UNIVERSITY OF CALIFORNIA, IRVINE

Cell-Type Specific Epigenetic Mechanisms in the NAc Regulating Cocaine-Induced Plasticity

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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1. Bagley JR, Miller BW, Arth C, **Campbell RR**, Nestler EJ, Haggerty SJ, Kippin TE, Szumlinski KK, Neve RL (2013) Nucleus accumbens histone deacetylases actively regulate cocaine-seeking in mice. Society for Neuroscience.

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Abstract of the Dissertation

Cell-Type Specific Epigenetic Mechanisms in the NAc Regulating Cocaine-Induced Plasticity

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Epigenetic mechanisms regulate critical processes of neuroplasticity that drive cocaine-seeking. Previous work from our lab identified the class I histone deacetylase (HDAC) HDAC3 acts as a negative regulator of cocaine-associated memory formation in the nucleus accumbens (NAc). However, it remains unknown whether this occurs via HDAC3's deacetylase function. It also unclear whether HDAC3 regulates cocaine-induced in a cell-type specific manner within the NAc. Cocaine induces distinct changes in the two major cell populations of the NAc (D1R- and D2R-medium spiny neurons) and activation of these cell types drive opposing behavioral responses to cocaine. In the present dissertation, we investigated whether HDAC3's deacetylase activity acts in a cell-type specific manner within the NAc to regulate behavioral responses to cocaine. In these experiments, we demonstrate that HDAC3's deacetylase activity drives molecular and cellular mechanisms of plasticity within the NAc. In addition, we show how HDAC3's activity within D1R-MSNs, not D2R-MSNs, affects cocaine-associated memory formation and cocaine seeking behaviors. We also demonstrate how HDAC3 regulates histone acetylation to drive changes in gene expression and synaptic plasticity within D1R-MSNs. Overall, this work illustrates how cocaine disrupts HDAC3-dependent mechanisms in a cell-type specific manner to drive cocaine-seeking behaviors.

I. Introduction

A. Drug Addiction, Mechanisms of Cocaine-Associated Memories and Cocaine Reinforcement

Substance Use Disorder (SUD) is a behavioral disorder characterized by loss of control over drug intake and compulsive drug use that persists despite periods of abstinence and adverse consequences. SUDs have led to widespread morbidity and long-lasting impacts throughout the United States. As of 2016, it is estimated that 7.8% of the adult U.S. population meets the criteria for diagnosis of a substance use disorder and $740 billion was spent annually within the United States to address all issues related to substance use[1]. Thus, SUDs are a growing concern for the country, however there are few effective treatments that currently exist. In an effort to create successful SUD treatments, research has focused on understanding how drugs of abuse persistently affect neuronal function that lead to long-lasting changes in behavior.

Early studies identified key neurotransmitter receptors and transporters responsible for the rewarding effects of drugs of abuse like cocaine, heroin, and alcohol. Both neuroimaging studies of human drug users and studies using animal models found that dopaminergic release from the ventral tegmental area (VTA) to several forebrain regions (e.g., nucleus accumbens, amygdala, ventral pallidum, hippocampus) and the frontal cortex is increased following administration of most drug substances[2]. These drug-induced increases in dopamine (DA) were found to mediate the reinforcing properties and reward-related learning that promote drug-consumption[3]. Although each drug class induces dopaminergic release originating from the ventral tegmental area (VTA) differently, (e.g. cocaine primarily increasing dopamine primarily through blocking DA reuptake) it is theorized that these drug-induced adaptations in DAergic signaling initiates the development of SUDs.

DAergic cell firing within the VTA is considered to encode for reward prediction signals through alternating between two firing modes: a tonic, low frequency pattern or a transient, high-frequency

phasic mode. Traditionally, tonic firing is considered to release DA that acts on high-affinity dopamine D2 receptors that determine motivational arousal, whereas phasic firing induces stimulation of D1R-receptors that are associated with conditioned learning[4]. The major projection site of VTA DA neurons is the nucleus accumbens (NAc), where DA interacts with dopamine receptors D1, D2, and D3R to promote action towards the rewarding stimulus[5]. Neuroadaptations within this system, by drugs of abuse, can thus affect motivation towards reinforcing cues and reward-predictive stimuli, including drug-related cues.

Cocaine acts as an instrumental reinforcer and potentiates transient phasic VTA DA signals projecting to the NAc[6,7]. This mechanism is considered to enhance neural signaling that encodes their reward value and increases the probability of future promoting behavioral responses that lead to drug-taking. Increases in dopamine release within the NAc occur not just from the pharmacological effects of cocaine[8]; experiments using fast-scan cyclic voltammetry in rats illustrated that dopamine release is increased within the NAc in response to presentation of learned cocaine-associated cues[8]. Similarly, in a positron emission tomography (PET) imaging human study, cocaine-addicted subjects exhibited increased dopamine following presentation of cocaine-cues within the striatum, measured by displacement of a competitive-D2R radioligands, which correlated with self-reported craving[9]. This enhanced DAergic signaling is theorized to underlie the incentive salience of drug-associated stimuli that are present during drug-taking through Pavlovian conditioning. More specifically, drug-associated cues can elicit approach towards and “wanting” of reinforcing drugs of abuse[10]. This is evident when re-exposure to drug associated cues promotes drug-seeking/reinstatement. For example, drug craving has been shown to incubate over extended periods of abstinence, with re-exposure to drug cues inducing drug-seeking behaviors and activation of brain regions important for goal-directed actions such as the NAc and prefrontal cortex [11,12].

As outlined above, drugs of abuse hijack the dopaminergic system, which normally encodes reward-learning, causing a heightened reward value of drugs and drug-associated stimuli. The dopaminergic system acts on several regions critical for emotional processing and memory consolidation, however it the modulation of glutamatergic inputs from the PFC and BLA onto the NAc is considered to promote SUD-related behaviors. The NAc integrates information from these structures to mediate goal-directed actions; drugs of abuse utilize these mechanisms to promote pathological motivation for drug-seeking from re-exposure to drug-associated cues.

B. Overview of the Nucleus Accumbens

Subregions of the Nucleus Accumbens

The Nucleus Accumbens (NAc) is a structure within the basal forebrain that is involved in action selection through motor behavior, decision-making, and motivational processes[13]. It generates goal-directed motivated behaviors through integrating cognitive and emotional information from its major inputs, including glutamatergic inputs from the prefrontal cortex, basolateral amygdala, and hippocampus, in addition to receiving dopaminergic input from the ventral tegmental area. These afferent projections are distributed across the two subregions of the NAc; the core and the shell. Discrete cortical regions project to the shell versus core; the medial lateral orbital frontal cortex (OFC) and infralimbic cortex sends information to the shell while the prelimbic and lateral OFC project to the core[14]. In addition, the core and shell have distinct efferent projections that regulate different aspects of motor behavior. The core sends outputs to the dorsolateral ventral pallidum, subthalamic nucleus and substantia nigra that then project to premotor cortical areas through the motor thalamus. The shell projects to subcortical limbic regions such as the lateral hypothalamus, ventromedial ventral pallidum and VTA. It should be noted that the core and shell have direct interconnections with one another (mostly afferents from the core to shell), however overall the differences in circuitry between the core and shell suggest they may control distinct aspects of motivated behaviors.

The differences in dopamine release dynamics, originating from the ventral tegmental area, within the NAc core and shell demonstrated how these two subregions differentially encode information about reward to promote Pavlovian learning and goal-directed actions[15]. With regard to reward processing, the NAcshell as opposed to the core, is primarily implicated in reward processing of unconditioned stimuli. For instance, critical dopamine release is increased or decreased only within the NAc shell by uncued rewarding and aversive tastants, respectively. Lesions to the NAcshell also attenuate unconditioned potentiating effects of psychostimulants[16]. Moreover, during non-contingent cocaine administration, despite increases in dopamine within both the core and shell, transient increases in DA release are greater within the shell compared to the core[17,18]. The NAccore is considered to regulate aspects of Pavlovian-associative cue learning. This was shown during a Pavlovian conditioning task where cue-evoked increases in dopamine release develops within the NAc core, but not the shell [19]. The above findings suggest that the core is important for aspects of reward-cue learning and the presentations whereas the Nacshell is important for processing the reward value of stimuli.

Cellular Composition

The Nucleus accumbens is a heterogenous structure with numerous types of interneurons and two major types of the output medium spiny neurons (MSNs). Although several types of these interneurons have recently been implicated in cocaine-associated behaviors, they comprise ~5% of the NAc. The GABAergic MSNs encompass approximately 90-95% of total neurons within the NAc and are subdivided into two distinct subpopulations based on the expression of dopamine receptors D1R and D2R as well as the expression profile of neuropeptides[20]. D1 receptor–containing MSNs (D1R-MSNs) coexpress dynorphin, substance P, and M4 cholinergic receptors, whereas D2 receptor MSNs (D2R-MSNs) express enkephalin, neurotensin, and A2a adenosine receptors. However, a small fraction of MSNs (2%–17%) do express both D1- and D2-receptors[21]. The two G protein-coupled dopamine receptors induce differential signaling cascades, with D1Rs

coupled to Gas/olf and D2R Gai/o, which act to stimulate or inhibit adenylyl cyclase and cAMP-dependent activity. At rest, the two types of MSNs are inhibited, however glutamatergic input shifts MSNs to a more depolarized state to drive neural activity. DA differentially modulates this activity, using D1R to further increase spiking in MSNs while D2 receptors inhibit the active "up-state" of MSNs[22]. In regard to MSN projections, in the dorsal striatum MSNs are segregated into a direct pathway, that projects to the globus pallidus internal (GPi) and substantia nigra pars reticulata (SNr), and then an indirect pathway, that projects to the globus pallidus external (GPe), with very little overlap in projections[23]. Although it was previously considered that these pathways are similarly segregated in the NAc, recent evidence demonstrates some overlap in MSN projections. D1-MSNs project to the GPi and midbrain structures, SNr and VTA, as well as the ventral pallidum (VP) and lateral hypothalamus while D2-MSNs project only to the VP[24]. Through modulation of these glutamatergic and dopaminergic inputs, cocaine has been shown to distinctly alter the cellular function of MSNs that affects their efferent activity to promote cocaine-induced behaviors.

C. Cocaine-Induced Synaptic Plasticity

Drugs of abuse restructure cellular properties within the mesocorticolimbic pathway to disrupt plasticity and promote mechanisms of drug craving and relapse. Depending on the drug class and administration paradigm, drugs of abuse can differentially alter the dendritic branching and spines in the brain. For example, opiates decrease the number and complexity of dendritic spines within the NAc, mPFC, and hippocampus whereas psychostimulants have been shown to increase dendritic complexity and spine density within NAc, mPFC and VTA[25]. These structural changes may facilitate changes in electrophysiology and synaptic strength seen following exposure to drugs of abuse.

In the VTA, acute exposure to cocaine induces persistent increases in excitatory synaptic strength due to transient reorganization of postsynaptic glutamatergic and GABA signaling to

promote DA release[26,27]. For example, during early stages of withdrawal following either chronic or acute cocaine exposure, GluA2-lacking Ca2+-permeable AMPAR signaling and AMPAR/NMDAR ratio[26] is increased within VTA DA neurons[26]. More specifically, chronic self-administration decreases $GABA_A$-R signaling that controls cellular excitability (Liu 2005) and reduces D2R autoreceptor function to regulate DA firing [28,29]. Interestingly, despite evidence demonstrating that chronic cocaine induces hyperexcitability of VTA dopamine neurons in early withdrawal, reductions in tonic extracellular dopamine in the ventral striatum following prolonged cocaine use is required to escalate cocaine intake[30–32]. Although difficult to reconcile, it is theorized that these low baseline levels of DA increase the signal-to-noise ratio to enhance phasic dopamine signaling in response to drug-associated cues [33,34].

Within the NAc, exposure to cocaine augments synaptic strength however the degree and time point varies depending on the affected subregion. Following acute cocaine exposure, the NAcc, but not NAcshell, exhibits changes in synaptic plasticity by increasing AMPAR signaling[35,36]. However, after chronic cocaine exposure, there is an initial suppression of synaptic strength in early stages of withdrawal in both the NAcc and Nacshell[37]. One underlying mechanism for this is the increase in newly-generated, silent immature NMDAR-only synapses[38,39]. Maturation and consolidation of these synapses increases synaptic strength with the subsequent insertion of AMPAR seen following prolonged withdrawal in both the NAcc and NAcshell[40]. These changes in NMDAR/AMAPAR signaling are required for both cue-induced cocaine seeking and incubation of cocaine craving.

Cocaine-induced synaptic potentiation of the NAc occurs, in part, due to excess glutamatergic release from the prelimbic prefrontal cortex (PrL). More specifically, presynaptic mGLUR2/3-dependent regulation of glutamate release is impaired and glial glutamate transporters regulating synaptic glutamate is reduced in the PrL-to-NAcc synapses following cocaine exposure[13]. This potentiated state impairs subsequent experimentally induced LTP, which is often referred to as

an occlusion effect of the potentiated synapses during withdrawal[36]. In line with this, re-instated drug seeking is associated with a marked increase in extracellular glutamate in the NAc derived from PrL afferents[41]. However, PrL-to NAcc synapses are de-potentiated following cocaine re-exposure; cocaine decreases surface AMPAR expression and reduced dendritic spine morphology within the NAc. It is suggested that the synaptic potentiation occurring during cocaine withdrawal and the transient potentiation in the presence of cocaine-associated cues may underlie drug-seeking and craving[42]. Following evidence that exposure to cocaine and cocaine-associated cues alters neural morphology and physiology within the mesocorticolimbic system to promote drug associated behaviors, the molecular mechanisms regulating cocaine-induced synaptic plasticity have been further investigated.

In regard to the morphological changes, several studies have shown that cocaine affects actin-filament dependent mechanisms of spine stabilization. Actin cycles from F-actin (filament complexes) to G-actin (monomer), causing an expansion or contraction of dendritic spines[43]. Chronic exposure to cocaine affects the activity of several actin-severing and polymerizing proteins, resulting in increased dendritic spine density within the NAc. This includes downregulation of activated GTPases RhoA and Rac1, and enhanced activation of the actin polymerizing protein WAVE1[44]. Disrupting the activity of these proteins presumably underlies the increases in F-actin seen following chronic cocaine. Evidence of this includes decreased levels of phosphorylated actin-severing protein cofilin (p-cofilin), which inhibits cofilin activity and is regulated by downstream Rac1 signaling, within the NAc following chronic cocaine[45,46]. Consistent with this, overexpressing constitutively active forms of Rac1 in the NAcshell blocks cocaine reward and cocaine-induced -increases in spine density, while increasing levels of constitutively active cofilin enhanced the rewarding effects of cocaine[46].

In terms of the glutamatergic related plasticity, silent synapses are produced by inserting NMDAR in a CREB-dependent manner into new synaptic sites [38]. For the maturation of these

synapses, increases in calcium-permeable AMPARs require constant protein translation and a mGluR1-dependent mechanism for insertion[47,48]. This was demonstrated using whole cell recordings of rats that underwent prolonged withdrawal; disruption of protein translation by bath applying pharmacological inhibitors reduced calcium-permeable-AMPAR mediated synaptic transmission. In addition, inhibition of protein translation eliminated mGluR1-mediated inhibition of NAc synaptic transmission[47]. As described above, cocaine utilizes mechanisms of actin polymerization, gene expression and protein translation to promote cellular plasticity within the NAc. In order for these long-term structural and functional changes to occur within neural systems, cocaine affects mechanisms of transcription key to drug-induced plasticity.

D. Transcriptional Mechanisms in Reward and Drug-Seeking Behaviors

Exposure to drugs of abuse alters gene expression within regions critical for drug-seeking behaviors in both human drug abusers and animal models of addiction [49]. Early work in the field examined how drugs of abuse, in particular cocaine, induce changes to a select set of genes implicated in learning and memory. More specifically, the expression profile and activity of transcription factors deemed markers of neuronal activity and plasticity were monitored following acute and chronic cocaine treatments. In general, genes implicated in memory formation are also upregulated following drug exposure. For example, Cyclic AMP response element binding protein (CREB), a critical learning-induced gene, is also upregulated in the NAc and other reward-related regions by cocaine and is believed to mediate tolerance to the rewarding effects of drugs to ultimately drive an escalation of drug intake [50,51]. CREB was one of the first transcription factors to be implicated in long-term memory formation (Yin, Tully) and recent work has suggested that levels of CREB within neurons also dictate their likelihood to be incorporated in a memory trace, potentially by increasing their excitability[52,53]. Similarly, CREB has been shown to regulate cocaine-induced cellular responses; overexpression of CREB in the NAc enhances surface expression and function of NMDARs in NAc MSNs (Huang 2008) and increases cellular

excitability within the NAcshell[37,54]. RNA-sequencing data has also shown that various forms of cocaine exposure enhances the expression of CREB-regulated genes throughout the mescorticolimbic system [55]. More recently, single-cell RNA sequencing shows revealed how cocaine induces cell-type specific transcriptional changes, that are dopamine (DA) and CREB-dependent[56]. Thus, much of the field has focused on understanding how other transcription factors interact with CREB to mediate cocaine-induced gene expression and the function of activated CREB-regulated genes in cocaine-related processes.

Several CREB-regulated genes that are implicated in drug-related behaviors include other key players in learning-induced plasticity, including the plasticity related- genes Erg1/2/3, Nr4a1/2, delta FosB, BDNF and Arc. Several of these genes, including Erg3 and BDNF are induced following both acute and chronic cocaine exposure[57–60]. In addition, overexpression of IEGs such as Erg3 and Nr4a1 have been shown to drive cocaine-reward and drug-seeking behaviors[60–62] . However, it should be noted that upregulation of these plasticity-inducing genes does not necessarily occur following every drug exposure; some of these genes are selectively expressed following acute or chronic drug exposure. One example of this is Delta FosB, a member of the Fos family of transcription factors that dimerize with Jun family members to form activator protein-1 (AP-1) complexes. Many of the Fos-family proteins (c-Fos, FosB, Fra1, Fra2) are induced transiently by acute exposure to drugs of abuse, however delta FosB is induced only after chronic drug exposure[50]. The accumulation of delta FosB persists after weeks of drug-abstinence and its expression within mesocorticolimbic regions promotes several drug-seeking behaviors (e.g. drug sensitization and reinforcement)[63,64]. Overall, FosB is considered a molecular switch for initiating and maintaining drug-induced states, in particular enhanced sensitization to the rewarding and reinforcing properties of cocaine.

Together, this work demonstrates that a number of plasticity-related genes important for memory formation are also key players in drug-seeking behavior. Now that the importance of

these genes in drug-seeking behavior has been clearly established, many in the field have worked to identify: 1) the upstream regulators that mediate IEG expression and activity, 2) the mechanisms by which IEGs and other cooperating transcription factors regulate gene expression and 3) the downstream gene targets of IEGs that promote drug-associated behaviors.

D. Epigenetics in reward and drug-seeking behavior

The epigenetic landscape of a cell can induce long-lasting changes to its transcriptional response towards stimuli. Epigenetics can be defined as the regulation of gene expression through the alteration of chromatin structure, without modifying the nucleotide base sequence. Chromatin is the protein-DNA assembly made up of repeating nucleosome units, which consist of 147 base pairs of DNA spooled around a histone octamer. By organizing and compacting DNA into the nucleus of the cell, modifications to these DNA-protein complexes can dynamically regulate transcription. Epigenetic mechanisms include histone modifications (e.g. histone acetylation, methylation), histone variant exchange, nucleotide modifications (e.g. methylation), noncoding RNAs, and chromatin remodeling[65,66]. These mechanisms were originally identified during stages of cell differentiation and cell division, including transgenerational passage of gene-regulatory information and the integration of environmental signals to coordinate transcriptional responses. In the learning and memory field, it is theorized that epigenetics may have a role in maintaining the engram (discrete cell populations that form neuronal ensembles representing the physical memory trace), possibly by increasing the responsiveness to stimulation through epigenetic priming[67]. Considering the role that learning and memory processes play in drug-associated behaviors, it is unsurprising that epigenetic mechanisms, which are critical for learning-induced plasticity, also regulate drug-seeking and drug-induced plasticity.

Histone Modifications

Residues in the tails of histones undergo covalent modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ribosylation, and many others, that change the way the histone and its surrounding DNA interact. The two primary histone modifications studied in relation to SUDs are histone acetylation and methylation. Histone acetylation negates the positive charge of lysine residues in the histone tail, which disrupts the interaction with negatively charged DNA and loosens DNA from compaction to increase likelihood of transcriptional activation[68,69]. This modification is regulated by "histone writers/erasers" called histone acetyltransferases (HATs) and histone deacetylases (HDACs), which contain multiple classes of enzymes with distinct expression and activity patterns throughout the brain. Because of this relationship, HDACs are hypothesized to act as molecular brake pads that repress gene expression at rest but are disengaged following an activity-dependent event to facilitate HAT activity[70]. This increase in acetylation, not only relaxes the chromatin to promote transcriptional access, but also allows for recognition of these marks by "histone readers". Histone readers can recognize acetylation due their bromodomains, which many chromatin-associated proteins, including HATs, contain to regulate transcription.

Methylation of histones can occur on lysine and arginine residues and occurs in different forms: lysine methylation can be mono- (me1), di- (me2), or trimethylated (me3) via histone methyltransferases, whereas histone arginine methylation can be monomethylated, symmetrically or asymmetrically demethylated by protein arginine methyltransferases[71,72]. Because methylation does not affect the charge of the histone residue, depending on the residue methylated and the degree of methylation, histone methylation can promote or repress transcription. Histone methylation marks are then read by proteins containing chromo-domains and domains with similar structure to chromodomains (i.e. chromobarrel, MBT,TTD, PWWP, Tudor) to further influence transcription. As discussed below, histone modifying enzymes are theorize to regulate persistent changes in cocaine-induced gene expression that promote cocaine-seeking behaviors.

Histone Acetylation

Cocaine affects global and gene-specific changes in histone acetylation are within the NAc. For example, acute and chronic cocaine exposure both increase acetylation of histone 3, lysine 14 residue (H3K14Ac) generally within the NAc and specifically at plasticity-related transcription factors, including *CREB*[73–76]. These changes in histone acetylation are thought to regulate gene expression, as cocaine-induced changes in H3K14Ac at the *cFos* promoter correlated with increases in *cFos* expression in the NAc[74]. Similarly, following acute cocaine conditioning, increases in H4K8 acetylation at the promoter of the immediate-early gene *Nr4a2* correlated with enhanced expression of *Nr4a2* in the NAc[74,77]. However, not all exposures to cocaine induce the same pattern of histone acetylation. For example, acute but not chronic non-contingent injection of cocaine transiently hyperacetylates histone H4 (H4ac) at the *cFos* promoter within the NAc[75]. This same pattern was shown in a separate study with histone H3 acetylation (H3Ac) and binding of the histone acetyltransferase CREB-binding protein (CBP) at the *cFos* promoter as well[74]. These cocaine-induced histone acetylation patterns are consistent with cocaine gene expression profiles: actue cocaine, but not chronic cocaine induces *cFos* expression.

Drug-induced changes in histone acetylation are not restricted to transcription factors; the genes encoding several synaptic related proteins have differential patterns of histone acetylation following drug exposure, as well. For example, chronic exposure of methamphetamine in the dorsal striatum decreased H4 acetylation and increased HDAC2 on *GluA1, GluA2 and GluN1* promoters. This is correlated with decreases in *GluA1, GluA2, GluN1* expression and altered electrophysiological glutamatergic responses within the striatum[78]. Drugs of abuse induce changes in histone acetylation within human tissue as well; increases in H3K27 acetylation on

GluA2 promoter are observed within the NAc of human heroin abusers[79]. With evidence that drugs of abuse affect histone acetylation and influence transcription within regions of reward processing, it is critical to identify the mechanisms that control this process. This includes the mechanisms of HATS and HDACs, including their interactions with transcription factors, that influence expression of select genes critical for cocaine-induced synaptic and behavioral plasticity.

Several HATs and HDACs have been implicated in cocaine-induced changes in transcription and behavior. Given the importance of CREB, it is unsurprising that the activity of the histone acetyltransferase CREB-binding protein (CBP) is among those that are important for cocaine-associated processes. CBP has been shown to regulate cocaine-induced-gene expression; as CBP binding is enhanced at the promoter of plasticity-related genes like FosB following cocaine exposure[75]. Further, loss of CBP within the NAc impairs cocaine-induced histone acetylation and gene expression, indicating that CBP-mediated transcription is critical for cocaine-induced transcription and cocaine-induced responding. Deletion of CBP also results in impaired behavioral responses to cocaine, such as cocaine-induced sensitization and memory formation[74]. These findings demonstrate that cocaine exposure drives changes in histone acetylation to influence gene expression and promote cocaine-associated behaviors.

Researchers have also examined how cocaine disrupts mechanisms of transcriptional repression via HDAC activity. Much of this initial work used pharmacological manipulations of HDAC activity, as many were available from the cancer field and were shown to have profound effects on neural processes of memory formation. For example, pretreatment with the non-specific HDAC inhibitor (HDACi) Suberoylanilide hydroxamic acid (SAHA) prior to an acute dose of cocaine was sufficient to enhance cocaine-induced changes to LTP (i.e. depress high frequency stimulation-induced potentiation) and gene expression [36]. Non-specific HDACis can also induce changes in behavior, including extinction of cocaine-induced CPP. Infusion of the HDACi Trichostatin A (TSA) into the NAcshell also increased motivation for cocaine intake; intravenous

infusion of TSA shifted the dose-response curve and increased the break point under progressive-ratio testing under cocaine-self administration conditions[73]. From these studies and others, it is clear that HDACs regulate cocaine-induced changes in histone acetylation and gene expression that can promote cocaine-associated synaptic plasticity and behavior.

More recent work has identified the roles of specific HDACs in mediating cocaine processes. Most notably, systemic injections of the Class I HDAC HDAC3 selective pharmacological inhibitor of the facilitates the extinction of cocaine-conditioned place preference[80]. Deletion of *HDAC3* within the NAc also enhanced cocaine-induced histone acetylation, gene expression, and the formation of cocaine-associated memory[77]. Although not tested directly, it is expected that the catalytic activity of HDAC3 regulates cocaine-induced transcription within the NAc to limit cocaine-associated behaviors. However, HDAC3 can also influence transcription through interactions with corepressor proteins including other HDACs. Whether it is HDAC3 expression or its deacetylase activity that is altered during prolonged periods of drug-taking/withdrawal that contributes to the dysregulation of cocaine-induced plasticity has not been examined.

It is possible that the effects of HDAC3 in cocaine-induced transcription come from its role in forming a complex with co-repressors rather than its catalytic activity *per se*. In fact, there is evidence that Class II HDACs, such as HDAC4 and HDAC5 function *in vivo* by interacting with Class I HDACs like HDAC3. Moreover, HDAC3 is critical for the formation of these repressive complexes. However, Class II HDACs reportedly do not have independent deacetylase activity, and have been shown in to influence gene expression through their interactions with other repressive transcription factors[81]. Cocaine dynamically regulates the cellular localization of both HDAC4 and HDAC5 in a phosphorylation-dependent manner, possibly disturbing protein-protein interactions among transcriptional corepressors to promote cocaine-induced transcription and cocaine-associated behaviors[82]. For instance, immediately following chronic cocaine exposure,

HDAC5 is initially hyperphosphorylated, however 24 hours following the last injection, dephosphorylation of HDAC5 increases in the NAc. This dephosphorylation serves to limit the formation of cocaine-reward associations, as overexpression of a non-phosphorylatable HDAC5 mutant into the NAc prevented nuclear accumulation of HDAC5 and impaired cocaine-induced CPP [82,83]. HDAC5 regulates other drug-associated behaviors; overexpression of nuclear HDAC5 in the NAc suppresses cue and cocaine-primed reinstatement under the cocaine self-administration task and regulates incubation of methamphetamine craving[84]. HDAC5 may regulate cocaine-associated behaviors partly through one of its target genes *Npas4*, which is induced following exposure to cocaine and cocaine-conditioned contexts and required for the formation of cocaine-associated memories[83]. Together, these findings suggest that mechanisms regulating HDAC5 localization and activity are disrupted in the development of SUDs. However, the mechanism by which this occurs is unclear.

Like HDAC5, the Class II HDAC HDAC4 has also been shown to regulate cocaine-related behaviors, and its cellular localization is also regulated by cocaine in a phosphorylation-dependent manner. Cocaine increases cytoplasmic-HDAC4 through phosphorylation and leads to enhanced MEF2-dependent transcription in KCl-induced striatal cultures. In contrast to HDAC3 and HDAC5, HDAC4 activity within the NAc promotes cocaine-associated memory and cocaine-induced sensitization[85]. This study highlights the importance in understanding how each individual HDAC enzyme contributes to cocaine-induced changes in histone acetylation and gene expression that regulate cocaine-induced plasticity.

The actions of histone “readers” such as bromomdomain and extraterminal family (BET) proteins have also been examined in regard to cocaine-induced plasticity. BET inhibition using pharmacological inhibitors prevents the formation of conditioned cocaine-associated reward and heroin self-administration behaviors[79,86]. In addition *Brd4,* a member of the BET family, was increased in the NAc following cocaine-self administration. It is speculated that these proteins

recognize histone acetylation marks and promote transcription of genes critical for cocaine-associated behaviors. For instance, Brd4 may regulate cocaine-induced gene expression of *BDNF*, as Brd4 binding on the BDNF promoter was increased following cocaine exposure[86]. The field is in the initial stages of understanding how BETs influence cocaine-induced gene expression and behavior, however these findings demonstrate how cocaine-induced histone acetylation may promote mechanisms of transcription.

Histone Methylation

The role of histone methylation and histone methylating enzymes have also been examined in relation to cocaine-induced gene expression and behaviors. In general, cocaine exposure decreases levels of H3K9ame2 and HMTs that catalyze this state within the NAc. For instance, chronic cocaine reduced expression of the lysine methyltransferase G9a and global levels of histone 3 lysine 9 dimethylation ((H3K9me2) in the NAc, with reduced levels at the promoters of several plasticity-related genes, include *Arc, Fosb,* and *BDNF IV*. G9a within the NAc regulates several drug-induced behaviors: overexpression of G9a impaired morphine reward and sensitization[87], and impairs cocaine-induced CPP. In addition, overexpression of G9a prevented chronic cocaine-induced increases in dendritic spine density within the NAc [49,88,89]. Histone methylation also plays a key role in cocaine-induced alterative splicing within the NAc. More specifically, Xu et. al. showed that cocaine induces changes in H3K36me3 at splice junctions, via histone methylase SET2, which alters splicing events in the NAc and is sufficient to drive cocaine-seeking behaviors[90]. Although few other histone lysine methyltransferases have been tested, it appears this activity at least within the nucleus accumbens functions to repress cocaine-induced plasticity. In regard to histone arginine methylation, only the asymmetric dimethylation of R2 on histone H3 (H3R2me2a), and its catalyzing enzyme protein-R-methyltransferase-6 (PRMT6) have been examined in cocaine action[71]. Repeated cocaine exposure reduces expression of both

H3R2Me2a and PRMT6 within in the NAc. Additionally, overexpressing PRMT6 enhanced cocaine-induced CPP, whereas overexpressing a miRNA targeting PRMT6 reduced cocaine induced-CPP. In line with histone lysine methylation, arginine methylation appears to be reduced within the NAc by cocaine in order to promote expression of genes critical for cocaine-induced behaviors.

These mechanisms discussed above illustrate the role of the epigenome in regulating drug-induced changes in in histone modifications and gene expression that affect synaptic plasticity and behavior. The field has theorized that drugs of abuse dysregulate epigenetic mechanisms to induce long-lasting changes in cellular and behavioral plasticity that lead to SUDs. Moving forward, it is important to further examine how the epigenome is dysregulated throughout different stages of drug use and drug-associated behaviors.

E. Cell Type Specific Cocaine-Induced Changes within the NAc

As described above, the NAc's major projection neuron types, medium spiny neurons (MSNs), and are further classified by the expression of D1R and D2R. These two MSN subtypes have been shown to promote differential behavior responses[20]. In regard to cocaine associated behaviors, optogenetic activation of D1Rs-MSNs enhances cocaine-induced CPP while activation of D2R-MSNs impairs cocaine-induced CPP[61]. In vivo calcium imaging of NAc MSNs also illustrated that D1R-MSNs display enhanced activity following exposure to the cocaine-conditioned chamber while D2R-MSNs have reduced activity [91]. Activation of D2R-MSN activity also impairs cocaine-self administration across various FR schedules, while inhibition of D2-MSNs enhances motivation for cocaine [92]. Thus, D1R vs D2R-MSNs appear to act in opposition of one another to influence cocaine-seeking, with D1R-MSNs promoting and D2R-MSNs inhibiting cocaine-associated behaviors.

Cocaine-induced cellular responses within D1R and D2R MSNs has also been examined to understand how activity of these cell subtypes is usurped to promote cocaine-induced behavioral responses. Cocaine potentiates excitatory transmission within D1R-MSNs, but not D2R-MSNs, to promotes cocaine seeking. For instance, acute cocaine exposure can occlude high frequency stimulation-induced long-term potentiation onto D1R-MSNs, but not D2R-MSNs, which can persist for up to 1 week [93,94]. Chronic cocaine similarly potentiates excitatory transmission within only D1R-MSNs. Few studies see changes in D2R-MSN synaptic activity, however behavioral measurements of perseverance and motivation for cocaine seeking negatively correlated with AMPAR/NMDAR ratios in D2R-, but not D1R-MSNs[95]. In examining changes in circuit function, cocaine blocks D2R-MSN to ventral pallidum (VP) long term depression (LTD), where restoring this D2R-MSN mediated inhibition onto the VP alleviates motivational deficits induced by cocaine exposure [96]. Cell-type specific changes in synaptic strength from cocaine exposure are suggested to occur, at least in part, due to cocaine-induced changes in dendritic morphology. For example, silent synapses are formed during early cocaine withdrawal onto D1R-MSNs (Kim 2011), but not D2R-MSNs. In addition, following long-term withdrawal, only D1R-MSNs have increased levels of spine density and have increased AMPAR insertion (becoming "unsilenced) [97,98]. From this evidence it appears that cocaine enhances excitatory transmission of the D1R-MSNs while simultaneously inhibiting D2R-MSN activity to promote cocaine-seeking behaviors.

It is theorized that robust molecular changes occur within D1R-MSNs and D2R-MSNs underlie cocaine-induced cellular responses that contribute to the reinforcing and sensitizing effects of drugs of abuse. For instance, chronic cocaine exposure differentially alters expression of several plasticity-related genes, such as *Egr3, Nr4a2, FosB, and CREB* within D1R versus D2R-MSNS[62]. In addition, overexpression of *Egr3* within D1R vs D2R MSNs induces differential response to cocaine: promoting cocaine-preference sensitization when overexpressed in D1R-MSNs while

impairing cocaine preference and sensitization within D2R-MSNs. More recently, there is evidence of sex-specific changes in the D2R- Egr3 activity to drive abstinenece induced cocaine-seeking[99]. It is possible that cocaine-induced changes in gene expression within D1R and D2R MSNs reflect cell type specific alterations within epigenetic mechanisms. For instance, the histone deacetylase Sirt1 and histone methyltransferase G9a also had distinct expression profiles within D1R vs D2R MSNs following chronic cocaine[62,100]. *Sirt1* expression increased and *G9a* decreased within D1R MSNs, while both genes had no significant changes in expression within D2R-MSNs. In a separate study, acute and chronic cocaine exposure can induce distinct profiles of histone acetylation and methylation within D1R and D2R MSNs[101]. These results indicate that cocaine may differentially affect epigenetic mechanisms within D1R vs D2R MSNs to induce distinct changes in gene expression and synaptic plasticity. However, no study to date has examined how activity of epigenetic enzymes may be differentially regulating loci-specific changes in histone modifications or gene expression within D1R- and D2R-MSNs. Identification of the distinct epigenetic mechanisms within these specific cell types required for gene expression involved in cocaine action will significantly contribute to our understanding of drug-seeking behaviors. **The experiment described here provide the first evidence that HDAC3's deacetylase activity within D1R-MSNs are critical in regulating cocaine-induced plasticity and behavior.**

Chapter 1: Cocaine-induced Changes on HDAC3-related Machinery in the NAc.

Rationale:

All major drugs of abuse alter epigenetic marks (e.g., histone acetylation and histone methylation) within the Nucleus Accumbens (NAc)[71,73,74,87,102]. Several epigenetic enzymes responsible for these marks are critical for drug-induced changes in gene expression within the NAc as well as drug-induced changes in behavior. Among them is the class I histone deacetylase (HDAC) HDAC3, which acts within the NAc to negatively regulate cocaine-associated changes in gene expression and cocaine-associated memory formation[77].

As an HDAC, HDAC3 acts in opposition of histone acetyl-transferases (HATs) by removing acetyl groups from lysine tails of histone proteins to condense chromatin structure and restrict gene expression. One histone modification in particular, acetylation of Lysine Site 8 on Histone 4 (H4K8Ac), is correlated with HDAC3 activity[103–106]. HDAC3 is hypothesized to gate memory processes through this deacetylase activity. In support of this, genetic deletion and pharmacological inhibition of HDAC3 alters learning-induced changes in gene expression that are thought to underlie memory formation[70,105,107]. However, HDACs can regulate gene expression independent of deacetylase activity. For instance, HDAC4 plays a role in memory and cocaine-processes despite having little catalytic activity on acetyl lysine residues[85,108]. Moreover, in non-neuronal tissues, HDAC3-mediated gene expression does not require HDAC3 enzymatic function. This can occur via HDAC's associations with HDAC4/HDAC5 along with the co-repressor nuclear receptor (NCoR) to form a multi-protein transcription repressor complex[83,109,110]. Despite this, it is unknown how cocaine alters the expression and activity of HDAC3 within the NAc to promote cocaine-seeking behaviors. The goal of this study was to determine whether the expression of genes critical for HDAC3 activity are altered by cocaine. In addition, whether any changes in HDAC3 activity or H4K8Ac are found on the promoters of cocaine-induced immediate early genes.

Materials and Methods:

Mice:

Male C57BL/6 J mice were all single-housed and at 8 and 15 weeks old at the time of behavioral testing (n=30). Mice were provided with food and water *ad libitum* for all experiments except food training. Lights were maintained on a 12:12 h light per dark cycle, with all behavioral tests performed during the light portion of the cycle. All experiments were conducted according to US National Institutes of Health Guidelines for Animal Care and Use, and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

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- experiments, cocaine-HCl was dissolved to a final concentration at 2.0 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).

Quantitative RT-qPCR

RT-qPCR was performed as described previously[105,111]. One millimeter punches were collected from NAc in a 500 M slice of tissue. RNA was isolated from punches 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, designed using the Roche Universal Probe Library:

Gene	Forward Primer	Reverse Primer	Roche Probe #
Hprt5	TGCTCGAGATGTCTGAAGG	ATCACATTGTGGCCCCTCTGT	-----
Fos	ggggcaaagtagagcagcta	agctccctcctccgattc	46

Nr4a1	agcttgggtgttgatgttcc	aatgcgattctgcagctctt	93
Nr4a2	ttgcagaatatgaacatcgaca	gttccttgagcccgtgtct	2
Nr4a3	gtgtcgggatggttaaggaa	gagggctcctgttgtagtgg	91
Hdac3	ttcaacgtgggtgatgactg	ttagctgtgttgctccttgc	32
Hdac4	gcacagttgcatgaacatatca	Ctccagtttccgctggtg	17
Hdac5	gcatgaactctcccaacgag	tctgggttgatactgcctctc	20
NCor1	gcacttggatccagctatgc	tatcctggggttggtgaaag	18
NCor2	cgcacaccggatcctaga	gtgtcagacggctggttgt	34
GriA1	agggatcgacatccagagag	tgcacatttcctgtcaaacc	62

Chromatin Immunoprecipitation

ChIP was performed as described previously [105]based on the protocol from the Millipore ChIP kit. Tissue was cross-linked with 1% formaldehyde (Sigma), lysed and sonicated, and chromatin was immunoprecipitated overnight with 5 µL of anti- HDAC3 (Millipore), anti-H4K8AC (Millipore) or 5 µL of anti-mouse IgG (negative control, Millipore). The immunoprecipitate was collected using magnetic protein A beads (Millipore). After washing, chromatin was eluted from the beads and reverse cross-linked in the presence of proteinase K before column purification of DNA. *Fos, Nr4a1* and *Nr4a2* promoter enrichment in ChIP samples was measured by quantitative real-time PCR using the Roche 480 LightCycler and SYBR green. Primer sequences for the promoters, designed by the Primer 3 program are listed below. Five µL of input, anti-HDAC3 IgG, or anti-mouse IgG immunoprecipitate from 4 separate mice in each condition were examined in duplicate. To normalize ChIP-qPCR data, we used the percent input method. The input sample was adjusted to 100% and both the IP and IgG samples were calculated as a percent of this input using the formula: 100*AE^(adjusted input – Ct (IP)). An in-plate standard curve determined amplification efficiency (AE).

Promoter	Forward Primer	Reverse Primer
Fos	TTCTCTGTTCCGCTCATGACGT	CTTCTCAGTTGCTAGCTGCAATCG
Nr4a1	Gatagaggggtgggctgaag	aaaagagctcagtccgacga
Nr4a2	TGAAGTCCGTGGTGATGCTA	CGGGACAACTGTCTCCACTT
Nr4a3	GAGGGAGGAGGAGGGTGACGTA	CATAGAGTGCCTGGAATGCGAGA

Western Blotting

1 mm punches in the NAc core of mice were collected from 500 µm slices. Protein was Tissue isolated by in 500 µl of homogenization buffer containing 320 mM sucrose, 5 mM HEPES buffer, 1% SDS, in the presence of phosphatase inhibitor cocktails I and II (Sigma) using an ultrasonic processor (Cole Parmer). Protein concentrations were determined using a Bio-Rad protein assay bovine serum albumin standards. 10–20 µg of protein was loaded onto 18% gradient Tris-HCl polyacrylamide gels for electrophoresis fractionation (Bio-Rad). Electrophoretic transfer was then performed overnight at 30 V at RT onto nitrocellluose membrane. Membranes were blocked for 1 h at room temperature in blocking solution (TBS Tween 20 Starting Block; ThermoFisher Scientific) and then incubated in primary antibodies (HDAC3 1:1000 (abcam), GAPDH, 1:1000 (abcam), cFos 1:500 (synaptic systems) , with agitation overnight at 4 degrees C. Membranes were then rinsed three times for 5 min each in PBS with agitation. Membranes were subsequently incubated in secondary antibody (1:5000 mouse anti-rabbit HRP light chain). Supersignal Westpico Chemiluminescent substrate (ThermoFisher Scientific) was used for chemiluminescent detection according to the manufacturer's instructions and analyzed using NIH ImageJ software. For Western blot analysis, band intensity for proteins was normalized to that of GAPDH for HDAC3 and Total H4 for H4K8Ac, to serve as protein loading controls. In addition, all quantifications were normalized to saline control animals set at 100%.

Statistical Analysis

All statistical analyses were performed using with Prism 6 (Graphpad Software Inc, La Jolla, CA, USA). Effects of chronic cocaine on mRNA and protein expression were analyzed with unpaired t-tests. Unpaired t-tests were also used for assessing the effects on chronic cocaine on HDAC3 and H4K8Ac occupancy at the promoters of Fos and Nr4a1/2/3. All analyses were two-tailed and required a value of 0.05 for significance. Error bars in all figures represent SEM. For all experiments, values $\pm$2 SD from the group mean were considered outliers and were removed from analyses.

Chronic cocaine does not alter expression of HDAC3- related machinery in the NAc

Genetic deletion experiments previously showed that HDAC3 regulates cocaine-associated memory processes in the NAc [77]. However, it is unclear how cocaine alters HDAC3-mediated mechanisms in the NAc to promote cocaine-induced plasticity. Thus, we first examined how chronic cocaine exposure affects the expression of genes HDAC3 is known to negatively regulate[77,104,105,112] in the NAc. Adult male mice underwent 7 days of intraperitoneal (I.P.) injections with either cocaine (20 mg/kg) or saline. One hour following their last injection, brain tissue was harvested (Fig. 1a). Consistent with previous studies, we found that cocaine increases the expression of *Nr4a1* ($t_{(15)} = 2.546$, $^*p < 0.05$), *Nr4a3* [113] ($t_{(16)} = 3.689$, $^*p < 0.05$) and *Fos* ($t_{(16)} = 3.97$, $^*p < 0.05$) [76,113,114]. However, no changes in Nr4a2 expression were found following this exposure paradigm and time point ($t_{(15)} = 1.488$, $p = 0.1574$) (Fig. 1b) These findings confirm that the expression of several HDAC3 targets are increased by cocaine in the NAc. Next, we observed whether chronic cocaine affects expression of genes involved in histone deacetylation within the HDAC3 complex. Interestingly, Hdac3 expression was unaffected by chronic cocaine exposure ($t_{(16)} = 1.708$, $p = 0.1070$). In addition, mRNA expression of genes known to form co-repressor complexes with Hdac3 were also unaffected; including *Hdac4* ($t_{(14)} = 1.483$, $p = 1.603$), *Hdac5* ($t_{(14)} = 01920$, $p = 0.1.371$), *NCor1* ($t_{(16)} = 0.4492$, $p = 0.6593$), and *NCor2* ($t_{(14)} = 0.7301$, $p = 0.4774$) (Fig. 1c). Consistent with these findings, HDAC3 protein levels, measured by Western

blots, were unaffected by chronic cocaine exposure($t_{(14)} = 0.308$, $p = 0.7626$). However, we were unable to recapitulate the effects of cocaine on cFOS protein expression ($t(12) = 0.2354$, $p = 0.8179$) . These findings suggest that cocaine does not alter the expression of HDAC3-related machinery to promote plasticity in the NAc.

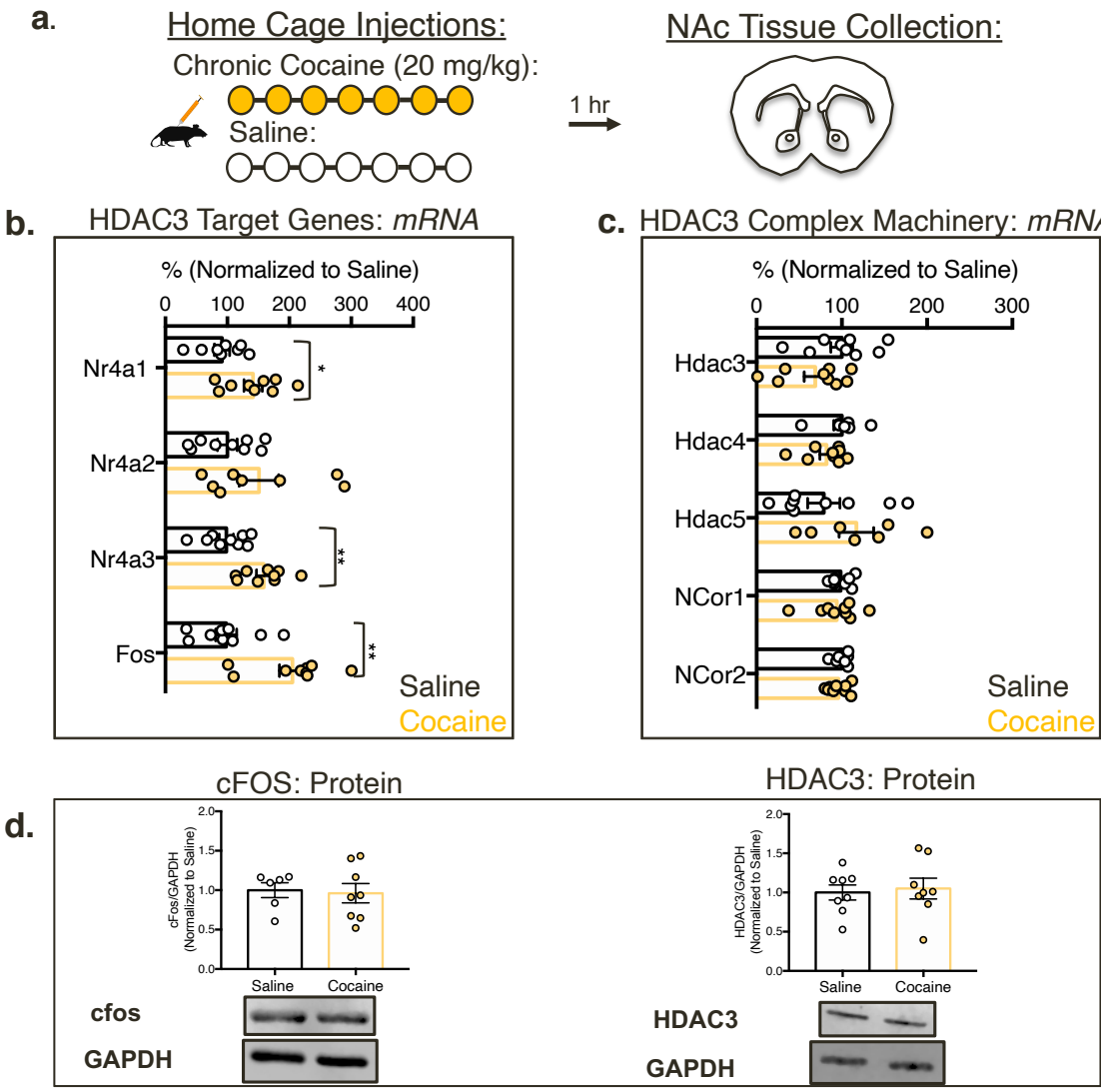

Fig. 1.1. Cocaine alters the activity, but not expression of HDAC3 to promote changes in gene expression. **a.** Adult male mice were I.P. injected for 7 days wither either cocaine (20 mg/kg) or saline and tissue was collected one hour following the last injection for either ChIP-qPCR or RT-qPCR. **b.** Chronic cocaine injections alters mRNA levels of Nr4a1($t_{(15)}=2.546$, $^*p<0.05$), Nr4a3 ($t_{(16)}=3.689$, $^*p<0.05$), and *Fos* ($t_{(16)}=3.97$, $^*p<0.05$) but not Nr4a2 ($t_{(15)}=1.488$, $p=0.1574$) in the NAc. **c.** Cocaine does not alter expression of Hdac3-related genes including *Hdac3* ($t_{(16)}=1.708$, $p=0.1070$), *Hdac4* ($t_{(14)}=1.483$, $p= 1.603$), *Hdac5* ($t_{(14)}=01920$, $p=0.1.371$), *NCor1* ($t_{(16)}=0.4492$, $p=0.6593$), and *NCor2* ($t_{(14)}=0.7301$, $p=0.4774$). **d.** HDAC3 protein levels were similarly unaffected by chronic cocaine exposure ($t_{(14)} = 0.308$, $p = 0.7626$) , however cocaine-induced changes in cFOS protein were not detected ($t_{(12)} = 0.2354$, $p = 0.8179$).

Chronic cocaine alters HDAC3 activity and H4K8Ac at promoters of IEGs in the NAc

Following the above results, we examined whether chronic cocaine alters the activity of HDAC3 to promote plasticity in the NAc. ChIP-qPCR was performed from NAc tissue of animals that underwent either 7 days of I.P. injections of cocaine (20 mg/kg) or saline. Tissue was collected 1 hour following the last injection (Fig 2a.). H4K8Ac and HDAC3 occupancy at the promoters of Nr4a1, Nr4a2, Nr4a3 and cFos. H4K8Ac is a putative target of HDAC3's deacetylase activity [105,106] and increases in acetylation at this H4 lysine site correlates with increases in gene expression[104,115]. has been Chronic cocaine decreased HDAC3 occupancy at the promoters of both Nr4a1(HDAC3 IP: $t_{(10)} = 2.648$, $p = 0.0244$) and Fos(HDAC3 IP: $t_{(9)} = 2.323$, $p = 0.0453$;) (Fig. 1b,c.). However, no changes in HDAC3 activity was affected in Nr4a2 (HDAC3 IP: $t_{(10)} = 0.03444$, $p = 0.9732$) or Nr4a3 (HDAC3 IP: $t_{(10)} = 0.03095$, $p = 0.9758$). H4K8Ac was found to increase only on the Nr4a1 promoter (H4K8Ac IP: $t_{(6)} = 2.491$, $p = 0.0471$) in contrast to Nr4a2 (H4K8Ac IP: $t_{(6)} = 0.1055$, $p = 0.09194$), Nr4a3 (H4K8Ac IP: $t_{(68} = 1.043$, $p = 03274$) and Fos(H4K8Ac IP: $t_{(7)} = 0.656$, $p = 0.5328$). This suggests that cocaine alters HDAC3 activity at target specific sites to promote changes in gene expression within the NAc.

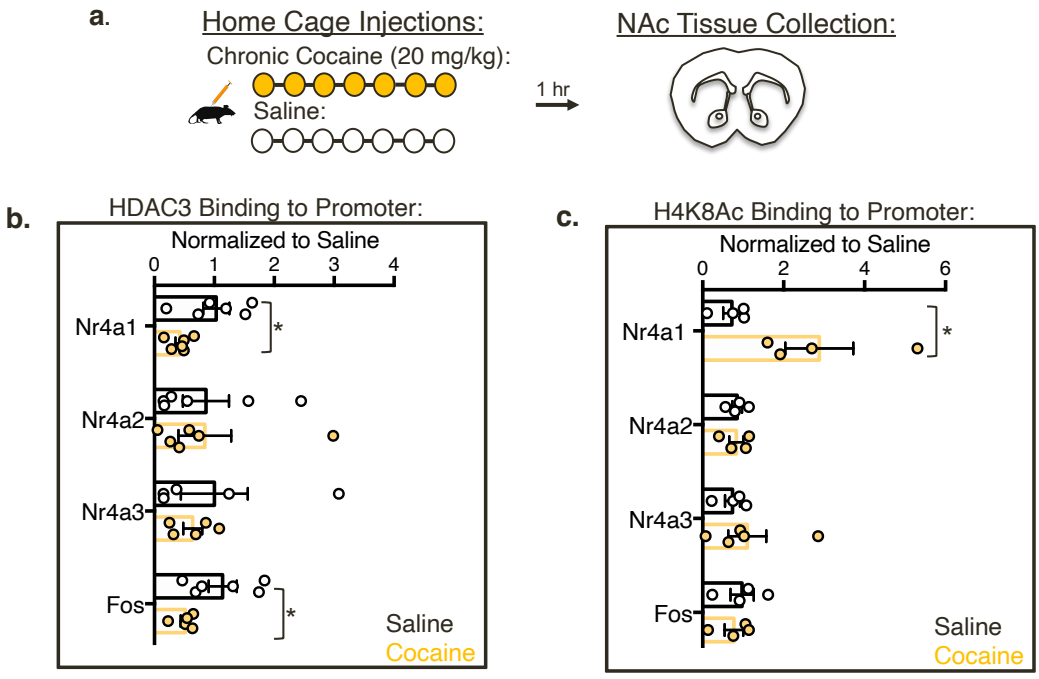

Fig. 1.2. Cocaine alters the HDAC3 activity and H4K8Ac at promoters of IEGs in the NAc. a. Adult male mice were I.P. injected for 7 days wither either cocaine (20 mg/kg) or saline and tissue was collected one hour following the last injection for either ChIP-qPCR or RT-qPCR. **b,c.** Chronic cocaine reduces HDAC3 binding and increases H4K8Ac binding to the promoter of Nr4a1(HDAC3 IP: $t_{(10)} = 2.648$, $p = 0.0244$; H4K8Ac IP: $t_{(6)} = 2.491$, $p = 0.0471$) ,reduces HDAC3 binding to the promoter of Fos (HDAC3 IP: $t_{(9)} = 2.323$, $p = 0.0453$; H4K8Ac IP: $t_{(7)} = 0.656$, $p = 0.5328$) but no changes were found on promoters of Nr4a2 (HDAC3 IP: $t_{(10)} = 0.03444$, $p = 0.9732$; H4K8Ac IP: $t_{(6)} = 0.1055$, $p = 0.09194$) or Nr4a3 (HDAC3 IP: $t_{(10)} = 0.03095$, $p = 0.9758$; H4K8Ac IP: $t_{(8)} = 1.043$, $p = 0.3274$).

Discussion

This study provides evidence, using molecular techniques such as Chromatin-immunoprecipitation, RT-qPCR and Western blotting, that HDAC3 activity, but not expression of HDAC3 is altered by chronic cocaine within the NAc. In addition, expression of genes known to form repressor complexes with HDAC3, HDAC4/5 and Ncor1/2, were similarly unaffected by cocaine. Our data also confirms that immediate early genes such as Nr4a1, *Nr4a3* and *Fos* expression are induced by chronic cocaine exposure. These changes in mRNA expression correlated with changes in HDAC3 binding and/or H4K8Ac at the Nr4a1 and Fos promoters following chronic cocaine. Together, these findings suggest that cocaine alters HDAC3 activity to drive target-specific changes in gene expression and plasticity within the NAc.

Although HDAC3 expression is unaltered by cocaine, our results demonstrate that HDAC3 activity is changed by cocaine exposure. It is unclear from this data alone how this may occur. It is possible upstream signaling cascades that mediate phosphorylation of HDAC3, which is known to alter HDAC3-mediate deacetylation, are affected by cocaine [116]. Additional studies will need to be conducted to test this. HDAC3 binding with NCOR1 or NCOR2, two proteins that have DNA-binding domains within the HDAC3 transcriptional repressor complex, could also be altered by cocaine. Although no changes in *Ncor1* or *Ncor2* expression were seen, follow up studies may examine whether these interactions HDAC3 have changed. Unfortunately, no studies were conducted to address this question as NCOR antibodies are often nonspecific. It is possible cocaine alters localization of HDAC3, similar to HDAC4 and HDAC5, however HDAC3 is considered a highly nuclear protein. Cocaine also may alter the activity patterns of HDAC3, instead of globally reducing it within the NAc, with different target genes affected by HDAC3. Future approaches with HDAC3 ChIP-sequencing would illustrate whether this may be occurring. Although the mechanism is still unclear, our data does illustrate the importance of HDAC3 in cocaine-induced expression in the NAc.

Here, we found that HDAC3 and H4K8Ac enrichment is altered at the promoters of a subset of IEGs induced by cocaine. Among these HDAC3-regulated genes is Nr4a1, which is known to have a critical role in regulating both memory formation in the hippocampus and cocaine action within the NAc. More specifically, AAV-overexpression of Nr4a1 in the hippocampus was found to enhance long-term memory following a subthreshold learning event[107]. In contrast, Carpenter et al. showed that CRISPR-CAS9 mediated increases in Nr4a1 expression attenuated cocaine memory formation and cocaine-seeking[60]. Although both findings indicate the importance of Nr4a1, the behavioral differences suggest that Nr4a1 and thus HDAC3 may have a region-specific or behavior-specific function in regulating plasticity. Another IEG with reduced HDAC3 occupancy following chronic cocaine was Fos. This was similarly seen following acute cocaine exposure [77] in the NAc. Interestingly, although HDAC3 occupancy was reduced at the Fos promoter, no changes in H4K8Ac were detected in this set of experiments. This may indicate that alternative lysine sites are acetylated or histone marks are altered following the removal of HDAC3 at the Fos promoter. In contrast with the memory-dependent changes seen in the hippocampus, our data suggests that HDAC3 does regulate expression of all Nr4a genes in the NAc following chronic cocaine[105,107,117]. For instance, despite changes in Nr4a3 were seen following chronic cocaine, HDAC3 activity was unaffected at Nr4a3 promoter. Although others have reported changes in Nr4a3 expression following cocaine exposure or a learning event[113,117], it seems that these changes are not HDAC3-dependent. However, Nr4a2 expression was not induced by chronic cocaine, nor were any changes in HDAC3 activity found at Nr4a2 promoter. This could indicate that molecular mechanisms, whether they are HDAC3 dependent or independent, prevent Nr4a2 expression following cocaine exposure within the NAc. Alternatively, work from the Lobo lab has shown, that 24 hours following chronic cocaine exposure, Nr4a2 is differentially expressed within the main cell types of the NAc[62]. Therefore, *Nr4a2* may be induced by cocaine, however our methods of global tissue analysis are not sufficient to capture these cell-

type specific changes. Overall, our findings suggest that HDAC3 activity may be a critical regulator of cocaine-induced molecular mechanisms in the NAc.

Chapter 2: Disrupting HDAC3's Deacetylase Activity in the NAc alters Cocaine-Induced Plasticity

Rationale:

Epigenetic enzymes responsible for regulating histone marks are critical in driving drug-induced changes in gene expression and behavior. The class I histone deacetylase (HDAC) HDAC3 is one epigenetic enzyme shown to negatively regulate cocaine-associated changes in gene expression and cocaine-associated memory formation. This is hypothesized to occur through HDAC3's deacetylase activity, gating neuroplasticity through repressing critical gene expression. In support of this, blocking HDAC3's deacetylase activity within the hippocampus and dorsal striatum enhanced memory formation processes. However, in contrast, disrupting HDAC3's activity within the hippocampus and prelimbic cortex has no effects on cocaine-associated memory formation. Thus, it is possible cocaine drives neural changes independent of HDAC3's deacetylase function to guide behaviors. Alternatively, HDAC3 may act in a region-specific manner to regulate cocaine-behaviors. In the studies described below, we aimed to understand the role of HDAC3's activity in the NAc by overexpressing either a deacetylase dead point mutant (HDAC3-Y298H-v5) or endogenous wild-type (HDAC3-WT) of HDAC3 and examining how molecular and behavioral responses to cocaine are affected.

Materials and Methods

Mice:

Male C57BL/6 J mice were all single-housed and at 8 and 15 weeks old at the time of behavioral testing. Mice were provided with food and water *ad libitum* for all experiments except food training. Throughout the operant food training, animals were food restricted to 95% of free feeding weight and given water *ad libitum*. Lights were maintained on a 12:12 h light per dark cycle, with all behavioral tests performed during the light portion of the cycle. All experiments were conducted according to US National Institutes of Health Guidelines for Animal Care and Use, and were

approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

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- experiments, cocaine-HCl was dissolved to a final concentration of either 0.5 mg/ml or 1.0 mg/mL mg/mL and administered in a volume of 10 ml/kg body weight, resulting in a final dose of 5 mg/kg or 10 mg/kg. Cocaine-HCl and saline were administered intraperitoneally (i.p). For the cocaine-induced locomotion experiments, animals were I.P injected at a final concentration of 10 mg/kg. Animals were I.P. injected with 20 mg/kg for both the electrophysiological recordings and molecular analysis experiments.

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. For the Empty Vector control, the HDAC3 coding sequence was not present, but all other elements remain. 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 The final titer of AAV-HDAC3-WT was 6.48×10^{12} GC/mL and the final titer of AAV-EV was 1.35×10^{13} GC/mL.

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). 0.5 µl of virus was infused bilaterally into the NAc [anteroposterior (AP): +1.3 mm; mediolateral (ML): ±1.1 mm; dorsoventral (DV): −4.5 mm relative to bregma. Immunofluorescence was used to confirm expression of HDAC3(Y298H). Viruses were infused at a rate of 6 µl /hr by using a 30 gauge Neuros Hamilton syringe (product #65459-01) mounted to either a Harvard Apparatus Nanomite Syringe Pump (product #MA1 70-2217) or Leica Biosystems Nanoinjector Motorized f/Stereotaxics (product #39462901). All infusions used the Leica Microsystems Angle Two Stereotaxic System. All animals were allowed to recover for a minimum of two weeks before handling.

Cocaine-Conditioned Place Preference

Following intracranial viral infusions and two weeks recovery, an unbiased conditioned place preference (CPP) was performed as described in previous studies. Briefly, all mice were handled for 2 minutes for 3 consecutive days prior to the experiment (days 1-3). Baseline preferences for three compartments in the CPP apparatus were assessed by placing the animals in the center compartment of the apparatus with free access to three distinct compartments for 15 min (day 4). Time spent in each compartment was recorded. Following this pretest, mice were conditioned over four consecutive days, receiving either cocaine-HCl (5 mg/kg or 10 mg/kg, IP; Sigma) or 0.9% saline (day 5-8). 24 hours following the last conditioning session, post-conditioning preference was tested in animals while they were in a drug-free state (day 9). Animals were allowed to freely explore all compartments of the CPP apparatus to assess preference, established as the difference between time spent in the cocaine-paired chamber and the saline-paired chamber, in seconds. Time spent was tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology).

In the electrophysiology experiments, animals underwent handling and pre-testing as described above. Following pre-conditioning testing, animals were injected with either cocaine-HCl (20

mg/kg or 5 mg/kg) or 0.9% saline prior to being confined to one conditioning compartment for 30 minutes. Electrophysiological recordings were conducted 24 hours following conditioning.

Cocaine-Induced Locomotion

This test examines the locomotor activating effects of cocaine in animals following experimenter-administered cocaine injections. In the HDAC3-Y298H-v5 experiments, following intracranial viral infusions and two weeks recovery,mice were handled for 2 min for 3 days (day 1-3) and were habituated to the activity apparatus (Plexiglas open field with sawdust bedding; base 16 cm × 32 cm) for 30 min per day for 2 consecutive days (days 4-5). Mice were then randomized into two different treatment groups (saline or cocaine) and locomotor activity was recorded for 30 minutes after an intraperitoneal injection of 10 mg/kg Cocaine-HCl or 0.9% saline for 5 days (day 6-10). In the HDAC3-WT experiments, following intracranial viral infusions and two weeks recovery, mice were handled for 2 min for 3 days (day 1-3) and habituated to the activity apparatus (Plexiglas open field with sawdust bedding; base 16 cm × 32 cm) for 30 min 1 day (day 4). All mice were had a saline day, where they were I.P injected with 0.9% saline (day 5. The following 5 days, all mice then had I.P. injections of 10 mg/kg Cocaine-HCl or 0.9% saline for 5 days (days 6-10). Locomotor activity (total distance travelled) was monitored and tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology, Leesburg, VA).

Operant Food Training

Subjects were mildly food restricted and trained to press a lever in an operant chamber (Med Associates, Fairfax, VT, USA) for food pellets (20 mg; TestDiet) under a fixed-ratio 1, time out 20 s (FR1TO20s) schedule of reinforcement. 2 retractable levers (1 active, 1 inactive) were used throughout each session. Once the response criteria was completed on the active lever, a food pellet was delivered. Responses on the inactive lever were recorded but had no scheduled consequences. All behavioral responses were automatically recorded by MedAssociates software.

Elevated Plus Maze

The plus-maze was conducted by an experimenter blind to the experimental groups. The maze consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm), that are connected by a central platform (5 × 5 cm). The maze was elevated 40 cm above the floor. During the test, mice were recorded for 5 min on the apparatus, with initially placing each mouse onto the central platform facing one of the open arms. Between subjects, the maze was cleaned with 70% ethanol. The percentage of time spent in the closed and open arms was scored using ANY-maze software.

Immunofluorescence

Following behavioral testing, animals were sacrificed and brain tissue was flash-frozen in isopentane and collected for immunohistochemistry. Twenty micrometer coronal sections were collected using a Leica CM 1850 cryostat at −20°C and mounted on slides. Slices were fixed in 4% PFA for 10 minutes, washed in 0.1-M PBS and permeated in 0.1% Triton X-100 in 0.1-M PBS. Slices were then blocked in blocking serum (8% NGS, 0.3% Triton X-100, in PBS; 1 hour) and incubated at 4°C overnight in primary solution (2% NGS, 0.3% Triton X-100; anti-v5: 1:1000, Abcam and HDAC3 clone Y415 antibody: 1:250; Abcam). The slices were then incubated in secondary solution (2 percent NGS, 0.3 percent Triton X-100; Alexa Fluor goat anti-rabbit 488). Lastly, tissue was incubated for 15-minute in a DAPI solution (1:10,000, Invitrogen). Slides were coverslipped using VectaShield Antifade mounting medium (Vector Laboratories).

The tissue was imaged by using Olympus Slide Scanner VSBX61. Fluorescence was quantified by using IMAGEJ. Briefly, background signal was collected from a soma-free region and subtracted from NAc signal. All values were normalized to v5-containing tissue.

Quantitative RT-qPCR

RT-qPCR was performed as described previously[105,111]. One millimeter punches were collected from NAc in a 500 M slice of tissue. RNA was isolated from punches 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, designed using the Roche Universal Probe Library:

Gene	Forward Primer	Reverse Primer	Roche Probe #
Hprt5	TGCTCGAGATGTCTGAAGG	ATCACATTGTGGCCCCTCTGT	-----
Fos	ggggcaaagtagagcagcta	agctccctcctccgattc	46
Nr4a1	agcttgggtgttgatgttcc	aatgcgattctgcagctctt	93
Nr4a2	ttgcagaatatgaacatcgaca	gttccttgagcccgtgtct	2
Nr4a3	gtgtcgggatggttaaggaa	gagggctcctgttgtagtgg	91

Slice Preparation and Recording

Parasagittal slices containing the NAc core were prepared from WT mice infused with either HDAC3-Y298H-v5 or EV (~2 months of age). Following isoflurane anaesthesia, mice were decapitated and the brain was quickly removed and submerged in ice-cold, oxygenated dissection medium containing (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 5 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$ and 10 glucose. Following removal of the cerebellum and lateral aspects of both hemispheres, parasagittal slices (320 µm) were cut from the blocked brain using a FHC vibrating tissue slicer (Model:OTS-5000). The tissue was then transferred to an interface recording chamber containing preheated artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 10 glucose and 10 µM picrotoxin to reduce feedforward inhibition. Slices were continuously perfused with this solution at a rate of 1.0–1.5 ml min^{-1}, while the surface of the slices were exposed to warm, humidified 95% O_2/5% CO_2 at 31±1 °C. Recordings began following at least 1.5 h of incubation.

Stimulation of glutamatergic afferent fibres within the NAc was achieved by placing a bipolar stainless steel stimulation electrode (25 µm diameter, FHC) just below the anterior commissure. Activation of fEPSPs were recorded using a glass pipette (2–3 MΩ) positioned caudal or caudal–ventral to the stimulation electrode. Thus, correct placement of electrodes within the NAc was confirmed by visual inspection of the slice and comparison with mouse brain atlas (Paxinos and Watson; 0.84–1.08 lateral to midline). Two parasagittal slices/hemisphere containing a large portion of the NAc core were obtained for each animal. Pulses were administered at 0.05 Hz using a current that elicited a 30–40% maximal response. Measurements of fEPSP slope (measured at 10–90% fall of the slope) were recorded during a minimum 20 min stable baseline period at which time LTP was induced by delivering three to five trains (intertrain interval of 1 min), each train containing three 'theta' bursts, with each burst consisting of four pulses at 100 Hz and the bursts themselves separated by 200 ms (TBS). The stimulation intensity was not increased during the delivery of TBS. Data were collected and digitized by NAC 2.0 Neurodata Acquisition System (Theta Burst Corp., Irvine, CA) and stored on a disk.

Data in the text are presented as means±s.d. Data in figures on LTP were normalized to the last 10 min of baseline. The LTP experiment and conventional measures of baseline synaptic transmission including paired-pulse facilitation and input/output curves were analyzed using a two-way repeated measure analysis of variance.

Statistical Analysis

All statistical analyses were performed using Prism 6 (Graphpad Software Inc, La Jolla, CA, USA). First, to assess whether HDAC3-Y298H-v5 alters Hdac3 expression using a Mann-Whitney test was used to analyze qPCR data. V5 protein expression data was analyzed using an unpaired t-test. We chose to normalize to the v5-conditions for these analyses because the empty vector control showed virtually no expression of the injected viruses, making normalization to the empty

vector (EV) control group impossible. ChIP-qPCR data analyzing the HDAC3-Y982H-v5's effects on promoter specific levels of H4K8Ac was conducting with upaired t-tests. For electrophysiological experiments testing whether disrupting HDAC3 activity (HDAC3-Y298H-v5) regulates cocaine-induced synaptic plasticity (20 mg/kg), two-way ANOVAs were conducted (treatment x virus) followed by a Bonferroni or Sidak's multiple comparisons test. The effects of HDAC3-Y298H-v5 or HDAC3-WT on NAc synaptic physiology using the lower cocaine conditioning dose (5 mg/kg) were analyzed using a One-Way ANOVA followed by a Tukey's multiple comparisons test. Effects on Paired Pulse or Input/output curve were assessed using Two-Way ANOVAs (interval stimulus x treatment; current x treatment) followed by a Tukey's multiple comparisons test. In CPP experiments, two-way ANOVAs were conducted to assess cocaine-associated memory formation. Locomotor activity during pre- and post- conditioning tests was assessed using unpaired t-tests. HDAC3-Y298H-v5 effects on cocaine-induced locomotion was assessed using multiple analysis two-way ANOVAs followed by a Sidak's multiple comparisons test. The effects of HDAC3-WT on cocaine-induced locomotion was assessed using multiple analysis two-way ANOVAs followed by a Sidak's multiple comparisons test. The role of HDAC3's activity on operant food training was assessed using repeated-measures two-way ANOVAs (lever x training) followed by a Sidak's multiple comparison test. The effects of manipulating HDAC3 (HDAC3-Y298H-v5 or HDAC3-WT) on anxiety-like behavior was assessed in elevated-plus maze using multiple analysis two-way ANOVAs followed by a Sidak's multiple comparisons test (arm x virus). All analyses were two-tailed and required a value of 0.05 for significance. Error bars in all figures represent SEM. For all experiments, values $\pm$2 SD from the group mean were considered outliers and were removed from analyses.

Results

Overexpressing HDAC3-Y298H-v5 alters HDAC3 activity in the NAc

HDACs directly regulate the state of chromatin through one of two ways: 1)their activity within corepressor complexes and 2) through the removal of acetyl groups from lysine tails of histone proteins. We hypothesized that HDAC3's deacetylase activity is a key function in regulating cocaine-induced processes within the NAc. To test this, we employed an AAV containing deacetylase-dead HDAC3 point mutant (HDAC3-Y298H-v5) that has been shown to affect HDA3 deacetylase activity and memory dependent processes(Fig. 2.1a,b). We first confirmed that AAV infusions of the HDAC3-Y298H-v5 in the NAc led to both increases in Hdac3 mRNA (Mann-Whitney test: U=1,EV-Control: n=8, median=0.359; HDAC3-Y398H-v5: n=7, median=11.8; $p<0.001$) and significant expression of V5 protein in comparison to EV-Controls ($t_{18}=9.898$, $p<0.0001$) (Fig 2.1c,d,e). Previous studies using this construct have confirmed increases in H4K8KAc acetylation *in vitro*, and *in vivo* within the hippocampus and the dorsal striatum[105,106]. Together, these data indicate that viral overexpression of HDAC3-Y298H-v5 is sufficient to examine the importance of HDAC3's deacetylase activity in the NAc.

Given that HDACs negatively regulate cocaine-induced and memory-related gene expression [36,73,80,83], we next assessed how disrupting HDAC3's deacetylase activity using HDAC3-Y298H-v5 affects cocaine-induced expression levels of plasticity-related genes. We hypothesized that HDAC3-Y298H-v5 would further enhance cocaine-induced gene expression of all Nr4a1/2/3 genes in the NAc. In these sets of experiments, animals underwent viral NAc infusions of either HDAC3-Y298H-v5 or EV and, following recovery, were I.P. injected with either cocaine (20mg/kg) or saline for 7 days. NAc tissue was collected 1 hour following the last injection and RT-qPCR was performed. To assess the effects of Y298H on cocaine-induced gene expression, all samples were normalized to Saline EV-Controls. In contrast to our hypothesis, we found discrete changes in gene expression in the NAc. More specifically, only Nr4a2 expression

was affected by disrupting HDAC3 deacetylase activity following chronic cocaine (Nr4a1 (t_{12}=0.4619, p=0.6524), Nr4a2 (t_{12}=2.183, *=p<0.05), Nr4a3 (t_{12}=0.6746, p=0.5127), (cfos (t_{12}=0.05628, p=0.9569). This effect on Nr4a2 is consistent with previous studies finding enhanced *Nr4a2* following genetic deletion of HDAC3 within the hippocampus and NAc [77,107] (Fig 2.1f). Together, these findings suggest that HDAC3's deacetylase activity regulates NAc gene expression in a target-specific manner.

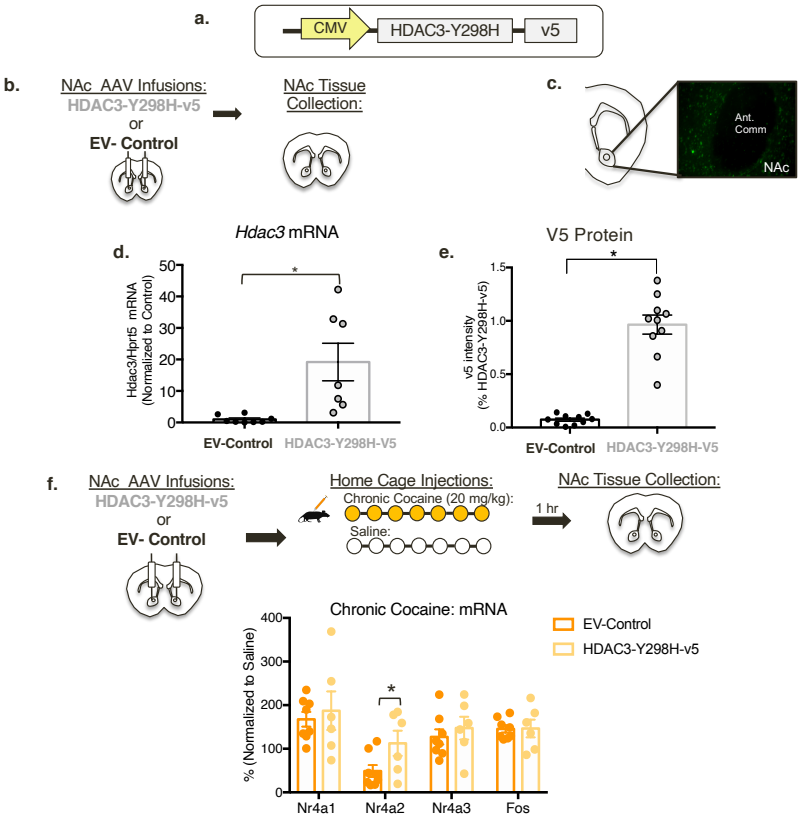

Fig. 2.1. HDAC3's deacetylase activity promotes cocaine-induced changes in *Nr4a2* expression in the NAc. a.,b. Adult male mice were infused with AAV either deacetylase dead HDAC3 point mutant (HDAC3-Y298H-v5) or EV-control. **c.,d.** Overexpression of HDAC3-Y298H-v5 was confirmed by qPCR (U=1,EV-Control: n=8, median=0.359; HDAC3-Y398H-v5: n=7, median=11.8; ***=p<0.001**)** and immunofluorescence (t_{18}=9.898, p<0.0001**)**. **e.** Mice infused with AAV either deacetylase dead HDAC3 point mutant (HDAC3-Y298H-v5) or EV-control were exposed with either chronic cocaine or saline home cage injections. 1 hour following the last injection, animals were sacrificed and tissue was collected for RT-qPCR. **f.** Disruption of HDAC3 activity enhances cocaine-induced changes in expression of *Nr4a2* mRNA following cocaine exposure (Nr4a1 (t_{12}=0.4619, p=0.6524), Nr4a2 (t_{12}=2.183, *=p<0.05), Nr4a3 (t_{12}=0.6746, p=0.5127), (cfos (t_{12}=0.05628, p=0.9569). For RT-qPCR, samples were normalized to HPRT5 and EV-Control saline samples.

Disruption of HDAC3's deacetylase activity restores cocaine-induced changes in synaptic plasticity

We next investigated whether HDAC3's deacetylase activity alters cellular responses to cocaine [35,118,119]. Mice were infused with viruses containing either HDAC3-Y298H-v5 or EV and then underwent either cocaine (20 mg/kg) or saline conditioning. 24 hours following conditioning,

animals were sacrificed and sagittal slices of the NAc were prepared for electrophysiology. Extracellular field potential recordings were collected from the NAc following stimulation of glutamatergic afferents (Fig. 2.2a). Cocaine alters synaptic plasticity within the NAc[35,36,118], and previous studies have shown that pharmacological HDAC inhibitors augment cellular responses to cocaine in the NAc [36]. We predicted that disrupting HDAC3's activity would display a further cocaine-induced LTP depression following theta-burst stimulation in comparison to EV-Control cocaine conditioned mice.

Consistent with previous studies, NAc slices of EV-Control cocaine-conditioned mice had occluded LTP in comparison to EV-Control saline-conditioned mice [36](Fig. 2.2b). NAc slices from HDAC3-Y298H-v5 cocaine-conditioned animals had restored LTP in comparison to cocaine-conditioned EV-controls(Fig. 2.2c, main effect of virus $F_{1,22}$=10.91, p=.0032; main effect of cocaine $F_{1,22}$=21.38, p=0.0001; virus x cocaine interaction $F_{1,22}$=15.77, p=0.0006). HDAC3-Y298H-v5 effects were specific to cocaine-conditioned mice, as HDAC3-Y298H-v5 saline-conditioned mice exhibited similar potentiation regardless of virus (p=0.9986). In contrast to our hypothesis, this data suggests that disrupting HDAC3 activity does not facilitate cocaine's effects but instead induces a physiological counteradaptation to cocaine within the NAc.

The effects of HDAC3-Y298H on baseline NAc neurotransmission were assessed using paired-pulse facilitation (PPF) to measure presynaptic plasticity and an input-output (I/O) curve to detect changes in excitability. PPF was similar across groups (Fig. 2.2d, main effect of interval stimulus: $F_{2,50}$=129.2, p<.001; no main effect of virus: $F_{3,25}$=1.034, p=0.3947; no interval stimulus x virus interaction: $F_{6,50}$=1.446, p=0.2163). Interestingly, HDAC3-Y298H-v5 had an effect on the I/O curve (main effect of current: $F_{5,125}$=118.5, p<.001; main effect of treatment: $F_{3,25}$=3.973, p=0.0192; current x treatment interaction: $F_{25,125}$=6.299, p=6.299). Consistent with previous studies, cocaine-conditioned slices had decreased NAc membrane excitability in comparison to EV-Controls, particularly at higher current injections (Fig. 2.2e, 500 uA: p=.0033)[120,121]. However,

cocaine-conditioned HDAC3-Y298H-v5 slices exhibited higher levels of excitability in comparison to cocaine-conditioned EV-Controls at higher current injections (500 uA: p=0.002; 600 uA: p<.0001). This collective data suggests that disrupting HDAC3 activity induces a physiological counteradaptation to cocaine, through altering membrane excitability, within the NAc.

Following this initial experiment, we wanted to follow this experiment by testing whether 1) HDAC3-Y298H has dose-dependent effects on cocaine-induced synaptic and 2) overexpressing HDAC3-WT alters cellular responses to cocaine in the NAc. Thus, mice were infused with viruses containing either HDAC3-Y298H, HDAC3-WT or EV and then underwent cocaine conditioning at 5 mg/kg. 24 hours following conditioning, animals were sacrificed and sagittal slices of the NAc were prepared for electrophysiology. Extracellular field potential recordings were collected from the NAc following stimulation of glutamatergic afferents (Fig. 2.3a). Following our HDAC3-Y298H-v5 data, we predicted that overexpressing HDAC3 would depress cocaine-induced LTP following theta-burst stimulation in comparison to EV-Control cocaine conditioned mice. Similar to our previous results, we first found that NAc slices of EV-Control cocaine-conditioned mice had occluded LTP in comparison to EV-Control saline-conditioned mice [36] and that HDAC3-Y298H-v5 enhances LTP Fig. 3c, $F_{3,31}$=6.749 p=0.0012; Cocaine EV vs Saline EV: p=0.0814; Cocaine EV vs Cocaine HDAC3-Y298H-v5: p=0.0007; Cocaine HDAC3-Y298H-v5 vs Cocaine HDAC3-WT: p=0.0339) (Fig. 2.3b,c). However, NAc slices from HDAC3-WT cocaine-conditioned animals had no changes in LTP in comparison to cocaine-conditioned EV-controls (Cocaine EV vs Cocaine HDAC3-WT: p=0.5485). Thus, in contrast to our hypothesis,

this data suggests that overexpressing HDAC3 does not alter cocaine-induced synaptic plasticity within the NAc.

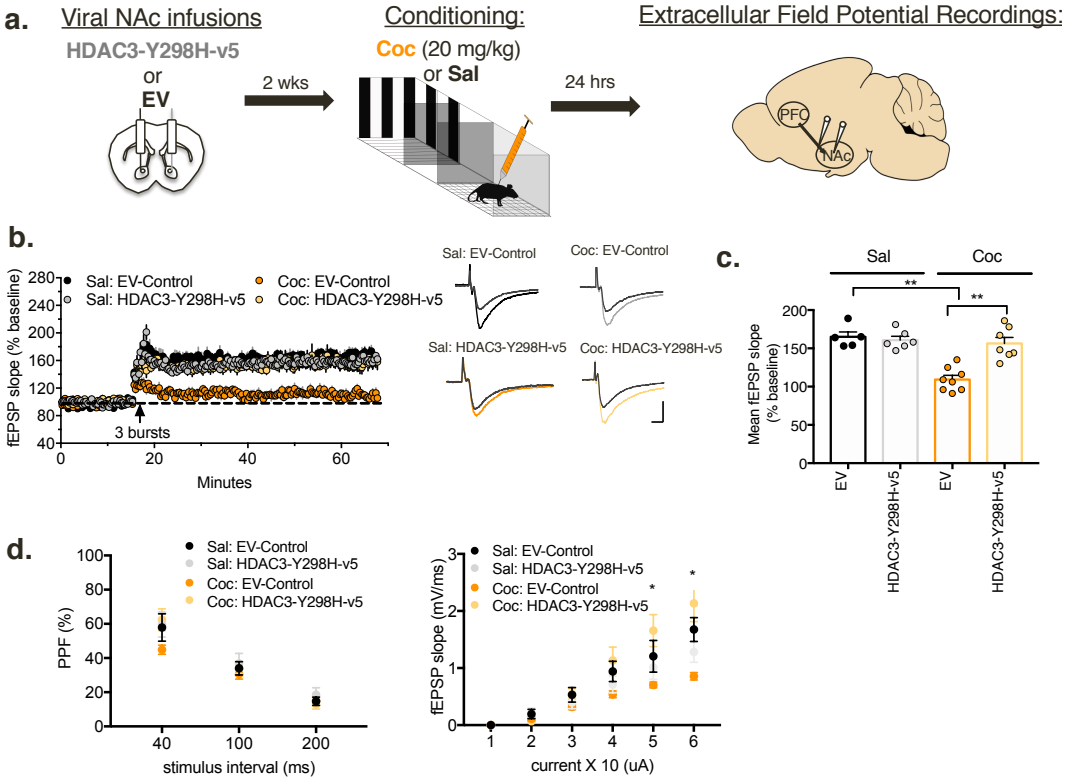

Fig. 2.2. Disrupting HDAC3's activity reverse cocaine-induced synaptic plasticity in the NAc. a. HDAC3-Y298H-v5 or EV-Control mice were injected with either cocaine (20 mg/kg) or saline prior to conditioning. 24 hours following conditioning, and extracellular field potential recordings were collected from the NAc following stimulation of glutamatergic afferents. **b,c.** NAc slices of EV-Control cocaine-conditioned mice had occluded LTP in comparison to EV-Control saline-conditioned mice. NAc slices from cocaine-conditioned animals infused with the HDAC3-Y298H-v5 virus had restored LTP in comparison to cocaine-conditioned EV-controls (main effect of virus $F_{1,22}$=10.91, p=.0032; main effect of cocaine $F_{1,22}$=21.38, p=0.0001; virus x cocaine interaction $F_{1,22}$=15.77, p=0.0006). These effects were specific to cocaine-conditioned mice, as saline-conditioned mice exhibited similar potentiation regardless of virus (p=0.9986). The effects of manipulating HDAC3 function on baseline neurotransmission were assessed using paired-pulse facilitation and with an input-output (I/O) curve. **d.** PPF was similar across groups, indicating that neither conditioning nor manipulation of HDAC3 activity had an effect on presynaptic release probability within the NAc (main effect of interval stimulus: $F_{2,50}$=129.2, p<.001; no main effect of virus: $F_{3,25}$=1.034, p=0.3947; no interval stimulus x virus interaction: $F_{6,50}$=1.446, p=0.2163). **e.** HDAC3-Y298H-v5 had an effect on intrinsic membrane excitability in the NAc (main effect of current: $F_{5,125}$=118.5, p<.001; main effect of treatment: $F_{3,25}$=3.973, p=0.0192; current x treatment interaction: $F_{25,125}$=6.299, p=6.299). Cocaine-conditioned slices had decreased NAc membrane excitability in comparison to EV-Controls, particularly at higher current injections (Fig. 3e, 500 uA: p=.0033). However, cocaine-conditioned HDAC3-Y298H-v5 slices exhibited higher levels of excitability in comparison to cocaine-conditioned EV-Controls at higher current injections (500 uA: p=0.002; 600 uA: p<.0001). These collected data suggests that disrupting HDAC3 activity induces a physiological counteradaptation within NAc neurons.

We further assessed whether any baseline affects in neurotransmission were affected by this cocaine conditioning dose of 5 m/kg as well as whether HDAC3-WT or HDAC3-Y398H alters any cocaine-induced changes in the NAc. Baseline neurotransmission was assessed using paired-pulse facilitation (PPF) to measure presynaptic plasticity and an input-output (I/O) curve to detect changes in excitability. PPF was similar across groups (Fig. 2.3d, main effect of interval stimulus: $F_{2,44}$=137.7, p<.001; no main effect of treatment: $F_{3,22}$=1.498, p=0.2428; no interval stimulus x virus interaction: $F_{6,44}$=1.3111, p=0.2725). HDAC3-WT had no effect on the I/O curve (main effect of current: $F_{6,192}$=248.2, p= <0.001; no main effect on treatment: $F_{3,32}$=0.583,

p=0.6793; no current x treatment interaction: $F_{18,192}$=0.7067, p.8020). Interestingly, in contrast to our 20 mg/kg data, this lower dose of cocaine (5 mg/kg) had no effects on membrane excitability in comparison to EV-Controls (Saline EV vs Cocaine EV: p=0.6713), nor did HDAC3-Y298H affect excitability (Cocaine EV vs Cocaine HDAC3Y398H: p=0.7801). This may point to a dose specific effect of cocaine on NAc excitability and perhaps cocaine-induced HDAC3 activity. Overall these data indicate that disrupting HDAC3 activity, but not overexpressing HDAC3 or enhancing HDAC3 activity alters synaptic processes in the NAc.

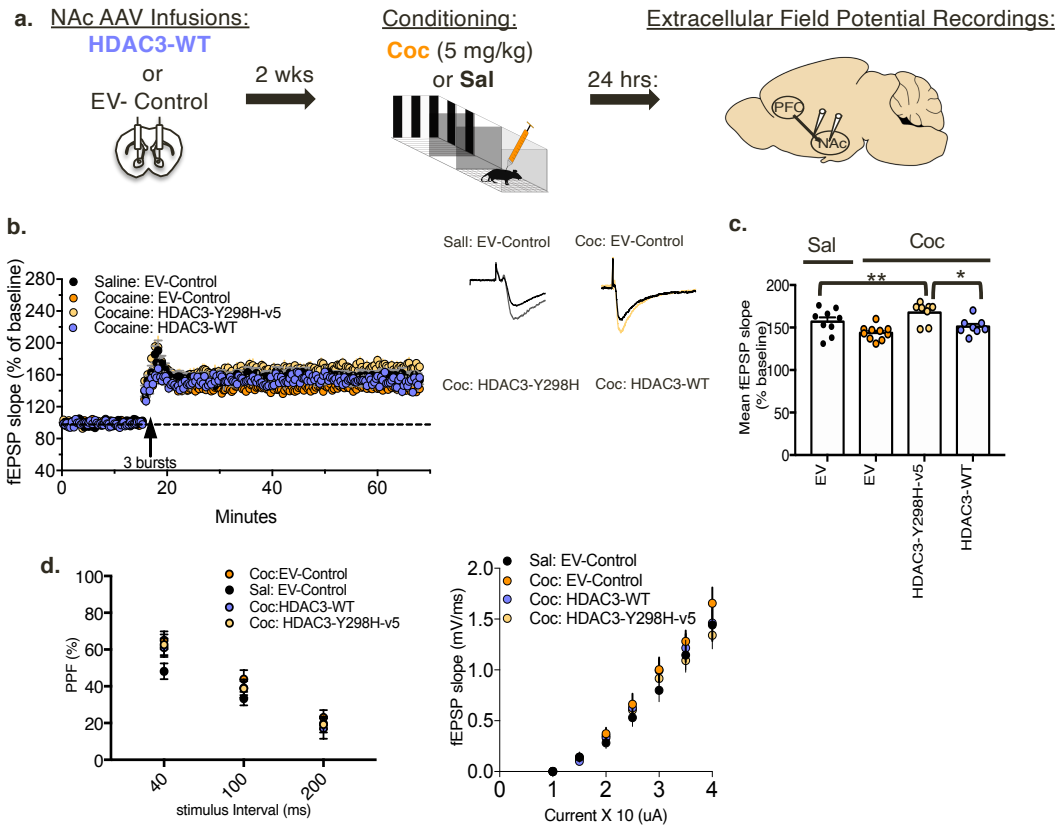

Figure 2.3. Overexpressing wild-type HDAC3 (HDAC3-WT) does not alter cocaine-induced synaptic plasticity in the NAc. a. HDAC3-WT or EV-Control mice were injected with either cocaine (5 mg/kg) or saline prior to conditioning. 24 hours following conditioning, and extracellular field potential recordings were collected from the NAc following stimulation of glutamatergic afferents. **b,c.** NAc slices of EV-Control cocaine-conditioned mice had occluded LTP in comparison to EV-Control saline-conditioned mice. In contrast to HDAC3-Y298H-v5, NAc slices from cocaine-conditioned animals infused with the HDAC3-WT virus had did not differ significantly between cocaine conditioned EV-control (One-way ANOVA:$F_{(2,24)}$=3.231, p=0.05, Tukey post-hoc: Saline:EV-Control vs Cocaine: EV-Control *=p<0.05 and Cocaine: EV-Control and Cocaine: HDAC3-WT p=0.4688. PPF was similar across groups, indicating that neither conditioning nor manipulation of HDAC3 had an effect on presynaptic release probability within the NAc (main effect of interval stimulus: $F_{2,36}$=108.2, p<.001; no main effect of virus: $F_{2,18}$=0.1472, p=0.31472; no interval stimulus x virus interaction: $F_{4,36}$=1.772, p=0.1559). **e.** HDAC3-Y298H-v5 had an effect on intrinsic membrane excitability in the NAc (main effect of current: $F_{6,150}$=186.2, p<.0001; no main effect of virus: $F_{2,25}$=0.5914, p=0.5611; no interval stimulus x virus interaction: $F_{12,150}$=0.5773, p=0.8579)

Global disruption of HDAC3 activity in the NAc does not affect cocaine-induced behaviors

Following the molecular and cellular effects seen from disrupting HDAC3 activity within the NAc, we tested whether HDAC3-Y298H-v5 affects behavioral responses to cocaine. First, we

examined the role of HDAC3's deacetylase activity in regulating cocaine-associated memory formation(Fig. 2.4a). Initially, mice were conditioned with a 5 mg/kg cocaine dose, as genetic deletion of Hdac3 in the NAc enhanced CPP-acquisition at this dose[77]. Prior to conditioning, HDAC3-Y298H-v5 and EV-mice showed no initial preference for either chamber (p= 0.9975). At a dose of 5 mg/kg, we found that HDAC3-Y298H-v5 had no effect on cocaine-induced CPP (main effect of conditioning $F_{1,18}$=27.68, p=<.0001; no main effect of virus $F_{1,18}$=0.3477, p=0.565; no conditioning x virus interaction $F_{1,18}$=1.72, p=0.2062) (Fig. 2.4b). HDAC3-Y298H-v5 had no effects on overall performance of the task, as no differences in locomotion were found on either pre-conditioning testing day (p=0.2634) or post-conditioning testing day (p=0.5511) (Fig. 2.5a,b). To test whether HDAC3's activity regulates cocaine action in a dose-dependent manner, in a separate cohort, HDAC3-Y298H-v5 or EV-Control mice underwent cocaine-induced CPP at a 10 mg/kg dose. Prior to conditioning, HDAC3-Y298H-v5 and EV-mice showed no initial preference for either chamber (p= 0.8786) (Fig. 2.5c,d). At a dose of 10 mg/kg, HDAC3-Y298H-v5 had no effect on cocaine-induced CPP (main effect of conditioning $F_{1,22}$=53.47, p=<.0001; no main effect of virus $F_{1,22}$=0.2522, p=0.625; no conditioning x virus interaction $F_{1,22}$=0.0001, p=0.9906)(Fig. 2.5c). No performance effects were found, as HDAC3-Y298H-v5 or EV-control mice had similar locomotion on pre-conditioning testing day (t_{22}= 0.3577, p=0.7255) and post-conditioning testing day (t_{22}= 0.3104, p=0.7592)(Fig. 2.5c). These results indicate that disrupting HDAC3's deacetylase activity throughout the NAc does not affect cocaine-associated memory formation. We next investigated the effects of HDAC3-Y298H-v5 on cocaine sensitivity(Fig. 2.4d). Previous studies have shown that genetic deletion of HATs or HDACs in the NAc alters locomotor responses to cocaine[74,85]. Thus, we hypothesized that disrupting HDAC3's deacetylase activity will enhance cocaine-induced locomotion. In contrast to our hypothesis, we found that HDAC3-Y298H had no effect on cocaine-induced or general locomotion (Fig. 4f: Two-way ANOVA RM: main effect of session: $F_{4,168}$=3.883, p=0.0048; main effect of treatment: $F_{3,42}$=28.99,

p<0.0001; interaction: $F_{12,168}$= 3.742, p<0.0001). Our data indicates that globally disrupting HDAC3 activity in the NAc does not affect cocaine-induced behaviors.

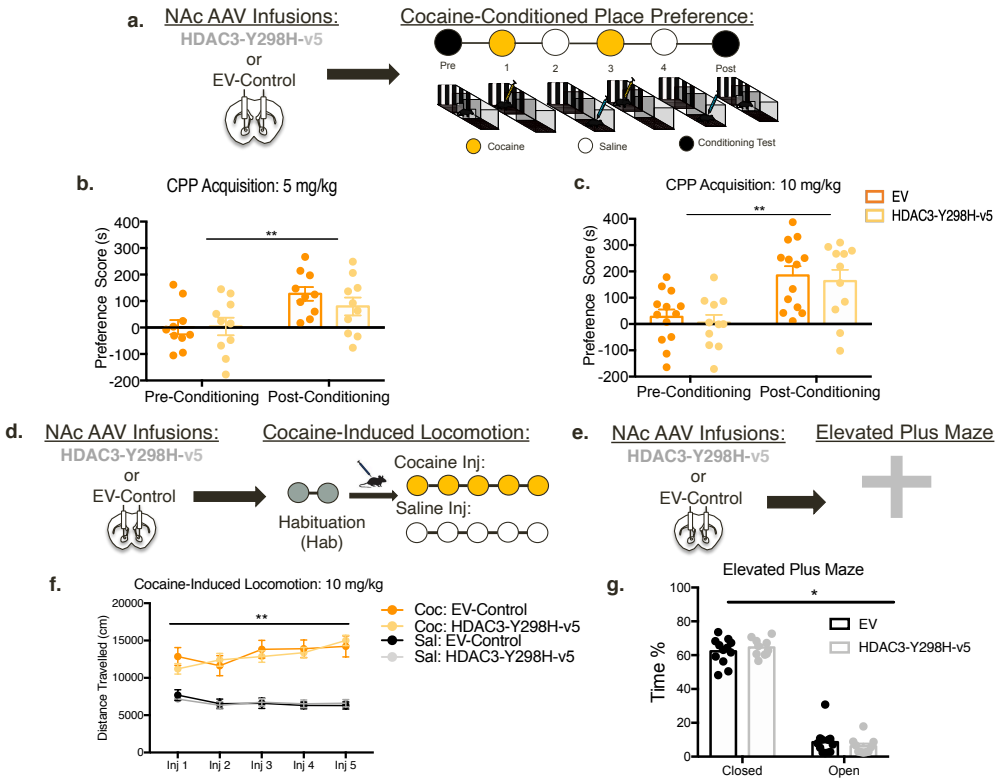

Figure 2.4. Global disruption of HDAC3's activity in the NAc does not alter behavioral responses to cocaine. a. AAVs containing either HDAC3-Y298H-v5 or an EV-control were infused into the NAc of adult male mice prior to cocaine-induced CPP. **b**. HDAC3-Y298H-v5 and EV-mice showed no initial preference for either chamber (p= 0.9975), and at a dose of 5 mg/kg, we found that HDAC3-Y298H-v5 had no effect on cocaine-induced CPP (main effect of conditioning $F_{1,18}$=27.68, p=<.0001; no main effect of virus $F_{1,18}$=0.3477, p=0.565; no conditioning x virus interaction $F_{1,18}$=1.72, p=0.2062). **c.** Mice that were infused with either HDAC3-Y298H-v5 or EV-Control into the NAc underwent cocaine-induced CPP at a 10 mg/kg dose. Mice had no initial preference for either chamber prior to conditioning (p= 0.8786), and no differences were seen between HDAC3-Y298H-v5 and EV- controls following 10 mg/kg cocaine conditioning (main effect of conditioning $F_{1,22}$=53.47, p=<.0001; no main effect of virus $F_{1,22}$=0.2522, p=0.625; no conditioning x virus interaction $F_{1,22}$=0.0001, p=0.9906). **d.,f.** Following AAV NAc infusions of HDAC3-Y298H-v5 or EV-control, adult male mice received 5 days of injections of either cocaine or saline prior to being placed in an open chamber to track the total amount of distance travelled. Animals that received cocaine exhibited similar locomotor responses regardless of virus (Two-way ANOVA RM: main effect: session: $F_{4,168}$=3.883, p=0.0048; main effect: treatment: $F_{3,42}$=28.99 , p<0.0001; interaction: $F_{12,168}$= 3.742, p<0..0001). **e.** AAVs containing either HDAC3-Y298H-v5 or an EV-control were infused into the NAc of adult male mice prior to elevated plus maze (EPM) test. **g.** HDAC3-Y398H-v5 and EV mice both spend significant amount of time in closed versus open arm in the EPM (two-way ANOVA RM: main effect of arm: $F_{1,20}$=511.4, p<0.0001; no main effect or virus: $F_{1,20}$=0.003, p=0.9568; no interaction: $F_{1,20}$=0.861, p=0.3645)

In separate set of studies, we examined the effects of enhancing HDAC3 activity via overexpression of HDAC3-WT on cocaine-induced behaviors. We first tested HDAC3-WT on cocaine-associated memory formation using cocaine-conditioned place preference. Following NAc viral infusions of either AAV containing HDAC3-WT or EV-Control, mice were conditioned with a 5 mg/kg cocaine dose(Fig. 2.6a). Prior to conditioning, HDAC3-WT and EV-mice showed no initial preference for either chamber (p= 0.9597). Following conditioning, no differences in cocaine-induced CPP were observed between HDAC3-WT and EV-Controls (main effect of conditioning $F_{1,16}$=14.49, p=0016; no main effect of virus $F_{1,16}$=0.3025, p=0.5899; no conditioning

x virus interaction $F_{1,16}$=0.0643, p=0.8031) (Fig.2.6b). Consistent with HDAC3-Y298H-v5, HDAC3-WT also had no effects on overall performance of the task, as no effects on locomotion, on either the pre-conditioning testing day (p=0.8233) or post-conditioning testing day (p=0.3981) were found (Fig.2.6c). The effect of overexpressing HDAC3 on cocaine sensitivity was also tested using the cocaine-induced locomotion paradigm. We hypothesized that, consistent with the idea of HDAC3 being a negative regulator of cocaine action, enhancing HDAC3 activity via HDAC3 overexpression would repress cocaine-induced locomotion. However, we found that HDAC3-WT had no effect on cocaine-induced or general locomotion (Fig.2.6d,e): Two-way ANOVA RM: main effect of session: $F_{6,126}$=32.73, p=0.0048; no main effect of virus: $F_{1,21}$=1.261, p=0.2742; no session x virus interaction: $F_{6,126}$ = 1.261, p=0.4548). Overall, these results indicate the global overexpression of HDAC3-WT does not alter behavioral responses to cocaine.

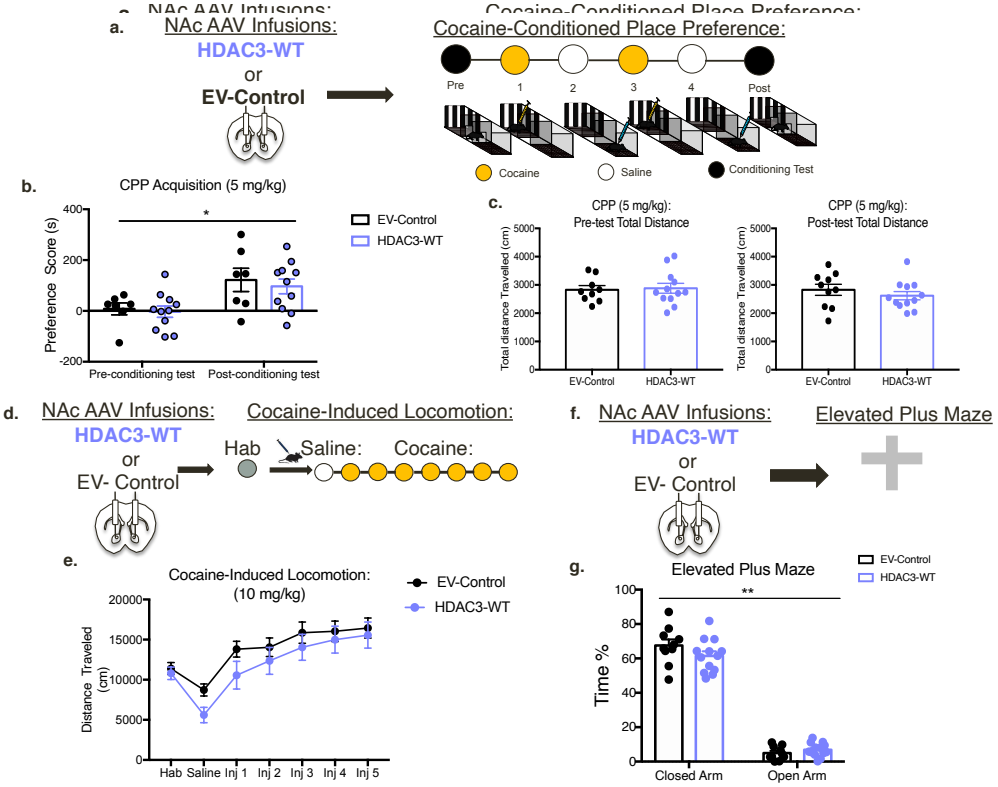

Figure 2.6. Global overexpression of wild-type HDAC3 (HDAC3-WT) in the NAc does not alter behavioral responses to cocaine. a. AAVs containing either HDAC3-WT or an EV-control were infused into the NAc of adult male mice prior to cocaine-induced CPP. **b.** HDAC3-WT and EV-mice showed no initial preference for either chamber (p= 0.9597), and at a dose of 5 mg/kg, we found that HDAC3-WT had no effect on cocaine-induced CPP (main effect of conditioning $F_{1,16}$=14.49, **p=<.01; no main effect of virus $F_{1,16}$=0.3025, p=0.5899; no conditioning x virus interaction $F_{1,16}$=0.0643, p=0.8031).d. HDAC3-WT and EV-control mice had no differences in total locomotion during the 5 mg/kg pre-conditioned testing day (unpaired t-test ($t_{(19)}$=0.2264, p=0.8233) or the post-conditioned testing day (unpaired t-test ($t_{(20)}$=0.8636 p=0.3981). **e.** Following AAV NAc infusions of HDAC3-WT or EV-control, adult male mice received 1day of saline and then 5 days of cocaine injections prior to being placed in an open chamber to track the total amount of distance travelled. Cocaine induced significantly increased locomotion regardless of virus, however virus had no effect on locomotion (Two-way ANOVA RM: main effect: session: $F_{6,126}$=32.73, p<0.001; no main effect of virus: $F_{1,21}$=1.261, p<0.2742; no session x virus interaction: $F_{6,126}$= 0.9605, p<0.4548). f. AAVs containing either HDAC3-Y298H-v5 or an EV-control were infused into the NAc of adult male mice prior to elevated plus maze (EPM) test. g. HDAC3-WT and EV mice both spend significant amount of time in closed versus open arm in the EPM (two-way ANOVA RM: main effect of arm: $F_{1,21}$=609.4, p<0.0001; no main effect or\f virus: $F_{1,21}$=0.8954, p=0.3548; no arm x virus interaction: $F_{1,21}$=2.896, p=0.1035).

Disruption of HDAC3's deacetylase activity in the NAc impairs food reinforcement

After finding no effects of disrupting HDAC3's activity on cocaine-related processes, the role of HDAC3 in natural reward-related processes was tested using operant training for food reinforcement. For these experiments, mice were virally infused mice with either HDAC3-Y298H-v5 or EV-Control and then underwent operant food training under a fixed ratio 1 schedule (FR1) under mild food-restricted conditions (85-90% original free feeding weight) (Fig. 2.7a). Both EV-Control mice and HDAC3-Y298H-v5 mice exhibited significant interactions across sessions (EV-control: Fig. 5b., training x levers $F_{4,64}$= 13.33, p=.0001); HDAC3-Y298H-v5: Fig 5c., training x levers $F_{4,40}$ = 7.785, p=.0001). In addition, both HDAC3-Y298H-v5 and EV-Control mice have no differences in acquiring, as both have similar discrimination indices (main effect of session: $F_{4,72}$=9.951, p<0.0001; no main effect of virus: $F_{1,18}$=3.11, p=0.0948; no session x virus interaction

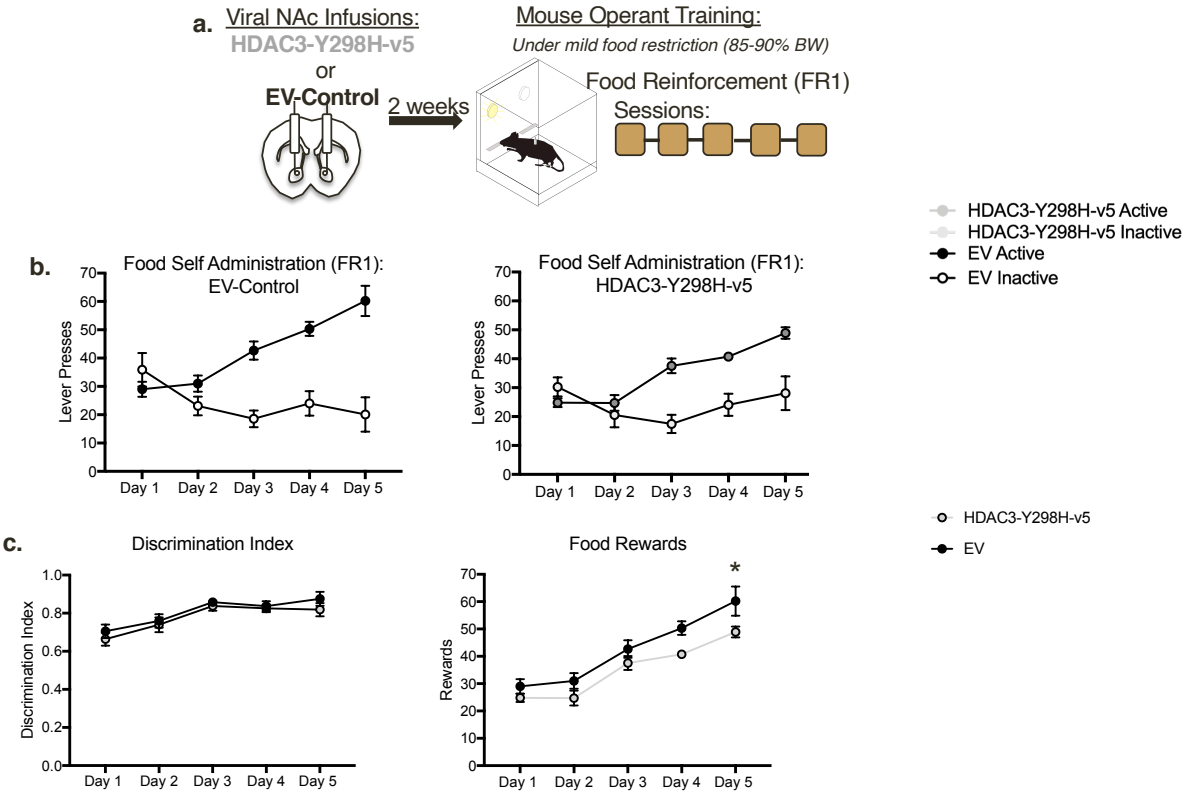

Figure 2.7. Disrupting HDAC3's deacetylase activity in the NAc reduces food reinforcement. a. Mice were virally infused mice with either HDAC3-Y298H-v5 or EV-Control and then underwent operant food training under a fixed ratio 1 schedule (FR1) under mild food-restricted conditions (85-90% original free feeding weight) . **b.** Both EV-Control mice and HDAC3-Y298H-v5 mice exhibited significant interactions across sessions (EV-control: training x levers $F_{4,64}$= 13.33, p=.0001); HDAC3-Y298H-v5: training x levers $F_{4,40}$ = 7.785, p=.0001). **c.** HDAC3-Y298H-v5 and EV-Control mice have similar discrimination indices (main effect of session: $F_{4,72}$=9.951, p<0.0001; no main effect of virus: $F_{1,18}$=3.11, p=0.0948; no session x virus interaction $F_{4,72}$=0.1652, p=0.9553). However, HDAC3-Y298H-v5 did reduce levels of lever pressing for food pellets (main effect of session: $F_{4,72}$=52.47, p<0.0001; main effect of virus: $F_{1,18}$=8.19, p=0.0104; no session x virus interaction $F_{4,72}$=0.8711, p=0.4856). Post hoc analysis revealed that HDAC3-Y298H-v5 having fewer food rewards than EV-Controls on Day 4 (p=0.0725) and significantly Day 5 on food training (p=0.0218).

$F_{4,72}$=0.1652, p=0.9553) (Fig. 2.7b). However, HDAC3-Y298H-v5 did reduce levels of lever pressing for food pellets (main effect of session: $F_{4,72}$=52.47, p<0.0001; main effect of virus: $F_{1,18}$=8.19, p=0.0104; no session x virus interaction $F_{4,72}$=0.8711, p=0.4856). Post hoc analysis revealed that HDAC3-Y298H-v5 having fewer food rewards than EV-Controls on Day 4 (p=0.0725) and significantly Day 5 on food training (p=0.0218) (Fig. 2.7c). However, it should be noted that all mice, regardless of virus, achieved the set criteria for the number of food rewards obtained during the final three sessions (>25 pellets/session), indicating there are not extensive deficits from HDAC3-Y298H-v5. Overall, this data provides initial evidence that HDAC3's deacetylase activity in the NAc regulates aspects of food reinforcement.

Disruption of HDAC3's deacetylase activity in the NAc has no effect on anxiety-like behavior

The role of HDAC3 activity in the NAc within baseline anxiety behaviors were also tested. Previous studies have shown that molecular mechanisms implicated in cocaine processes also play roles in stress-induced anxiety and depressive behaviors. Thus, to ensure any effects seen within cocaine or reinforcement behaviors were not related to altered baseline anxiety, the effects of HDAC3-Y298H-v5 and HDAC3-WT on the elevated plus maze test were also assessed (Fig.2.5g, 2.5g). Baseline anxiety-like behaviors was unaffected by global overexpression of HDAC3-Y298H-v5 in the NAc (Fig. 2.6h: two-way ANOVA RM: main effect of arm: $F_{1,20}$=511.4, p<0.0001; no main effect of virus: $F_{1,20}$=0.003, p=0.9568; no interaction: $F_{1,20}$=0.861, p=0.3645). In addition, HDAC3-WT did not alter preference for closed versus opens in EPM (Fig.2.6g: two-way ANOVA RM: main effect of arm: $F_{1,21}$=608.44, p<0.0001; no main effect of virus: $F_{1,21}$=0.8954, p=0.3548; no arm x virus interaction: $F_{1,21}$=2.896, p=0.1035). These studies indicate that neither global disruption of HDAC3 activity or overexpression of HDAC3 in the NAc affects baseline anxiety-like behaviors.

Discussion

In these set of experiments, we show that disrupting HDAC3's deacetylase activity, but not overexpressing HDAC3, mediates cocaine-induced plasticity within the NAc. We also identify a key target of HDAC3's enzymatic function in the NAc, *Nr4a2*. In addition, we found cellular responses to cocaine (i.e. excitability) are altered by disrupted NAc HDAC3 deacetylase activity. Despite these effects on cocaine-induced molecular and cellular processes in the NAc, disrupting HDAC3 activity have no effect on behavioral response to the NAc. Yet, we show some initial evidence that HDAC3's deacetylase activity may be regulating processes of reinforcement. Together, our data support the idea that HDAC3's deacetylase activity regulate processes of experience dependent plasticity within the NAc.

Recent studies have demonstrated that HDAC3 is a negative regulator of cocaine-induced behaviors[77,80]. Although it was presumed these effects were due to blocking HDAC3's deacetylase activity, the approaches used in these experiments (i.e. pharmacological inhibitors or genetic deletions) may also disrupt HDAC3-protein interactions with NCoR or SMRT and Class II HDACs 4 and 5. Consistent with this, the interaction between HDAC3 and its corepressor NCoR is sufficient to enhance memory and localization of both HDAC 4 and 5 alter cocaine-related behaviors [83,85,104]. In addition, HDAC3 has shown to regulate gene expression independent of its deacetylase activity[122]. Thus, our dominant-negative point mutant virus (AAV-HDAC3(Y298H)-v5) approach that selectively abrogates the deacetylase activity of HDAC3 without affecting its protein–protein interactions allows us to determine the importance of HDAC3's enzymatic activity in the NAc [81,105,106].

Here, we have shown that HDAC3's deacetylase activity regulates cocaine-induced changes in gene expression. More specifically, disrupting HDAC3's deacetylase activity was sufficient to alter NAc expression of *Nr4a2* following chronic cocaine exposure. This supports studies found in the hippocampus that HDAC3s regulates Nr4a2 expression[107]. *Nr4a2* in the

hippocampus is a regulator of age-related memory processes and *Nr4a1*, a family member of *Nr4a2*, has recently been implicated in cocaine-induced processes in the NAc. Thus, it is possible that HDAC3 is regulating critical processes of plasticity in the NAc through gating *Nr4a2* expression, however additional studies would need to confirm this. However, the lack of changes in Nr4a1 is surprising, considering that studies in the hippocampus identified Nr4a1 as a bonafide target of HDAC3. It is possible that HDAC3's deacetylase function regulates gene targets in a region-specific manner, as upstream signaling cascades or protein-interactions that occur within the NAc may be distinct from those within the hippocampus. Our lack of changes in cocaine-induced expression of Fos, Nr4a1 and Nr4a3 expression following HDAC3-Y298H-v5 could be accounted for a host of reasons. One being that it is possible that the cocaine-induced histone modifications observed at this time point create a permissible state for transcription of these key plasticity related genes at later time points within withdrawal. In addition, changes in Hdac expression may occur later within withdrawal as a counteradaptive change in response to cocaine. Despite these differences, our data does suggest that HDAC3's deacetylase activity regulates aspects of histone acetylation and cocaine-induced gene expression within the NAc.

Our data also indicates that HDAC3's deacetylase activity can alter cocaine-induced synaptic changes within the NAc. It is unclear whether the restoration of long-term potentiation results from HDAC'3 deacetylase activity accelerating processes of cocaine action, such as increases in spine density or unsilencing synapses within the NAc, to causes these changes[37,123–125]. It is also possible these effects may have occurred due to changes in ion channel expression or activity, as there were increases in excitability from the NAc of cocaine-conditioned HDAC3-Y298H-v5 animals within the 20 mg/kg conditioning experiment. Other studies have found similar effects on cocaine-related excitability when overexpressing the transcription factor CREB in the NAc[120]. Given their relationship, it is possible that disruption of HDAC3 activity may promote

CREB-dependent transcription and excitability[126]. Overall, these findings shed light on possible mechanisms of action underlying changes in NAc cellular responses.

Although some studies have examined the effects of general HDAC inhibition on drug-induced LTP[36,119], this is the first to study the effects of disrupting enzymatic function of a specific HDAC on cocaine-induced synaptic plasticity. In contrast to earlier studies, which found that chronic exposure of pharmacological HDAC inhibitor enhanced cocaine-induced depression of LTP, we found that disrupting HDAC3 activity restored LTP in the NAc of cocaine-conditioned mice. These discrepancies may be due to differences in approaches (one week I.P. injections of SAHA vs. NAc viral infusions of HDAC3-Y298H-v5)[36]. Alternatively, they could indicate that different mechanisms and molecular players underlie these distinct effects, as SAHA is a HDAC inhibitor that non-specifically targets both Class I and Class II HDAC activity. Previous studies have also found HDAC3-Y298H-v5 within the hippocampus results in the increases in LTP in naïve animals, whereas HDAC3-Y298H-v5 had no effect on LTP in saline conditioned mice. It is unclear whether this could be from differences in circuitry (stimulation of the Schaffer collateral-commissural projections to the CA1b stratum radiatum versus cortical glutamatergic afferents projecting to the NAc) or perhaps in the HDAC3-Y298H-v5 affected cell-types of these two regions. However, our finding that lack of effect on HDAC3-Y298H-v5 on LTP in saline conditioned animals is consistent with our saline gene expression data. Thus, in the NAc, disrupting HDAC3's deacetylase activity may create a permissive environment for increased transcription and cellular activity, however additional dopaminergic dependent activity may be required to promote persistent molecular and cellular adaptations. This is consistent with previous findings showing that global deletion of HDAC5 does not affect cocaine-CPP unless animals had prior cocaine experience[102]. Future studies parsing apart the different roles of each HDAC in cocaine-induced synaptic plasticity and region-specific differences in HDAC3 activity will thus be critical in better understanding the mechanisms underlying these contrasting results.

Despite observing changes in molecular and cellular responses to cocaine in the NAc, overexpressing HDAC3-Y298H-v5 had no effects on behavioral responses to cocaine. This could indicate that HDAC3's deacetylase activity has no role in regulating cocaine-induced behavioral responses within the NAc. In support of this, prior work from our lab has also shown that HDAC3-Y298H-v5 within the prelimbic cortex, infralimbic cortex and ventral hippocampus has no effect on cocaine-conditioned place preference behaviors. However, this would be incongruous with our molecular and cellular data. Instead, we hypothesize that our viral tool to target all cell types within the NAc is potentially masking the role HDAC3 has in regulating cocaine-induced behaviors. The two major output neurons subtypes of the NAc, dopamine 1 receptor (D1R-) and dopamine 2 receptor (D2R-) medium spiny neurons (MSNs), have opposing roles in regulating cocaine-induced behaviors. Furthermore, these cell types have shown to have distinct changes in histone acetylation and gene expression following cocaine exposure. One of these genes that is differentially expressed within D1R vs D2R MSNs following cocaine exposure is Nr4a2. Thus, is it possible that a cell-type specific approach would be more suitable to investigate HDAC3's role in regulating behavioral responses to cocaine.

In contrast to cocaine-induced behaviors, global disruption of HDAC3 activity in the NAc was sufficient to impair food reinforcement. From my results alone, it is unclear what may be the underlying mechanism causing this impairment. Similar behavioral findings within the field are from studies using HDAC inhibitors, with reported impairments in cocaine-induced locomotion and cocaine reinforcement. Work from Kennedy and Nestler et al. points to the effects on histone methylation produced from HDAC inhibition. It is possible disrupting HDAC3 activity results in counteradaptive molecular mechanisms that regulate reinforcement. Alternatively, cocaine similarly impair reward and reinforcement processes for natural foods. Therefore, our AAV manipulation may be affecting similar processes that cocaine does to impair food reinforcement. These results do not necessarily negate our hypothesis of HDAC3 having a cell-type specific in

regulating cocaine-induced behaviors. Although D1R and DR2-MSNs are known to have distinct roles in regulating cocaine action, MSNs have reported overlapping roles in reinforcement. For instance, Cole and Everritt et al. show mice will seek to optogenetically self-stimulate D1R or DR2-MSNs in an instrumental task. Thus HDAC3 may have a more defined and distinct role in cocaine action in comparison to reinforcement.

Despite confirming that our virus overexpresses HDAC3 and v5 expression is detected from our AAV NAc infusions, we fail to detect any synaptic or behavioral changes following this manipulation. This similar effect has been seen in mouse experiments with the MHb. We interpretation of this data is that, without additional overexpression of other CoRepressor proteins such as NCor1 and NCor2, additional HDAC3 expression does not affect molecular mechanisms to drive changes in neuronal function. Consistent with this idea, similar results are seen with overexpression of HDACs, such as HDAC4, or other histone modifying enzymes such as Set2. Thus, additional tools that will anchor HDAC3 to promoter specific regions of the genome, such as the CRISPR-dCas9 system will greatly bolster the field's understanding how increased HDAC3 activity alters drug-induced molecular and cellular mechanisms.

Conclusion

Here, we find that disrupting HDAC3's deacetylase activity within the NAc alters molecular and cellular responses to cocaine. Although no changes in cocaine-induced behavior were found following global manipulation of HDAC3 activity in the NAc, there were some effects on food reinforcement. Our results suggest that our tools to investigate the role of HDAC3 may be insufficient. Moving forward, we hypothesize that HDAC3 operates in a cell-type specific manner within the NAc to drive cocaine-seeking behaviors.

Chapter 3: Cell Type Specific Role of HDAC3's Deacetylase Activity on Cocaine-Associated Memory Formation

Rationale:

The two major cell types and output neurons of the NAc are categorized based on the expression of dopamine D1- receptor and D2-receptors. These D1R – and D2R- cell-types have distinct molecular and cellular responses to cocaine. For instance, chronic cocaine has shown to induce distinct changes in histone acetylation and immediate early gene expression within D1R- vs D2R- MSNs. In addition, cocaine has shown to potentiate D1R, but not D2R-expressing cells of the NAc. Activation of these two cell populations induce distinct changes in cocaine-seeking behaviors. It is unknown how cocaine alters HDAC3 within these two cell types and whether HDAC3 is differentially affected within MSNs to drive cocaine-induced behaviors. Here, we first confirmed that HDAC3 is expressed within these two cell types and how cocaine affects Hdac3 expression within D1R vs D2R MSNs. Next, we evaluated the effects of disrupting HDAC3's activity within these cell types, using Cre-dependent viral vectors and Cre driver mice, on cocaine-induced behaviors. Overall, our data suggests that cocaine alters the activity, but not expression of HDAC3, within D1R-MSNs to drive cocaine-induced changes in plasticity and behavior.

Materials and Methods

Mice:

C57BL/6 J mice, D1R- Cre and D2R- Cre mice were all single-housed and at 8 and 15 weeks old at the time of behavioral testing. Drd1-Cre(EY262Gsat) and Drd2-Cre (ER44Gsat) mice were crossed with C57BL/6 J mice to breed hemizygous Drd1-Cre and Drd2-Cre mice for all experiments. Mice were provided with food and water *ad libitum* for all experiments except food training. Lights were maintained on a 12:12 h light per dark cycle, with all behavioral tests performed during the light portion of the cycle. All experiments were conducted according to US

National Institutes of Health Guidelines for Animal Care and Use, and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

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- experiments, cocaine-HCl was dissolved to a final concentration of either 0.5 mg/ml or 1.0 mg/mL mg/mL and administered in a volume of 10 ml/kg body weight, resulting in a final dose of 5 mg/kg or 10 mg/kg. Cocaine-HCl and saline were administered intraperitoneally (i.p). For the cocaine-induced locomotion experiments, animals were I.P injected at a final concentration of 10 mg/kg. Animals were I.P. injected with 20 mg/kg for both the electrophysiological recordings and molecular analysis experiments.

AAV Production

The production of these plasmids was described in Lopez et al. 2019[112]. Briefly, Cre-dependent versions of HDAC3-Y298H-v5 were generated by cloning HDAC3-Y398H-v5 into a modified pAAV-hSyn-DIO-eGFP (Addgene #50457, a generous gift from Dr. Bryan Roth) with the addition of β-globin intron. GFP element was removed from the original vector and replaced with a V5-tag. To create the HDAC3 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. Plasmids were generated by Dr. Alberto Lopez and packaged by either Dr. Thekla Hemstedt was serotyped with AAV 2.1. The final titer of AAV-DIO-HDAC3(Y298H) was 6.48×10^{12} GC/mL and the final titer of AAV-EV-mcherry was purchased Addgene with a titer of 4×10^{13} GC/mL.

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). 0.5 µl of virus was infused bilaterally into the NAc [anteroposterior (AP): +1.3 mm; mediolateral (ML): ±1.1 mm; dorsoventral (DV): −4.5 mm relative to bregma. Immunofluorescence was used to confirm expression of HDAC3(Y298H). Viruses were infused at a rate of 6 µl /hr by using a 30 gauge Neuros Hamilton syringe (product #65459-01) mounted to either a Harvard Apparatus Nanomite Syringe Pump (product #MA1 70-2217) or Leica Biosystems Nanoinjector Motorized Stereotaxics (product #39462901). All infusions used the Leica Microsystems Angle Two Stereotaxic System. All animals were allowed to recover for a minimum of two weeks days before handling.

Cocaine-Conditioned Place Preference

Following intracranial viral infusions and two weeks recovery, an unbiased conditioned place preference (CPP) was performed as described in previous studies. Briefly, all mice were handled for 2 minutes for 3 consecutive days prior to the experiment (days 1-3). Baseline preferences for three compartments in the CPP apparatus were assessed by placing the animals in the center compartment of the apparatus with free access to three distinct compartments for 15 min (day 4). Time spent in each compartment was recorded. Following this pretest, mice were conditioned over four consecutive days, receiving either cocaine-HCl (5 mg/kg or 10 mg/kg, IP; Sigma) or 0.9% saline (day 5-8). 24 hours following the last conditioning session, post-conditioning preference was tested in animals while they were in a drug-free state (day 9). Animals were allowed to freely explore all compartments of the CPP apparatus to assess preference, established as the difference between time spent in the cocaine-paired chamber and the saline-paired chamber, in seconds. Time spent was tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology).

Cocaine-Induced Locomotion

This test examines the locomotor activating effects of cocaine in animals following experimenter-administered cocaine injections. Mice were handled for 2 min for 3 days (day 1-3) and were

habituated to the activity apparatus (Plexiglas open field with sawdust bedding; base 16 cm × 32 cm) for 30 min per day for 2 consecutive days (days 4-5). Following intracranial viral infusions and two weeks recovery, mice were randomized into two different treatment groups (saline or cocaine) and locomotor activity was recorded for 30 minutes after an intraperitoneal injection of either 5 mg/kg or 10 mg/kg Cocaine-HCl or 0.9% saline for 5 days (day 6-10). Locomotor activity (total distance travelled) was monitored and tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology, Leesburg, VA).

Elevated Plus Maze

The plus-maze was conducted by an experimenter blind to the experimental groups. The maze consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm), that are connected by a central platform (5 × 5 cm). The maze was elevated 40 cm above the floor. During the test, mice were recorded for 5 min on the apparatus, with initially placing each mouse onto the central platform facing one of the open arms. Between subjects, the maze was cleaned with 70% ethanol. The percentage of time spent in the closed and open arms was scored using ANY-maze software.

Immunofluorescence

Following behavioral testing, animals were sacrificed and brain tissue was flash-frozen in isopentane and collected for immunohistochemistry. Twenty micrometer coronal sections were collected using a Leica CM 1850 cryostat at −20°C and mounted on slides. Slices were fixed in 4% PFA for 10 minutes, washed in 0.1-M PBS and permeated in 0.1% Triton X-100 in 0.1-M PBS. Slices were then blocked in blocking serum (8% NGS, 0.3% Triton X-100, in PBS; 1 hour) and incubated at 4°C overnight in primary solution (2% NGS, 0.3% Triton X-100; rabbit anti-v5: 1:1000, Abcam). The slices were then incubated in secondary solution (2 percent NGS, 0.3 percent Triton X-100; Alexa Fluor goat anti-rabbit 488). Lastly, tissue was incubated for 15-minute in a DAPI solution (1:10,000, Invitrogen). Slides were coverslipped using VectaShield Antifade mounting medium (Vector Laboratories).

To confirm cell-type specific expression of DIO-HDAC3-Y298H-v5, Drd1-Cre and Drd2-Cre mice were virally infused with DIO-HDAC3-Y298H-v5 into the NAc. Following 2 weeks of recovery and for viral expression, mice were perfused with 4% PFA and brain tissue was harvested. 35 uM slices were collected and mounted on slides and underwent immunofluorescence. Briefly, slices were washed in 0.1-M PBS and permeated in 0.1% Triton X-100 in 0.1-M PBS. Slices were then blocked in blocking serum (8% NGS, 0.3% Triton X-100, in PBS; 1 hour) and incubated at 4°C overnight in primary solution (2% NGS, 0.3% Triton X-100; mouse anti-v5: 1:1000, Invitrogen; Rabbit anti-pro-Enkephalin: 1:1000, Immunostar; Rabbit anti-Substance P, 1:1000, Millipore). The slices were then incubated in secondary solution (2 percent NGS, 0.3 percent Triton X-100; Alexa Fluor goat anti-rabbit 488; anti-mouse 577). Lastly, tissue was incubated for 15-minute in a DAPI solution (1:10,000, Invitrogen). Slides were coverslipped using VectaShield Antifade mounting medium (Vector Laboratories). The tissue was imaged by using Olympus Slide Scanner VSBX61 and further processed using IMAGEJ.

Quantitative RT-qPCR

RT-qPCR was performed as described previously[105,111]. One millimeter punches were collected from NAc in a 500 M slice of tissue. RNA was isolated from punches 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, designed using the Roche Universal Probe Library:

Gene	Forward Primer	Reverse Primer	Roche Probe #
Hprt5	TGCTCGAGATGTCTGAAGG	ATCACATTGTGGCCCCTCTGT	-----
Hdac3	ttcaacgtgggtgatgactg	ttagctgtgttgctccttgc	32

In Situ Hybridization

We performed RNAscope ISH for *Hdac3*, *Drd1*, and *Drd2* mRNA. Sixty minutes after the last injection, we briefly anesthetized mice with pentobarbital (100 mg/kg intraperitoneal), perfused mice with 1x-PBS and extracted whole brain tissue. Brains were then incubated in 4% PFA for 24 hours, and 30% sucrose solution for at least 48 hours. Brains were then flash frozen in isopentane and stored at −80°C until use. NAc coronal sections (35 µm) were mounted directly onto Superfrost Plus slides (Fisher Scientific). We used an RNAscope Multiplex Fluorescent Reagent Kit II (Advanced Cell Diagnostics) and performed the ISH assay according to the user manual for fixed-frozen tissue. Each RNAscope target probe used contains a mixture of 20 ZZ oligonucleotide probes that are bound to the target RNA, as follows: Hdac3-C1 probe, Drd1-C2 probe and Drd2-C3 probe. Slides were incubated in a 1:10,000 DAPI solution for 15 minutes and washed with 1x-PBS two times prior to coverslipping. Immediately following last washes, slides were coverslipped with a VECTASHIELD fluorescent mounting medium (H-1400, Vector Laboratories). 60x NAc fluorescent images were captured using a confocal microscope (Leica SP8).

For analysis, number of Hdac3 puncta in Drd1 vs Drd2 cells were counted using Imaris software. A percentage of the number of Hdac3 puncta was calculated for each slice as follows: # of Hdac3 puncta in Drd1-cells or Drd2 cells/(Total # of Hdac3 in Drd1+Drd2 cells). An average was calculated based on this percentage for each slice per animal (1-3 slices). We then compared the Average % of Hdac3 colocalization for each cell type in cocaine-treated versus saline-treated animals.

Statistical Analysis

All statistical analyses were performed using with Prism 6 (Graphpad Software Inc, La Jolla, CA, USA. Hdac3 expression in Drd1 versus Drd2 cells using RNAScope was analyzed with two-way

ANOVAs (treatment x virus) followed by a Sidak's multiple comparisons test. Unpaired t-tests were conducted To assess whether DIO-HDAC3-Y298H affects HDAC3 expression with RT-qPCR. In CPP experiments, two-way ANOVAs were conducted to assess cocaine-associated memory formation. Locomotor activity during pre- and post- conditioning tests was assessed using unpaired t-tests. Cocaine-induced locomotion was assessed using multiple analysis two-way ANOVAs followed by a Sidak's multiple comparisons test. The effects of manipulating HDAC3 on anxiety-like behavior was assessed in elevated-plus maze using multiple analysis two-way ANOVAs followed by a Sidak's multiple comparisons test (arm x virus). All analyses were two-tailed and required a value of 0.05 for significance. Error bars in all figures represent SEM. For all experiments, values ± 2 SD from the group mean were considered outliers and were removed from analyses.

Results

Chronic cocaine alters Hdac3 expression in Drd1 vs Drd2 cells of the NAc

Given that the two major cell types (D1R-MSNs and D2R-MSNs) of the NAc have opposing roles in regulating cocaine-related behavior, we hypothesized that Hdac3 has a cell-type specific role within the NAc to regulate cocaine-induced behaviors. To test whether cocaine alters *Hdac3* expression in a cell-type specific manner, we performed *in situ* hybridization on NAc tissue. Using NAc tissue from animals that underwent either chronic injections of cocaine (20 mg/kg) or saline, we examined *Hdac3* expression *Drd1* versus *Drd2* expressing cells (Fig. 3.1a,b). We first confirmed that cocaine does not affect *Hdac3* expression in the NAc (Fig 3.1c: unpaired t-test $(t(12) = 0.6217, p = 0.545)$. However, when examining the effects of cocaine on Hdac3 expression within Drd1 and Drd2 cells of the NAc, we found stark differences (Fig. 3.1d). We found cocaine

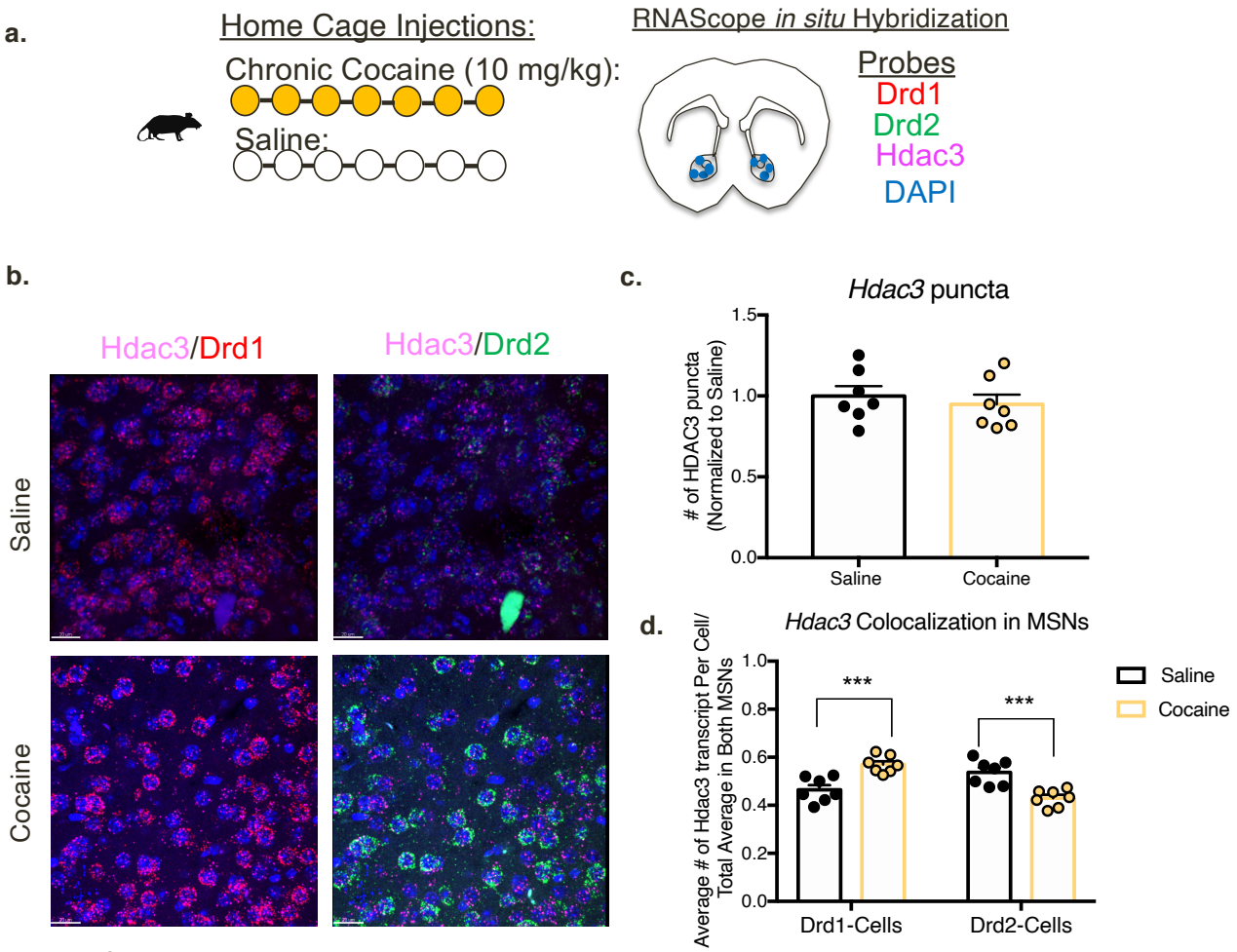

Fig. 3.1. Chronic cocaine increases Hdac3 expression in Drd1 and decreases Hdac3 expression in Drd2 cells of the NAc. a. Animals underwent chronic injections of either saline or cocaine and NAc tissue was collected to examine Hdac3 mRNA localization/expression in Drd1 vs Drd2 cells using *in situ* hybridization. **b.**Triplex detection of *Drd1* (red), *Drd2* (green), and *Hdac3* (pink) mRNAs in NAc after chronic cocaine or saline injections. Representative images of selected cells *(Hdac3*-positive, white arrows*).* Images on the left *(Hdac3+ Drd1)* show merged channels for *Hdac3* (pink) and *Drd1* (red) signals. Images on the right *(Hdac3 + Drd2)* show the merged channels for *Hdac3* (pink) and *Drd2* (green) from the same brain sections. **c.** Cocaine exposure does not alter Hdac3 expression. Counts of Hdac3 puncta detected in the NAc following cocaine or saline exposure. (unpaired t-test $(t_{(12)}=0.6217,\ p=0.545)$ **d.** Graphs indicating the Average # of *Hdac3*-transcripts (n = cocaine: 7, saline: 7.) coexpressed in *Drd1 (Hda3c + Drd1)* or *Drd2 (Hdac3 + Drd2)* mRNA in the NAc. Hdac3 transcript is detected in Drd1-containing cells versus Drd2-containing cells following cocaine exposure (Two-Way-ANOVA: interaction $F_{1,12}=19.97$, p=0.0008; Sidak's test, p<0.0005

increases the amount of Hdac3 puncta within Drd1-containing cells and decreases Hdac3 expression within Drd2- containing cells Two-Way-ANOVA: interaction $F_{1,12}=19.97$, p=0.0008; Sidak's test, p<0.0005). This may indicate that Drd1 cellular activity leads to increased expression of *HDAC3* as a counteradaptive response to cocaine action, however because these molecular changes do not occur within Drd2 cells, Hdac3 expression is decreased. This suggests that HDAC3 has a cell-type specific role in regulating cocaine-induced changes within D1R- but not D2R- cells of the NAc.

Disrupting HDAC3 activity in a cell type specific manner using DIO-HDAC3-Y298H-v5

To investigate the cell-type specific role of HDAC3 within MSNs, we used Cre-dependent versions HDAC3-Y298H-v5 (DIO-HDAC3-Y298H) and Cre-driver mouse lines. Overexpression of HDAC3-Y298H-v5 in the NAc of both D1R-Cre and D2R-Cre mice was confirmed using IHC to detect V5 protein expression in D1R-Cre or D2R-Cre mice that were infused with AAV containing DIO-HDAC3-Y298H-v5 (Fig. 3.2a-d). This was also confirmed using RT-qPCR(Fig. 3.2e-f). D1R-Cre and D2R-Cre mice that were infused with DIO-HDAC3-Y298H-v5 have higher expression of HDAC3-v5 and HDAC3 in the NAc compared to DIO-mcherry controls(Fig 3.2e).

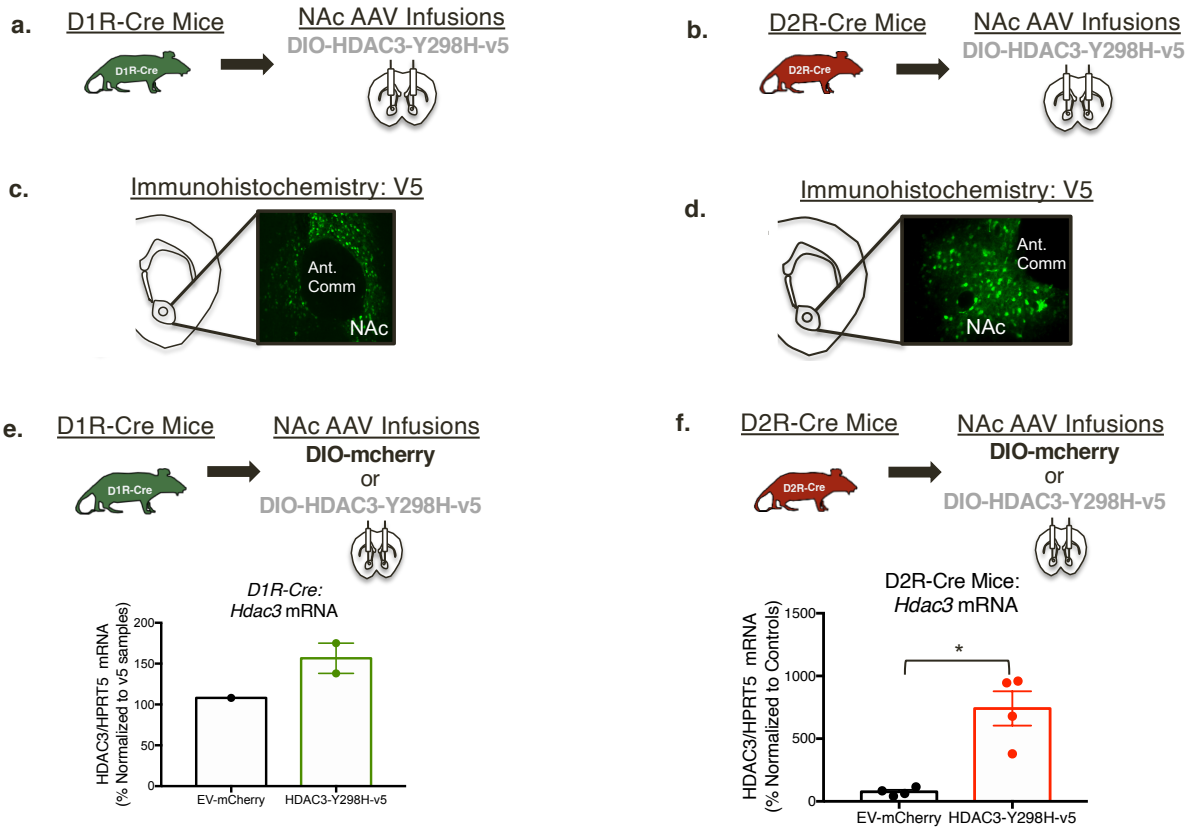

Fig. 3.2. DIO-HDAC3-Y298H-v5 sufficiently expresses in the NAc. a.,b. D1R-Cre or D2R-Cre adult mice were infused with AAV either deacetylase dead HDAC3 point mutant (HDAC3-Y298H-v5) and immunohistochemistry confirmed V5 protein detection. **c.,d. Viral NAc infusions were performed in D1R-Cre or D2R-Cre mice. Mice were infused with AAV containing either DIO-HDAC3-Y298H-v5 or DIO-mcherry.** Overexpression of HDAC3-Y298H-v5 was confirmed by qPCR in both D1R-Cre and D2R-Cre mice, as animals with DIO-HDAC3-Y298H-v5 infusions had significantly higher expression of *Hdac3* than DIO-EV-mCherry controls (U=0, EV-mCherry: n=4, median=0.004713; HDAC3-Y398H-v5: n=4, median=100; *=p<0.05**)**.

D1R-MSN specific disruption of HDAC3's deacetylase activity in the NAc enhances cocaine-induced CPP

Once we confirmed that Hdac3 is expressed within both cell types, however its expression is not altered by cocaine within either cell type, we wanted to determine whether Hdac3's activity regulates cocaine-induced behaviors through its actions within either cell type. To test this, D1R and D2R Cre mice underwent AAV infusions containing either a Cre-dependent HDAC3-Y298H-v5 or mCherry construct and underwent cocaine-CPP (Fig. 3.3, 3.4). We found that male and female mice with disrupted HDAC3 activity in D1R-MSNs had enhanced cocaine-memory formation compared to controls following 5 mg/kg conditioning (Fig 3.3b. male: (main effect of conditioning $F_{1,18}$=11.79, p=0.003; main effect of virus $F_{1,18}$=10.57, p=0.0044; no conditioning x virus interaction $F_{1,18}$=1.394, p=0.2532; Fig. 3.3c female: main effect of conditioning $F_{1,19}$=1.097, p=0.3081; main effect of virus $F_{1,19}$=10.62, p=0.0041; no conditioning x virus interaction $F_{1,19}$=1.021, p=0.3250) and 10 mg/kg conditioning (Fig. 3.3d 3.3e. male: main effect of conditioning $F_{1,16}$=10.3, p=0.055; main effect of virus $F_{1,16}$=5.704, p=0.0296; conditioning x virus interaction $F_{1,16}$=04.591, p=0.0479; female: main effect of conditioning $F_{1,16}$=10.3, p=0.055; main effect of virus $F_{1,16}$=5.704, p=0.0296; conditioning x virus interaction $F_{1,16}$=04.591, p=0.0479). This data suggests that cocaine alters HDAC3 activity within D1R-MSNs to drive cocaine-associated memory processes.

In contrast, disrupting HDAC3 activity within D2R-cells had no effect on cocaine-induced CPP in males or females (Fig 3.4a.). D2R-Cre mice that were infused with either DIO-HDAC3-Y298H-v5 or DIO-mCherry-Control into the NAc underwent cocaine-induced CPP at a 10 mg/kg dose. Following 10 mg/kg cocaine conditioning, HDAC3-Y298H-v5 enhanced CPP acquisition in males (Fig 3.4b main effect of conditioning $F_{1,18}$=41.83, p<0.0001; no main effect of virus $F_{1,18}$=0.000247, p=0.9875; no conditioning x virus interaction $F_{1,16}$=0.5622, p=0.4631) and in

females (Fig 3.4c: main effect of conditioning $F_{1,16}$=114.5, p<0.0001; no main effect of virus $F_{1,16}$=0.7306, p=0.4053; no conditioning x virus interaction $F_{1,16}$=0.6666, p=0.4262). These results indicate that HDAC3's activity within D2-MSNs does not affect cocaine-associated memory formation.

We also confirmed there were no effects on overexpressing HDAC3-Y298H-v5 within D1R-MSNs and D2R-MSNs on performance throughout all CPP testing days. D1R-Cre male mice, DIO-HDAC3-Y298H-v5 and DIO-mCherry-control had no significant effect on total distance during the 5 mg/kg pre-conditioned testing days (unpaired t-test ($t_{(18)}$ = 0.181, p = 0.8584)) or the

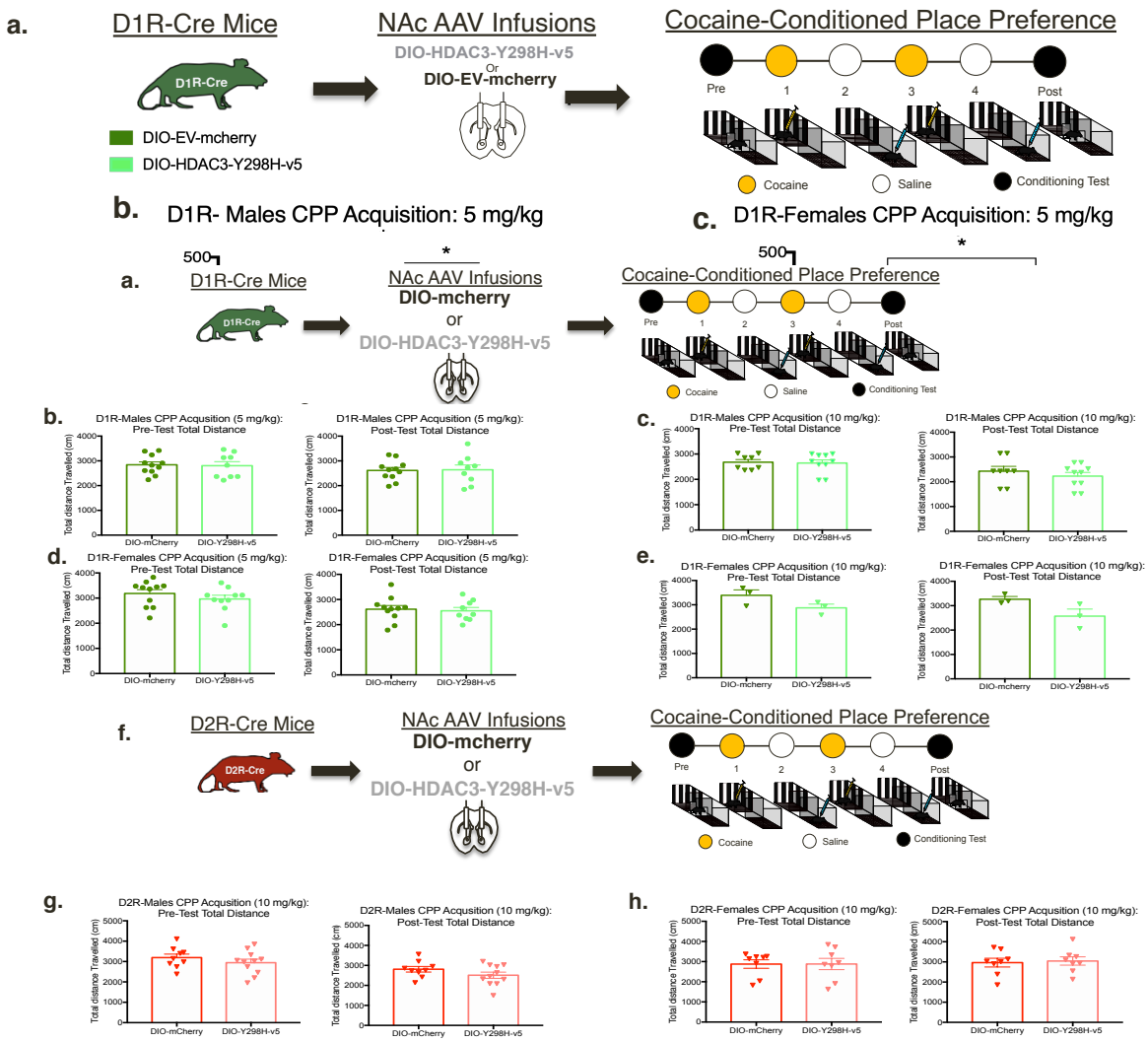

Fig 3.5. Disrupting HDAC3's activity in either D1R and D2R-cells has no effect on locomotion during pre/post-conditioning test CPP days. a. AAVs containing either DIO-HDAC3-Y298H-v5 or DIO-mCherry-control were infused into the NAc of adult D1R-Cre mice prior to cocaine-induced CPP. **b**. In D1R-Cre male mice, DIO-HDAC3-Y298H-v5 and DIO-mCherry-control had no significant effect on total distance during the 5 mg/kg pre-conditioned testing day (unpaired t-test ($t_{(18)}$=0.181, p=0.8584)) or the post-conditioned testing day (unpaired t-test ($t_{(18)}$=0.129, p=0.8988. **c.** DIO-HDAC3-Y298H-v5 and DIO-mCherry-control male mice had no significant effect on the amount of total distance travelled in the preconditioning testing (unpaired t-test ($t_{(16)}$=0.1976, p=0.8459) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test ($t_{(16)}$=0.8315, p= 0.4179). **d**. In D1R-Cre female mice, DIO-HDAC3-Y298H-v5 and DIO-mCherry-control had no significant effect on total distance during the 5 mg/kg pre-conditioned testing day (unpaired t-test ($t_{(18)}$=0.5221, p=0.6079)) or the post-conditioned testing day (unpaired t-test ($t_{(18)}$=0.3102, p=0.7599. **e.** DIO-HDAC3-Y298H-v5 and DIO-mCherry-control female mice had no significant effect on the amount of total distance travelled in the preconditioning testing (unpaired t-test ($t_{(4)}$=1.89, p=0.1317)) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test ($t_{(4)}$=2.226, p=0.0900)). **f.** AAVs containing either DIO-HDAC3-Y298H-v5 or DIO-mCherry-control were infused into the NAc of adult D2R-Cre mice prior to cocaine-induced CPP. **g.** DIO-HDAC3-Y298H-v5 and DIO-mCherry-control male D2R-Cre mice had no significant effect on the amount of total distance travelled in the preconditioning testing (unpaired t-test ($t_{(18)}$=0.181, p=0.9968) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test ($t_{(18)}$=1.403, p=0.1777)). **h.** DIO-HDAC3-Y298H-v5 and DIO-mCherry-control female mice had no significant effect on the amount of total distance travelled in the preconditioning testing (unpaired t-test ($t_{(14)}$=0.01474, p=0.9884) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test ($t_{(18)}$=0.2737, p=0.7883).

post- conditioned testing day (unpaired t-test *(t*(18) = 0.129, *p* = 0.8988. This was also seen within male D1R-Cre mice on 10 mg/kg testing days (preconditioning testing (unpaired t-test *(t*(16) = 0.1976, *p* = 0.8459) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test *(t*(16) = 0.8315, *p* = 0.4179). Similarly, in D1R-Cre female mice, DIO-HDAC3-Y298H-v5 and DIO-mCherry-control had no significant effect on total distance during the 5 mg/kg pre-conditioned testing day (unpaired t-test *(t*(18) = 0.5221, *p* =0.6079)) or the post-conditioned testing day (unpaired t-test *(t*(18) = 0.3102, *p* = 0.7599. This was consistent within the 10 mg/kg testing days on female D1R-Cre mice (preconditioning testing (unpaired t-test *(t*(4) = 1.89, *p* = 0.1317)) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test *(t*(4) = 2.226, *p* = 0.0900)).

Within D2R-Cre male mice, DIO-HDAC3-Y298H-v5 and DIO-mCherry-control had no effect on the amount of total distance travelled in the preconditioning testing (unpaired t-test *(t*(18) = 0.181, *p* = 0.9968) or post-conditioning testing in CPP 10 mg/kg (unpaired t-test *(t*(18) = 1.403,

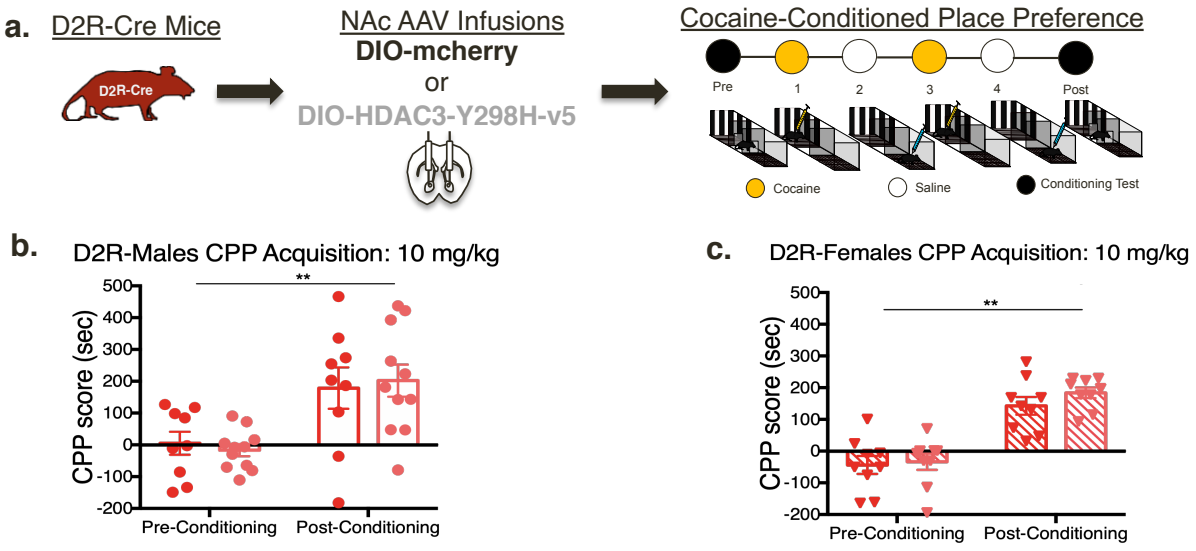

Fig 3.3. D2R-MSN specific disruption of HDAC3's deacetylase activity in the NAc does not alter cocaine-induced behaviors. a. AAVs containing either DIO-HDAC3-Y298H-v5 or an DIO-mCherry-control were infused into the NAc of adult mice prior to cocaine-induced CPP. **b.** D2R-Cre mice that were infused with either DIO-HDAC3-Y298H-v5 or DIO-mCherry-Control into the NAc underwent cocaine-induced CPP at a 10 mg/kg dose. Following 10 mg/kg cocaine conditioning, DIO-HDAC3-Y298H-v5 enhanced CPP acquisition in males (main effect of conditioning $F_{1,18}$=41.83, p<0.0001; no main effect of virus $F_{1,18}$=0.000247, p=0.9875; no conditioning x virus interaction $F_{1,16}$=0.5622, p=0.4631) **c.** and in females (main effect of conditioning $F_{1,16}$=114.5, p<0.0001; no main effect of virus $F_{1,16}$=0.7306, p=0.4053; no conditioning x virus interaction $F_{1,16}$=0.6666, p=0.4262).

p = 0.1777)). This was similarly seen in female on preconditioning testing (unpaired t-test *(t*(14) =

0.01474, p = 0.9884 and post-conditioning testing days **(**unpaired t-test *(t*(18) = 0.2737, p = 0.7883). Similarly, D2R-HDAC30Y298H-v5 had no effect on locomotion during pre-conditioning or post-conditioning testing (Supp Fig gc., hd). Thus the effects of DIO-HDAC3-Y298H-v5 on CPP testing were not related to impairments in locomotor activity but reflect changes in cocaine-associated memory formation.

Disruption of HDAC3's deacetylase activity in D1R- and D2R-MSNs in the NAc does not affect cocaine-induced locomotion

Next, we examined whether the effects of disrupting HDAC3's activity within either D1R or D2R cells had an effect on cocaine-induced locomotion. AAV were infused into the NAc of D1R or D2R-Cre male and female mice, which contained either a Cre-dependent HDAC3-Y298H-v5 or mCherry construct, and locomotor activity was examined following I.P. injections of cocaine or saline. Given our CPP data, we hypothesized that overexpressing HDAC3-Y298H-v5 within D1R-MSNs would enhance cocaine-induced locomotion. A lower dose of cocaine (5 mg/kg) was selected to ensure no ceiling effects would occur. However, we did not see any effects on D1-Y298H-v5 on cocaine-induced locomotion. D1R-Cre mice that received cocaine injections exhibited significantly higher locomotor responses versus saline regardless of virus (Three Way ANOVA: main effect of cocaine: $F_{1,4}$=43.13, p<0.0001; no main effect of virus: $F_{1,4}$=0.1575, p=0.6923; no main effect on session: $F_{4,4}$=2.152, p=0.0800). (Fig 5a. D1R: Two-Way ANOVA RM: main effect: session: $F_{4,88}$=7.805, p<0.0001; main effect: treatment: $F_{3,22}$=4.862, p=0.0096; interaction: $F_{12,88}$= 0.8491, p=0.6004; D2R: Supp. Fig. 4e). Next, we tested the effects of disrupting HDAC3 activity within D2R-MSNs on cocaine-induced locomotion. Mice underwent similar testing conditions, however the cocaine dose was 10 mg/kg. This is because much of the literature has shown that D2R-MSNs related mechanisms inhibit cocaine behavioral responses. Therefore, in order to test whether D2-Y298H-v5 impaired cocaine-induced locomotion, we selected a higher

dose to prevent any floor effects. D2R-Cre mice that received cocaine exhibited significantly higher locomotor responses versus saline regardless of virus (Three Way ANOVA: main effect of cocaine: $F_{1,4}$=265.7, p<0.0001; no main effect of virus: $F_{1,4}$=3.312, p=0.0738; no main effect on session: $F_{4,4}$=1.819, p=0.1370). The combined data suggests that HDAC3 within either MSN subtype does not regulate cocaine-induced locomotion.

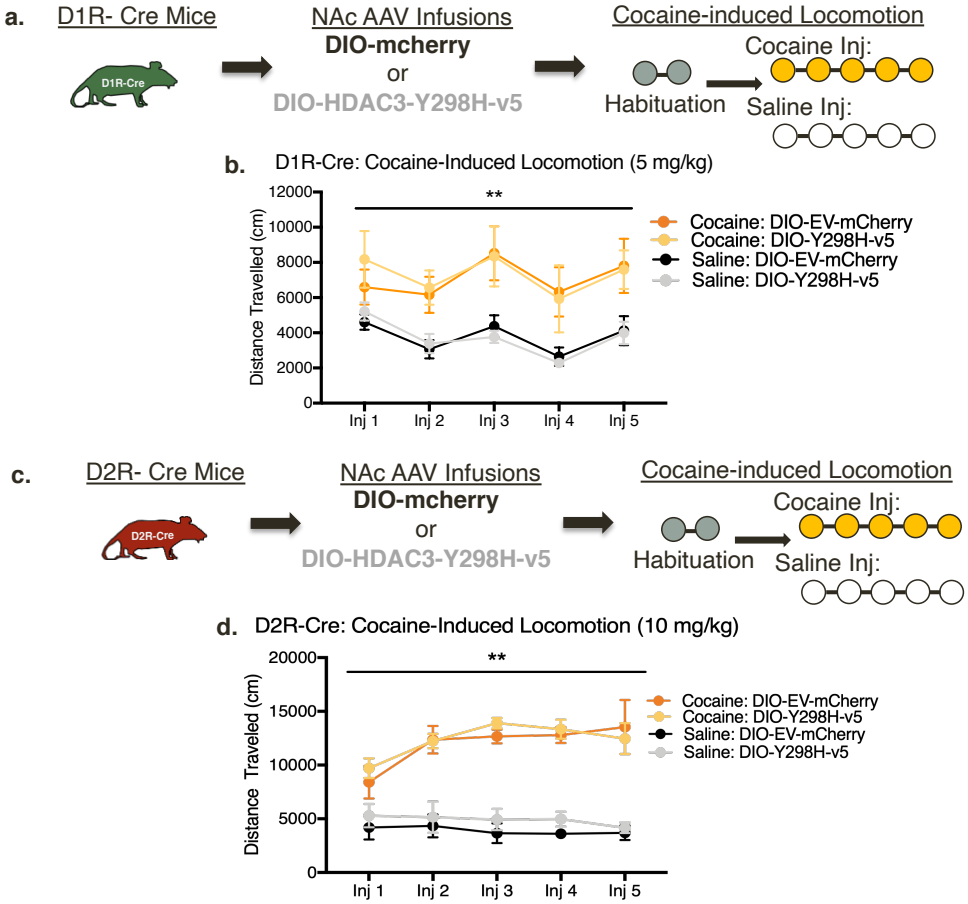

Fig 3.6 Disrupting HDAC3's activity in either D1R and D2R-cells has no effect on cocaine-induced locomotion. a. AAVs containing either DIO-HDAC3-Y298H-v5 or DIO-mcherry were infused into the NAc of D1R-Cre adult male and female mice. Animals next underwent cocaine-induced locomotion test, where mice were subjected to 5 days I.P. injections of either cocaine (5 mg/kg) or saline and placed in an open chamber to track distance travelled per session. **b**. D1R-Cre mice that received cocaine exhibited significantly higher locomotor responses versus saline regardless of virus (Three Way ANOVA: main effect of cocaine: $F_{1,4}$=43.13, p<0.0001; no main effect of virus: $F_{1,4}$=0.1575, p=0.6923; no main effect on session: $F_{4,4}$=2.152, p=0.0800). **c.** Following AAV NAc infusions of DIO-HDAC3-Y298H-v5 or DIO-mCherry-control, adult male and female D2R-Cre mice received 5 days of injections of either cocaine or saline prior to being placed in an open chamber to track the total amount of distance travelled. **d.** D2R-Cre mice that received cocaine exhibited significantly higher locomotor responses versus saline regardless of virus (Three Way ANOVA: main effect of cocaine: $F_{1,4}$=265.7, p<0.0001; no main effect of virus: $F_{1,4}$=3.312, p=0.0738; no main effect on session: $F_{4,4}$=1.819, p=0.1370)

Disruption of HDAC3's deacetylase activity in D1R- and D2R-MSNs in the NAc does not affect anxiety-like behaviors

Next, we tested the effects of disrupting HDAC3 activity within D1- and D2R-MSNs on baseline anxiety-like behavior using the elevated plus maze test (EPM). AAVs containing either DIO-HDAC3-Y298H-v5 or an DIO-EV-mcherry were infused into the NAc of adult mice prior to EPM. **).** In regards to D1R-MSNs, D1R-males had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,9}$=515.6, p<0.0001; no main effect of virus: $F_{1,9}$=0.8755, p=0.3739; no interaction: $F_{1,9}$=1.338, p=0.2771). Similarly, D1R-females had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,8}$=137.2, p<0.0001; no main effect of virus: $F_{1,8}$=0.3649, p=0.5625; no interaction: $F_{1,8}$=0.7629, p=0.4079). This suggests that HDAC3's deacetylase activity within D1R-MSNs doesn't affect baseline anxiety-like behavior. D2R-MSN manipulations had similar results. D2R-males had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,18}$=382.2, p<0.0001; no main effect of virus: $F_{1,18}$=0.2811, p=0.624 no interaction: $F_{1,18}$=0.05258, p=0.8212). Similarly, D1R-females had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,16}$=175.6, p<0.0001; no main effect of virus: $F_{1,16}$=0.04625, p=0.8324; no interaction: $F_{1,16}$=1.588, p=0.2257). D2R-males had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,18}$=382.2, p<0.0001; no main effect of virus: $F_{1,18}$=0.2811, p=0.624 no interaction: $F_{1,18}$=0.05258, p=0.8212). D1R-females had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,16}$=175.6, p<0.0001; no main effect of virus: $F_{1,16}$=0.04625, p=0.8324; no interaction: $F_{1,16}$=1.588, p=0.2257).

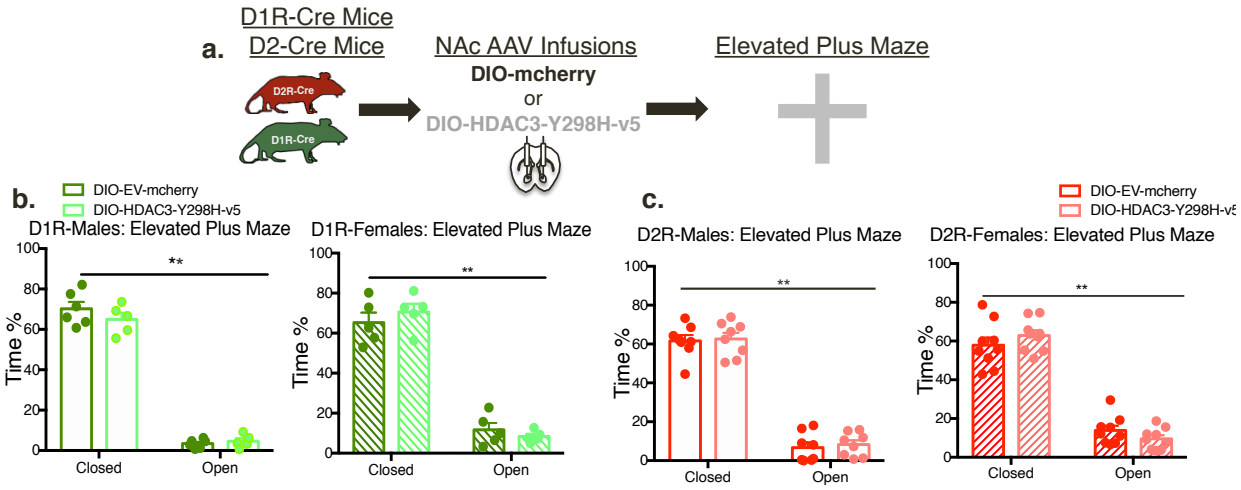

Fig. 3.7 Disruption of HDAC3 Activity in D1R and D2R-MSNs has no effect on baseline anxiety-like behavior. a. AAVs containing either DIO-HDAC3-Y298H-v5 or an DIO-EV-mcherry were infused into the NAc of adult D1R-Cre or D2R-Cre mice prior to elevated plus maze (EPM) test. **b,left.** D1R-males had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,9}$=515.6, p<0.0001; no main effect of virus: $F_{1,9}$=0.8755, p=0.3739; no interaction: $F_{1,9}$=1.338, p=0.2771). **b, right**. Similarly, D1R-females had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,8}$=137.2, p<0.0001; no main effect of virus: $F_{1,8}$=0.3649, p=0.5625; no interaction: $F_{1,8}$=0.7629, p=0.4079). **c,left.** D2R-males had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,18}$=382.2, p<0.0001; no main effect of virus: $F_{1,18}$=0.2811, p=0.624 no interaction: $F_{1,18}$=0.05258, p=0.8212). **c, right**. Similarly, D1R-females had spent significantly more time closed arms versus open arms, regardless of virus (two-way ANOVA RM: main effect of arm: $F_{1,16}$=175.6, p<0.0001; no main effect of virus: $F_{1,16}$=0.04625, p=0.8324; no interaction: $F_{1,16}$=1.588, p=0.2257).

Discussion

We found that cocaine alters expression of HDAC3 in D1R versus D2R-MSNs of the NAc. This, along with our lack of behavioral effects from global manipulation of HDAC3 activity in the NAc, led to the hypothesis that HDAC3 regulates cell-type specific processes from promoting cocaine-seeking behaviors. Cell-type specific changes in cocaine-induced gene expression have previously been reported within the NAc [62,127,128], however this the first to report D1R-specific changes in Hdac3 expression. Our data showing increases in D1R-Hdac3 expression following the last chronic cocaine injections suggest that a counteradaptive response occurs in D1R-MSNs. That is, Hdac3 expression is increased as a negative feedback loop due to the enhanced transcriptional activity seen within D1R-MSNs[62] and decreased HDAC3 activity in the NAc following cocaine.

This is supported by our behavioral data, where we find that disrupting HDAC3 activity within D1R-, but not D2R-MSNs, enhances cocaine-memory formation. In D2R-MSNs, it is possible that other HDAC3 functions or other corepressor proteins play a more critical role in regulating cocaine-induced transcriptional changes. Other studies have found D1R- but not D2R-

MSN molecular mechanisms in the NAc that regulate behavior [129,130]. However, perhaps HDAC3's deacetylase activity creates a permissive state for transcription to occur in both D1R- and D2R-MSNs, yet the signaling and activity that is required for transcription to occur is absent/reduced in D2R-MSNs following cocaine exposure[91]. Therefore, altered HDAC3 activity alone in D2R-MSNs is insufficient to cause robust behavioral changes. Future studies examining HDAC3's role in D1R- vs D2R-MSNs in different behavioral paradigms may provide more insight into the exact mechanism at play.

Chapter 4: Disrupting D1R- HDAC3's Deacetylase Activity may alter Abstinence-Induced Cocaine-Seeking

Rationale:

Cocaine induces cellular adaptations within the brain that can lead to persistent drug-seeking behaviors. There is increasing evidence that epigenetic mechanisms are critical in regulating cocaine seeking in mice. Pharmacological studies have shown that inhibition of HDAC reduce cocaine reinforcement, motivation, and cocaine-induced reinstatement under intravenous self-administration conditions[131,132]. Brain region specific manipulations indicate that the activity of epigenetic enzymes within the nucleus accumbens (NAc) can influence cocaine-seeking behaviors. This includes studies showing that nuclear overexpression of Class I histone deacetylase HDAC5 in the NAc attenuates cue-induced seeking and work showing that overexpression of HDAC4 in the NAc reduces cocaine motivation[73,83].

Characterization of the activity of different cell subtypes within the NAc has identified that molecules have cell-type specific roles in regulating behavioral responses to cocaine. This includes histone modifying enzymes, such as G9a[49,89]. The role of histone deacetylases in cell-type specific regulation of cocaine seeking has yet to be examined. We have shown that HDAC3 is key in regulating cocaine-induced plasticity, and that disrupting its activity within D1R- but not D2R- MSNs affects cocaine-associated memory formation. Here, we tested whether disrupting HDAC3's activity within D1R-MSNs, using Cre-dependent viral vectors and Cre driver mice affects cocaine reinforcement and abstinence-induced cocaine seeking. Our data provides initial evidence that HDAC3 within D1R-MSNs regulates cocaine-seeking.

Materials and Methods

Mice:

Male and female D1R- Cre and D2R- Cre mice were all single-housed and at 8 and 15 weeks old at the time of behavioral testing. Drd1-Cre(EY262Gsat) and Drd2-Cre (ER44Gsat) mice were crossed with C57BL/6 J mice to breed hemizygous Drd1-Cre and Drd2-Cre mice for all experiments. Mice were provided with food and water *ad libitum* for all experiments except food training. Lights were maintained on a 12:12 h light per dark cycle, with all behavioral tests performed during the light portion of the cycle. All experiments were conducted according to US National Institutes of Health Guidelines for Animal Care and Use, and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

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- experiments, cocaine-HCl was dissolved to a final concentration of either 1 mg/ml and further diluted based on the animal's body weight, at a final dose of 0.5 mg/kg/infusion.

AAV Production

The production of these plasmids was described in Lopez et al. 2019[112]. Briefly, Cre-dependent versions of HDAC3-Y298H-v5 were generated by cloning HDAC3-Y398H-v5 into a modified pAAV-hSyn-DIO-eGFP (Addgene #50457, a generous gift from Dr. Bryan Roth) with the addition of β-globin intron. GFP element was removed from the original vector and replaced with a V5-tag. To create the HDAC3 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. Plasmids were generated by Dr. Alberto Lopez and packaged by either Dr. Thekla Hemstedt was serotyped with AAV 2.1. The final titer of AAV-DIO-HDAC3(Y298H) was 6.48×10^{12} GC/mL and the final titer of AAV-EV-mcherry was purchased Addgene with a titer of 4×10^{13} GC/mL.

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). 0.5 µl of virus was infused bilaterally into the NAc [anteroposterior (AP): +1.3 mm; mediolateral (ML): ±1.1 mm; dorsoventral (DV): −4.5 mm relative to bregma. Immunofluorescence was used to confirm expression of HDAC3(Y298H). Viruses were infused at a rate of 6 µl /hr by using a 30 gauge Neuros Hamilton syringe (product #65459-01) mounted to either a Harvard Apparatus Nanomite Syringe Pump (product #MA1 70-2217) or Leica Biosystems Nanoinjector Motorized Stereotaxics (product #39462901). All infusions used the Leica Microsystems Angle Two Stereotaxic System. All animals were allowed to recover for a minimum of two weeks days before handling.

Intravenous Self-Administration

For catherization surgeries, mice were anesthetized with an isoflurane (1– 3%)/oxygen vapor mixture during surgery and implanted with intravenous catheters. The catheter tubing was passed subcutaneously into the jugular vein, Following the surgery, animals recovered for ≥48 h prior to self-administration. Mice were mildly food restricted to 85–90% of their free-feeding bodyweight throughout training. Subjects were permitted to acquire intravenous cocaine self-administration during 2 hour daily sessions for 10 consecutive days. Cocaine was delivered through the intravenous catheter by a Razel syringe pump (Med Associates). Each session was performed using two retractable levers (1 active, 1 inactive). Completion of the response criteria on the active lever resulted in the delivery of an intravenous cocaine infusion (0.03 ml infusion volume; FR1TO20s schedule for Days 1-3; FR2TO20s for Days 4-10) at a dose of 0.5 mg/kg/infusion. Responses on the inactive lever were recorded but had no scheduled consequences. Catheters were flushed daily with physiological sterile saline solution (0.9% w/v) containing heparin (100

USP U/ml). Subjects and their data were removed from the study if the catheter integrity was compromised as determined by visual leakage or intravenous propofol assessment (propofol sodium, Patterson Vet). Behavioral responses were automatically recorded by Med Associates software.

Cocaine-Seeking Tests: Following 10 days of cocaine SA mice underwent 1-day or 21 days of abstinence. Mice were subjected to a single 1-hr SA session under extinction conditions, in which an active lever response resulted in a presentation of a cue but not drug delivery. Mice were sacrificed immediately following the seeking session and NAc tissue was collected for downstream analysis.

Immunofluorescence

Following behavioral testing, animals were sacrificed and brain tissue was flash-frozen in isopentane and collected for immunohistochemistry to confirm v5 expression in HDAC3-Y298H-v5 infused mice. Twenty micrometer coronal sections were collected using a Leica CM 1850 cryostat at −20°C and mounted on slides. Slices were fixed in 4% PFA for 10 minutes, washed in 0.1-M PBS and permeated in 0.1% Triton X-100 in 0.1-M PBS. Slices were then blocked in blocking serum (8% NGS, 0.3% Triton X-100, in PBS; 1 hour) and incubated at 4°C overnight in primary solution (2% NGS, 0.3% Triton X-100; anti-v5: 1:1000, Abcam). The slices were then incubated in secondary solution (2 percent NGS, 0.3 percent Triton X-100; Alexa Fluor goat anti-rabbit 488). Lastly, tissue was incubated for 15-minute in a DAPI solution (1:10,000, Invitrogen). Slides were coverslipped using VectaShield Antifade mounting medium (Vector Laboratories). The tissue was imaged by using Olympus Slide Scanner VSBX61.

Results

Establishing Paradigm for Assaying Cocaine Reinforcement and Cocaine-Seeking using IVSA

Intravenous drug self-administration in mice is a translationally relevant model for drug intake that has more recently been implemented within the field[133–135]. An extension of this paradigm is the cocaine seeking test, where under extinction conditions lever presses are recorded however active lever pressing does not result in drug delivery, to determine cocaine-seeking[60,136]. To determine whether this IVSA paradigm can be employed in subsequent studies, a pilot study was conducted(Fig 4.1a). Following catheterization and recovery from surgery, mice were food restricted to 85-90% of free feeding body weight and underwent 10 consecutive session of cocaine IVSA. Mice learned to respond for the active lever, which delivers a cocaine infusion (0.5 mg/kg/infusion) under FR1TO20s conditions for Session 1-3 and FR2TO10s conditions for Session 4-10 (Fig 4.1b:Two-way Repeated Measures ANOVA: Main effect on Session: $F_{(9,81)}$=5.477, p<0.0001; Main effect on Lever: $F_{(1,9)}$=142.4, p<0.0001; Session x Lever Interaction: $F_{(9,81)}$=3.716, p<.0001). This is also see using in a plot of the discrimination index of mice [83](Fig. 4.1c) and as mice continue to cocaine intake across each session(Fig 4.1d). In cocaine seeking tests, mice continue to press for the active lever significantly more than the inactive, despite no reinforcement delivery(t_{10}=7.054, p<0.0001). This occurs even following 30 days of

abstinence(t_{10}=6.023, p<0.0001). This model can thus be used to determine how HDAC3 regulates aspects of cocaine reinforcement and cocaine seeking following forced abstinence.

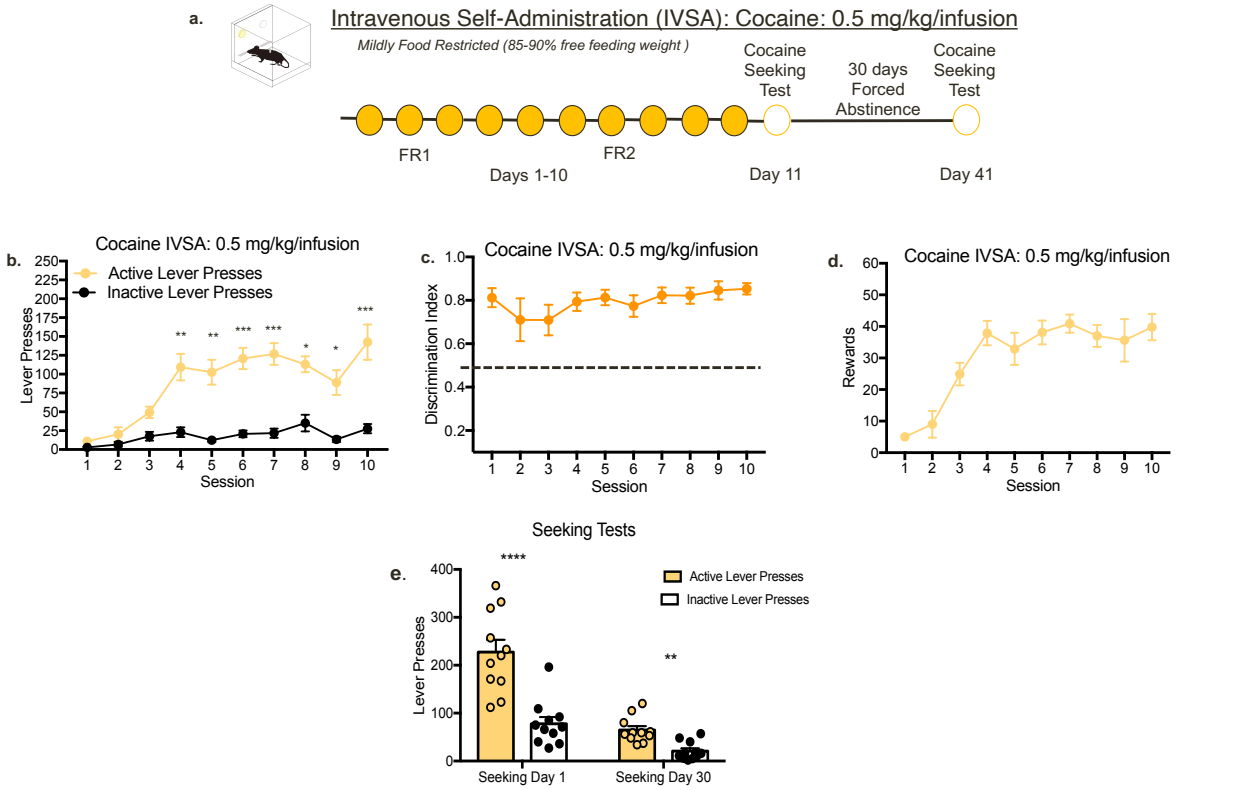

Fig 4.1. Establishing Paradigm for Assaying Cocaine Reinforcement and Cocaine-Seeking using IVSA. a. Following catheterization and recovery from surgery, mice were food restricted to 85-90% of free feeding body weight and underwent 10 consecutive session of cocaine IVSA. Cocaine seeking tests were conducted WD1 and WD30. **b.** Mice learned to respond for the active lever, which delivers a cocaine infusion (0.5 mg/kg/infusion) under FR1TO20s conditions for Session 1-3 and FR2TO10s conditions for Session 4-10 (Fig 4.1a:Two-way Repeated Measures ANOVA: Main effect on Session: $F_{(9,81)}$=5.477, p<0.0001; Main effect on Lever: $F_{(1,9)}$=142.4, p<0.0001; Session x Lever Interaction: $F_{(9,81)}$=3.716, p<.0001). C. Mice discriminate between active lever and in active lever. d. Mice self-administer cocaine across each session. e. During cocaine seeking tests, mice continue to press for the active lever significantly more than the inactive, despite no drug delivery(t_{10}=7.054, p<0.0001). This occurs following 30 days of abstinence(t_{10}=6.023, p<0.0001).

Disruption of HDAC3-Y298H-v5 in D1R-MSNs affects abstinence-induced cocaine seeking

HDAC inhibition has been shown to regulate aspects of cocaine reinforcement and affect molecular mechanisms within the NAc To determine whether D1R-specific HDAC3 activity regulates cocaine-related learning processes, we tested the effects of cocaine reinforcement. Given our CPP data, we hypothesized that DIO-HDAC3-Y298H-v5 within D1-Cre mice would enhance cocaine-reinforcement. Male and female D1R-Cre mice underwent AAV infusions containing either a Cre-dependent HDAC3-Y298H-v5 or mCherry construct and underwent 10 days of intravenous cocaine-self administration IVSA; FR1→FR2; 0.5 mg/kg/infusion). D1-HDAC3-Y298H-v5 mice (Two-way Repeated Measures ANOVA: Main effect of Session:

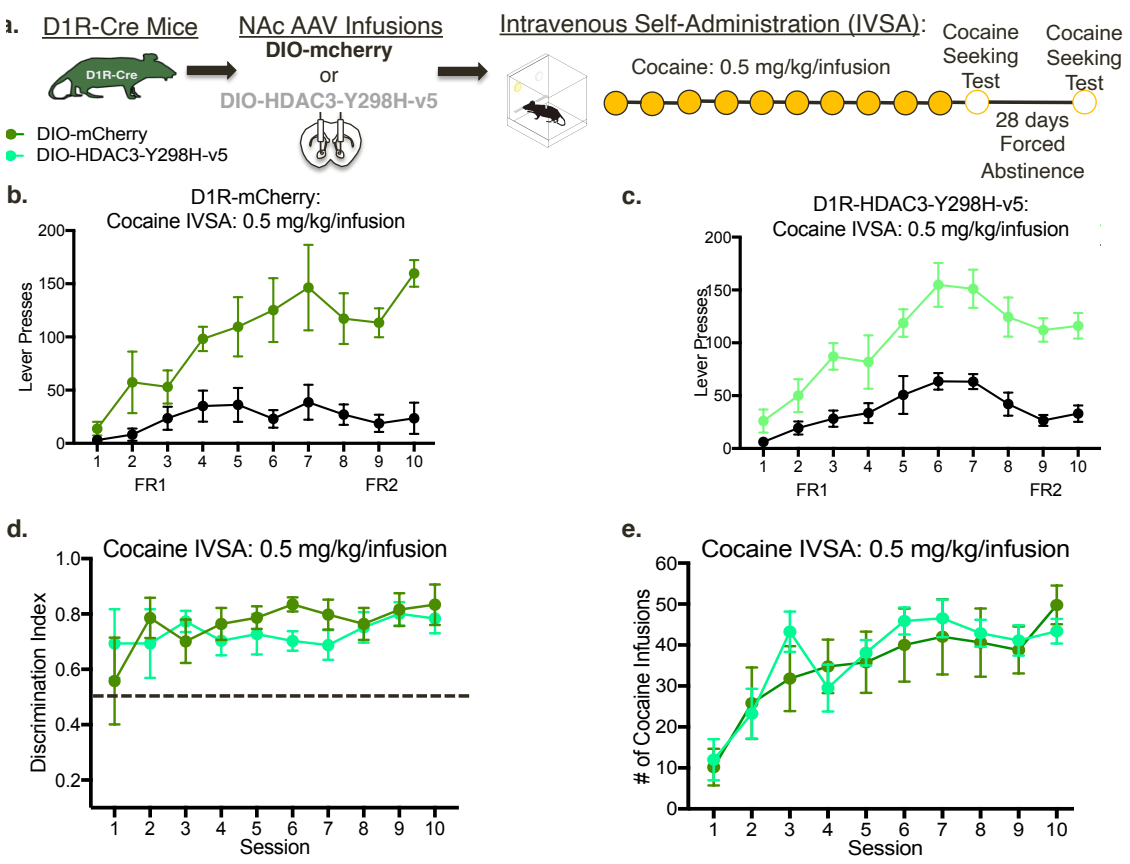

Fig. 4.2. Disrupting HDAC3 activity in NAc D1R-MSNs alters cocaine seeking in mice following cocaine IVSA. a. D1R-Cre mice were infused AAVs containing either DIO-mcherry or DIO-HDAC3-Y298H-v5 and two weeks following AAV infusions underwent catherization surgery. Following recovery, mice underwent cocaine IVSA conditions (FR1-→FR2; 0.5 mg/kg/infusion) for 10 days and then underwent cocaine seeking tests 24 hours and 30 days following last IVSA session. **b.,c** Both D1R-mcherry and D1R-Y2898H mice learned to self-administer cocaine(D1-HDAC3-Y298H-v5 mice (Two-way Repeated Measures ANOVA: Main effect of Session: $F_{(9,108)}$=12.25, p<.0.001; Main effect of lever $F_{(9,12)}$=43.21, p<.0001: Interaction: $F_{(9,108)}$=2.294, p=0.0213), and D1-mcherry mice (Two-way Repeated Measures ANOVA: Main effect of Session: $F_{(9,72)}$=8.211, p<.0001; Main effect of lever $F_{(1,8)}$=15.86, p<.0001: Interaction: $F_{(9,72)}$=4.16, p=0.0002 **d.** Disrupting HDAC3 activity (overexpressing HDAC3-Y298H-v5) in NAc D1-MSNs had no effect on discrimination index (Two-way Repeated Measures ANOVA: no main effect on virus: $F_{(1,11)}$=0.5701, p=0.4661; no main effect on session: $F_{(9,99)}$=4.260, p=0.4661; no Interaction: $F_{(9,99)}$=0.6726, p=0.7318). or **e.** cocaine intake (FR1, 0.5mg/kg/inf): (Two-way Repeated Measures ANOVA: Main effect on Session: $F_{(9,99)}$=11.31, p<0.0001; no main effect on Virus: $F_{(9,99)}$=11.31, p<0.0001; no Interaction: $F_{(9,99)}$=0.4373, p=0.9118)

$F_{(9,108)}$=12.25, p<.0001; Main effect of lever $F_{(9,12)}$=43.21, p<.0001: Interaction: $F_{(9,108)}$=2.294, p=0.0213), and D1-mcherry mice (Two-way Repeated Measures ANOVA: Main effect of Session: $F_{(9,72)}$=8.211, p<.0001; Main effect of lever $F_{(1,8)}$=15.86, p<.0001: Interaction: $F_{(9,72)}$=4.16, p=0.0002) learn to discriminate between the active lever and inactive lever. Disrupting HDAC3 activity (overexpressing HDAC3-Y298H-v5) in NAc D1-MSNs had no effect on cocaine intake (Two-way Repeated Measures ANOVA: Main effect on Session: $F_{(9,99)}$=11.31, p<0.0001; no main effect on Virus: $F_{(9,99)}$=11.31, p<0.0001; no Interaction: $F_{(9,99)}$=0.4373, p=0.9118, or on discrimination index (Two-way Repeated Measures ANOVA: no main effect on virus: $F_{(1,11)}$=0.5701, p=0.4661; no main effect on session: $F_{(9,99)}$=4.260, p=0.4661; no Interaction:

$F_{(9,99)}$=0.6726, p=0.7318). This data demonstrates that, in contrast to our hypothesis, disrupting HDAC3 activity within D1R-MSNs is not sufficient to alter cocaine reinforcement.

Following cocaine IVSA, we tested whether D1-Y298H-v5 affects abstinence induced cocaine seeking. Animals underwent two cocaine seeking tests under extinction conditions, at 24 hours and 30 days following last IVSA session. D1R-specific overexpression of Y298H resulted in decreased cocaine seeking 24h after the last cocaine session (t_{10}=1.635 p=0.1303) and this trend persisted in the 30 days following the last session (t_{10}=1.999, p=0.07). Unlike our CPP data, this initial data suggests that disrupting HDAC3 activity within D1R MSNs may impair cocaine-seeking following prolonged forced abstinence.

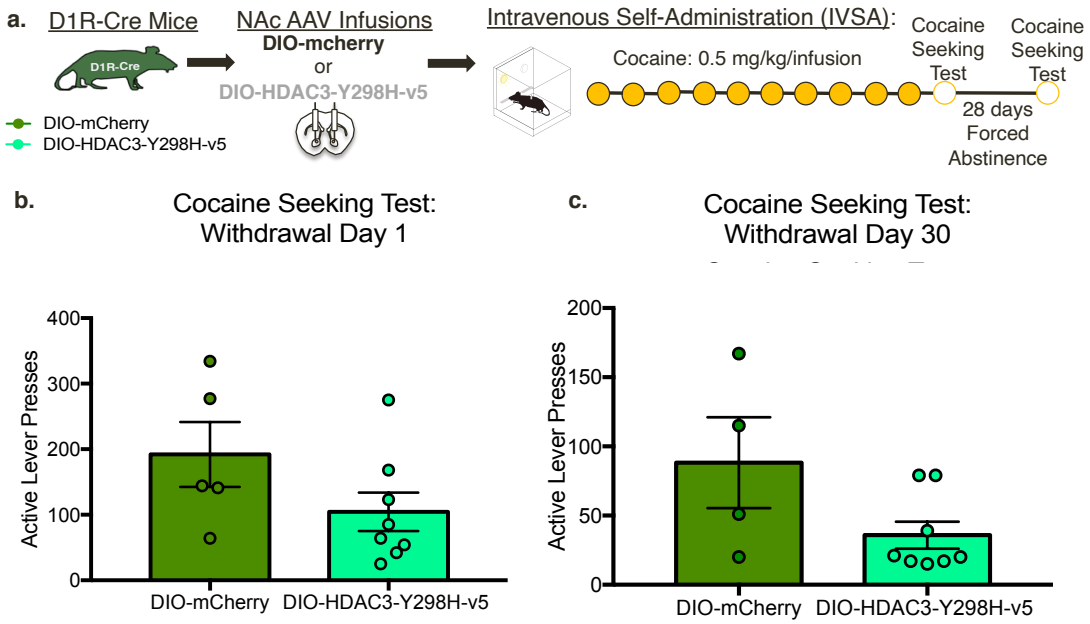

Fig. 4.3. Disrupting HDAC3 activity in NAc D1R-MSNs may alter cocaine seeking following forced abstinence. In cocaine seeking tests, D1R-Y298H mice show blunted cocaine seeking (Two-way Repeated Measures ANOVA: no main effect on virus: $F_{(1,11)}$=0.5701, p=0.4661; no main effect on session: $F_{(9,99)}$=4.260, p=0.4661; no Interaction: $F_{(9,99)}$=0.6726, p=0.7318) 24h after the last cocaine session (t_{10}=1.635 p=0.1303). and further trending decreases in 30 days withdrawal: (t_{10}=1.999, p=0.07).

Discussion

Here we present initial evidence that disrupting HDAC3 activity in D1R-MSNs may alter abstinence induced cocaine-seeking. In addition, we find that overexpressing a HDAC3 point mutant within D1R MSNs has no effect on cocaine reinforcement or cocaine intake. Although

follow-up studies must be conducted, and are currently underway, to make any conclusions about the effects on this manipulation, several hypotheses arise from this set of data.

Consistent with our data, several other studies examining the role of transcriptional mechanisms within the NAc have similarly shown no effect on cocaine intake or cocaine reinforcement but changes in abstinence-induced cocaine seeking. It is difficult to discern whether this is indicative that these molecules, like HDAC3, do not affect cocaine intake or whether the paradigm at present is not sensitive to detect the role of these subtle changes. At present, the employed IVSA paradigm uses a fixed-ratio schedule. Although we do see an escalation of intake when increasing the schedule from FR1 to FR2, new protocols using IVSA in mice have shown that variable ratio is achievable and presents more robust self-administration and cocaine intake[135]. It is possible with these updated parameters, we may be able to detect more subtle changes on cocaine intake. However, cocaine-seeking tests do provide a window for viewing how molecular mechanisms regulate both early and late abstinence seeking.

It is possible that changes in HDAC3's activity within D1R-MSNs may affect not 1 WD but 30 WD cocaine-seeking. Furthermore, in contrast to our CPP data, we see that disrupting HDAC3 activity within D1R-MSN may impair cocaine seeking. As discussed above, this may indicate that HDAC3 alters paradigm specific processes within D1R-MSNs. Additional experiments will need to be conducted to confirm if this trend is a significant effect, however there are several explanations if true. It is possible that because these cocaine-seeking tests are under extinction conditions we are capturing persistent effects on HDAC3 manipulation facilitating extinction. Previous studies have shown that HDAC inhibition can facilitate extinction under CPP conditions. This may mean that HDAC3 within D1R-MSNs accelerates molecular mechanisms related to extinction, such as synaptic AMPAR levels [137,138]. Additional experiments could be conducted to have animals undergo extinction and reinstatement conditions to confirm whether this may in fact

be occurring. Alternatively, it is possible that we are illustrating how HDAC3-dependent mechanisms differ when cocaine is self-administered, as differences in experimenter-administered and cocaine-self administration are reported[51,137,139]. Thus, we may see how HDAC3 has a role in regulation of calcium permeable-AMPA receptor (CP-AMPAR) expression, as synaptic incorporation of the GluA2-lacking AMPAR is reported to accumulate only after longer withdrawal conditions following cocaine IVSA[94,137]. Follow up studies examining whether HDAC3 regulation affects synaptic processes and synaptic localization of glutamate receptors would further expand on this idea.

Overall this presents initial evidence for how HDAC3 may act within D1R-MSNs to affect cocaine-seeking processes follow cocaine self-administration. Consistent with other work from the field, we present paradigm specific changes in cocaine action from HDAC3, a powerful regulator of plasticity, within the NAc.

Conclusions

Drugs of abuse induce long-lasting changes in neural mechanisms that can result in persistent drug use and drug craving, despite long periods of abstinence or negative consequences. These circuit-wide changes in cellular function are driven by persistent changes in molecular mechanisms. Identifying these molecular mechanisms will be critical for developing therapeutics for addiction.

Epigenetic mechanisms are powerful regulators of molecular mechanisms that drive neural function and behavior. Drugs of abuse, such as cocaine, have been shown to affect and recruit epigenetic modifications and modifiers throughout the reward system. HDAC3 is a histone deacetylase shown to be critical in regulating cocaine-induced adaptations. Genetic knockdown has confirmed its role within the NAc in regulating cocaine-processes, however it has never been

clear how cocaine affects HDAC3 to promote plasticity. The work in this dissertation presents evidence that cocaine alters HDAC3's deacetylase activity within D1R-MSNs to drive cocaine-induced plasticity within the NAc. These changes in neuroplasticity are sufficient to regulate cocaine-induced behaviors.

Throughout this dissertation we present key data to support this conceptual framework for HDAC3 in cocaine action. From data collected in **Chapter 1,** our data illustrates how cocaine alters HDAC3 activity but not HDAC3 expression to drive cocaine-induced changes in gene expression. This characterization of cocaine's actions on HDAC3 led to a set of viral studies in Chapter 2 to determine what processes is HDAC3's deacetylase activity involved in. In **Chapter 2's** data, we demonstrate how disrupting HDAC3's activity, but not overexpressing HDAC3, is sufficient to reverse cocaine-induced plasticity within the NAc. We also find that disrupting HDAC3's activity can affect cocaine-induced expression of *Nr4a2*. However, we did not ultimately see this as in driving changes in cocaine-induced behaviors. This led to a hypothesis that HDAC3's activity within D1R-MSNs drives cocaine-induced behaviors. In both **Chapter 3** and **Chapter 4**, we provide initial evidence that HDAC3 operates within a cell-type specific manner to promote cocaine-associated memory formation and abstinence induced cocaine-seeking.

What is unclear at this time is whether HDAC3 has cell-type specific targets at baseline or following cocaine exposure. In addition whether cocaine-induces changes in HDAC3 activity within D1R-MSNs but not D2R-MSNs that leads to cell-type specific changes in gene expression. Additional studies will and should be conducted to further elucidate the molecular targets and mechanisms that HDAC3 affects in D1R-MSNs. However, we speculate that signaling cascades activated within D1R-MSNs but not D2R-MSNs that promote gene expression are what drive these cell-type specific effects on behavior. As we hypothesize that HDAC3 creates a permissive state for transcription, however without the additional transcriptional machinery required, gene expression does not occur.

Overall, this data emphasizes the importance in investigating cell-type specific mechanisms that drive cocaine-seeking behaviors. Given the distinct functions of D1R- and D2R-MSNs in cocaine action, these cell types provide a great insight into how a powerful regulator such as HDAC3 can operate in one cellular context versus another.

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