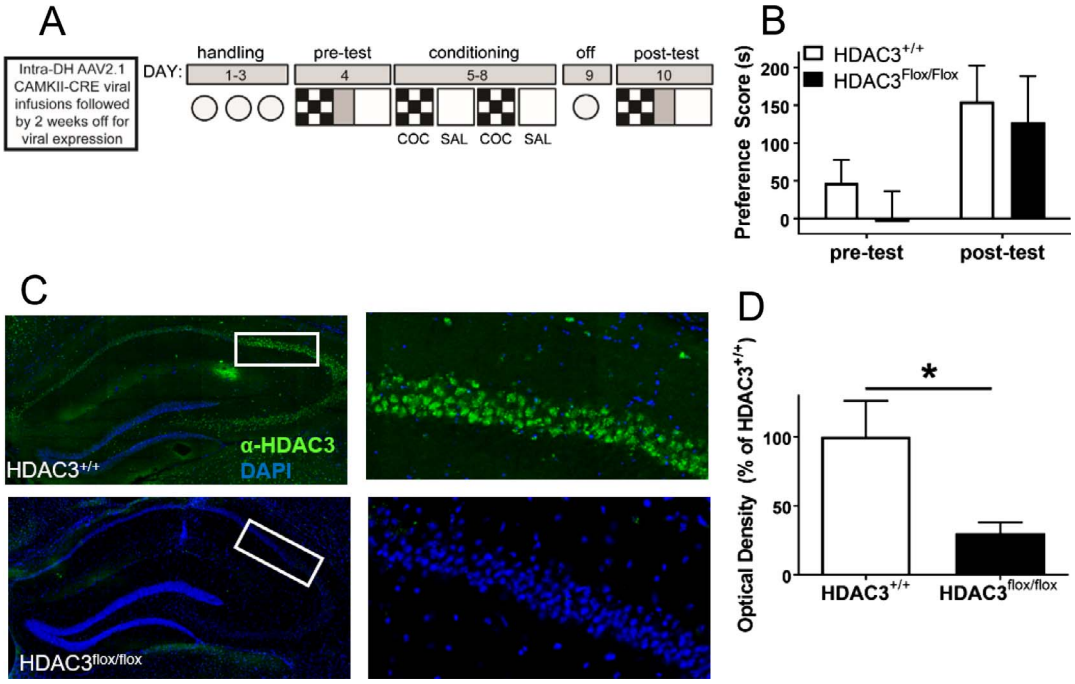


Fig. 4. Focal homozygous gene deletion of Hdac3 in the dorsal hippocampus has no effect on the formation of cocaine-induced CPP memory. (A) Schematic of the cocaine-CPP procedure. (B) Cocaine-CPP expression indicated by mean CPP score (time spent in cocaine-paired (CS+) minus saline-paired (CS−) ± s.e.m). At 5 mg/kg cocaine-HCl conditioning dose, $Hdac3^{flox/flox}$ mice exhibited similar CPP score to $Hdac3^{+/+}$ controls during the post-test. (C) Representative immunofluorescence image showing expression of HDAC3 (green) in the dorsal hippocampus after infusion of AAV2.1 Cre infused in $Hdac3^{+/+}$ and $Hdac3^{flox/flox}$ mice. Cells were counterstained with DAPI (blue). (D) Quantification of immunostaining confirmed that HDAC3 was significantly reduced in $Hdac3^{flox/flox}$ mice compared to $Hdac3^{+/+}$ controls, $Hdac3^{+/+}$ n = 12, $Hdac3^{flox/flox}$ n = 11. $^{*}p < 0.05$.

handled for 1 min each day for 3 days prior to the start of the experiment (days 1–3). The CPP procedure for all of the experiments was performed using an unbiased, counterbalanced protocol. Baseline preferences for each of the experiments were assessed by placing the animals in the center compartment of the place preference apparatus and allowing free access to all compartments for 15 min (pre-test; day 4). The training stage began one day after the pre-test. Conditioning was carried out over the subsequent 4 days with the guillotine doors closed, thus confining animals to one of the two outer compartments of the CPP apparatus for 30 min (days 5–8). An unbiased design was used so that half of the animals were given cocaine prior to placement in the checkered compartment and half received cocaine prior to placement in white compartment on training day 1 (CS+). The next day, treatment and compartment were reversed for each animal (training day 2), mice were injected with saline before placement in the alternate compartment (CS−). Injections were altered for subsequent conditioning sessions. For the acquisition experiments shown in Figs. 3–5, animals received a total of two 30 min pairings with cocaine in one compartment and two 30 min pairings with saline in the other. Forty-eight hours after the last conditioning session, preference (15 min; post-test 1; day 10) was assessed in all animals as described above in a drug-free state. For the extinction experiments shown in Figs. 6 and 7, animals underwent conditioning followed by post-test 1 48 h after the last conditioning session as described above. Two weeks following viral infusion into the DH or IL, animals underwent repeated drug-free preference tests daily (15 min; post-tests 2–5; days 25–28). CPP score was calculated as time (s) spent in cocaine-paired (CS+) minus saline-paired (CS−) compartments. The time spent in each of the large outer compartments was analyzed by automated software from MPEG videos using EthoVision 3.1 software (Noldus Technology; see Malvaez et al., 2011).

2.9. Statistical analysis

Graphpad Prism 7.02 (GraphPad Software) was used for all statistical analysis. Habituation was analyzed using a two-way ANOVA to compare total distance traveled across the habituation sessions. Training and testing data were analyzed using a Student's *t* test to compare either exploration or DI between control and test animals. Test data was also analyzed using one-tailed *t*-test comparing DI values to zero to determine whether or not significant discrimination was observed. CPP experiments were analyzed using two-way analysis of variance (ANOVAs) followed by *Bonferroni's post-hoc tests* with Preference Scores (PS) at tests as within-subjects variables and group/genotype (Empty Vector vs. Y298H point mutant; $Hdac3^{+/+}$ vs. $Hdac3^{flox/flox}$). This statistical test was used to make specific comparisons when significant interactions and/or main group effects were observed. Test data was also analyzed using one-tailed *t*-test comparing PS values to zero to determine whether or not significant preference was observed. For extinction experiments, Student's *t*-tests were first conducted to determine if animals formed a significant preference on post-test 1 prior to viral infusion. RT-qPCR values were obtained as described above. Differences in RT-qPCR values were assessed with Student's *t*-tests. For all analyses, an α value of 0.05 was required for significance.

3. Results

3.1. Expression of the point mutant virus AAV-HDAC3(Y298H)

To target HDAC3 deacetylase activity we developed a point mutant (Y298H) expressed from adeno-associated virus (AAV2.1-HDAC3 (Y298H)-v5). Replacing a histidine for a tyrosine (Y298H) abolishes the enzymatic activity of HDAC3 (Kwapis et al., 2017; Lahm et al., 2007; Sun et al., 2013). We also added a V5 epitope tag to measure immunoreactivity and quantify viral mRNA expression of the HDAC3 point mutant independently of endogenous HDAC3 (Fig. 1A). Mice were given bilateral infusions of either the point mutant virus (AAV-HDAC3(Y298H)-v5) or control (AAV-EV). Two weeks later (allowing for optimal expression) (Barrett et al., 2011; Kwapis et al., 2017; McQuown et al., 2011; Rogge et al., 2013), mice were sacrificed and V5

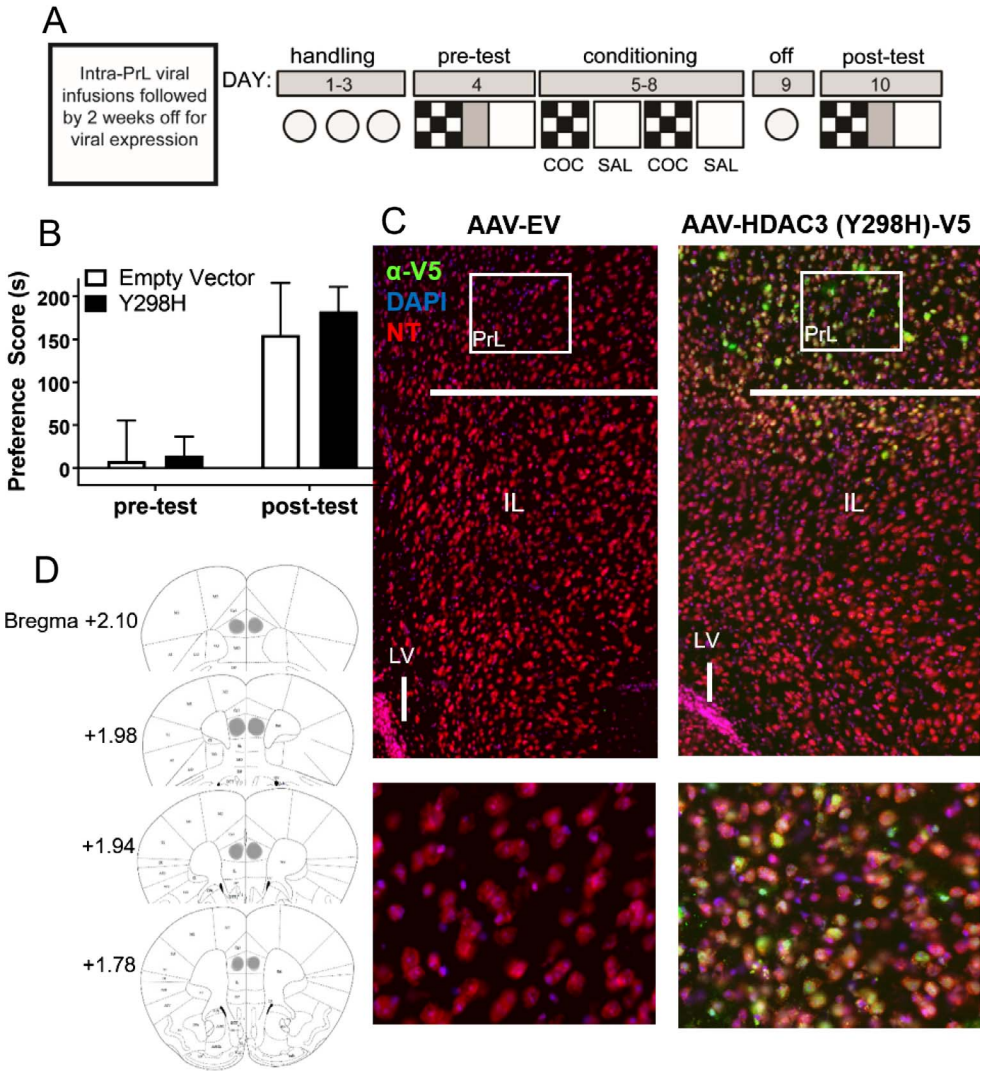


Fig. 5. Blocking HDAC3 activity in the prelimbic cortex (PrL) has no effect on the formation of cocaine-induced CPP memory. (A) Schematic of the cocaine-CPP procedure. (B) Cocaine-CPP expression indicated by mean CPP score (time spent in cocaine-paired (CS+) minus saline-paired (CS−) ± s.e.m). At 5 mg/kg cocaine-HCl conditioning dose, AAV-HDAC3(Y298H)-v5 mice exhibited similar CPP score to EV controls during the post-test, AAV-EV n = 12, HDAC3(Y298H)-v5 n = 12. (C) Representative immunofluorescence image showing expression of the V5 epitope tag (green) after infusion of AAV-HDAC3(Y298H)-v5 targeting the PrL. No V5 staining was observed with the AAV-EV control virus. Cells were counterstained with DAPI (blue) and neurons were counterstained with NeuroTrace (red). V5 expression was largely confined to the prelimbic region of the medial prefrontal cortex. (D) Targeted viral infusion in the PrL. The shaded regions of the mouse atlas images illustrate the representative extend of viral infusion.

and HDAC3 were measured by immunoreactivity and RT-qPCR. To confirm that our viral infusions target the DH, we assessed immunoreactivity to the V5 epitope on AAV-HDAC3(Y298H). We observed successful transduction of AAV-HDAC3(Y298H)-v5 throughout areas CA1 and CA3 of the DH of all point mutant virus-infused animals, without V5 staining in animals infused with the AAV-EV control virus (Fig. 1B). We also measured V5 and wildtype HDAC3 in DH tissue using RT-qPCR. Primers against both the V5-containing HDAC3(Y298H) transcript and the endogenous *Hdac3* transcript (which recognizes both endogenous *Hdac3* and mutated *Hdac3(Y298H)-v5* mRNA) confirmed significantly higher levels in mice infused with AAV-HDAC3(Y298H)-v5 (v5: $t_{(7)} = 4.14$; $^{*}p < 0.01$, Empty Vector n = 5; Y298H n = 4; HDAC3: $t_{(9)} = 3.12$, $^{*}p = 0.012$, Empty Vector n = 6; Y298H n = 6) (Fig. 1C and D).

3.2. Blocking HDAC3 activity in the dorsal hippocampus with AAV-HDAC3(Y298H)-v5 enhances long-term memory for object location

Previous studies have shown that HDAC3 inhibition enhances memory such that a subthreshold learning event that would not result in long-term memory is transformed into an event leading to long-term memory (Malvaez et al., 2013; McQuown et al., 2011). To test whether the deacetylase-dead point mutant version of HDAC3 affects long-term memory, mice received bilateral infusions of either the point mutant virus ((AAV-HDAC3(Y298H)-v5) or control (AAV-EV). Two weeks later, mice were trained on the OLM task. During training, mice were placed in an arena with two identical objects for a 3 min training session, which does not result in long-term memory (Malvaez et al., 2013; Stefanko et al., 2009), and then tested 24 h later in the same arena with one familiar object moved to a novel location (Fig. 2A). In this experiment as well as all subsequent experiment, every animal included in the behavioral analyses had viral infusion confirmed by immunohistochemistry. Empty Vector mice did not show significant discrimination (DI = 3.66 ± 3.4%, $t_{(12)} = 1.085$, $p > 0.1$, n = 8) confirming that 3 min was a subthreshold training period (Fig. 2B). In contrast, Y298H mice showed significant memory for object location, evident by a significantly greater discrimination index (DI = 28.76 ± 4.45%; $t_{(12)} = 4.587$, $^{*}p < 0.001$, n = 6; Fig. 2B). Groups did not differ in total exploration time of the two objects ($t_{(12)} = 1.024$; $p > 0.05$; Fig. 2C).

Next, we tested whether infusing the point mutant virus in the DH affected long-term memory in a standard novel object recognition task (ORM; Fig. 2D). In this task, one of the familiar objects is replaced with a novel object, but there is no change in context or object location. As shown in Fig. 2E, after subthreshold training (3 min), both Empty Vector and Y298H mice spent similar amounts of time with both the familiar and novel objects on test day ($t_{(14)} = 0.55$; $p > 0.05$, Empty Vector n = 9; Y298H n = 7). Groups did not differ in total exploration time of the two objects ($t_{(14)} = 0.88$; $p > 0.05$; Fig. 2F). Together, the data in Fig. 2 suggest that infusing the deacetylase-dead point mutant

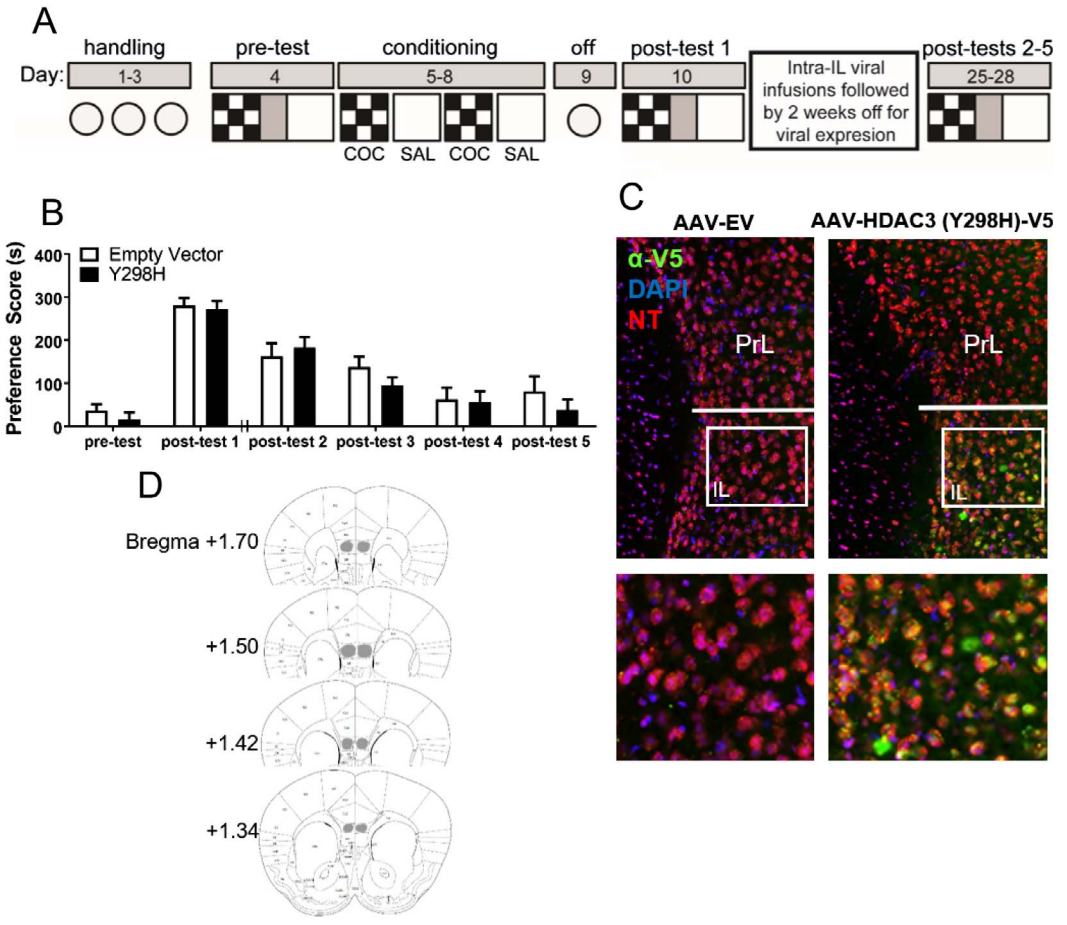


Fig. 6. Blocking HDAC3 activity in the infralimbic cortex (IL) has no effect on the extinction of cocaine-induced CPP memory. (A) Schematic of the cocaine-CPP procedure. (B) Cocaine-CPP expression indicated by mean CPP score (time spent in cocaine-paired (CS+) minus saline- paired (CS−) ± s.e.m). Animals were initially trained on 20 mg/kg cocaine-HCl. Subsequently, animals received intra-IL AAV-EV or AAV-HDAC3(Y298H)-v5 viral infusions and extinction memory was examined. AAV-HDAC3(Y298H)-v5 mice exhibited similar CPP score to EV controls during post-tests 2–5, AAV-EV n = 17, HDAC3(Y298H)-v5 n = 16. (C) Representative immunofluorescence image showing expression of the V5 epitope tag (green) after infusion of AAV-HDAC3(Y298H)-v5 targeting the IL. No V5 staining was observed with the AAV-EV control virus. Cells were counterstained with DAPI (blue) and neurons were counterstained with NeuroTrace (red). V5 expression was largely confined to the infralimbic region of the medial prefrontal cortex. (D) Targeted viral infusion in the IL. The shaded regions of the mouse atlas images illustrate the representative extend of viral infusion.

HDAC3 virus in the DH results in a selective enhancement of long-term memory for the object location (Fig. 2B) but not the object itself (Fig. 2E).

3.3. Disrupting HDAC3 activity in the dorsal hippocampus with AAV-HDAC3(Y298H)-v5 has no effect on the formation of cocaine-associated memory

It has been shown in rats that the DH plays a role in drug-associated

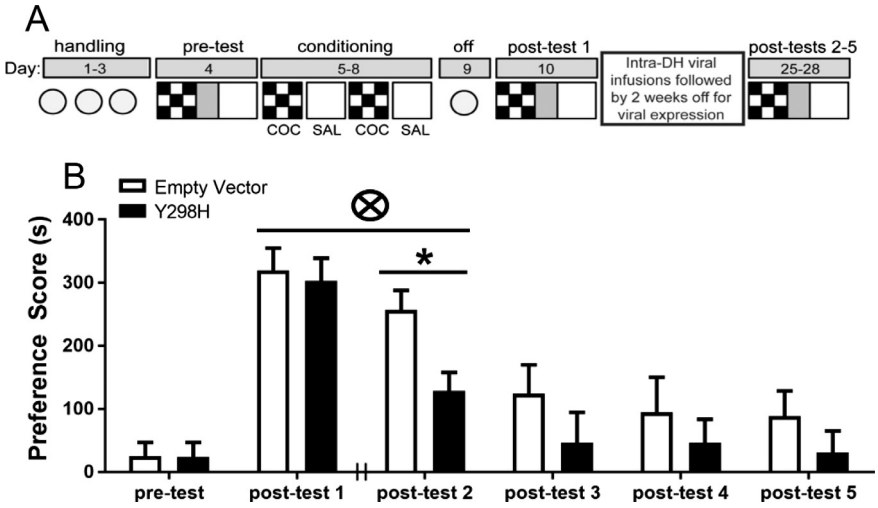


Fig. 7. Blocking HDAC3 activity in the dorsal hippocampus (DH) enhances extinction of cocaine-induced CPP memory. (A) Schematic of the cocaine-CPP procedure. (B) Cocaine-CPP expression indicated by mean CPP score (time spent in cocaine-paired (CS+) minus saline-paired (CS−) ± s.e.m). Animals were initially trained on 20 mg/kg cocaine-HCl. Subsequently, animals received intra-DH AAV-EV or AAV-HDAC3(Y298H)-v5 viral infusions and extinction memory was examined. Animals infused with AAV-HDAC3(Y298H)-v5 in the DH showed significantly reduced cocaine-CPP on post-test 2 compared to animals given intra-DH EV control. indicates a significant treatment by test interaction and * indicates a significant decrease in PS of AAV- HDAC3(Y298H)-v5 compared to AAV-EV control animals during post-test 2, *Bonferroni post- hoc*, $p < 0.05$, AAV-EV n = 10, HDAC3(Y298H)-v5 n = 12.

memory processes. Specifically, excitotoxic lesions of the DH in rats has been shown to disrupt acquisition of cocaine-CPP (Meyers, Zavala, & Neisewander, 2003). Given our own initial finding showing that manipulating HDAC3 in the hippocampus affects long-term memory for object location (Fig. 2B), and evidence suggesting that the DH plays a role in drug-associated memory formation, we set out to examine whether disrupting HDAC3 activity in the DH with AAV-HDAC3(Y298H)-v5 would affect the acquisition of cocaine-CPP. The schematic of the CPP procedure is shown in Fig. 3A (fully described in Materials and Methods). The training dose of cocaine-HCl used for this experiment as well as all future acquisition experiments was 5 mg/kg. We used this lower dose in order to avoid a ceiling effect (see Rogge et al., 2013). Neither the Empty Vector nor the Y298H group displayed an initial preference for either context before conditioning (pre-test: Empty vector: $t_{(14)} = 0.347$; Y298H: $t_{(14)} = 1.841$, $p > 0.05$). After conditioning with two pairings of cocaine, both the Empty Vector and Y298H groups established a preference for the cocaine-paired environment at post-test (Fig. 3B). A two-way repeated-measures ANOVA revealed a significant main effect of conditioning ($F_{(1,28)} = 16.36$, $p < 0.001$) but not group ($F_{(1,28)} = 0.18$, $p > 0.05$) and no treatment-by-test interaction ($F_{(1,28)} = 0.05$, $p > 0.05$). *Bonferroni post-hoc* analysis showed no difference between Empty Vector and Y298H mice on pre-test ($t_{(56)} = 0.47$, $p > 0.05$) or post-test ($t_{(56)} = 0.19$, $p > 0.05$, n = 15 per group) (Fig. 3 B). These results demonstrate that AAV-HDAC3(Y298H) infusion into the DH had no effect on the acquisition of cocaine-associated memory.

3.4. Deletion of HDAC3 in the dorsal hippocampus has no effect on the formation of cocaine-associated memory

We next examined whether deleting the entire *Hdac3* gene in the DH would affect cocaine-associated memory formation. In this experiment, DH-specific, homozygous deletions of *Hdac3* were generated in adult mice. Because HDAC3 is expressed in neurons, oligodendrocytes, and glia (Baltan, Bachleda, Morrison, & Murphy, 2011; Broide et al., 2007), the use of AAV serotype 2.1, which preferentially transduces neurons (Burger et al., 2004), allowed for the deletion of *Hdac3* specifically in neurons. Also, we used CAMKII-Cre and the CAMKII promoter should restrict Cre expression to forebrain excitatory neurons (Kojima et al., 1997). $Hdac3^{+/+}$ and $Hdac3^{flox/flox}$ mice underwent cocaine-induced CPP to examine the effect of DH-specific Hdac3 deletion on CPP memory formation. The schematic of the CPP procedure is shown in Fig. 4A. As shown in Fig. 4B, both $Hdac3^{+/+}$ and $Hdac3^{flox/flox}$ mice established a preference for the cocaine-paired environment at post-test. When the data was analyzed by factorial ANOVA (treatment × genotype), there was a significant main effect of conditioning ($F_{(1,21)} = 12.03$, $p < 0.01$) but not genotype ($F_{(1,21)} = 0.52$, $p > 0.05$) and no interaction ($F_{(1,21)} = 0.12$, $p > 0.05$). *Bonferroni post-hoc* analysis showed no difference between $Hdac3^{+/+}$ and $Hdac3^{flox/flox}$ mice on pre-test ($t_{(42)} = 0.80$, $p > 0.05$) or post-test ($t_{(42)} = 0.42$, $p > 0.05$, $Hdac3^{+/+}$ n = 12, $Hdac3^{flox/flox}$ n = 11) (Fig. 4B). The extent of HDAC3 deletion in $Hdac3^{flox/flox}$ mice is shown in Fig. 4C. Viral infusions and focal deletions were bilateral and Hdac3 deletions were restricted to the DH in all $Hdac3^{flox/flox}$ mice included in the data presented in subsequent figures. To confirm that our viral infusion appropriately targeted the DH and resulted in the deletion of *Hdac3*, we measured immunoreactivity to HDAC3. Fig. 4C shows HDAC3 expression in the DH after immunofluorescence staining with anti-HDAC3 antibody. DAPI staining confirmed the presence of nuclei in the DH of both genotypes. Quantified HDAC3 immunoreactivity in the DH of all $Hdac3^{+/+}$ and $Hdac3^{flox/flox}$ mice used in this study is shown in Fig. 4D. HDAC3 immunoreactivity was significantly reduced in the DH of $Hdac3^{flox/flox}$ mice compared with $Hdac3^{+/+}$ (mean percentage of $Hdac3^{+/+}$ ± s.e.m: $Hdac3^{+/+} = 100 \pm 26.0$; $Hdac3^{flox/flox} = 30.2 \pm 7.8$, $t_{(9)} = 2.790$, $^{*}p < 0.05$).

3.5. Disrupting HDAC3 activity in the PrL with AAV-HDAC3(Y298H)-v5 has no effect on the formation of cocaine-associated memory

Next, we tested whether disrupting HDAC3 activity in the prelimbic cortex (PrL) with HDAC3-(Y298H)-v5 would affect cocaine-CPP memory formation. The PrL has been shown to play a critical role in cocaine-CPP acquisition (Isaac, Nonneman, Neisewander, Landers, & Bardo, 1989; Tzschentke & Schmidt, 1998, 1999). Further, the neuroplasticity of the prefrontal cortex can be abnormally altered by addictive drugs of abuse such as cocaine through the regulation of gene expression (Krasnova et al., 2008; Marie-Claire et al., 2003). Thus, we hypothesized that the deacetylase activity of HDAC3 may play a critical role in the PrL for cocaine-associated memory formation. The schematic of the CPP procedure was the same as in Fig. 3 and the schematic is depicted in Fig. 5A. Neither the Empty Vector nor the Y298H group that received an infusion the AAV-HDAC3-(Y298H)-v5 virus displayed an initial preference for either context before conditioning (pretest: Empty Vector: $t_{(11)} = 0.166$; Y298H: $t_{(11)} = 0.655$, $p > 0.1$). After conditioning with two pairings of cocaine, both the Empty Vector and Y298H groups established a preference for the cocaine-paired environment at post-test (Fig. 5B). A two-way repeated-measures ANOVA revealed a significant main effect of conditioning ($F_{(1,22)} = 24.10$, $p < 0.0001$) but not group ($F_{(1,22)} = 0.11$, $p > 0.05$) and no treatment-by-test interaction ($F_{(1,22)} = 0.10$, $p > 0.05$). *Bonferroni post-hoc* analysis showed no difference between AAV-EV and HDAC3(Y298H)-v5 mice on pre-test ($t_{(44)} = 0.11$, $p > 0.05$) or post-test ($t_{(44)} = 0.45$, $p > 0.05$, n = 12 per group) (Fig. 5B). To confirm that our viral infusions specifically target the PrL, we measured immunoreactivity to the V5 epitope on AAV-HDAC3(Y298H). We observed successful transduction of AAV-HDAC3(Y298H)-v5 of all point mutant virus-infused animals throughout the PrL, while avoiding spread into the infralimbic cortex (IL) region of the medial prefrontal cortex (mPFC). As expected, no V5 staining was observed in animals infused with the AAV-EV control virus (Fig. 5C). Fig. 5D illustrates the targeted viral infusion in the PrL. Infusion was restricted to the PrL in all animals included in the data presented. These results demonstrate that, like the DH, AAV-HDAC3(Y298H) infusion into the PrL also had no effect on the acquisition of cocaine-associated memory.

3.6. Blocking HDAC3 activity in the infralimbic cortex with AAV-HDAC3(Y298H)-v5 has no effect on the extinction of cocaine-associated memory

Next, we examined whether selectively blocking HDAC3 deacetylase activity in the IL enhances the extinction of cocaine-CPP. The IL has been shown to be involved in the extinction of cocaine seeking (LaLumiere, Niehoff, & Kalivas, 2010; Peters, Kalivas, & Quirk, 2009; Peters, LaLumiere, & Kalivas, 2008). Animals underwent cocaine-CPP conditioning with a 20 mg/kg cocaine-HCl training dose. The reason for this higher dose of cocaine in this extinction experiment as well as all subsequent extinction studies was to prevent extinction floor effects and establish a strong enough preference that could endure the two weeks necessary for viral expression following virus infusion. Following cocaine-CPP conditioning, animals showed a significant preference at post-test 1 (post-test 1; $t_{(64)} = 15.11$; $p < 0.001$). Then we bilaterally infused HDAC3(Y298H)-v5 or AAV-EV into the IL, and, mice underwent drug-free post-tests (extinction training) two weeks following infusions (Fig. 6A). Disrupting HDAC3 activity in the IL with HDAC3(Y298H) had no effect on the extinction of CPP memory. Using a two-way repeated-measures ANOVA, we found a significant main effect of test ($F_{(1, 155)} = 35.91$, $p < 0.001$), as expected, but no significant treatment-by-test interaction ($F_{(5, 155)} = 0.62$, $p > 0.05$) nor main treatment group effect ($F_{(1, 31)} = 0.75$, $p > 0.05$) (AAV-EV: n = 17, HDAC3(Y298H)-v5: n = 16 per group) (Fig. 6B). To confirm that our viral infusions specifically target the infralimbic, we measured immunoreactivity to

the V5 epitope on AAV-HDAC3(Y298H). We observed successful transduction of AAV-HdAC3(Y298H)-v5 of all point mutant virus-infused animals throughout the IL, while avoiding spread into the PrL region of the mPFC. No V5 staining was observed in animals infused with the AAV-EV control virus (Fig. 6C). Fig. 6D shows the targeted viral infusion in the IL. Infusion was restricted to the IL in all animals included in the data presented.

3.7. Blocking HDAC3 activity in the dorsal hippocampus with AAV-HDAC3(Y298H)-v5 facilitates extinction of cocaine-associated memory

Next, we examined whether selectively blocking HDAC3 deacetylase activity in the DH enhances the extinction of cocaine-CPP. As described above, animals underwent cocaine-CPP conditioning and showed a significant preference at post-test 1 (post-test 1; $t_{(42)} = 9.79$; $p < 0.0001$). Then we bilaterally infused HDAC3(Y298H)-v5 or AAV-EV into the DH and had animals undergo drug-free post-tests (extinction training) two weeks following infusions (Fig. 7A). Disrupting HDAC3 activity in the DH with HDAC3(Y298H) resulted in a significant extinction of CPP on post-test 2, as revealed by ANOVA comparing Preference Score (PS) of the AAV-EV and HDAC3(Y298H)-v5 groups across post-tests 1 and 2 showing a significant main effect of test ($F_{(1, 20)} = 25.58$, $p < 0.001$) and a significant treatment-by-test interaction ($F_{(1, 20)} = 5.67$, $p = 0.02$). Both the AAV-EV control and the HDAC3(Y298H) animals showed similar CPP preferences on post-test 1, but after viral manipulation, the animals that received the point mutant virus showed a significant decrease in PS on post-test 2 compared to the AAV-EV control animals as shown by *Bonferroni post-hoc* analysis ($t_{(40)} = 2.73$, $p = 0.02$; AAV-EV n = 10, HDAC3(Y298H)-v5 n = 12) (Fig. 7B). These findings demonstrate that mice infused with AAV-HDAC3(Y298H)-v5 showed enhanced extinction of cocaine-associated behavior.

4. Discussion

It has been shown that systemic administration of a HDAC3-selective inhibitor enhances long-term object-location memory as well as extinction of cocaine-associated memory (Malvaez et al., 2013). Further, HDAC3 inhibition promotes a distinct pattern of histone acetylation linked to gene expression in the hippocampus and infralimbic cortex (Malvaez et al., 2013). Our lab has examined the role of HDAC3 in the DH and nucleus accumbens in OLM as well as cocaine-associated memory, respectively (McQuown et al., 2011; Rogge et al., 2013). However, whether or not the deacetylase domain of HDAC3 is necessary for these memory processes in specific brain regions remained unclear. Here, we examined the specific role that the deacetylase domain of HDAC3 plays in long-term OLM formation, cocaine-context associated memory formation, as well as the extinction of cocaine-context associated memory.

In these experiments, we used a deacetylase-dead point mutant virus (AAV-HDAC3(Y298H)-v5 that selectively blocks the deacetylase activity of HDAC3 (Lahm et al., 2007; Sun et al., 2013). We found that selectively blocking the deacetylase domain of HDAC3 in the DH enhanced long-term memory for object location after a subthreshold training period. This finding was specific to objection location memory formation because the deacetylase domain of HDAC3 in the DH appeared to play no role in the acquisition/consolidation of cocaine-associated memory formation. We also investigated the role of HDAC3 in the prelimbic cortex with regards to cocaine-CPP memory formation and found that the deacetylase activity of HDAC3 in that brain region did not seem to be critical for cocaine-CPP memory acquisition. Finally, we found that blocking HDAC3 activity in the DH also facilitated the extinction of cocaine-CPP, whereas this same manipulation had no effect in the infralimbic cortex, a region implicated in extinction learning (Laurent & Westbrook, 2009; Lebrón, Milad, & Quirk, 2004; Quirk, Garcia, & Gonzalez-Lima, 2006; Quirk, Russo, Barron, & Lebron, 2000; Sierra-Mercado, Corcoran, Lebron-Milad, & Quirk, 2006). These results suggest that the deacetylase domain of HDAC3 plays a selective role in specific brain regions underlying long-term memory formation of object location as well as cocaine-associated memory formation and extinction.

We recently showed that this specific deacetylase-dead point mutant virus blocks the deacetylase activity of HDAC3 and promotes learning induced-histone acetylation (Kwapis et al., 2017). Kwapis et al. (2017) found that selectively blocking HDAC3 deacetylase activity in either the DH or basal nucleus of the amygdala enhanced context fear without having an effect on tone fear. However, blocking HDAC3 activity in the lateral nucleus of the amygdala enhanced tone, but not context fear memory. Thus, HDAC3 activity regulates different aspects of fear memory in the basal and lateral subregions of the amygdala. Together, the findings presented in Kwapis et al. (2017) along with the present study show that the deacetylase domain of HDAC3 is important for memory processes.

In contrast, Sun et al. (2013) showed that the deacetylase domain of HDAC3 is unnecessary for HDAC3 function in the liver. They found that a deacetylase-dead HDAC3 mutant was able to almost completely rescue repressed lipogenic gene expression as well as lipid metabolism. Further, they showed that interaction with the corepressor NCOR is required for deacetylase-independent function of HDAC3. Thus, despite evidence for the important role of the deacetylase domain of HDAC3, HDAC3 also has critical nonezymatic roles in transcriptional processes.

In our previous study, McQuown et al. (2011), we found that focal deletion of HDAC3 in the DH results in enhanced long-term memory for OLM, but not ORM. Likewise, in the current study, we found that selectively blocking HDAC3 deacetylase activity (using a deacetylase-dead point mutant of HDAC3) in the DH impaired OLM, but had no effect on ORM. In rodents, the involvement of the hippocampus in object recognition has been a point of controversy (Dere, Huston, & De Souza Silva, 2007; Ennaceur, 2010; Mumby, 2001; Winters, Saksida, & Bussey, 2008). Our laboratory previously found a significant impairment in ORM when we used muscimol post-training to inactivate the hippocampus. However, when the hippocampus was inactivated before retrieval of ORM, no effect was observed (Haettig et al., 2011). Therefore, it seems that the hippocampus is necessary for consolidation but not retrieval of ORM in the same mouse task used in the current study. Multiple studies from our lab (Barrett et al., 2011; McQuown et al., 2011; Vogel-Ciernia et al., 2013) and others (Balderas et al., 2008) similarly demonstrate that a dorsal hippocampus manipulation only affects OLM, and not ORM. In our experiments, we manipulate a gene of interest (CBP, Barrett et al., 2011; HDAC3, McQuown et al., 2011; BAF53b, Vogel-Ciernia et al., 2013), which leaves communication between the DH and other brain regions intact while selectively disrupting local plasticity. Similarly, blocking protein synthesis with anisomycin fails to impair ORM consolidation (Balderas et al., 2008). Blocking hippocampal activity with muscimol, on the other hand, impairs the consolidation of ORM. Muscimol inactivates hippocampal cells, preventing communication with key brain regions necessary for ORM acquisition/consolidation. Thus, it seems that manipulating gene expression or blocking protein synthesis within the DH is not sufficient to disrupt acquisition/consolidation mechanisms required for long-term memory for object recognition. Indeed, studies have suggested that long-term memory for object recognition relies on peri-postrhinal and insular cortices, rather than the DH (Balderas et al., 2008; Roozendaal et al., 2010).

Previous studies from our lab used HDAC3 inhibitors given systemically in order to acutely block HDAC3 enzymatic activity (Malvaez et al., 2013). Here, we use viral manipulations to chronically inhibit the deacetylase activity of HDAC3. This chronic inhibition may allow for compensatory mechanisms to come on board in the brain region in which our manipulation was performed, resulting in normal learning. The deacetylase domain of HDAC3 in the DH may play a role in extinction of cocaine-CPP memory, since infusing the point mutant

HDAC3 virus into the DH resulted in facilitated extinction. It is interesting to note that blocking the deacetylase activity of HDAC3 in the DH enhanced extinction learning but had no effect on acquisition/consolidation using the CPP model. One possible explanation for this is that the learning that occurs during extinction is believed to be more susceptible to manipulations compared to the initial conditioning. Studies have shown that the rate of extinction can be slower than the rate of initial acquisition (Rescorla, 2002), suggesting that extinction is more challenging and therefore more susceptible to disruption. This seems quite plausible when it comes to robust cocaine-associated memories because the original formation of these memories involves the powerful psychostimulant cocaine, making it easier for animals to form this original memory versus a subsequent extinction memory.

With regards to the acquisition/consolidation of drug-associated memories, we found that whether we used a deacetylase-dead point mutant virus or deleted the entire *Hdac3* gene using homozygous *Hdac3* deletion in the DH, our manipulations failed to have any effects on the acquisition/consolidation of cocaine-CPP memory. The DH has been correlated with information processing (for review, see Fanselow & Dong, 2010) and excitotoxic lesions of the DH in rats has been shown to disrupt acquisition of cocaine-CPP (Meyers et al., 2003). Though the DH has been suggested to play a role in the expression of drug context-induced cocaine seeking (Fuchs, Eaddy, Su, & Bell, 2007; Fuchs et al., 2005) and is required for processing contextual information, the DH is not required for the association of an auditory conditioned stimulus with the unconditioned stimulus, according to the fear literature (Maren, 2001). Thus, while the DH processes information about context, it is not believed to be the site of associative convergence between context and shock (Maren, 2001). Therefore, although HDAC3 appears to play a necessary role in acquisition/consolidation of cocaine-CPP memory in brain regions such as the nucleus accumbens which plays a central role in reward circuits and is the primary mediator of the reinforcing properties of drugs of abuse and associative processing of drug-paired conditioned stimuli (Kalivas & McFarland, 2003; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Rogge et al., 2013), our present findings suggest that the deacetylase domain of HDAC3 in the DH does not appear to mediate the formation of cocaine-associated memories.

Our results show that the deacetylase region of HDAC3 plays a role in specific brain regions implicated in the extinction of cocaine-associated memory. We first investigated the role of HDAC3 enzymatic activity in the infralimbic cortex because this brain region has been deemed a central locus for the extinction of memories (LaLumiere et al., 2010; Peters et al., 2008). Stafford, Raybuck, Ryabinin, and Lattal (2012) highlighted the role of histone deacetylation mechanisms in the IL in fear extinction by showing that the Class I histone deacetylase inhibitor NaB resulted in enhanced extinction when infused into the IL, but not the PrL. When we infused the point mutant HDAC3 virus into the IL, we found no effects on the rate of extinction of cocaine-associated memory. The Class I HDAC family consists of HDAC-1, -2, -3, and -8 (Haberland, Montgomery, & Olson, 2009). Ressler and colleagues found that the HDAC-1, -2, and -3 inhibitor, RGFP963, enhances consolidation of cued fear extinction, but RGFP966, a selective inhibitor of HDAC3, did not (Bowers, Xia, Carreiro, & Ressler, 2015). Further, Rumbaugh et al. (2015) showed that RGFP963 and another HDAC-1, -2, and -3 inhibitor, RGFP968, were most effective at stimulating synaptogenesis, while RGFP966 was ineffective. These results highlight the important differences in findings observed depending on whether the deacetylase domain of HDAC3, the entire Class 1 HDAC family, or a subset of Class I HDACs are targeted and suggest that targeting several isoforms of Class I HDACs may yield more robust effects on learning and memory processes.

In sum, our findings highlight the role of the deacetylase domain of HDAC3 in specific brain regions implicated in object location memory and the formation and extinction of cocaine-associated memory. It is important to note that previous studies have acutely targeted HDAC3 using systemic manipulations that cross the blood-brain barrier and target many brain regions (*e.g.* Malvaez, Sanchis-Segura, Vo, Lattal, & Wood, 2010; Malvaez et al., 2013; Stafford et al., 2012). Here, we show how chronically manipulating the deacetylase region of HDAC3 in key brain regions involved in object location learning and the formation and extinction of cocaine-associated memories affect these memory processes. Our findings extend previous findings that the deacetylase activity of HDAC3 plays a critical role in long-term memory processes and speaks to the specific role of HDAC3 deacetylase activity with regards to both brain region as well as memory process.

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