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Epigenetic regulation of immediate-early gene *Nr4a2/Nurr1* in the medial habenula during reinstatement of cocaine-associated behavior.

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HIGHLIGHTS

- Cocaine-primed reinstatement alters HDAC3-mediated regulation of Nr4a2 in the MHb.
- HDAC3 overexpression in the MHb is not sufficient to prevent relapse-like behavior.
- Loss of NR4A2 function in the MHb blocks MHb activity and relapse-like behavior.

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ABSTRACT

Propensity to relapse following long periods of abstinence is a key feature of substance use disorder. Drugs of abuse, such as cocaine, cause long-term changes in the neural circuitry regulating reward, motivation, and memory processes through dysregulation of various molecular mechanisms, including epigenetic regulation of activity-dependent gene expression. Underlying drug-induced changes to neural circuit function are the molecular mechanisms regulating activity-dependent gene expression. Of note, histone acetyltransferases and histone deacetylases (HDACs), powerful epigenetic regulators of gene expression, are dysregulated following both acute and chronic cocaine exposure and are linked to cocaine-induced changes in neural circuit function. To better understand the effect of drug-induced changes on epigenetic function and behavior, we investigated HDAC3-mediated regulation of *Nr4a2/Nurr1* in the medial habenula, an understudied pathway in cocaine-associated behaviors. *Nr4a2*, a transcription factor critical in cocaine-associated behaviors and necessary for MHb development, is enriched in the cholinergic cell-population of the MHb; yet, the role of NR4A2 within the MHb in the adult brain remains elusive. Here, we evaluated whether epigenetic regulation of *Nr4a2* in the MHb has a role in reinstatement of cocaine-associated behaviors. We found that HDAC3 disengages from *Nr4a2* in the MHb in response to cocaine-primed reinstatement. Whereas enhancing HDAC3 function in the MHb had no effect on reinstatement, we found, using a dominant-negative splice variant (NURR2C), that loss of NR4A2 function in the MHb blocked reinstatement behaviors. These results show for the first time that regulation of NR4A2 function in the MHb is critical in relapse-like behaviors.

1. Introduction

Repeated exposure to drugs of abuse causes long-lasting changes in neural circuit function, ultimately leading to the persistent behavioral adaptations that characterize substance use disorder (Volkow et al., 2003). These changes are underlined, in part, by drug-induced dysregulation of the various molecular mechanisms regulating gene expression. Mounting evidence has demonstrated that cocaine exposure alters

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gene expression throughout the circuitry regulating reward, motivation, and drug-seeking behavior through changes in chromatin modifications. For example, cocaine changes histone acetylation profiles that modulate gene expression required for long-lasting synaptic plasticity and associative processes, ultimately driving drug-seeking behavior (Maze and Nestler, 2011).

Two major families of enzymes are known to regulate histone acetylation in an activity dependent manner: histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, HATs and HDACs promote or repress gene expression via addition or removal of acetyl groups to histone tails, respectively (Roth and DenuAllis, 2003). Both HAT and HDAC families of enzymes have been linked to cocaine-induced changes to neural circuit function and cocaine-seeking behaviors. Of note, histone deacetylase 3 (HDAC3), one of the most densely expressed HDACs in the brain, negatively regulates gene expression, synaptic plasticity, and associative behaviors (McQuown and Wood, 2012). Specifically, disruption of HDAC3 in the striatum profoundly affects acquisition and extinction of cocaine-associated behaviors (Malvaez et al., 2018; Rogge et al., 2013). It is possible that cocaine's ability to generate long-lasting changes in neural plasticity and cocaine-associated behavior results from altering endogenous epigenetic machinery and may even be associated with propensity to relapse drug-seeking behavior, even following long periods of abstinence. Identifying key gene targets of epigenetic modifiers, such as HDAC3, is a critical next step in elucidating how changes in epigenetic structure confer changes to neural circuit function, and ultimately, provide a deeper understanding of epigenetic regulation of drug-associated behaviors.

Nr4a2/Nurr1 is an immediate-early gene, transcription factor, and known HDAC3 target (Hawk and Abel, 2011; Kwapis et al., 2017). Previous work from the Wood lab and others has demonstrated that enhancements to associative memory and synaptic plasticity via HDAC3 disruption are dependent on subsequent increases in *Nr4a2* expression. Specifically, while deletion of *Hdac3* in the dorsal hippocampus enhances memory in a hippocampus-dependent task, simultaneous deletion of *Hdac3* and *Nr4a2* blocks enhancements to long-term memory (McQuown et al., 2011). In the mesolimbic dopamine pathway, NR4A2 is required for dopaminergic neuronal development and regulates several enzymes in dopaminergic signaling, such as *tyrosine hydroxylase*, *dopamine transporter*, *vmat2*, and *amino acid decarboxylase* (Schweitzer et al., 2012; Johnson et al., 2011; Sacchetti et al., 2001; Saucedo-Cardenas et al., 1998). NR4A2 has also been previously linked to cocaine-induced changes to the nucleus accumbens and ventral tegmental area. Post-mortem analysis of human cocaine abusers found decreases in NR4A2 in the ventral tegmental area (Bannon et al., 2002, 2004). Moreover, NR4A2 is dysregulated in the nucleus accumbens following chronic cocaine exposure in a cell-type specific manner (Leo et al., 2007; Chandra et al., 2015). While NR4A2 is necessary for memory processes and key for normal dopaminergic signaling in the mesolimbic pathway, a causal link between NR4A2 function and cocaine-associated behaviors remains to be established.

Most work linking epigenetic changes to drug-associated behaviors have primarily focused on regions within the mesolimbic dopamine reward pathway, including the dorsal and ventral striatum, ventral tegmental area, and prefrontal cortex (Malvaez et al., 2011, 2018; Feng et al., 2014). However, the medial habenula (MHb) has recently been identified as a hub for various drug-associated behaviors. The MHb is a known regulator of nicotine aversion, withdrawal, and nicotine-seeking behaviors (Fowler et al., 2011; Salas et al., 2009; Fowler and Kenny, 2012). Several studies have also implicated the MHb in cocaine-associated behaviors, linking MHb activity, particularly its cholinergic population, to reinstatement of cocaine-seeking and cocaine-associated memory (López et al., 2018; McCallum and Glick, 2009; James et al., 2011). Although enriched with both HDAC3 and NR4A2, few studies have investigated epigenetic regulation within the MHb and its link to drug-associated behaviors.

Recent work from our laboratory has shown increased Histone 4 Lysine 8 acetylation (H4K8Ac) in the MHb during cocaine-primed reinstatement (López et al., 2018). Increases in H4K8Ac have been previously correlated with changes in both HDAC3 function and *Nr4a2* expression in the amygdala, hippocampus, and nucleus accumbens in response to associative learning (Rogge et al., 2013; Kwapis et al., 2017; Malvaez et al., 2013). Whereas NR4A2 is critical for habenula development and is induced during associative learning (Quina et al., 2009; Ressler et al., 2002), the function of NR4A2 in the adult habenula remains unknown. Moreover, it remains unknown if cocaine-induced changes to histone acetylation in the MHb are linked to changes in HDAC3 activity, if these changes occur at *Nr4a2*, and if NR4A2 is subsequently required for cocaine-primed reinstatement in the MHb.

To address these questions, we examined the roles of HDAC3 and NR4A2 in the cholinergic neurons of the MHb. Using LSL-TdTomato:ChAT-Cre reporter mice, we examine HDAC3's response to cocaine-primed reinstatement in cholinergic neurons of the MHb and, using a viral approach, examine the role of HDAC3 enzymatic activity in cocaine-associated behaviors. Lastly, we use a recently discovered endogenous dominant-negative variant of NR4A2 (NURR2C) to determine if NR4A2 function in the MHb is necessary for cocaine-primed reinstatement of conditioned place preference. The results from these studies provide further evidence for the role of epigenetic regulation of drug-associated behaviors and further bolster the role of the MHb in regulating cocaine relapse-like behaviors.

2. Materials and methods

2.1. Animals

Subjects were adult male and female heterozygous ChAT-IRES-Cre (ChAT-Cre) or double heterozygous LSL-TdTomato:ChAT-Cre (TdChAT) mice (2–5 months old; Jackson Laboratory). Mice were bred and maintained as described in Supplemental Information. All experiments were conducted according to the National Institutes of Health guideline for animal care and use. Experiments were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. AAV production

Plasmids containing *AAV-hSyn-DIO-GFP, AAV-hSyn-DIO-HDAC3$^{W.T.}$-V5^{-}*, or *hSyn-DIO-V5-NURR2C* were generated and packaged into AAV constructs as described in the Supplementary Information and described previously (Hemstedt et al., 2017).

2.3. Co-immunoprecipitation and Western blotting

HT22 cells were transfected with V5-NURR2C with or without NR4A2. 48 h following transfection, cells were harvested and precipitates made using a V5-specific antibody. Precipitates were then immunoblotted using a NR4A2-specific antibody, to assay for NR4A2 immunoreactivity, and a V5-specific antibody, to assay for precipitation specificity.

2.4. Stereotaxic surgeries

Animals were bilaterally injected with 0.5 μL of either *AAV-hSyn-DIO-GFP, AAV-hSyn-DIO- HDAC3$^{W.T.}$-V5*, or *hSyn-DIO-V5-NURR2C* in the MHb. Immunofluorescence was used to confirm expression of V5-tagged constructs.

2.5. CPP reinstatement paradigm

Cocaine-induced CPP was performed as previously described (López et al., 2018; White et al., 2016) and further described in Supplemental Information. Briefly, animals were conditioned in a 3-chambered CPP

apparatus using cocaine-HCl (counterbalanced, unbiased) and tested for conditioned preference 24 h final conditioning in a drug-free state. Following extinction, animals received either a cocaine or saline prime and immediately tested for reinstatement. For FACS-ChIP-qPCR experiments, animals were sacrificed 45 min following cocaine-primed reinstatement. For all CPP sessions, total locomotion was monitored for all groups (See Supplemental Information, Supplemental Figure 1).

2.6. Fluorescence activated cell sorting (FACS)

FACS was performed to isolate TdTomato:ChAT + neurons as previously described (Finegersh and Homanics, 2016) and further in Supplemental Information. Briefly, tissue containing the MHb was harvested from TdChAT mice following cocaine primed reinstatement (n = 2–3 animals/sample). Single-cell lysates were generated and MHb cells were positively sorted based on TdTomato expression (See Supplemental Information, Supplemental Figure 2).

2.7. Chromatin immunoprecipitation qPCR (ChIP-qPCR)

ChIP was performed based on the Millipore ChIP kit protocol (Supplemental Information). Digested chromatin was immunoprecipitated with either an HDAC3-specific antibody (Millipore) or anti-mouse IgG (negative control, Millipore). Promoter-specific HDAC3 enrichment was measured using qPCR.

2.8. Immunohistochemistry

Animals were euthanized and brains flash frozen in dry ice-chilled isopentane. 20 μM coronal sections were collected using a Leica CM 1850 cryostat. Immunohistochemistry was used to validate expression of V5-tagged constructs in the vMHb and confirm expression of TdTomato in the vMHb.

2.9. Statistical analysis

All statistical analysis was conducted using Prism 8. For all data, * indicates $p \leq 0.05$, ** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$, ‡ indicates $p \leq 0.05$ main effect of NURR2C, | indicates break in Western blot.

3. Results

3.1. HDAC3 occupancy is altered in MHb cholinergic neurons during cocaine-primed reinstatement of CPP

In a previous study, our lab has found that cocaine-primed reinstatement increases H4K8Ac throughout the MHb (López et al., 2018), suggesting a disengagement of HDAC3 enzymatic activity. We also demonstrated that activity in cholinergic neurons in the MHb is sufficient to drive reinstatement behavior. However, it is unknown if HDAC3 function is altered in the same cholinergic population. Thus, to examine HDAC3 in the cholinergic population of the MHb, we crossed the LSL-TdTomato mouse line to the ChAT-Cre mouse line. This crossbred TdChAT mouse line limits expression of the fluorescent TdTomato reporter to cholinergic neurons, especially in the ventral MHb (vMHb; Supp. Fig. 1A). Using RT-qPCR, we confirmed that TdTomato:ChAT + neurons can be sorted from the vMHb of TdChAT mice (Supp. Fig. 1B). These results demonstrate the ability to use TdChAT mice combined with FACS to isolate the cholinergic neurons of the MHb.

To determine if HDAC3 occupancy is altered during cocaine-primed reinstatement, TdChAT animals underwent cocaine-primed reinstatement. TdChAT animals acquire (Fig. 1A) and extinguish (Fig. 1B) cocaine-induced CPP. 24 h following the final extinction session, animals were primed with either saline or cocaine-HCl (5 mg/kg, I.P.) and immediately tested. Cocaine-primed animals show a significant reinstatement of preference score compared to saline-primed controls (Fig. 1C). 45 min following reinstatement session, animals were sacrificed, habenula tissue was harvested, and single-cell TdTomato + lysates were FACS sorted. Previous work from our lab and others has demonstrated 45 min is a peak time-point for monitoring changes in HDAC3 occupancy and changes to histone acetylation. Cocaine-primed animals show a significantly decreased HDAC3 occupancy at the promoter of *Nr4a2* (Fig. 1D) compared to saline-primed controls in the cholinergic neurons of the MHb. No significant change was observed at other HDAC3 target genes known to be critical in associative plasticity or cocaine response including *Ppp1r1b* (*Darpp32*; Fig. 1E), *Cebpb* (NR4A2-target gene; Fig. 1F), and *Nr4a3* (Nr4a family member; Fig. 1G). HDAC3 disengages from the promoter of *Nr4a2* in the cholinergic neurons of the MHb in response to cocaine-primed reinstatement. Thus, we next examined whether changes in either HDAC3 or NR4A2 function in the MHb are necessary for reinstatement behaviors.

3.2. HDAC3 overexpression has no effect on MHb-mediated cocaine-associated behaviors

To determine if HDAC3-mediated negative regulation is sufficient to prevent cocaine-primed reinstatement, a Cre-independent HDAC3$^{W.T.}$ was generated and shown to cause significant overexpression of *Hdac3* in HT22 cells (Supplemental Information, Supplemental Figure 3). This construct was used to develop a Cre-dependent approach to overexpress wild-type HDAC3 (DIO-HDAC$^{W.T.}$-V5) in the cholinergic neurons of the MHb (Fig. 2A and B). We infused either DIO-GFP or DIO-HDAC3$^{W.T.}$-V5 into the MHb of ChAT-Cre mice. DIO-GFP and DIO-HDAC3$^{W.T.}$-V5 expressing mice equally acquired (Fig. 2C) cocaine-induced CPP. Overexpression of HDAC3$^{W.T.}$-V5 in the MHb had no effect on extinction of cocaine-induced CPP (Fig. 2D). 24 h following the final extinction session, both GFP and HDAC3$^{W.T.}$-V5 expressing animals received a cocaine prime (5 mg/kg, I.P.) and were immediately tested for reinstatement. HDAC3$^{W.T.}$-V5 in the MHb had no effect on cocaine-primed reinstatement of CPP (Fig. 2E).

3.3. Overexpression of NR4A2 dominant-negative, NURR2C, alters MHb cholinergic neuronal activity and impairs cocaine-primed reinstatement of CPP

HDAC3 has been previously shown to be a powerful negative regulator of plasticity and associative processes, through negative regulation of *Nr4a2*. While *Nr4a2* is enriched in the vMHb, little is known about NR4A2 function in the adult habenula. Previous work has shown that an endogenously occurring *Nr4a2* splice-variant, NURR2C, blocks NR4A2-dependent transcription (Michelhaugh et al., 2005). However, it is unknown if NURR2C functions as a direct dominant-negative to NR4A2 activity. First, to determine if NURR2C directly interacts with NR4A2, we expressed V5-NURR2C with or without untagged NR4A2 in HT22 cells. 48 h following transfection, we immunoprecipitated V5-NURR2C using a V5-specific antibody and immunoblot analysis of V5-precipitates using an NR4A2-specific antibody identified a ~65kD NR4A2-immunoreactive band only in samples co-expressing NR4A2 and V5-NURR2C (Fig. 3A, see full Western blot in Supplemental Figure 4), demonstrating a direct NURR2C/NR4A2 interaction. NR4A2-targetting antibody also identified a ~55kD immunoreactive band. As NURR2C is an endogenous occurring splice-variant of NR4A2, NR4A2 antibodies are likely capable of recognizing NURR2C and the identified band at ~55kD is likely V5-NURR2C. Indeed, immunoblot analysis of V5-precipitates using a V5-specific antibody, identified the same ~55kD band (Fig. 3B, see full Western blot in Supplemental Figure 4). Moreover, immunoblot analysis using V5-specific antibody did not yield a band at 62kD (the predicted weight of endogenous NR4A2), demonstrating that the V5 antibody is specific for V5-NURR2C and the identified V5-NURR2C:NR4A2 interaction is not due to V5 reactivity

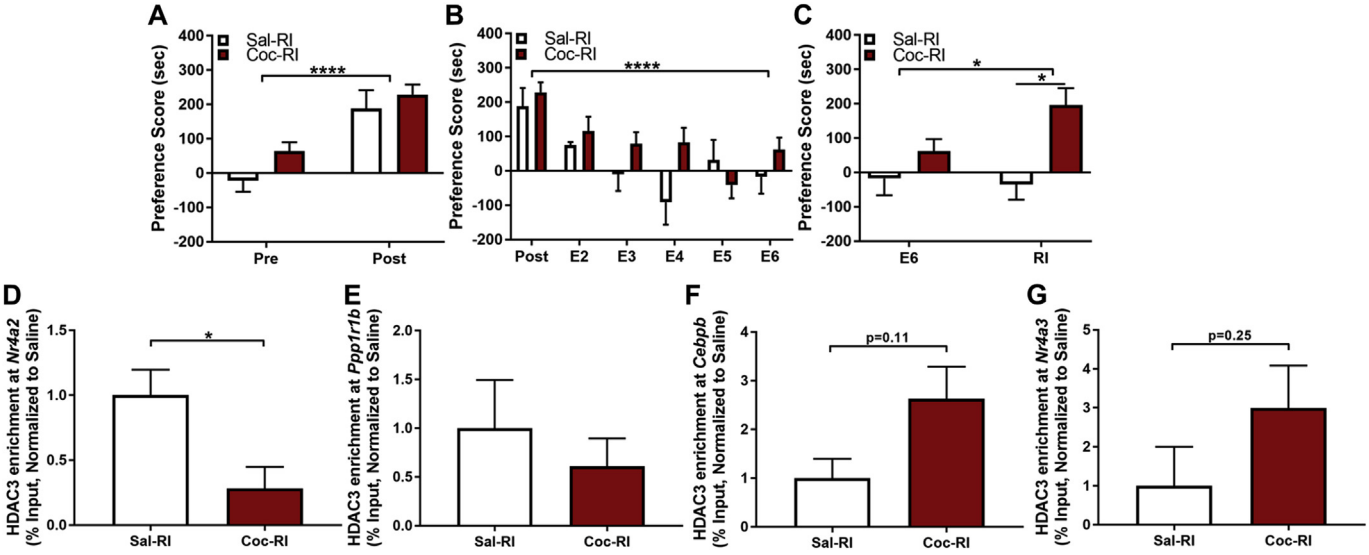

Fig. 1. HDAC3 occupancy is altered in the MHb in response to cocaine-primed reinstatement. TdChAT mice **(A)** acquire cocaine-induced CPP (Two-way ANOVA, main effect of Conditioning: $F_{(1,\ 16)} = 29.33$, $p < 0.0001$), with no differences saline-reinstated (Sal-RI, n = 7) and cocaine-reinstated animals (Coc-RI, n = 11) (Two-way ANOVA, No main effect of Reinstatement-Priming: $F_{(1,16)} = 3.347$, $p = 0.0860$; although there is a trend, there is no Interaction between effects, $F_{(1,16)} = 0.4218$, $p = 0.5253$ and the trend is likely being driven by variability in Pre-Test score). **(B)** CPP can be extinguished with repeated drug-free exposures to conditioning apparatus (Two-way ANOVA, main effect of Extinction $F_{(5,80)} = 8.023$, $p < 0.0001$). **(C)** Cocaine-primed animals significantly reinstate previously extinguished cocaine-induced CPP compared to saline-primed controls (Two-way ANOVA, main effect of Cocaine-Priming: $F_{(1,16)} = 8.389$, $p = 0.0105$; trending Interaction $F_{(1,16)} = 4.173$, $p = 0.0579$; Sidak's Post-hoc analysis shows a significant difference between saline-primed animals and cocaine-primed animals during reinstatement session $t_{(32)} = 3.544$, $p = 0.0025$ but not during E6 $t_{(32)} = 1.215$, $p = 0.4120$). For FACS-ChIP-qPCR, 2–3 animals were pooled per sample (Sal-RI, n = 3; Coc-RI, n = 4). ChIP-qPCR indicates HDAC3 occupancy is significantly lower in cocaine-primed animals at the **(D)** *Nr4a2* promoter ($t_{(5)} = 2.802$, $p = 0.0379$), with no differences at the promoters of **(E)** *Ppp1r1b* ($t_{(5)} = 0.737$, $p = 0.4961$), **(F)** *Cebpb* ($t_{(5)} = 1.919$, $p = 0.1131$), and **(G)** *Nr4a3* ($t_{(5)} = 1.298$, $p = 0.2508$).

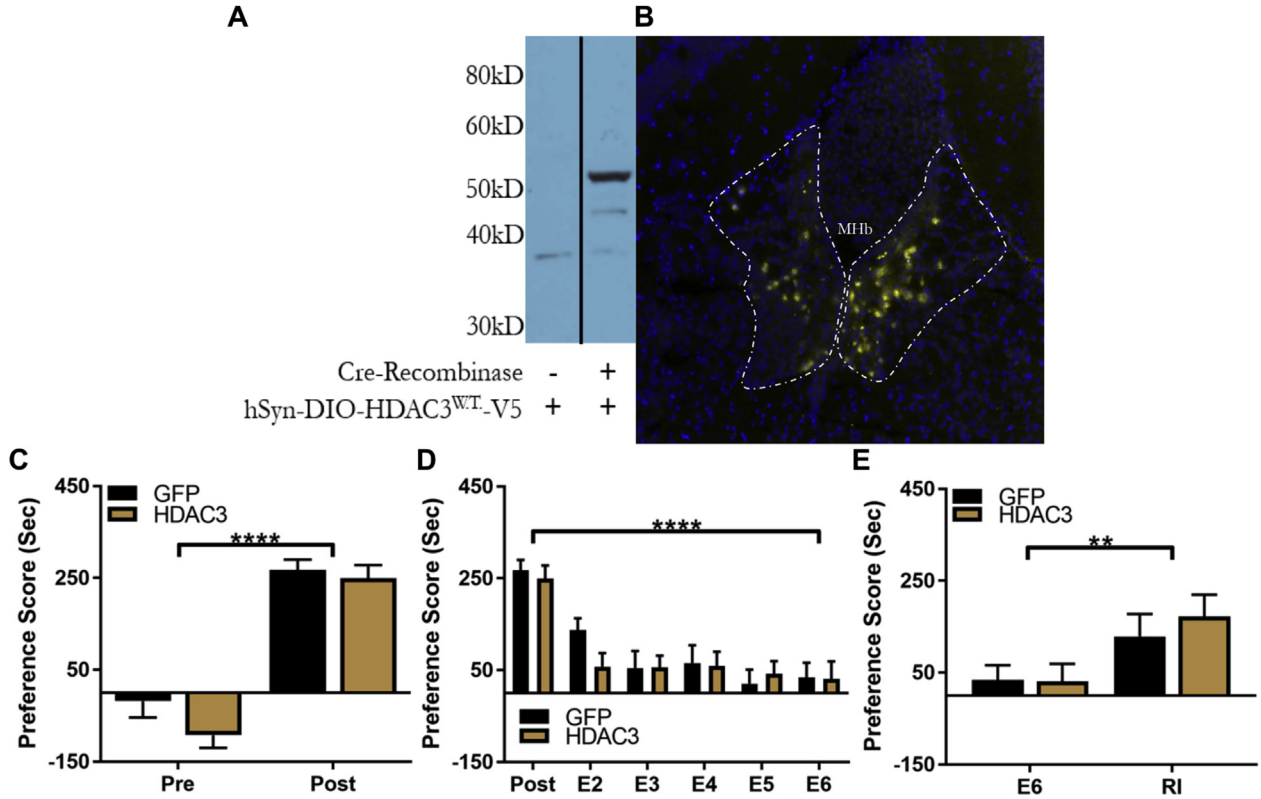

Fig. 2. HDAC3$^{W.T.}$ in the MHb does not affect cocaine-associated behaviors (A). Western blot probing for V5-tagged constructs expressed in HT22 cells. DIO Expression is limited to co-expression of Cre-recombinase and detected V5+ signal occurs at the predicted molecular weight of HDAC3 (~50kD). **(B)** Representative image of DIO-HDAC3$^{W.T.}$-V5 expression (*red*) in the vMHb of ChAT-Cre mouse. Note limited expression to the vMHb, where the cholinergic MHb population is enriched. Animals infused with DIO-GFP (n = 15) and DIO-HDAC3$^{W.T.}$(n = 13) equally **(C)** acquire cocaine-induced CPP (main effect of Acquisition, $F_{(1.26)} = 134.9$, $p < 0.0001$) and equally **(D)** extinguish (main effect of Extinction $F_{(5,130)} = 28.8$, $p < 0.0001$). Both GFP and V5-HDAC3 equally **(E)** reinstate preference following a cocaine-prime (main effect of Reinstatement $F_{(1,26)} = 12.72$, $p = 0.0014$).

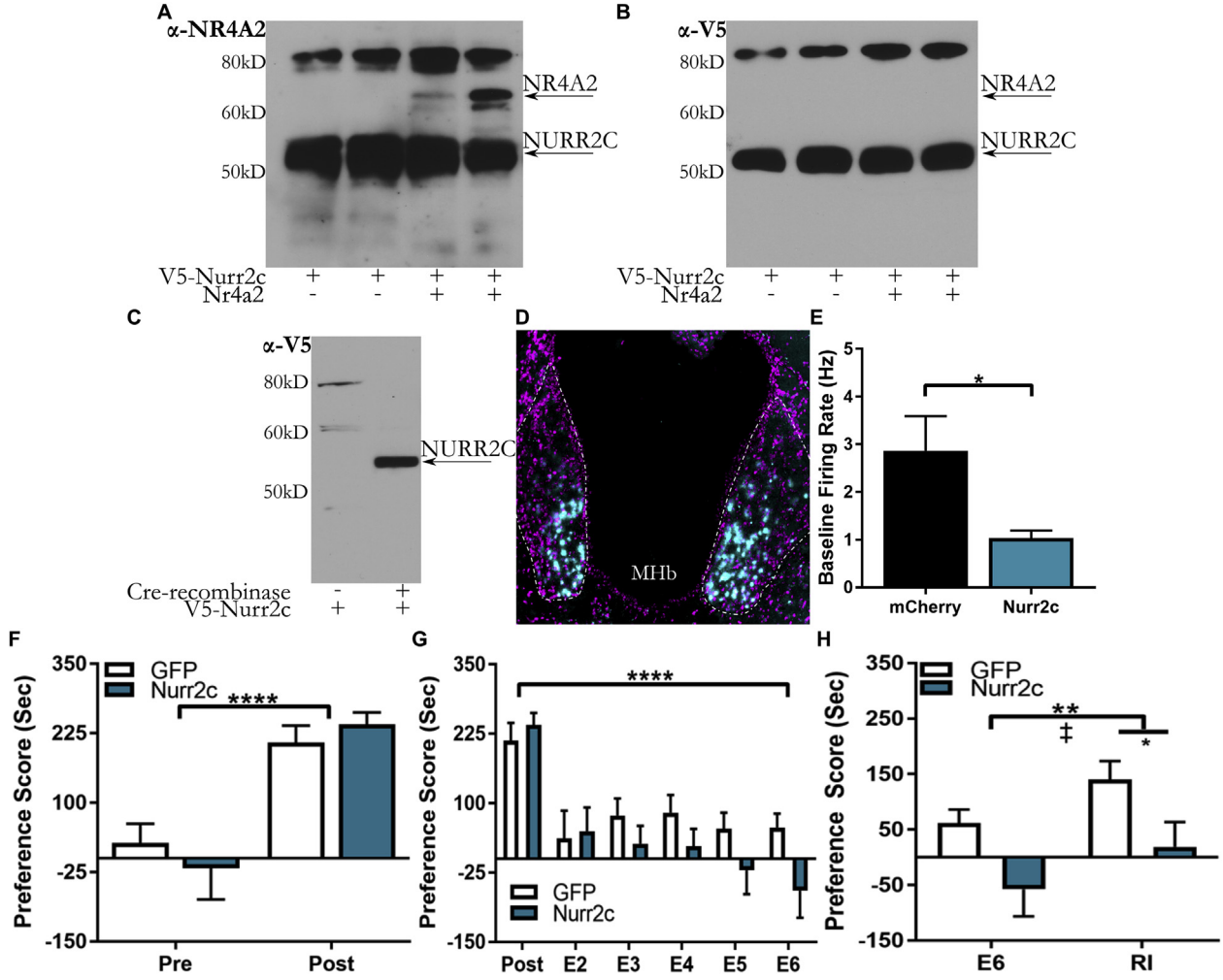

Fig. 3. Expression of NURR2C in the vMHb alters reinstatement behaviors. HT22 cells either expressing V5-NURR2C alone (*lane 1* and *lane 2*) or co-expressing V5-NURR2C and untagged NR4A2 (*lane 3* and *lane 4*). Cells underwent immunoprecipitation using *anti*-V5 and Western blot probing for **(A)** NR4A2 (note NR4A2-immunoreactivity ~65kD) or **(B)** V5 (Note V5-NURR2C immunoreactivity ~55kD and absence of NR4A2-immunoreactivity ~65kD). **(C)**. Western blot probing for V5-tagged constructs expressed in HT22 cells. DIO Expression is limited to co-expression of Cre-recombinase and detected V5+ signal occurs at the predicted molecular weight of NURR2C (55kD). Construct expressing wild-type NR4A2 used as positive control. **(D)** Representative image of DIO-V5-NURR2C expression (*green*) in the MHb of ChAT-Cre mouse. Note limited expression to the vMHb, where the cholinergic MHb population is enriched. **(E)** Overexpression of NURR2C blunts baseline firing rate of MHb cholinergic neurons ($t_{(14.25)} = 2.473$, $p = 0.0266$). Animals infused with DIO-GFP ($n = 16$) and DIO-V5-NURR2C ($n = 13$) equally **(F)** acquire cocaine-induced CPP (main effect of Acquisition, $F_{(1,27)} = 61.98$, $p < 0.0001$) and equally **(G)** extinguish (main effect of Extinction $F_{(5,130)} = 21.48$, $p < 0.0001$; no Main effect of NURR2C $F_{(1,27)} = 1.618$, $p = 0.2143$, although there is an Interaction $F_{(5,130)} = 2.371$, $p = 0.0214$, Sidak's posthoc analysis indicates there is no significant difference between GFP and NURR2C expressing animals on any extinction session). Following extinction, NURR2C expressing animals showed a **(H)** significantly blunted cocaine-primed reinstatement compared to GFP controls (main effect of Reinstatement $F_{(1,17)} = 10.36$, $p = 0.0033$; main effect of NURR2C $F_{(1,27)} = 6.599$, $p = 0.0160$; Sidak's posthoc analysis significant difference between GFP and NURR2C during reinstatement $t_{(54)} = 2.3$, $p = 0.05$).

with endogenous NR4A2.

To determine the role of NR4A2 in vMHb function, we generated a Cre-dependent NURR2C expressing vector (Fig. 3C). This viral strategy allows limited overexpression of V5-NURR2C to the cholinergic neurons of the vMHb in ChAT-Cre mice (Fig. 3D**,** see full Western blot in Supplemental Figure 4). To determine the effect of loss of NR4A2 function on MHb cholinergic function, we co-expressed DIO-mCherry + DIO-V5-NURR2C or DIO-mCherry alone in the vMHb of ChAT-Cre mice. 4 weeks following viral infusion, loose seal clamp recordings were taken from mCherry labeled neurons in coronal slices through the MHb. Cells co-expressing DIO-mCherry + DIO-V5-NURR2C had a significantly decreased firing rate compared to cells expressing DIO-mCherry alone (Fig. 3E), suggesting NR4A2 function is necessary for maintenance of MHb firing.

To determine the role of NR4A2 in MHb-mediated relapse-like behaviors, we infused either DIO-GFP or DIO-V5-NURR2C into the MHb of ChAT-Cre mice. Animals expressing DIO-GFP or DIO-V5-NURR2C showed no difference on acquisition (Fig. 3F) or extinction (Fig. 3G) of cocaine-induced CPP. Following extinction, animals received a cocaine prime (5 mg/kg, I.P.) and were immediately tested for reinstatement of conditioned preference. NURR2C expressing animals showed a blunted preference following cocaine-primed reinstatement compared to GFP expressing controls (Fig. 3H).

4. Discussion

In this series of experiments, we demonstrate that during cocaine-primed reinstatement, HDAC3 disengages from *Nr4a2* in the cholinergic neurons of the MHb. We detected no changes in other known HDAC3 target genes enriched in the MHb that are also known regulators of cocaine response and plasticity (including *Cebpb*, *Ppp1r1b,* and *Nr4a3*). Furthermore, we demonstrate that overexpression of $HDAC3^{W.T.}$ alone in the vMHb is unable to prevent cocaine-primed reinstatement. Lastly, using the dominant-negative NURR2C, we demonstrate that loss of

NR4A2 function in the vMHb significantly blunts cocaine-primed reinstatement. This work sheds new light on the epigenetic regulation of a key transcription factor, NR4A2, within the adult habenula and its link to cocaine-associated behaviors.

Previous work has demonstrated that the MHb is engaged by and regulates reinstatement of cocaine-induced CPP and cocaine self-administration (López et al., 2018; James et al., 2011). Specifically, our lab has demonstrated that H4K8Ac, a permissive epigenetic mark negatively regulated by HDAC3 and positively correlated with increased *Nr4a2*, is increased in the MHb following cocaine-primed reinstatement. In this study, we demonstrated that in response to cocaine-primed reinstatement, there is decreased HDAC3 occupancy at the promoter of *Nr4a2* in the cholinergic neurons of the MHb, thus, suggesting *Nr4a2* is a subsequent target for previously observed increases in H4K8Ac. Although we assayed HDAC3 occupancy at other known HDAC3 target genes (including *Cebpb, Ppp1r1b,* and *Nr4a3*), we found no significant change in response to cocaine-primed reinstatement in the cholinergic neurons of the MHb. Previous work from the Wood lab and others has demonstrated that HDAC3 is a negative regulator of long-term memory in the hippocampus and amygdala and a regulator of cocaine-associated behaviors in the NAc (Kwapis et al., 2017; Malvaez et al., 2013). Moreover, HDAC3-mediated negative regulation of memory in the hippocampus and striatum occurs primarily through negative regulation of *Nr4a2* (Rogge et al., 2013; McQuown et al., 2011; Alaghband et al., 2017). However, due to the global changes in H4K8Ac in the MHb observed during cocaine-primed reinstatement, it is unlikely that *Nr4a2* is the only gene with decreased HDAC3 occupancy. To fully characterize the array of HDAC3-regulated genes that are altered in the MHb during cocaine-primed reinstatement, future studies should employ a less hypothesis-driven approach, such as next generation ChIP-Sequencing. Nevertheless, these results provide further evidence for HDAC3 functioning as a molecular brake pad on memory through negative regulation of *Nr4a2*.

Additionally, overexpression of HDAC3$^{W.T.}$ had no effect on the acquisition or extinction of cocaine-induced CPP. While it is possible the conditioning dose (10 mg/kg) is too strong to detect subtle changes in CPP acquisition, effects on acquisition would likely be observed through changes in CPP extinction. Overexpression of HDAC3$^{W.T.}$ in the vMHb also had no effect on cocaine-primed reinstatement. The inability of HDAC3$^{W.T.}$ to block MHb-mediated reinstatement was surprising, as HDAC3 is a powerful negative regulator of associative behaviors, has been linked to cocaine-associated behaviors and plasticity, and we demonstrate changes in HDAC3 occupancy in the vMHb during cocaine-primed reinstatement. However, previous work has demonstrated that HDAC3 deacetylase activity is dependent on the formation of a complex with co-repressors NCoR and SMRT (Guenther et al., 2001; Sun et al., 2013). Thus, it is possible that simultaneous overexpression of NCoR is necessary to obtain an effect of HDAC3$^{W.T.}$ overexpression. Yet, recent work has shown that overexpression of HDAC3$^{W.T.}$ in the dorsal striatum prevented habit formation (Malvaez et al., 2018). Future studies should more thoroughly evaluate what role HDAC3 in the MHb may have on the regulation of acquisition, extinction, and reinstatement of CPP. With the advent of CRISPR/dCas9 technology, future studies will be able to specifically target HDAC3$^{W.T.}$ to the *Nr4a2* locus to determine if maintained HDAC3 negative regulation of *Nr4a2* in the vMHb is sufficient to prevent cocaine-primed reinstatement.

Lastly, we evaluate what role NR4A2 may have on vMHb regulated behaviors. NR4A2 is an immediate early gene and transcription factor that has a versatile role throughout the CNS. It has been shown to be required for long-term memory formation in the hippocampus (McNulty et al., 2012; Peña de Ortiz et al., 2000; Colón-Cesario et al., 2006). Also, NR4A2 is necessary for proper development of dopaminergic neurons and is dysregulated throughout the ventral midbrain and ventral striatum following chronic cocaine administration, both in humans and animal models (Bannon et al., 2002, 2004; Chandra et al., 2015). Whereas NR4A2 is required for proper MHb development (Quina et al., 2009), its role in the adult MHb remains unknown. Previous work demonstrates that *Nr4a2* is induced in the MHb following an associative learning paradigm, but does not provide a causal link between NR4A2 function and MHb-mediated behaviors (Ressler et al., 2002). Here, we demonstrate for the first time that splice-variant NURR2C directly interacts with endogenous NR4A2. Moreover, our data demonstrate that overexpression of dominant-negative NURR2C in the MHb decreases baseline firing in the cholinergic neurons of the MHb and subsequently blunts cocaine-primed reinstatement, providing evidence that NR4A2 function is required for MHb-mediated relapse-like behaviors. Yet, how NR4A2 affects MHb circuit function and which gene targets of NR4A2 are subsequently engaged during cocaine-primed reinstatement remain key open questions. *Cebpb,* an NR4A2 target gene (Johnson et al., 2011), is one potential candidate gene for conferring changes in the epigenome to changes in MHb circuit function. *Cebpb* expression is activity dependent and is required for plasticity and hippocampal long-term memory (Alberini et al., 1994; Taubenfeld et al., 2001). Moreover, CEBP/β is a transactivator of cholinergic signaling genes enriched in the vMHb (such as *Vacht* and *Chat* (Robert et al., 2002)). However, as we detected no changes in HDAC3 occupancy during cocaine-primed reinstatement, these and other NR4A2 target genes may regulate vMHb function at a different time point, if at all.

The MHb has become a focus for addiction researchers over the past decade. The presented studies further link MHb function with relapse-like behaviors by identifying a key molecular mechanism, in HDAC3-dependent regulation of *Nr4a2*, underlying cocaine-primed reinstatement of conditioned place preference. Moving forward, identifying the downstream targets of HDAC3 and NR4A2 that may confer cocaine-induced changes to MHb circuit function will provide a novel avenue of studies regarding the molecular and circuit mechanisms regulating MHb activity during relapse and relapse-like behaviors.

Author contributions

A.J.L., T.J.H, Y.J., P.H.H, R.R.C., J.L.K, A.O.W., O.C., V.S. performed experiments, D.P.M, G.L., and M.A.W. provided feedback on project and participated in writing the manuscript. A.J.L. designed study, analyzed data, interpreted results, and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2019.04.016.

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